THE IMPACT OF CARBON DIOXIDE ON FRESHWATER FISH BEHAVIORS

BY

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THESIS

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ABSTRACT

Freshwater systems may be impacted by acidification due to increases in dissolved carbon dioxide (CO_2) by different factors. Recent work in the marine environment suggests that increased CO_2 levels due to climate change can negatively affect a fish's ability to detect predators, home to natal environments, and perform aerobically; thus, elevated CO_2 may also have negative impacts on freshwater communities, an area that remains understudied. The first study was to quantify the impacts of elevated CO_2 on fathead minnow (*Pimephales promelas*) and silver carp (*Hypophthalmichthys molitrix*) alarm cue behaviors. Fathead minnow responses to conspecific skin extracts were significantly impaired following exposure to elevated CO_2 levels for at least 96 hours, while silver carp behavior was not altered. However, high pCO_2 exposed fathead minnow irregular behaviors to skin extracts did re-establish after 14 days of returning fish to ambient CO_2 levels. The second study defined the behavioral response of individual *Lepomis macrochirus* following exposures to elevated carbon dioxide (CO_2). For this, *L. macrochirus* were first held at ambient pCO_2 (160 µatm pCO_2) for 7 d, then exposed to elevated pCO_2 (8300 µatm pCO_2) for 5 d, then returned to ambient conditions for a further 5 d to recover. Following each exposure period, several behavioral metrics were quantified (boldness, lateralization, and activity). Average velocity, velocity in the thigmotaxis zone and proportion of activity in the thigmotaxis zone increased with pCO_2 exposure. During recovery, average velocity of *L. macrochirus* decreased. In addition, individual rank was repeatable during the pre-exposure and recovery period in three of the 17 metrics investigated (average velocity in the middle zone, average velocity near object, and total shuttles to the object zone), suggesting that elevated pCO_2 may disrupt performance. Together, these results suggest elevated CO_2 may impact some freshwater fish species behavior and managers should use this information to manage fisheries appropriately.

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CHAPTER 1: EFFECTS OF ELEVATED CARBON DIOXIDE ON ALARM CUE RESPONSES IN FRESHWATER FISHES

Abstract

Freshwater environments have the potential to be impacted by acidification due to increases in dissolved carbon dioxide (CO_2). Recent work in the marine environment suggests that increased CO_2 levels due to climate change can negatively affect a fish's ability to detect predators, home to natal environments, and perform aerobically. The potential for elevated CO_2 to have similar negative impacts on freshwater communities remains understudied. The objective of our study was to quantify the effects of elevated CO_2 on the behaviors of fathead minnows (*Pimephales promelas*) and silver carp (*Hypophthalmichthys molitrix*) following exposure to conspecific skin extracts (alarm cues). In fathead minnows, their response to conspecific skin extracts were significantly impaired following exposure to elevated CO_2 levels for at least 96 h, while silver carp behaviors were unaltered. However, fathead minnow behaviors did return to pre-CO_2 exposure in high CO_2 exposed fish following 14 d of holding at ambient CO_2 levels. Overall, this study suggests there may be potential impacts to freshwater fishes alarm cue behaviors following CO_2 exposure but these responses may be species-specific, and will likely be abated should the CO_2 stressor be removed.

Introduction

Fishes have a keen sense of olfaction (Hara 1993), which is important across a variety of life stages (Lima and Dill, 1990), and for a number of processes, including predator avoidance (Fuiman and Magurran, 1994), kin recognition (Gerlach *et al*., 2008) and habitat selection (Dittman *et al*., 1996). Olfaction is particularly important for fishes within the superorder Ostariophysi, as it has been shown that chemical cues used by this group can elicit predator avoidance behaviors (e.g., refuging, shoaling, darting and freezing) when fish are exposed to injured conspecifics or heterospecifics, due to alarm cues or pheromones that they possess within the skin (Pfeiffer *et al*., 1985; Chivers and Smith, 1998; Brown *et al*., 2000). As is the case for many life processes in fish, environmental stressors, such as reduced pH (Leduc *et al*., 2013), runoff (Fisher *et al*., 2006), or pollutants (Hara *et al*., 1976) can negatively influence olfaction capabilities in fish, thereby reducing their ability to sense and/or respond to information contained within chemical cues.

One environmental stressor that potentially could influence olfaction in Ostariophysi is the rise of carbon dioxide (CO_2) concentrations in freshwater, which also has a concomitant effect of reducing pH. Acidification of water in the context of acid rain (i.e., drop of pH from 8.0 to near 4.0 due to the addition of strong acids, such as nitric or sulfuric acid) has been shown to negatively impact olfaction and alarm cue responses in fishes, largely through two mechanisms (Lemly and Smith, 1985; Brown *et al*., 2002; Leduc *et al*., 2004; Leduc *et al*., 2009; Leduc *et al*., 2013). First, one of the main chemical components of the alarm pheromone is hypoxan-thine-3(N)-oxide (H_3NO), and the structure of this molecule can be altered in water at a pH < 6.0 making it undetectable (Brown *et al*., 2002). Second, olfaction can be negatively impacted by reduced pH because the sensitivity or affinity of olfactory receptors may be reduced

at low pH, evidenced by the fact that fathead minnows showed a reduced feeding response to amino acids at pH 6.0 (Lemly and Smith, 1985). These studies have shown that acidification of freshwater due to factors such as acid rain, can potentially impact olfaction, as well as the alarm cue responses of freshwater fishes.

Recently, research related to ocean acidification in the context of climate change has shown that not only can the reduction in pH related to ocean acidification disrupt the alarm cue responses of fish, but the rise in dissolved CO_2 can also negatively impact olfaction (Munday *et al*., 2010; Dixson *et al*., 2010). In the marine environment, the partial pressure of CO_2 (pCO_2) in water can increase due to many factors (e.g., atmospheric levels of CO_2, seeps, upwellings, etc.), and, pCO_2 in the oceans has increased over the past several decades due to increases in atmospheric CO_2 (Ciais *et al*., 2013). The increase in dissolved CO_2 causes respiratory acidosis in fishes (Heuer and Grosell, 2014), and this acidosis results in a disruption of cellular ionic gradients. It has been well established that this change in cellular ionic gradients alters $GABA_A$ receptor function, which, in turn, impacts fish behavior (Nilsson *et al.*, 2012). More specifically, exposure to elevated pCO_2 has been found to have a range of negative impacts for fish including a loss of anti-predator responses (Allan *et al.*, 2013), changes in auditory preferences (Simpson *et al.*, 2011), increased activity levels (Munday *et al.*, 2010; Ferrari *et al.*, 2011), poor prey detection and feeding (Cripps *et al.*, 2011), and negatively alters visual risk assessment (Ferrari *et al.*, 2012). More importantly, Dixson *et al.*, (2010) showed that settlement-stage orange clownfish *Amphiprion percula* larvae were attracted to the smell of a predator, and could not distinguish between a predator and non-predator odor cues, following exposure to elevated levels of pCO_2 for a short duration (11 d post-hatch), which could have important consequences for survival. Laboratory tests have also shown that marine juvenile damselfish exposed to CO_2-

acidified water for 4 d displayed impaired responses to conspecific alarm cues (Ferrari *et al*., 2011). Despite these findings, behavioral changes driven by elevated pCO_2 have been found to be variable across fish species, as no change to behaviors such as predator avoidance, lateralization, and swimming kinematics have been observed in some fish species despite extended exposures to high pCO_2 (Jutfelt and Hedgärde, 2013; Maneja *et al.*, 2013; Sundin and Jutfelt, 2015). The rise in CO_2 and concomitant reduction in pH does inhibit olfaction in some marine species and therefore, it may be possible that similar response exist for freshwater fishes.

In freshwater, pCO_2 levels can vary across watersheds (Cole *et al*., 1994), as well as on episodic, seasonal and diel cycles (Maberly 1996; Riera *et al*., 1999). For example, levels of pCO_2 in freshwater are naturally variable, and ranged from 107–4,128 µatm across 62 lakes measured globally (Cole *et al.*, 1994), and, within a lake, free CO_2 may increase 7-fold above atmospheric concentrations in Fall, Winter and early Spring (Maberly 1996). Furthermore, freshwater fishes may experience elevated pCO_2 due to a number of mechanisms including a rise in atmospheric CO_2 (Phillips *et al*., 2015), increased terrestrial primary productivity (Ameth *et al*., 2010), hatchery rearing (Colt and Orwicz, 1991), or the deployment of a non-physical barrier that use zones of elevated CO_2 gas to prevent fish movements (Noatch and Suski, 2012). Understanding how potential increases in pCO_2, coupled with a reduction in pH, are critical for predicting consequences of elevated pCO_2 on the behavior of freshwater fish (Hasler *et al*., 2016).

Based on this background, the objectives of this study were to (1) determine how exposure to elevated of pCO_2 would change olfactory predator avoidance behaviors of fathead minnows (*P. promelas*) and silver carp (*Hypophthalmichthys molitrix*); and, (2) if impaired olfaction behavior occurred, determine if 'normal' behaviors re-establish after fish are returned

to ambient conditions. To accomplish these goals, fathead minnows and silver carp were exposed to one of three different levels of CO_2 (ambient, low, high) for at least 4 d and were then exposed to conspecific skin extracts. Naïve fish in holding tanks were returned to ambient pCO_2 for at least 11 d prior to undergoing the same behavioral trials.

Methods and Materials

Experimental animals

Adult fathead minnows were obtained from Logan Hollow Fish Farm (Murphysboro, IL) and transported to the University of Illinois Aquatic Research Facility for experimentation (Urbana, IL; travel time 3.25 h), while experiments with hatchery-reared silver carp took place at the Upper Midwest Environmental Sciences Center (UMESC; La Crosse, WI). Fathead minnows were placed in a 3 w/v% salt (NaCl) bath for 30 s to disinfect and promote fish health upon arrival at the aquatic facility (Swann and Fitzgerald, 1991). Fathead minnows were then divided into three groups of approximately 200 and held in separate 379 L plastic holding tanks supplied with oxygen through an air stone attached to an air blower and water from a 0.04 ha, earthen-pond. About 1.2 g/L of salt was added and manually flushed out each day for the first two days of laboratory acclimation to further reduce stress and promote fish health (Swann and Fitzgerald 1991). Fathead minnows were given a total of 5 d to recover from transport and acclimate to laboratory conditions prior to the onset of experiments. Waste was siphoned and 50 % water changes occurred one to three times daily to ensure ammonia levels remained low (measured using: Hach Company, kit 224100, Loveland, CO, USA) . Silver carp at UMESC were collected from a common holding tank, separated into groups of 20, and placed into re-

circulating flow-through 230 L tanks supplied with well water. For both fathead minnow and silver carp, water quality was monitored daily for the duration of the experiment: temperature, dissolved oxygen (DO) (YSI, 550A Yellow Springs Instruments, Irvine, CA, USA), total alkalinity (TA) (Hach Company, Titrator model 16,900 and kit 94399, Loveland, CO, USA), and pH (WTW pH 3310 meter with a SenTix 41 probe, Germany); the pH probe was calibrated daily during this study (Moran 2014). In addition, pCO_2 was measured daily during silver carp trials using an infrared CO_2 sensor (Vaisala, Carbon Dioxide Transmitter Series GMT220, Finland) wrapped in a semi-permeable polytetrafluoroethylene cover (Johnson *et al.*, 2010; Munday *et al.*, 2014) (Table 1). During fathead minnow trials, pCO_2 was quantified by entering temperature, pH and alkalinity data into CO2Calc (Robbins *et al*., 2010; http://pubs.usgs.gov/of/2010/1280/) using all other parameters as constants. All fish were fed commercial pellet feed until satiation every day.

p*CO₂ exposure treatments*

In the treatment tanks (379 L for fathead minnows and 227 L for silver carp), fish were exposed to one of three different CO_2 treatments: control (ambient) (≈750 µatm), low pCO_2 (≈1500 µatm) and high pCO_2 (≈7000 µatm; Table 1). These treatment levels were chosen because (a) Kates *et al*., (2012) found that short-term exposure to 70 mg L^{-1} of CO_2 (≈150,000 µatm) altered ventilation rates and caused behaviors indicative of 'stress' (e.g., surface ventilations, coughing, loss of equilibrium) suggesting that a holding level below 150,000 µatm would prevent such consequences, (b) many marine acidification studies that have demonstrated an impact of CO_2 exposure on olfactory responses targeted ≈1500 µatm for high CO_2 exposure,

which is a future projection of pCO_2 in marine ecosystems (Jutfelt *et al*., 2013; Foresgren *et al*., 2013; Allan *et al*., 2014) and (c) Heurer and Grossell (2014) indicated that the use of multiple CO_2 levels within a single study can help define mechanisms of CO_2 impacts. Even though no previous ocean acidification studies on fish have used pCO_2 higher levels as high as 7000 µatm as an experimental treatment, pCO_2 in freshwater ecosystems fluctuates widely and can experience higher levels of pCO_2 than marine ecosystems (Leduc *et al*., 2013), making 7000 µatm a relevant and valuable level for holding. Target pCO_2 levels were held constant using a Pinpoint pH Regulator Kit (American Marine Inc., Ridgefield, CT, USA) (Munday *et al*., 2012; Allan *et al*., 2014) adjusted to add CO_2 to the water when water pH rose above a set level (Gattuso *et al*., 2010). A homogenous mixture of CO_2 was achieved in the holding tanks by using an air stone connected to a 1.80 amp air compressor (Sweetwater, Aquatic Eco-Systems, Apopka, FL, USA), which also prevented hypoxia. Fathead minnows were held in the treatment tanks for 4–12 d, while silver carp were held for 4–10 d prior to commencing behavioral testing. This exposure duration was chosen based on previous work that has shown the potential for olfactory behavioral impairments in fish to occur following 96 h of continuous exposure to elevated pCO_2 (Munday *et al*., 2010; Ferrari *et al*., 2011). Following the behavioral tests (described below), all holding tanks were returned to ambient pCO_2 for 11–14 d for fathead minnows and 14–17 d for silver carp by replacing CO_2-rich water in the tank with water at ambient levels.

Alarm cue extraction

Alarm cue stimuli preparation methods were adapted from Mathis and Smith (1993). Stimuli were prepared from 90 fathead minnows and 45 silver carp that had a mean fork length of 5.04 ± 0.70 standard error (SE) and 11.67 ± 2.05 cm, respectively. Males and females in breeding condition, identified by the presence of gametes, were not used as breeding males do not produce alarm cues (Smith 1973). Donor fathead minnows and silver carp were euthanized by snipping off their heads with scissors and skin from both sides of each fish was removed using a scalpel. The length and width of each skin sample was measured, and the total area of skin collected was approximately 274.4 cm^2 and 891.6 cm^2 for fathead minnows and silver carp, respectively. Skin samples were immediately placed in 600 ml of chilled ultra-pure water (approximately 5°C) and homogenized with a Polytron homogenizer (T18 Basic Ultra-Turrax, IKA, Germany). The homogenate was filtered through glass wool to remove scales and other solid particles, and then was further diluted by the addition of 1,800 ml of ultra-pure water (total volume was 2,400 ml), and stored at -20°C in 30 ml aliquots until use. Additionally, 30 ml aliquots of ultra-pure water were stored at -20°C and used as a control (Little *et al*., 2011).

