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ORIGINAL PAPER

Molecular, behavioral, and performance responses of juvenile largemouth bass acclimated to an elevated carbon dioxide environment

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Abstract Aquatic hypercarbia, either naturally occurring or anthropogenically induced, can have extensive impacts on aquatic environments and resident organisms. 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. To better understand the impacts of prolonged hypercarbia exposure, largemouth bass were held at ambient CO_2 (13 mg L^{-1}) and elevated CO_2 (31 mg L^{-1}; ≈21,000 µatm) for 58 days. Following this acclimation period, fish were subjected to three separate, yet complementary, experiments: (1) acute hypercarbia challenge of 120 mg L^{-1} CO_2 for 1 h to quantify physiological and molecular responses; (2) hypercarbia avoidance challenge to compare CO_2 agitation and avoidance responses; and (3) swim performance challenge to quantify burst swimming performance. Acclimation to 31 mg L^{-1} CO_2 resulted in a significant constitutive upregulation of *c-fos* expression in erythrocytes, combined with significant constitutive expression of *hsp70* in both gill and erythrocytes, relative to controls. Largemouth bass acclimated to elevated CO_2 also had a reduced glucose response (relative to controls) following an acute CO_2 exposure, indicating a reduced stress response to CO_2 stressors. In addition, largemouth bass acclimated to elevated CO_2 conditions required 50 % higher CO_2 concentrations to illicit agitation behaviors and displayed prolonged burst swimming abilities in high CO_2 environments relative to controls. Together, results demonstrate that largemouth bass exposed to chronic hypercarbia may possess a physiological advantage during periods of elevated CO_2 relative to naïve fish, which may permit increased performance in hypercarbia.

Keywords Acclimation · Behavior · Hypercarbia · Invasive species · Performance · Stress

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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 in the marine environment driven by global climate change (Cooley and Doney 2009; Raven et al. 2005). Predicted levels of dissolved CO_2 by the year 2300 (approximately, 1900 µatm) have been shown to impair the ability of larval and juvenile marine fishes to avoid predators, locate settlement and refuge, and respond to auditory cues, potentially resulting in population declines and failure to recruit (Dixson et al. 2010; Munday et al. 2009, 2010; Simpson et al. 2011). In addition, while

the dynamics of CO_2 in freshwater are variable and future climate-related changes are difficult to predict (Cole et al. 1994, 2007; Raymond et al. 2013), freshwater fishes can be subjected to aquatic hypercarbia through a number of potential mechanisms that include 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. Previous research has shown, however, 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 an increase in hypercarbic environments in the future is likely (Feely et al. 2008), particularly in the marine environment, examining the impact of chronically elevated CO_2 exposure in fishes is becoming increasingly important.

Aquatic organisms have a variety of mechanisms to respond to environmental stress related to aquatic hypercarbia. More specifically, animals can display behavioral avoidance to escape poor water quality environments and potentially avoid the energetic cost necessary to inhabit suboptimal habitats (Kieffer and Cooke 2009). Similarly, animals can respond by altering molecular and physiological systems 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 allow these organisms to cope with chronic hypercarbia exposure (Dennis et al. 2014; Iwama et al. 2004; Rimoldi et al. 2009). For example, *c-fos* is one of the few gene transcripts that has been associated with hypercarbia stress in fishes (Dennis et al. 2014; Rimoldi et al. 2009) and is believed to modify ventilatory behavior in response to elevated CO_2 exposure (Rimoldi et al. 2009; Tankersley et al. 2002). Previous research has also shown that increased *hsp70* transcript expression can occur following the exposure to a variety of environmental stressors, including increasing temperatures (Healy et al. 2010), seawater exposure (Niu et al. 2008), and elevated CO_2 (Dennis et al. 2014), and is believed to preserve protein function under these conditions (Iwama et al. 2004).

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 have been well studied, relatively little work has been performed to define the physiological, behavioral, and performance impacts of chronic hypercarbia exposure in 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 end points, such as swimming performance or avoidance responses (Melzner et al. 2009; Ross et al. 2001). Additionally, prolonged exposure of animals to sublethal environmental variation can impart reversible physiological and molecular alterations without changes to genotypes, defined as phenotypic plasticity, which may provide a selective advantage and/or benefit an animal's ability to tolerate environmental stress (Leroi et al. 1994; Piersma and Drent 2003; Chevin et al. 2010). While previous studies have demonstrated phenotypic plasticity in fishes as a response to variables such as temperature (Angilletta 2009; Lurman et al. 2009) and hypoxia (Gaulke et al. 2014), few studies have examined this potential with hypercarbia, especially in terms of physiological and performance metrics. More importantly, freshwater ecosystems are predicted to experience an increase in dissolved CO_2 associated with future climate change (Sobek et al. 2005; Hasler et al. 2016), and little information exists on the response of freshwater fishes to extended exposure to elevated CO_2. Therefore, determining the impact of chronic hypercarbia exposure and subsequent acclimation capacity on largemouth bass will provide critical information on (1) how chronic hypercarbia exposure impacts 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.

