IMPACT OF HYPERCARBIA ON JUVENILE FISH PHYSIOLOGY, BEHAVIOR, PERFORMANCE, AND ACCLIMATION POTENTIAL

BY

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THESIS

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ABSTRACT

Asian carp are non-native invasive fishes that have quickly become the most abundant fishes in many portions of the Midwestern United States. While Asian carp are currently contained within the Mississippi River basin by a pair of electrified barriers, these fish have the potential to negatively impact the Great Lakes ecosystem if this barrier is breached. As such, novel barrier technologies would provide an additional mechanism to prevent Asian carp from invading the Great Lakes, and provide redundancy and safety to the current electric barrier. Therefore, the overall goal of this thesis was to quantify the impact of hypercarbia on the physiology, behavior, and performance of juvenile fish, with an emphasis on determining the effectiveness of carbon dioxide as a chemical deterrent of juvenile Asian carp movement. The first study used a combination of molecular and behavioral experiments to determine the effectiveness of carbon dioxide as a chemical deterrent for larval and juvenile fishes. Results from this study indicated that larval and juvenile fishes induced stress-related gene transcripts following an acute exposure to hypercarbia, and juvenile fishes will actively avoid elevated CO_2 waters that are greater than 200 mg/L, indicating that a CO_2 chemical barrier has potential to deter the movement of larval and juvenile Asian carp. The second study held largemouth bass at ambient CO_2 (13 mg/L) and elevated CO_2 (31 mg/L) for 58 days, and then used a combination of physiological, behavioral, and performance experiments to determine the acclimation capacity of fishes to chronic hypercarbia. Results from this study clearly indicated 1) acclimation to chronic hypercarbia causes plastic alterations in molecular and physiological parameters, 2) CO_2-acclimated fish displayed a reduced stress response to an acute hypercarbia exposure compared to naïve fishes, 3) CO_2-acclimated fish were less impacted by additional hypercarbia stressors, as shown by increased tolerance of CO_2 in behavioral and swimming performance tests. This study

suggested that largemouth bass exposed to chronic hypercarbia may possess a beneficial advantage over naïve fishes, due to increased oxygen uptake capacity and acid-base regulation, during periods of elevated carbon dioxide. Together, these two studies provide insight into the potential efficacy of a CO_2 chemical deterrent for Asian carp by examining two potential caveats that could decrease the usefulness of the barrier. Additionally, results of this study can provide important information on the molecular, physiological, behavioral, and performance impacts of hypercarbia on freshwater fishes.

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CHAPTER 1: GENERAL INTRODUCTION

Non-native invasive species can have tremendous negative economic and ecological impacts on a receiving environment (Pimentel et al., 2005; Ricciardi and MacIsaac, 2011; Ricciardi, 2013). Ricciardi (2013) defines non-native invasive species as individuals that have the ability to aggressively spread into novel geographic regions and cause long-lasting adverse effects on the invaded environment. Damaging ecosystem impacts caused directly or indirectly by nuisance species can lead to depletions in animal populations that can eventually lead to extinction (Clavero and García-Berthou, 2005; Ricciardi and MacIsaac, 2011; Ricciardi, 2013). For example, the opossum shrimp (*Mysis diluviana*) was deliberately introduced into a lake to supplement the diet of non-indigenous salmon. The introduced shrimp were able to avoid predation by salmon, through differential habitat selection, and outcompeted salmon in obtaining another prey species, zooplankton. As shrimp populations increased in the pond, zooplankton densities declined and salmon populations crashed due to this shift in the food web. As a result, eagle and grizzly bear populations that relied on spawning salmon as a food source nearly disappeared (Ricciardi and MacIsaac, 2011). Currently, approximately 42 % of species listed as 'threatened' or 'endangered' under the Endangered Species Act are considered to be primarily at risk due to invasive species (Pimentel et al., 2005). Biological invasions around the globe are predicted to have cost the global economy approximately $1.4 trillion (US) annually, or roughly 5 % of the global economy (Ricciardi, 2013).

Aquatic invasive species have ecologically and economically devastated two distinct areas in eastern North America: the Laurentian Great Lakes and the Mississippi River basin (Patel et al., 2010). Approximately 182 non-indigenous species have been documented as introduced into the Great Lakes basin since 1840 (i.e., nearly one invader every 28 weeks),

which is currently the highest introduction rate for a freshwater ecosystem (Ricciardi, 2006). Over the past 50 years, the invasions of a few prominent aquatic species [e.g., sea lamprey (*Petromyzon marinus*), alewife (*Alosa pseudoharengus*), and zebra mussels (*Dreissena polymorpha*)] have caused tremendous ecological and economic turmoil in the Laurentian Great Lakes (Rasmussen et al., 2011; Ricciardi and MacIsaac, 2011). Similarly, the Mississippi River basin is at risk due to the recent invasion of round goby (*Apollonia melanostomus*) and invasive mussels, which threatens an area that has the highest diversity of freshwater fishes and mussels in North America (Rasmussen et al., 2011). The economic instability that additional invasive species establishment, and subsequent negative impacts on native populations, could cause to the Laurentian Great Lakes and the Mississippi River basin would be substantial. For example, sport fishing contributes approximately $69 billion in revenue to the economy of the United States and invasive species have contributed a loss of $5.4 billion annually to this business due to the ecological, economic, and social impacts that nuisance species have on an invaded environment (Pimentel et al., 2005). Thus, preventing the spread of invasive species between the Mississippi River basin and the Laurentian Great Lakes is necessary for the protection of endangered species and maintenance of species diversity, as well as a growing commercial and recreational fishing industry. As management strategies to monitor and control already established invasive species can be incredibly costly, the most effective means to deter the movement of invasive species into novel environments is through utilization of prevention technologies (Lodge et al., 2006; Finnoff et al., 2007).

Asian Carp

Silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*), hereafter collectively referred to as Asian carp, are two invasive species that have potential to spread into

the Laurentian Great Lakes. Asian carp are invasive, filter feeding fishes that have quickly become the most abundant fishes in many rivers throughout the Midwestern United States and have potential to negatively impact non-native freshwater environments (Kolar et al., 2007; Patel et al., 2010). As planktivorous filter feeders, Asian carp primarily rely on phytoplankton and zooplankton as food sources; however these fishes can also utilize bacteria and detritus when needed (Kolar et al., 2007; Patel et al., 2010). Asian carp can have direct impacts on the aquatic environment through food selection. For example, the turbidity of water could increase following zooplankton prey selection by Asian carp, as phytoplankton populations spike due to decreases in zooplankton abundance. Alterations in water quality can then negatively impact larval fishes (i.e., reduced prey abundance), reduce the growth of aquatic macrophytes (i.e., reduced sunlight), and eventually alter the structure of the food web (Kolar et al., 2007). Recently, Irons et al. (2007) found that body condition of bigmouth buffalo (*Ictiobus cyprinellus*) and gizzard shad (*Dorosoma cepedianum*) decreased following establishment of Asian carp within the La Grange Reach of the Illinois River. Several spawning and life history traits of Asian carp (e.g., short generation time, high fecundity, ability to spawn multiple times throughout the year, ability to swim great distances) allow these invaders to quickly establish populations in novel environments (Kolar et al., 2007; DeGrandchamp et al., 2008; Patel et al., 2010). While larval and juvenile Asian carp have only been confirmed in the southern portions of the Illinois River (DeGrandchamp et al., 2008; Irons et al., 2011), the leading edge of potentially spawning adults is approximately 35 km away from the only known permanent and continuous connection between the Mississippi River and Great Lakes basins (Patel et al., 2010). Rasmussen et al. (2011) stated that this connection, created by the Chicago Area Waterway

System (CAWS), is considered the greatest risk for potential exchanges of aquatic invasive species between these two basins.

Asian Carp Management Strategies and the Electric Barrier

The cornerstone of management strategies to prevent the spread of Asian carp from getting into the Laurentian Great Lakes has been the construction and operation of a pair of electrified barriers in the CAWS (Conover et al., 2007; Patel et al., 2010; Rasmussen et al., 2011). The electrical dispersal barrier is located approximately 40 km from Lake Michigan and emits a low-voltage, pulsing electrical current designed to repel fish, as the voltage required to achieve lethal damage to fish would likely cause severe trauma or mortality to humans and other animals that contact the water (Rasmussen et al., 2011). While the electric barriers in the CAWS are believed to have been successful at deterring the movement of Asian carp from getting into the Great Lakes, previous research has shown that these barriers may not be absolutely effective at preventing the spread of fishes upstream under all potential scenarios. More specifically, the effectiveness of electricity to stun fish depends on several factors including fish species (i.e., specific conductivity of the fish), water quality (e.g., conductivity, temperature), and electrical parameters (i.e., current type and characteristics, distance from electrode, direction of current) (Noatch and Suski, 2012). The size (volume) of the fish can also impact the ability of electricity to incapacitate fishes, as greater amounts of electrical energy is needed to immobilize fishes as they become smaller in size (Reynolds, 1996; Dolan and Miranda, 2003). Dettmers et al. (2005) found that small fishes may also be able to utilize protective cover near steel-hulled barges to avoid shock, as the electric field can be distorted and reduced around these objects. Routine maintenance, power interruptions, and accumulation of debris can also impact the effectiveness of the electric deterrent system leaving the canal vulnerable to aquatic invaders (Patel et al.,

2010; Rasmussen et al., 2011). Flooding events can also create temporary connections between the Des Plains River and the CAWS potentially allowing fish to completely by-pass the electrical barriers (Patel et al., 2010). Therefore, given that no non-physical barrier is 100 % effective at preventing the upstream movement of fishes, and the electric barrier may be susceptible to small fishes, there is a *critical need* to develop additional prevention mechanisms to deter the movement of juvenile fishes to ensure that Asian carp, regardless of size, will be prevented from spreading into the Laurentian Great Lakes.

Potential Solutions to Supplement the Electric Barrier

Several management solutions exist to increase the effectiveness of the current Asian carp containment system and prevent the movement of fishes from the Mississippi River into the Great Lakes. The best permanent solution to preventing the exchange of aquatic invasive species from these two basins is through complete hydrological and ecological separation that involves the closure of the CAWS (Patel et al., 2010; Rasmussen et al., 2011). Unfortunately, a complex set of political and engineering issues provide several obstacles for supporters of hydrological separation. More specifically, the CAWS provides the backbone for drainage of wastewater, flood control, commercial and recreational fishing, and commercial shipping throughout the greater metropolitan area of Chicago (Patel et al., 2010). Another potential strategy for the containment of Asian carp is to optimize the operating parameters of the current electrical barrier. According to the 2012 Asian Carp Control Strategy Framework, construction of a permanent Barrier I to replace the demonstration barrier was expected to begin in 2013 to provide additional safety and redundancy to the electrical barrier system. However, the same deficiencies explained in the previous section would still apply for this potential solution. A barrier that can not only deter, but also immobilize, fish is necessary to supplement the current

electrical barrier. Fish deterrents (e.g., strobe lights, bubble curtains, and pheromones) are typically used to cause a behavioral response in fishes to avoid specific areas, however these technologies lack the ability to immobilize individuals (Sorensen and Stacey, 2004; Hamel et al., 2008; Noatch and Suski, 2012). An acoustic disturbance of sufficient frequency and pressure should be able to deter fishes, however this technology is technically demanding and expensive (Noatch and Suski, 2012). Another potential solution would be the utilization of chemical toxicants (i.e., chlorine, ozone, nitrogen, and carbon dioxide) to deter the movement of fishes (Noatch and Suski, 2012). In particular, nitrogen (to induce hypoxia) and carbon dioxide (to induce hypercarbia) gas have shown promise as chemical means to deter the movement of fish, as these gases have previously been shown to induce behavioral and physiological impacts on fishes (Clingerman et al., 2007; Hasler et al., 2009). A chemical barrier should impact fish both as a behavioral deterrent, where fish actively avoid poor water conditions, and as a physiological barrier, where fish that are resistant to the avoidance aspect of the chemical barrier will eventually lose equilibrium before breaching the barrier (Noatch and Suski, 2012). While research on the efficacy of nitrogen (i.e., low oxygen) to deter the movement of Asian carp does not show strong promise as a barrier (Suski et al., unpublished data), elevated carbon dioxide (CO_2) has been proven to induce avoidance behaviors and eventual loss of equilibrium in adult Asian carp (Kates et al., 2012).

Carbon Dioxide

Elevated carbon dioxide has shown promise as a fish deterrent that could potentially be used to supplement the current electrical barrier; however a number of caveats and unknowns need to be investigated before full-scale implementation of a CO_2 chemical deterrent system. Initially, elevated carbon dioxide would act as a behavioral modifier with fish choosing to avoid

water with high CO_2 concentrations (Clingerman et al., 2007; Kates et al., 2012). Previous research has shown that fish are able to sense CO_2 in the environment by utilizing chemoreceptors in their gills (Gilmour, 2001), potentially motivating fish to search for areas of improved water quality. If fish are resistant to the avoidance aspect of the CO_2 chemical barrier, the prolonged exposure to hypercarbia will eventually lead to unconsciousness due to impairments in brain electrical activity, as brain pH decreases to maintain acid-base equilibrium in the arterial blood (Iwama et al., 1989; Yoshikawa et al., 1991; Yoshikawa et al., 1994). Previous research has shown that acute hypercarbia exposure can cause a variety of physiological impacts on fish including respiratory acidosis (Iwama et al., 1989; Bernier and Randall, 1998), metabolic acidosis (Bernier and Randall, 1998), ion imbalance (Brauner et al., 2000), and stress (Iwama et al., 1989; Ross et al., 2001; Kates et al., 2012). Kates et al. (2012) demonstrated that adult Asian carp are capable of inducing a suite of physiological responses at approximately 30 mg $L^{-1}CO_2$, display reflex responses (i.e., irregular behaviors and ventilation rates) at 70 mg L^{-1} CO_2, and initiate active avoidance behaviors at a CO_2 concentration of approximately 100 mg L^{-1}. However, the ability of carbon dioxide to elicit a stress response and impede the movements of early-life stage Asian carp has not been defined and must be investigated to determine the efficacy of a CO_2 chemical barrier to deter the movement of larval and juvenile Asian carp.

The potential for fish to acclimate to an elevated carbon dioxide environment could be an additional caveat that could undermine the efficacy of a CO_2 chemical barrier. Acclimation to challenging environments, defined as an alteration of a phenotype in response to a single environmental change (Huey et al., 1999), can give acclimated individuals a performance advantage over naïve individuals that have not encountered this environment previously (Leroi et

al., 1994). If fishes have the capacity to acclimate to an elevated CO_2 environment, this could give individuals an enhanced ability to tolerate CO_2 (i.e., greater concentration required to elicit agitation and avoidance responses, increased swimming performance during hypercarbia exposure) potentially allowing fish to breach the barrier before succumbing to the anesthetic effect of hypercarbia. Thus, it will be necessary to determine the capacity of fishes to acclimate to hypercarbic environments, and whether alterations in physiology, behavior, and performance have potential to undermine the effectiveness of a CO_2 chemical barrier. Obtaining information on these two potential caveats will help determine whether elevated carbon dioxide could be an effective long-term deterrent for Asian carp movements, regardless of size, and provide much needed safety and redundancy to the electric barrier in the CAWS.

Conclusion

Based on this background, the overall goal of my thesis was to quantify the impact of hypercarbia on juvenile fish physiology, behavior, performance, and acclimation potential. In order to achieve this goal, two separate, yet complementary studies, were performed. The first study quantified the behavioral and molecular responses of larval and juvenile fish to acute hypercarbia exposure, while the second study quantified the capacity of juvenile fishes to acclimate to an elevated CO_2 environment. Together, the results of these two studies will also help define the ability of CO_2 to serve as a non-physical barrier to deter the movement of small fishes.

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CHAPTER 2: MOLECULAR AND BEHAVIORAL RESPONSES OF EARLY-LIFE STAGE FISHES TO ELEVATED CARBON DIOXIDE

Abstract

Asian carp are non-native invasive fishes that have quickly become the most abundant fishes in many portions of the Midwestern United States. While Asian carp are currently contained to the Mississippi River basin by three electrified barriers, these fish have the potential to negatively impact the Great Lakes ecosystem if this barrier is breached, and these barriers may be particularly vulnerable to the passage of small fishes. As such, novel barrier technologies would provide an additional mechanism to prevent Asian carp from invading the Great Lakes, and provide redundancy and safety to the current electric barrier. The current study used a combination of molecular and behavioral experiments to determine the effectiveness of carbon dioxide as a chemical deterrent for larval and juvenile fishes, with an emphasis on Asian carp. Juvenile silver carp, bighead carp, bluegill and largemouth bass showed avoidance of elevated CO_2 environments of approximately 200 mg/L. Additionally, exposure to 120 mg/L CO_2 resulted in the induction of *hsp70* mRNA in eight day-old silver carp fry, while gill *c-fos* transcripts increased following hypercarbia exposure in all juvenile species examined. Together, results show that CO_2 has potential to deter the movement of larval and juvenile fishes.

Introduction

Invasions of non-native nuisance species can have a tremendous negative impact on the receiving environment, ranging from economic to ecological damage (Pimentel et al. 2005; Ricciardi and MacIsaac 2011; Ricciardi 2013). For example, it is estimated that aquatic invasive

species are responsible for a loss of approximately $5.4 billion from the $69 billion sport fishing industry within the United States (Pimentel et al. 2005). Two areas in eastern North America, the Laurentian Great Lakes and the Mississippi River basin, have been particularly devastated by aquatic invasive species (Patel et al. 2010). Conservation biologists have struggled to minimize the economic and ecological cost of established aquatic invaders (e.g., alewife (*Alosa pseudoharengus*), round goby (*Apollonia* meianostomus), sea lamprey (*Petromyzon marinus*), and zebra mussels (*Dreissena polymorpha*)) within these two regions over the past 50 years (Rasmussen et al. 2011; Ricciardi and MacIsaac 2011). Given the ecological and economic burdens that are attached to invasive species, people within the Great Lakes are particularly concerned by the potential invasion of silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*), non-native invasive fishes currently contained within the Mississippi River basin. Silver carp and bighead carp (hereafter collectively referred to as Asian carp) have quickly become the most abundant fishes in many portion of the Midwestern United States, continue to grow in population size, and have potential to negatively impact freshwater environments (Kolar et al. 2007; Patel et al. 2010). At present, the extent of the potential impact of Asian carp on the Great Lakes ecosystem is not known (Cooke and Hill 2010), but has potential to be detrimental (Conover et al. 2007; Patel et al. 2010; Rasmussen et al. 2011).

The most effective means to minimize the impact of invasive species on receiving environments is to prevent initial arrival and spread rather than attempting to extirpate invasive species after establishment (Lodge et al. 2006; Finnoff et al. 2007). Currently, the cornerstone of management strategies to prevent the spread of Asian carp from the Mississippi River basin to the Great Lakes has been the construction and operation of three electrified barriers in the Chicago Area Waterway System (CAWS) (Conover et al. 2007; Patel et al. 2010; Rasmussen et

al. 2011). While these electric dispersal barriers are believed to have been successful to date at preventing the movement of Asian carp from entering the Great Lakes, previous research has shown that these barriers may not be absolutely effective at deterring all fish movement under all circumstances (Sparks et al. 2010). More specifically, electric barriers are prone to shut downs due to routine maintenance, power interruptions, and accumulation of debris that leaves them vulnerable to aquatic invaders (Patel et al. 2010; Rasmussen et al. 2011), and fish could also utilize 'protective cover' provided by steel-hulled barges moving through the CAWS to bypass the existing barriers (Dettmers et al. 2005). More importantly, the effectiveness of an electric deterrent field can also vary based on several factors including the targeted fish species, water chemistry, electrical parameters, and distance from the electrodes (Noatch and Suski 2012). In particular, small fishes may be less vulnerable to electric fields than larger fish due to the decreased ability of electricity to immobilize fishes as they become smaller in size, indicating that existing electric barriers may not be as effective at influencing small fish relative to larger individuals (Reynolds 1996; Dolan and Miranda 2003). Given that, the leading edge of Asian carp is approximately 35 km away from the electric dispersal barriers (Patel et al. 2010), there is a critical need to develop additional prevention techniques to ensure that Asian carp will be prevented from spreading in the Laurentian Great Lakes, with technologies effective against small fishes of particular importance.

Several management solutions are available to increase the effectiveness of the current Asian carp containment system and prevent the movement of invasive fishes from the Mississippi River basin into the Great Lakes. The best permanent solution to preventing the exchange of aquatic invasive species from these two basins is through complete hydrological and ecological separation that involves the closure of the CAWS (US Army Corps of Engineers

2014). However due to a complex set of biological, socio-economic, political, and engineering issues, this solution will likely take decades to implement (Patel et al. 2010; Rasmussen et al. 2011). As such, additional non-physical deterrent systems implemented in the short term would help supplement the current electric barrier while longer-term solutions to aquatic invasive species are being pursued. Previous research has shown that carbon dioxide gas (CO_2) added to water at approximately 100 mg/L resulted in adult fishes, including Asian carp, 'choosing' to leave an area (Kates et al. 2012). Exposure of adult fishes to concentrations of CO_2 below 100 mg/L resulted in reflex responses (e.g., irregular behaviors, decreased ventilation rates) along with a host of physiological disturbances (Kates et al. 2012), suggesting that CO_2 added to water has the potential to influence the movement of Asian carp. The ability of carbon dioxide to impede the movements of larval and juvenile fishes, however, has not been defined and must be investigated to determine the efficacy of a CO_2 chemical barrier in deterring the movement of smaller size classes of fishes, and evaluating CO_2 as a barrier technology that can effectively supplement existing barrier technologies.

Therefore, the overall goal of this study was to quantify the behavioral and molecular responses of larval and juvenile fish to acute hypercarbia exposure, with an emphasis on defining the ability of CO_2 to serve as a non-physical barrier to deter the movement of small fishes. To accomplish this goal, three separate yet complementary studies were performed. The first study determined the capacity for carbon dioxide to elicit a stress response (i.e., activation of stress genes) in developing fry, while the second experiment quantified physiological disturbance (i.e., stress gene activation) in juvenile fish following exposure to a range of CO_2 concentrations. The final study exposed juvenile fishes to an elevated CO_2 environment to determine if elevated CO_2 would cause juveniles to avoid an area.

