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Fish behavior in elevated CO_2: implications for a movement barrier in flowing water

Eric V. C. Schneider · **Caleb T. Hasler** · **Cory D. Suski**

Abstract Preventing the spread of invasive fishes is an important aspect of management programs, but is challenging due to the behavior of fish and the nature of aquatic environments. The use of dissolved carbon dioxide (CO_2) has recently gained traction as a non-physical barrier for invasive fishes due to its ability to elicit avoidance behaviors in fish. Research to date has focused on the development of CO_2 barriers using static water environments. Because CO_2 barriers have been proposed for flowing water (i.e., in rivers or shipping canals), understanding the dynamics between fish and elevated CO_2 in flowing water is essential. Our study aims to define threshold levels required to alter behavior of bluegill (*Lepomis macrochirus*) and largemouth bass (*Micropterus salmoides*) in flowing water, and to quantify behavioral metrics of fish exposed to < 200 [ambient], 25,000, 50,000, and 100,000 μatm pCO_2. We also sought to quantify the impacts of repeated CO_2 exposure on fish behavior. Bluegill showed increased activity at 25,000 μatm, while largemouth bass showed increased activity at 100,000 μatm. When repeatedly exposed to cycles of 50,000 μatm pCO_2, bluegill exhibited increased activity followed by a diminished response after the second exposure. Results from this study define threshold levels required to elicit behavioral responses, and show that the effects that multiple exposures of elevated pCO_2 can decline, possibly due to habituation. Results will help shape the development and deployment of a CO_2 barrier to control the movements of invasive fishes.

Keywords Carbon dioxide · Acidification · Behavior · Climate change · Barrier · Invasive species

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Introduction

Management of invasive species, particularly in well-connected freshwater systems, often includes the creation of physical or non-physical barriers (Fausch et al. 2009). Non-physical barriers for invasive fishes include deterrents such as strobe lights, sound-emitting devices, and chemicals that alter fish behavior and result in avoidance (Noatch and Suski 2012). Avoidance is a typical behavioral response for fish to distance themselves from aversive stimuli (e.g., poor

water quality, sound), while environmental conditions such as extreme temperature, hypoxia, high salinity, and hypercarbia are common variables that can also lead to avoidance of areas (Tierney 2016). Further, the degree to which an individual avoids adverse conditions is a product of both the concentration and exposure duration of the noxious stimulus, such that avoidance often only occurs once threshold levels are achieved (Tierney 2016). For example, hardhead (*Mylopharodon conocephalus*) avoided water below 17 °C when allowed to traverse through a gradient of temperatures in an experimental tank (Cocherell et al. 2014). More broadly, commercially targeted fish in Lake Erie were shown to avoid hypoxic zones and alter their behavior and residence patterns accordingly (Kraus et al. 2015). The specific behavioral avoidance response that is elicited by adverse water conditions is likely dependent on a variety of internal and external factors, including concentration, contact or exposure time, and the surrounding environment (e.g., water temperature, flow condition).

Carbon dioxide (CO_2) is a naturally occurring compound shown to induce avoidance behaviors in fish (Kates et al. 2012; Dennis et al. 2016b; Donaldson et al. 2016). Elevated in aquatic systems through both natural and anthropogenic sources, CO_2 is readily detected by fishes even at low concentrations (Cummins et al. 2014). Fish likely avoid hypercarbic zones because CO_2 is a waste product from aerobic respiration and is indicative of poor water quality (Noatch and Suski 2012; Tierney 2016). For example, despite the ability of fish to regulate intra- and extra-cellular pH to some degree, elevated pCO_2 may reduce their ability to uptake and transport oxygen, thus having implications for aerobic metabolism (Heuer and Grosell 2014). In laboratory studies in static tanks, fish display behavioral and physiological modifications such as erratic ventilations and increased plasma glucose as part of the stress response at low CO_2 levels, whereas at higher concentrations fish will display avoidance (Kates et al. 2012), highlighting the negative impact that environmental CO_2 can have on fish. Due to elevated CO_2 levels inducing avoidance in fish, CO_2 has been recommended for investigation as a potential non-physical fish deterrent (Noatch and Suski 2012; United States Army Corps of Engineers 2014).