Largemouth bass are an ideal species to use to answer questions related to CO_2 stressors in freshwater for the following reasons: (1) largemouth bass have been shown to be phenotypically plastic and can acclimate to a variety of environmental stressors [i.e., hypoxia (Gaulke et al. 2014), water temperatures (Díaz et al. 2007)]; (2) previous research on acute physiological responses to hypercarbia has been performed on largemouth bass (Kates et al. 2012); (3) assessing the impact of chronic CO_2 exposure may be useful for aquaculture managers rearing largemouth bass; and (4) largemouth bass are one of the most popular targets for recreational anglers in the USA, making them economically important (US Department of the Interior 2011).

The objective of this study was to quantify the capacity of fish to acclimate to elevated CO_2 environments. To accomplish this, juvenile largemouth bass were first held in

either elevated CO_2 or ambient CO_2 conditions for 8 weeks. Following this acclimation period, largemouth bass were subjected to three separate, yet complementary, 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 hypercarbia 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.

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 3 weeks prior to start of the 8-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 3-week outdoor holding period, largemouth bass were carefully netted from the holding tank, measured (total length in mm), weighed to the nearest 0.1 g, 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 6 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. This level of CO_2 was chosen for extended holding, as 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 inducing irregular activities (i.e., surface ventilations, coughing, loss of equilibrium), and, as such, the CO_2 concentration used in the H treatment was chosen to maximize the likelihood of molecular/physiological acclimation. Concentrations of CO_2 were held constant using a 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 that target CO_2 concentrations were achieved. Aeration through the use of a blower was maintained in each tank to ensure that fish were not subjected to hypoxia (maintained at 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 altering the activity of ion transport mechanisms in gills, as well as causing changes to plasma ion concentrations (Deigweiher et al. 2008; Fivelstad et al. 2003).

Mean water quality measurements taken from the acclimation tanks are presented in Table 1. Values for pCO_2 were calculated using the program CO2calc (version 1.2.0, US 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 as 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 1, these data should be interpreted cautiously as water chemistry may not have been at equilibrium at the time of measurement. The temperature in the H tank (15.4 ± 0.2 °C) was not statistically different from the L tank (15.1 ± 0.2 °C) during the 58-day acclimation period (t test, $t = 0.28$, $P > 0.05$). The mean sizes of largemouth bass at the beginning of the acclimation period were not

Table 1 Water quality measurements at the conclusion of the 8-week acclimation period, acute hypercarbia challenge, and burst swim performance challenge

	Temp (°C)	Dissolved oxygen (mg L^{-1})	pH	Total alkalinity (mg L^{-1})	Dissolved CO_2 (mg L^{-1})	pCO_2 (μatm)
Eight-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* 20,980 ± 711
Acute hypercarbia challenge	*L* 14.9 ± 0.1	*L* 8.5 ± 0.1	LC 8.42 ± 0.01	LC 150 ± 2	LC 15 ± 1	LC 654 ± 14
			LS 6.19 ± 0.01	LS 154 ± 2	LS 123 ± 4	LS 116,440 ± 2915
	H 14.7 ± 0.1	*H* 8.4 ± 0.1	HC 6.82 ± 0.01	HC 146 ± 2	HC 32 ± 1	HC 25,730 ± 958
			HS 6.19 ± 0.01	HS 140 ± 3	HS 117 ± 3	HS 104,360 ± 3063
Hypercarbia agitation/avoidance challenge	*L* 16.4 ± 0.2	*L* 8.9 ± 0.05	*L* 7.67 ± 0.08	*L* 153 ± 2	*L* 19 ± 1	*L* 6740 ± 1407
	H 14.8 ± 0.1	*H* 8.9 ± 0.1	*H* 6.69 ± 0.04	*H* 158 ± 1	*H* 38 ± 2	*H* 39,120 ± 3130
Swim performance challenge	*L* 15.5 ± 0.2	*L* 8.5 ± 0.1	LC 8.22 ± 0.02	LC 160 ± 1	LC 15 ± 0.4	LC 1130 ± 64
			LS 6.18 ± 0.01	LS 155 ± 3	LS 122 ± 2	LS 119,220 ± 3421
	H 15.6 ± 0.2	H 8.3 ± 0.1	HC 6.91 ± 0.04	HC 164 ± 2	HC 33 ± 1	HC 24,100 ± 2162
			HS 6.19 ± 0.01	HS 157 ± 3	HS 118 ± 2	HS 118,880 ± 2867

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 four 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

statistically different across treatments: (*L* 148 ± 1.0 mm; *H* 148 ± 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 that 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 freshwater 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 habituated to their containers for 24 h, while 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. Exposure of fishes to CO_2 concentrations of 120 mg L^{-1} have previously been shown to induce behavioral (i.e., hypercarbia agitation and avoidance responses), reflex (i.e., ventilation and irregular activities), and physiological disturbances in adult largemouth bass (Kates et al. 2012), and 120 mg L^{-1} was therefore selected for the acute hypercarbia exposure, as it 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 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 methanesulfonate (MS-222) buffered with 500 mg L^{-1} sodium bicarbonate].

