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Molecular and behavioral responses of early-life stage fishes to elevated carbon dioxide

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Abstract Bigheaded carps are non-native invasive fishes that have quickly become the most abundant fishes in many portions of the Midwestern United States. While the spread of bigheaded carps into the Great Lakes is currently impeded 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 bigheaded carps from invading the Great Lakes, and provide much needed redundancy 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 bigheaded carps. Juvenile silver carp (*Hypophthalmichthys molitrix*), bighead carp (*H. nobilis*), bluegill (*Lepomis macrochirus*) and largemouth bass (*Micropterus salmoides*) showed avoidance of elevated CO_2 environments at approximately 200 mg/L. Additionally, exposure to 120 mg/L CO_2 resulted in the induction of *hsp70* mRNA in 8 days old silver carp fry, while gill *c-fos* transcripts increased following hypercarbia exposure in all juvenile species examined. Together, our results show that CO_2 has potential to deter the movement of larval and juvenile fishes.

Keywords Bigheaded carps · Hypercarbia · Avoidance · Barriers · Stress · Genes · Juvenile · Larval

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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 negatively impacting the abundance and distribution of native fishes, which, in turn, has led to 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 shaped 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 melanostoma*), sea lamprey (*Petromyzon marinus*), and dreissenid mussels] within these two regions over the past 50 years

(Rasmussen et al. 2011; Ricciardi and MacIsaac 2011). Given the ecological and economic burdens associated with invasive species, residents of the Great Lakes are particularly concerned by the potential invasion of silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*), non-native invasive fishes that have established populations within the Mississippi River basin and are currently threatening to spread into the Great Lakes basin. Silver carp and bighead carp (hereafter collectively referred to as bigheaded carps) have quickly become the most abundant fishes in many portions 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). More specifically, bigheaded carps may have direct impacts on the aquatic environment by selectively feeding on small zooplankton and algae, altering water quality (i.e., increased turbidity), which can then negatively impact aquatic macrophytes, larval fishes, and eventually alter the structure of the food web (Kolar et al. 2007). Bigheaded carps have also been shown to have indirect impacts on native fishes, such as bigmouth buffalo (*Ictiobus cyprinellus*) and gizzard shad (*Dorosoma cepadianum*) which have similar diets, with Irons et al. (2007) documenting a decrease in body condition in these fishes following establishment of bigheaded carp within the Illinois River. At present, the extent of the potential impact of bigheaded carps on the Great Lakes ecosystem is not known (Cooke and Hill 2010), but clearly 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 introduction 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 bigheaded carps 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 bigheaded carps into the Great Lakes, previous research has shown that these barriers may not be completely effective in deterring 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 leaving them vulnerable to aquatic invaders (Patel et al. 2010; Rasmussen et al. 2011). Fishes 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 vary based on the targeted fish species, water chemistry, electrical parameters, and distance from 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 deterring small fish relative to larger individuals (Reynolds 1996; Dolan and Miranda 2003). Given that the leading edge of the bigheaded carp invasion is approximately 35 km downstream from the electric dispersal barriers (Patel et al. 2010), there is a critical need to develop additional control techniques to ensure that bigheaded carps will be prevented from spreading into the Great Lakes, with technologies effective against small fishes of particular importance.

Several management solutions are available to increase the effectiveness of the current bigheaded carps 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 between these two basins is 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. A barrier that can not only deter, but 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 (i.e., underwater speakers, hydro-guns) 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, carbon dioxide gas (CO_2) applied to water (i.e., hypercarbia) has shown promise as a chemical means to deter fish movements, by inducing behavioral and physiological impacts on fish (Clingerman et al. 2007; Hasler et al. 2009). Initially, elevated CO_2 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). If fish are resistant to the avoidance aspect of the CO_2 chemical barrier, prolonged exposure to hypercarbia may lead to unconsciousness due to impairments in brain electrical activity (Iwama et al. 1989; Yoshikawa et al. 1991, 1994). Previous research has shown that CO_2 added to water at approximately 100 mg/L resulted in adult fishes, including bigheaded carps, '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; Dennis et al. 2014), suggesting that CO_2 added to water has the potential to influence the movement of bigheaded carps. 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 all size classes of fishes.