While the avoidance of fishes to elevated CO_2 is well documented (Kates et al. 2012; Noatch and Suski

2012; United States Army Corps of Engineers 2014; Donaldson et al. 2016), three unresolved issues are impairing the ability to successfully design and implement a CO_2 barrier in a field setting. First, to date, studies have not been performed to specifically define avoidance thresholds of pCO_2 (CO_2 pressure at which there is no response seen below, but a consistent, predictable response above). As such, recommended targets for CO_2 to induce avoidance have been based on group mean responses, around which considerable variation exists. For instance, Dennis et al. (2016a) showed largemouth bass (*Micropterus salmoides*) avoided CO_2 at a mean concentration of ~ 140 mg L^{-1}, however this avoidance occurred at a range from ~ 50 to ~ 375 mg L^{-1}, which shows a degree of individual variation. Second, nearly all research on the development of a CO_2 barrier has occurred in static water conditions. While the exact design of a CO_2 barrier in the field has not been identified, deployment would likely need to occur into a river or shipping channel via a lock, or a deployment at a 'choke point', rather than in static water (United States Army Corps of Engineers 2014). If a CO_2 barrier were to be deployed into a flowing water environment, fish residing downstream from the deployment could conceivably be challenged with multiple successive pulses of CO_2 as CO_2-rich water from the lock is released to allow vessel traffic. The effects on multiple exposures to elevated CO_2 have also not been investigated in fish, but responses to recurring stimuli have been shown to either amplify or weaken (Reinert et al. 2002), making this an important study objective. Finally, the deployment of a CO_2 barrier into flowing water may lead to differences in behavioral responses of fishes relative to observations made to date in static conditions, which may have implications for the design and deployment of such a barrier. More specifically, differences in avoidance behaviors may lie in how fish interact with turbulent flowing water (Montgomery and Coombs 2011) and how the turbulence may distribute CO_2 and create a more dynamic environment (Massel 1999), either of which could lead to a barrier being more or less effective in flowing water than under static conditions. Understanding the relationships between CO_2 and fish behavior will allow us to optimize barrier effectiveness.

The objectives of this study were to: (1) identify the threshold—in this case the first pCO_2 that elicits

significant behavioral responses that were not present during the preceding pCO_2 tested-in native fish species in flowing water; (2) quantify changes in behavioral responses such as distance moved, velocity, acceleration and percent time active that fish (largemouth bass and bluegill, *Lepomis macrochirus*) display during short-term exposures to elevated levels of pCO_2; and (3) quantify changes to the same behavioral responses of fishes repeatedly exposed to elevated pCO_2. Based on previous research, we expected to find a threshold at or below 50,000 μatm pCO_2 that would result in a significant increase in the behavioral metrics listed above. In addition, we expected a compounding effect of successive exposures to CO_2 to occur that would result in the same behavioral responses at potentially lower pCO_2. Previous work has shown that an individual's responses to a repetitive stimulus may be compounded or attenuated (Reinert et al. 2002), and more specifically that fish avoid areas of elevated CO_2 even after multiple exposures (Kates et al. 2012; Dennis et al. 2016b). One proposed deployment strategy involves the addition of CO_2 into a shipping lock (United States Army Corps of Engineers 2014), as locks can serve as a chokepoint in a waterway to reduce the volume of water to be treated with CO_2. As a result, the repetitive cycle of lock openings for vessel traffic would result in plumes of CO_2-rich water moving downstream. Largemouth bass and bluegill are both regionally prevalent and important native species and were used as surrogates for Asian carp due to limitations in obtaining properly sized carp. Further, work to date indicates few differences across species in the response of fish to CO_2 (Kates et al. 2012; Donaldson et al. 2016), suggesting that results obtained from largemouth bass and bluegill would be generalizable to other fishes. To address objectives 1 and 2, fish were exposed to varying partial pressures of CO_2 (from ambient to 100,000 μatm) and video recorded to measure behavioral responses (e.g., distance moved, percent of time active, acceleration and average velocity) to the change in pCO_2. For objective 3, fish were repeatedly exposed to 50,000 μatm, a pCO_2 known to induce behavioral responses, to quantify any changes in behavioral metrics. Together, these results will provide insight into how and when fish respond to elevated pCO_2, aiding the development of a CO_2 barrier to deter fish movement.