Following cessation of ventilation, fish were measured and 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 1. The mean size of largemouth bass subjected to acute hypercarbia challenge was not statistically different across treatments: (LC 159 ± 2 mm; LS 157 ± 2 mm; HC 156 ± 2 mm; HS 157 ± 1 mm) (one-way analysis of variance (ANOVA), $F = 0.56$, $P = 0.64$). Sample size for largemouth bass subjected to acute hypercarbia challenge was $N = 10$ for fish acclimated to ambient CO_2 and $N = 9$ for fish acclimated to 30 mg L^{-1} CO_2.

Hypercarbia avoidance challenge

Prior to the 8-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 1 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) provides 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 habituate 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. To avoid potential complications with residual CO_2 that could influence results, behavioral data were only collected from a fish once, and behavioral trials concluded when a fish shuttled into the opposite holding tank. 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 one of the indoor acclimation tanks. The mean size of largemouth bass subjected to the 'initial' hypercarbia avoidance challenge was not statistically different across acclimation groups: (*L* 141 ± 2 mm; *H* 140 ± 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 1. The size of largemouth bass subjected to the 'final' hypercarbia avoidance challenge did not differ statistically across acclimation groups (*L* 153 ± 2 mm; *H* 150 ± 1 mm) (*t* test, $t = 0.23$, $P > 0.05$). The sample size for largemouth bass subjected to the 'final' hypercarbia avoidance challenge was $N = 12$ for each acclimation group.

Swim performance challenge

Following the 8-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 habituate for 2 h at a water velocity of 0.5 body lengths per second (BL s^{-1}), a duration of time that should allow recovery from handling stressors (Suski et al. 2007) and that has been used previously with studies of swimming performance in fishes (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

Table 2 Quantitative real-time PCR primer sets for juvenile largemouth bass

Species	Gene	Sequence 5′ → 3′	Melting temperature	Fragment length (bp)
Largemouth Bass	*c-fos*	*F:* GTCTCCATTCCTCCTGTCCA	59	113
		R: GGTTGTGGTGAAGGTTGAC	57	
	hsp70	*F:* ACTGATTGGGAGAAAGCTGG	59	136
		R: CCTCTGGGCTGAAGGTTTTG	60	
	18s	*F:* TTATTCCCATGACCCGCCG	62	156
		R: GGTGAGGTTTCCCGTGTTGA	62	

Sequence, melting temperature, and fragment length information for each primer pair are presented in the table

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 the target-dissolved CO_2 concentrations were verified using the CO_2 digital titrator. Fish subjected to ambient CO_2 concentrations experienced an identical procedure, except for 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 burst swimming velocity (BL s^{-1}) 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 1. The mean size of largemouth bass subjected to the burst swimming challenge was not statistically different across treatments: (LC 161 ± 2 mm; LS 158 ± 1 mm; HC 160 ± 2 mm; HS 158 ± 2 mm) (one-way ANOVA, $F = 0.66$, $P = 0.58$). The sample size for largemouth bass subjected to the burst swimming challenge was $N = 8$ for all treatments.

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 and 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 the yield and purity of the extracted RNA. The RNA integrity was confirmed using gel electrophoresis. Extracted RNA was frozen at −80 °C. The 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 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 the 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 of largemouth bass subjected to the hypercarbia avoidance challenge were performed using a two-way ANOVA with test period (initial or final), acclimation, and their interaction (test period × acclimation) entered as fixed effects, while fish identification number was entered as a random effect. A Tukey–Kramer HSD post hoc test was again used to separate means where appropriate (Sokal and Rohlf 1995).

Comparisons of burst swimming performance (i.e., burst swimming 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 the 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-pair multiple comparison test was applied to separate means (Douglas and Michael 1991). All means are reported as ±SE where appropriate. Two-way Kruskal–Wallis test and Hartley's F_{max} test calculations were accomplished by hand using Zar (1984) as a template, while all other statistical analyses were performed using the JMP version 9.0.2 (SAS Institute Inc., Cary, NC, USA). All tests were run at a significance level (α) of 0.05.