The 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. For these first two studies, a suite of functionally distinct gene transcripts were examined to provide a broad perspective of the molecular stress responses (i.e., handling stress, hypoxia, hypercarbia) in larval and juvenile fishes following acute hypercarbia exposure. The final study exposed juvenile fishes to a hypercarbic environment to determine if elevated CO_2 would cause juveniles to avoid an area.

Materials and methods

Hypercarbia challenge: fry

Experimental animals

Silver and bighead carp fry were used for the fry hypercarbia challenge experiments. Mature bighead carp and silver carp males ($n = 7$) and females ($n = 4$) 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 450 L round incubation tanks with center air diffusion and were supplied with water from a nearby pond. Water temperature in the incubation tanks was maintained at 27 °C (±2 °C) and larvae were not given supplemental food prior to experimentation. Approximately 8 days following fertilization (183–189 h), hatched fry were carefully netted from the incubation tank and subjected to a hypercarbia challenge.

Hypercarbia challenge

The experimental design used to expose 8 days old, 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 180, 266 mL perforated, individually numbered plastic cups. The cups 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. A total of three replicate coolers were utilized for each treatment to minimize the potential of a cooler effect, and ten 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 (°C) and dissolved oxygen concentrations (mg/L O_2) 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 (mg/L CO_2) and total alkalinity (TA) concentrations (mg/L $CaCO_3$) 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). 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 generated by a compressed air blower was provided throughout the challenge to ensure that fry were not subjected to hypoxia [mean = 7.60 mg/L O_2 ± 0.02 mg/L standard error (SE)]. At the conclusion of the treatment, 3–4 perforated cups were randomly removed from each cooler, using a random number generator to select cups, until a total of ten 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 1–14 days per manufacturer's recommendations, and subsequently frozen at −80 °C until gene expression analyses. Fry in the ambient (control) treatment were allowed to remain in their cups undisturbed (i.e., 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 fish species were used in this juvenile hypercarbia challenge experiment: largemouth bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*), 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 using 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 bigheaded 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 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 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. Following this 24 h acclimation period, each container was randomly assigned to one of six treatments identical to the hypercarbia challenge described above for 8 days 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). 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). Fish in the control (ambient) treatment were allowed to remain undisturbed in their containers (i.e., 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 (N = 10 for each experimental treatment) 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 (0.01 g). 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).

Hypercarbia avoidance: juveniles

Experimental animals

Four fish species were used for the juvenile hypercarbia avoidance experiment: largemouth bass, bluegill, silver carp, and bighead carp, and the fish used in this experiment were from the same population as those used in the juvenile hypercarbia challenge experiment listed above.

Hypercarbia avoidance challenge

Hypercarbia avoidance was quantified using a 'shuttle box' choice arena (Loligo Inc., Hobro, Denmark), consisting of two holding tanks (1.5 m diameter, 0.5 m depth) connected by a narrow central tunnel (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 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 'shuttle box' 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. Following the acclimation period, the buffer chamber associated with the holding tank that contained the fish received a continuous addition of dissolved CO_2 gas (i.e., increasing CO_2 concentration on that side of the 'shuttle box'), while the buffer chamber for the holding tank without the fish received a continuous addition of compressed air to strip CO_2 from the water (i.e., maintaining ambient CO_2 concentration on the other side of the 'shuttle box'). During the addition of CO_2, the time was recorded when the fish shuttled to the opposite holding tank (i.e., side of the 'shuttle box' with ambient CO_2 concentration), or when the fish lost equilibrium. Concurrently, water quality measurements were collected from water flowing into the buffer chamber associated with the tank that was receiving inputs of CO_2. If/when a fish shuttled to the opposite holding tank (i.e., tank receiving compressed air), both buffer chambers were supplied with compressed air for 10 min to strip CO_2 from the 'shuttle box' choice arena (i.e., decreasing CO_2 concentration in both

holding tanks and allowing water chemistry return to control conditions). After this 10 min period, the tank in which the fish had settled was treated with CO_2 gas (i.e., increasing CO_2 concentration), while the opposite tank was supplied with compressed air to maintain ambient CO_2 concentrations on that side of the 'shuttle box'. The trial was repeated in this manner until the individual shuttled a total of 6 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. For each species, ten individuals were subjected to the hypercarbia avoidance challenge.