Methods

Fish holding and husbandry

Sub-adult largemouth bass and bluegill were obtained from a local hatchery (Logan Hollow Fish Farm, Murphysboro, IL, USA) and transported to the University of Illinois Aquatic Research Facility in Urbana, Illinois. Upon arrival, fish were placed into one of two indoor holding tanks (separated by species, approximately 1200 L volume each) and acclimated for 1 week prior to experimentation. During this period fish were fed pelleted commercial fish feed to satiation daily (Purina Aquamax, St. Louis, MO, USA). Water within these tanks was obtained from a nearby earthen-bottom pond (used as a water source in previous behavior studies, e.g., Tix et al. 2016a) and approximately 10% water changes were performed daily for each tank. Mechanical filtration (Fluval 406 Canister Filter), UV filtration (Vecton-4: V2 400 15 Watt UV Filter), and supplemental aeration in each tank were used to maintain water quality. Ammonia (LaMotte Company, Ammonia Nitrogen Kit no. 3351-02, Chestertown, MD, USA), total alkalinity (Hach Company, Titrator 16,900, kit 2272700, Love-land, CO, USA), pH (WTW pH3310 m, SenTix probe, Germany), dissolved oxygen and temperature (YSI ProODO, Yellow Springs Instruments, Irvine, CA, USA) were monitored daily (Table 1). Food was withheld for 24 h prior to trials to ensure a post-absorptive state was reached before trials began (Roche et al. 2013).

Threshold levels and behavioral responses

To determine threshold pCO_2 required to elicit behavioral responses in bluegill and largemouth bass, and to quantify those responses, a behavioral choice arena (Fig. 1; Loligo Systems, Viborg, Denmark) was used (Jutfelt et al. 2016; Tix et al. 2016b). This choice arena has successfully been used for studies of behavior and avoidance in fishes, making it the ideal tool for this study (e.g., Jutfelt and Hedgärde 2013). However, as noted below, the water in the arena was homogeneous and thus "choice" was not monitored (Tix et al. 2016b). Briefly, the behavioral choice arena is a rectangular tank consisting of an upstream inflow (through which the water source can be changed between two header tanks), a succession of

Table 1 Water quality parameters measured in each 1200 L holding tank through the duration of experimentation

Species	Temperature (°C)	Dissolved oxygen (mg L^{-1})	pH	Ammonia (mg L^{-1})	Total alkalinity (mg L^{-1} of $CaCO_3$)
Bluegill	17.7 ± 0.2	8.2 ± 0.2	8.7 ± 0.1	0.5 ± 0.0	204.4 ± 2.1
Largemouth bass	17.4 ± 0.4	7.6 ± 0.4	8.5 ± 0.3	0.4 ± 0.0	194.2 ± 5.1

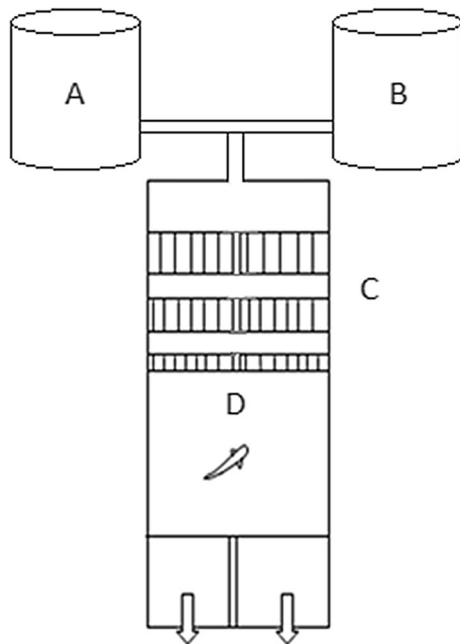Values are means ± 1 SE. All measurements were taken once each morning

Fig. 1 Diagram of the flow-through behavior tank used. Header tanks (A and B) gravity-fed water into the tank. Honeycomb flow straighteners (C) eliminated turbulence and created an even and laminar flow through the behavioral arena (D). As indicated by the arrows, water flows from the top of the diagram to the bottom

honeycomb inserts to generate laminar flow (4 cm s^{-1} during these experiments), a test chamber for fish monitoring (L 32 cm × W 40 cm × D 10 cm), and a downstream outflow. Water is gravity-fed into the choice arena from the two header tanks, and valves on the upstream end of the choice arena dictate which header tank is supplying water to the test chamber. While this arena has the capability to generate two separate flow channels that would allow water from each header tank to enter the test chamber simultaneously such that fish can 'choose' between flows from the two header tanks, this set-up was not used in the

current study. Rather, water from a single header tank was manipulated to a target pCO_2 (described below), and that water was then delivered to the entire test chamber such that fish were unable to avoid CO_2-rich water. The entire choice arena was surrounded by a visual screen, and noise levels were nil to prevent outside stimuli from affecting fish behavior.