Behavioral trials

To quantify behavioral responses of fathead minnows and silver carp to skin extracts, a flume channel (Choice Tank, Loligo Systems, Denmark; Jutfelt and Hedgärde, 2013) containing a 32 × 40 cm arena with a water depth of 15 cm was used. Two 208 L vertical header tanks, outfitted with an air stone to facilitate aeration, as well as a small fountain pump to facilitate mixing of water, were attached to the flume, and water flowed from the header tanks into the

choice channel by gravity. One header tank was identified as a 'control' tank, while the second tank was identified as the 'treatment' tank, and the treatment tank received skin extracts (skin extracts were always added to the same 'treatment' tank to prevent contamination of the 'control' tank). A valve downstream of the header tanks allowed the flume to receive water from either header tank with minimal interruption to water flow. Both vertical header tanks were filled with equal amounts of ambient freshwater (pond water for fathead minnows and well water for silver carp).

At the commencement of the 'acclimation' period, and prior to a fish being placed into the arena, one 30 ml aliquot of ultra pure water (described above) was added to the control tank and given 10 min to mix. A valve on the control tank was then opened and the choice area received water at a flow rate of 6.7 L min^{-1} (verified with a flow meter; 807 series Rotameter, Georg Fischer, Schaffhausen, Switzerland). The outflowing water from the arena was captured at the outlet in a 49.2 L plastic tub and returned to the header tank *via* a 124 W submersible pump, thus creating a recirculating system. A single fish was carefully netted from one of the selected holding tanks (treatment was selected randomly using a random number generator), placed into the arena, and allowed 1 h to acclimate. A 1 h acclimation period was chosen as preliminary trials indicated that this period of time was sufficient to reduce increased freezing and darts behaviors following introduction to the arena, and for the fish to begin exploring the choice area; previous studies have also used a similar 1 h acclimation period *(e.g., De Robertis et al*., 2003). A single fish was tested at a time (as opposed to testing multiple individuals concurrently) to obtain a "pure response" not influenced by conspecifics (Lawrence & Smith, 1989), the entire arena was surrounded by dark plastic wrapping and noise level in the immediate area of the arena was limited to reduce the potential for external stimuli to influence fish

behavior. During the final 10 min of the 1 h acclimation period, fish position, behavior and activity were recorded using a camera (iDS uEye 1480-C camera, iDS, Obersulm, Germany) (Little *et al*., 2011; Poulsen *et al*., 2014). Two fish that remained stationary during the acclimation period were removed from the arena and excluded from the study (Munday *et al*., 2010).

After the acclimation period, one 30 ml aliquot of prepared skin extract was added to the 'treatment' header tank and was allowed to mix for 10 min. Water with the skin extract was then allowed to flow from the header tank into the arena (also at a rate of 6.7 L min^{-1}). Once water containing skin extracts entered the choice arena (determined to be 30 s using a preliminary dye test), fish were again recorded for 10 min (Little *et al*., 2011; Poulsen *et al*., 2014). Following this 10 min recording period, the fish was removed from the choice tank, and measured for total length (mm) and weight (g) (Table 2). This procedure was repeated for 27 fish until a sample size of $N = 9$ for each treatment was achieved for both fathead minnows and silver carp, and fish were only used once and then were euthanized. Note that, during the skin extract exposure period, water was not returned to the header tank using the submersible pump, and between trials, the tank was thoroughly rinsed.

To quantify the potential for changed behaviors to return to 'normal', fathead minnows were held for an additional 11 to 14 d in water at ambient pCO_2 (≈400 μatm) using protocols outlined above, and behavioral trials were repeated for 29 naïve fish (i.e., previously assessed fish were not re-used). Similarly, silver carp were allowed to recover for 14 to 17 d in ambient pCO_2 water (≈950 μatm) and behavior trials were repeated for 27 naïve fish. This duration of recovery was chosen as Hamilton *et al.*, (2014) found that anxiety behaviors of juvenile

California rockfish (*Sebastes diploproa*) altered by exposure to increased levels of pCO_2 returned to normal after returning to ambient seawater for 12 d.

Data acquisition and statistical analyses

Analyses of total distance travelled, velocity, and active time were generated using videos with the program Lolitrack (Loligo Systems, Denmark; Lawrence and Smith, 1989; Poulsen *et al*., 2014). Total distance travelled and velocity were transformed into body lengths (BL) and BL/s, respectively to standardize metrics across fish lengths. In addition, each video was manually analyzed for darts (rapid movement lasting at least 1 s) and freezes (> 30 s motionless) using protocols defined by Chivers and Smith (1998), and these two metrics were then summed together to generate irregular activities. In addition to darts and freezes, jumps were also quantified as part of silver carp irregular activities, as jumps are known to be a fright response in silver carp (Kolar *et al*., 2007).

To determine if elevated pCO_2 had an effect on the response to skin extract, Generalized Linear Mixed Models (GLMMs) were performed, with appropriate error, distributions, and link-functions. For the GLMMs, a Poisson distribution was used only for count data (Quinn and Keough, 2002) (i.e., total irregular activities). For fathead minnows, data were parsed by pCO_2 treatment and therefore, activity, velocity, and total distance travelled were analyzed using GLMMs with normal distributions. These four metrics (irregular activities, activity, distance travelled and velocity) were entered as response variables, exposure (acclimation or stimulus levels), treatment period (CO_2 or recovery) were included as fixed effects, and fish ID was included as a random effect for each treatment. The use of a random effect (a repeated measures

design) was necessary because multiple measurements were taken from each fish across trials (acclimation and stimulus), meaning that each measurement was not independent and potentially correlated within an individual (Laird and Ware, 1982; Lindstrom and Bates, 1990). For silver carp, data were not parsed by pCO_2 treatment due to obtaining normality in residuals, therefore the models included the four metrics entered as response variables, exposure (acclimation or stimulus levels) and treatment (control, low, high) were included as fixed effects with fish ID as a random effect. Including pCO_2 treatment as a fixed effect was done because of poorly distributed residuals when the fixed effect was not included.

GLMMs for continuous response variables were fitted using the 'glmer' function from the 'lme4' library in R (Venables Ripley 2002; Bates 2010), and, for models with count response variables, which were also over-dispersed, the 'glmmPQL' function from the 'MASS' library was used (Bolker *et al*., 2009). A visual analysis of fitted residuals, using a normal probability plot (Anscombe and Tukey, 1963) was used to assess normality, and visual inspection of the distribution of residuals, was used to assess homogeneity of variances. If expectations of normality or homogeneity of variance were not met, a log transformation of the response variables (i.e., a log-linear model) was used to adjust residuals and achieve normality (Keene *et al*.,1995). For models of count variables using GLMMs, significance was tested at the 95% level. For the GLMMs containing continuous variables, to define the importance of fixed effects, the sim function ('arm' package in R) was used to generate $N = 1000$ posterior simulations of each fixed effect. The resulting posterior distribution of effect estimates were assessed to determine significance of the effects (i.e., distributions of fixed effects whose 95% credible intervals did not overlap 0 were said to be significant). To complete multiple comparisons between levels of significant factors, changes in means and 95% credible intervals

of simulated changes in model intercepts were compared. All data are reported as means ± standard error, SE, where appropriate.

Results

Both fathead minnows and silver carp responded to the skin extracts of conspecifics. Specifically, for fathead minnows held at ambient pCO_2, the number of irregular activities (e.g., darts and freezes) increased 4.5 fold and 2.4 fold during the treatment and recovery periods after being exposed to skin extracts, respectively (Table 3; Figure 1A). Similarly, silver carp responded to skin extracts with increased irregular activities (e.g., darts, freezes and jumps) by 1.6 fold during the stimulus relative to the acclimation period in both the exposure and recovery periods (Table 3; Figure 2). In addition to responding with increased irregular activities, fathead minnows held at ambient pCO_2 also had faster swimming velocity and greater distance travelled, as fish swam 0.34 ± 0.11 BL s^{-1} faster (Table 4; Figure 5A), and travelled 138 ± 46 BL more (Table 4; 5; Figure 4A), respectively, following exposure to skin extracts.

Exposure to elevated pCO_2 resulted in changes to the responses of fathead minnows to skin extracts, but not silver carp. More specifically, unlike fish held at control conditions (750 µatm), fathead minnows treated with high pCO_2 (7,000 µatm) displayed no irregular responses to skin extract exposure (Table 3; Figure 1C). Fathead minnows exposed to low pCO_2 (1,500 µatm), still displayed a response to conspecific skin extracts in the form of irregular activities; however, these appeared to be lower than the increases observed in fish held at control conditions (2.4–4.5 fold increase) as the number of irregular activities increased by only 1.1–2.3 fold relative to the acclimation period when exposed to skin extracts (Table 3; Figure 1B).

Furthermore, the changes in distance travelled (Tables 4, 5; Figure 4B,C), and swimming velocity (Table 4; Figure 4B,C) that was observed for fathead minnows held at control conditions were no longer visible when fish were exposed to both levels of elevated CO_2 (1,500 and 7,000 µatm). For silver carp, all behavior metrics did not differ relative to the acclimation period following exposure to skin extracts, regardless of pCO_2 treatment (Table 3; 4; 6).

Returning fathead minnows to water at ambient pCO_2 for 11–14 days caused some behavioral impairments induced by CO_2 exposure to abate. During the recovery period, fathead minnows previously exposed to high pCO_2 demonstrated a 2.7-fold increase in irregular activities after exposure to skin extract (Figure 1; Table 3). Fathead minnows exposed only to control values of pCO_2 were also monitored and showed a response to skin extracts during the recovery period too, as activity decreased by about double during the stimulus period (Figure 3; Table 4; 5).

Discussion

Alarm cue behaviors in fathead minnows exposed to conspecific skin extracts were altered when fish were held in water with elevated pCO_2 for 4–12 days. Specifically fathead minnows exposed to high pCO_2 (7,500 µatm) did not show irregular behaviors and neither low (1,500 µatm) or high CO_2 treated fish showed changes in velocity or distance travelled (Table 3; 4; 5; Figure 1; 3; 4; 5) following exposure to elevated pCO_2. These results are similar to what has been found in marine ecosystems where marine fishes are unable to detect predator olfactory cues following exposure to elevated pCO_2 (Munday *et al*., 2009; Dixson *et al*., 2010). For example, Munday *et al*., (2010) discovered that clownfish larvae (*A. percula*) were attracted to,

rather than repelled by, predator odors after just 2 d at 850 µatm pCO_2. One possible explanation to why fish exposed to elevated pCO_2 have a reduced alarm cue response may be due to reduced sensitivity of the olfactory receptors. Specifically, with respect to acidification, rainbow trout (*Oncorhynchus mykiss),* Atlantic salmon (*Salmo salar*) and fathead minnows exposed to a pH < 6.0 for 30 min to 72 h were unable to respond to amino acids and ovulated female urine, suggesting that olfactory receptors were inhibited by reduced pH (Lemly and Smith, 1985; Royce-Malmgren and Watson, 1987; Moore 1994). Previous research has also shown that mucus can increase on the olfactory epithelium in low pH and can disrupt olfaction capabilities (Lemly and Smith, 1987; Klaprat *et al*., 1988). With respect to exposure to elevated pCO_2, Nilsson *et al*., (2012) showed function of the $GABA_A$ receptors, a major inhibitory neurotransmitter receptor, was reversed causing it to become excitatory (efflux of anions) rather than inhibitory (normal influx of anions). These abrupt changes in ion gradients result in changes in behaviors of fishes and could explain why fathead minnows were unable to respond to conspecific alarm cues following CO_2 exposure. Together these results clearly demonstrate that in fathead minnows, conspecific alarm cue responses are affected by elevations in pCO_2.

Fathead minnows may be able to recover and respond to conspecific skin extracts after pCO_2 exposure and subsequent return to ambient freshwater. Fathead minnows that showed behavioral impairments after exposure to ≈ 7000 µatm displayed a 3-fold increase in irregular activities during the recovery period (Table 3; Figure 1C). It has been shown that when an environmental stressor is removed, fish physiology and behavior may return to pre-exposure levels. Some examples of this include recovery following changes in temperature (Galloway and Kieffer, 2003), dissolved oxygen (Suski *et al*., 2006) and ammonia (Suski *et al*., 2007). Previous work has also shown that behaviors altered by exposure to elevated pCO_2 can be reversed

following removal of the CO_2 and pH stressors, but this potential for recovery has not been well studied. For example, Munday *et al*., (2010) showed predator avoidance behaviors were re-established in larval *P. wardi* after returning them to ambient seawater for 2 d following exposure to elevated pCO_2. Other work has also shown that when tested in pH < 6.0 using sulfuric acid, fathead minnow olfaction was impaired, but, when re-tested in ambient freshwater (pH 8.0), olfactory impairments were abolished (Brown *et al*., 2002). The most likely explanation for recovery of fish response that were previously exposed to elevated pCO_2 is a return of $GABA_A$ receptors to normal functioning (i.e., inhibitory rather than excitatory) as Nilsson *et al*., (2012) observed. To better understand the mechanisms underlying changes in the alarm cue responses of freshwater fishes in response to elevated pCO_2 and the recovery of such responses, future studies should include $GABA_A$ receptor antagonist such as gabazine (Nilsson *et al*., 2012), as well as methods to isolate pH and elevated pCO_2 as independent contributors to altered responses.

Interestingly, silver carp showed no impairment to alarm cue responses despite 4–10 d of exposure to low or high pCO_2 (3000 µatm and 8000 µatm respectively; Table 3; 4; Figure 2). Other fish species have demonstrated conservative responses to alarm cues following exposure to environmental stressors. For example, juvenile rainbow trout (*O. mykiss*) were able to recognize a novel odor as dangerous despite application of tests in acidified water of pH 6.0 (Leduc *et al*., 2004). Species like silver carp, which may live in environments where variations in pH and pCO_2 occur regularly, may be more adapted to elevated pCO_2 and reduced pH than species that live in more stable environments such as large bodies of water (Hirata *et al*., 2003; Melzner *et al*., 2009). Species, like cardinal tetras (*Cheirodon axelrodi*), have acclimated to have physiological tolerances to acidified water of pH 3.1 and survived for 5 weeks (Dunson *et al*.,

1977). Similarly, Gonzalez *et al*., (1987) showed *Enneacanthus obesus* had no change in body sodium concentration after 5 weeks in a pH of 4.0. Some freshwater species, like silver carp, may be able to adapt to elevated pCO_2 and still be able to respond appropriately to conspecific alarm cues and thus there may be minimal impacts to populations and overall mortality. Results from this study support the idea that impacts to conspecific alarm cues may be species dependent to exposures to elevated pCO_2.

