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Molecular responses of fishes to elevated carbon dioxide

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ABSTRACT

Hypercarbia, or elevated carbon dioxide, is an environmental challenge that can have detrimental effects on the physiology and performance of aquatic organisms. With aquatic hypercarbia predicted to become more prevalent in the future due to global climate change, it is important to quantify how hypercarbia impacts aquatic organisms, especially fish. The impact of hypercarbia on the behavior and physiology of fishes has been well studied, but relatively few studies have examined the molecular processes that underlie resulting behavioral and physiological changes. In an effort to define the molecular response of fishes to acute hypercarbia exposure, bluegill (*Lepomis macrochirus*) and silver carp (*Hypophthalmichthys molitrix*) were exposed to either 30 mg L^{-1} CO_2 ($pCO_2 \approx 15{,}700$ μatm) or ambient (10 mg L^{-1} CO_2; $pCO_2 \approx 920$ μatm) conditions for 1 h and the expression of a variety of genes, across three tissues, were compared. Exposure to 30 mg L^{-1} CO_2 in bluegill and silver carp resulted in an increase in *c-fos*, *hif1-α*, and *gr-2* transcripts, while silver carp alone showed increases in *hsp70* and *hsc70-2* mRNA. This study demonstrates that acute hypercarbia exposure impacts gene expression in a species and tissue specific manner, which can be useful in identifying potential mechanisms for hypercarbia tolerance between species, and pinpoint specific tissues that are sensitive to hypercarbia exposure.

1. Introduction

Environmental stress is a ubiquitous challenge for aquatic organisms, and exposure to stress can translate into a suite of molecular, biochemical, and behavioral changes. The perception of an external stressor initially involves the activation of receptors and release of corticosteroids, which can lead to changes in the molecular expression of processes, involved in oxygen transport, metabolism, osmoregulation, and eventually changes in the whole animal performance and behavior (Barton, 2002). Following the perception of a stressor, organisms have the ability to alter genetic and physiological systems to maintain homeostasis in the face of environmental challenges (Barton, 2002; McEwen and Wingfield, 2003). In addition, animals can display behavioral avoidance to escape poor quality environments and avoid potential energetic costs associated with inhabiting sub-optimal areas (Kieffer and Cooke, 2009). Due to anthropogenic-driven environmental challenges, such as global climate change and degraded water quality, determining the capacity of aquatic organisms to respond to environmental stressors will become increasingly important in the future.

Aquatic hypercarbia, both naturally-occurring and anthropogenically induced, can be an environmental challenge for aquatic organisms. Elevated dissolved CO_2 concentrations can occur in both freshwater and marine environments, especially in estuarine waters and coastal upwelling zones (Feely et al., 2008; Thomsen et al., 2010), due to a wide range of factors including thick surface vegetation, insufficient water mixing, and the respiratory processes of microbes (Heisler et al., 1982; Ultsch, 1996). In addition to natural occurrences, aquatic hypercarbia can be created through anthropogenic means, such as through intensive aquaculture practices (Colt and Orwicz, 1991; Kristensen et al., 2009) or novel chemical fish deterrent systems (Clingerman et al., 2007; Kates et al., 2012). Due to the wealth of sources for aquatic hypercarbic environments, many studies have investigated the impact of elevated CO_2 on marine and freshwater fishes and demonstrated a variety of physiological impacts on fishes including respiratory acidosis (Iwama et al., 1989; Bernier and Randall, 1998), metabolic acidosis (Bernier and Randall, 1998), ion imbalance (Brauner et al., 2000), and activation of stress hormones (Iwama et al., 1989). Exposure to hypercarbia has also been shown to influence ventilation amplitude and frequency in an effort to offset elevated internal CO_2 concentrations (Gilmour, 2001; Gilmour and Perry, 2007). Elevated dissolved CO_2 concentrations can also impair the ability of larval and juvenile marine fishes to detect auditory cues, locate refuge and settlement areas, and avoid predation, resulting in potential reductions in fish populations and recruitment (Munday et al., 2009, 2010; Dixson et al., 2010; Simpson et al., 2011). More importantly, hypercarbic environments are expected to become more prevalent in the future due to global climate change (Feely et al., 2008), making elevated environmental CO_2 a growing concern that has potential to impart negative impacts on both individual fish (Baumann et al., 2012; Esbaugh et al., 2012) and fish populations (Munday et al., 2010).

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While the physiological and behavioral impacts of hypercarbia exposure for fishes are well understood, currently relatively little work has been performed to define the molecular underpinnings that drive many of the physiological and behavioral adjustments that have been observed following hypercarbia exposure (Rimoldi et al., 2009). Therefore, determining the molecular response of fishes to elevated carbon dioxide will provide critical information on 1) how hypercarbia exposure affects different fish species at the molecular level; 2) what specific genetic pathways are being activated following hypercarbia exposure; and 3) mechanisms behind species-specific tolerance to elevated CO_2 environments.