Different pCO_2 were achieved with the common method of bubbling CO_2 gas into the water of one header tank *via* an air stone until a target pCO_2 was reached (endpoints were measured with an infrared CO_2 m: GMT220, Vaisala, Vantaa, Finland; Johnson et al. 2010), while the second header tank remained at ambient pCO_2 (< 200 µatm, which is common for fresh waters, see Cole et al. 1994) with a normal air stone bubbling in air from a blower (Sweetwater Air Pump, Pentair Aquatic Eco-Systems). Both header tanks contained identical recirculation pumps (Magnetic Drive Pump, Pentair Aquatic Eco-Systems) to ensure uniform mixing within the tank. The pCO_2 that fish were exposed to during this study were: < 200 (ambient); 25,000; 50,000; and, 100,000 µatm. The order of partial pressures used during trials in the study was determined using a random number generator. Previous work has suggested that 50,000 µatm is a potential target for a non-physical barrier to effectively deter fish movements (Cupp et al. 2016; Donaldson et al. 2016). Thus, the range of pCO_2 used in the study were chosen because (a) exposure of fishes to these pressures have previously been shown to induce behavioral responses (Kates et al. 2012), and, (b) these pressures extend beyond levels previously identified as targets for a non-physical barrier for fishes, allowing us to quantify behaviors that occur both above and below proposed target pressures for a barrier (Dennis et al. 2016a; Donaldson et al. 2016).

After the target pCO_2 in the header tank was reached, a single fish was placed in the test chamber of the behavioral choice arena. Based on preliminary

trials and observations, both *M. salmoides* and *L. macrochirus* required approximately 3–5 min to become acclimated to the choice tank, evidenced by a decrease in overall swimming activity, fewer wall strikes and a reduced number of jumps. This is a duration consistent with similar work in past studies (Tix et al. 2016a). To ensure a calm state for fish, a conservative 10-min acclimation period was used between the time the fish was introduced into the test chamber and the onset of the experiment. During this acclimation period, water moved from the header tank held at ambient pCO_2, through the choice tank and into a reservoir at the downstream end of the tank where it was pumped back into the header tank to create a closed-loop. A top-mounted video camera (iDS uEye 1480-C camera, iDS, Obersulm, Germany) began recording fish position and activity immediately following the 10-min acclimation period, and this recording lasted for 3 min. The first minute of this recording, while the fish was in water at ambient pCO_2, was referred to as 'pre-exposure' and represented baseline (control) activity levels. Immediately following the 1-min pre-exposure period, the inflow of water to the choice arena was changed such that water from the header tank treated to the target pCO_2 was delivered to the test chamber. Fish were exposed to water at the target pCO_2 for 1 min, during which time behavior and activity were recorded, and this was referred to as the 'CO_2 Exposure' period. Preliminary trials using food coloring confirmed our ability to switch water between header tank sources. Finally, after this 1 min exposure to a CO_2 treatment, the header tank providing water to the choice arena was switched back to provide water at ambient pCO_2, and the fish was again exposed to ambient water for 1 min (referred to as 'post-exposure'), and behavior/activity were recorded. Together, this study design generated a total of 3 min of video observations of fish that occurred prior to, during, and after exposure to the four pCO_2 treatments.

Sample sizes for each pCO_2 were 10 fish per species (n = 40 for each species), and this sample size was similar to previous work asking similar questions with this equipment and study design (Kates et al. 2012). An identical procedure was followed to act as an experimental control to account for the effects of confinement in the choice arena. For this control, both header tanks were filled with water at ambient pCO_2 and fish were exposed to ambient water throughout the three exposure periods. Results from this preliminary experiment showed no difference in fish behavior across time periods, indicating no impact on fish behavior due to the switching of flow between header tanks. Data for this preliminary experiment are provided in Supplementary Materials.