A reduced alarm cue response after exposure to elevated CO_2 has many implications for management and the ecology of freshwater fish species. Freshwater fishes may be exposed to elevated pCO_2 due to natural environmental variation (reviewed by Hasler *et al.*, 2016), climate change (Phillip *et al.*, 2015), hatchery rearing (Colt and Orwicz, 1991), and zones of elevated pCO_2 deployed as fish barriers (Kates *et al.*, 2012; Noatch and Suski, 2012). If fathead minnows were subjected to an increase in pCO_2 concentrations, they may lose their ability to appropriately respond to conspecific alarm cues such as skin extracts from a predator event. Thus, this impaired alarm cue response may have implications to mortality and population dynamics (Leduc *et al*., 2013), and could potentially alter community composition (Chown and Gaston, 2016). However, if the CO_2 stimulus was removed, behaviors such as darts and freezes may return to normal. Interestingly, silver carp appear to be more robust to changes in environmental pCO_2, and elevations in pCO_2 may have minimal effects on their ability to detect and respond to alarm cues. Additional research is needed to define the mechanisms underlying these differences in the responses of freshwater fish species to elevated pCO_2. Together, these results provide information about the possible consequences and responses of freshwater fish to environmental changes such as elevations in pCO_2 and acidification.

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CHAPTER 2: THE EFFECTS OF EXPOSURE TO ELEVATED CARBON DIOXIDE ON BEHAVIOUR IN *LEPOMIS MACROCHIRUS*

Abstract

Carbon dioxide is a common stressor that can have negative outcomes for many fish behaviours. The current study defined the behavioural response of individual *Lepomis macrochirus* following exposures to elevated carbon dioxide (CO_2). For this, *L. macrochirus* were first held at ambient pCO_2 (160 µatm pCO_2) for 7 d, then exposed to elevated pCO_2 (8300 µatm pCO_2) for 5 d, then returned to ambient conditions for a further 5 d to recover. Following each exposure period, several behavioural metrics were quantified (boldness, lateralization, and activity). Average velocity, velocity in the thigmotaxis zone and proportion of activity in the thigmotaxis zone increased with pCO_2 exposure. During recovery, average velocity of *L. macrochirus* decreased relative to pre-exposure. In addition, individual rank was repeatable during the pre-exposure and recovery period in three of all the metrics investigated (average velocity in the middle zone, average velocity near object, and total shuttles to the object zone), suggesting that elevated pCO_2 may disrupt performance. Overall, this study suggests behaviours of freshwater fish such as *L. macrochirus* may not be as seriously impacted by increases in pCO_2 as has been shown for marine fishes, and behavioural disruptions that do occur may return to normal should the CO_2 stimulus be removed.

Introduction

The behaviour of fishes is important for survival, growth, and reproduction, and can be influenced by both internal and external stimuli (Keenleyside, 1979). Physiology of fishes (e.g.,

enzyme activity, metabolism) can also be impacted by environmental variables (Fry 1947), and can directly influence fish behaviour (Kramer 1987; Beitinger 1990; Bonga 1997). Thus, there is a clear bidirectional link between a fish's physiology and its behaviour that can be affected by abiotic and biotic factors. For example, boldness-type behaviours in individuals may result in increased access to food resources; however, these behaviours may also result in higher risk-taking actions, which may be costly in high predation areas (Wilson and Godin 2009). Conversely, shyer or anxiety-related behaviours may be beneficial for individuals in avoiding predation, but these behaviours may result in fewer reproductive opportunities in social groupings of fish (Wilson *et al.* 1993). Innate behaviours such as turning preference (i.e., lateralization) are also thought to be important for predator avoidance behaviours (Dadda *et al.* 2010), and play an important role in cognitive tasks (Dadda and Bisazza 2006) and in shoaling behaviours (Bisazza and Dadda 2005). Disruptions in innate behaviours due to environmental stimuli can influence fitness and mortality in fishes (Cantalupo *et al.*, 1995; Toms *et al.*, 2010; Chapman *et al.*, 2011), and thus support the notion that fish behaviours are influenced by environmental changes as has been seen in several studies (Tuomainen and Candolin 2011; Wong and Candolin 2015).

One potential human-induced environmental stimulus that may impact fish behaviour is an increase in carbon dioxide (CO_2) levels. Research from the marine environment has shown that the partial pressure of CO_2 (pCO_2) in seawater increases as atmospheric pCO_2 rises (Sabine *et al.*, 2004). Elevation in seawater pCO_2 causes a concomitant decrease in water pH, which combined with higher pCO_2, causes respiratory acidosis in fishes (Heuer and Grosell, 2014). The decrease in the blood pH of fish results in a disruption of cellular ionic gradients, and, in marine fish, it has been established that this disruption alters the function of the neural gamma-

aminobutyric acid type A ($GABA_A$) receptor, which has consequences for fish behaviour, specifically anxiety and olfactory-linked behaviors (Nilsson *et al.*, 2012; Hamilton *et al.*, 2014). For example, Dixson *et al.*, (2010) showed that settlement-stage orange clownfish *Amphiprion percula* (Lacepède 1802) larvae were actually attracted to the smell of a predator, and could not distinguish between a predator and non-predator, following exposure to elevated pCO_2 for a short duration (11 days post-hatch). Exposure to heightened levels of CO_2 can also lead to altered brain asymmetry and changes in behavioural lateralization, which have been documented for damselfishes (Ward's damsel *Pomacentrus wardi* Whitley 1927, *Neopomacentrus azysron* Bleeker 1877) (Domenici *et al.*, 2012; Nilsson *et al.*, 2012). In addition to studies on obligate marine fish species, exposure to elevated pCO_2 in marine-adapted three-spined stickleback *Gasterosteus aculeatus* L. 1758 have resulted in a loss of lateralization and a decrease in boldness and learning (Jutfelt *et al.*, 2013). In addition to these outcomes, exposure to elevated CO_2 levels has also been shown to have a range of other negative impacts for fish including a loss of antipredator responses (Allan *et al.*, 2013), changes in auditory preferences (Simpson *et al.*, 2011), higher activity levels (Munday *et al.*, 2010; Ferrari *et al.*, 2011), poor prey detection and feeding (Cripps *et al.*, 2011), and visual risk assessment (Ferrari *et al.*, 2012). However, CO_2-induced behavioural changes have been found to be variable across fish species, as behaviours (e.g., predator avoidance, lateralization, and swimming kinematics) of some marine fish species were unchanged after exposure to high pCO_2 (Jutfelt and Hedgärde, 2013; Maneja *et al.*, 2013; Sundin and Jutfelt, 2015). Clearly, much research has been completed on marine fish species and the potential for elevated CO_2 levels to influence fish behaviour.

Currently, the behavioural responses of freshwater fishes to elevated pCO_2 have not been well defined; in fact, no empirical study has been completed at naturally relevant levels of CO_2.

Freshwater fishes can experience elevated CO_2 levels through a number of mechanisms, including climate change (Phillips *et al*., 2015; Hasler *et al*. 2016), hatchery rearing (Colt and Orwicz, 1991), or the deployment of non-physical barriers that use CO_2 gas to prevent fish movements (Kates *et al*. 2012; Noatch and Suski 2012). In a recent study by Philips *et al.* (2015), it was hypothesized that the Laurentian Great Lakes may experience a future rise in pCO_2 similar to what has been observed in the oceans. Furthermore, fishes reared in hatchery settings frequently are exposed to elevated pCO_2 due to overcrowding and poor water quality, and water supersaturated with pCO_2 beyond natural levels has been shown to be an adequate fish deterrent (Noatch and Suski 2012). In addition, pCO_2 in freshwaters are naturally variable, for instance, pCO_2 ranged from 107–4,128 µatm across 62 lakes measured globally and many freshwater systems are considered supersaturated with CO_2 (Cole *et al.*, 1994). Therefore, freshwater fishes may be uniquely adapted to higher pCO_2 than their marine counterparts, although this has not been directly quantified. The consequences of elevated pCO_2 on the behaviour of freshwater fish are unknown, and, in light of possible changes in pCO_2 that may expose freshwater fish to elevated levels, there is a critical need to fill this gap (Hasler *et al*., 2016).

Another aspect of CO_2 exposure that is relevant for fishes is their capacity to recover after exposure to high pCO_2. With exception of climate change induced elevated pCO_2, many of the CO_2-related stressors that fish experience are transient, for instance high CO_2 levels in a hatchery (Colt and Orwicz, 1991) or exposure to elevated pCO_2 due to a fish barrier (Noatch and Suski 2012). Thus, it is conceivable that fish may escape from these stressors, either through avoidance or due to their release from a hatchery. Previous studies suggest that changes in behaviour may recover after exposure to high pCO_2, as Hamilton *et al.*, (2014) found that anxiety behaviours of juvenile California rockfish *Sebastes diploproa* Gilbert 1890 altered by exposure

to increased levels of pCO_2 returned to normal after returning to ambient seawater for 12 days. Therefore, understanding whether behavioural changes recover after exposure to high pCO_2 can have important implications for managers deploying CO_2 barriers or releasing hatchery-reared fish.

Due to the limited understanding of how the behaviours of freshwater fish are affected by high pCO_2, the objectives of this study were to (1) quantify the consequences of exposure to various levels of CO_2 on lateralization, boldness, and anxiety-linked behaviors in bluegill *Lepomis macrochirus* Rafinesque 1810; (2) determine whether affected behaviours recover (i.e., return to pre-exposure levels) after fish were returned to ambient conditions; and (3) determine if exposure to high CO_2 altered the performance rank of individuals. To accomplish these goals, uniquely identified *L. macrochirus* were subjected to a series of behavioural assays before, during, and following exposure to various levels of CO_2.

Materials and Methods

Experimental animals and husbandry

Adult male and female *Lepomis macrochirus* (N = 16; total length, 11.3 ± 1.2 cm [mean ± standard deviation; S.D.]; weight, 22.6 ± 6.7 g) were obtained from the Illinois Department of Natural Resources Little Grassy Fish Hatchery, Makanda, IL, and transported to the University of Illinois Aquatic Research Facility, Champaign-Urbana, IL. *Lepomis macrochirus* were held in a single 379 L plastic flow-through holding tank supplied with compressed air (Sweetwater, Aquatic Eco-Systems, Apopka, FL) through an air stone, and a continuous flow of water from a 0.04 ha earthen-pond. Fish were given seven days to acclimate to the new holding conditions

prior to the onset of experiments, and to allow for recovery from handling and hauling stressors (Milligan, 1996). To identify individuals and allow for repeated tests across different levels of CO_2 exposures, each *L. macrochirus* was provided a unique coloured tag using visible elastomer implant tags (Northwest Marine Technology Inc. Shaw Island, WA; Wilson and Godin, 2009). Tags were implanted using a syringe (29 gauge, 1 cc) and tags were arranged into different combinations of two colors and four tagging areas all within the muscle of the dorsal fin (upper or lower on left or right side of the dorsal spine). Tagging procedures lasted less than 30 s and occurred 24 h before the initial testing period. Following tagging, fish were returned to the common 379 L plastic flow-through holding tank. An ultraviolet water sterilizer (v2 Vecton 600, Tropical Marine Centre, Bristol, UK) was attached to a small pump to reduce bacterial growth in the tank, which also circulated water in the tank. Throughout the study, fish were fed red worms *Eisenia fetida* Savigny 1826 *ad libitum* daily until 24 h before each trial period. Water quality measurements in the holding tank were taken daily (Table 7), and included: temperature, dissolved oxygen (YSI, 550A Yellow Springs Instruments, Irvine, CA, USA), ammonia (LaMotte Company, Ammonia Nitrogen kit No. 3351-02, Chestertown, MD, USA), total alkalinity (TA) (Hach Company, Titrator model 16,900 and kit 94399, Loveland, CO, USA), and pH (WTW pH 3310 meter with a SenTix 41 probe, Germany); the pH probe was calibrated daily during this study. In addition pCO_2 was measured daily using an infrared CO_2 sensor (Vaisala, Carbon Dioxide Transmitter Series GMT220, Finland) wrapped in a semi-permeable polytetrafluoroethylene cover (Johnson *et al.*, 2010; Munday *et al.*, 2014) (Table 7).

pCO_2 exposure treatments

Over the course of the study, three pCO_2 exposure treatments were applied to the common holding tank. Behavioural assays (see below) were carried out following each exposure. Prior to the first set of behavioural tests (detailed below), fish were held for 7 d at ambient pCO_2, which was 160 µatm (± 43 µatm standard deviation; SD). Following completion of the first set of tests, the pCO_2 in the common holding tank was raised to 8300 µatm (± 400 µatm standard deviation; SD) for 5 d. This holding duration and pCO_2 were chosen as a 4 d holding period at 850 µatm pCO_2 has elicited changes in the behaviour of marine fishes (Munday *et al.*, 2010) and an extended exposure of *L. macrochirus* to this pCO_2 caused small physiological changes, but not a loss in equilibrium (Kates *et al.*, 2012). The target pCO_2 were held constant throughout the experiment using a Pinpoint pH Regulator Kit (American Marine Inc., Ridgefield CT) (Munday *et al.*, 2012; Allan *et al.*, 2013) programmed to bubble in gaseous CO_2 when water pH rose above a target level (Gattuso *et al.*, 2010). A homogenous mixture of CO_2 was achieved in the tank by using an air stone connected to a 1.80 amp air compressor (Sweetwater, Aquatic Eco-Systems, Apopka, FL), which also prevented hypoxia. Following the second set of behavioural tests, pCO_2 in the common tank was returned to ambient conditions 44 (± 13 µatm standard deviation; SD) for 5 d, after which behavioural tests were carried out for the third time. The two behavioral tests described below were conducted on the same day, and which test came first and what individual was chosen was randomly chosen during the three trial periods.

Lateralization test

Lepomis macrochirus were assessed for turning preference *via* a lateralization test after the three experimental treatments described above. To quantify lateralization, a double T-maze (15.2 × 45.7 cm main runway with a 15.2 × 45.7 cm runway at each end that were parallel to one another and attached perpendicular to the main runway) was used because it minimizes handling of fish (i.e., fish can be gently encouraged back along the main channel) and allows for multiple choice events in a single trial (Bisazza *et al.*, 1998; Jutfelt *et al.*, 2013). Twenty-four hours prior to each trial, all *L. macrochirus* were subdivided (N = 3 to 4) into 5.7 L containers held within the common holding tank to limit exposure to repeated netting during the behavioural trials. During the lateralization trial, one individual *L. macrochirus* was quickly taken out of the containers and placed in the double T-maze. The double T-maze had a dark curtain around the outside to reduce external stimuli. Water within the maze was filled to a depth of 8 cm, and the pCO_2 in the maze was identical to that of the common holding tank at each sampling period (Hamilton *et al.*, 2014). After a 2 min acclimation period (Domenici *et al.*, 2012), *L. macrochirus* were gently encouraged to move forward within the maze using a small PVC rod until the fish reached one end of the double T-maze and was forced to turn right or left (Domenici *et al.*, 2012; Jutfelt *et al.*, 2013). The choice made (i.e., turn to either the left or right) for each event was noted at the point when the fish left the main channel and entered one of the perpendicular side channels, and each animal was tested 10 times within a given trial (Domenici *et al.*, 2012). Following completion of the final choice event, animals were removed from the maze and returned to their individual containers within the common holding tank for additional testing (see below) or returned to the common holding tank if both tests were completed for that individual.