For the first two studies, a suite of functionally distinct gene transcripts were examined to provide a broad perspective of the molecular stress responses in larval and juvenile fishes following acute hypercarbia exposure, compared to many previous studies that tend to focus on candidate genes within a targeted gene family (Ali et al. 2003; Lund et al. 2003; Rimoldi et al. 2012). These gene transcripts have previously been utilized to quantify the molecular response to acute hypercarbia stress in adult bluegill (*Lepomis macrochirus*) and silver carp (Dennis et al. 2014). A brief description of the function and relevance of each gene transcript in regards to the determination of acute hypercarbia stress in fishes is presented below. One of the few gene transcripts that has been associated with environmental hypercarbia is *c-fos*, an immediate early gene that is rapidly induced following the perception of elevated CO_2, along with decreased pH in the environment (Sato et al. 1992; Tankersley et al. 2002). The c-Fos protein enhances the transcription of multiple genes (Curran and Franza Jr 1988) and is suspected to modify ventilatory behavior in response to hypercarbia exposure (Tankersley et al. 2002; Rimoldi et al. 2009). Another transcription factor that modifies the expression of multiple genes, specifically in response to hypoxia stress, is hypoxia-inducible factor 1 alpha (*hif1-α*) (Nikinmaa and Rees 2005). Previous research has shown that acute hypercarbia exposure can decrease blood pH resulting in a reduction in blood PO_2 (Bernier and Randall 1998; McKenzie et al. 2003), likely as a consequence of Root effects on oxygen binding efficiency and carrying capacity of erythrocytes. As such, *hif1-α* transcripts were quantified to determine whether oxygen concentrations within tissues were impacted following an acute hypercarbia exposure. Exposure to elevated CO_2 environments, along with many other types of stressors, can result in disruptions in blood chemistry, such as elevations in stress hormones (i.e., cortisol and catecholamines) and metabolic compounds (i.e., glucose and lactate) (Iwama et al. 1989; Ross et al. 2001; Barton

2002). Glucocorticoid receptor isoform 2 (*gr-2*) transcript expression was quantified to determine whether cortisol may be influencing transcriptional responses to an acute hypercarbia exposure. The GR-2 protein, localized on the cell membrane of all tissues, binds to free-cortisol circulating in the blood stream resulting in the alteration of several gene transcripts involved in ion regulation, metabolism, growth, and immune response (Mommsen et al. 1999). Another gene transcript that can be induced following exposure to a wide variety of environmental stressors is heat shock protein 70 (*hsp70*). The product of this gene transcript, Hsp70, mediates the repair and degradation of altered proteins and is typically induced following a stressor of sufficient duration and intensity to cause proteins to denature, aggregate, or otherwise lose functionality (Iwama et al. 2004). Therefore, *hsp70* transcripts were quantified to determine whether an acute hypercarbia exposure impacted protein functioning within larval and juvenile fishes.

Materials and methods

Hypercarbia Challenge – Fry

Experimental Animals

Two fish species were used for this experiment, silver carp and bighead carp. Mature bighead carp and silver carp females were collected from the Missouri River, on June 6, 2012, and induced to spawn at a local fish hatchery (Osage Catfisheries Inc., Osage Beach, MO, USA). Following spawning, developing eggs and resulting fry were housed in an incubation tank supplied with water from a nearby pond. Approximately eight days following fertilization, hatched fry were carefully netted from the incubation tank and subjected to a hypercarbia challenge.

Hypercarbia Challenge

The experimental design used to expose eight day-old (183-189 hrs.), hatched fry of both carp species to differing hypercarbic environments follows the general experimental outline described in Landsman et al. (2011). Briefly, groups of 30 fry were transferred into 266 mL perforated, individually numbered plastic cups, until a total of 180 cups were prepared. The cups, each containing 30 fry, were then randomly assigned to one of six treatments, each contained in separate, aerated, 15 L coolers: 1) 30 min exposure to ambient water ; 2) 60 min exposure to ambient water; 3) 30 min exposure to 70 mg/L CO_2 ; 4) 60 min exposure to 70 mg/L CO_2 ; 5) 30 min exposure to 120 mg/L CO_2 ; or 6) 60 min exposure to 120 mg/L CO_2. Therefore, fry were either exposed to one of three CO_2 concentration ranges: control (i.e., ambient CO_2), low CO_2 (i.e., 70 mg/L CO_2), or high CO_2 (i.e., 120 mg/L CO_2) for either 30 min or 60 min. A total of three replicate coolers were utilized for each treatment to minimize the potential of a cooler effect, and 10 cups were placed within a single cooler. Prior to the start of any treatment, baseline water quality measurements were taken from each cooler. Water temperature and dissolved oxygen concentrations were measured with a portable meter (YSI, 550A Yellow Springs Instruments, Irvine, CA, USA), pH was determined using a handheld pH meter (WTW pH 3310 meter with a SenTix 41 probe, Germany), while dissolved CO_2 and total alkalinity (TA) concentrations were quantified using a digital titration kit (Hach Company, titrator model 16900, kit No. 2272700 for CO_2 and kit No. 2271900 for total alkalinity). Water quality measurements for the hypercarbia challenge on eight day-old fry are presented in Table 1. Values for pCO_2 were calculated using the program CO2calc (version 1.2.0, U.S. Geological Survey, Reston, VA, USA) using temperature, pH, and total alkalinity (Robbins et al. 2010). The amount of time required for carbonate chemistry within an open system to reach equilibrium

can be as long as a few days (Riebesell et al. 2010), and, with the rate and volume of CO_2 gas addition to our containers, it was challenging to generate accurate pCO_2 data. As such, the digital titrator was used as the main tool to quantify dissolved CO_2 levels in each treatment, and was used to standardize CO_2 treatment across studies. While pCO_2 values are shown in Table 1, these data should be interpreted cautiously as the water carbonate chemistry may not have reached equilibrium at the time of measurement. It is important to note, however, that this method of gas application is representative of real-world situations where CO_2 gas would be added to water to serve as a barrier to fish movement. Target concentrations of carbon dioxide were achieved within 2 min by bubbling compressed CO_2 gas directly into the cooler via an airstone, and a small fountain pump was used to ensure homogenous CO_2 concentrations in each cooler. The concentration of dissolved CO_2 was verified using the digital titrator, and the pH range that corresponds to each desired CO_2 concentration was determined using the handheld pH meter. Target CO_2 concentrations were maintained manually throughout the challenge by monitoring pH levels and providing additions of CO_2 when necessary. Aeration, by use of a compressed air blower, was provided throughout the challenge to ensure that fry were not subjected to hypoxia (mean = 7.6 mg/L O_2 ± 0.02 mg/L standard error, SE; Table 1). At the conclusion of the treatment, 3-4 perforated cups were randomly removed from each cooler, using a random number generator to select which cups, until a total of 10 cups were sampled from each of the treatments. All fry within a single cup were immediately transferred to a 1.5 mL microcentrifuge tube filled with 1 mL of RNAlater® (AM7021, Life Technologies, Grand Island, NY, USA) and chilled on ice. Fry were refrigerated for between 1-14 days as per manufacturer's recommendations, and subsequently frozen at -80 °C until gene expression analyses.

Water quality measurements were collected at the conclusion of hypercarbia stress challenge to ensure the proper water chemistry and target CO_2 concentrations were maintained throughout the trial (Table 1). Fry in the ambient (control) treatment were allowed to remain in their cups undisturbed, with no change in water parameters, during the entire duration of the challenge, and were sampled in an identical manner to fry from the CO_2 treatment groups.

Hypercarbia Challenge – Juveniles

Experimental Animals

Four species of juvenile fishes were used in this experiment: largemouth bass (*Micropterus salmoides*), bluegill, silver carp, and bighead carp. Juvenile largemouth bass and bluegill were purchased from a commercial supplier (Logan Hallow Fish Farm, Murphysboro, IL, USA) and delivered to the Aquatic Research Facility at the University of Illinois, Champaign-Urbana, Illinois, on September 29, 2012. These fishes were housed outdoors in round plastic tanks (1280 L, 1.7 m diameter) supplied with water from a 0.04 ha natural, earthen-bottom pond with abundant vegetation. Water from the pond was allowed to drain from the tanks back to the pond providing nitrogenous waste removal and water replacement. Supplemental aeration was also provided to each of the tanks through the use of a low-pressure air blower. Juvenile largemouth bass and bluegill were fed pelleted food (Dense Culture Food, F2C, Aquatic Ecosystems, Apopka, FL, USA) until satiation every other day and solid waste was removed via siphoning every other day. Juvenile silver carp and bighead carp were cultured and housed at the Upper Midwest Environmental Sciences Center (UMESC) in La Crosse, Wisconsin, USA and fish husbandry was provided by U.S. Geological Survey (USGS) biologists. At UMESC, fish were housed in round plastic tanks contained within an indoor

recirculating aquaculture systems optimized to culture juvenile silver carp and bighead carp. Juvenile silver carp and bighead carp cultured at UMESC were from the same stock of Asian carp fry used in the Fry Hypercarbia Challenge described above, providing an opportunity to compare the hypercarbia stress response between different life-stages of fish that were of similar genetic origin. Fish from all locations received a minimum of 48 h acclimation time, without food, prior to experiments to ensure sufficient time for recovery from disturbances associated with hauling, acute stress, and digestion (Milligan 1996; Suski et al. 2006).

Hypercarbia Challenge

Juvenile largemouth bass and bluegill were subjected to the hypercarbia challenge between October 9, and October 29, 2012, while experiments involving juvenile silver carp and bighead carp occurred between December 6, and December 10, 2012. Prior to the start of the hypercarbia challenge, fish were carefully netted from the holding tank and placed into individual opaque, sensory deprivation containers continuously supplied with fresh water from a central basin. Water was allowed to overflow from each container and drain back into the central basin forming a closed, recirculating system (Vanlandeghem et al. 2010; Kates et al. 2012). The containers were sized appropriately to house fish of each species (1.9 L per largemouth bass, 1.9 L per bluegill, 0.7 L per silver carp, 0.7 L per bighead carp), contained an airstone connected to a blower to ensure sufficient oxygenation, and were outfitted with a tight-fitting lid to ensure that fish could not escape during the challenge. Fish were allowed to acclimate to their containers for 24 h, and dissolved oxygen concentrations during this acclimation period remained at 8.8 ± 0.1 mg/L (Table 1). Following this 24 h acclimation period, each container was randomly assigned to one of six treatments identical to the

hypercarbia challenge described above for eight day-old fry. Hypercarbia was achieved within 2 min by bubbling compressed CO_2 gas into the water in the central basin to the desired dissolved CO_2 concentration and then pumping this water to the specific containers being treated (Clingerman et al. 2007; Kates et al. 2012). The concentration of dissolved CO_2 was verified using a CO_2 digital titration kit with water samples taken from an extra test chamber within the system, ensuring that water quality monitoring did not impact test subjects. Target CO_2 concentrations were maintained manually throughout the challenge by monitoring pH levels and providing additions of CO_2 gas when necessary. Aeration was maintained throughout the hypercarbia challenge to ensure that fish were not subjected to hypoxia (mean = 8.8 ± 0.1 mg/L O_2; Table 1). Fish in the control (ambient) treatment were allowed to remain undisturbed in their containers with no change in water parameters during the entire duration of the experiment. At the conclusion of the hypercarbia challenge, water flow to the container was ceased, and test subjects were euthanized by an overdose of anesthetic [250 mg/L tricaine methanesulphonate (MS-222) buffered with 500 mg/L sodium bicarbonate] added directly into the container.

Following cessation of ventilation, fish were measured (total length in mm) and weighed to the nearest tenth of a gram. Samples of gill filaments, hereafter referred to as gill tissue, were excised and stored in a 1.5 mL microcentrifuge tube filled with 1 mL of RNAlater® (AM7021, Life Technologies, Grand Island, NY, USA). Tissue samples were refrigerated between 1-14 days, as per manufacturer's recommendations, and then frozen at -80 °C until further processing. Water quality measurements were collected from individual containers at the conclusion of each test subject's hypercarbia challenge to confirm that proper water chemistry conditions and target CO_2 concentration were achieved during the challenge, and are presented in Table 1. Fish size did not vary across treatments within species: largemouth bass, 99 ± 1 mm, bluegill, 104 ± 1

mm, silver carp, 73 ± 1 mm, and bighead carp, 73 ± 1 mm; one-way analyses of variance, F values < 1.7644, P values > 0.1370).

Hypercarbia Avoidance – Juveniles

Experimental Animals

Four species of juvenile fishes were used for this experiment: largemouth bass, bluegill, silver carp, and bighead carp, and the fish used in this experiment were identical to those used in the hypercarbia experiments listed above. Thus, the study sites and fish holding systems described above for each fish species in the avoidance trials is identical to the holding conditions described above.

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Hypercarbia Avoidance Challenge

Quantification of hypercarbia avoidance was performed using a 'shuttle box' choice arena (Loligo Inc., Hobro, Denmark), consisting of two holding tanks (1.5m diameter, 0.5m depth) connected by a narrow central tunnel (Serrano et al. 2010; Kates et al. 2012). Two external buffer chambers, each associated to a specific holding tank, were filled with water that can be treated and then returned to their respective holding tank without influencing water quality in the opposite tank. Water quality in each holding tank was manipulated independently using a computer and software package (ShuttleSoft 2.6.0, Loligo Inc., Hobro, Denmark). The buffer chambers continuously received water from the holding tanks through the use of an external pump. Concurrently, water was driven by gravitational force from the buffer chamber back into the holding tank creating a circular current within each of the tanks. Two pH probes, one in each holding tank, were placed in the current to obtain real-time pH measurements during

the trial. A darkened curtain around the holding tanks ensured the presence of an observer did not influence fish activity. Fish location was monitored remotely from above with a video camera.

The hypercarbia avoidance challenge followed the same procedure for hypercarbia avoidance outlined in Kates et al. (2012). Briefly, the hypercarbia avoidance challenge began by randomly selecting a fish species and then carefully netting the test subject from the holding tank. A coin flip was used to randomize which of the two holding tanks the fish was placed. Individual fish were allowed 2 h to acclimate to the 'shuttle box' choice arena under ambient water quality conditions. Initial water quality parameters during the acclimation period for all species were 15.8 °C (± 0.2 °C), 8.7 mg/L O_2 (± 0.2 mg/L), pH 7.46 (± 0.08), 21 mg/L CO_2 (± 1 mg/L), 147 mg/L $CaCO_3$ (± 2 mg/L), and pCO_2 10260 µatm (± 1760 µatm). Upon completion of the acclimation period, the external buffer chamber associated to the holding tank in which the fish had settled received a continuous addition of dissolved CO_2 gas, while the external buffer chamber for the holding tank without the fish received a continuous addition of compressed air to strip CO_2 from the water. Water from these external buffer chambers were pumped into their respective holding tanks, thereby ensuring a gradual but steady increase in CO_2 for the holding tank with the fish, while water quality in the opposite tank remained at the ambient dissolved CO_2 concentration. During the addition of CO_2 to the holding tank, the time was recorded when the fish shuttled to the opposite holding tank via the central tunnel, or when the fish lost equilibrium. Simultaneously, water quality measurements were collected from water flowing into the buffer chamber, associated with the tank that was receiving inputs of carbon dioxide. Calculation of pCO_2 values during the avoidance trial was challenging because dissolved CO_2 was stripped from the system between trials, but pH remained constant due to the presence of

carbonic acid; as such, the only method to obtain CO_2 concentrations was through the use of the digital titrator. If/when a fish shuttled to the other holding tank, both external buffer chambers received compressed air for 10 min to strip CO_2 from the treated tank. After this 10 min period, whichever tank the fish had settled was treated with CO_2 gas. The trial was repeated in the manner until the individual shuttled at total of six times (or until the fish lost equilibrium), typically resulting in multiple CO_2 measurements that elicited hypercarbia avoidance responses (i.e., shuttling) for each subject. At the conclusion of the hypercarbia avoidance challenge, fish were removed from the system and euthanized, as described previously, to be weighed and measured. Fish sizes within species were as follows: largemouth bass, 104 ± 1 mm; bluegill, 105 ± 1 mm; silver carp, 67 ± 1 mm; and bighead carp, 71 ± 1 mm.

Laboratory analyses

RNA isolation and cDNA synthesis

All laboratory procedures below adhere to the current guidelines for publication of quantitative real-time PCR (qPCR) studies outlined by Bustin et al. (2009). All tissue samples, submerged in 1mL of TRI Reagent (Ambion, Life Technologies, Grand Island, NY, USA), were disrupted and homogenized for 1 min using a mechanical homogenizer (Tissue-Tearor®, Biospec Products Inc., model No. 935370, Bartlesville, OK, USA). Total RNA from these tissue samples were then isolated using an Ambion Ribopure Kit (AM1924, Life Technologies, Grand Island, NY, USA), which involved the addition of bromochloropropane at 4 °C to effectively separate genomic DNA and proteins from RNA during purification. Extracted RNA was then treated with a Ambion DNA-free™ DNA Removal Kit (AM1906, Life Technologies, Grand Island, NY, USA) to eliminate any remaining sources of genomic DNA contamination.

Following DNase treatment, yield and purity of extracted RNA was determined using a Nanodrop ND-1000 UV-Vis spectrophotometer (Peqlab, Erlangen, Germany). RNA integrity was confirmed through the use of gel electrophoresis. Extracted RNA was subsequently frozen at -80 °C until cDNA synthesis.

To synthesize cDNA, MultiScribe Reverse Transcriptase, RNase Inhibitor, and random primers were used according to the manufacturer's protocol included in the High-Capacity cDNA Reverse Transcription kit (ABI #4374966, Life Technologies, Grand Island, NY, USA) using 2 µg of total RNA for a reaction volume of 20 µl. Enzyme activation was achieved using an Eppendorf Mastercycler® Pro thermal cycler (Eppendorf, Hamburg, Germany) set at 25 °C for 10 min, followed by a 2 h incubation period at 37 °C, and then a last step for 5 min at 85 °C to denature the enzyme. All cDNA was then stored long-term at -20 °C until qPCR analysis.

qPCR primers

Gene specific qPCR primers for all four species were designed using NCBI's Primer-BLAST (Ye et al. 2012) using sequences that were available in the GenBank database. Specific sequences that were used to create qPCR primers for juvenile largemouth bass are as follows: *c-fos* (accession no. KC493364.1), glucocorticoid receptor isoform 2 (*gr-2*, accession no. KC493363.1), hypoxia inducible factor 1 alpha (*hif1-α*, accession no. JX901057.1), heat shock protein 70 (*hsp70*, accession no. KC493362.1), and *18s* (accession no. JQ896299.1). Juvenile bluegill qPCR primers were created using sequences on NCBI's GenBank: *c-fos* (accession no. KC493364.1), *gr-2* (accession no. KC493363.1), *hif1-α* (accession no. KC493362.1), *hsp70* (accession no. KC493361.1), and *ef1-α* (accession no. AF485331.1). Silver carp sequences that were used to create qPCR primers for fry and juvenile silver carp and bighead carp are as follows: *c-fos* (accession no. KC493359.1), *gr-2* (accession no. KC493358.1), *hif1-α* (accession

no. HM146310.1), *hsp70* (accession no. KC493357.1), and *18s* (accession no. JQ896300.1). Largemouth bass, bluegill, silver carp, and bighead carp qPCR primer sequences, melting temperature, and fragment length information are described in Table 2.

qPCR analysis

All qPCR reactions were performed using 1 µl of stock cDNA (diluted 1:50 using RNase-free water), 1 µl of each qPCR primer at a 1µM concentration, 2 µl of RNase-free water, and 5 µl of RealMasterMix™ Fast SYBR ROX Kit (Kit no. 2200840, 5 PRIME Inc., Gaithersburg, MD, USA), for a total reaction volume of 10 µl. Gene expression analyses were then conducted using an ABI 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) using the following protocol: one 2 min cycle at 50 °C, one 10 min cycle at 95 °C, followed by 40 cycles of 1) 15 s at 95 °C and 2) 1 min at 60 °C. Following the completion of these 40 cycles, all PCR products underwent a melt curve analysis (one 15 s cycle at 95 °C, one 15 s cycle at 60 °C, and finally one 15 s cycle at 95 °C) to confirm the presence of a single amplicon. Gel electrophoresis (2% agarose gel containing ethidium bromide) was performed to determine that the amplicon was the correct length and the only product generated by the reaction.

Relative standard curves for all target (*c-fos*, *gr-2*, *hif1-α*, and *hsp70*) and reference (*18s*, *ef1-α*) genes were created using multiple, highly induced samples to compare threshold cycle to cDNA concentration for each qPCR primer pair. Relative cDNA concentration for each sample was then normalized using either *18s* or *ef1-α*, as mRNA concentrations of these reference genes remained constant across treatments (ANOVA $P > 0.05$). Several RNA samples that had not undergone cDNA synthesis were chosen and qPCR analyses were performed with each qPCR primer pair to detect potential genomic DNA contamination. Negligible DNA was confirmed

through an observed difference of at least 5 Cts between RT-positive and RT-negative samples (Mancebo et al. 2013), along with the observation that RT-negative and NTC samples were outside the detection limit of the standard curve (Lewis et al. 2010).

Statistical analysis

Comparisons of stress gene expression in eight day-old silver carp and bighead carp fry exposed to differing CO_2 concentration and exposure durations were performed using a two-way analysis of variance (ANOVA) with CO_2 concentration (ambient, 70 mg/L CO_2, and 120 mg/L CO_2), duration of exposure (30 min or 60 min), and their interaction (CO_2 concentration × duration of exposure) entered as fixed effects, along with cooler number entered as a random effect (Sokal and Rohlf 1995). If the interaction term was significant, or if any of the main effects were significant, a Tukey-Kramer honestly significant differences (HSD) post hoc test was applied to separate means (Sokal and Rohlf 1995). Comparisons of stress gene expression in the gills of juvenile fishes exposed to differing CO_2 environments were made using a two-way ANOVA with CO_2 concentration, duration of exposure, and their interaction entered as fixed effects (Sokal and Rohlf 1995). A Tukey-Kramer HSD post hoc test was applied to separate means if the interaction term, or any of the main effects, was significant (Sokal and Rohlf 1995). Real-time PCR data, generated in both the eight day-old fry and the juvenile fish hypercarbia challenge experiments, were log transformed, if necessary, to meet assumptions of normality and homogeneity of variances (Zar 1984). Normality was confirmed through visual analysis of fitted residuals using a normal probability plot (Anscombe and Tukey 1963), while homogeneity of variances was assessed using Hartley's F_{max} test (Hartley 1950) and through visual analysis of fitted residuals using a residual by predicted plot. If either of these assumptions were still

violated following transformation, a two-way Kruskal-Wallis test (Zar 1984; Sokal and Rohlf 1995) was performed in lieu of a two-way ANOVA. If the interaction term was significant, or if any of the main effects were significant, a Steel-Dwass all-pairs multiple comparison test was applied to separate means (Douglas and Michael 1991). Quantitative comparisons of gene expression data across species and across life stages were not performed as baseline expression of both candidate genes and reference genes can differ between species/life stage, and qPCR primers are different for each species. However, qualitative comparisons of gene expression between species and life stages were performed. Comparisons of CO_2 concentration that induced shuttling were made across species using a repeated measures ANOVA (with fish identification number entered as a random effect to account for multiple measurements collected from each individual), followed by a Tukey-Kramer HSD post hoc test to separate means (Sokal and Rohlf 1995). All means are reported ± SE where appropriate. Two-way Kruskal-Wallis test calculations and analysis were accomplished by hand using Zar (1984) as a template, while all other statistical analyses were performed using JMP version 9.0.2 (SAS Institute Inc., Cary, NC, USA). All tests were run at a significance level (α) of 0.05.