Following the three exposure periods described above, fish were removed from the test chamber, measured for total length (mm) and weight (g), and released into a recovery holding tank. Fish size did not differ across treatments within species (largemouth bass: 148.6 mm ± 2.1 mm [range 111–167 mm], ANOVA, $F_{3,36} = 1.28$, $p = 0.295$; bluegill: 78.4 mm ± 1.7 mm [range 64–110 mm], $F_{3,36} = 0.77$, $p = 0.517$). Before, during and after each trial, water quality parameters were measured in the behavioral tank to ensure accuracy and precision of all CO_2 treatments using equipment described above (Table 2).

Repeat exposures

Preliminary analyses of data from the single exposure experiment described above revealed that behavioral changes occurring at 25,000 μatm were minor, while behavioral changes occurring at pCO_2 levels of 50,000 μatm were more consistent and pronounced. As such, fish used in the study to define the impacts of repeated CO_2 exposure on behavior were repeatedly exposed to 50,000 μatm pCO_2. For this study, identical procedures to those described above were followed regarding the setup of the experiment, sample sizes (10 fish per treatment, n = 20 for each species: both bluegill and largemouth bass were used) and methods for handling both fish and CO_2. For this study, following the 3 min of behavior monitoring during the CO_2 exposure trial, water from the header tank held at ambient pCO_2 was recirculated through the choice arena for an additional 10 min, essentially acting as a second acclimation period during which no observations were recorded. Then, fish were exposed to the treatment water again using the same 3-min protocol described above. A fish was recorded in this cycle five times (i.e., fish were exposed to pCO_2 of 50,000 μatm five times in 55 min) before it was removed from the system and weighed/measured; fish sizes did not differ across treatments within species (largemouth bass: 145.3 mm ± 3.1 mm [range 124–170 mm], ANOVA, $F_{1,19} = 0.02$, $p = 0.878$;

Table 2 Water quality parameters measured in the behavioral tank during the *threshold* experiment trials (during which fish were exposed to varying levels of pCO_2)

Species	pCO_2 treatment (µatm)	Temperature (°C)	Titrated CO_2 (mg L^{-1})	pCO_2 (µatm)
Bluegill	Ambient	15.8 ± 0.1	10.4 ± 0.4	168 ± 58
	25,000	16.3 ± 0.1	34.7 ± 0.7	$24,807 \pm 320$
	50,000	17.1 ± 0.3	45.6 ± 1.8	$49,904 \pm 567$
	100,000	18.0 ± 0.2	120.4 ± 3.1	$95,078 \pm 661$
Largemouth bass	Ambient	16.6 ± 0.2	7.6 ± 0.8	38 ± 15
	25,000	15.8 ± 0.2	29.0 ± 2.2	$25,546 \pm 488$
	50,000	16.7 ± 0.3	53.6 ± 2.7	$51,552 \pm 351$
	100,000	16.7 ± 0.1	103.3 ± 7.2	$98,060 \pm 821$

Values are means $\pm$ 1 SE measured once during each individual exposure period. pCO_2 was measured with an infrared CO_2 m and converted to µatm. Water velocity through the behavioral tank was consistently 4 cm s^{-1}

bluegill: 83.3 mm $\pm$ 3.5 mm [range 69–136 mm], $F_{1,18} = 0.13$, $p = 0.722$). A second supplementary study was performed to act as a control for confinement. For this, the identical procedure to that describe above was followed, except that fish were exposed to ambient water throughout the three exposure periods in all five successive cycles. Data for this study showed no impact of changing water source between header tanks on fish behavior, and are available in Supplementary Materials (Fig. S1, S2). Similar water quality parameters were monitored as previously described (Table 3).

Data analyses

Videos of fish behavior and activity were analyzed using LoliTrack video software (Loligo Systems, Viborg, Denmark). Responses quantified by the software were distance moved (cm), proportion of time active, acceleration (m s^{-2}) and velocity (m s^{-1}) in each of the pre-, during, and post-CO_2 treatments. To determine if behavioral metrics were correlated with each other, a Pearson correlation test was performed using the package 'corrplot' in R (R Development Core Team 2010; Wei and Simko 2016). A correlation matrix was generated and showed that distance moved and percent time active were correlated, and velocity and acceleration were correlated (Fig. S3). Therefore, we removed percent time active and acceleration from further analysis and this data has not been shown. Further, for the purposes of the discussion: distance moved and percent time active will collectively be referred to as 'movement', while velocity and acceleration will collectively be referred to as 'speed'.