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 1 mL 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 was 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 using 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 used to create qPCR primers for juvenile largemouth bass included: *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 used to create qPCR primers for fry and juvenile silver carp and bighead carp included: *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 1.

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 1 µM 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

Table 1 Quantitative real-time PCR primer sets for silver carp, bighead carp, largemouth bass, and bluegill

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	

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

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 8 days 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 or 60 min), and their interaction (CO_2 concentration × duration of exposure) entered as fixed effects and cooler number as a random effect (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). 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). 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 avoidance responses (i.e., lowest, greatest, and mean CO_2 concentration necessary to induce shuttling; time spent in elevated CO_2 by individuals prior to shuttling for either a) the entire duration of the hypercarbia avoidance trial or b) at the greatest CO_2 concentration; total CO_2 exposure time; total number of successful shuttles) were made across species using a one-way ANOVA (with fish identification number entered as a random effect to account for multiple measurements collected from each individual, as needed), followed by a Tukey–Kramer HSD post hoc test to separate means (Sokal and Rohlf 1995).

Data were log10 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 one-way or two-way Kruskal–Wallis test (Zar 1984; Sokal and Rohlf 1995) was performed. 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).

All means are reported ± 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 JMP version 9.0.2 (SAS Institute Inc., Cary, NC, USA). All tests were run at a significance level (α) of 0.05.

Results

Water quality measurements at the conclusion of the hypercarbia challenge on 8 days old silver carp and bighead carp, along with juvenile silver carp, bighead carp, bluegill, and largemouth bass, are presented in

Table 2 Water quality measurements at the conclusion of the hypercarbia challenge for both fry and juvenile fishes

Location	Species	Life stage	Final temp (°C)	Final dissolved oxygen (mg/L)	Final pH	Final total alkalinity (mg/L)	Final dissolved CO_2 (mg/L)	Final pCO_2 (μatm)
Osage Beach, MO	BHC	Fry	24.8 ± 0.0	7.6 ± 0.0	C1—8.13 ± 0.03	C1—246 ± 3	C1—40 ± 1	C1—2454 ± 170
	SLC				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—11,499 ± 545
					L2—7.39 ± 0.01	L2—241 ± 3	L2—74 ± 0	L2—13,100 ± 373
					H1—6.88 ± 0.01	H1—243 ± 3	H1—123 ± 2	H1—42,266 ± 460
					H2—6.86 ± 0.01	H2—241 ± 3	H2—123 ± 1	H2—43,567 ± 400
Urbana, IL	LMB	Juveniles	17.0 ± 0.1	8.9 ± 0.1	C1—8.28 ± 0.02	C1—148 ± 2	C1—15 ± 0	C1—919 ± 40
	BLG				C2—8.26 ± 0.01	C2—147 ± 2	C2—15 ± 0	C2—961 ± 40
					L1—6.64 ± 0.01	L1—153 ± 2	L1—74 ± 1	L1—41,827 ± 939
					L2—6.64 ± 0.01	L2—159 ± 2	L2—73 ± 1	L2—43,181 ± 1134
					H1—6.45 ± 0.01	H1—145 ± 3	H1—112 ± 1	H1—61,967 ± 2241
					H2—6.44 ± 0.01	H2—150 ± 2	H2—115 ± 1	H2—65,370 ± 1734
La Crosse, WI	SLC	Juveniles	16.0 ± 0.0	8.4 ± 0.0	C1—8.37 ± 0.02	C1—156 ± 3	C1—15 ± 1	C1—782 ± 26
	BHC				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—73,127 ± 1243
					L2—6.41 ± 0.01	L2—162 ± 2	L2—70 ± 1	L2—74,270 ± 1023
					H1—6.00 ± 0.01	H1—161 ± 2	H1—122 ± 1	H1—188,993 ± 2745
					H2—6.02 ± 0.01	H2—166 ± 1	H2—120 ± 1	H2—186,435 ± 2978

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) 8 days 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. Water quality measurements are presented as the mean value, along with ±1 standard error to display variation in measurements

Table 2. Values for pCO_2, presented in Table 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). pCO_2 data should be interpreted cautiously, however, as water carbonate chemistry may not have reached equilibrium at the time of measurement (Gattuso et al. 2010), and, 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 treatments across studies.