Results

Bighead carp fry upregulated *hsp70* transcripts approximately 4-fold following a 60 min exposure to 120 mg/L CO_2 when compared with bighead carp fry in ambient water, although this difference was not statistically significant (Fig. 2.1a; Table 2.4). The abundance of *hsp70* mRNA in silver carp fry, however, significantly increased 3-fold following exposure to 120 mg/L CO_2 relative to fry in the ambient treatment (Fig. 2.1b; Table 2.4). Bighead and silver carp fry transcript levels for *c-fos* and *gr-2* did not differ significantly between treatment groups

following hypercarbia exposure (Table 2.3, 2.4). Silver carp fry in the 30 min exposure duration group had a significantly lower expression of *hif1-α* mRNA, approximately 20 %, compared to silver carp fry in the 60 min exposure duration group (Table 2.3, 2.4), however there was no significant interaction or CO_2 concentration effect. The abundance of *hif1-α* transcripts in bighead carp fry did not change significantly from control fry following exposure to hypercarbia (Table 2.3, 2.4).

Following a 60 min exposure to either 70 mg/L CO_2 or 120 mg/L CO_2, the concentration of *c-fos* transcripts from gill tissue in juvenile bighead carp was elevated approximately 6-fold compared to juvenile bighead carp that only received ambient water (Fig. 2.2a; Table 2.6). Similarly, silver carp juveniles increased gill *c-fos* mRNA expression roughly 3-fold following a 30 min exposure to 120 mg/L CO_2, while extending the duration of exposure to 60 min resulted in an approximate 7-fold increase in *c-fos* mRNA abundance at both 70 mg/L and 120 mg/L CO_2 when compared to the ambient treatment (Fig. 2.2b; Table 2.6). The expression of gill *c-fos* mRNA in juvenile bluegill and largemouth bass increased nearly 18-fold and 12-fold, respectively, following exposure to either 70 mg/L or 120 mg/L CO_2 when compared to fish exposed to ambient CO_2 (Fig. 2.2c,d; Table 2.7). The abundance of gill *hif1-α* transcripts in juvenile silver carp decreased significantly (43 %) in the 30 min exposure group compared to the 60 min exposure group (Table 2.5, 2.6). Juvenile silver carp gill *hif1-α* mRNA was also significantly higher (approximately 65 % greater) following exposure to 70 mg/L CO_2 relative to silver carp exposed to 120 mg/L CO_2 (Table 2.5, 2.6). Juvenile largemouth bass in the 30 min exposure group had a 12 % decrease in expression of *hif1-α* mRNA in gills compared to largemouth bass in the 60 min exposure group (Table 2.5, 2.7), however there was no significant interaction or CO_2 concentration effect. The abundance of *hif1-α* transcripts in the gill tissue of

juvenile bighead carp and bluegill did not differ among the groups (Table 2.5, 2.6, 2.7). Similarly, gill transcript levels for *gr-2* and *hsp70* for all four species did not differ significantly across groups following the hypercarbia challenge (Tables 2.5, 2.6, 2.7).

All four species displayed hypercarbia avoidance behaviors (i.e., shuttling) between concentrations of approximately 150 mg/L to 220 mg/L CO_2, with no significant difference across species ($F_{[3]} = 1.84$, P = 0.16) (Fig. 2.3). As such, exposure of juvenile bluegill, largemouth bass, silver carp, and bighead carp to elevated CO_2 within the shuttle box resulted in voluntary shuttling from water containing high CO_2 concentrations to the tank with lower CO_2 concentrations.

Discussions

Following an acute exposure to concentrations of approximately 200 mg/L CO_2, all four juvenile fish species displayed active avoidance from areas of high dissolved CO_2 concentrations by voluntarily swimming to water with lower CO_2 concentrations. More specifically, CO_2 concentrations of 160 mg/L resulted in juvenile bluegill, largemouth bass and silver carp shuttling to water with lower CO_2 concentrations, while juvenile bighead carp required 210 mg/L CO_2 to actively avoid areas of high CO_2. Aquatic organisms have a variety of molecular and behavioral mechanisms to respond to reductions in water quality, with avoidance behaviors being advantageous in situations where a) continual inhabitance in sub-optimal environments can have detrimental energetic costs and b) movement toward higher quality environments is possible (Kieffer and Cooke 2009). The ability to sense CO_2 in the environment is an inherent trait shared among diverse organisms, both prokaryotes and eukaryotes alike (Cummins et al. 2014). Many studies have assessed avoidance behaviors that are initiated upon elevation of

dissolved CO_2 concentrations in a variety of aquatic vertebrates and invertebrates (Jones et al. 1985; Ross et al. 2001; Clingerman et al. 2007; Bierbower and Cooper 2010). Previous research has shown that several fish species are able to detect elevated CO_2 concentrations by utilizing external chemoreceptors in their gills (Gilmour 2001), potentially allowing these organisms to discern high quality habitats from degraded environments. Given that fish can sense CO_2 in their environment, the efficacy of using CO_2 to influence the movement of fishes has been well studied in the last two decades. For example, Clingerman et al. (2007) were able to use CO_2 concentrations ranging from 60-120 mg/L to direct the movement of adult rainbow trout (*Oncorhynchus mykiss*) from a "growout" tank into a "harvest" tank providing aquaculture managers a more efficient, economical, and less laborious transfer process. Avoidance responses in adult silver carp, largemouth bass, and bluegill have been previously documented by Kates et al. (2012), with all three species choosing to move away from a high CO_2 environment at approximately 100 mg/L. Results from this current study suggest that juvenile fishes likely require greater concentrations of dissolve CO_2 to induce active avoidance behaviors compared to adult fishes, but avoidance behaviors were still observed. Together, results from the current study show that juvenile silver carp, bighead carp, largemouth bass, and bluegill all demonstrated active avoidance of elevated CO_2 waters once concentrations reached approximately 200 mg/L.

Juvenile fish exposed to a range of hypercarbic environments exhibited gene expression changes in gill tissue suggesting disruption to homeostasis across a number of stress pathways. More specifically, juvenile silver carp and bighead carp increased abundance of *c-fos* gill mRNA nearly 6-fold following exposure to low and high CO_2 concentrations for 60 min compared to fish only exposed to ambient CO_2 levels. Additionally, *c-fos* transcripts were induced in the gill tissue of juvenile bluegill and largemouth bass following exposure to CO_2 when compared to fish

in the control (ambient CO_2) groups. Following exposure to an acute stressor, a variety of physiological systems (e.g., the hypothalamic-pituitary-interrenal (HPI) axis, osmoregulation, induction of heat shock proteins, and oxygen transport) can be altered, all in an effort to maintain homeostasis (Barton 2002; Prunet et al. 2008; Kassahn et al. 2009). The physiological response of acute hypercarbia exposure to juvenile and adult teleosts has been well studied (reviewed in Perry and Gilmour 2006; Brauner and Baker 2009), and includes physiological alterations (e.g., disturbance to blood acid-base status, increased stress hormones, and changes in hematological features) as well as reflex responses (e.g., changes in ventilation frequency and magnitude). However, relatively little work has been performed on the molecular precursors that drive many of the physiological and behavioral changes that have been documented following acute hypercarbia exposure (Rimoldi et al. 2009). Previous studies have shown that gene transcripts such as *gr* and *hsp70* respond to a variety of stressors including temperature shock (Healy et al. 2010), wastewater exposure (Wang et al. 2007; Ings et al. 2011), handling stress (Wiseman et al. 2007; López-Patiño et al. 2014), and even infectious diseases (Stolte et al. 2009), while other gene transcripts, such as *hif1-α* and *c-fos*, typically respond to specific environmental stressors like hypoxia (Nikinmaa and Rees 2005; Rimoldi et al. 2012) or hypercarbia (Sato et al. 1992; Tankersley et al. 2002; Rimoldi et al. 2009), respectively. In the current study, exposure of juvenile fish to an acute hypercarbia stressor resulted in the induction of *c-fos* mRNA in the gills. Previous research has shown that *c-fos* mRNA is rapidly induced following hypercarbia exposure in a variety of organisms, ranging from teleosts (Rimoldi et al. 2009) to rodents (Sato et al. 1992; Tankersley et al. 2002). Once translated, the c-Fos protein regulates the expression of a multitude of genes in response to a specific stressor (Curran and Franza Jr. 1988; Kassahn et al. 2009), in this case hypercarbia, all in an effort to maintain physiological homeostasis. While

previous research has shown brain *c-fos* gene expression patterns differs among mouse strains that vary in their ventilatory response to acute hypercarbia exposure (Tankersley et al. 2002), additional studies will need to be performed to determine whether gill *c-fos* expression modulates the ventilatory response of fishes to hypercarbia stress, potentially providing an additional mechanism to eliminate CO_2 from the blood stream (Gilmour 2001; Perry and Gilmour 2006). Additionally, juvenile largemouth bass and bluegill appeared to be more responsive/sensitive to hypercarbia exposure compared to juvenile Asian carp (i.e., greater relative increase in *c-fos* transcripts, expression of *c-fos* mRNA at lower cocentrations of CO_2). Similarly, Dennis et al. (2014) examined gene expression in adult bluegill and silver carp following a 1 h exposure to 30 mg/L CO_2 and found that *c-fos* transcripts were induced 12-fold and 8-fold in gill and erythrocyte tissues in bluegill compared to silver carp who exhibited 3-fold increases in *c-fos* mRNA in erythrocytes. The authors suggested this difference in gene expression may contribute to the greater CO_2 tolerance of adult bluegill compared to silver carp documented by Kates et al. (2012). As such, juvenile Asian carp may have difficulties maintaining homeostasis when exposed to hypercarbic environments compared to juvenile native fishes, which might be advantageous in developing a barrier to negatively impact only invasive juvenile Asian carp movement. Together, results from the current study clearly indicate that juvenile fishes respond, through changes in *c-fos* mRNA expression, to elevations of dissolved CO_2.

Similar to the juvenile fish species studied, eight day-old hatched bighead carp and silver carp fry exhibited gene expression alterations following an acute hypercarbia exposure. More specifically, silver carp fry upregulated the expression of *hsp70* transcripts approximately 2-fold following exposure to 70 mg/L CO_2 and nearly 3-fold when CO_2 levels were increased to 120

mg/L. Additionally, exposure of bighead carp fry to 120 mg/L CO_2 resulted in the 4-fold induction of *hsp70* mRNA, although this increase was not statistically significant relative to ambient controls. Previous work quantifying the impacts of external, chemical stressors on larval fishes have mainly focused on mortality rates as the response variable of interest and were usually performed over long periods of time (i.e., 1-7 days) (Kikkawa et al. 2004; Landsman et al. 2011; Baumann et al. 2012). In the present study, larval fishes were subjected to an acute hypercarbia exposure meant to simulate CO_2 exposure durations that larval fish would experience by drifting through a field-deployed CO_2 barrier. In addition, the candidate genes examined provided a comprehensive, broad view of sub-lethal stress in larval fishes. These candidate gene transcripts (*c-fos, hif1-α, gr-2,* and *hsp70*) have been used successfully in past studies to quantify 'stress' in larval fishes exposed to hypoxia (Liu et al. 2013), heavy metal exposure (Sassi et al. 2012), and insecticide exposure (Beggel et al. 2012). Results from the current study suggest that a 60 min exposure to 120 mg/L CO_2 was sufficient to cause physiological disturbance in eight day-old Asian carp larvae, as seen through increases in *hsp70* mRNA. Heat shock protein transcripts are typically induced to maintain homeostasis within the cell by facilitating the proper folding of nascent proteins, acting as a molecular chaperone, and by mediating the repair and degradation of altered or denatured proteins following a stressor (Iwama et al. 2004), suggesting that an acute hypercarbia exposure to larval Asian carp potentially had an impact on protein functioning. Previous research has shown that *hsp70* transcripts can be upregulated in teleost fish embryos following temperature shock, with the authors suggesting that increased *hsp70* mRNA may have been playing a protective role against heat damage and allowed embryos to develop normally (Takle et al. 2005). While *hsp70* mRNA expression was likely important for larval Asian carp to maintain homeostasis and proper cellular

functioning under acute hypercarbia stress, additional research is needed to determine whether *hsp70* expression allows larvae to continue to develop correctly. Interestingly, juvenile and larval fishes utilized different gene transcripts to respond to an acute hypercarbia stressor with juvenile fishes inducing a hypercarbia-linked transcription factor (*c-fos*) compared to larval fishes activating 'general' stress transcripts (*hsp70*). This suggests that eight-day old larval fish may not be capable of responding to this stressor in a hypercarbia-specific manner, or larval fishes may rely on other stress-related gene transcripts to respond to acute CO_2 exposure, and as a potential consequence need to induce *hsp70* to maintain protein functioning under hypercarbia stress. However, additional research will be necessary to determine if larval fishes are incapable of mounting a hypercarbia-specific stress response, and whether this makes larval fishes more susceptible to hypercarbia exposure compared to juvenile fishes. Together, results from the current study clearly demonstrate that even eight day-old Asian carp larvae respond, through elevations in *hsp70* mRNA, to acute hypercarbia exposure.

Interestingly, neither *gr*-2 nor *hif1-α* transcripts responded to an acute exposure of hypercarbia for the larval and juvenile fishes studied. Expression of *gr-2* mRNA was assessed to quantify whether elevations in cortisol due to a stress response might be directly influencing transcriptional regulation. The product of this gene, the GR-2 protein, activates the expression of multiple gene pathways (e.g., increased metabolism, decreased growth, and ion maintenance) following exposure to a stressor by binding free cortisol circulating in the blood stream (Mommsen et al. 1999). While Stouthart et al. (1998) found that common carp (*Cyprinus carpio*) embryos had a fully functional HPI axis at the time of hatching, several previous studies have shown that larval fishes often do not have a fully developed stress response until approximately one week post hatching (Alsop and Vijayan 2009; Applebaum et al. 2010; Zubair

et al. 2012), potentially explaining the lack of *gr-2* response for larval fishes. Dennis et al. (2014) showed that gill *gr-2* transcripts were upregulated 2-fold in adult silver carp exposed to 30 mg/L CO_2, as such, it was surprising that a similar result did not occur for juvenile Asian carp. However, the induction of a hypercarbia specific gene transcript (*c-fos*) in the gills of juvenile silver carp that was not observed in adult silver carp (Dennis et al. 2014) may alleviate the need to induce other stress-related transcripts (*gr-2, hsp70*), or juvenile/larval fishes may simply utilize a different gene variant of GR. In addition, the decrease in blood pH that accompanies hypercarbia exposure (Iwama et al. 1989) can theoretically decrease oxygen binding efficiencies due to Root and Bohr effects, and hypercarbic environments have also been shown to reduce ventilation rates (Gilmour 2001) in fishes, potentially negatively impacting oxygen uptake. Previous research has shown, however, that oxygen consumption does not change dramatically following hypercarbia exposure (Ishimatsu et al. 2008), potentially providing an explanation for the lack of *hif1-α* transcript upregulation observed in the current study.

When taken together, results from this study demonstrate the potential utility of CO_2 as a non-physical barrier to prevent the movement of larval and juvenile fishes. Juvenile fishes will choose to avoid areas of high CO_2, and both larval and juvenile fishes experience physiological disturbances when placed in elevated CO_2 environments. If fish are unwilling or unable to avoid exposure to elevated CO_2 over extended time periods, individuals will likely succumb to the anesthetic effect of hypercarbia exposure and lose equilibrium (Iwama et al. 1989; Kates et al. 2012), demonstrating efficacy of CO_2 as a non-physical barrier. Prior to implementation of a CO_2 barrier in a field setting, however, there are a number of studies that must be completed to address knowledge gaps and increase confidence in this technology to influence fish movement.

For example, subsequent studies should examine the responses of free-swimming fish within a pond or lock chamber rather than in a laboratory setting, as was done in the current study. Similarly, it is critical to quantify the behavior of both fish and CO_2 in flowing, dynamic water systems (e.g., assessing the preference/avoidance behaviors of fish in flowing water). In addition, because the addition of CO_2 to water results in a concomitant reduction in pH, the impact of aquatic hypercarbia on non-target organisms (e.g., microbes, macroinvertebrates, amphibians, reptiles, mammals, and humans), along with a more detailed examination of impacts on water chemistry (e.g., leaching of metals, shifts in carbonate chemistry, and impacts on permanent structures), must be investigated prior to field deployment. It is also important to emphasize that no non-physical barrier is 100 % effective at deterring fish (Noatch and Suski 2012), and a CO_2 chemical deterrent would likely function best when used in tandem with other nonphysical and physical barriers (e.g., electric barrier). Finally, results generated across several studies suggest that a CO_2 barrier would not be species-specific, and using CO_2 to influence the movement of a particular target species would likely also impact non-target fish movement as well.

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CHAPTER 3: MOLECULAR, BEHAVIORAL, AND PERFORMANCE RESPONSES OF JUVENILE LARGEMOUTH BASS ACCLIMATED TO AN ELEVATED CARBON DIOXIDE ENVIRONMENT

Abstract

Aquatic hypercarbia, or elevated dissolved carbon dioxide, either naturally occurring or anthropogenically induced can have extensive impacts on aquatic environments and the organisms that rely on these habitats. While the impact of acute hypercarbia exposure on the behavior and physiology of fishes has been well studied, relatively little work has examined the physiological impact and acclimation capacity of fishes to chronic hypercarbia. In an effort to better understand the impacts of prolonged hypercarbia, largemouth bass were held at ambient CO_2 (13 mg l^{-1}; ≈ 1100 μatm) and elevated CO_2 (31 mg l^{-1}; ≈ 21000 μatm) for 58 days, which has previously been shown to be sufficient to induce plastic phenotypic changes. Following the acclimation period, fish from each group were subjected to three separate, yet complimentary, experiments: 1) acute hypercarbia challenge of 120 mg l^{-1} CO_2 for 1 h, and physiological and molecular parameters were assessed; 2) hypercarbia avoidance challenge to compared CO_2 agitation and avoidance responses; and 3) swim performance challenge to quantify the impact of chronic and acute hypercarbia exposure on burst swimming performance. Acclimation to 31 mg l^{-1} CO_2, and acute exposure to 120 mg l^{-1} CO_2, resulted in alterations in a variety of molecular and physiological parameters. Swimming performance and CO_2 agitation results suggest that CO_2 acclimation confers tolerance to additional hypercarbia exposures relative to naïve fish. This study demonstrates that largemouth bass exposed to chronic hypercarbia, either naturally or

anthropogenically, may possess a beneficial advantage of increased oxygen uptake capacity and acid-base regulation during periods of elevated carbon dioxide.

Introduction

Aquatic hypercarbia, or elevated dissolved carbon dioxide (CO_2), can be an environmental stressor for many aquatic organisms. While hypercarbia can occur naturally in the environment (e.g., coastal upwelling zones, estuarine waters) (Feely et al., 2008; Thomsen et al., 2010), anthropogenically driven hypercarbia is becoming increasingly problematic. Of particular concern is the expected increase in dissolved CO_2 concentrations driven by global climate change (Cooley and Doney, 2009; Raven et al., 2005). Predicted levels of dissolved CO_2 by year 2300 (approximately 1900 µatm) have been shown to impair the ability of larval and juvenile marine fishes to avoid predators, locate settlement and refugia, and respond to auditory cues, potentially resulting in population declines and failure to recruit (Dixson et al., 2010; Munday et al., 2009; Munday et al., 2010; Simpson et al., 2011). In addition to global climate change, fish can be subjected to aquatic hypercarbia through intensive aquaculture practices (Colt and Orwicz, 1991; Kristensen et al., 2009) or novel chemical fish deterrents (Clingerman et al., 2007; Kates et al., 2012). For example, Blancheton (2000) suggests that CO_2 concentrations below 40 mg l^{-1} should be safe for fish in aquaculture systems, however previous research has shown that the health and condition of fish can be impacted by chronic exposure to approximately 20 mg l^{-1} CO_2 (Fivelstad et al., 1999). Given that hypercarbic environments are expected to become more prevalent in the future (Feely et al., 2008), examining the impact of chronic elevated CO_2 exposure in fishes is becoming increasingly important.

Aquatic organisms have a variety of potential mechanisms to respond to environmental stress related to aquatic hypercarbia. More specifically, animals can display behavioral avoidance to escape poor quality environments and potentially avoid the energetic cost necessary to inhabit sub-optimal habitats, a concept known as the Bogert effect (Bogert, 1949; Kieffer and Cooke, 2009). Similarly, animals can respond by altering molecular and physiological systems to enable animals to maintain homeostasis and cope with the hypercarbic stressor (Barton, 2002; McEwen and Wingfield, 2003). This process initially starts through the activation of receptors and release of stress hormones (i.e., cortisol), which can lead to changes in the physiological processes involved in oxygen transport, metabolism, ion regulation, and eventually result in whole animal performance and behavioral modifications (Barton, 2002). Finally, genetic mechanisms, such as the induction of heat shock protein 70 (*hsp70*) and *c-fos* transcripts, can enable protein functioning under stressful conditions (*hsp70*) or enhance the response to hypercarbia (*c-fos*) allowing these organism to cope with hypercarbia (Iwama et al., 2004; Rimoldi et al., 2009).

While the physiological (Bernier and Randall, 1998; Brauner and Baker, 2009; Perry and Gilmour, 2006) and behavioral impacts (Dixson et al., 2010; Munday et al., 2009) of acute hypercarbia exposure on fishes has been well studied, relatively little work has been performed to define the physiological, behavioral, and performance impacts of chronic hypercarbia exposure on fishes. Due to the threat of ocean acidification, current research focuses on the impact of chronic hypercarbia in marine fishes through the examination of acid-base status (Michaelidis et al., 2007; Petochi et al., 2011), ion regulation (Deigweiher et al., 2008; Melzner et al., 2009; Petochi et al., 2011), energy metabolism (Michaelidis et al., 2007; Santos et al., 2013), and stress (Petochi et al., 2011; Santos et al., 2013). Despite the quantity of research

conducted on hypercarbia, few studies have examined how chronic CO_2 exposure influences biologically and ecologically important endpoints, such as swim performance or avoidance responses (Melzner et al., 2009; Ross et al., 2001). Additionally, prolonged exposure of animals to sub-lethal environmental stressors can induce physiological and molecular alterations, defined as phenotypic plasticity (Chevin et al., 2010), that can actually benefit an animal's performance and ability to tolerate environmental stress, known as beneficial acclimation (Leroi et al., 1994). While previous studies have examined beneficial acclimation in fishes in response to temperature shock (Angilletta, 2009; Lurman et al., 2009) and hypoxia (Gaulke et al., 2014), few studies have examined the acclimation capacity of fishes to hypercarbia, especially in terms of physiological and performance metrics. Therefore, determining the impact of chronic hypercarbia exposure and subsequent acclimation capacity of fish will provide critical information on 1) how chronic hypercarbia exposure affects fish, 2) if prolonged exposure to hypercarbia imparts physiological alterations that benefit an individual's response to hypercarbia, and 3) how freshwater species respond to chronic hypercarbia stress.