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 up-regulation, for fish in the ambient CO_2 and elevated CO_2 acclimation group, respectively, relative to controls (Fig. 1a; Table 3). Largemouth bass in the group acclimated to elevated CO_2 also had approximately threefold higher constitutive expression of *c-fos* mRNA in erythrocytes compared to fish in the ambient CO_2 group (Fig. 1a; Table 3). Concentrations of *hsp70* transcripts in both gill tissue and erythrocytes for largemouth bass acclimated to elevated CO_2 were twofold and five- to eightfold greater (respectively) than largemouth bass held at ambient CO_2 (Fig. 1b; Table 3).

One-hour exposure to 120 mg L^{-1} CO_2 caused a threefold increase in plasma cortisol concentrations for largemouth bass, regardless of the acclimation group (Fig. 2a; Table 4). One-hour exposure to 120 mg L^{-1} CO_2 also resulted in a fivefold increase in plasma glucose in fish acclimated to ambient CO_2, but only a threefold increase in fish acclimated to 30 mg L^{-1} (Fig. 2b; Table 4). Regardless of the acclimation group, largemouth bass exposed to 120 mg L^{-1} CO_2 for 1 h experienced increased plasma

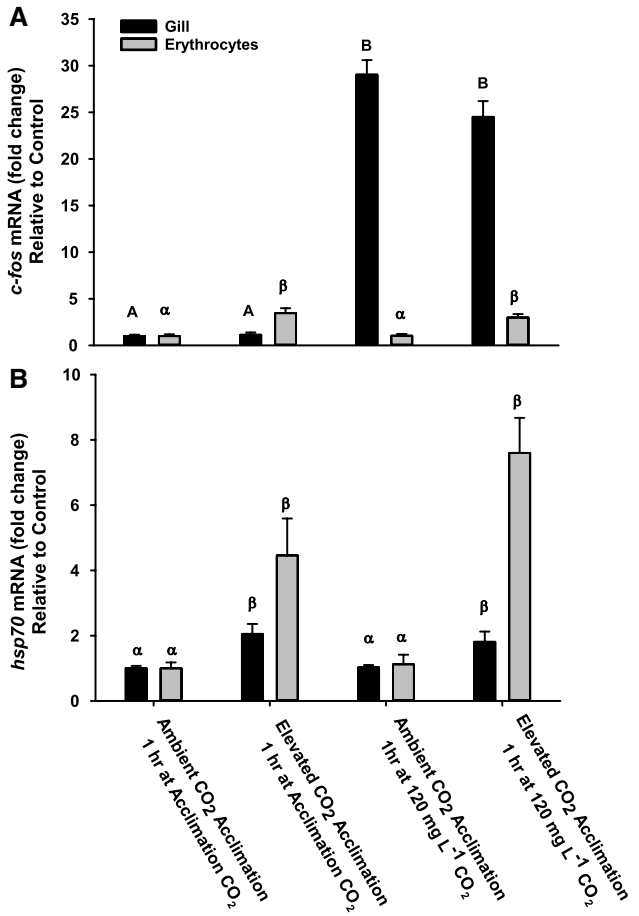

Fig. 1 Tissue-specific relative mRNA expression for *c-fos* (**a**) and *hsp70* (**b**) in largemouth bass exposed to an acute hypercarbia challenge. Relative mRNA expression for gill tissue is shown by *black bars*, while erythrocyte mRNA expression is shown by *gray bars*. Four groups of fish were subjected to the acute hypercarbia challenge: control fish exposed to ambient water for 1 h, CO_2-acclimated fish exposed to acclimation water (30 mg L^{-1} CO_2) for 1 h, control fish exposed to 120 mg L^{-1} CO_2 for 1 h, and CO_2-acclimated fish exposed to 120 mg L^{-1} CO_2 for 1 h. Dissimilar *uppercase 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 mean ± 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. The sample size is $N = 10$ for largemouth bass in the ambient CO_2 acclimation group and $N = 9$ for the elevated CO_2-acclimated group

lactate concentrations (Fig. 2c; Table 4). Plasma sodium concentrations did not differ between acclimation groups or the acute exposure treatments (Tables 4, 5). Largemouth bass acclimated to elevated CO_2 showed a 5 % reduction in plasma chloride compared to fish acclimated to ambient CO_2 (Tables 4, 5). Similarly, an acute exposure to 120 mg L^{-1} CO_2 resulted in a 5 % decrease in plasma chloride concentrations relative to controls (Tables 4, 5). Regardless of the acclimation group, largemouth bass exposed to an acute hypercarbia stressor showed a significant increase in hematocrit (Tables 4, 5).