Bighead carp fry upregulated *hsp70* transcripts approximately fourfold 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 (two-way ANOVA, $F_{[2]} = 0.38$, $P > 0.05$) (Fig. 1a). However, the abundance of *hsp70* mRNA in silver carp fry significantly increased threefold following exposure to 120 mg/L CO_2 relative to fry in the ambient treatment (two-way ANOVA, $F_{[2]} = 5.61$, $P = 0.0211$) (Fig. 1b). Bighead and silver carp fry transcript levels for *c-fos* and *gr-2* did not differ significantly between treatment groups following hypercarbia exposure (two-way ANOVAs, F values <3.40, $P > 0.05$) (Table 3). 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 (two-way ANOVA, $F_{[1]} = 11.52$, $P = 0.0081$) (Table 3); 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 in response to an exposure to hypercarbia relative to bighead carp fry exposed to ambient CO_2 (two-way ANOVA, $F_{[2]} = 0.71$, $P > 0.05$) (Table 3).

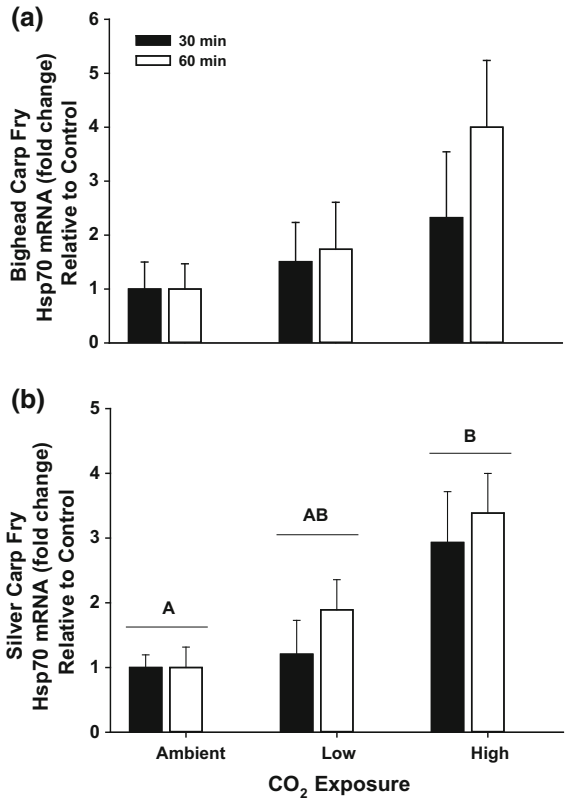

Fig. 1 Relative mRNA expression for *hsp70* in bighead carp 8 days old fry (**a**) and silver carp 8 days old fry (**b**) exposed to two hypercarbia concentrations. Relative mRNA expression of fry exposed for 30 min are in *black bars*, and fry exposed for 60 min are in *white bars*. *Horizontal lines* denote a significant CO_2 concentration effect across exposure durations within a species. Data are mean ± 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

The concentration of *c-fos* transcripts from gill tissue in juvenile bighead carp was elevated approximately sixfold in fishes subjected to 60 min exposure to both 70 mg/L CO_2 and 120 mg/L CO_2 compared to juvenile bighead carp that only received ambient water (two-way Kruskal–Wallis test, $\chi^2_{[2]} = 6.01$, $P = 0.0496$) (Fig. 2a). Similarly, silver carp juveniles increased gill *c-fos* mRNA expression roughly threefold following a 30 min exposure to 120 mg/L CO_2, and approximately sevenfold at 60 min exposures to both 70 mg/L and 120 mg/L CO_2 when compared to the ambient treatment (two-way Kruskal–Wallis test, $\chi^2_{[2]} = 5.75$, $P = 0.0056$) (Fig. 2b). 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 (two-way Kruskal–Wallis test, $\chi^2_{[2]} > 37.60$, $P < 0.0001$) (Fig. 2c, d). 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 (two-way ANOVA, $F_{[1]} = 7.32$, $P = 0.0092$) (Table 4). 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 (two-way ANOVA,

Table 3 Relative gene expression values from bighead carp and silver carp fry exposed to two concentrations and durations of elevated CO_2

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 ± 0.07^{+}	0.72 ± 0.09^{+}	0.83 ± 0.08^{+}	1.00 ± 0.11†	1.04 ± 0.10†	1.23 ± 0.17†