Based on this background, the objective of the current study was to characterize stress-related gene expression patterns (i.e., upregulation/downregulation, tissue-specific expression, species-specific expression) in fishes exposed to an elevated carbon dioxide environment. In an effort to better understand the transcriptional changes induced upon an acute hypercarbia stressor, a suite of functionally distinct gene transcripts were examined to provide a broad perspective of the molecular stress responses in adult fishes, compared to many previous studies that often focus on candidate genes within a target gene family (Ali et al., 2003; Lund et al., 2003; Rimoldi et al., 2012). One of the few gene transcripts that have been linked to hypercarbia exposure is *c-fos*, an immediate early gene transcript that is rapidly induced once animals are exposed to hypercarbia (Sato et al., 1992; Tankersley et al., 2002; Rimoldi et al., 2009). The product of this gene, the c-Fos protein, is a nuclear factor that enhances the transcription of multiple genes (Curran and Franza, 1988) and may potentially modify ventilation behavior in response to hypercarbia (Rimoldi et al., 2009). Hypoxia-inducible factor 1 alpha (*hif1-α*) is another transcription factor that enhances the expression of several genes, however this gene transcript is typically induced following hypoxia stress (Nikinmaa and Rees, 2005). Previous research has demonstrated that acute hypercarbia in fishes will decrease blood pH (Iwama et al., 1989), which theoretically results in a loss of efficiency in hemoglobin oxygen uptake and delivery to the tissues (Root and Bohr effects). For adult bluegill and silver carp, Kates et al. (2012) found that exposure to 30 mg L^{-1} CO_2 resulted in an increased hematocrit concentration with the authors suggesting that this resulted from impaired oxygen uptake coupled with reductions in ventilation rates. Therefore, *hif1-α* mRNA expression was quantified to determine whether oxygen transport or uptake was impacted due to blood acidosis caused by an acute exposure to hypercarbia. Along with potential impairments to oxygen delivery mechanisms, previous work has also shown that acute exposure to elevated CO_2 environments can result in disruptions in blood chemistry, such as increased cortisol and glucose concentrations (Iwama et al., 1989; Ross et al., 2001). To determine whether cortisol may be directly influencing transcriptional regulation, glucocorticoid receptor isoform 2 (*gr-2*) mRNA expression was examined. The product of this gene, the GR-2 protein, is localized on the cell membrane of all tissues and binds free cortisol in the blood stream resulting in the activation of several effector genes that regulate responses to a general stressor (i.e., ion maintenance, increased metabolism, decreased growth, and changes in behavior) (Mommsen et al., 1999). Heat-shock protein 70, which mediates the repair and degradation of altered proteins (Iwama et al., 2004), can also be induced following a stressor of sufficient intensity and duration to cause proteins to denature or become altered and lose functionality. As such, heat-shock protein 70 (*hsp70*) and heat-shock cognate 70 isoform 2 (*hsc70-2*) transcripts were examined to determine whether exposure to an acute hypercarbia stressor impacted cellular protein functioning resulting in the upregulation of heat shock proteins. While the synthesis of these transcripts (mRNA) does not confirm any functional impact and does not always correlate with changes in protein concentrations, they do represent the initial cellular response to an acute stressor. We hypothesized that an acute hypercarbia exposure would increase transcript abundance of *c-fos*, *hif1-α*, and *gr-2* due to molecular responses targeting hypercarbia-specific, hypoxia-specific, and general-stress related transcription factors. Expression of *hsp70* and *hsc70-2* transcripts, however, were expected to remain at control levels following hypercarbia exposure due to previous research on acute thermal stress showing that *hsp70* can take several hours to reach peak expression levels (Lund et al., 2003; Lewis et al., 2010). We also hypothesized that gene expression within the gill tissue would be altered to a greater extent than the heart or erythrocyte tissue due to the gills' direct contact with the hypercarbic stressor.

To test these hypotheses, two species of fish, bluegill (*Lepomis macrochirus*) and silver carp (*Hypophthalmichthys molitrix*), were given an acute sub-lethal hypercarbia challenge, and the expression of several genes, across three tissues, were quantified. Silver carp and bluegill were ideal species to use in this study for the following reasons: 1) these are evolutionarily divergent species (Betancur et al., 2013), making general trends in gene expression data applicable across a broad range of species; 2) previous research by Kates et al. (2012) has shown that during a common hypercarbia exposure, bluegill reduced ventilation rates while all silver carp lost consciousness during the trial suggesting that silver carp have greater sensitivity to elevated CO_2 relative to bluegill; and 3) assessing the impact of realistic acute elevated CO_2 concentrations on bluegill may be useful for aquaculture managers rearing bluegill. Results will also have important implications for understanding how fish respond at the molecular level to acute hypercarbia exposure, and how these changes in gene expression might confer hypercarbia tolerance.

2. Materials and methods

2.1. Experimental animals

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, in October–November, 2010. Silver carp were collected from a variety of locations in east central Illinois along the Illinois River by using standard pulsed direct current (DC) boat electroshocking in a 5.5 m flat bottom aluminum boat. As per standardized long-term collection protocols, electroshocking parameters (i.e. voltage, amperage, and wavelength) were adjusted daily to maintain a performance of approximately 3000 Watts (W) based on water chemistry parameters on site (Gutreuter et al., 1995). Once captured, silver carp were transported to the Aquatic Research Facility in a 640 L truck bed hauler in ambient water supplied with compressed oxygen gas to near saturation to minimize transport stress. At the Aquatic Research Facility, all fish were housed outdoors in round plastic holding tanks (1280 L, 1.7 m diameter) connected to a 0.04 ha natural, earthen-bottom pond with abundant vegetation. Water was supplied to the tanks from the pond, and allowed to drain back into the pond providing sufficient water replacement and nitrogenous waste removal. Tanks also received supplemental aeration from a low-pressure air blower. Bluegill were acclimated to the outdoor holding system for 4 weeks and fed pelleted food (Dense Culture Food, F2C, Aquatic Ecosystems, Apopka, FL, USA) until satiation every other day, while silver carp were only held for a period of 2–4 days prior to being used in this experiment and did not receive supplemental food during laboratory confinement. All fish received a minimum of 48 h acclimation time, without food, prior to experiments to ensure sufficient time for recovery from disturbances associated with capture, hauling, acute stress, and food digestion (Milligan, 1996; Lund et al., 2003; Suski et al., 2006). During holding, water temperatures averaged 14.1 °C (±1.1 °C, standard error, SE) and dissolved oxygen averaged 9.5 mg L^{-1} (±0.4 mg L^{-1} SE).