Novel object and thigmotaxis behavioural tests

Lepomis macrochirus were also assessed for boldness and anxiety-linked behaviours after the three experimental treatment periods described above. Novel object tests were conducted in a 61 cm diameter opaque, non-reflective arena surrounded by a dark curtain to reduce external stimuli. Water in this novel object experimental arena was filled to a height of 10 cm and had the same respective pCO_2 that the selected fish were exposed to at that time before each trial (Hamilton *et al.*, 2014). A novel object was placed in the center of the tank prior to the beginning of the trial and covered with a white plastic cover attached to fishing line. Note that a different novel object was used for each experimental treatment to avoid colour preference (habituation) of the fish (Jutfelt *et al.*, 2013; Hamilton *et al.*, 2014). A 3 cm Rubik's cube was used during the pre-exposure test, a multi-coloured 3 cm tall Lego® man was used during the post-exposure test, and a 2.5 cm diameter multi-coloured bouncy ball was used during the recovery test. At the beginning of each trial, an individual *L. macrochirus* was carefully netted from the 5.7 L containers held within the common holding tank and placed into the arena in the center of the tank, facing in the direction of the covered novel object. Each fish was given 10 min to acclimate to the tank before the cover on the novel object was gently raised up using the attached fishing line. The position of the fish was recorded for 10 min using an overhead-mounted video camera (iDS uEye 1480-C camera, iDS, Obersulm, Germany) (Maximino *et al.*, 2010; Hamilton *et al.*, 2014). Assessments of boldness and anxiety parameters were quantified using the program Lolitrack (Loligo Systems, Denmark; Poulsen *et al.*, 2014). Using the analysis software, the arena was divided into three equal-sized, concentric zones (center or near the object, middle, and outer or thigmotaxis/near the wall; Ou *et al.*, 2015) and these zones were at least equal to or larger than the body length (BL) of the fish (Schnörr *et al.*, 2012). Following the 10 min

observation period, the trial was terminated, and fish were returned to the common holding tank or were given the lateralization test based on if they completed that test yet.

Data analysis

Lateralization behavioural data analyses were adapted from Bisazza *et al.*, (1998) and Domenici *et al.*, (2012). To define a preference in turning side for an individual fish, a relative lateralization index (L_R) was calculated according to:

$$L_R = \frac{Turn\ to\ the\ right - Turn\ to\ the\ left}{Turn\ to\ the\ right + Turn\ to\ the\ left} \times 100$$

Mean L_R was used to evaluate "turning preference" of the fish (i.e., bias in left or right turns). Values of between -100 (i.e., fish that turned left during all 10 choice events) and 100 (fish that turned right during all 10 choice events) were calculated. An L_R of zero represented a non-bias in turning preference (i.e., fish that turned left and right in equal measure) (Bisazza *et al.*, 2000). The absolute lateralization index (L_A) for each individual was also calculated as the absolute value of L_R and plotted separately. The L_A estimates for each *L. macrochirus* were between 0 (i.e., individual turned in equal proportion to the right and to the left) to 100 (i.e., individual that turned right or left during all 10 choice events; Domenici *et al.*, 2012).

For the 'novel object and thigmotaxis behavioural test', parameters such as active time, total time in each of the three zones, total shuttles between zones, and average velocity were analyzed. Previous studies have used active time to quantify boldness in fish (e.g., *G. aculeatus*, (Bell, 2005) and zebrafish *Danio rerio* F. Hamilton 1822 (Moretz *et al.*, 2007)). Using video analysis also allowed for the measurement of average velocity and distance travelled, which has

been used to assess anxiety and boldness behaviours in fish (Cachat *et al.*, 2011a; Cachat *et al.*, 2011b). To further quantify boldness and anxiety, the proportion of time spent in the center zone (i.e., time spent investigating the novel object; Jutfelt *et al.*, 2013), and in the outer zone (i.e., wall hugging or thigmotaxis; (López-Patiño *et al.*, 2008; Schnörr *et al.*, 2012)), was measured, respectively. Boldness was assessed by calculating the proportion of activity and time spent within the center zone, divided by the test duration (Hamilton *et al.*, 2014). Thigmotaxis was defined as proportion of activity as well as the proportion of time spent in the outer zone (Schnörr *et al.*, 2012). The proportion of total activity in the outer most zone of the tank was calculated as the ratio between the total activity in the outer zone and the total activity within the whole test arena (includes center, middle, and outer/thigmotaxis zones) and multiplied by 100. The proportion of total activity corrects for potential individual differences in activity (Bouwknecht and Paylor, 2008). The proportion of time spent in the outer zone was calculated as the total time spent in the outer zone, divided by the test duration, and multiplied by 100. Video data were first transformed from pixels to cm and then into BL to standardize distance, velocity, and acceleration measurements.

Statistical Analysis

To determine if elevated pCO_2 had an effect on lateralization, we performed Generalized Linear Mixed Models (GLMMs), with appropriate error and distributions and link-functions (Quinn and Keough, 2002), where the L_R and L_A were the response variables, treatment (pre-exposure, post-exposure, recovery) was included as a fixed effect, and fish ID was included as a random effect. The use of a random effect (essentially a repeated measures design) was

necessary because multiple measurements were taken from each animal across trials, meaning that each measurement was not independent and potentially correlated within an individual (Laird and Ware, 1982; Lindstrom and Bates, 1990). We fitted these GLMMs with linear mixed effects regression models (LMER) (glmer from the lme4 library in R; Bates 2010). For the L_R model, a binomial error distribution was used due to the test outcome choice being left or right. This model used the number of turns to the left as a response variable and the number of total turns was used as a weight. The same model was used for the L_A, with the exception that the maximum number of turns to the preferred side was used as the response variable and a Poisson error distribution was used with no weights because the data were counts.

To determine if pCO_2 affected thigmotaxis or boldness behaviours, GLMMs were again used. Similar to above, treatment was included as a fixed factor and fish ID was included as a random effect. For shuttle parameters, a GLMM using Penalized Quasi-Likelihood (PQL) (glmmPQL from the MASS library in R (Venables and Ripley, 2002) was used with a quasipoisson error distribution to account for overdispersion of the data (Bolker *et al.*, 2009). The same model was used for the proportion of time spent in each zone except with a weight of total time added. For all other parameters a GLMM-PQL was used with a quasi error distribution to again account for overdispersion of data (Crawley, 2002).

To determine if exposure to elevated levels of pCO_2 influenced the repeatability of individual's rank within each behavioural metric, a Spearman's coefficient of rank correlation tests was conducted (Zar 1999). These comparisons of each individual's rank were compared for pre-exposure and post-post-exposure, and pre-exposure and recovery.

For all statistical tests, analyses were performed in R Studio (Venables and Smith, 2010), and differences were considered significant if α was less than 0.05. Fish total length was initially included as a covariate in the analytical models, but was not significant, and therefore excluded from the final models (Engqvist, 2005).

Results

Prior to being exposed to elevated pCO_2, *L. macrochirus* displayed a right turning preference, and this preference was unaffected by exposure to elevated pCO_2 and subsequent return to ambient conditions (Table 8; Fig. 6A). Similarly, L_A was not altered by exposure to elevated pCO_2 in that there was no change in the strength of turning side preference regardless of preference in *L. macrochirus* (Table 8; Fig. 6B).

Following the initial acclimation period and exposure to the novel object, *L. macrochirus* explored the arena and approached the object. However, many of the parameters measured were not significantly different after exposure to elevated pCO_2 or following the subsequent recovery period (Table 9; Fig 6, 7, 8). The proportion of time spent in each zone (i.e., thigmotaxis, middle, and near the object) did not differ significantly across the three treatment periods (Table 9; Fig. 7). Similarly, the level of exploration in the tank was not affected by pCO_2 exposure, as the proportion of total activity during the monitoring period, total shuttles between zones in the tank and average velocity by the object did not differ significantly across treatment periods (Table 9; Fig. 8A, B, C). The proportion of activity in the middle zone of the tank, and also near the novel object, during the entire monitoring period also did not differ significantly across treatments

(Table 9). In addition, the average velocity of each *L. macrochirus* within each zone was not significantly different after exposure to elevated pCO_2 (Table 9).

Lepomis macrochirus did display some boldness-linked behaviours after being exposed to elevated pCO_2. Average velocity during the pre-exposure period increased significantly from 0.23 ± 0.07 to 0.30 ± 0.10 BL s^{-1} (means ± SEM) after elevated pCO_2 exposure, and then decreased to 0.24 ± 0.07 BL s^{-1} during the recovery period and was significantly different than the post-exposure period (Table 9; Fig. 9A). Within the thigmotaxis zone, the average velocity during the pre-exposure period significantly increased from 0.22 ± 0.09 to 0.30 ± 0.10 BL s^{-1} during the post-exposure period and decreased to 0.24 ± 0.07 BL s^{-1} after fish were moved to ambient conditions, although this change was not significantly different from the post-exposure period (Table 9; Fig. 9B). The proportion of time spent being active in the thigmotaxis zone during the trial also increased from 0.20 ± 0.10 during the pre-exposure to 0.28 ± 0.10 s in the post-exposure period (Table 9; Fig. 9C). During the recovery period, time spent active in the thigmotaxis zone was 0.26 ± 0.12 s, which did not differ significantly from the post-exposure period (Table 9; Fig. 9C).

The repeatability of an individual's rank across the three treatment periods was used to assess intra-individual variability across treatments. Ranks for only three of the behavioural metrics during the pre-exposure period (average velocity in the middle zone, average velocity near object, and total shuttles to the object zone) were similar to the rankings during the recovery period (return to ambient conditions following elevated pCO_2 treatment), but not during the period of elevated CO_2 exposure (Table 10). However, in all but the average velocity near the object, the rank coefficients (r_s) were less than 0.50, indicating that only a strong association was found for one of the three behavioural metrics.

Discussion

Five days of exposure to elevated pCO_2 (~8300 µatm) resulted in changes to some, but not all, of the behavioural metrics measured for *L. macrochirus*. More specifically, average velocity in the whole arena, as well as the average velocity and the proportion of time active in the thigmotaxis zone, increased after exposure to elevated pCO_2 relative to the ambient pCO_2 treatment; these increases in velocity and activity point to a shift to bolder and more active behaviours. While the current study is the first to examine CO_2-induced behavioural changes for an obligate freshwater fish species (i.e., *L. macrochirus*), previous work on marine, temperate and anadromous species also suggests that exposure of fishes to elevated pCO_2, even for short durations, can alter behaviour. For example, Munday *et al.,* (2010) found that post-settlement stage *P. wardi* exposed to 850 µatm CO_2 for 4 d displayed bolder and more active behaviour by venturing further away from a shelter. Similarly, several studies have shown that fish display an increase in activity after exposure to elevated pCO_2 (Cripps *et al.*, 2011; Ferrari *et al.*, 2011; Munday *et al.*, 2013). At present, the purported mechanism responsible underlying these behavioural changes is that the $GABA_A$ neural receptor function is reversed by becoming excitatory (i.e., an efflux of anions) rather than inhibitory (i.e., influx of anions) (Nilsson *et al.*, 2012). This hypothesis has been tested for several fish species, and the treatment of fishes with gabazine (a $GABA_A$ receptor inhibitor) prevents the CO_2-induced changes in behaviour (Nilsson *et al.*, 2012; Lai *et al.*, 2015; Ou *et al.*, 2015). Although the role of $GABA_A$ receptors as a mediator of CO_2-induced behvaioural effects was not tested in the present study, previous work on marine fish suggested that altered $GABA_A$ function may also explain the tendency for increased boldness in *L. macrochirus* and warrants further investigation. Ultimately, increases in boldness-type behaviours and activity may translate to higher risk-taking behaviours, which may

alter predator-prey relationships and ultimately have consequences for fitness or mortality rates in fish exposed to high pCO_2.

Interestingly, of the three behavioural metrics shown to change following exposure to high pCO_2, only average velocity in the whole arena returned to levels observed during the pre-exposure period following 5 d of recovery at ambient pCO_2 (44 µatm), with the other two metrics (average velocity and the proportion of time active in the thigmotaxis zone) remaining elevated. Previous work has shown, that behavioural changes induced by exposure to elevated pCO_2 can be reversed following removal of the CO_2 stimulus, but this potential for recovery has not been well studied. For example, predator avoidance behaviours were re-established in larval *P. wardi* after returning to ambient seawater for 2 d (Munday *et al.*, 2010), and anxiety behaviours in juvenile *S. diploproa* affected by CO_2 exposure returned to normal after 12 d in ambient conditions (Hamilton *et al.*, 2014). Following the removal of the CO_2 stimuli, previously disrupted cellular ion gradients likely re-establish, allowing for changes in the $GABA_A$ receptor to return to normal functioning (i.e., inhibitory rather than excitatory), thus restoring normal neuronal activity and behaviour. It may be possible that additional recovery time at ambient conditions (i.e., beyond 5 d) may be necessary to reverse the physiological effects of CO_2 exposure (i.e., blood acidosis that is responsible for ionic gradient disruption), and that all measured behavioural metrics may return to baseline levels following a longer recovery period. These hypotheses were not tested in the current study, however, they do put forth some interesting avenues to consider with respect to the impacts of physiology on behaviour.