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. Data are the mean ± SE, calculated relative to the expression of *18s* for bighead carp and silver carp fry. $P < 0.05$ for all significant comparisons

$F_{[2]} = 3.36$, $P = 0.0423$) (Table 4). 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 (two-way ANOVA, $F_{[1]} = 7.86$, $P = 0.0070$) (Table 4), however there was no significant interaction (CO_2 concentration × duration) and CO_2 concentration, as a main effect, was also not statistically significant. The abundance of *hif1-α* transcripts in the gill tissue of juvenile bighead carp and bluegill did not differ among the groups (two-way ANOVAs, $F_{[5]} < 2.30$, $P > 0.05$) (Table 4). Similarly, gill transcript levels for *gr-2* and *hsp70* for all four species did not differ significantly across groups following the hypercarbia challenge (two-way ANOVAs, $F_{[5]} < 11.06$, $P > 0.05$) (Table 4). 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 ANOVA, $F < 1.7644$, $P > 0.05$.

Initial water quality measurements during the acclimation period of the juvenile hypercarbia avoidance experiment for bluegill, largemouth bass, silver carp, and bighead carp 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), and 147 mg/L $CaCO_3$ (±2 mg/L). 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. Fish sizes within species for the hypercarbia avoidance challenge were as follows: largemouth bass, 104 ± 1 mm; bluegill, 105 ± 1 mm; silver carp, 67 ± 1 mm; and bighead carp, 71 ± 1 mm.

Juvenile bluegill, largemouth bass, silver carp, and bighead carp 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 (one-way repeated measures ANOVA, $F_{[3]} = 1.84$, $P > 0.05$) (Fig. 3). Juvenile bighead carp spent approximately 60 % more time in elevated CO_2 prior to shuttling to the opposite side of the 'shuttle box' compared to juvenile silver carp, bluegill, and largemouth bass (one-way repeated measures ANOVA, $F_{[3]} = 6.01$, $P = 0.0020$) (Fig. 3). The threshold CO_2 concentration that elicited avoidance behaviors did not differ across the juvenile species (bighead carp, 180 ± 32 mg/L CO_2, silver carp, 125 ± 32 mg/L CO_2, bluegill, 99 ± 22 mg/L CO_2, largemouth bass, 111 ± 23 mg/L CO_2; one-way ANOVA, $F_{[3]} = 1.66$, $P > 0.05$). The greatest CO_2 concentration that individuals experienced prior to eliciting an avoidance behavior did not statistically differ across the juvenile species (bighead carp, 257 ± 6 mg/L CO_2, silver carp, 248 ± 20 mg/L CO_2, bluegill, 222 ± 20 mg/L CO_2, largemouth bass, 280 ± 30 mg/L CO_2; one-way Kruskal–Wallis