Exposure of largemouth bass to elevated CO_2 within the shuttle box induced an agitation response, followed by active CO_2 avoidance as animals left the area of high CO_2. More specifically, largemouth bass acclimated to ambient CO_2 displayed agitation responses at approximately 60 mg L^{-1} CO_2, while largemouth bass acclimated to elevated CO_2 did not display agitated responses until 105 mg L^{-1} (Fig. 3a; Table 6). This difference in agitation responses between acclimation groups was driven by the twofold increase in CO_2 concentration necessary to induce agitation in largemouth bass that were exposed to elevated CO_2 for 8 weeks. Largemouth bass acclimated to elevated CO_2 in the 'final' time period also required a greater CO_2 concentration to display an active avoidance response (approximately 160 mg L^{-1} CO_2) relative to fish in the 'initial' time period (approximately, 110 mg L^{-1} CO_2); however, this difference was not statistically significant (Fig. 3b; Table 6). 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 threefold decrease in the duration of the swimming trial (Fig. 4a) and a twofold decrease in burst swimming velocity (Fig. 4b) 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: $F_{[3]} = 8.97$, $P = 0.0003$).

Discussion

An 8-week exposure to 30 mg L^{-1} CO_2 induced a number of alterations to molecular and physiological parameters in juvenile largemouth bass. More specifically, largemouth bass acclimated to an elevated CO_2 environment had constitutively higher expression of *c-fos* mRNA in erythrocytes, along with elevated *hsp70* gill and erythrocyte mRNA, compared to control fish. In addition, largemouth bass acclimated to elevated CO_2 exhibited plasma chloride concentration 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 biological properties, a process commonly known as phenotypic plasticity (Chevin et al. 2010). Heat shock protein transcripts are typically induced to maintain homeostasis within a cell by facilitating folding of nascent proteins, as well as repairing

Table 3 Two-way analysis of variance (ANOVA), or equivalent non-parametric 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 that were either acclimated to ambient CO_2 concentrations or 30 mg L^{-1} CO_2 for 8 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

Bold text indicates statistical significance ($P < 0.05$)

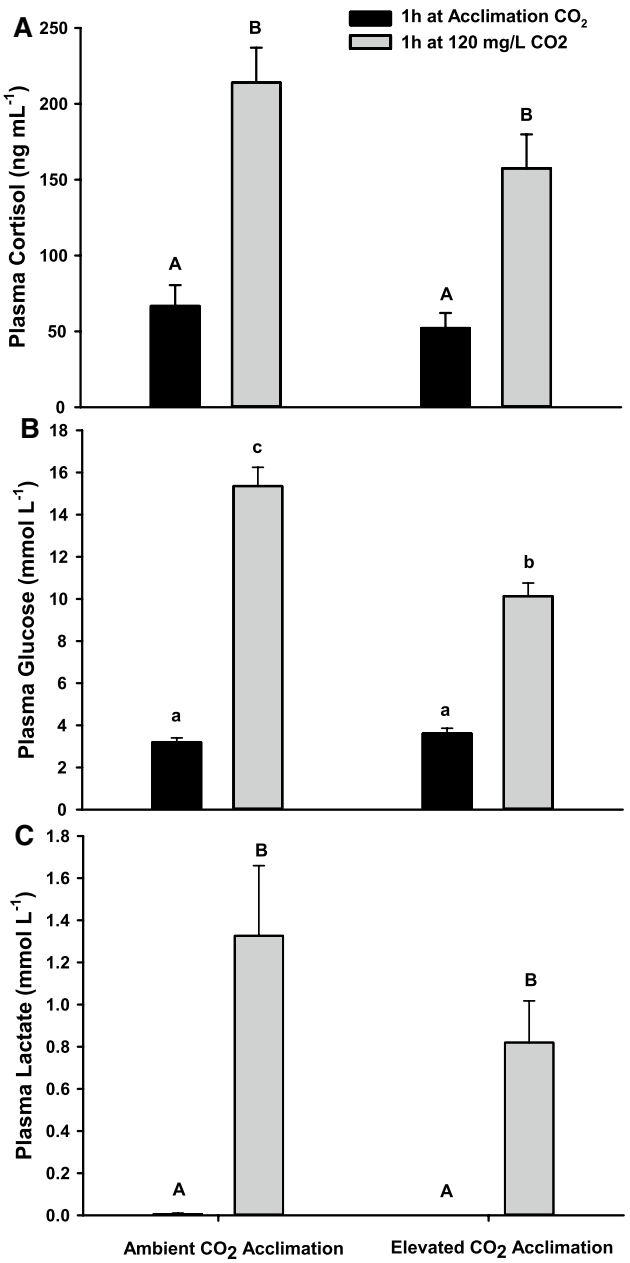

Fig. 2 Concentrations of plasma cortisol (**a**), plasma glucose (**b**), and plasma lactate (**c**) for largemouth bass exposed to the acute hypercarbia challenge. Data for largemouth bass exposed acclimation water for 1 h are shown by *black bars*, while fish exposed to a 1-h exposure to 120 mg L^{-1} CO_2 are shown by *gray bars*. Dissimilar *uppercase letters* (*A*, *B*) denote statistically significant differences between the acute exposure treatments (i.e., control and 120 mg L^{-1} CO_2), while dissimilar *lowercase letter* (*a*, *b*, *c*) denote statistically significant difference among all groups. *Error bars* shown 1 standard error (SE). Sample size is $N = 10$ for largemouth bass in the ambient CO_2 acclimation group and $N = 9$ for the elevated CO_2-acclimated group