2.2. Hypercarbia challenge

Prior to the start of the hypercarbia challenge, fish were carefully netted from the holding tank and placed into individual opaque, sensory

deprivation containers continuously supplied with fresh water from a central basin. Water was allowed to overflow from each container and drain back into the central basin forming a closed, recirculating system (Vanlandeghem et al., 2010; Kates et al., 2012). Containers were sized appropriately to house fish of each species (2.25 L per bluegill and 24 L per silver carp), contained an airstone to maintain adequate oxygenation, and were outfitted with a tight-fitting lid to ensure that fish could not escape during the trial. Fish were allowed to acclimate to their containers for 24 h, and dissolved oxygen concentrations during this acclimation period remained at 9.9 ± 0.3 mg L^{-1} (SE). Following the 24 h acclimation period, baseline water quality measurements were taken from an extra test chamber within the system to ensure that monitoring of water quality did not impact test subjects. Water temperature and dissolved oxygen concentrations were measured with a portable meter (YSI, 550A Yellow Springs Instruments, Irvine, CA, USA), and pH was quantified using a handheld pH meter (WTW pH 3310 meter with a SenTix 41 probe, Germany). Hypercarbia was achieved by bubbling compressed CO_2 gas into the water in the central basin, which was then pumped into the individual containers. Dissolved CO_2 concentrations, verified using a CO_2 digital titration kit (Hach Company, Titrator model 16,900 and kit No. 2272700, Loveland, CO, USA), and pH measurements, obtained through the use of the handheld pH meter, were used to develop a relationship between these two water parameters (American Public Health Association American Water Works Association and Water Environment Federation, 1998; Summerfelt and Sharrer, 2004; Clingerman et al., 2007). For the hypercarbia challenge, dissolved CO_2 concentrations were allowed to increase from an ambient level of approximately 10 mg L^{-1} (mean = 10 mg L^{-1} CO_2; mean pH = 8.74 ± 0.47 SE; $pCO_2 \approx 920$ μatm) to approximately 30 mg L^{-1} (mean = 32 ± 2 mg L^{-1} CO_2 SE; mean pH = 7.04 ± 0.02 SE; $pCO_2 \approx 15{,}700$ μatm) within 2 min. Ambient values of dissolved CO_2 were consistent with previous studies from freshwater environments (Brauner et al., 2000; Clingerman et al., 2007). Target CO_2 concentrations were maintained manually throughout the challenge by monitoring pH levels and providing additions of CO_2 gas when necessary. Aeration was maintained throughout the hypercarbia challenge to ensure that fish were not subjected to hypoxic environments (mean = 9.84 ± 0.09 mg L^{-1} O_2 SE). After a 1 h exposure to this concentration of dissolved CO_2, the flow of water to each individual container was ceased and fish were euthanized by an overdose of anesthetic [250 mg L^{-1} tricaine methanesulfonate (MS-222) buffered with 500 mg L^{-1} sodium bicarbonate] to the individual containers.

Following cessation of ventilation, fish were weighed to the nearest gram, measured (total length in mm), and blood was drawn from the caudal vasculature along the spine of the fish using a 22-gauge needle and a 1 mL syringe rinsed with lithium heparin. Immediately after blood collection, the whole blood was centrifuged at 2000 × gravity (*g*) for 2 min to separate erythrocytes from plasma. Erythrocytes were transferred to 1.5 mL microcentrifuge tubes and immediately placed in liquid nitrogen until later storage at −80 °C. Samples of gill filaments and cardiac muscle, hereafter referred to as gill and heart tissue, were excised and stored in 1.5 mL microcentrifuge tubes filled with 1 mL of ice-cold RNAlater® (AM7021, Life Technologies, Grand Island, NY, USA). Tissue samples were refrigerated between 1 and 14 days, as per the manufacturer's recommendations, and then frozen at −80 °C until further processing (AM7021, Life Technologies, Grand Island, NY, USA). Water quality measurements were collected from individual containers at the conclusion of each test subject's challenge to confirm that proper water chemistry conditions and target carbon dioxide concentrations were achieved during the challenge. Fish in the control (ambient) treatment were allowed to remain undisturbed in their containers with no change in water parameters during the entire duration of the challenge, and were then sampled in an identical manner. Water temperatures across treatments were 14.9 ± 0.1 °C for bluegill and 15.3 ± 0.1 °C for silver carp, and fish size did not vary across treatments within species: bluegill, 151 ± 4 mm, and silver carp, 473 ± 8 mm; one-way analyses of variance, F values <2.5, P values >0.05).

2.3. Laboratory analyses

2.3.1. RNA isolation and cDNA synthesis

All laboratory procedures below adhere to current guidelines for the publication of quantitative real-time PCR (qPCR) studies, as outlined by the following document: The Minimum Information for Publication of Quantitative Real-Time PCR Experiments 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). Erythrocyte total RNA was isolated using an Ambion RiboPure Blood Kit (AM1928, Life Technologies, Grand Island, NY, USA) with the following changes made to the protocol to maximize RNA integrity and quantity: 1) erythrocytes were thawed on ice as RNAlater® was not used prior to storage in liquid nitrogen, and 2) extracted RNA was treated with Ambion DNase (AM1906, Life Technologies, Grand Island, NY, USA) to eliminate any genomic DNA. Total RNA from gill and heart tissues were isolated using an Ambion RiboPure Kit (AM1924, Life Technologies, Grand Island, NY, USA), which involves the addition of bromochloropropane at 4 °C to effectively separate genomic DNA and proteins from RNA during purification. Following RNA isolation, yield and purity of extracted RNA were determined using a Nanodrop ND-1000 UV–Vis spectrophotometer (Peqlab, Erlangen, Germany). RNA integrity was quantified for several tissue samples in the control and hypercarbia challenge using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) to determine the quality of the extracted RNA. The majority of RNA isolates had RNA integrity numbers (RIN) greater than 5 (mean RIN = 7.4 ± 0.2) indicating good quality RNA suitable for qPCR analyses (Fleige and Pfaffl, 2006). 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 1 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 at 85 °C for 5 min to stop the reaction. All cDNA was then stored long-term at −20 °C until qPCR analysis.