Based on this background, the objective of this study was to quantify the capacity of fish to acclimate to elevated carbon dioxide environments. To accomplish this, juvenile largemouth bass were first held in either elevated CO_2 or ambient CO_2 conditions for eight weeks. Following this acclimation period, largemouth bass were subjected to three separate, yet complimentary, experiments to define their capacity to acclimate to elevated CO_2. The first experiment quantified the impact of extended CO_2 exposure on molecular and physiological disturbance in largemouth bass following acute hybercarbia exposure. The second study examined whether prolonged exposure to an elevated CO_2 environment influenced thresholds of CO_2 agitation or avoidance. The final study quantified swim performance of CO_2-acclimated

largemouth bass under control and hypercarbic conditions. Together, these three experiments provide valuable insight into the acclimation potential and subsequent response to hypercarbic environments. Largemouth bass were an ideal species to use in this study for the following reasons: 1) largemouth bass have been shown to be a highly plastic species that can acclimate to a variety of external stressors [i.e., hypoxia (Gaulke et al., 2014), temperature shock (Díaz et al., 2007)]; 2) previous research on acute physiological responses to hypercarbia has been performed on largemouth bass (Kates et al., 2012); and 3) assessing the impact of chronic CO_2 exposure may be useful for aquaculture managers rearing largemouth bass.

Results

Following a 1 h exposure to 120 mg l^{-1} CO_2, *c-fos* transcripts in the gill tissue of largemouth bass exhibited a 25-fold and 29-fold, for fish in the ambient CO_2 and elevated CO_2 acclimation group respectively, relative to largemouth bass in the control treatments (Fig. 3.1A, Table 3.3). Largemouth bass in the elevated CO_2 acclimation group also had constitutively higher expression of *c-fos* mRNA in erythrocytes, approximately 3-fold, compared to fish in the ambient CO_2 acclimation group (Fig. 3.1A, Table 3.3). Concentrations of *hsp70* transcripts in both gill tissue and erythrocytes for largemouth bass acclimated to 30 mg l^{-1} CO_2 were 2-fold and 5- to 8-fold greater (respectively) than largemouth bass held at ambient CO_2 (Fig. 3.1B, Table 3.3).

One-hour exposure to 120 mg l^{-1} CO_2 caused a 3-fold increase in plasma cortisol concentrations for largemouth bass, regardless of acclimation group (Fig. 3.2A, Table 3.5). While plasma glucose concentrations were not statistically different between the acclimation groups following a 1 h exposure to control conditions, largemouth bass acclimated to ambient

CO_2 had a greater induction of plasma glucose (5-fold) compared to largemouth bass acclimated to 30 mg l^{-1} CO_2 (3-fold) following a 1 h exposure to 120 mg l^{-1} CO_2 (Fig. 3.2B, Table 3.5). Regardless of acclimation group, largemouth bass exposed to 120 mg l^{-1} CO_2 for 1 h experienced increased plasma lactate concentrations (Fig. 3.2C, Table 3.5). Plasma sodium concentrations did not differ between acclimation groups or the acute exposure treatments (Table 3.4, 3.5). For plasma chloride, largemouth bass acclimated to elevated CO_2 had a 5% reduction compared to fish acclimated to ambient CO_2 concentrations (Table 3.4, 3.5). Similarly, an acute exposure to 120 mg l^{-1} CO_2 resulted in a 5% decrease in plasma chloride concentrations relative to fish exposed to control conditions (Table 3.4, 3.5). Regardless of acclimation group, largemouth bass exposed to an acute hypercarbia stressor increased hematocrit concentrations, approximately 12% for fish acclimated to ambient CO_2 and 25% for fish acclimated to 30 mg l^{-1} CO_2 (Table 3.4, 3.5).

Exposure of largemouth bass to elevated CO_2 within the shuttle box induced an agitation response, followed by active CO_2 avoidance by voluntarily leaving an area of high CO_2. More specifically, largemouth bass in the 'initial' time period and fish acclimated to ambient CO_2 in the 'final' time period displayed agitation responses at approximately 95 mg l^{-1} CO_2, while largemouth bass acclimated to elevated CO_2 did not display agitated responses until 160 mg l^{-1} (Fig. 3.3A, Table 3.6). Largemouth bass in the 'final' time period also required a greater CO_2 concentration to display an active avoidance response (approximately 210 mg l^{-1} CO_2) relative to fish in the 'initial' time period (approximately 135 mg l^{-1} CO_2), however there was no effect of prolonged CO_2 exposure on avoidance (Fig. 3.3B, Table 3.6). During the 'final' time period, largemouth bass acclimated to elevated CO_2 spent 60% more time within the CO_2 treated holding tank (35 min) than largemouth bass acclimated to ambient CO_2 (Fig. 3.4A, Table 3.6). Similarly,

largemouth bass acclimated to 30 mg l^{-1} CO_2 were able to shuttle approximately 4 times per trial before losing equilibrium, while largemouth bass in the 'initial' time period and fish acclimated to ambient CO_2 in the 'final' time period only shuttled 3 times per trial (Fig. 3.4B, Table 3.6). By comparing 'initial' and 'final' CO_2 agitation and avoidance responses of individuals, we determined that only individual CO_2 agitation responses were repeatable following an eight-week acclimation period, regardless of acclimation treatment (Fig. 3.5). All other responses measured for during the hypercarbia avoidance challenge were not correlated between the 'final' and 'initial' measurements (Fig. 3.5).

Exposure of largemouth bass to elevated CO_2 within the swim tunnel resulted in a reduction of burst swimming performance, but only for fish that remained at ambient CO_2 concentrations during acclimation. More specifically, exposure to 120 mg l^{-1} CO_2 resulted in largemouth bass acclimated to ambient CO_2 exhibiting a 3-fold decrease in the duration of the swimming trial (Fig. 3.6A) and velocity (Fig. 3.6B) relative to largemouth bass exposed to control conditions or elevated CO_2-acclimated largemouth bass subjected to 120 mg l^{-1} CO_2 (duration: $F_{[3]} = 8.42$, $P = 0.0004$; velocity: $F_{[3]} = 8.37$, $P = 0.0004$).

Discussion

Acclimation to an elevated CO_2 environment for eight-weeks impacted hypercarbia agitation and avoidance responses of juvenile largemouth bass. More specifically, CO_2-acclimated largemouth bass tolerated greater CO_2 concentrations (160 mg l^{-1}) prior to displaying agitation responses (i.e., surface ventilations, twitching, erratic/elevated swimming) compared to control largemouth bass. Similarly, CO_2-acclimated fish maintained equilibrium for a longer period of time compared to ambient CO_2-acclimated fishes (i.e., total CO_2 exposure time and

number of shuttles greater in elevated CO_2-acclimated fish). Behavioral and performance assays can be useful tools to identify adverse aquatic conditions for fishes, such that researchers can 1) readily demonstrate sub-lethal concentration related response (i.e., CO_2 agitation and avoidance response, burst swimming performance) and 2) relate results to ecologically-relevant activities (i.e., predator avoidance, capturing prey, locating optimal habitat) that can impact fitness and survival of organisms (Plaut, 2001; Ross et al., 2001; Wolter and Arlinghaus, 2003). In the current study, elevated CO_2-acclimated fish displayed greater tolerance to an elevated CO_2 environment (i.e., greater CO_2 concentration necessary to induce agitation, spent more time in CO_2 prior to loss of equilibrium) compared to control fish. Molecular and physiological alterations that occurred during hypercarbia acclimation may have increased the capacity of largemouth bass to respond to an additional elevated CO_2 stressor, such as the hypercarbia avoidance challenge. The physiological response of fishes to an acute hypercarbia exposure has been well studied, and includes an initial elevation in blood P_{CO2}, reduction in blood pH, and a subsequent increase in bicarbonate (HCO_3^-) to increase blood pH to basal levels (reviewed in Brauner and Baker, 2009; Perry and Gilmour, 2006). Oxygen uptake and release by erythrocytes can be impaired by reductions in plasma pH due to acute hypercarbia exposure, as a consequence of Root-Bohr effects, resulting in reductions in plasma PO_2 (Bernier and Randall, 1998; McKenzie et al., 2003). As such, elevated hematocrit levels shown in CO_2-acclimated largemouth bass following exposure to 120 mg l^{-1} CO_2 likely improved oxygen uptake and transport (Wells, 2009) compared to control fishes, potentially allowing CO_2-acclimated fishes to maintain equilibrium for a longer period of time and display agitation responses at greater CO_2 concentrations. Alterations in plasma parameters (i.e., plasma cortisol, plasma ions, hematocrit, etc.) (Bernier and Randall, 1998; Iwama et al., 1989) and gene transcript expression (Dennis et

al., 2014; Rimoldi et al., 2009), have also been shown to be impacted by acute hypercarbia exposure. Heat shock protein transcripts are typically induced to maintain homeostasis within a cell by facilitating folding of nascent proteins, as well as repairing proteins damaged by various stressors (Iwama et al. 2004). Thus, elevated constitutive levels of erythrocyte *hsp70* transcripts likely conferred a greater ability for oxygen uptake and transport compared to control fish by maintaining erythrocyte functioning (Iwama et al., 2004; Wells, 2009), providing another potential mechanism for how CO_2-acclimated fish were able to display agitation responses at greater concentrations of CO_2 compared to naïve fishes. Previous research on common carp (*Cyprinus carpio*) has shown that blood acidosis, and subsequent reductions in brain pH, was the underlying cause for the anesthetic effect of hypercarbia (Yoshikawa et al., 1994). As such, CO_2-acclimated largemouth bass may have been able to withstand hypercarbia for a longer period of time before losing equilibrium relative to control fish due to elevations in plasma sodium and depressed plasma chloride concentrations observed following acute hypercarbia stress (i.e., through potential activation of ion pumps to regulate blood acid-base status) (Evans et al., 2005). Interestingly, the concentration of CO_2 that caused avoidance was identical for both groups of largemouth bass, suggesting that there is a potential CO_2 threshold at which fishes choose to swim away from elevated CO_2 zones, independent of acclimation capacity; however research with multiple CO_2 acclimation concentrations will be necessary to definitively address this phenomena. Therefore, the results of the current study clearly demonstrate that CO_2-acclimated fish have increased tolerance to additional hypercarbic stressors relative to naïve fish, however there was no difference in avoidance responses.

Burst swimming performance (i.e., time until exhaustion, swimming velocity) of CO_2-acclimated largemouth bass was not impacted by a prolonged exposure to 30 mg l^{-1} CO_2,

however CO_2-acclimated fish were able to maintain swimming performance following an acute hypercarbia stressor unlike naïve fish. Several studies have shown a depressed swimming performance following exposure to environmental stressors, such hypoxia (Herbert and Steffensen, 2005) and heavy metals (Rajotte and Couture, 2002). Previous research has shown that locomotory performance was not compromised by differing levels of hypercarbia exposure in European eel (*Anguilla Anguilla*) and Atlantic cod (McKenzie et al., 2003; Melzner et al., 2009), and the authors suggested that new blood acid-base equilibria driven by ion regulation, established to maintain blood pH and PO_2 levels, might prevent adverse effects on exercise performance by maintaining tissue oxygen supply. Dahlberg et al. (1968) subjected juvenile largemouth bass to 50 mg l^{-1} CO_2 and found that swimming speed was not impacted, however a similar exposure in juvenile coho salmon (*Oncorhynchus kisutch*) resulted in a depressed swimming performance. The physiological and molecular alterations (i.e., elevation of plasma sodium, decrease in plasma chloride) that occurred in largemouth bass following acclimation likely allow these fish to maintain swimming performance following exposure to 120 mg l^{-1} CO_2 compared to control fish. For example, reduced plasma chloride concentrations have been observed in many fish species following hypercarbia exposure (Fivelstad et al., 2003; McKenzie et al., 2003; Petochi et al., 2011) with the authors suggesting this was due to increased activity of Cl^-/HCO_3^- exchangers, which actively pumps HCO_3^- from the external environment in exchange for plasma Cl^- to restore blood pH to normal. While increased plasma sodium likely also contributes to plasma pH regulation, as the accumulation of plasma sodium may be due to increased activity of Na^+/H^+ exchanges in the gills, which actively pump hydrogen ions into the environment in exchange for sodium (Evans et al., 2005) increasing blood pH. The improved ability of CO_2-acclimated largemouth bass to regulate plasma anions and cations in response to

acute hypercarbia likely allows these organisms to maintain blood pH, and blood PO_2, compared to naïve largemouth bass, potentially providing a mechanism to explain how CO_2-acclimated fish maintained burst swimming speed following acute exposure to 120 mg l^{-1} CO_2 compared to control fish that experienced a severe reduction in burst swimming velocity.

Following an eight-week exposure to 30 mg l^{-1} CO_2, juvenile largemouth bass exhibited alterations in a variety of physiological parameters. More specifically, CO_2-acclimated largemouth bass had constitutively higher expression of *c-fos* erythrocyte mRNA, and *hsp70* gill and erythrocyte mRNA, compared to control fish. In addition, largemouth bass acclimated to elevated CO_2 showed plasma chloride concentrations reductions of approximately 5% relative to largemouth bass acclimated to ambient CO_2. Previous studies on a wide range of organisms have shown that prolonged exposure of individuals to altered environmental conditions can induce plastic changes to a number of different biological properties, a process commonly known as phenotypic plasticity (Chevin et al., 2010). For example, 5 weeks of exposure to heavy metals in two marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*) resulted in elevations in metal binding protein transcripts (Götze et al., 2014), and 7 weeks of exposure to hypoxia (3 mg l^{-1} O_2) resulted in an enhanced acute hypoxia stress response (e.g., increased hematocrit and hemoglobin) for largemouth bass (Gaulke et al., 2014). Previous research examining heat shock protein responses in two intertidal sculpins has shown that tidepool sculpin (*Oligocottus maculosus*), which reside in upper and lower tidepools, had higher constitutive Hsp70 levels compared to fluffy sculpin (*Oligocottus snyderi*), which reside exclusively in lower tidepools during low tide (Nakano and Iwama, 2002), likely permitting tidepool sculpin to maintain a higher capacity to repair proteins that may be damaged due to fluctuating temperatures. As such, the higher constitutive *hsp70* transcript abundance in largemouth bass

subjected to extended hypercarbia may allow these fish to minimize the negative impact of protein functioning that would occur due to hypercarbia. Once translated, the c-Fos protein regulates the expression of a multitude of genes in response to a specific stressor (Curran and Franza Jr., 1988; Kassahn et al., 2009), in this case chronic hypercarbia, all in an effort to maintain physiological homeostasis. Thus, a larger constitutive pool of *c-fos* transcripts in erythrocytes may allow cells to maintain proper functioning (i.e., oxygen delivery), which can become impaired under hypercarbia stress due to Root effects (Bernier and Randall, 1998). In addition, several studies have shown that plasma chloride decreases following acute and chronic hypercarbia stress (Fivelstad et al., 2003; McKenzie et al., 2003; Petochi et al., 2011), and these authors suggested this was due to increased activity of Cl^-/HCO_3^- ion exchangers to increase blood pH, potentially driven by elevated external CO_2 concentrations. Interestingly, it does not appear that largemouth bass in the chronic hypercarbia treatment were experiencing chronic stress, evidenced by baseline cortisol, glucose, and lactate concentrations that were similar between acclimation groups, along with no difference between lengths and weights in the two acclimation groups. Other studies that have acclimated fishes to an elevated CO_2 environment have shown similar results, such as Santos et al. (2013) and Petochi et al. (2011), who showed that European sea bass (*Dicentrarchus labrax*) acclimated for 45-60 days exposure to hypercarbia did not elevate plasma cortisol, glucose, or lactate compared to control fish. Together, results from the current study clearly demonstrate that juvenile largemouth bass have the capacity to acclimate to an elevated CO_2 environment by altering molecular and physiological parameters related to protein folding, ventilation control, and blood acid-base regulation.

Prolonged exposure to an elevated CO_2 environment altered the physiological responses of juvenile largemouth bass to an acute CO_2 challenge. More specifically, plasma glucose levels differed between acclimation groups following acute hypercarbia exposure, such that control fish had a greater induction of plasma glucose (5-fold) compared to CO_2-acclimated fish (3-fold). Three types of responses to an acute stressor are possible for individuals following extended exposure to a chronic stress, in this case long-term exposure to 30 mg l^{-1} CO_2: 1) an attenuated response, 2) an identical response, or 3) an exacerbated response (Barton, 2002). Previous studies comparing the acute stress response of chronically stressed vs. naïve populations have shown that responses can be varied, and can depend on the type/duration of stressor, and the species tested (Barton et al., 1985; Dickens and Romero, 2013; Norris et al., 1999; Pickering and Pottinger, 1987). The elevation of plasma glucose concentrations is a secondary stress parameter that fuels metabolically-active tissues (Barton, 2002; Wendelaar Bonga, 1997) suggesting that an acute hypercarbia stressor is not as energetically demanding for fish that have been previously acclimated to an elevated CO_2 environment. Interestingly, the molecular and physiological alterations shown in CO_2-acclimated fish following a 1 h exposure to 120 mg l^{-1} suggest that CO_2-acclimated largemouth bass were more tolerant (i.e., less stressed) relative to naïve fish. More specifically, CO_2-acclimated largemouth bass mounted a 70% greater induction of *hsp70* transcripts in erythrocytes following acute hypercarbia exposure relative control fish. Dalvi et al. (2012) showed a similar trend with catfish (*Horabagrus brachysoma*) acclimated at 30°C compared to 20°C, where Hsp70 levels were higher in 30°C acclimated fish compared to 20°C acclimated fish when exposed to an identical heat shock. The authors concluded that the elevated Hsp70 level in warm-acclimated fish resulted in fish that were more thermo-tolerant (i.e., later induction of Hsp70, greater induction at comparable heat stress) compared to cold-

acclimated fishes. Thus, the constitutive and resulting exacerbated *hsp70* erythrocyte transcript response following acute hypercarbia exposure may give CO_2-acclimated largemouth bass a higher capacity to repair cellular proteins, and potentially confer additional tolerance to hypercarbia compared to naïve fish. Attenuation of plasma cortisol and lactate following an acute hypercarbic stressor in CO_2-acclimated largemouth bass relative to control fish may 1) decrease the energetic cost of mounting a stress response (i.e., release of cortisol), which could then be reallocated for growth or reproduction, and 2) decrease the H^+ burden on the blood that can increase following release of lactate into the bloodstream (Bernier and Randall, 1998). Exacerbated hematocrit levels in CO_2-acclimated largemouth bass following acute exposure to hypercarbia likely improved oxygen uptake (Wells, 2009), while elevations in plasma sodium likely contributed to blood acid-base regulation, due to potential increases in Na^+/H^+ exchangers at the gills (Evans et al., 2005). Together, results from the current study clearly indicate that CO_2-acclimated largemouth bass had a molecular and physiological response to acute hypercarbia that was unique compared to ambient CO_2-acclimated fish, and these plastic changes potentially conferred greater tolerance to an acute hypercarbia stressor.

Juvenile largemouth bass from both acclimation groups exhibited several molecular and physiological changes across a number of stress pathways following an acute hypercarbia exposure. More specifically, largemouth bass *c-fos* gill transcripts were upregulated nearly 30-fold following exposure to 120 mg l^{-1} CO_2. In addition, plasma cortisol, glucose, lactate, and hematocrit increased in largemouth bass following exposure to acute hypercarbia, while plasma chloride concentrations decreased approximately 5% in fish exposed to 120 mg l^{-1} CO_2. Studies examining acute hypercarbia exposure in Atlantic salmon (*Salmo salar*) smolts and European sea bass show elevations in plasma cortisol levels representing the immediate response of these fish

to environmental change (Fivelstad et al., 1999; Petochi et al., 2011), and elevation of cortisol is commonly used as an indicator of stress in fishes suggesting that largemouth bass in this study were impacted by the acute hypercarbia exposure (Barton, 2002; Wendelaar Bonga, 1997). Secondary responses to stress, such as increasing plasma glucose and lactate concentrations (Barton, 2002; Wendelaar Bonga, 1997) have been observed in rainbow trout (Bernier and Randall, 1998) and largemouth bass (Kates et al., 2012) following acute hypercarbia exposure. Increasing plasma glucose concentrations can help fuel aerobic tissues, such as heart or gills that are under additional stress due to external and internal acidosis (Wendelaar Bonga, 1997), while elevations in plasma lactate concentrations may signal decreasing tissue oxygen supply as lactate is produced when tissues switch from aerobic to anaerobic metabolism (Bernier and Randall, 1998). Hematocrit concentrations have also been shown to increase approximately 30% following an acute exposure to 30 mg l^{-1} CO_2 for European sea bass and largemouth bass (Kates et al., 2012; Petochi et al., 2011), similar to the 12-25% increase observed in the current study, with the authors suggesting that increased hematocrit levels improves oxygen transport during acute hypercarbia exposure. Brauner et al. (2000), along with Petochi et al. (2011), also observed reductions in plasma Cl^- following hypercarbia exposure in Atlantic salmon and European sea bass, respectively, and the authors suggested this was due to elevated activity of Cl^-/HCO_3^- exchangers. In addition to these plasma parameters, *c-fos* gill transcripts were also induced following an acute exposure to 120 mg l^{-1} CO_2. Previous research has shown that *c-fos* transcripts are responsive to acute elevations in CO_2, and this response has been observed in variety of organism from rodents (Sato et al., 1992; Tankersley et al., 2002) to fishes (Rimoldi et al., 2009). Rimoldi et al. (2009) exposed European sea bass for 1 h at 70 mg l^{-1} CO_2 and found that brain *c-fos* transcripts doubled in hypercarbia treated fish compared to control fishes, and the

authors suggested this increased *c-fos* expression may be involved in the ventilatory response to hypercarbia. Therefore, the results of this current study clearly demonstrate that exposure of largemouth bass to 120 mg l^{-1} CO_2 for 1 h period caused alterations in molecular and physiological parameters, likely due to disruption in acid-base regulation.