A number of the behaviours monitored in this study did not change following 5 d of exposure to elevated pCO_2. Overall, no change in lateralization (i.e., turning preference) and limited changes in boldness and anxiety-linked behaviours in *L. macrochirus* (see above) were

found following exposure to elevated pCO_2. Boldness behaviours, such as time spent investigating the novel object (i.e., time near the object and velocity near the object), total shuttles between zones and the proportion of activity in the arena (as assessments of overall activity), did not change after exposure to elevated pCO_2. Although previous studies found that lateralization was altered in *G. aculeatus* following a 20 d exposure to 1000 µatm pCO_2 (Näslund *et al.*, 2015) and in a coral reef fish *N. azysron* exposed to 880 µatm pCO_2 for 4 d, and boldness type behaviours are generally elevated by exposure to elevated pCO_2 (see above examples), CO_2-mediated behavioural changes are not ubiquitous among all fishes (Heuer and Grosell, 2014). For example, L_R and activity in temperate wrasse *Ctenolabrus rupestris* L. 1758 were unaffected after 28 d of exposure to 995 µatm pCO_2 (Sundin and Jutfelt 2015). Similarly, juvenile Atlantic cod *Gadus morhua* L. 1758 displayed no change in L_R or L_A, or emergence from a shelter (an indicator of boldness) despite exposure to 995 µatm pCO_2 for 28 d (Jutfelt and Hedgärde 2015). In addition, swimming speed of larval cobia *Rachycentron canadum* L. 1766 was unaltered after exposure to 2100 µatm for 3 weeks (Bignami 2013). At present, the mechanism for this lack of a behavioural response in freshwater fishes following exposure to high pCO_2 has not been defined, however, it may be due to a fish's ability to acclimatize to changes in the environment, or possibly because some behaviours may be affected more than others following exposure to elevated pCO_2 (Näslund *et al.*, 2015). While the mechanism for and quantity of CO_2 in sea water is relatively well defined (Nilsson *et al.*, 2012; Hamilton *et al.*, 2014) and is quite stable in some areas of the ocean (Sabine *et al.*, 2004), CO_2 in freshwater systems is extremely variable due to a number of different factors (e.g., stream order, terrestrial primary productivity, precipitation, substrate) and can vary across a range of time scales (e.g., diel and seasonal) (reviewed by Hasler *et al.*, 2016). For example, pCO_2 in freshwater lakes can

vary from 3.1-fold below to 16-fold above atmospheric pCO_2, with a mean of ~ 1000 µatm (Cole *et al.*, 2007). As such, freshwater fishes have likely been exposed to fluctuations in pCO_2 over extended, evolutionary time periods, potentially making them more robust against CO_2-induced behavioural changes compared to some marine fishes. Potentially, this difference in exposures characterized by higher and more variable pCO_2 may explain, at least in part, the lack of behavioural responses for *L. macrochirus* in the current study. Together, results from the present study and those of previous studies demonstrate that behavioural responses to elevated pCO_2 are varied, and suggest that freshwater fish may have the potential to be more robust to increases in pCO_2.

One unanticipated finding from the current study was the amount of variation in the behavioural responses of individual *L. macrochirus* across treatments. Specifically, individual rank in behaviours was only repeatable across treatments for three of the 17 metrics, and only when comparing the pre-exposure ranks to the 'recovery' ranks. This indicates that, for most behaviours measured, they were not in a repeatable fashion (i.e., performance was not consistent across the trials). Interestingly, however, performance for two of the bold-associated metrics (shuttles to the object zone, and velocity in the object zone) were repeatable during trials when fish were held at ambient pCO_2, but not during the high CO_2 exposure period, indicating that elevated pCO_2 scrambled the rankings of individual's boldness performance. Taken together with the increase in boldness behaviours described above (e.g., average velocity in the whole arena, as well as the average velocity and the proportion of time active in the thigmotaxis zone), and should boldness in the wild correlate with fitness (Brown *et al*., 2007), this may suggest that that exposure to elevated pCO_2 may alter fitness of individual wild fish. Furthermore, two of the three metrics were repeatable during ambient holding conditions but not during high CO_2

exposure were measures of velocity (i.e., lab measured locomotory performance), which may be related to field locomotory performance (Irschick 2003; Irschick *et al.*, 2008). In wild freshwater fish swimming speed is repeatable (Hanson *et al.*, 2010) and therefore, a change in locomotory performance during high CO_2 exposure further supports the notion that exposure to elevated pCO_2 may change individual fitness in wild fish (reviewed in Dommenici and Blake 1997). Failure to conduct studies that account for intra-individual differences in behavioural responses (and specifically performance among conspecifics) may mask important changes among individuals.

In summary, results of the present study have a number of implications for fisheries management and ecology, in the context of exposure to elevated pCO_2 for freshwater fishes. Freshwater fishes have potential to be exposed to elevated pCO_2 due to natural environmental variation (reviewed by Hasler *et al.*, 2016), climate change (Phillip *et al.*, 2015), hatchery rearing (Colt and Orwicz, 1991), and zones of elevated pCO_2 deployed as fish barriers (Kates *et al.*, 2012; Noatch and Suski, 2012). If a freshwater species such as *L. macrochirus* is exposed to sufficiently high pCO_2 for extended periods, data from the current study indicate that fish lateralization and bold/anxiety behaviours may not be largely altered, but fish may show an increase in activity and/or velocity of up to 30 %, resulting in additional swimming activity. In turn, these increases in velocity and activity may result in elevated energy consumption, potentially translating into increase foraging to maintain condition (Jobling, 1995). Depending on variables such as prey abundance, competition, and abundance of predators, this elevation in activity, and potentially boldness, could have a number of possible outcomes ranging from increased predation risk to reduced growth or reproduction (Safina and Burger, 1985; Werner and Anholt, 1993). Should the CO_2 stimulus be removed, however, behaviours like velocity

would be expected to return to normal. In addition, the limited amount of significant findings shown here contrasts with a large amount of research from the marine environment showing a suite of negative changes to fish behaviour following extended high pCO_2 exposure (Munday *et al.*, 2009; Dixson *et al.*, 2010; Jutfelt *et al.*, 2013). Additional work is therefore needed to define the biological implications for increased CO_2 levels in freshwater, such as long-term mortality, predator-prey interactions, fitness, and risk to endangered species populations. Most importantly, mechanisms underlying possible acclimatization (and perhaps adaptation) of freshwater fish to changes in pCO_2 needs to be further explored. Together, the current data set, coupled with additional studies related to mechanism(s) and consequences, can help better define the responses of freshwater fishes to a common environmental stimulus such as CO_2.

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CHAPTER 3: GENERAL CONCLUSIONS

Understanding the possible negative consequences of elevated pCO_2 has become increasingly important particularly in marine environments with respect to climate change. However, less focus has been given to understanding how elevated pCO_2 may impact freshwater ecosystems. For instance, freshwater fish may be impacted by elevated pCO_2 in overcrowded hatchery settings, new non-physical barrier technologies such as CO_2 and climate change. Freshwater pCO_2 systems are also naturally variable unlike the marine environment. Therefore, freshwater fishes may be uniquely adapted to higher pCO_2 than their marine counterparts, although this has not been directly quantified. The two separate, yet complimentary studies described in this thesis provide insight into how elevated pCO_2 may impact freshwater fish behavior: are conspecific alarm cue responses impacted after prolonged exposure to elevated pCO_2; and how are personality behaviors in the same individuals altered after prolonged exposure to elevated pCO_2. Both of these studies also included a recovery component as fish have been shown to avoid elevated pCO_2 and pCO_2 in freshwater systems varies constantly.

The first data chapter demonstrated that low and high concentrations of pCO_2 may impact fathead minnows ability to respond to conspecific skin extracts but not silver carp. The first chapter also showed fathead minnows that were exposed to high concentrations of elevated pCO_2 may be able to recover as irregular activities increased during the stimulus compared to the acclimation. This study shows that there may be species specific differences in how elevated pCO_2 impacts alarm cue responses but they may be able to recover after returning to ambient water.

The second study in this thesis demonstrated that individual *L. macrochirus* lateralization, boldness and anxiety may not be altered by prolonged elevated pCO_2 exposure. Average velocity, velocity in the thigmotaxis zone and proportion of activity in the thigmotaxis zone however did increase with pCO_2 exposure. Although, during the recovery period the average velocity decreased among individuals, suggesting recovery. In addition, individual rank was repeatable during the pre-exposure and recovery period in only three of the total metrics investigated (average velocity in the middle zone, average velocity near object, and total shuttles to the object zone), suggesting that elevated pCO_2 may disrupt performance. Overall, this second study suggests behaviors of freshwater fish such as *L. macrochirus* may not be as seriously impacted by increases in pCO_2 as has been shown for marine fishes, and behavioural disruptions that do occur may return to normal should the CO_2 stimulus be removed.

Collectively, the results from these two studies present a few ways elevated pCO_2 may alter freshwater fish behavior. I recommend researchers look into what physiological mechanisms may be altered and are creating these behavioral differences as well as what other behaviors and species may or may not be impacted by elevated pCO_2 exposure.

TABLES AND FIGURES

Table 1. Mean and standard error summary of daily water chemistry (temperature [°C], dissolved oxygen [mgL^{-1}], pH, pCO_2 [µatm], alkalinity [mgL^{-1}]) in the three treatment groups (control, low, high) during CO_2 exposure and recovery periods for both species (fathead minnows and silver carp).

Species	Procedure	Treatment	Temperature (°C)	Dissolved Oxygen (mgL^{-1})	pH	pCO_2 (µatm)	Alkalinity (mgL^{-1})
Fathead minnows	CO_2	Control	17.1 ± 0.3	9.29 ± 0.2	8.32 ± 0.04	582 ± 67	178 ± 4
		Low	16.6 ± 0.2	9.35 ± 0.2	8.21 ± 0.07	793 ± 141	171 ± 2
		High	17.2 ± 0.3	9.53 ± 0.2	7.27 ± 0.04	6895 ± 631	175 ± 3
	Recovery	Control	16.2 ± 0.5	9.89 ± 0.2	8.50 ± 0.04	345 ± 33	174 ± 5
		Low	16.3 ± 0.4	9.85 ± 0.2	8.43 ± 0.06	443 ± 87	172 ± 4
		High	16.5 ± 0.5	9.95 ± 0.2	8.43 ± 0.04	394 ± 34	168 ± 4
Silver carp	CO_2	Control	12.8 ± 0.6	6.63 ± 0.3	7.63 ± 0.05	1100 ± 77	133 ± 2
		Low	12.7 ± 0.6	6.67 ± 0.2	7.37 ± 0.03	3060 ± 300	133 ± 2
		High	12.8 ± 0.6	6.59 ± 0.2	7.05 ± 0.03	7910 ± 812	133 ± 2
	Recovery	Control	12.1 ± 0.1	6.47 ± 0.2	7.67 ± 0.02	840 ± 24	137 ± 1
		Low	12.1 ± 0.5	6.25 ± 0.2	7.55 ± 0.11	960 ± 24	138 ± 1
		High	12.1 ± 0.5	6.48 ± 0.2	7.54 ± 0.14	1020 ± 80	137 ± 1

Table 2. Mean ± standard error with ranges summary of each species (fathead minnow and silver carp) fork length (mm) and weight (g) measurements after each behavior trial by procedure (CO_2 or recovery exposure) and treatment (control, low, high). Total number of fish used from each treatment is represented by n.

Species	Procedure	Treatment	n	Fork Length (mm)	Weight (g)
Fathead Minnows	CO_2	Control	9	54.0 ± 2.1 (range: 45-65)	1.6 ± 0.2 (range: 0.9-2.6)
		Low	9	51.1 ± 1.7 (range: 43-57)	1.3 ± 0.1 (range: 0.9-2.0)
		High	9	52.3 ± 1.6 (range: 48-60)	1.3 ± 0.1 (range: 0.9-2.1)
	Recovery	Control	9	58.4 ± 1.9 (range: 52-67)	1.9 ± 0.2 (range: 1.3-3.0)
		Low	10	61.7 ± 1.7 (range: 51-70)	2.2 ± 0.2 (range: 1.5-3.1)
		High	10	53.8 ± 0.8 (range: 50-57)	1.4 ± 0.1 (range: 1.1-1.7)
Silver Carp	CO_2	Control	9	127.3 ± 3.4 (range: 119.4-144.8)	18.7 ± 1.9 (range: 13.2-30.5)
		Low	9	132 ± 3.6 (range: 122.9-157.5)	22.2 ± 2.2 (range: 14.8-32.5)
		High	9	130 ± 2.5 (range: 114.3 ± 139.7)	19.5 ± 1.1 (range: 11.7-22.8)
	Recovery	Control	9	117.4 ± 1.8 (range: 111.8 ± 127)	13.1 ± 0.7 (range: 11.1-16.6)
		Low	9	125.6 ± 3.9 (range: 111.8 ± 139.7)	17.2 ± 1.4 (range: 11.8-22.3)
		High	9	123.6 ± 3.9 (range: 114.3 ± 144.8)	16.0 ± 1.2 (range: 12.2-22.4)

Table 3. Statistical outputs (Value, Standard Error, DF, *t*-value and *p*-value) for each output (Intercept, Stimulus, Recovery and Stimulus x Recovery for fathead minnows and Intercept, Low, High, Pre-stimulus, Low × Pre-stimulus and High x Pre-stimulus for silver carp) of Poisson generalized linear mixed effects models from R using penalized quasi-likelihood for each response. Fathead minnows were divided by CO_2 treatment (control and control recovery, low and low recovery and high and high recovery) while silver carp were divided by treatment group (all CO_2 exposure or all recovery fish). The intercept value represents the "Pre-stimulus" values for fathead minnows and "control stimulus" for silver carp. For each parameter value it represents the change in the model intercept associated with that response.

Response	Species	Treatment	Model	Parameter	Value	Standard Error	DF	*t*-value	*p*-value
Irregular activities	Fathead minnows	Control	Poisson GLMPQL	Intercept	0.30	0.60	16	0.51	0.62
				Stimulus	**1.71**	**0.61**	**16**	**2.81**	**0.01**
				Recovery	0.35	0.79	16	0.44	0.66
				Stimulus x Recovery	-0.50	0.81	16	-0.62	0.55
Irregular activities	Fathead minnows	Low	Poisson GLMPQL	Intercept	-0.05	0.53	17	-0.10	0.92
				Stimulus	**1.20**	**0.57**	**17**	**2.10**	**0.05**
				Recovery	0.73	0.65	17	1.13	0.27
				Stimulus x Recovery	-0.46	0.71	17	-0.65	0.52
Irregular activities	Fathead minnows	High	Poisson GLMPQL	Intercept	1.32	0.36	17	3.72	0.002
				Stimulus	-0.30	0.37	17	-0.81	0.43
				Recovery	-0.72	0.54	17	-1.34	0.2
				Stimulus x Recovery	**1.61**	**0.51**	**17**	**3.15**	**0.006**

Table 3 (cont.)

Response	Species	Treatment	Model	Parameter	Value	Standard Error	DF	t-value	p-value
Irregular activities	Silver carp	CO_2	Poisson GLMPQL	Intercept	1.14	0.39	24	2.94	0.007
				Low	-0.04	0.55	24	-0.07	0.95
				High	-0.10	0.55	24	-0.18	0.86
				Pre-stimulus	**-0.75**	**0.36**	**24**	**-2.06**	**0.05**
				Low x Pre-stimulus	-1.07	0.66	24	-1.61	0.12
				High x Pre-stimulus	0.23	0.51	24	0.44	0.66
Irregular activities	Silver carp	Recovery	Poisson GLMPQL	Intercept	0.94	0.25	24	3.73	0.001
				Low	-0.74	0.44	24	-1.67	0.11
				High	0.45	0.32	24	1.39	0.18
				Pre-stimulus	**-1.06**	**0.49**	**24**	**-2.14**	**0.04**
				Low x Pre-stimulus	0.96	0.65	24	1.33	0.20
				High x Pre-stimulus	-0.13	0.72	24	-0.20	0.84

Table 4. Statistical outputs of generalized linear mixed effects models. Fathead minnows were divided by CO_2 treatment (control and control recovery, low and low recovery and high and high recovery) while silver carp were divided by treatment group (all CO_2 exposure or all recovery fish). 95 % credible intervals were calculated using posterior simulations of each fixed effect. The intercept value represents the baseline values (e.g., pre-stimulus for fathead minnows and control stimulus for silver carp). Values for factors represent the percent change in the model intercept associated with the factor. Significance was determined if 95% credible intervals did not overlap zero and are bolded.