2.3.2. Creation of species specific qPCR primers

Species-specific gene sequences, needed to create qPCR primers for non-model species, were obtained using degenerate primers designed from conserved regions of the target gene from multiple species to obtain partial sequences for the target genes (*c-fos*, *hif1-α*, *gr-2*, *hsp70*, *hsc70-2*). Degenerate primers for *c-fos* were obtained using sequences from grass carp (*Ctenopharyngodon idella*) (accession no. DQ298418) and goldfish (*Carassius auratus*) (accession no. AB111051) for silver carp, while orange-spotted grouper (*Epinephelus coioides*) and European seabass (*Dicentrarchus labrax*) (accession no. DQ838581) sequences were utilized for bluegill. Hypoxia-inducible factor 1 alpha (*hif1-α*) degenerate primers for bluegill were determined using sequences from zebrafish (*Danio rerio*) (accession no. AY326951) and European perch (*Perca fluviatilis*) (accession no. EF100706). Glucocorticoid receptor 2 (*gr-2*) degenerate primers were determined using sequences from common carp (*Cyprinus carpio*) (accession no. AM183668) for silver carp, while Nile tilapia (*Oreochromis niloticus*) (accession no. XM_003446939) and rainbow trout (*Oncorhynchus mykiss*) (accession no. NM_001124482) were used for bluegill. Degenerate primers for heat shock protein 70 (*hsp70*) were determined using sequences from grass carp (accession no. GU475146) for silver

carp, while southern platyfish (*Xiphophorus maculates*) (accession no. AB062114) and Japanese medaka (*Oryzias latipes*) (accession no. AF286875) sequences were used for bluegill. Heat-shock cognate 70 isoform 2 (*hsc70-2*) degenerate primers for silver carp were obtained using sequences from rainbow trout (accession no. NM_001124745) and mummichog (*Fundulus heteroclitus macrolepidotus*) (accession no. DQ202280).

All PCRs were performed using an Eppendorf Mastercycler with Taq DNA polymerase (M0285S, New England BioLabs, Ipswich, MA, USA), the extracted cDNA from each tissue, and the specific degenerate primer pair for the gene of interest. Each PCR reaction were held under the following conditions: one cycle at 95 °C for 30 s followed by 40 cycles of 95 °C for 25 s, 60 °C for 60 s, and 68 °C for 45 s. At the conclusion of the 40 cycles, the reaction was ceased at 68 °C for 5 min. Gel electrophoresis (1.5% agarose gel containing ethidium bromide) was used to separate the PCR products. The targeted DNA fragments were extracted from the gel matrix using a MinElute Gel Extraction kit (#28606, QIAGEN Inc., Valencia, CA, USA) and then purified using a QIAquick PCR Purification kit (#28106, QIAGEN Inc., Valencia, CA, USA). The purified DNA fragments were sequenced using a capillary sequencer (ABI 3730XL, Life Technologies, Grand Island, NY, USA) at the Core DNA Sequencing Facility at the University of Illinois at Urbana-Champaign (Urbana, Illinois). The resulting sequence information for bluegill and silver carp were submitted to the National Center for Biotechnology Information, National Institute of Health's GenBank and are reported in Table 1. These sequences, along with other previously published sequences available on GenBank, were then utilized to create qPCR primers for the target and reference (*ef1*-α) genes using the primer designing software Primer Express (v.2.0.0.0, Life Technologies, Grand Island, NY, USA) and qPCR primers are listed in Table 1.

2.3.3. qPCR analysis

All qPCR reactions were performed using 2 μL cDNA (diluted 1:100 with RNase-free water), 2 μL of each qPCR primers at a 1 μM concentration, 4 μL of RNase-free water, and 10 μL of 2× Power SYBR Green Master Mix (Life Technologies, Grand Island, NY, USA), for a total volume of 20 μL. Gene expression analyses were then conducting using an ABI 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) under the following conditions: 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 60 s. Following the conclusion of these 40 cycles, all PCR products underwent a melt curve analysis (1 cycle at 95 °C for 15 s, 1 cycle at 60 °C for 15 s, and then 1 cycle at 95 °C for 15 s) to confirm the presence of a single amplicon. Gel electrophoresis (3% 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 and reference 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 were then normalized using *ef1*-α as a reference gene due to mRNA levels of EF1-α remaining constant between the control and hypercarbia challenge for all tissues and species (ANOVA $P > 0.05$). Several RNA samples that had not undergone cDNA synthesis were chosen and qPCR was 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).

2.4. Statistical analysis

Comparisons of species-specific gene expression across tissues for each treatment were made using a two-way analysis of variance (ANOVA) with tissue (gill, heart, and erythrocytes), treatment (control and hypercarbia), and their interaction (treatment × tissue) 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 difference (HSD) post hoc test was applied to separate means (Sokal and Rohlf, 1995). Inter-species comparisons in gene expression were not examined as baseline expression of candidate genes and reference genes differ between species and qPCR primers are different for each species, making quantitative comparisons across species challenging. However, qualitative comparisons of gene expression between species were examined. All means are reported ±SE where appropriate and all statistical analyses were performed using JMP version 9.0 (SAS Institute Inc., Cary, North Carolina), all tests were run at a significance level (α) of 0.05.

Table 1
Quantitative real-time PCR primer sets for bluegill and silver carp.