Our results show that individual responses to CO_2 exposure prior to acclimation, measured during the 'initial' hypercarbia avoidance challenge, were not predictive of individual responses after acclimation. More specifically, initial and final measurements from individual largemouth bass were only correlated for the CO_2 concentration that induced agitation response, and there was no relationship between the initial and final measurements in the other metrics examined. Several recent laboratory studies have examined the role of individual variation in locomotory performance across taxa (Bennett et al., 1989; Kolok, 1992; Walsberg et al., 1986), and this variability has been shown to be repeatable in multiple fish species (Hanson et al., 2008; Kolok, 1992). For example, Killen (2014) determined that temperature preference for individual common minnow (*Phoxinus phoxinus*) could be reliably assayed multiple times in a one-week period using the 'shuttle box' choice arena. In the current study, only the CO_2 agitation response for both acclimation groups of largemouth bass was deemed to be repeatable following an eight-week acclimation period. For CO_2 agitation response, this means that largemouth bass that 'initially' tolerated a high CO_2 concentration before displaying an agitation response also required a high CO_2 concentration to display agitation following the eight-week acclimation. While the CO_2 agitation response for control fish was the same for the 'initial' and 'final' time periods, largemouth bass acclimated to elevated CO_2, as a group, required a greater concentration of CO_2 to elicit an agitation response. Thus, CO_2 agitation was a plastic behavioral trait that increased following hypercarbia acclimation, and the variation in individual responses was

maintained. For all other parameters measured during the hypercarbia avoidance trial, no repeatability was detected in these metrics between the 'initial' and 'final' measurements. A number of potential situations can lead to this result: 1) CO_2 avoidance/tolerance is not an inherent trait of individual fish, such that the genetic make-up of the individual does not determine the outcome of this trait, 2) accumulated hypercarbia exposure during the challenge interferes with fish behavior, or 3) eight-week acclimation period altered the behavior of fish. While repeatability of these behavioral metrics in individual fish was low, the overall difference in group means between CO_2-acclimated and naïve fish was less than the variability in individual responses, suggesting that the majority of fish will respond to CO_2, and not just a sub-population, which has important connotations for how populations may respond to elevated CO_2 environments.

This study can provide insight into how aquatic organisms might acclimate to chronic hypercarbic conditions and continue to thrive. Elevated dissolved CO_2 concentrations occur naturally in both freshwater and marine environments, especially in estuarine waters, coastal upwelling zones (Feely et al., 2008; Thomsen et al., 2010), while global climate change, resulting in increasing water temperature, elevated pCO_2, and decreased pH levels, is of particular ecological and economic concern (Cooley and Doney, 2009; Raven et al., 2005). Should fishes be exposed to elevated concentrations of carbon dioxide for extended periods, results from the current study suggest they would experience alterations in molecular (e.g., elevated *c-fos* and *hsp70* transcripts) and physiological (e.g., reductions of plasma chloride) parameters, which could result in free-swimming fishes that display improved tolerance (e.g., reduced stress response, greater CO_2 concentrations necessary to induce behavioral responses) and performance (e.g., sustained burst swimming speed) within hypercarbic environments.

While the acclimation CO_2 concentration (≈ 21000 μatm) used in the current study is approximately 10 times greater than the expected rise in CO_2 levels predicted by the year 2300 (Caldeira and Wickett, 2003), the concentration of CO_2 used in the current study could still be useful to researchers looking into the acclimation capacity of fishes to hypercarbic environments, and the ecologically significant physiological and behavioral alterations documented in this study can be useful for researchers studying the impact and acclimation capacity of highly plastic fishes to chronic hypercarbia exposure. In addition to elevated CO_2 resulting from climate change, hypercarbic conditions in aquaculture can be created by an overabundance of fish within tanks (Colt and Orwicz, 1991; Kristensen et al., 2009), and can be a series issue for fish farmers and aquaculture managers. For example, chronic exposure to 20 mg l^{-1} CO_2 in Atlantic salmon smolts can result in reduced condition and health decreasing the sale price of these fish (Fivelstad et al., 1999). Hatchery fish may also be maladapted for release into natural environments, if these fish acclimate to elevated CO_2 conditions within the hatchery, compounding negative impacts that hatchery fish can have on natural fish populations (Araki and Schmid, 2010). In addition, the potential for carbon dioxide to influence the movement of free-swimming fishes has been examined (Clingerman et al., 2007; Kates et al., 2012). In particular, Kates et al. (2012) demonstrated that elevated carbon dioxide influenced the movement of invasive Asian carp (*Hypophthalmichthys* sp.) which showed potential for a field-implemented CO_2 chemical barrier to deter the movement of invasive fishes. While the results of this study show that fish have the capacity to acclimate to elevated CO_2 environments, CO_2-acclimated fish still choose to avoid areas of high CO_2 at concentrations near 200 mg l^{-1} and eventually succumbed to the anesthetic effects of hypercarbia exposure suggesting that a CO_2 barrier still has potential to influence movement of fishes acclimated to hypercarbia. As hypercarbic

environments become more prevalent due to global climate change, knowledge on the hypercarbia acclimation capacity of aquatic organisms and the resulting impact on physiological and behavioral traits will be of vital importance for conservation managers.

Materials and methods

Experimental Animals

Juvenile largemouth bass were acquired from Logan Hollow Fish Farm (Murphysboro, IL, USA) and delivered to the University of Illinois Aquatic Research Facility (Champaign, IL, USA) on September 8, 2013. Upon arrival at the research facility, fish were housed outdoors in round plastic tanks (1280 l, 1.7 m diameter) supplied with water from a 0.04 ha natural, earthen-pond with abundant vegetation. Juvenile largemouth bass were fed pelleted food (Dense Culture Food, F2A, Pentair Aquatic Eco-Systems, Apopka, FL, USA) until satiation every other day and solid waste was removed via siphoning. Fish were held for three weeks prior to start of the eight-week acclimation treatment. Across this initial outdoor holding period, water temperatures averaged 21.7°C (± 0.4°C, standard error, SE) and dissolved oxygen averaged 8.0 mg l^{-1} (± 0.1 mg l^{-1}).

Acclimation treatments

Following the three-week outdoor holding period, largemouth bass were carefully netted from the holding tank, measured (total length in mm), weighed to the nearest tenth gram, and transferred, at random, to one of two indoor acclimation tanks: ambient CO_2 (L) or high CO_2 (H). The L tank was aerated continuously with air stones attached to a compressed air blower, and a fountain pump in the tank ensured adequate water mixing. Solid wastes were removed

from this tank by siphoning every other day, while nitrogenous waste removal was achieved by pumping water through a container of activated carbon and complete replacement of water every six days. Ammonia levels during the acclimation period did not exceed 1 ppm (mean ammonia = 0.2 ± 0.01 ppm) (kit #3351-02, LaMotte Company, Chestertown, MD, USA). Water quality measurements were taken daily, and included temperature, dissolved oxygen (YSI 550A, Yellow Springs Instruments, Irvine, CA, USA), and pH (WTW pH 3310 meter with a SenTix 41 probe, Germany). Dissolved CO_2 and total alkalinity (kit #2272700 and 2271900, Hach Company, Loveland, CO, USA) were quantified using a digital titrator (model 16900, Hach Company, Loveland, CO, USA). Dissolved CO_2 concentrations were 13 mg l^{-1} (± 0.2 mg l^{-1}) in the L tank. The H tank held fish at 31 mg l^{-1} CO_2 (± 0.6 mg l^{-1}) by bubbling compressed CO_2 gas into the tank. Kates et al. (2012) showed that acute exposure to 30 mg l^{-1} CO_2 was sufficient to induce physiological alterations in adult largemouth bass without impacting ventilation rate or irregular activities (i.e., surface ventilations, coughing, loss of equilibrium), as such the CO_2 concentration used in the H treatment was chosen to maximize the likelihood of molecular/physiological acclimation. CO_2 concentrations were held constant using Pinpoint® pH controller (Pentair Aquatic Eco-Systems, Apopka, FL, USA), which regulated pH/CO_2 levels using a pH probe connected to a solenoid valve attached to a tank of compressed CO_2 gas. Dissolved CO_2 concentrations were measured daily to confirm target CO_2 concentrations were achieved. Aeration through the use of a blower was maintained in the H tank to ensure that fish were not subjected to hypoxia (8.5 ± 0.1 mg l^{-1} O_2). Each acclimation tank was held at their respective dissolved CO_2 concentration for 58 days, as previous research has shown 6-8 weeks was sufficient to induce plastic physiological changes to elevated CO_2 (Deigweiher et al., 2008; Fivelstad et al., 2003). Mean water quality measurements taken from the acclimation tanks are

presented in Table 3.1. Values for pCO_2 were calculated using the program CO2calc (version 1.2.0, U.S. Geological Survey, Reston, VA, USA) using water temperature, pH, and total alkalinity measurements (Robbins et al., 2010). The amount of time required to reach carbonate chemistry equilibrium within an open system can take as long as a few days (Riebesell et al., 2010), and, with the volume and rate of CO_2 gas addition to the holding tanks, it was challenging to generate accurate pCO_2 data. As such, the digital titrator was used at the main tool to quantify dissolved CO_2, and was used to standardize CO_2 treatments between experiments. While pCO_2 values are presented in Table 3.1, these data should be interpreted cautiously as water chemistry may not have been at equilibrium at the time of measurement. Temperature in the H tank (15.4°C ± 0.2°C) was not statistically different than the L tank (15.1°C ± 0.2°C) during the 58-day acclimation period (t-test, $t > 0.28$). Mean size of largemouth bass at the beginning of the acclimation period were not statistically different across treatments: (L: 148 mm ± 1.0 mm; H: 148 mm ± 0.7 mm) (t-test, $t > 0.62$, $P > 0.05$). Fish were withheld from supplemental food for at least 48 h prior to experimentation to ensure food digestion would not impact molecular, physiological, or performance metrics.

Acute Hypercarbia Challenge

Following 58 days of acclimation, largemouth bass from both treatments were subjected to an acute hypercarbia challenge. Fish were carefully netted from the acclimation tanks and placed into individual opaque, sensory deprived containers (4.0 l per largemouth bass) continuously supplied with fresh water from a central basin. Water was allowed to overflow from each container and drain back into the central basin, creating a closed, recirculating system (Kates et al., 2012). The containers were sized appropriately, contained an airstone for aeration,

and were outfitted with a tight-fitting lid to ensure that fish could not escape during the challenge. Fish were acclimated to their containers for 24 h, and dissolved oxygen concentrations remained at 9.3 ± 0.1 mg l^{-1}. Largemouth bass collected from the L tank were supplied with water at ambient CO_2 conditions (14 ± 0.8 mg l^{-1} CO_2) during this 24 h period, while fish collected from to the H tank were supplied with water at 32 ± 0.6 mg l^{-1} CO_2. This experimental design (i.e., maintaining acclimation water quality conditions for baseline values) is similar to previous studies investigating the effect of extended holding in different water conditions on fish (Iwama and Heisler, 1991; Logan and Somero, 2011; Melzner et al., 2009). Following this 24 h period, each container was randomly assigned to one of two treatments: 1) 1 h exposure to acclimation CO_2 concentrations (i.e., control) or 2) 1 h exposure to 120 mg l^{-1} CO_2. Concentrations of CO_2 concentrations of 120 mg l^{-1} and below have previously been shown to induce behavioral (i.e., hypercarbia agitation and avoidance responses), reflex (i.e., ventilation and irregular activities), and physiological alterations for adult largemouth bass (Kates et al., 2012), and the acute hypercarbia exposure was expected to induce a molecular and physiological response in juvenile largemouth bass. Acute hypercarbia was achieved within 2 min by bubbling CO_2 gas into the central basin and then pumping this water into specific containers (Kates et al., 2012). Dissolved CO_2 concentrations, presented in Table 3.1, were verified using a CO_2 digital titrator using water samples taken from an extra test chamber within the system. Aeration was maintained throughout the trial to ensure fish were not exposed to hypoxic conditions (8.4 ± 0.1 mg l^{-1} O_2). Fish exposed to ambient CO_2 concentrations (i.e., control treatment) remained undisturbed in their containers with no manipulation in water chemistry. At the conclusion of the acute hypercarbia challenge, water flow was ceased, and test subjects were euthanized by an

overdose of anesthetic [250 mg l^{-1} tricaine methanesulphonate (MS-222) buffered with 500 mg l^{-1} sodium bicarbonate].

Following cessation of ventilation, fish were measured, weighed, and blood was drawn from the caudal vasculature using a 22-gauge needle and 1 ml syringe rinsed with lithium heparin. To quantify hematocrit, whole blood was transferred to two 75 mm microhematocrit tubes (Drummond Scientific, Broomall, PA, USA) and spun for 2 min at 4400 × gravity (*g*) in a microhematocrit centrifuge (LW Scientific Zippocrit, Atlanta, GA). The remaining whole blood was centrifuged for 2 min at 2000 *g* to separate red blood cells from plasma. Plasma was transferred to 1.5 ml microcentrifuge tubes, and then plasma and red blood cells were immediately stored in liquid nitrogen. Gill filaments, hereafter referred to as gill tissue, were excised and stored in a 1.5 ml microcentrifuge tube filled with 1 ml of RNAlater® (AM7021, Life Technologies, Grand Island, NY, USA). Tissue samples were refrigerated for 1-7 days and then stored at -80°C. Water quality measurements were collected at the conclusion of each test subject's challenge to confirm that proper water chemistry conditions were achieved during the challenge, and are presented in Table 3.1. Mean size of largemouth bass subjected to the acute hypercarbia challenge were not statistically different across treatments: (LC: 159 mm ± 2 mm; LS: 157 mm ± 2 mm; HC: 156 mm ± 2 mm; HS: 157 mm ± 1 mm) (one-way analysis of variance (ANOVA), $F < 0.56$, $P > 0.64$).

Hypercarbia Avoidance Challenge

Prior to the eight-week acclimation, 24 largemouth bass were implanted with a passive integrated transponder tag (PIT tag) (TX1411SSL, VeriTeQ, Delray Beach, FL, USA) and allowed to recover for one week. Following this recovery period, individuals were subjected to

an ‘initial’ hypercarbia avoidance challenge. Quantification of hypercarbia agitation and avoidance parameters was performed using a ‘shuttle box’ choice arena (Loligo Inc., Hobro, Denmark) (Serrano et al., 2010). Kates et al. (2012) provide a description of the ‘shuttle box’ choice arena, along with a general protocol for the hypercarbia avoidance challenge. Briefly, the hypercarbia avoidance challenge began by carefully netting a PIT tagged fish, confirmed through the use of a handheld electronic PIT tag reader (Pocket Reader EX, Biomark, Inc., Boise, ID, USA), and randomly placing the fish into one of the two ‘shuttle box’ holding tanks. Individuals were allowed 2 h to acclimate to the ‘shuttle box’. Upon completion of the acclimation period, the buffer chamber associated with the holding tank that contained the fish received a continuous addition of CO_2, while the buffer chamber associated with the holding tank that did not contain the fish received a continuous addition of compressed air to strip CO_2 from the water. During the addition of CO_2, the time was recorded when the fish became agitated (i.e., surface ventilations, twitching, elevated/erratic swimming), shuttled to the opposite holding tank via the tunnel, or lost equilibrium. Concurrently, water quality measurements were taken from water flowing into the buffer chamber. If/when the fish shuttled to the opposite tank, both buffer chambers received compressed air for 10 min to strip CO_2 from the water, verified through the use of the digital titrator. After this 10 min period, a continuous addition of CO_2 was applied to the buffer chamber associated with the holding tank that the fish had settled. This process was repeated until the fish shuttled six times (or until the subject lost equilibrium), typically resulting in multiple measurements for each individual. At the conclusion of the hypercarbia avoidance challenge, fish were removed from the system to be weighed and measured, and were then gently placed into one of the indoor holding tanks. PIT tagged largemouth bass were then randomly assigned into each of the indoor acclimation tanks. Mean size of largemouth bass subjected to

the ‘initial’ hypercarbia avoidance challenge were not statistically different across acclimation groups: (L: 141 mm ± 2 mm; H: 140 mm ± 1 mm) (t-test, $t > 0.77$, $P > 0.05$).

Following the 58 day acclimation period, individual PIT tagged largemouth bass were randomly selected and subjected to a ‘final’ hypercarbia avoidance challenge. The ‘final’ hypercarbia avoidance challenge was identical to the ‘initial’ hypercarbia avoidance challenge, and fish ID # was read prior to the ‘final’ treatment to identify individuals allowing inter-trial comparisons. Initial water quality parameters during the 2 h acclimation period for fish subjected to the hypercarbia avoidance challenge are presented in Table 3.1. Mean size of largemouth bass subjected to the ‘final’ hypercarbia avoidance challenge were not statistically different across acclimation groups: (L: 153 mm ± 2 mm; H: 150 mm ± 1 mm) (t-test, $t > 0.23$, $P > 0.05$).

Swim Performance Challenge

Following the eight-week acclimation, largemouth bass were subjected to a burst swimming performance challenge, which was quantified using a swim tunnel respirometer (SW10160, Loligo Inc., Hobro, Denmark) (Melzner et al., 2009). Individual fish, randomly selected from the acclimation tanks, were weighed and measured (total length, width, and depth in mm), and then gently placed into the swimming chamber. Fish were allowed to acclimate for 2 h at a water velocity of 0.5 body lengths per second (Gregory and Wood, 1998). Ten minutes prior to the conclusion of the acclimation period, the ‘flush’ pump, which circulates water from the respirometer to the water bath, was turned off to isolate the swimming chamber from the external water bath. The individual was then randomly assigned to one of two swim performance challenges: 1) swim challenge at acclimation CO_2 or 2) swim challenge at 120 mg l^-

[1] CO_2. Hypercarbic conditions were achieved within 2 min by bubbling compressed CO_2 gas into the water bath. The 'flush' pump was activated 5 min later to expose the test subject to hypercarbia, and target dissolved CO_2 concentrations were verified using the CO_2 digital titrator. Fish subjected to ambient CO_2 concentrations were experience an identical procedure, except the addition of CO_2. Following the 2 h acclimation period, water velocity was steadily increased at a rate of 10 cm s^{-1} min^{-1} (Reidy et al., 1995), resulting in a burst swimming challenge. The burst swimming challenge was considered complete when the test subject became impinged on the back of the swimming chamber for 10 s, at which point the time and water velocity was recorded. Upon conclusion of the swim performance challenge, individuals were euthanized and water quality measurements were taken from the external water bath, and are presented in Table 3.1. Mean size of largemouth bass subjected to the burst swimming challenge were not statistically different across treatments: (LC: 161 mm ± 2 mm; LS: 158 mm ± 1 mm; HC: 160 mm ± 2 mm; HS: 158 mm ± 2 mm) (one-way ANOVA, $F < 0.66$, $P > 0.58$).

Quantification of Physiological Parameters

Plasma cortisol was quantified using a commercially available kit (ADI-900-071, Enzo Life Sciences Inc., Farmingdale, NY, USA). Plasma sodium concentrations were determined using a flame photometer (model 2655-00, Cole-Palmer Instrument Company, Chicago, IL, USA), while plasma chloride concentrations were quantified using a chloridometer (model 4435000, Lab-conco Corporation, Kansas City, MO, USA). Following the methods of Lowry and Passonneau (1972), plasma lactate and glucose concentrations were determined enzymatically in a 96-well microplate analyzed with a commercially available spectrophotometer (Spectra Max Plus 384, model No. 05362, Molecular Devices, Union City, CA, USA).

Quantification of Molecular Parameters

All tissue samples, submerged in 1 ml of TRI Reagent (Ambion, Life Technologies, Grand Island, NY, USA), were homogenized for 1 min using a mechanical homogenizer (Tissue-Tearor®, model No. 935370, Biospec Products Inc., Bartlesville, OK, USA). Total RNA from red blood cells, hereafter referred to as erythrocytes, were isolated using an Ambion RiboPure Blood Kit (AM1928, Life Technologies, Grand Island, NY, USA) with the following modifications to the protocol to maximize RNA integrity and quantity: 1) erythrocytes were thawed on ice, as RNAlater® was not utilized prior to storage in liquid nitrogen, and 2) Ambion DNase (AM1906, Life Technologies, Grand Island, NY, USA) was applied to extracted RNA to eliminate any genomic DNA. Total RNA from gill tissue was isolated and extracted, using an Ambion RiboPure Kit (AM1924, Life Technologies, Grand Island, NY, USA), and then Ambion DNase was applied to remove any remaining genomic DNA. Following DNase treatment, a Nanodrop ND-1000 UV-Vis spectrophotometer (Peqlab, Erlangen, Germany) was used to quantify yield and purity of the extracted RNA. RNA integrity was confirmed using gel electrophoresis. Extracted RNA was frozen at -80°C. Synthesis of cDNA was accomplished using a High-Capacity cDNA Reverse Transcription kit (ABI No. 4374966, Life Technologies, Grand Island, NY, USA), such that 2 µg of total RNA was present in a reaction volume of 20 µl. An Eppendorf Mastercycler® Pro thermal cycler (Eppendorf, Hamburg, Germany) was used to run the following cDNA synthesis reaction: 1) 10 min at 25°C to activate enzymes, 2) 2 h at 37°C for incubation, and 3) 5 min at 85°C to denature enzymes. All cDNA was then stored at -20°C.

Juvenile largemouth bass qPCR primer sequences, melting temperature and fragment length information are provided in Table 3.2. All qPCR reactions were performed using 1 µl of stock cDNA (diluted 1:25 using RNase-free water), 1 µl of each qPCR primer pair (1 µM concentration), 2 µl of RNase-free water, and 5 µl of RealMasterMix™ Fast SYBR ROX kit (No. 2200840, 5 PRIME Inc., Gaithersburg, MD, USA). An ABI 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) was utilized to conduct gene expression analyses using the following protocol: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, followed by 40 cycles at 1) 95°C for 15 s and 2) 60°C for 1 min. After the completion of these 40 cycles, all qPCR products underwent a melt curve analysis (1 cycle at 95°C for 15 s, 1 cycle at 60°C for 15 s, and 1 cycle at 95°C for 15 s) to confirm the presence of a single amplicon. Relative standard curves for the reference (*18s*) and all target (*c-fos* and *hsp70*) genes were created using several, highly induced samples to compare cDNA concentration to threshold cycle for each qPCR primer pair. Relative cDNA concentration was normalized using *18s*, as mRNA concentrations of this reference gene remained constant across all treatments (ANOVA $P > 0.05$). To detect potential genomic DNA contamination, an identical qPCR analysis was performed on extracted RNA that had not been reverse-transcribed. Genomic DNA contamination was determined to be negligible if 1) at least 5 Cts difference was observed between RT-positive and RT-negative samples (Mancebo et al., 2013), and 2) RT-negative and NTC samples were outside the detection limit of the standard curve (Lewis et al., 2010).