proteins damaged by various stressors (Iwama et al. 2004). As such, the higher constitutive *hsp70* transcript abundance in largemouth bass subjected to extended hypercarbia may allow these fish to minimize the negative impacts on protein function that could occur due to hypercarbia exposure. The c-fos protein, meanwhile, regulates the expression of a multitude of genes in response to elevated CO_2 (Curran and Franza 1988; Kassahn et al. 2009) and may induce changes in ventilatory behavior (Rimoldi et al. 2009; Tankersley et al. 2002) in an effort to maintain physiological homeostasis. While the impact of *c-fos* transcript elevation in erythrocytes is currently not known, a greater constitutive pool of *c-fos* transcripts in erythrocytes may allow these cells to maintain proper function (i.e., oxygen delivery). 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 suggest that this was due to increased Cl^-/HCO_3^- ion exchange to increase blood pH, potentially driven by elevated external CO_2. 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 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 acclimated for 45–60 days exposure to hypercarbia did not have elevated 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, with alterations to a number of molecular and physiological parameters occurring.

Table 4 Two-way (ANOVA) examining the impact of elevated CO_2 on blood parameters of juvenile largemouth bass, acclimated to either ambient CO_2 or 30 mg L^{-1} CO_2 for 8 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

Bold text indicates statistical significance ($P < 0.05$)

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. The physiological response of fishes to an acute hypercarbia exposure has been well studied, and includes an initial elevation in blood pCO_2, 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)]. The ventilation frequency of fishes (Perry and Gilmour 2006), along with elevations of stress indicators (i.e., plasma cortisol and glucose) (Bernier and Randall 1998; Iwama et al. 1989), have also been shown to be impacted by acute hypercarbia exposure. 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 associated with stress response can help fuel aerobic tissues, such as heart or gills, that are under stress (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

Table 5 Response in physiological parameters of juvenile largemouth bass subjected to an acute hypercarbia challenge

Parameter	Ambient CO_2-acclimated fish		Elevated CO_2-acclimated fish	
	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†**

Largemouth bass acclimated for 8 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 *uppercase letters* (*A*, *B*) denote statistically significant differences between the acclimation groups, while dissimilar characters (*plus symbol*, *dagger symbol*) denote statistical differences between the acute exposure treatments (i.e., control and 120 mg L^{-1} CO_2). Sample size for largemouth bass subjected to the acute hypercarbia challenge was $N = 10$ for control fish and $N = 9$ for CO_2-acclimated fish

Bold text indicates statistical significance ($P < 0.05$)