Response	Species	Treatment	Model	Parameter	Estimate	95% Credible Interval
log (activity)	Fathead minnow	Control	Linear Mixed Effects Model	Intercept	4.98	4.40, 5.50
				Stimulus	0.60	-0.03, 1.26
				Recovery	0.13	-0.58, 0.93
				Stimulus x Recovery	**-1.58**	**-2.52, -0.74**
log (activity)	Fathead minnow	Low	Linear Mixed Effects Model	Intercept	5.07	4.55, 5.57
				Stimulus	-0.06	-0.67, 0.60
				Recovery	-0.21	-0.89, 0.45
				Stimulus x Recovery	-0.25	-1.16, 0.59
log (activity)	Fathead minnow	High	Linear Mixed Effects Model	Intercept	5.27	4.72, 5.82
				Stimulus	0.04	-0.58, 0.70
				Recovery	0.08	-0.68, 0.81
				Stimulus x Recovery	-0.52	-1.38, 0.41

Table 4 (cont.)

Response	Species	Treatment	Model	Parameter	Estimate	95% Credible Interval
log (distance travelled)	Fathead minnow	Control	Linear Mixed Effects Model	Intercept	4.30	3.48, 5.05
				Stimulus	**1.11**	**0.25, 2.04**
				Recovery	0.14	-0.88, 1.24
				Stimulus x Recovery	**-1.81**	**-3.09, -0.57**
log (distance travelled)	Fathead minnow	Low	Linear Mixed Effects Model	Intercept	4.54	3.86, 5.24
				Stimulus	0.02	-0.80, 0.87
				Recovery	-0.45	-1.45, 0.45
				Stimulus x Recovery	-0.14	-1.37, 1.03
log (distance travelled)	Fathead minnow	High	Linear Mixed Effects Model	Intercept	4.92	4.28, 5.57
				Stimulus	-0.02	-0.74, 0.71
				Recovery	-0.04	-0.94, 0.81
				Stimulus x Recovery	-0.19	-1.13, 0.81
log (velocity)	Fathead minnow	Control	Linear Mixed Effects Model	Intercept	-0.69	-1.02, -0.37
				Stimulus	**0.51**	**0.10, 0.95**
				Recovery	0.00	-0.43, 0.46
				Stimulus x Recovery	-0.23	-0.81, 0.37
log (velocity)	Fathead minnow	Low	Linear Mixed Effects Model	Intercept	-0.58	-0.84, -0.33
				Stimulus	0.13	-0.19, 0.47
				Recovery	-0.20	-0.54, 0.16
				Stimulus x Recovery	0.06	-0.40, 0.49
log (velocity)	Fathead minnow	High	Linear Mixed Effects Model	Intercept	-0.35	-0.63, -0.08
				Stimulus	-0.06	-0.38, 0.24
				Recovery	-0.12	-0.51, 0.25
				Stimulus x Recovery	0.35	-0.08, 0.80

Table 4 (cont.)

Response	Species	Treatment	Model	Parameter	Estimate	95% Credible Interval
log (activity)	Silver carp	CO_2	Linear Mixed Effects Model	Intercept	5.75	5.54, 5.97
				Low	0.03	-0.27, 0.35
				High	0.12	-0.19, 0.43
				Pre-stimulus	-0.05	-0.29, 0.19
				Low x Pre-stimulus	-0.10	-0.45, 0.22
				High x Pre-stimulus	0.09	-0.26, 0.44
log (distance travelled)	Silver carp	CO_2	Linear Mixed Effects Model	Intercept	4.59	4.22, 5.00
				Low	0.10	-0.45, 0.67
				High	0.17	-0.38, 0.71
				Pre-stimulus	-0.08	-0.47, 0.30
				Low x Pre-stimulus	-0.21	-0.77, 0.34
				High x Pre-stimulus	0.16	-0.38, 0.74
log (velocity)	Silver carp	CO_2	Linear Mixed Effects Model	Intercept	-1.16	-1.35, -0.96
				Low	0.05	-0.25, 0.32
				High	0.05	-0.22, 0.33
				Pre-stimulus	-0.03	-0.19, 0.14
				Low x Pre-stimulus	-0.10	-0.32, 0.16
				High x Pre-stimulus	0.07	-0.17, 0.32
log (activity)	Silver carp	Recovery	Linear Mixed Effects Model	Intercept	5.66	5.40, 5.90
				Low	-0.21	-0.55, 0.14
				High	0.13	-0.24, 0.50
				Pre-stimulus	0.06	-0.20, 0.31
				Low x Pre-stimulus	0.09	-0.32, 0.46
				High x Pre-stimulus	-0.03	-0.32, 0.38

Table 4 (cont.)

Response	Species	Treatment	Model	Parameter	Estimate	95% Credible Interval
log (distance travelled)	Silver carp	Recovery	Linear Mixed Effects Model	Intercept	4.53	4.17, 4.94
				Low	-0.40	-0.98, 0.14
				High	0.10	-0.47, 0.65
				Pre-stimulus	0.05	-0.39, 0.44
				Low x Pre-stimulus	0.16	-0.42, 0.78
				High x Pre-stimulus	-0.01	-0.55, 0.54
log (velocity)	Silver carp	Recovery	Linear Mixed Effects Model	Intercept	-1.12	-1.26, -0.97
				Low	-0.19	-0.40, 0.01
				High	-0.03	-0.22, 0.16
				Pre-stimulus	-0.01	-0.17, 0.14
				Low x Pre-stimulus	0.07	-0.13, 0.29
				High x Pre-stimulus	0.02	-0.19, 0.24

Table 5. Changes in the intercept estimate for response variables with a significant interactive effect (see Table 4) for control fathead minnows (*Pimephales promelas*) during the CO_2 exposure and recovery period. Both mean and 95% credible intervals were calculated from estimates obtained using posterior simulations of each fixed effect. Significance was determined if the 95% credible intervals did not overlap zero and are bolded.

Response	Treatment	Mean	95% Credible Interval
log (activity)	CO_2	0.61	-0.02, 1.24
	Recovery	**-0.98**	**-1.62, -0.35**
log (distance)	**CO_2**	**1.11**	**0.21, 2.06**
	Recovery	-0.71	-1.58, 0.17

Table 6. Total activity (s), total distance travelled in body lengths (BL), and velocity (BL/s) for silver carp (*Hypophthalmichthys molitrix*) during acclimation and stimulus periods following CO_2 exposure and recovery. Silver carp were exposed to either ambient (750 µatm), low (1,500 µtam), or high (7,000 µatm) CO_2 levels for 4-10 d and then held for an additional 11-14 d at ambient (750 µatm CO_2) conditions. Data are presented as means ± SE (N = 9). No significant effect of monitoring period or treatment were detected within the CO_2 or recovery treatment periods (GLMM, see Tables 3, 4).

Procedure	Treatment	Monitoring Period	Activity (s)	Total Distance (BL)	Velocity (BL/s)
CO_2	Control	Acclimation	315.8 ± 34.5	106.4 ± 18.5	0.32 ± 0.03
		Stimulus	329.1 ± 27.0	109.8 ± 15.7	0.32 ± 0.03
	Low	Acclimation	289.4 ± 28.2	93.2 ± 18.3	0.30 ± 0.03
		Stimulus	338.0 ± 31.0	121.7 ± 17.9	0.34 ± 0.03
	High	Acclimation	396.6 ± 36.0	151.9 ± 21.8	0.36 ± 0.03
		Stimulus	366.6 ± 28.4	128.1 ± 16.3	0.34 ± 0.02
Recovery	Control	Acclimation	310.4 ± 24.8	105.7 ± 16.3	0.33 ± 0.02
		Stimulus	315.2 ± 39.5	112.7 ± 18.3	0.33 ± 0.02
	Low	Acclimation	284.7 ± 31.8	88.1 ± 15.0	0.29 ± 0.02
		Stimulus	257.1 ± 38.9	75.8 ± 15.6	0.27 ± 0.02
	High	Acclimation	344.9 ± 29.0	115.9 ± 15.8	0.32 ± 0.02
		Stimulus	333.3 ± 25.5	110.0 ± 13.9	0.32 ± 0.02

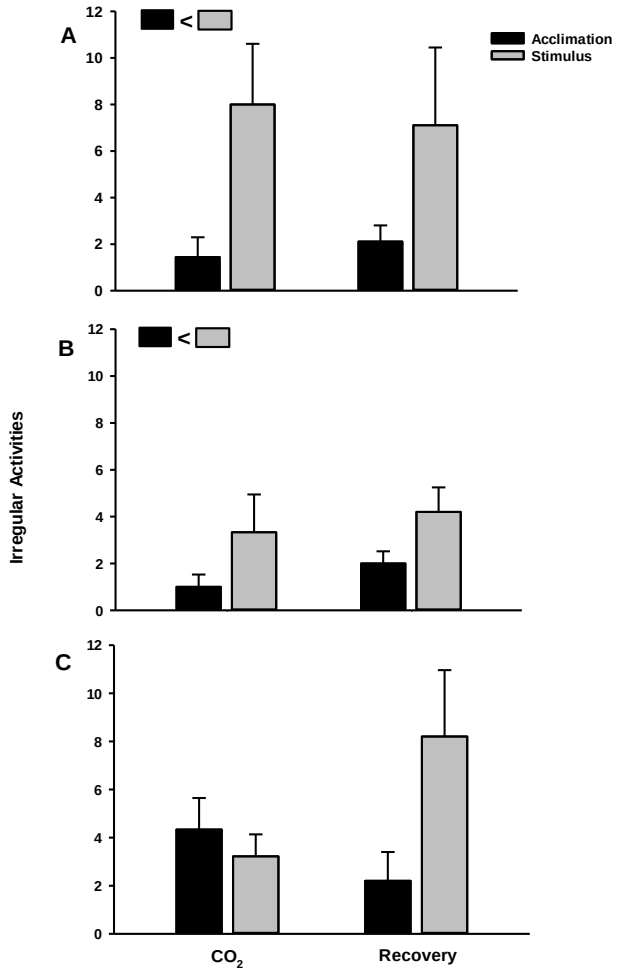

Figure 1. The number of irregular activities (i.e., darts and freezes) for fathead minnows (*Pimephales promelas*) during the acclimation and stimulus periods following CO_2 exposure and recovery. Fathead minnows were exposed to either (A) control (750 µatm), (B) low (1,500 µtam), or (C) high (7,000 µatm) CO_2 levels for 4-12 d and then held for an additional 11-14 d at ambient (750 µatm CO_2) conditions. Data are presented as means ± SE (N = 9-10). For panels A and B, the grey and black boxes with a less than symbol represent a significant effect of monitoring period between the acclimation and stimulus period (GLMM, see Table 3). For panel C, a significant interactive effect of stimulus and recovery was detected and bars that do not share a letter are significantly different from one another (GLMM, see Table 3).

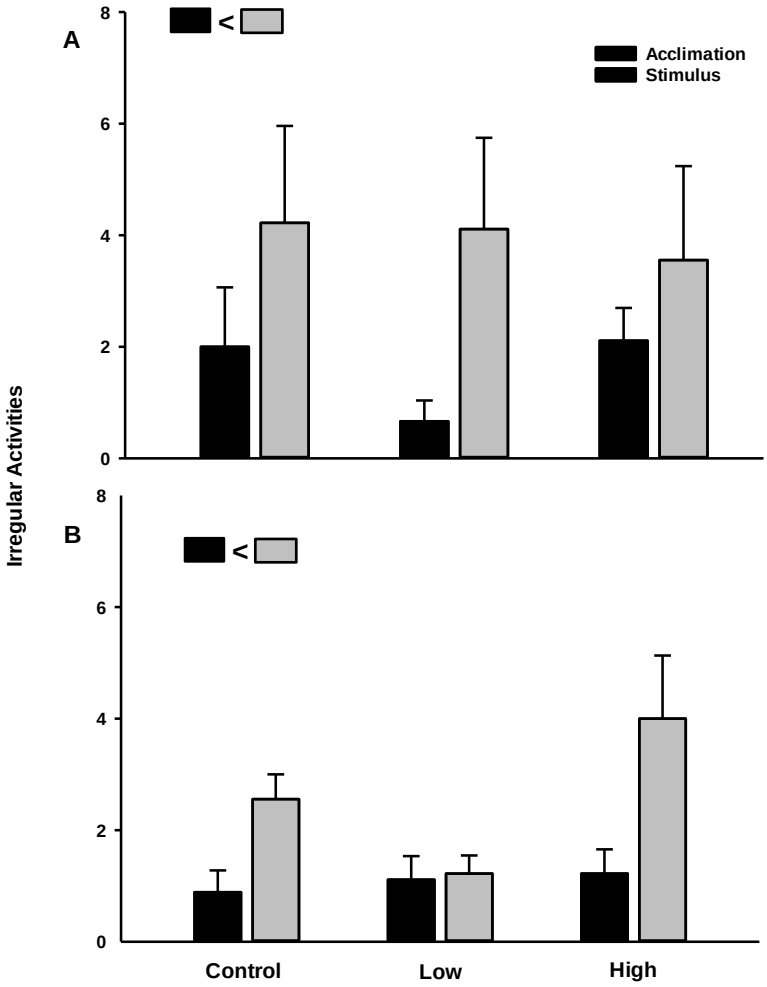

Figure 2. The number of irregular activities (darts, freezes, and jumps) for silver carp (*Hypophthalmichthys molitrix*) during acclimation and stimulus periods following CO_2 exposure and recovery. Silver carp were (A) exposed to either ambient (750 µatm), low (1,500 µtam), or high (7,000 µatm) CO_2 levels for 4-10 d and then (B) held for an additional 11-14 d at ambient (750 µatm CO_2) conditions. Data are presented as means ± SE (N = 9). The grey and black boxes with the less than symbol represent a significant effect of monitoring period between acclimation and stimulus (GLMM, see Table 3).