Statistical Analysis

Comparisons of physiological parameters in largemouth bass exposed to an acute hypercarbia challenge were performed using a two-way analysis of variance (ANOVA) with

acclimation (H and L), acute exposure (acclimation CO_2 and 120 mg l^{-1} CO_2), and their interaction (acclimation × acute exposure) entered as fixed effects. If the interaction term was significant, or if any of the main effects were significant, a Tukey-Kramer honestly significant differences (HSD) post-hoc test was applied to separate means (Sokal and Rohlf, 1995). Comparisons of stress gene expression in the gills and erythrocytes of largemouth bass exposed to an acute hypercarbia challenge were also made using a two-way ANOVA with acclimation, acute exposure, and their interaction entered as fixed effects. A Tukey-Kramer HSD post-hoc test was applied to separate means where appropriate (Sokal and Rohlf, 1995).

Comparisons of CO_2 agitation and avoidance responses (i.e. CO_2 concentration necessary to induce agitation or avoidance responses, total time spent in elevated CO_2 zone, and total number of shuttles) of largemouth bass subjected to the hypercarbia avoidance challenge were performed using a two-way repeated measures ANOVA with test period (initial or final), acclimation, and their interaction (test period × acclimation) entered as fixed effect, while fish identification number was entered as a random effect to account for multiple measurements collected from each individual. A Tukey-Kramer HSD post-hoc test was again used to separate means where appropriate (Sokal and Rohlf, 1995). To determine the repeatability of CO_2 agitation and avoidance responses of individual fish, Spearman's rank correlation tests were conducted on the different test periods, with acclimation groups being analyzed separately (Zar, 1984).

Comparisons of burst swimming performance (i.e., final velocity and time until exhaustion) in largemouth bass exposed to an acute hypercarbia challenge were made using a two-way ANOVA with acclimation, acute exposure, and their interaction entered as fixed

effects, followed by a Tukey-Kramer HSD post-hoc test to separate means (Sokal and Rohlf, 1995).

For all experiments, data were log transformed, if necessary, to meet assumptions of normality and homogeneity of variances (Zar, 1984). A visual analysis of fitted residuals, using a normal probability plot (Anscombe and Tukey, 1963), was used to assess normality, while Hartley's F_{max} test (Hartley, 1950), combined with visual inspection of the distribution of fitted residuals, were used to assess homogeneity of variances. A two-way Kruskal-Wallis test (Sokal and Rohlf, 1995; Zar, 1984) was performed in lieu of a two-way ANOVA if either normality or homogeneity of variance assumptions were violated. If the interaction term, or any of the main effects, were significant, a Steel-Dwass all-pairs multiple comparison test was applied to separate means (Douglas and Michael, 1991). All means are reported ± SE where appropriate. Two-way Kruskal-Wallis test calculation and analyses were performed by hand using Zar (1984), as a template, while all other statistical analyses were performed using JMP version 9.0.2 (SAS Institute Inc., Cary, NC, USA). All tests were run at a significance level (α) of 0.05.

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CHAPTER 4: GENERAL CONCLUSIONS

Understanding the effects of hypercarbia on aquatic organisms is becoming an increasingly important topic, both ecologically and economically, as global climate change makes hypercarbic environments more prevalent. On a smaller scale, aquaculture practices can also subject fishes to elevated CO_2 environments, which can influence the business aspect of the fishery (e.g., lower sale price) and potentially impacts the success of stocking and rehabilitating wild fish populations with hatchery fish. While aquatic hypercarbia is traditionally viewed under negative connotations, few research groups have attempted to utilize the well-studied impacts of hypercarbia exposure on fish physiology and behavior to solve applied natural resources problems. Recently, our lab group has been exploring the potential application of an elevated chemical deterrent to prevent the spread of invasive Asian carp into the Great Lakes. The two separate, yet complementary studies described in this thesis provides insight into the efficacy of a CO_2 chemical deterrent for Asian carp by examining two potential caveats that could decrease the usefulness of the barrier: 1) are small fishes behaviorally and physiologically impacted by acute exposure to hypercarbic environments; and 2) are fishes able to acclimate to elevated CO_2 environments and does this acclimation benefit animal performance or tolerance to acute hypercarbia exposure.

The first data chapter of this thesis demonstrated that larval and juvenile Asian carp were impacted by acute hypercarbia exposure, and responses were similar to adult Asian carp suggesting that a CO_2 barrier has potential to impede the movement of juvenile and adult fishes. Given that the electrical barrier is potentially susceptible to small fishes, the utility of a CO_2 chemical deterrent to supplement the existing barrier shows promise. This first study also provided insight into some of the molecular alterations that occur following acute hypercarbia

exposure, and displayed any differences among species and life-stages which could be of interest to researchers examining ontogenetic or interspecies comparisons following hypercarbia exposure.

The second data chapter of the thesis demonstrated that largemouth bass were able to induce plastic molecular and physiological alterations following chronic hypercarbia exposure and were relatively more hypercarbia tolerant (i.e., lower stress response following acute hypercarbia challenge, swimming performance not impacted by acute hypercarbia stressor, and required greater CO_2 concentrations to become agitated). In terms of a CO_2 barrier, fish may choose to remain nearby the barrier in sub-optimal environmental conditions (i.e., elevated CO_2) due to preferred habitat (e.g., refuge, spawning ground). While results of this study show that CO_2 acclimated fish were more tolerant to additional hypercarbia exposure compared to naïve fish, CO_2 acclimated fish still succumbed to the anesthetic effect of hypercarbia and choose to swim away from elevated CO_2 at the same concentration as naïve fish suggesting that a CO_2 barrier may be effective long-term. This study also serves as a starting-point for future research on the acclimation capacity of freshwater fishes, as global climate change will make chronic hypercarbic environments more prevalent in the future. Aquaculture managers may also be able to determine the impact of chronic hypercarbia exposure on largemouth bass on ecologically important metrics, such as behavior and swimming performance, which potentially makes these hatchery fish maladaptive for release into wild populations.

Collectively, the results of these two studies present a holistic view of the impacts of acute and chronic hypercarbia exposure in juvenile fishes ranging from genes to whole-animal performance. I also recommend that researchers examining global climate change, ocean acidification, or other types of irreversible chronic stressors, perform experiments that link

physiology, performance, and population-level metrics in order to better understand how individual animals can cope with the stressor and how whole populations of fish will respond.

TABLES AND FIGURES

Table 2.1. Water quality measurements at the conclusion of the hypercarbia challenge for both fry and juvenile fishes. Water quality measurements were taken from three separate locations: Osage Beach, MO; Urbana, IL; and La Crosse, WI. At Osage Beach, bighead carp (BHC) and silver carp (SLC) eight day-old fry were subjected to a hypercarbia challenge and final water quality measurements are presented in the table. Juvenile largemouth bass (LMB) and bluegill (BLG) were subjected to a similar hypercarbia challenge at the Aquatic Research Facility in Urbana, IL. Experiments on juvenile SLC and BHC occurred at the Upper Midwest Environmental Science Center in La Crosse, WI. Water quality for the six treatments used in the hypercarbia challenge are presented in the table as follows: C1 – ambient CO_2 exposure for 30 min; C2 – ambient CO_2 exposure for 60 min; L1 – 30 min exposure to 70 mg/L CO_2; L2 – 60 min exposure to 70 mg/L CO_2; H1 – 30 min exposure to 120 mg/L CO_2; and H2 – 60 min exposure to 120 mg/L CO_2.

Location	Species	Life Stage	Final Temp (°C)	Final Dissolved Oxygen (mg/L)	Final pH	Final Total Alkalinity (mg/L)	Final dissolved CO2 (mg/L)	Final pCO2 (µatm)
Osage Beach, MO	BHC SLC	Fry	24.8 ± 0.01	7.6 ± 0.02	C1 – 8.13 ± 0.03	C1 – 246 ± 3	C1 – 40 ± 1	C1 – 2454 ± 170
					C2 – 8.16 ± 0.01	C2 – 244 ± 4	C2 – 40 ± 1	C2 – 2177 ± 55
					L1 – 7.45 ± 0.02	L1 – 242 ± 2	L1 – 77 ± 1	L1 – 11499 ± 545
					L2 – 7.39 ± 0.01	L2 – 241 ± 3	L2 – 74 ± 0.4	L2 – 13100 ± 373
					H1 – 6.88 ± 0.01	H1 – 243 ± 3	H1 – 123 ± 2	H1 – 42266 ± 460
					H2 – 6.86 ± 0.01	H2 – 241 ± 3	H2 – 123 ± 1	H2 – 43567 ± 400
Urbana, IL	LMB BLG	Juveniles	17.0 ± 0.1	8.9 ± 0.05	C1 – 8.28 ± 0.02	C1 – 148 ± 2	C1 – 15 ± 0.2	C1 – 919 ± 40
					C2 – 8.26 ± 0.01	C2 – 147 ± 2	C2 – 15 ± 0.2	C2 – 961 ± 40
					L1 – 6.64 ± 0.01	L1 – 153 ± 2	L1 – 74 ± 1	L1 – 41827 ± 939
					L2 – 6.64 ± 0.01	L2 – 159 ± 2	L2 – 73 ± 1	L2 – 43181 ± 1134
					H1 – 6.45 ± 0.01	H1 – 145 ± 3	H1 – 112 ± 1	H1 – 61967 ± 2241
					H2 – 6.44 ± 0.01	H2 – 150 ± 2	H2 – 115 ± 1	H2 – 65370 ± 1734
La Crosse, WI	SLC BHC	Juveniles	16.0 ± 0.03	8.4 ± 0.04	C1 – 8.37 ± 0.02	C1 – 156 ± 3	C1 – 15 ± 1	C1 – 782 ± 26
					C2 – 8.38 ± 0.01	C2 – 153 ± 3	C2 – 15 ± 1	C2 – 747 ± 22
					L1 – 6.41 ± 0.01	L1 – 161 ± 3	L1 – 72 ± 1	L1 – 73127 ± 1243
					L2 – 6.41 ± 0.01	L2 – 162 ± 2	L2 – 70 ± 1	L2 – 74270 ± 1023
					H1 – 6.00 ± 0.01	H1 – 161 ± 2	H1 – 122 ± 1	H1 – 188993 ± 2745
					H2 – 6.02 ± 0.01	H2 – 166 ± 1	H2 – 120 ± 1	H2 – 186435 ± 2978

Table 2.2. Quantitative real-time PCR primer sets for silver carp, bighead carp, largemouth bass, and bluegill. Sequence, melting temperature, and fragment length information for each primer pair is presented in the table.

Species	Gene	Sequence 5′ → 3′	Melting Temperature	Fragment Length (bp)
Silver Carp	*c-fos*	*F:* CTGTTTTCCAGCATGCCCTC	63	126
		R: GACAGAGCGAGCAGTTTCCA	62	
	hif1-α	*F:* CTCTGACCTACCTTGTGCTC	55	187
		R: GTATTCGTACACCGACTTGT	55	
	gr-2	*F:* AGAAGCCTGTCTTTAGCGTG	57	109
		R: CATTCGCTGGCCCTCTGTTG	66	
	hsp70	*F:* GAACCCTCTCCTCCAGCTCT	60	160
		R: ATCTTGGCGTCTCTCAAGGC	60	
	18s	*F:* ACCACGAGTCTTTGGGTTCC	61	129
		R: GTCAATCCTTTCCGTGTCC	58	
Bighead Carp	*c-fos*	*F:* CTGTTTTCCAGCATGCCCTC	63	126
		R: GACAGAGCGAGCAGTTTCCA	62	
	hif1-α	*F:* CTCTGACCTACCTTGTGCTC	55	187
		R: GTATTCGTACACCGACTTGT	55	
	gr-2	*F:* AGAAGCCTGTCTTTAGCGTG	57	109
		R: CATTCGCTGGCCCTCTGTTG	66	
	hsp70	*F:* GAACCCTCTCCTCCAGCTCT	60	160
		R: ATCTTGGCGTCTCTCAAGGC	60	
	18s	*F:* ACCACGAGTCTTTGGGTTCC	61	129
		R: GTCAATCCTTTCCGTGTCC	58	
Largemouth Bass	*c-fos*	*F:* GTCTCCATTCCTCCTGTCCA	59	113
		R: GGTTGTGGTGAAGGTTGAC	57	
	hif1-α	*F:* CACACTGAGCAGACTCCCAAC	60	115
		R: AAGGTTTTGGTGTCCAGAGG	58	
	gr-2	*F:* TGCCGCTTCAGGAAATGTC	59	114
		R: GCTGCTGATAGGCTCTGATG	58	
	hsp70	*F:* ACTGATTGGGAGAAAGCTGG	59	136
		R: CCTCTGGGCTGAAGGTTTTG	60	
	18s	*F:* TTATTCCCATGACCCGCCG	62	156
		R: GGTGAGGTTTCCCGTGTTGA	62	
Bluegill	*c-fos*	*F:* GTCTCCATTCCTCCTGTCCA	59	113
		R: GGTTGTGGTGAAGGTTGAC	57	
	hif1-α	*F:* CACACTGAGCAGACTCCCAAC	60	115
		R: AAGGTTTTGGTGTCCAGAGG	58	
	gr-2	*F:* TGCCGCTTCAGGAAATGTC	59	114
		R: GCTGCTGATAGGCTCTGATG	58	
	hsp70	*F:* CAAAGGGGAGGACAAAACC	57	138
		R: GAGTCGTTGAAGTACGCCG	59	
	ef1-α	*F:* TGGAGACAGCAAGAACGACC	60	128
		R: CAATGTGAGCAGTGTGGCAG	60	

Table 2.3. Relative gene expression values from bighead carp and silver carp fry exposed to two concentrations and durations of elevated CO_2. The treatments used are as follows: C1 – ambient CO_2 exposure for 30 min; C2 – ambient CO_2 exposure for 60 min; L1 – 30 min exposure to 70 mg/L CO_2; L2 – 60 min exposure to 70 mg/L CO_2; H1 – 30 min exposure to 120 mg/L CO_2; and H2 – 60 min exposure to 120 mg/L CO_2. Dissimilar characters (+, †) denote statistically significant differences within a species for fry exposed for 30 min compared to 60 min. Results of statistical analyses are presented in Table 2.4.

Gene	C1	L1	H1	C2	L2	H2
			Bighead Carp Fry			
c-fos	1.00 ± 0.26	0.85 ± 0.15	0.98 ± 0.11	1.00 ± 0.17	0.61 ± 0.21	1.27 ± 0.29
gr-2	1.00 ± 0.10	0.77 ± 0.06	0.72 ± 0.10	1.00 ± 0.08	0.86 ± 0.04	0.80 ± 0.05
hif1-α	1.00 ± 0.07	0.84 ± 0.04	1.05 ± 0.14	1.00 ± 0.04	0.94 ± 0.09	0.90 ± 0.08
			Silver Carp Fry			
c-fos	1.00 ± 0.11	0.70 ± 0.25	1.05 ± 0.19	1.00 ± 0.23	1.09 ± 0.19	1.80 ± 0.36
gr-2	1.00 ± 0.07	1.00 ± 0.05	0.95 ± 0.08	1.00 ± 0.12	0.98 ± 0.06	0.96 ± 0.09
hif1-α	$1.00 \pm 0.07^{+}$	$0.72 \pm 0.09^{+}$	$0.83 \pm 0.08^{+}$	$1.00 \pm 0.11^{\dagger}$	$1.04 \pm 0.10^{\dagger}$	$1.23 \pm 0.17^{\dagger}$

Table 2.4. Two-way analysis of variance (ANOVA), with cooler number entered as a random effect, examining the impact of differing elevated CO_2 concentrations and durations on candidate gene expression for silver carp and bighead carp fry.

Candidate Gene	Main effects	df	*F*	*P*
	Bighead Carp – 8 day old fry			
c-fos	Concentration	2	0.64	0.5466
	Duration	1	0.01	0.9277
	Concentration × duration	2	0.30	0.7500
hif11-α	Concentration	2	0.71	0.5140
	Duration	1	0.03	0.8722
	Concentration × duration	2	0.76	0.4928
gr-2	Concentration	2	3.31	0.0763
	Duration	1	0.40	0.5387
	Concentration × duration	2	0.13	0.8795
hsp70	Concentration	2	0.38	0.6914
	Duration	1	0.68	0.4271
	Concentration × duration	2	0.09	0.9178
	Silver Carp – 8 day old fry			
c-fos	Concentration	2	2.59	0.1216
	Duration	1	3.40	0.0931
	Concentration × duration	2	1.48	0.2706
hif1-α	Concentration	2	1.20	0.3466
	Duration	**1**	**11.52**	**0.0081**
	Concentration × duration	2	1.96	0.1980
gr-2	Concentration	2	0.16	0.8576
	Duration	1	0.003	0.9566
	Concentration × duration	2	0.01	0.9856
hsp70	**Concentration**	**2**	**5.61**	**0.0211**
	Duration	1	0.90	0.3634
	Concentration × duration	2	0.84	0.7348

Table 2.5. Relative gene expression values from the gills of juvenile bighead carp, silver carp, bluegill, and largemouth bass exposed to two concentrations and durations of elevated CO_2. The six treatments used in the hypercarbia challenge are as follows: C1 – ambient CO_2 exposure for 30 min; C2 – ambient CO_2 exposure for 60 min; L1 – 30 min exposure to 70 mg/L CO_2; L2 – 60 min exposure to 70 mg/L CO_2; H1 – 30 min exposure to 120 mg/L CO_2; and H2 – 60 min exposure to 120 mg/L CO_2. Dissimilar characters (+, †) denote statistically significant differences between juveniles within a species exposed for 30 min compared to 60 min. Dissimilar letters (a, b) denote statistically significant differences in gene expression between fish that were exposed to differing CO_2 concentrations. Results for statistical tests are given in Table 2.6 and Table 2.7.

Gene	C1	L1	H1	C2	L2	H2
Juvenile Bighead Carp						
gr-2	1.00 ± 0.09	1.06 ± 0.11	1.20 ± 0.15	1.00 ± 0.27	1.07 ± 0.09	1.22 ± 0.33
hif1-α	1.00 ± 0.11	1.24 ± 0.20	1.23 ± 0.21	1.00 ± 0.22	1.33 ± 0.18	1.32 ± 0.23
hsp70	1.00 ± 0.19	2.34 ± 1.27	0.88 ± 0.19	1.00 ± 0.30	1.03 ± 0.17	1.29 ± 0.52
Juvenile Silver Carp						
gr-2	1.00 ± 0.17	1.02 ± 0.22	0.69 ± 0.05	1.00 ± 0.07	1.41 ± 0.23	1.06 ± 0.25
hif1-α	$1.00 \pm 0.20^{+,ab}$	$1.09 \pm 0.26^{+,b}$	$0.67 \pm 0.07^{+,a}$	$1.00 \pm 0.09^{†,ab}$	$1.89 \pm 0.35^{†,b}$	$1.11 \pm 0.13^{†,a}$
hsp70	1.00 ± 0.54	0.83 ± 0.20	0.94 ± 0.21	1.00 ± 0.19	2.01 ± 0.63	1.28 ± 0.13
Juvenile Bluegill						
gr-2	1.00 ± 0.09	1.10 ± 0.18	0.88 ± 0.20	1.00 ± 0.27	0.82 ± 0.15	0.86 ± 0.12
hif1-α	1.00 ± 0.13	0.74 ± 0.10	0.62 ± 0.08	1.00 ± 0.18	1.02 ± 0.11	1.03 ± 0.13
hsp70	1.00 ± 0.16	1.39 ± 0.23	1.21 ± 0.35	1.00 ± 0.26	0.93 ± 0.13	0.86 ± 0.12
Juvenile Largemouth Bass						
gr-2	1.00 ± 0.05	1.02 ± 0.08	1.13 ± 0.06	1.00 ± 0.07	0.96 ± 0.05	0.90 ± 0.04
hif1-α	$1.00 \pm 0.04^{+}$	$0.88 \pm 0.05^{+}$	$0.96 \pm 0.04^{+}$	$1.00 \pm 0.06^{†}$	$1.11 \pm 0.05^{†}$	$1.08 \pm 0.06^{†}$
hsp70	1.00 ± 0.07	0.95 ± 0.05	1.11 ± 0.09	1.00 ± 0.16	0.85 ± 0.11	0.86 ± 0.08

Table 2.6. Two-way analysis of variance (ANOVA), or equivalent nonparametric two-factor ANOVA, examining the impact of differing elevated CO_2 concentrations and durations on candidate gene expression in the gill tissue of juvenile bighead carp and silver carp.

Candidate Gene	Main effects	df	*F or* χ^2	*P*
		Juvenile Bighead Carp		
c-fos (χ^2)	**Entire model**	**5**	**37.82**	**<0.0001**
	Concentration	**2**	**19.64**	**<0.0001**
	Duration	**1**	**12.17**	**0.0005**
	Concentration × duration	**2**	**6.01**	**0.0496**
hif11-α	Entire model	5	0.73	0.6048
	Concentration	2	1.67	0.1983
	Duration	1	0.04	0.8428
	Concentration × duration	2	0.12	0.8890
gr-2	Entire model	5	0.36	0.8725
	Concentration	2	0.71	0.4949
	Duration	1	0.20	0.6534
	Concentration × duration	2	0.07	0.9293
hsp70	Entire model	5	0.65	0.6641
	Concentration	2	0.90	0.4143
	Duration	1	0.34	0.5606
	Concentration × duration	2	0.67	0.5158
		Juvenile Silver Carp		
c-fos (χ^2)	**Entire model**	**5**	**18.40**	**<0.0001**
	Concentration	**2**	**28.94**	**<0.0001**
	Duration	**1**	**22.80**	**<0.0001**
	Concentration × duration	**2**	**5.75**	**0.0056**
hif1-α	**Entire model**	**5**	**3.33**	**0.0111**
	Concentration	**2**	**3.36**	**0.0423**
	Duration	**1**	**7.32**	**0.0092**
	Concentration × duration	2	1.55	0.2229

Table 2.6 (cont.)

gr-2	Entire model	5	10.78	0.0559
	Concentration	2	4.96	0.0838
	Duration	**1**	**5.18**	**0.0228**
	Concentration × duration	2	0.64	0.7270
hsp70	Entire model	5	11.06	0.0503
	Concentration	2	2.59	0.2737
	Duration	**1**	**8.31**	**0.0039**
	Concentration × duration	2	0.15	0.9260

Table 2.7. Two-way analysis of variance (ANOVA), or equivalent nonparametric two-factor ANOVA, examining the impact of differing elevated CO_2 concentrations and durations on candidate gene expression in the gill tissue of juvenile bluegill and largemouth bass.