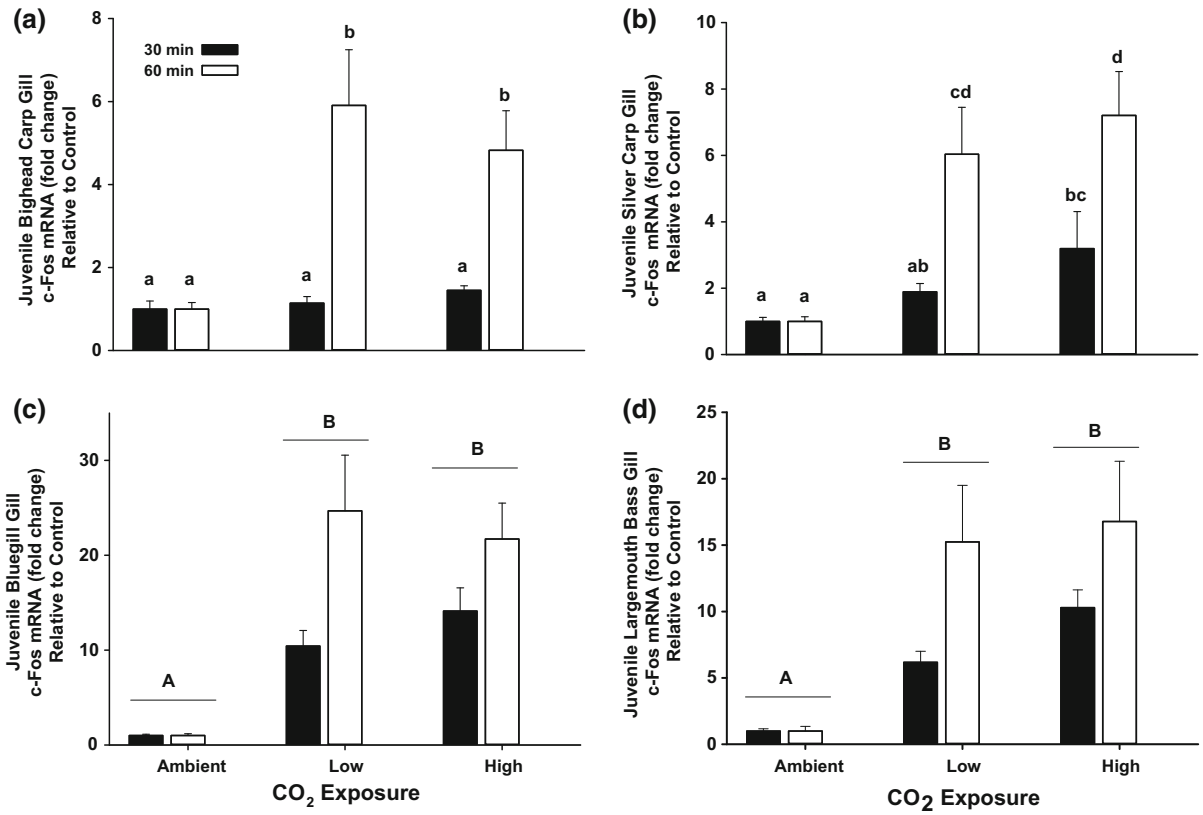

Fig. 2 Relative expression of *c-fos* mRNA extracted from the gill tissue of juvenile bighead carp (**a**), silver carp (**b**), bluegill (**c**), and largemouth bass (**d**) exposed to a two hypercarbic treatments. Relative mRNA expression of juvenile fish that had an exposure duration of 30 min are 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 mean ± 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

test, $\chi^2_{[3]} = 3.74$, $P > 0.05$). Juvenile bighead carp spent approximately 70 % more time at the highest CO_2 concentration that elicited avoidance reactions compared to juvenile bluegill (one-way ANOVA, $F_{[3]} = 4.18$, $P = 0.0123$) (Fig. 4). Juvenile bluegill nearly had triple the amount of successful shuttles compared to juvenile bighead carp, 4.6 shuttles compared to 1.7 shuttles on average (one-way ANOVA, $\chi^2_{[3]} = 14.15$, $P = 0.0027$) (Fig. 4). Finally, the total duration of time that individuals spent in elevated CO_2 during the entire hypercarbia avoidance challenge was not significantly different across the juvenile species examined (bighead carp, 29 ± 4 min, silver carp, 32 ± 3 min, bluegill, 31 ± 3 min, largemouth bass, 36 ± 4 min; one-way ANOVA, $F_{[3]} = 0.74$, $P > 0.05$).

Discussions

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), and many studies have assessed avoidance behaviors that are initiated upon encountering elevated CO_2 concentrations in a variety of aquatic vertebrates and invertebrates (Jones et al. 1985; Ross et al. 2001;

Table 4 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

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	$1.00 \pm 0.20^{+,a,b}$	$1.09 \pm 0.26^{+,b}$	$0.67 \pm 0.07^{+,a}$	$1.00 \pm 0.09^{\dagger,a,b}$	$1.89 \pm 0.35^{\dagger,b}$	$1.11 \pm 0.13^{\dagger,a}$
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	$1.00 \pm 0.04^{+}$	$0.88 \pm 0.05^{+}$	$0.96 \pm 0.04^{+}$	$1.00 \pm 0.06^{\dagger}$	$1.11 \pm 0.05^{\dagger}$	$1.08 \pm 0.06^{\dagger}$
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

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. Data are the mean ± SE, calculated relative to either *ef1-α* for juvenile bluegill or *18s* for juvenile bighead carp, silver carp, and largemouth bass. $P < 0.05$ for all significant comparisons