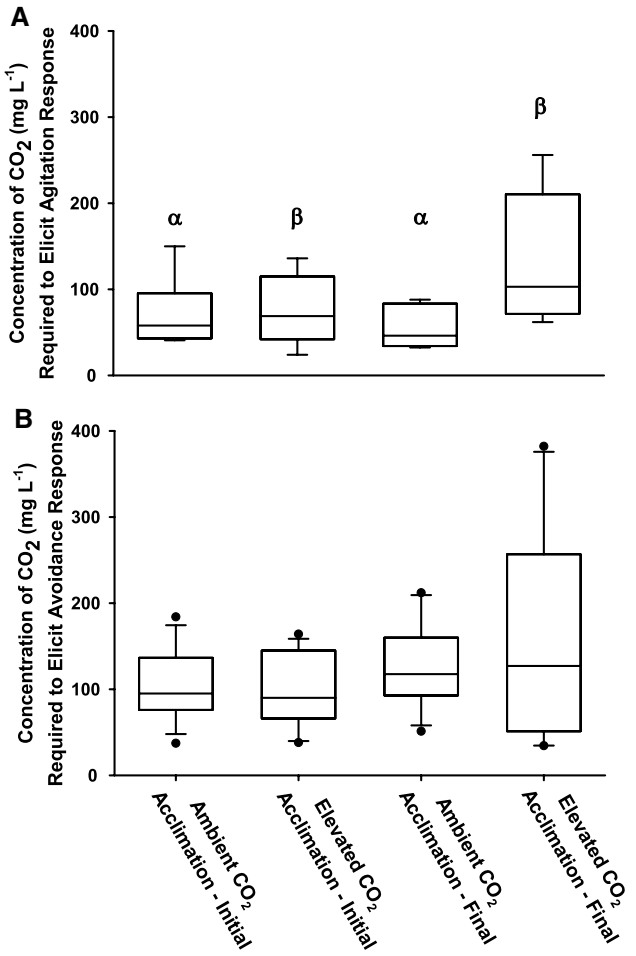

Fig. 3 Concentration of CO_2 at which largemouth bass displayed either an agitated activity (i.e., surface ventilation, twitching, or erratic/elevated swimming) (**a**) or active avoidance of CO_2 by moving out of a high CO_2 environment to a lower CO_2 environment (**b**) during the course of the hypercarbia avoidance challenge. Two groups of fish were subjected to an 'initial' hypercarbia avoidance challenge (i.e., before an 8-week exposure to acclimation CO_2 conditions). Following 58 days of acclimation, these fish were subjected to a 'final' hypercarbia avoidance challenge (i.e., after an 8-week exposure to acclimation CO_2 conditions). Dissimilar *Greek letters* (α, β) denote statistically significant differences between the acclimation groups. For *box plots*, the *top* and *bottom* of the *box* represent the 75th and 25th percentiles (respectively), while the *horizontal line within the box* represents the median; *whiskers* (*error bars*) *above and below the box* represent the 90th and 10th percentiles. Sample size for largemouth bass subjected to the 'initial' and 'final' hypercarbia avoidance challenge was $N = 12$ for the ambient CO_2 and elevated CO_2-acclimated group

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 improve oxygen transport during acute hypercarbia exposure. It is currently unknown whether hematocrit levels in this study were elevated due to the release of additional red blood cells from the spleen or swelling of the existing blood cells. As such, additional research is necessary to determine whether increased hematocrit in largemouth bass exposed to CO_2 actually improved their oxygen transport capability by releasing more blood cells. 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 the elevated activity of Cl^-/HCO_3^- exchangers allowing the uptake of HCO_3^- from the external environment in exchange for plasma Cl^- to buffer further reductions in blood pH. In addition to these plasma parameters, *c-fos* gill transcripts were also induced following an acute exposure to 120 mg L^{-1} CO_2. 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 that 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.

Acclimation to an elevated CO_2 environment for 8 weeks impacted a number of behavioral and physiological responses in juvenile largemouth bass. More specifically, CO_2-acclimated largemouth bass required 50 % higher CO_2 compared to ambient CO_2-acclimated fishes during shuttle box trials and, while not statistically significant, largemouth bass acclimated to 30 mg L^{-1} CO_2 tolerated a greater CO_2 concentration (160 mg L^{-1}) prior to displaying active avoidance by voluntarily shuttling to the other compartment of the shuttle box. 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. For example, the reduced concentration of plasma glucose in largemouth bass acclimated to 30 mg L^{-1} CO_2 relative to control fish following exposure to an acute hypercarbia stressor suggests that this type of stressor is not as energetically demanding for fish that have been acclimated to an elevated CO_2 environment, as glucose is released as secondary stress parameter that fuels aerobic tissues (Barton 2002; Wendelaar Bonga 1997), thus potentially allowing elevated CO_2-acclimated fish to withstand hypercarbia challenges of greater intensity before inducing behavioral responses to hypercarbia stress. Elevated hematocrit levels shown in CO_2-acclimated largemouth bass following exposure to 120 mg L^{-1} CO_2 may have improved oxygen uptake and transport (Wells 2009), which was potentially impacted by reductions in plasma pH due to acute hypercarbia exposure (Bernier and Randall

Table 6 Two-way 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 8-week acclimation period

Parameter	Main effects	*df*	*F*	*P*
CO_2 concentration-induced agitation	Test period	1	1.81	0.1907
	Acclimation	1	**5.81**	**0.0236**
	Test period × acclimation	1	3.65	0.0677
CO_2 concentration-induced avoidance	Test period	1	1.72	0.1961
	Acclimation	1	0.05	0.8272
	Test period × acclimation	1	0.05	0.8208

Bold text indicates statistical significance ($P < 0.05$)