Species	Gene	Sequence 5′ → 3′	Accession number	Fragment length (bp)
Bluegill	*c-fos*	*F*: CCA AAG GCA TCC ACT TTC ACT T *R*: AGA AGA TCG AGT TGG AGG TTG TG	KC493364	63
	hif1-α	*F*: GAC AGC CAC AGA TGA GCA GAC T *R*: AGA TCA AAA CCA GGT ATG GAA TAG GT	KC493362	66
	gr-2	*F*: TGC CGC TTC AGG AAA TGT CT *R*: TCT TGT TTT TCC TCG CTT CCA	KC493363	57
	hsp70	*F*: AGA GAC TGA TTG GGA GAA AGA TG *R*: TCC GTC TCC GAC CAC CTT G	KC493361	82
	hsc70-2	*F*: TCC TCA GAT TGA GGT GAC TTT TGA *R*: CAC CGC AGA CAC GTT CAT G	AB436469	60
	ef1-α	*F*: AAG CCT GGT ATG GTT GTG ACC TT *R*: GCA TCT CAA CAG ACT TGA CCT CAG T	GU385741	66
Silver Carp	*c-fos*	*F*: CAA GAC TGG GAG CCA CTC TAC A *R*: GAC GAC AGG CGT GCA AAG A	KC493359	65
	hif1-α	*F*: GAG CCC CCT CTG ACC TAC CT *R*: CGA TGT TTG AGG GAT GAG GAA	HM146310	60
	gr-2	*F*: GCT GTC AGT CAG CAG AAA GAT CA *R*: AAC GGC TCT TCT GAA GAG GTC AT	KC493358	72
	hsp70	*F*: GGC GGT GAG GAC TTT GAC A *R*: GTG CTT CCT CTT GAA TTC TTC TAC AA	KC493357	62
	hsc70-2	*F*: AAC CAC TTC ATC TCC GAG TTC AA *R*: GCC CTT TTG TTG TCG TGA T	KC493356	61
	ef1-α	*F*: TGA GAT GCA CCA TGA ATC TCT TG *R*: TTG ACA GAC ACG TTC TTA ACG TTG A	GU385741	77

3. Results

Following a 1 h exposure to 30 mg L^{-1} CO_2, *c-fos* transcripts in the gill tissue of bluegill exhibited a twelve-fold increase relative to the control treatment (Table 2, Fig. 1A). Similarly, the abundance of *c-fos* mRNA in erythrocytes of bluegill exposed to hypercarbia increased seven-fold relative to control fish, although this difference was not statistically significant (Table 2, Fig. 1A). Hypercarbia exposure did not result in an increase in *c-fos* levels for bluegill heart tissue relative to the control treatment (Table 2, Fig. 1A). For silver carp, 1 h exposure to 30 mg L^{-1} CO_2 resulted in a four-fold increase in *c-fos* mRNA abundance in erythrocytes, but no significant changes in *c-fos* expression were seen in any other tissues examined (Table 3, Fig. 1A). Concentrations of *hif1*-α mRNA in the erythrocytes of both bluegill and silver carp doubled following a 1 h exposure to 30 mg L^{-1} CO_2, while gill and heart tissue *hif1*-α transcripts did not significantly deviate from control levels in either species (Table 2,3, Fig. 1B). In bluegill, erythrocyte *gr-2* mRNA was induced two-fold following the hypercarbia treatment, while gill and heart tissue transcripts did not differ significantly from control values (Table 2, Fig. 1C). Across all three tissues combined, *gr-2* mRNA abundance doubled in silver carp exposed to 30 mg L^{-1} CO_2, though changes within tissues were not significant (Table 3, Fig. 1C).

Concentrations of *hsp70* transcripts for bluegill exposed to hypercarbia did not differ significantly from control values for all tissues examined (Table 2, Fig. 2A). Following a 1 h exposure to 30 mg L^{-1} CO_2, silver carp exhibited a five-fold induction in *hsp70* transcripts in erythrocytes, while gill and heart tissue mRNA abundance remained unchanged (Table 3, Fig. 2A). Across all tissues for bluegill, transcript abundance for *hsc70-2* did not deviate from control fish values following hypercarbia exposure (Table 2, Fig. 2B). Expression of *hsc70-2* transcripts from silver carp heart and erythrocytes were upregulated, five and two-fold, respectively, following a 1 h exposure to hypercarbia (Table 3, Fig. 2B). In silver carp gill tissue, however, *hsc70-2* abundance relative to control fish did not change following an exposure to 30 mg L^{-1} CO_2 (Table 3, Fig. 2B).

Table 2
Two-way analysis of variance (ANOVA) examining the impact of elevated CO_2 on candidate gene expression among several tissues of bluegill.

Candidate gene	Main effects	SS	df	F	P
c-fos	Entire model	3.89	5	13.06	<0.0001
	Tissue	0.92	2	7.74	0.0016
	Treatment	1.83	1	30.71	<0.0001
	Tissue × treatment	0.80	2	6.67	0.0035
	Error	2.09	35		
hif1-α	Entire model	0.13	5	5.48	0.0008
	Tissue	0.03	2	3.56	0.0389
	Treatment	0.06	1	12.63	0.0011
	Tissue × treatment	0.03	2	3.66	0.0358
	Error	0.18	36		
gr-2	Entire model	0.22	5	9.81	<0.0001
	Tissue	0.09	2	10.53	0.0002
	Treatment	0.03	1	7.22	0.0106
	Tissue × treatment	0.10	2	11.26	0.0001
	Error	0.17	38		
hsp70	Entire model	0.07	5	0.73	0.6083
	Tissue	0.04	2	0.94	0.3995
	Treatment	0.004	1	0.22	0.6418
	Tissue × treatment	0.03	2	0.70	0.5037
	Error	0.69	37		
hsc70-2	Entire model	0.03	5	0.49	0.7811
	Tissue	0.01	2	0.51	0.6071
	Treatment	0.009	1	0.83	0.3683
	Tissue × treatment	0.004	2	0.20	0.8221
	Error	0.41	38		