Candidate Gene	Main effects	df	*F or* χ^2	*P*
		Juvenile Bluegill		
c-fos (χ^2)	**Entire model**	**5**	**40.18**	**<0.0001**
	Concentration	**2**	**37.60**	**<0.0001**
	Duration	1	0.08	0.7787
	Concentration × duration	2	2.51	0.2856
hif11-α	Entire model	5	2.30	0.0581
	Concentration	2	0.99	0.3797
	Duration	**1**	**5.55**	**0.0222**
	Concentration × duration	2	1.86	0.1660
gr-2	Entire model	5	0.48	0.7932
	Concentration	2	0.34	0.7140
	Duration	1	0.75	0.3897
	Concentration × duration	2	0.46	0.6344
hsp70	Entire model	5	0.66	0.6529
	Concentration	2	0.48	0.6241
	Duration	1	1.67	0.2023
	Concentration × duration	2	0.33	0.7188
		Juvenile Largemouth Bass		
c-fos (χ^2)	**Entire model**	**5**	**38.68**	**<0.0001**
	Concentration	**2**	**37.56**	**<0.0001**
	Duration	1	0.08	0.7787
	Concentration × duration	2	1.00	0.6058
hif1-α	**Entire model**	**5**	**2.61**	**0.0346**
	Concentration	2	0.12	0.8846
	Duration	**1**	**7.86**	**0.0070**
	Concentration × duration	2	2.47	0.0937

Table 2.7 (cont.)

gr-2	Entire model	5	1.53	0.1959
	Concentration	2	0.09	0.9138
	Duration	1	3.84	0.0553
	Concentration × duration	2	1.82	0.1723
hsp70	Entire model	5	0.95	0.4568
	Concentration	2	0.60	0.5501
	Duration	1	1.95	0.1681
	Concentration × duration	2	0.79	0.4574

Table 3.1. Water quality measurements at the conclusion of the eight-week acclimation period, acute hypercarbia challenge, and burst swim performance challenge. For the hypercarbia agitation and avoidance challenge, water quality parameters were taken during the 2 h acclimation period. Water quality measurements from tanks containing juvenile largemouth bass acclimated to ambient CO_2 are designated by an L, while an H denotes water quality measurements taken from tanks containing CO_2-acclimated largemouth bass. For the acute hypercarbia challenge and the swim performance challenge, water quality parameters were collected from 4 total treatments: LC – control fish exposed to ambient CO_2 water, LS – control fish exposed to 120 mg L^{-1} CO_2, HC – CO_2-acclimated fish exposed to control water, and HS –CO_2-acclimated fish exposed to 120 mg L^{-1} CO_2

	Temp (°C)	Dissolved Oxygen (mg L^{-1})	pH	Total Alkalinity (mg L^{-1})	Dissolved CO2 (mg L^{-1})	pCO2 (µatm)
8-week Acclimation Period	L - 15.1 ± 0.2	L - 8.5 ± 0.1	L - 8.20 ± 0.02	L - 147 ± 1	L - 13 ± 0.2	L - 1130 ± 42
	H - 15.4 ± 0.2	H - 8.5 ± 0.1	H - 6.93 ± 0.01	H - 147 ± 1	H - 31 ± 1	H - 20980 ± 711
Acute Hypercarbia Challenge	L - 14.9 ± 0.1	L - 8.5 ± 0.1	LC - 8.42 ± 0.01 LS - 6.19 ± 0.01	LC - 150 ± 2 LS - 154 ± 2	LC - 15 ± 1 LS - 123 ± 4	LC - 654 ± 14 LS - 116440 ± 2915
	H - 14.7 ± 0.1	H - 8.4 ± 0.1	HC - 6.82 ± 0.01 HS - 6.19 ± 0.01	HC - 146 ± 2 HS - 140 ± 3	HC - 32 ± 1 HS - 117 ± 3	HC - 25730 ± 958 HS - 104360 ± 3063
Hypercarbia Agitation / Avoidance Challenge	L - 16.1 ± 0.3	L - 8.9 ± 0.1	L - 7.59 ± 0.10	L - 156 ± 2	L - 20 ± 1	L - 8100 ± 1980
	H – 16.0 ± 0.3	H - 8.9 ± 0.1	H - 7.26 ± 0.14	H - 153 ± 2	H - 28 ± 2	H - 21570 ± 4010
Swim Performance Challenge	L - 15.5 ± 0.2	L - 8.5 ± 0.1	LC - 8.22 ± 0.02 LS - 6.18 ± 0.01	LC - 160 ± 1 LS - 155 ± 3	LC - 15 ± 0.4 LS - 122 ± 2	LC - 1130 ± 64 LS - 119220 ± 3421
	H - 15.6 ± 0.2	H - 8.3 ± 0.1	HC - 6.91 ± 0.04 HS - 6.19 ± 0.01	HC - 164 ± 2 HS - 157 ± 3	HC - 33 ± 1 HS - 118 ± 2	HC - 24100 ± 2162 HS - 118880 ± 2867

Table 3.2. Quantitative real-time PCR primer sets for juvenile largemouth bass. Sequence, melting temperature, fragment length, and accession number for each primer pair is presented in the table.

Gene	Sequence 5′ → 3′	Melting Temperature	Fragment Length (bp)	Accession Number
c-fos	*F:* GTCTCCATTCCTCCTGTCCA	59	113	KC493364.1
	R: GGTTGTGGTGAAGGTTGAC	57		
hsp70	*F:* ACTGATTGGGAGAAAGCTGG	59	136	KC493362.1
	R: CCTCTGGGCTGAAGGTTTTG	60		
18s	*F:* TTATTCCCATGACCCGCCG	62	156	JQ896299.1
	R: GGTGAGGTTTCCCGTGTTGA	62		

Table 3.3. Two-way analysis of variance (ANOVA), or equivalent nonparametric two-factor ANOVA, examining the impact of exposure to elevated CO_2 on candidate gene expression in the gill and erythrocytes of juvenile largemouth bass, who were either acclimated to ambient CO_2 concentrations or 30 mg L^{-1} CO_2 for eight weeks.

Candidate Gene	Main effects	df	*F or* χ^2	*P*
	Juvenile Largemouth Bass - Gills			
c-fos	**Entire model**	**3**	**369.93**	**<0.0001**
	Acclimation	1	0.65	0.4259
	Acute Exposure	**1**	**1107.28**	**<0.0001**
	Acclimation × Acute exposure	1	1.87	0.1795
hsp70 (χ^2)	**Entire model**	**3**	**17.87**	**0.0005**
	Acclimation	**1**	**16.46**	**<0.0001**
	Acute Exposure	1	0.35	0.5518
	Acclimation × Acute exposure	1	1.06	0.3041
	Juvenile Largemouth Bass - Erythrocytes			
c-fos	**Entire model**	**3**	**19.67**	**<0.0001**
	Acclimation	**1**	**58.52**	**<0.0001**
	Acute Exposure	1	0.11	0.7367
	Acclimation × Acute exposure	1	0.40	0.5292
hsp70	**Entire model**	**3**	**20.86**	**<0.0001**
	Acclimation	**1**	**56.39**	**<0.0001**
	Acute Exposure	1	3.49	0.0703
	Acclimation × Acute exposure	1	3.02	0.0913

Table 3.4. Response in physiological parameters of juvenile largemouth bass subjected to an acute hypercarbia challenge. Largemouth bass acclimated for eight-weeks in either ambient CO_2 (L Tank) or 30 mg L^{-1} CO_2 (H Tank) were exposed for 1 h at either acclimation conditions (Control) or 120 mg L^{-1} CO_2. Dissimilar upper case letters (A, B) denote statistically significant differences between the acclimation groups, while dissimilar characters (+, †) denote statistical differences between the acute exposure treatments (i.e., Control and 120 mg L^{-1} CO_2). Results of statistical analyses are presented in Table 3.5.

Parameter	Control Fish (L Tank)		CO_2-Acclimated Fish (H Tank)	
	Control	120 mg L^{-1} CO_2	Control	120 mg L^{-1} CO_2
Plasma Sodium (mequiv. L^{-1})	124 ± 3	123 ± 3	125 ± 3	131 ± 2
Plasma Chloride (mequiv. L^{-1})	**115 ± 1$^{A,+}$**	**110 ± 1^{A,†}**	**109 ± 1$^{B,+}$**	**105 ± 1^{B,†}**
Hematocrit (%)	**28.7 ± 1.3^{+}**	**32.4 ± 0.8†**	**27.8 ± 0.9^{+}**	**34.9 ± 0.9†**

Table 3.5. Two-way analysis of variance (ANOVA) examining the impact of exposure to elevated CO_2 on blood parameters of juvenile largemouth bass, who were either acclimated to ambient CO_2 concentrations or 30 mg L^{-1} CO_2 for eight weeks.

Parameter	Main effects	df	*F*	*P*
Plasma cortisol	**Entire model**	**3**	**17.84**	**<0.0001**
	Acclimation	1	3.68	0.0635
	Acute Exposure	**1**	**47.46**	**<0.0001**
	Acclimation × Acute exposure	1	1.39	0.2471
Plasma glucose	**Entire model**	**3**	**152.32**	**<0.0001**
	Acclimation	**1**	**7.33**	**0.0105**
	Acute Exposure	**1**	**419.51**	**<0.0001**
	Acclimation × Acute exposure	**1**	**19.39**	**0.0001**
Plasma lactate	**Entire model**	**3**	**11.02**	**<0.0001**
	Acclimation	1	1.66	0.2060
	Acute Exposure	**1**	**29.00**	**<0.0001**
	Acclimation × Acute exposure	1	1.59	0.2162
Plasma sodium	Entire model	3	1.60	0.2066
	Acclimation	1	2.51	0.1224
	Acute Exposure	1	0.98	0.3284
	Acclimation × Acute exposure	1	1.44	0.2390
Plasma chloride	**Entire model**	**3**	**17.77**	**<0.0001**
	Acclimation	**1**	**30.68**	**<0.0001**
	Acute Exposure	**1**	**22.24**	**<0.0001**
	Acclimation × Acute Exposure	1	0.15	0.7038
Hematocrit	**Entire model**	**3**	**12.56**	**<0.0001**
	Acclimation	1	1.70	0.2009
	Acute Exposure	**1**	**34.43**	**<0.0001**
	Acclimation × Acute exposure	1	1.37	0.2498

Table 3.6. Two-way repeated measures analysis of variance (ANOVA), with fish identification number was entered as a random effect, examining the CO_2 agitation and avoidance response of juvenile largemouth bass before and after the eight-week acclimation period.

Parameter	Main effects	df	*F*	*P*
CO_2 Concentration - Induced Agitation	**Test Period**	**1**	**12.45**	**0.0008**
	Acclimation	1	1.55	0.2165
	Test Period × Acclimation	**1**	**10.53**	**0.0016**
CO_2 Concentration – Induce Avoidance	**Test Period**	**1**	**20.26**	**<0.0001**
	Acclimation	1	0.42	0.5191
	Test Period × Acclimation	1	0.12	0.7288
Total Time Spent in Elevated CO_2	**Test Period**	**1**	**24.48**	**<0.0001**
	Acclimation	**1**	**14.29**	**0.0005**
	Test Period × Acclimation	**1**	**14.29**	**0.0005**
Number of Successful Shuttles	**Test Period**	**1**	**8.12**	**0.0066**
	Acclimation	1	1.86	0.1800
	Test Period × Acclimation	**1**	**4.43**	**0.0410**

Figure 2.1

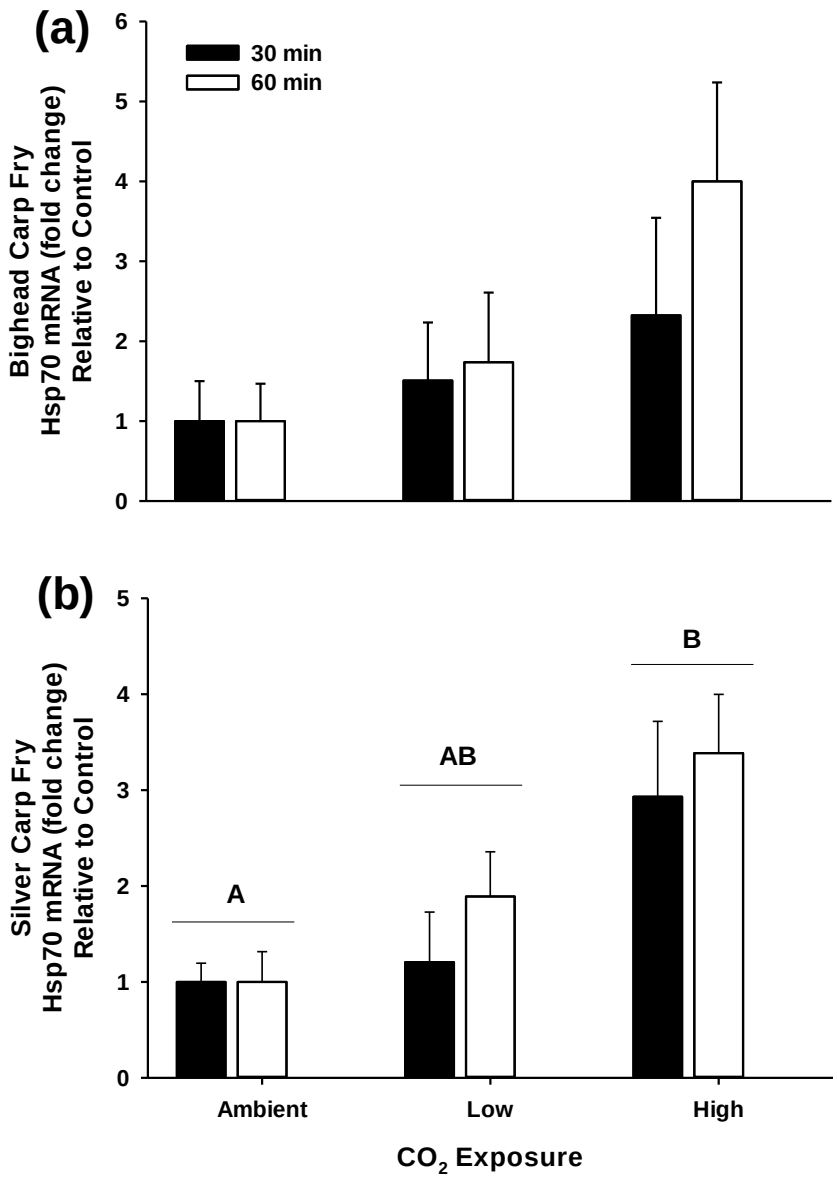

Figure 2.1: Relative mRNA expression for *hsp70* in bighead carp eight day-old fry (a) and silver carp eight day-old fry (b) exposed to two hypercarbia concentrations. Relative mRNA expression of fry exposed for 30 min are in black bars, and fry exposed for 60 min are in white bars. Horizontal lines denote a significant CO_2 concentration effect across exposure durations within a species. Data are means ± SE, calculated relative to the expression of the reference gene (i.e., *18s*). For clarity, data are expressed relative to the mean of fry exposed to ambient water conditions for each species and exposure duration.

Figure 2.2

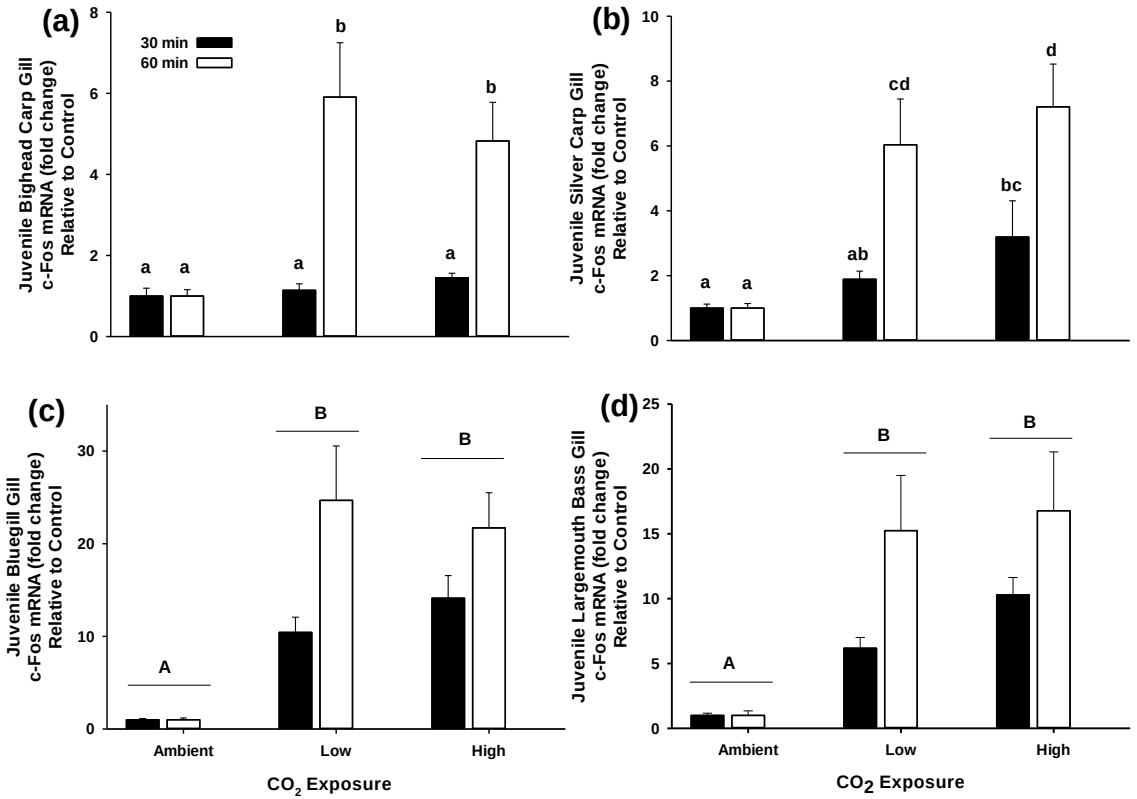

Figure 2.2: Relative expression of *c-fos* mRNA extracted from the gill tissue of juvenile bighead carp (a), silver carp (b), bluegill (c), and largemouth bass (d) exposed to a two hypercarbic treatments. Relative mRNA expression of juvenile fish that had an exposure duration of 30 min are shown in black bars, while white bars show the mRNA expression of juvenile fish exposed for 60 min. Horizontal lines denote a significant CO_2 concentration effect across exposure durations within a species. Dissimilar letters indicate significant differences between bars within a species. Data are means ± SE, calculated relative to the expression of the reference gene (i.e., either *18s* or *ef1-α*). For clarity, data are expressed relative to the mean of juvenile fish exposed to ambient water conditions.

Figure 2.3

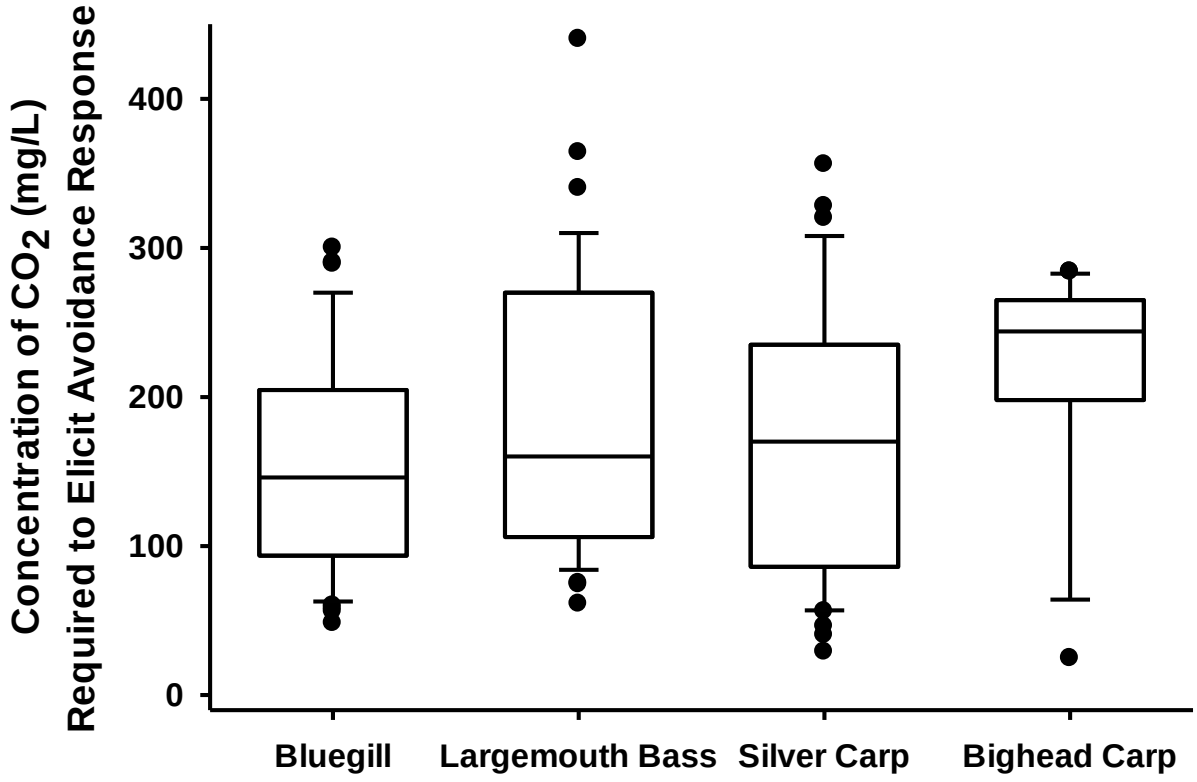

Figure 2.3: Concentration of CO_2 at which bluegill, largemouth bass, silver carp, and bighead carp displayed active shuttling behavior from a high CO_2 environment to a lower CO_2 environment during the course of the hypercarbia avoidance trial. Sample size is ten fish for all four species, and approximately four measurements (shuttles) were collected from each subject.