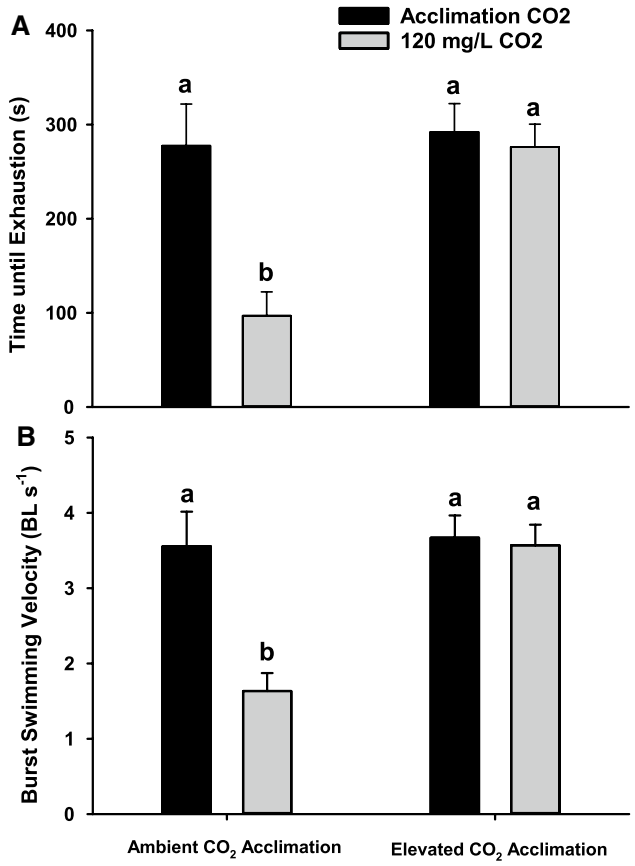

Fig. 4 Time of exhaustion (**a**) and burst swimming velocity (**b**) at the conclusion of the burst swim performance challenge for largemouth bass acclimated to two different CO_2 concentrations. Largemouth bass exposed to acclimation water are shown by *black bars*, while fish exposed to 120 mg L^{-1} CO_2 are shown by *gray bars*. Dissimilar *lowercase letters* (*a*, *b*) denote statistically significant difference among the four groups. Data are shown at mean ± SE. Eight largemouth bass is the sample size for each group

1998). Improved oxygen update and transport may allow CO_2-acclimated fishes to display agitation and avoidance responses at greater CO_2 concentrations. Additionally, constitutively elevated *hsp70* transcripts in the CO_2-acclimated group likely conferred a greater ability for oxygen uptake and transport in erythrocytes of CO_2-acclimated largemouth bass relative to control fish, as greater heat shock protein transcript expression may allow proteins within erythrocytes to function properly under acute stressors (Iwama et al. 2004; Wells 2009). Interestingly, the concentration of CO_2 that induced avoidance was not statistically different between the acclimation groups, suggesting that there may be a potential CO_2 threshold at which fishes choose to swim away from elevated CO_2 zones, independent of acclimation history; research with multiple CO_2 acclimation concentrations will be necessary to definitively address this hypothesis. 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, possibly driven by physiological changes following acclimation; however, these changes did not influence the avoidance response.

Burst swimming performance (i.e., time until exhaustion, burst swimming velocity) of CO_2-acclimated largemouth bass was not impacted by prolonged exposure to 30 mg L^{-1} CO_2; however, largemouth bass acclimated to a high CO_2 environment were able to maintain swimming performance following an acute hypercarbia stressor, unlike control fish. Physiological alterations (i.e., decrease in plasma chloride) that occurred in largemouth bass following acclimation to the elevated CO_2 environment likely allowed these fish to maintain swimming performance following exposure to 120 mg L^{-1} CO_2 compared to control fish. Similar conclusions were reached in previous studies of European eel (*Anguilla Anguilla*) and Atlantic cod (McKenzie et al. 2003; Melzner et al. 2009), as both of these species showed no reduction in locomotory performance following extended hypercarbia exposure. 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. Thus, an improved ability to regulate plasma anions in response to hypercarbia acclimation likely allows elevated CO_2-acclimated largemouth bass 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 reduction in burst swimming velocity.

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 and 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 CO_2 for extended periods, results from the current study suggest that 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 CO_2 pressure used for acclimation in the current study (≈21,000 μatm) is approximately ten times greater than the expected rise in CO_2 levels for marine environments predicted by the year 2300 (Caldeira and Wickett 2003), pCO_2 levels freshwater environments are much more dynamic and variable, and can naturally reach levels that are tenfold higher than present-day atmospheric concentrations (Cole et al. 1994; Telmer and Veizer 1999) making the use of such concentrations realistic and applicable. The concentrations of CO_2 used in the current study are also useful to researchers looking to define 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 fishes to chronic hypercarbia exposure (Heuer and Grosell 2014). 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 serious issue for fish farmers and aquaculture managers. Hatchery-reared fish that have acclimated to elevated CO_2 conditions in rearing tanks may also experience challenges when released into the wild, compounding the negative impacts that hatchery fish can have on natural fish populations (Araki and Schmid 2010). Exposure to elevated CO_2, even for as short as 4–11 days, has also been shown to influence predator avoidance (Dixson et al. 2010) and homing (Munday et al. 2009) in marine fishes. In addition, the potential for CO_2 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 CO_2 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 160 mg L^{-1}, suggesting that a CO_2 barrier still has potential to influence movement of fishes acclimated to hypercarbia. Finally, it is currently not known if the changes observed in this study are reversible (plastic) and if animals would return to control phenotypes following a return to ambient CO_2 levels. As hypercarbic environments become more prevalent due to global climate change, knowledge of the capacity for aquatic organisms to acclimate to elevated CO_2, as well as the resulting impact on physiological and behavioral traits, will be of vital importance for conservation managers.

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