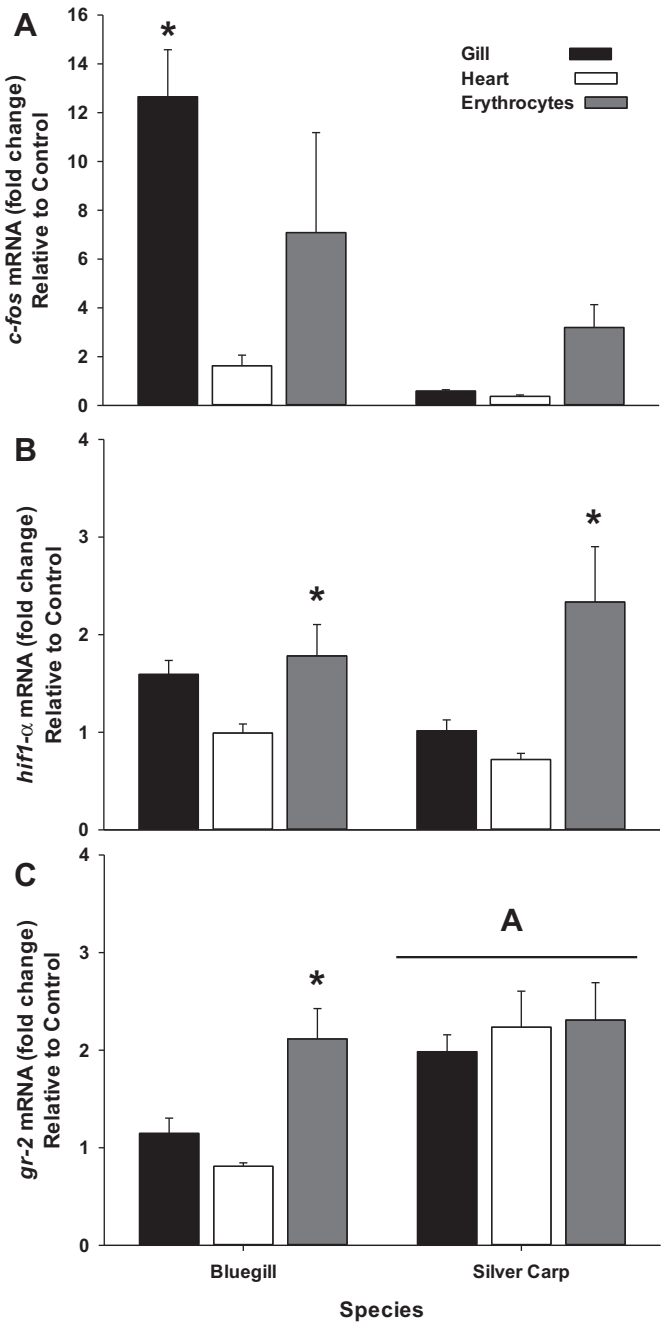

Fig. 1. Tissue-specific relative mRNA expression for *c-fos* (a), *hif1*-α (b), and *gr-2* (c) in bluegill and silver carp exposed to 30 mg L^{-1} CO_2 for 1 h. Relative mRNA expression extracted from gill tissues are shown in black bars, heart tissues are shown in white bars, and erythrocytes are shown in gray bars. An asterisk denotes a statistically significant difference between control and treatment values within a species. Horizontal lines denote a significant treatment effect across all tissues within a species for a response variable. Data are the means ± SE, calculated relative to the expression of *ef1*-α for all tissues and species examined. For clarity, data are expressed relative to the mean of fish exposed to ambient water conditions for each species, gene, and tissue. $P < 0.05$ for all significant comparisons.

4. Discussion

Following an acute hypercarbia exposure, both bluegill and silver carp upregulated a variety of gene transcripts in response to an acute hypercarbic stressor. More specifically, exposure to 30 mg L^{-1} CO_2 (≈15700 μatm) significantly increased expression of *c-fos*, *hif1*-α, and *gr-2* mRNA transcripts in bluegill and silver carp. Additionally, the expression of *hsp70* and *hsc70-2* mRNA increased 2–5 fold following hypercarbia exposure in silver carp alone. Following the perception of

Table 3
Two-way analysis of variance (ANOVA) examining the impact of elevated CO_2 on candidate gene expression among several tissues of silver carp.

Candidate gene	Main effects	SS	df	F	P
c-fos	Entire model	0.83	5	6.97	<.0001
	Tissue	0.41	2	8.65	0.0008
	Treatment	0.006	1	0.27	0.6070
	Tissue × treatment	0.36	2	7.50	0.0018
	Error	0.93	39		
hif1-α	Entire model	0.29	5	4.62	0.0024
	Tissue	0.10	2	4.06	0.0260
	Treatment	0.02	1	1.72	0.1983
	Tissue × treatment	0.12	2	4.78	0.0146
	Error	0.44	35		
gr-2	Entire model	0.40	5	6.29	0.0003
	Tissue	0.001	2	0.04	0.9654
	Treatment	0.40	1	31.26	<0.0001
	Tissue × treatment	0.003	2	0.12	0.8896
	Error	0.47	37		
hsp70	Entire model	1.17	5	9.75	<0.0001
	Tissue	0.33	2	6.92	0.0029
	Treatment	0.26	1	10.69	0.0024
	Tissue × treatment	0.56	2	11.72	0.0001
	Error	0.86	36		
hsc70-2	Entire model	1.47	5	24.61	<0.0001
	Tissue	0.41	2	17.11	<0.0001
	Treatment	0.50	1	42.19	<0.0001
	Tissue × treatment	0.42	2	17.55	<0.0001
	Error	0.43	36		