Figure 3.1

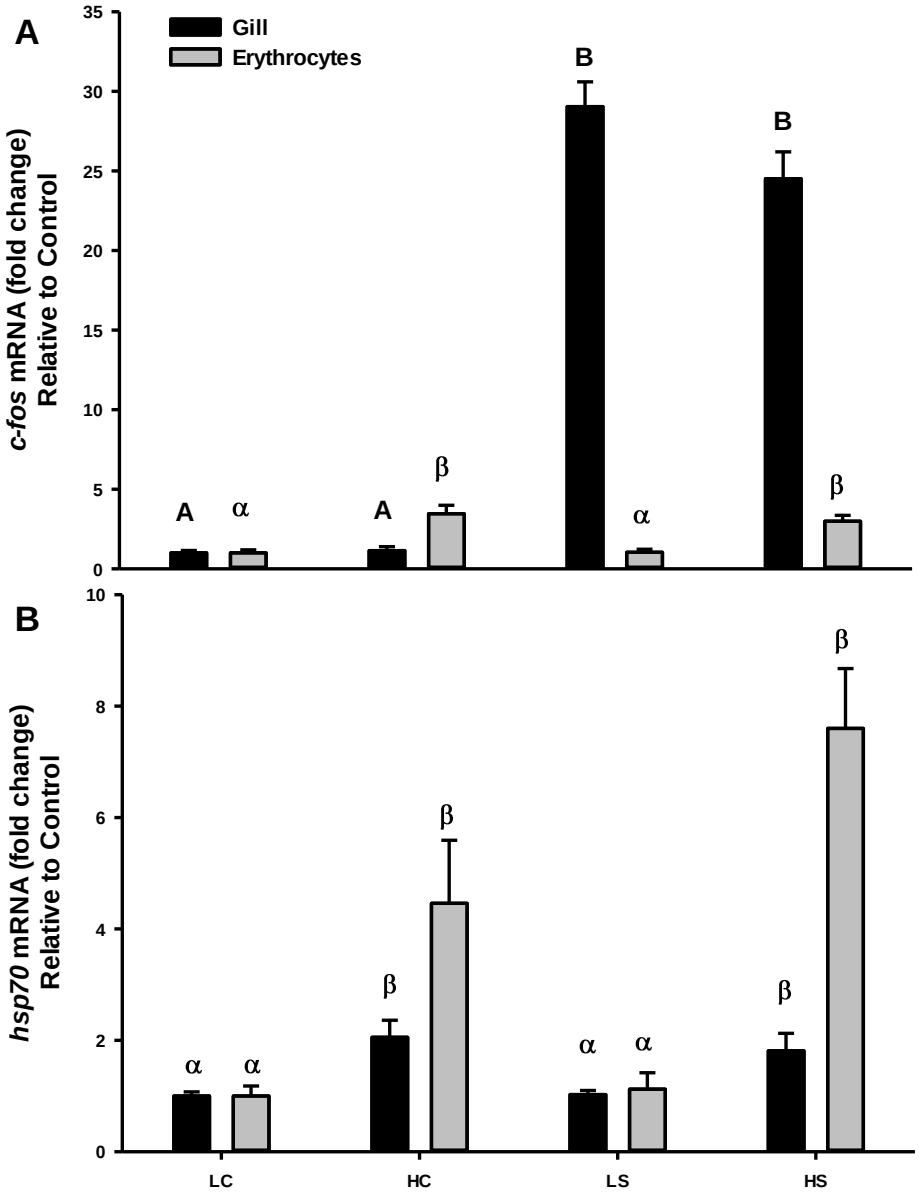

Figure 3.1. Tissue-specific relative mRNA expression for *c-fos* (A) and *hsp70* (B) in largemouth bass exposed to the acute hypercarbia challenge. Relative mRNA expression for gill tissue is shown in black bars, while erythrocyte mRNA expression is shown in gray bars. Four groups of fish were subjected to the acute hypercarbia challenge: LC – control fish exposed to ambient water for 1 h, HC –CO_2-acclimated fish exposed to acclimation water (30 mg l^{-1} CO_2) for 1 h, LS –control fish exposed to 120 mg l^{-1} CO_2 for 1 h, and HS – CO_2-acclimated fish exposed to 120 mg l^{-1} CO_2 for 1 h. Dissimilar upper case letters (A, B) denote statistically

significant differences between the acute exposure treatments (i.e., control and 120 mg l^{-1} CO_2), while dissimilar Greek letters (α, β) denote statistically significant differences between the acclimation groups. Data are means ± SE, calculated relative to the expression of the reference gene (i.e., *18s*). For clarity, data are expressed relative to the mean of ambient CO_2-acclimated largemouth bass exposed to ambient water for 1 h.

Figure 3.2

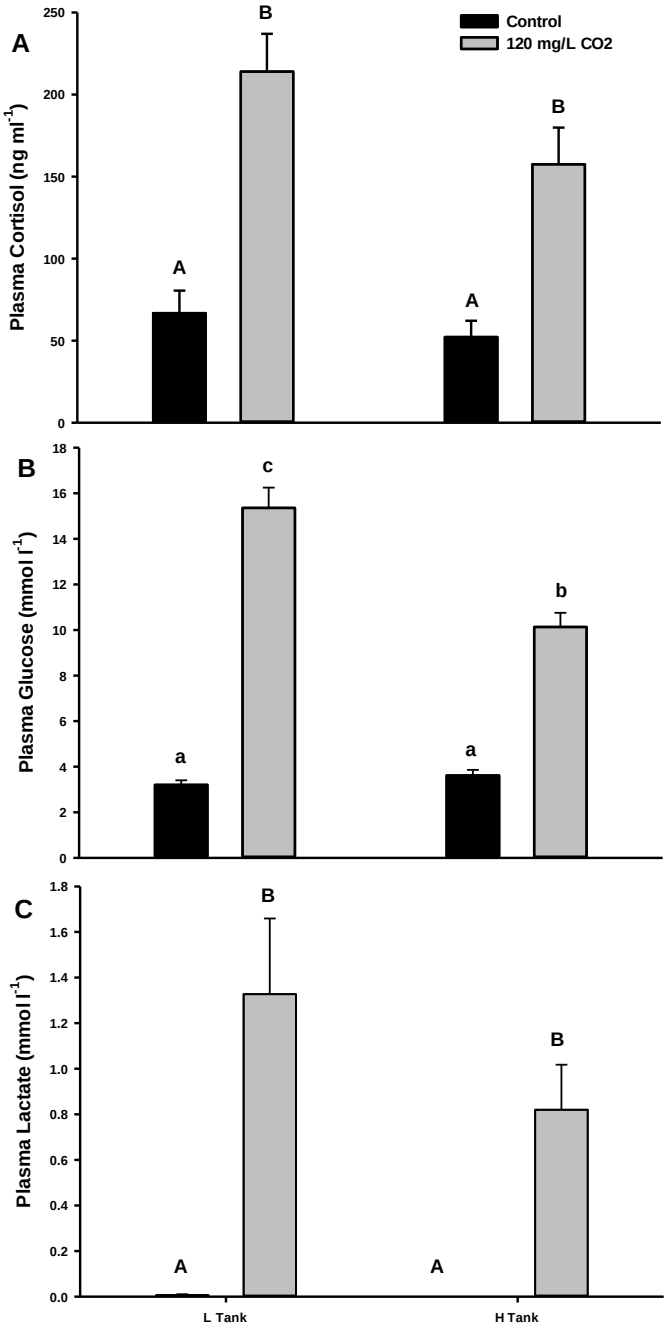

Figure 3.2. Concentrations of plasma cortisol (A), plasma glucose (B), and plasma lactate (C) for largemouth bass exposed to the acute hypercarbia challenge. Data for largemouth bass exposed acclimation water for 1 h are shown in black bars, while fish exposed to a 1 h exposure to 120 mg l^{-1} CO_2 are shown in gray bars. Results for largemouth bass acclimated to ambient

CO_2 conditions are shown above 'L Tank' category, while largemouth bass acclimated to 30 mg l^{-1} CO_2 are shown above the 'H Tank' category. Dissimilar upper case letters (A, B) denote statistically significant differences between the acute exposure treatments (i.e., control and 120 mg l^{-1} CO_2), while dissimilar lower case letter (a, b, c) denote statistically significant difference among all groups. Error bars shown 1 standard error (SE).

Figure 3.3

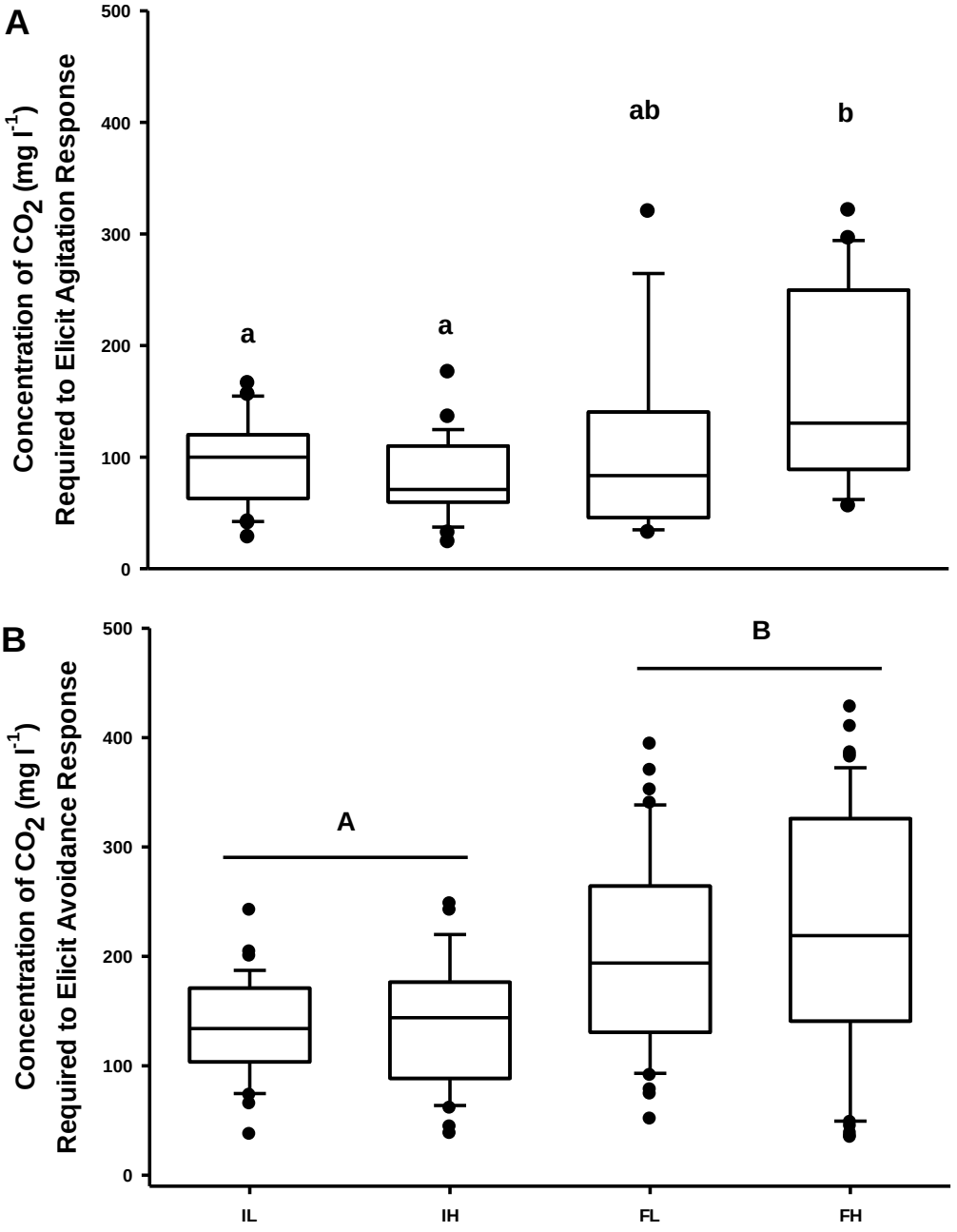

Figure 3.3. Concentration of CO_2 at which largemouth bass displayed either an agitated activity (i.e., surface ventilation, twitching, or erratic/elevated swimming) (A) or active avoidance of CO_2 by moving out of a high CO_2 environment to a lower CO_2 environment (B) during the course of the hypercarbia avoidance challenge. Two groups of fish were subjected to an 'initial' hypercarbia avoidance challenge (i.e., IL –control group, IH –CO_2 acclimation group).

Following 58 days of acclimation, these fish were subjected to a 'final' hypercarbia avoidance challenge (i.e., FL – control group, FH –CO_2 acclimation group). Horizontal lines denote a significant test period effect across the groups, while dissimilar lower case letters (a, b) denote a statistically significant difference between the four groups. Sample size is ten fish, and approximately four measurements (shuttles) were collected from each subject.

Figure 3.4

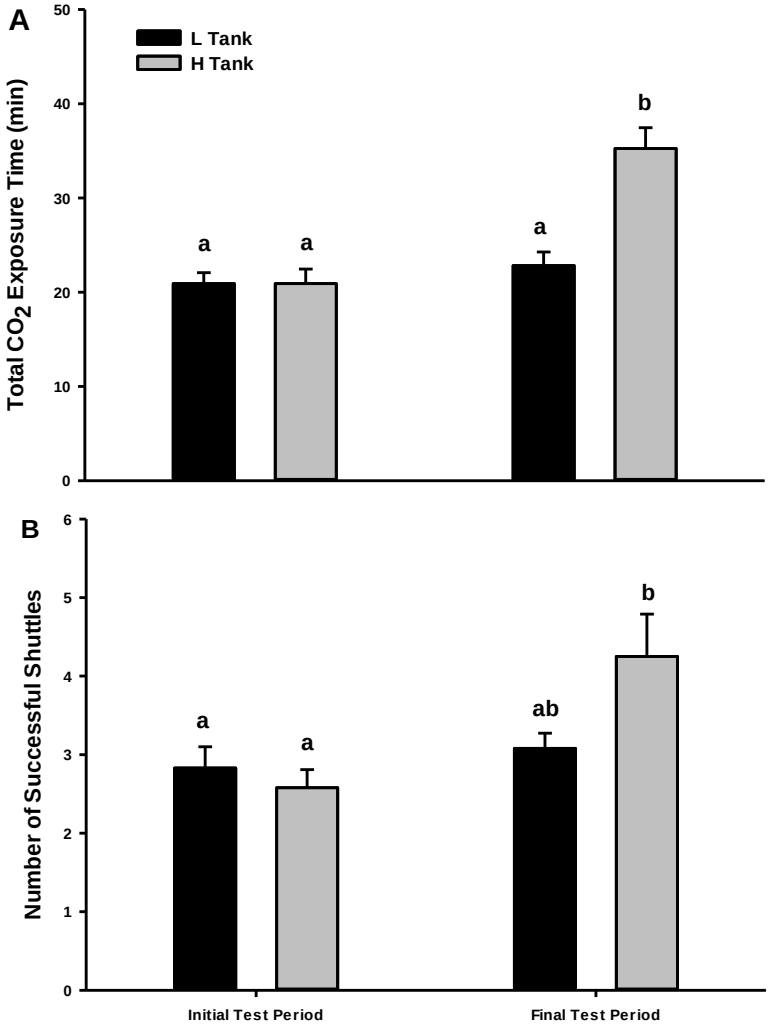

Figure 3.4. Total CO_2 exposure time (A) and total number of successful shuttles (B) observed in largemouth bass subjected to the hypercarbia avoidance challenge. Largemouth bass assigned to the control (L Tank) group are shown in black bars, while largemouth bass assigned to the CO_2 acclimation (H Tank) group are shown in gray bars. Results for largemouth bass subjected to the 'initial' hypercarbia avoidance challenge are shown above the 'Initial Test Period' category, while fish subjected to the 'final' hypercarbia avoidance challenge are shown above the 'Final Test Period' category. Dissimilar lower case letters (a, b) denote a statistically significant different between the four groups. Sample size is ten fish per group, and data is presented as mean ± SE.

Figure 3.5

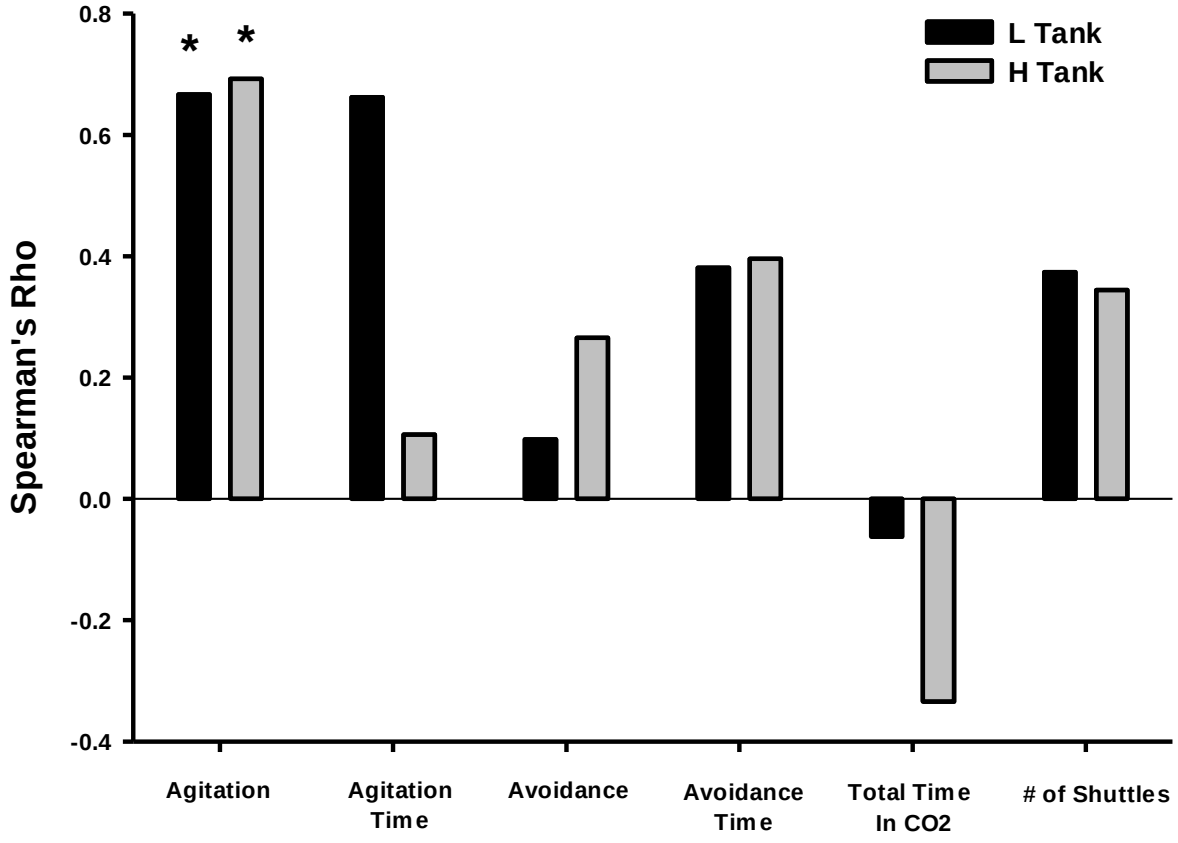

Figure 3.5. Spearman's ρ values, denoting correlation of individual largemouth bass CO_2 agitation and avoidance responses between the 'initial' and 'final' hypercarbia avoidance challenge. Largemouth bass assigned to the control (L Tank) group are shown in black bars, while largemouth bass assigned to the CO_2 acclimation (H Tank) group are shown in gray bars. Hypercarbia agitation and avoidance responses with significant amounts of correlation between the 'initial' and 'final' hypercarbia avoidance challenge, indicating repeatability of these responses, are marked with asterisks.

Figure 3.6

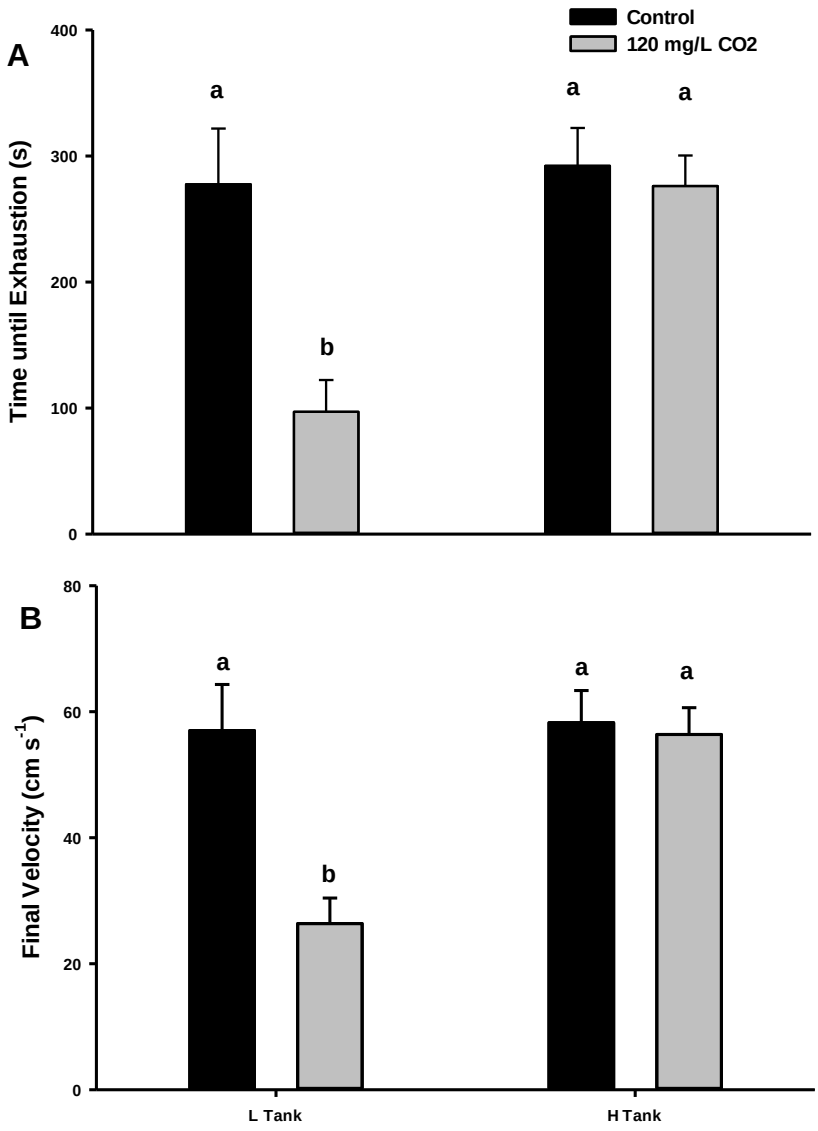

Figure 3.6. Time of exhaustion (A) and final velocity (B) at the conclusion of the burst swim performance challenge for largemouth bass acclimated to two different CO_2 concentrations. Largemouth bass exposed to acclimation water are shown in black bars, while fish exposed to 120 mg l^{-1} CO_2 are shown in gray bars. Results for largemouth bass acclimated to ambient CO_2 conditions are shown above the 'L Tank' category, while largemouth bass acclimated to 30 mg l^{-1} CO_2 are shown above the 'H Tank' category. Dissimilar lower case letters (a, b) denote statistically significant difference among the four groups. Data are shown at means ± SE. Eight largemouth bass is the sample size for each group.