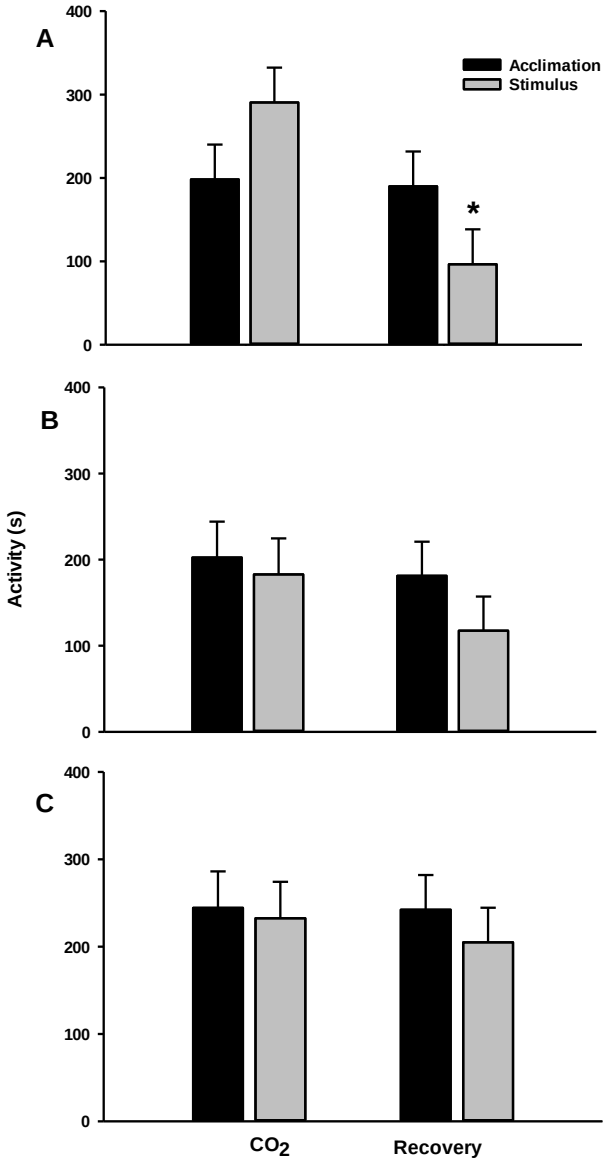

Figure 3. Mean activity (s) for fathead minnows (*Pimephales promelas*) during the acclimation and stimulus periods following CO_2 exposure and recovery. Fathead minnow were exposed to either (A) ambient (750 µatm) (B) low (1,500 µtam), or (C) high (7,000 µatm) CO_2 levels for 4-12 d and then held for an additional 11-14 d at ambient (750 µatm CO_2) conditions. Data are presented as the means ± SE. An asterisk represents a significant interaction between acclimation and stimulus during the recovery period (GLMM, see Tables 4, 5).

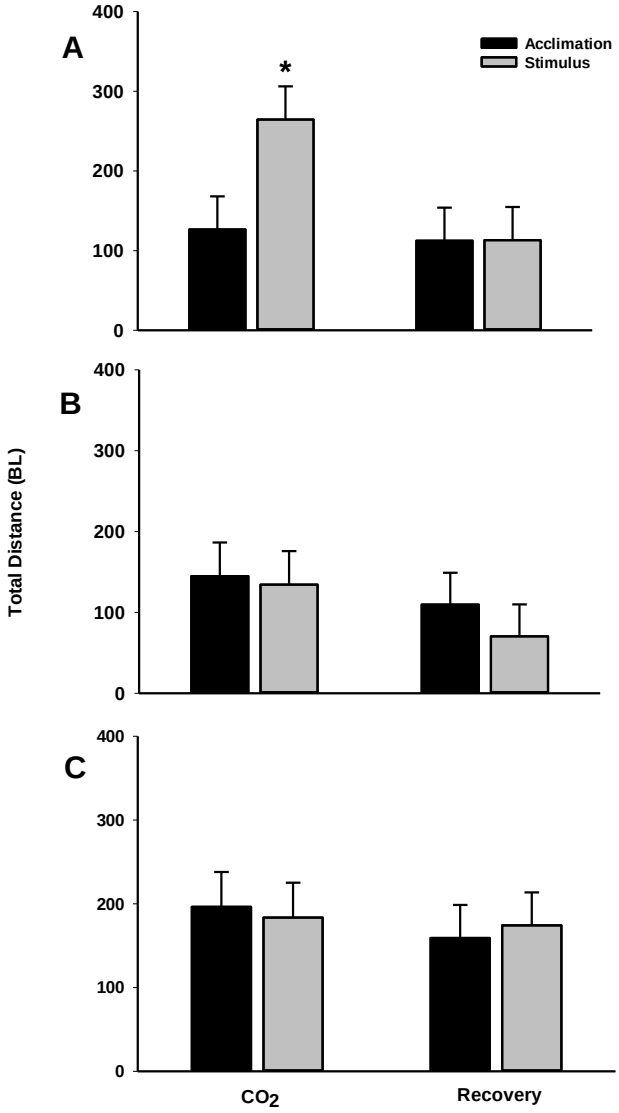

Figure 4. Mean total distance (BL) for fathead minnows (*Pimephales promelas*) during the acclimation and stimulus periods following CO_2 exposure and recovery. Fathead minnow were exposed to either (A) ambient (750 µatm) (B) low (1,500 µtam), or (C) high (7,000 µatm) CO_2 levels for 4-12 d and then held for an additional 11-14 d at ambient (750 µatm CO_2) conditions. Data are presented as the means ± SE. An asterisk represents a significant interaction between acclimation and stimulus during the recovery period (GLMM, see Tables 4, 5).

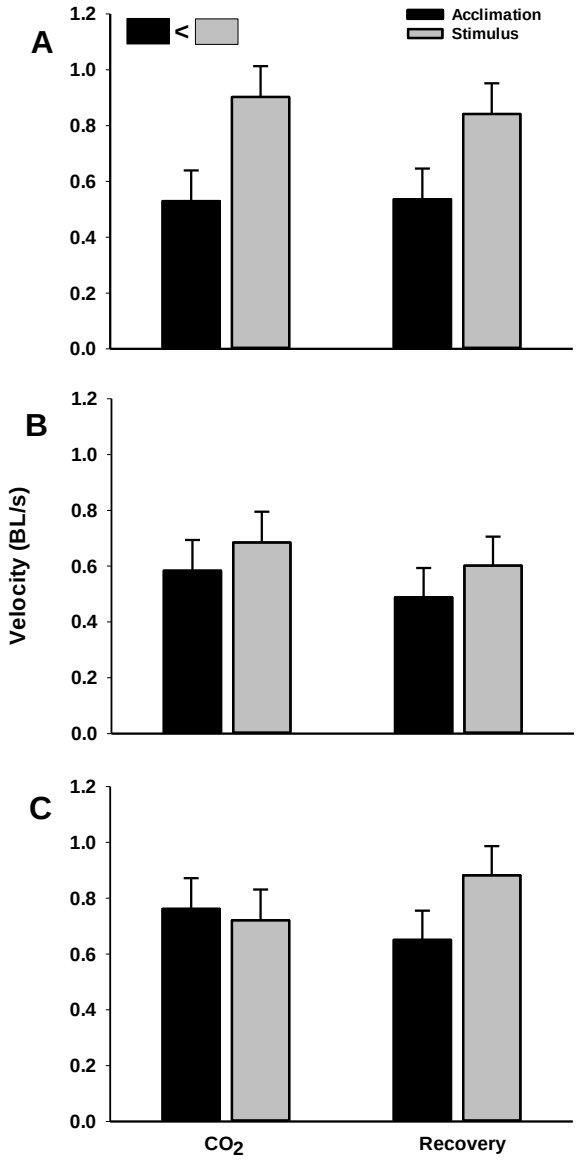

Figure 5. Mean swimming velocity (BL/s) for fathead minnows (*Pimephales promelas*) during the acclimation and stimulus periods following CO_2 exposure and recovery. Fathead minnows were exposed to either (A) ambient (750 µatm) (B) low (1,500 µtam), or (C) high (7,000 µatm) CO_2 levels for 4-12 d and then held for an additional 11-14 d at ambient (750 µatm CO_2) conditions. Data are presented as the means ± SE. The grey and black boxes with the less than symbol represent a significant effect of monitoring period between acclimation and stimulus (GLMM, see Tables 4).

Table 7. Water quality measurements [temperature (°C), dissolved oxygen (mg L^{-1}), pH, dissolved CO_2 (mg L^{-1}), total alkalinity (mg L^{-1}) and pCO_2 (µatm)] sampled during each treatment period. Pre-exposure represents the 7 d period where *Lepomis macrochirus* were held at ambient conditions, and post-exposure represents the 5 d period where *L. macrochirus* were held at elevated pCO_2. The recovery treatment was the period where *L. macrochirus* were returned to ambient pCO_2 for 5 d. Values reported as means ± SD.

Treatment period	Temperature (C°)	Dissolved Oxygen (mg L^{-1})	pH	Dissolved Carbon Dioxide (mg L^{-1})	Total Alkalinity (mg L^{-1})	pCO_2 (µatm)
Pre-exposure	20.88 ± 0.82	8.38 ± 0.52	9.05 ± 0.13	4.40 ± 0.73	149.13 ± 6.17	160 ± 43
Post-exposure	21.20 ± 1.16	8.61 ± 0.20	7.15 ± 0.09	24.83 ± 3.92	148.00 ± 4.90	8300 ± 400
Recovery	16.50 ± 1.48	9.46 ± 0.29	9.51 ± 0.10	1.40 ± 0.55	149.20 ± 7.40	44 ± 13

Table 8. Statistical outputs [Value, Standard Error, DF (degrees of freedom), *t*-value and *p*-value] for each output (Intercept, Pre-exposure×Post-exposure and Pre-exposure×Recovery) of generalized linear mixed effects regression models from R for relative lateralization (L_R) and absolute lateralization (L_A). L_R was analyzed using a binomial distribution while L_A was analyzed using a poisson distribution. The intercept value represents the "pre-exposure" values. For pre-exposure *vs*. post-exposure and pre-exposure *vs*. recovery each parameter value represents the change in the model intercept associated with that response. Significant parameters are highlighted in bold.

Response	Model	Parameter	Estimate	Standard Error	DF	z-value	*p*-value
		Intercept	**-0.687**	**0.183**	**30**	**-3.748**	**< 0.001**
	Binomial	Pre-exposure vs. Post-exposure	-0.431	0.25	30	-1.728	0.084
L_R	GLMER	Pre-exposure vs. Recovery	-0.116	0.241	30	-0.482	0.63
		Intercept	**1.964**	**0.094**	**30**	**20.966**	**< 0.001**
	Poisson	Pre-exposure vs. Post-exposure	0.084	0.13	30	0.648	0.517
L_A	GLMER	Pre-exposure vs. Recovery	0.017	0.132	30	0.132	0.895

Table 9. Statistical outputs [Value, Standard Error, DF (degrees of freedom), *t*-value and *p*-value] for each output (Intercept, Pre-exposure *vs*. Post-exposure and Post-exposure *vs*. Recovery) of generalized linear mixed effects models from R using penalized quasi-likelihood for each response. The intercept value represents the "post-exposure" values. For pre-exposure *vs*. post-exposure and post-exposure *vs*. recovery each parameter value represents the change in the model intercept associated with that response. Significant parameters are highlighted in bold.

Response	Model	Parameter	Value	Standard Error	DF	*t*-value	*p*-value
Prop Time Active During Trial	Quasi GLM	Intercept	0.367	0.04	28	9.14	0
		Pre-exposure vs. Post-exposure	-0.071	0.049	28	-1.465	0.154
		Post-exposure vs. Recovery	-0.039	0.048	28	-0.799	0.4311
Prop Time Active in Thigmotaxis zone During Trial		Intercept	0.284	0.028	28	10.21	0
		Pre-exposure vs. Post-exposure	**-0.082**	**0.036**	28	**-2.278**	**0.0305**
		Post-exposure vs. Recovery	-0.024	0.036	28	-0.661	0.514
Prop Time Active in Middle Zone During Trial		Intercept	0.055	0.024	28	2.282	0.03
		Pre-exposure vs. Post-exposure	0.041	0.032	28	1.295	0.206
		Post-exposure vs. Recovery	-0.015	0.032	28	-0.462	0.647

Table 9 (cont.)

Response	Model	Parameter	Value	Standard Error	DF	*t*-value	*p*-value
Prop Time Active by Object During Trial	Quasi GLM	Intercept	0.028	0.011	28	2.546	0.017
		Pre-exposure vs. Post-exposure	0	0.013	28	-0.38	0.707
		Post-exposure vs. Recovery	0	0.013	28	-0.016	0.988
Average Velocity		Intercept	0.303	0.021	28	14.504	0
		Pre-exposure vs. Post-exposure	**-0.077**	**0.028**	**28**	**-2.776**	**0.01**
		Post-exposure vs. Recovery	**-0.059**	**0.028**	**28**	**-2.113**	**0.044**
Average Velocity Thigmotaxis Zone		Intercept	0.301	0.023	28	13.144	0
		Pre-exposure vs. Post-exposure	**-0.084**	**0.032**	**28**	**-2.63**	**0.014**
		Post-exposure vs. Recovery	-0.058	0.032	28	-1.806	0.082
Average Velocity Middle Zone		Intercept	0.253	0.041	28	6.221	0
		Pre-exposure vs. Post-exposure	-0.085	0.047	28	-1.803	0.082
		Post-exposure vs. Recovery	-0.072	0.047	28	-1.518	0.14

Table 9 (cont.)

Response	Model	Parameter	Value	Standard Error	DF	*t*-value	*p*-value
Average Velocity by Object	Quasi GLM	Intercept	0.214	0.049	28	4.4	0
		Pre-exposure vs. Post-exposure	-0.1	0.051	28	-1.95	0.061
		Post-exposure vs. Recovery	-0.063	0.051	28	-1.232	0.228
Total Shuttles	Quasi Poisson GLMM	Intercept	2.593	0.276	28	9.409	0
		Pre-exposure vs. Post-exposure	-0.177	0.272	28	-0.652	0.52
		Post-exposure vs. Recovery	-0.327	0.284	28	-1.154	0.258
Total Shuttles to Thigmotaxis Zone		Intercept	1.636	0.233	28	7.011	0
		Pre-exposure vs. Post-exposure	-0.06	0.244	28	-0.246	0.808
		Post-exposure vs. Recovery	-0.28	0.259	28	-1.081	0.289
Total Shuttles to Middle Zone		Intercept	1.838	0.296	28	6.212	0
		Pre-exposure vs. Post-exposure	-0.202	0.294	28	-0.685	0.499
		Post-exposure vs. Recovery	-0.363	0.308	28	-1.177	0.249

Table 9 (cont.)