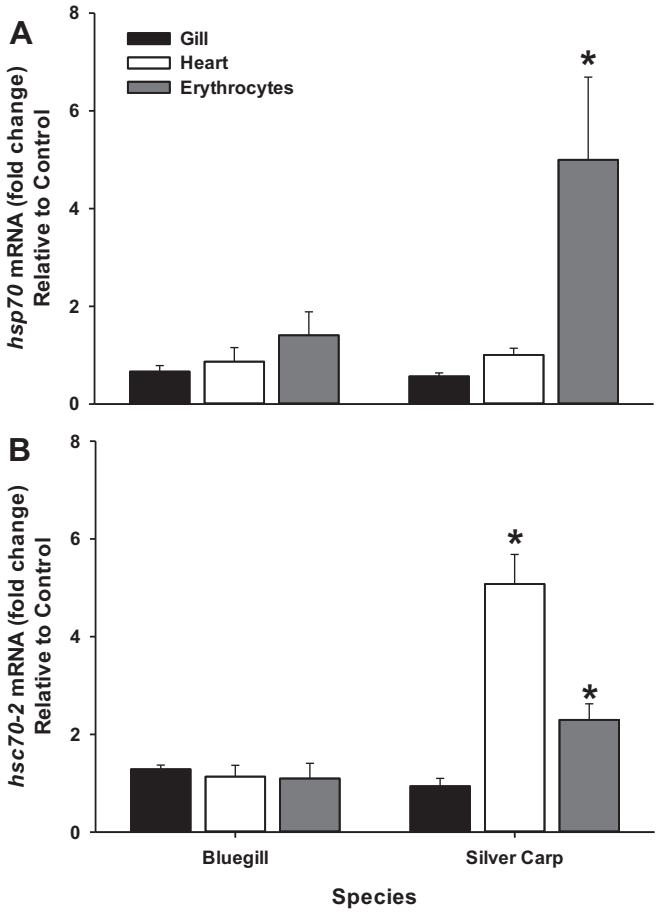

Fig. 2. Tissue-specific relative mRNA expression for *hsp70* (a) and *hsc70-2* (b) in bluegill and silver carp exposed to 30 mg L^{-1} CO_2 for 1 h. Relative mRNA expression extracted from gill tissues are shown in black bars, heart tissues are shown in white bars, and erythrocytes are shown in gray bars. An asterisk denotes a statistically significant difference between control and treatment values within a species. Data are the means ± SE, calculated relative to the expression of *ef1-α* for all tissues and species examined. For clarity, data are expressed relative to the mean of fish exposed to ambient water conditions for each species, gene, and tissue. $P < 0.05$ for all significant comparisons.

a stressor, organisms can experience changes to a number of physiological systems, including the hypothalamic–pituitary–interrenal (HPI) axis (i.e., the glucocorticoid responses), cellular protein repair and degradation, and alterations in behavior patterns, all in an effort to maintain homeostasis (Barton, 2002; Prunet et al., 2008; Kassahn et al., 2009). The physiological response of fishes to acute hypercarbia exposure has been well studied (reviewed in Perry and Gilmour, 2006; Brauner and Baker, 2009), and includes an initial increase in blood P_{CO_2}, decrease in blood pH, followed by an elevation of bicarbonate (HCO_3^-) to return blood pH levels to normal. Acute hypercarbia exposure has also been shown to impact the ventilation frequency of fishes (Perry and Gilmour, 2006), as well as increase levels of hematocrit and stress hormones (i.e., cortisol), all of which may function to maintain homeostasis (Iwama et al., 1989; Bernier and Randall, 1998). For silver carp and bluegill, in the current study, exposure to an acute CO_2 stressor activated a number of different physiological pathways including those related to general stress, protein repair and degradation, and oxygen transport with responses seen across a range of tissues. For example, *hif1-α* mRNA has previously been shown to be upregulated following exposure to hypoxia, cold, and heavy metal contaminants in an effort to maintain proper oxygen transport (van Heerden et al., 2004; Heise et al., 2006; Terova et al., 2008). Previous research on freshwater fishes such as rainbow trout (Iwama et al., 1989), silver carp and bluegill (Kates et al., 2012), have shown that hypercarbia exposure results in an increase in hematocrit concentration and the authors proposed that this resulted from impaired oxygen uptake along with reductions in ventilation rates observed in these fish species. Thus, the 2-fold increase in erythrocyte *hif1-α* mRNA found in both bluegill and silver carp was likely an additional mechanism, other than increasing hematocrit, to improve oxygen binding and transport (Bracken et al., 2003; Nikinmaa and Rees, 2005). Similarly, exposure to infectious disease and salinity stress have been shown to induce expression of glucocorticoid receptors, which serves to activate immune responses and help maintain water and ion concentrations in these fishes (Acerete et al., 2007; Aruna et al., 2012). Previous work by Kates et al. (2012) showed that silver carp and bluegill plasma ions did not differ from baseline following exposure to 30 mg L^{-1} CO_2, while all four fish species examined displayed increased glucose levels following an acute hypercarbia exposure. Therefore, the upregulation of *gr-2* transcripts for bluegill and silver carp exposed to hypercarbia likely occurred to help maintain ion balance and release additional energy (Evans et al., 2005; Stolte et al., 2009). Together, results from the current study clearly indicate that an acute exposure to 30 mg L^{-1} CO_2 (≈15,700 μatm) altered the expression of genes that comprised many components of the cellular stress response in both bluegill and silver carp.