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 to 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. In the current study, 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. Results from this study suggest that juvenile fishes likely require greater concentrations of dissolved CO_2 to induce active avoidance behaviors compared to adult fishes, but avoidance behaviors were still observed. Interestingly, juvenile bighead carp had greater tolerance to elevated CO_2 zones (i.e., greater CO2 concentration to elicit avoidance responses, greater duration of exposure to elevated CO_2 between shuttles) compared to juvenile silver carp, bluegill, and largemouth bass. However, this greater tolerance to elevated CO_2 for juvenile bighead carp appears to have a trade-off, as the number of successful shuttles that individual bighead carp were able to perform during the hypercarbia avoidance challenge was significantly less than the other juvenile fishes examined, and all bighead

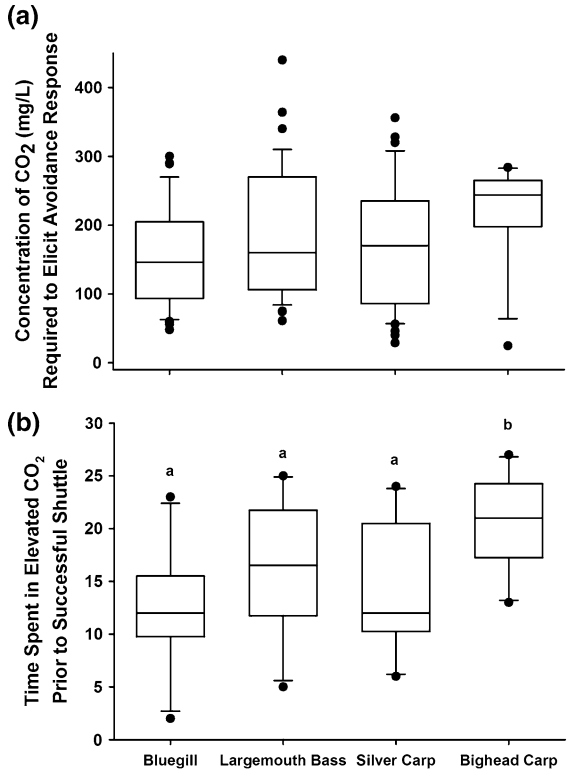

Fig. 3 Concentration of CO_2 at which bluegill, largemouth bass, silver carp, and bighead carp displayed active shuttling behavior (**a**) and the duration of time individuals spent in elevated CO_2 prior to shuttling (**b**) from a high CO_2 environment to a lower CO_2 environment during the course of the hypercarbia avoidance trial. *Error bars* show 1 SE around the mean. *Dissimilar lower case letters* (*a*, *b*) denote statistically significant differences between species. Sample size is ten fish for all four species, and approximately four measurements were collected from each subject

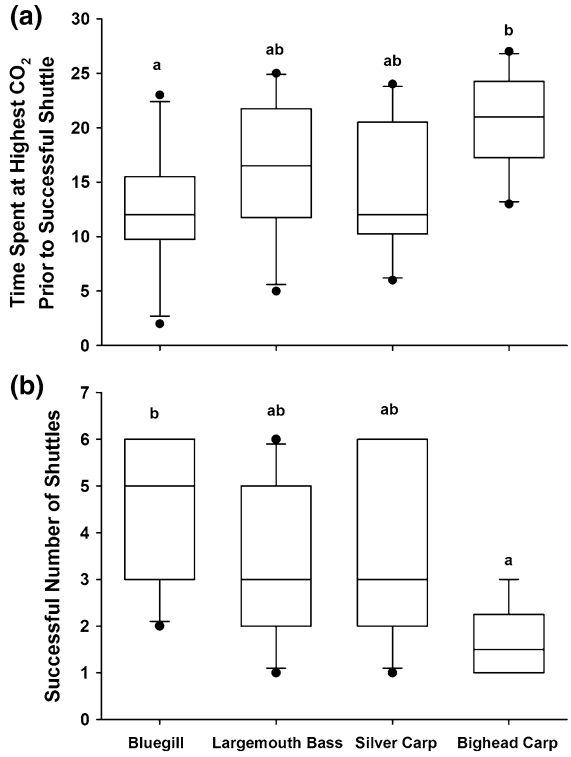

Fig. 4 Duration of time that juvenile bluegill, largemouth bass, silver carp, and bighead carp spent at the highest CO_2 concentration prior to shuttling (**a**) and the total number of successful shuttles (**b**) observed in fishes subjected to the hypercarbia avoidance challenge. *Dissimilar lower case letters* (*a*, *b*) denote statistically significant differences between species. *Error bars* show 1 SE around the mean. Sample size is ten fish for all four species