Response	Model	Parameter	Value	Standard Error	DF	*t*-value	*p*-value
Total Shuttles to Object Zone	Quasi Poisson GLMM	Intercept	0.528	0.39	28	1.353	0.187
		Pre-exposure vs. Post-exposure	-0.405	0.323	28	-1.254	0.22
		Post-exposure vs. Recovery	-0.331	0.316	28	-1.047	0.304
Prop Time Spent Thigmotaxis Zone		Intercept	-0.222	0.078	28	-2.83	0.008
		Pre-exposure vs. Post-exposure	-0.076	0.102	28	-0.743	0.464
		Post-exposure vs. Recovery	0.074	0.098	28	0.753	0.458
Prop Time Spent Middle Zone		Intercept	-2.055	0.317	28	-6.475	0
		Pre-exposure vs. Post-exposure	0.449	0.345	28	1.301	0.204
		Post-exposure vs. Recovery	-0.525	0.44	28	-1.193	0.243
Prop Time Spent Object Zone		Intercept	-3.476	0.437	28	-7.951	0
		Pre-exposure vs. Post-exposure	-0.471	0.371	28	-1.269	0.215
		Post-exposure vs. Recovery	-0.064	0.331	28	-0.193	0.848

Table 10. Statistical outputs (r_s, *F*-ratio and *p*-value) for each output (Pre-exposure vs. Post-exposure and Pre-exposure vs. Recovery) of the Spearmans rank correlation for each behavioural metric.

Response	Parameter	r_s	*F*-ratio	*P*-value
L_R	Pre-exposure vs. Post-exposure	0.042	0.609	0.448
	Pre-exposure vs. Recovery	0.003	0.043	0.839
L_A	Pre-exposure vs. Post-exposure	0.025	0.354	0.562
	Pre-exposure vs. Recovery	0.017	0.246	0.627
Prop Time Active During Trial	Pre-exposure vs. Post-exposure	0.1	0.131	0.723
	Pre-exposure vs. Recovery	0.009	0.122	0.732
Prop Time Active in Thigmotaxis zone During Trial	Pre-exposure vs. Post-exposure	0.032	0.428	0.524
	Pre-exposure vs. Recovery	0.033	0.446	0.516
Prop Time Active in Middle Zone During Trial	Pre-exposure vs. Post-exposure	0.157	2.429	0.143
	Pre-exposure vs. Recovery	0.21	3.464	0.085
Prop Time Active by Object During Trial	Pre-exposure vs. Post-exposure	0.147	2.238	0.159
	Pre-exposure vs. Recovery	0.209	3.436	0.087
Average Velocity	Pre-exposure vs. Post-exposure	0.026	0.345	0.567
	Pre-exposure vs. Recovery	0.046	0.626	0.443
Average Velocity Thigmotaxis Zone	Pre-exposure vs. Post-exposure	0.016	0.206	0.657
	Pre-exposure vs. Recovery	0.051	0.693	0.42

Table 10 (cont.)

Response	Parameter	rs	F-ratio	*p*-value
Average Velocity Middle Zone	Pre-exposure vs. Post-exposure	0.001	0.013	0.912
	Pre-exposure vs. Recovery	**0.281**	**5.068**	**0.042**
Average Velocity by Object	Pre-exposure vs. Post-exposure	0.092	1.31	0.273
	Pre-exposure vs. Recovery	**0.514**	**13.769**	**0.003**
Total Shuttles	Pre-exposure vs. Post-exposure	0.07	0.974	0.342
	Pre-exposure vs. Recovery	0.165	2.567	0.133
Total Shuttles to Thigmotaxis Zone	Pre-exposure vs. Post-exposure	0.128	1.917	0.19
	Pre-exposure vs. Recovery	0.053	0.723	0.411
Total Shuttles to Middle Zone	Pre-exposure vs. Post-exposure	0.081	1.152	0.303
	Pre-exposure vs. Recovery	0.216	3.58	0.081
Total Shuttles to Object Zone	Pre-exposure vs. Post-exposure	0.211	3.48	0.085
	Pre-exposure vs. Recovery	**0.371**	**7.664**	**0.016**
Prop Time Spent Thigmotaxis Zone	Pre-exposure vs. Post-exposure	0.009	0.119	0.735
	Pre-exposure vs. Recovery	0.218	3.619	0.079
Prop Time Spent Middle Zone	Pre-exposure vs. Post-exposure	0.004	0.054	0.82
	Pre-exposure vs. Recovery	0.154	2.369	0.148
Prop Time Spent Object Zone	Pre-exposure vs. Post-exposure	0.203	3.31	0.092
	Pre-exposure vs. Recovery	0.237	4.028	0.066

L_R, relative lateralization; L_A, absolute lateralization

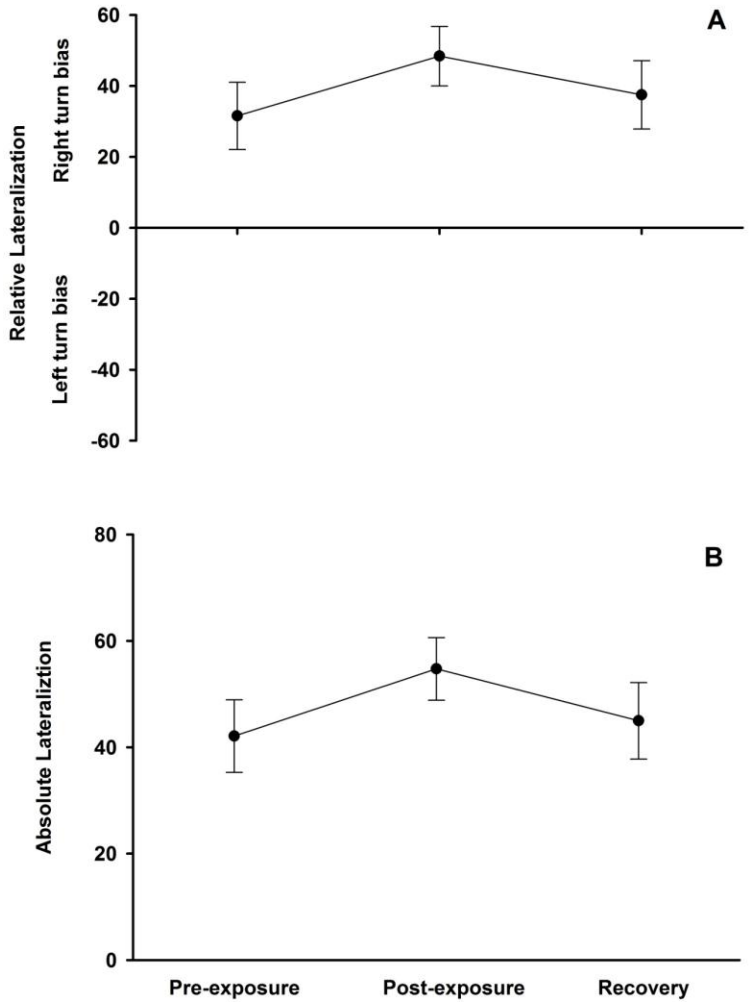

Figure 6. Lateralization index for *Lepomis macrochirus* held at ambient pCO_2 (156 µatm, pre-exposure), following 5 d at 8300 µatm CO_2 (post-exposure), or after a return to ambient pCO_2 for 5 d (44 µatm, recovery). Individual fish (N = 16) were tested in a double T-maze over the three treatment periods and each fish made a total of 10 turning choices per trial. The relative lateralization index (L_R; A) where positive values indicate a right-turning bias while negative values indicate a left-turning bias and a value of 0 indicates no preference in turning side. The absolute lateralization (L_A; B) indicates the strength of the side preference independent of the preferred turning side. Data are presented as means ± SE. Treatment groups were not significantly different from one another (see Table 7).

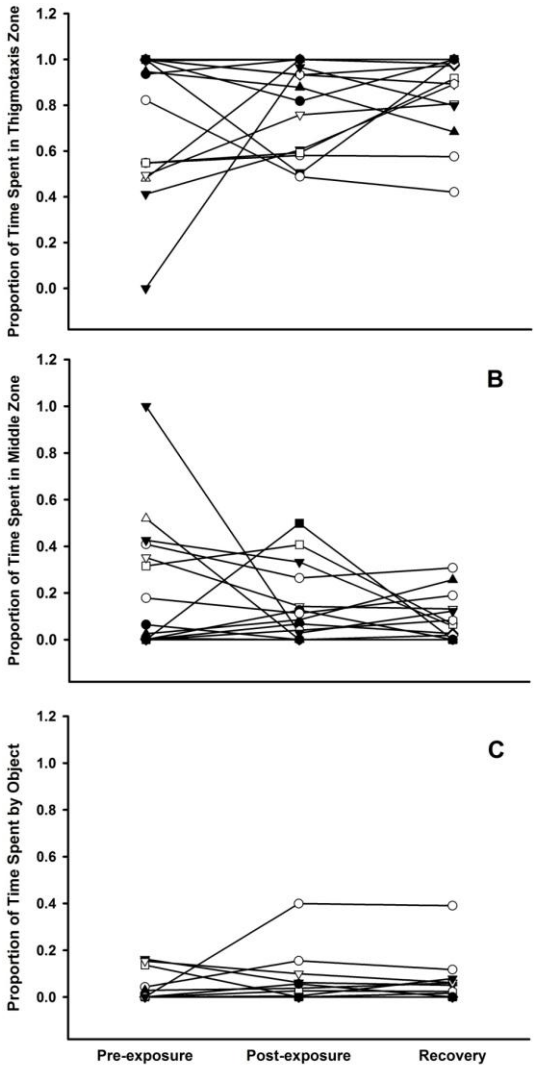

Figure 7. The proportion of time spent in the thigmotaxis zone (A), in the middle zone (B) and by the novel object (C) for *Lepomis macrochirus* held at ambient pCO_2 (156 µatm, pre-exposure), following 5 d at 8300 µatm CO_2 (post-exposure), or after a return to ambient pCO_2 for 5 d (44 µatm, recovery). The figure shows responses for each individual (N = 15), with each individual bluegill represented by a single line and a common symbol across all three treatments. No significant effects of treatment were detected (see Table 8).

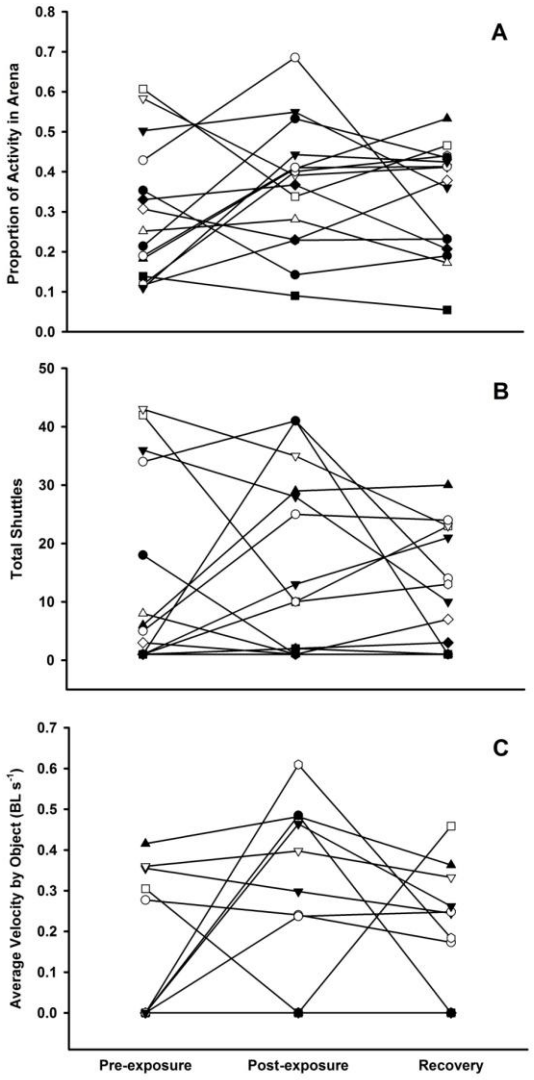

Figure 8. The proportion of activity in the novel object arena (A), total amount of shuttles between the thigmotaxis, middle, and near the object zones (B) and the average velocity (BL s^{-1}) in the novel object zone (C) for *Lepomis macrochirus* held at ambient pCO_2 (156 µatm, pre-exposure), following 5 d at 8300 µatm CO_2 (post-exposure), or after a return to ambient pCO_2 for 5 d (44 µatm, recovery). The figure shows responses for each individual (N = 15), with each individual bluegill represented by a single line and a common symbol across all three treatments. No significant effects of treatment were detected (see Table 8)

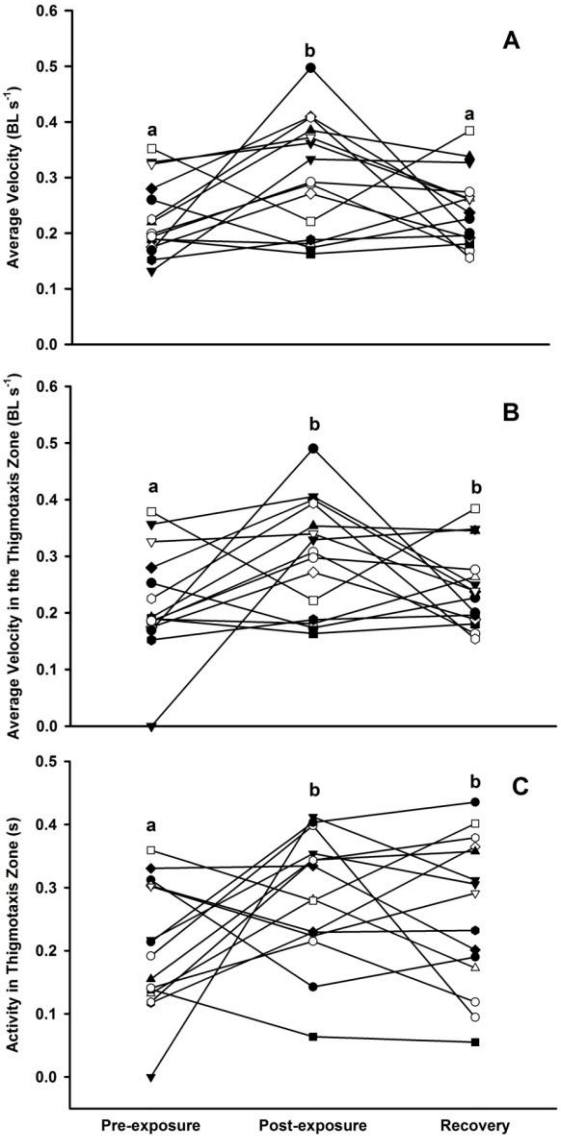

Figure 9. Average velocity (BL s^{-1}) in the novel object arena (A), average velocity (BL s^{-1}) in the thigmotaxis zone (B), and proportion of activity in the thigmotaxis zone (C) for *Lepomis macrochirus* held at ambient pCO_2 (156 µatm, pre-exposure), following 5 d at 8300 µatm CO_2 (post-exposure), or after a return to ambient pCO_2 for 5 d (44 µatm, recovery). The figure shows responses for each individual (N = 15), with each individual bluegill represented by a single line and a common symbol across all three treatments. Factors with different letters represent significant differences between the means (see Table 8).