While both bluegill and silver carp exhibited alterations in gene expression following an acute exposure to 30 mg L^{-1} CO_2, these two species showed inter-specific differences in the gene transcripts that were upregulated, and the results from the current study may explain hypercarbia tolerance differences observed previously in these two species. More specifically, gill tissue in bluegill increased expression of *c-fos* mRNA roughly 12-fold, while silver carp upregulated transcripts in two genes (*hsp70* and *hsc70-2*), which encode proteins involved in mediating the repair and degradation of altered proteins, following a 1 h exposure to 30 mg L^{-1} CO_2. Inter-specific variation in the genetic response to external stressors, even between two closely related species or populations, is not uncommon and has previously been used to explain differences in stress tolerance between organisms. For example, Healy et al. (2010) showed that, following heat shock, adult killifish of the northern sub-species (*F. heteroclitus macrolepidotus*) significantly increased specific *hsps*, including *hsp70-1* and several small *hsps*, in contrast to killifish from the southern sub-species (*F. heteroclitus heteroclitus*), which

upregulated *hsp70-2*. The authors hypothesized that this expression pattern was partially responsible for the observed difference in swimming performance and metabolic rate during heat stress across these two sub-species. Previous research, by Kates et al. (2012), demonstrated that adult silver carp and bluegill differed in their hypercarbia tolerance, with bluegill being more tolerant to hypercarbia compared to silver carp. For example, following a 30 min exposure to CO_2 concentrations of 70 mg L^{-1}, bluegill exhibited a 50% decrease in ventilation rate while all silver carp lost equilibrium as a result of the hypercarbia stressor (Kates et al., 2012). Similarly, exposure to 30 mg L^{-1} CO_2 caused 40% of silver carp to exhibit irregular activities (episodic or intermittent ventilations, twitching, surface ventilations, coughing, or loss of equilibrium) while none of the bluegill examined showed irregular activities (Kates et al., 2012). Interestingly, the interspecific differences in gene expression between adult silver carp and bluegill found in this study may provide potential mechanisms to explain the observed differences in hypercarbia tolerance. Previous research has shown that *c-fos* mRNA is rapidly expressed in the brain of many organisms following exposure to acute hypercarbia (Sato et al., 1992; Tankersley et al., 2002; Rimoldi et al., 2009). Once translated, the c-Fos protein then enhances the transcription of many genes in response to a stressor, in this case acute hypercarbia (Curran and Franza, 1988). Thus, significant increases in *c-fos* mRNA for bluegill tissues, which are substantially reduced in silver carp tissues, suggest that silver carp adults likely use more 'general' stress mechanisms (*gr-2* and *hsps*) to maintain homeostasis following hypercarbia exposure compared to adult bluegill who utilized a hypercarbia-linked transcription factor (*c-fos*). While this observation alone does not explain observed differences in hypercarbia tolerance between these two fish species, additional studies are warranted to examine the role of *c-fos* in the ventilatory response of silver carp and bluegill to hypercarbia. Additionally, only silver carp induced expression of *hsp70* and *hsc70-2* following exposure to 30 mg L^{-1} CO_2. While previous research by Lund et al. (2003) and Lewis et al. (2010) had shown that heat shock protein transcripts could take several hours (>4 h) to reach peak expression levels following a thermal stressor, the current study showed adult silver carp *hsp70* and *hsc70*-2 mRNA increased after only a 1 h exposure to hypercarbia. Heat shock protein transcripts are typically induced to maintain homeostasis within the cell by repairing or degrading altered or denatured proteins following a stressor (Iwama et al., 2004), suggesting that the impact of hypercarbia on protein functioning was more detrimental for silver carp compared to bluegill and potentially results in silver carp being more hypercarbia sensitive. Together, results from this current study clearly indicate that bluegill and silver carp display species-specific differences in gene expression patterns following acute hypercarbia exposure, and these differences in gene expression may provide a potential explanation for observed differences in acute hypercarbia.

The concentration of carbon dioxide used for this study, approximately 30 mg L^{-1} (≈15,700 μatm), provides a realistic starting point for researchers looking into the impact of hypercarbia on fishes. Of particular concern on an ecological and economic global perspective is the expected increase in dissolved CO_2 concentrations in water driven by global climate change (Raven et al., 2005; Cooley and Doney, 2009). While the CO_2 concentrations used in this study are roughly 8 times greater than predicted increases in CO_2 due to global climate change by the year 2300 (Caldeira and Wickett, 2003), the gene transcripts examined in this study could be used as a starting point for other researchers who want to determine the sub-lethal stress response of aquatic organisms to hypercarbic environments. Hypercarbia is also a serious issue for fish farmers and aquaculture managers, as an overabundance of fish within tanks can lead to elevated CO_2 environments (Colt and Orwicz, 1991; Kristensen et al., 2009). For example, Atlantic salmon (*Salmo salar*) smolts exposed to a chronic exposure of 20 mg L^{-1} CO_2 displayed reduced condition factor, which decreases sale price of these fishes (Fivelstad et al., 1999). Carbon dioxide also has the potential to be used as a barrier to influence the movement of fishes, as shown by both Clingerman et al. (2007) and Kates et al. (2012) who generated hypercarbic environments to direct fish movements.

5. Conclusions

In conclusion, the current study clearly demonstrates that a variety of gene transcripts are upregulated, across three tissues, for bluegill and silver carp following exposure to an acute hypercarbia stressor. Expression of *c-fos*, *hif1*-α, and *gr-2* mRNA were upregulated in bluegill and silver carp tissues following a 1 h exposure to 30 mg L^{-1} CO_2, while only silver carp induced expression of heat shock protein transcripts. Differences in gene expression observed between species, especially in bluegill *c-fos* and silver carp *hsp70* and *hsc70-2*, may offer a potential mechanism underpinning the difference in hypercarbia tolerance observed previously by Kates et al. (2012). As hypercarbic environments become more prevalent in the future, knowledge on the impacts of hypercarbia on aquatic organisms will be of utmost importance to conservation biologists and managers.

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