carp lost consciousness during the challenge after approximately 30 min in elevated CO_2 waters. This would suggest that a CO_2 barrier would need to apply approximately 215 mg/L CO_2 to waters to effectively deter bigheaded carps. 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 bluegill, largemouth bass, silver carp, and bighead carp 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 sixfold 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) group. 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). Previous studies have shown that gene transcripts such as *gr*-2 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 1988; Kassahn et al. 2009), in this case hypercarbia. 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 bigheaded carps (i.e., greater relative increase in *c-fos* transcripts, expression of *c-fos* mRNA at lower concentrations 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 threefold 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 bigheaded carps 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 bigheaded carps movement. Results from the juvenile hypercarbia avoidance challenge support this statement, as juvenile bighead carp were the only species to have all individuals lose equilibrium during the trial (after approximately 30 min exposure to elevated CO_2 waters) and had the least number of successful shuttles (<2). While additional research is necessary to link CO_2 detection to increased *c-fos* expression (although see Sato et al. (1992)), delayed avoidance responses observed in juvenile bighead carp could potentially be due to delayed *c-fos* expression (i.e., upregulation at only 60 min exposure) in the gills of bighead carp compared to juvenile silver carp, bluegill, and largemouth bass.

Similar to the juvenile fish species studied, 8 days 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 twofold following exposure to 70 mg/L CO_2 and nearly threefold 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 fourfold induction of *hsp70* mRNA, although this increase was not statistically significant relative to ambient controls. The candidate gene transcripts used in this study (*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 8 days old bigheaded carps larvae, as seen through increases in *hsp70* mRNA. Heat shock protein transcripts are typically induced to maintain proper functioning of the cell by facilitating the 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 bigheaded carps 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 bigheaded

carps to maintain 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 8-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.

Interestingly, neither *gr-2* nor *hif1-α* transcripts responded to an acute exposure of hypercarbia for the larval (i.e., silver carp and bighead carp fry) and juvenile fishes (i.e., bluegill, largemouth bass, silver carp, and bighead carp) 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 1 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 twofold 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 bigheaded carps. 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 bigheaded carps. Juvenile silver carp and bighead carp will likely choose to avoid areas of high CO_2, and both larval and juvenile bigheaded carps experience physiological disturbances when placed in elevated CO_2 environments. If these 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. There are a number of strengths that would support the use of elevated CO_2 as a barrier to deter invasive fish movement and spread. For example, CO_2 is relatively easy to apply to water (i.e., airstones attached to CO_2 gas cylinders) and can be placed in remote areas, as little infrastructure is required to operate a CO_2 barrier. While the CO_2 concentrations to elicit multiple, repeatable avoidance reactions in fish are significantly greater than what fishes would experience in the wild, these concentrations can be achieved by pumping CO_2-infused water (i.e., injecting CO_2 gas into highly pressurized water resulting in water supersaturated with CO_2) and large bodies of water can relatively quickly reach and maintain CO_2 concentrations used in this study (Suski et al. *Unpublished Data*). A CO_2 barrier could also be designed to either just deter fish movement, at which the CO_2 concentration to be targeted may be the slightly above the threshold that induces avoidance behaviors (approximately 130 mg/L CO_2 in the current study), or block fish passage, at which the CO_2 concentration to be targeted may be the greatest CO_2 concentration that

induces avoidance responses (approximately 250 mg/L CO_2 in the current study) and allow the contact with elevated CO_2 be sufficiently long enough to cause fishes to lose equilibrium. A CO_2 barrier should also be effective at deterring fish movement across species and life-stage, which, for areas that historically were never hydrologically connected (i.e., Great Lakes and Mississippi River basins), may make CO_2 a more effective deterrent compared to other non-physical deterrents, such as sound or light.

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, especially where fish may naturally wish to inhabit (i.e., areas that may protect from predation or contain high quality spawning habitat) to ensure that a CO_2 barrier would still be effective. 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. Despite these challenges, elevated CO_2 has the potential to act as a non-physical barrier to influence the movement of early-life stage bigheaded carps, which could work to enhance existing control efforts to prevent the spread of invasive carps into the Great Lakes.

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