The full analysis to quantify the impacts of varying pCO_2 on fish behaviors consisted of linear mixed-effects models (JMP13.0, SAS Institute Inc.). The models were created to determine how behavioral metrics changed across treatment pCO_2 level and CO_2 exposure period (pre-exposure, CO_2 exposure, and

Table 3 Water quality parameters measured in the header tank during *repeat exposures* experiment trials (during which fish were exposed to either ambient or 50,000 µatm pCO_2 over five successive cycles to determine if any behavioral responses occur), shown means $\pm$ 1 SE measured once during each individual exposure period

Species	pCO_2 treatment (µatm)	Temperature (°C)	Titrated CO_2 (mg L^{-1})	pCO_2 (µatm)
Bluegill	Ambient	16.4 ± 0.5	9.0 ± 1.1	179.4 ± 106
	50,000	16.8 ± 0.4	54.0 ± 4.5	$50,775 \pm 557$
Largemouth bass	Ambient	16.3 ± 0.2	9.3 ± 1.2	111 ± 39
	50,000	16.7 ± 0.4	54.4 ± 4.6	$50,664 \pm 523$

pCO_2 was measured with an infrared probe (previously described) and converted to µatm. Water velocity through the behavioral tank was consistently 4 cm s^{-1}

post-exposure). Treatment pCO$_2$, exposure period, and their interaction, were included in the predictive models as fixed factors, and fish ID was included as a random effect because each individual was measured three times (Pre-exposure, CO$_2$ exposure and Post-exposure) during each trial (Laird and Ware 1982; Lindstrom and Bates 1990). If the interaction term was significant, the two main effects were ignored and a Tukey test was performed at a significance level of 0.05. Residuals were inspected and, if necessary, data were log-transformed to ensure model assumptions were met (Zuur et al. 2009). Significance (α) was tested at the 95% confidence level.

To quantify the impacts of repeated CO$_2$ exposures on fish behaviors, linear mixed-effects models were used to predict behavioral responses from treatment pCO$_2$, CO$_2$ exposure period (pre-exposure, CO$_2$ exposure, and post-exposure) and exposure number (i.e., first exposure, second exposure, etc.), and a Tukey test was used where applicable. Treatment pCO$_2$, CO$_2$ exposure period, exposure number, and the interaction between these variables were included in the predictive model as fixed factors. Fish ID was included as a random effect. Significance (α) was tested at the 95% confidence level.

Results

Threshold levels

In bluegill, exposure to elevated pCO$_2$ resulted in a significant increase in distance moved and velocity, relative to the pre-exposure period as evidenced by a Tukey test (Table 4, Fig. 2). There was no significant pCO$_2$ or interaction effect, however some notable trends occurred. For the control treatment, CO$_2$-exposure activity levels were similar to pre-exposure levels, but increased between 100 and 300% for the 25,000, 50,000 and 100,000 μatm treatments, indicating a heightened state of agitation. After the water to which bluegill were exposed returned to ambient pCO$_2$ levels, behavioral metrics did not differ significantly from pre-exposure levels, indicating a return to baseline activity levels (Table 4, Fig. 2).

Largemouth bass also displayed an increase in distance moved and velocity as pCO$_2$ treatment increased above ambient (Table 4, Fig. 3). However, no statistical increase relative to control occurred at

pCO$_2$ below 100,000 μatm. Exposure to 100,000 μatm pCO$_2$ resulted in a significantly higher distance moved (300% increase) and average velocity (80% increase) relative to the respective pre-exposure period in each of the metrics as per a Tukey test (Tables 4, 5).

Repeat exposures

When bluegill experienced five successive exposures of 50,000 μatm pCO$_2$, several trends were observed. Distance moved increased sevenfold during the first exposure number relative to the pre- and post-exposure periods of the same exposure number (Table 5, Fig. 4). Velocity displayed a significant increase, both doubling, during the CO$_2$ exposure period of the first cycle followed by a return to 'baseline' levels throughout all the following exposure numbers (Fig. 4). Largemouth bass showed no change in behavior across the exposures at control or 50,000 μatm pCO$_2$ (Table 5, Fig. 5). Although not statistically significant, largemouth bass displayed a doubling of distance moved following the initial exposure to 50,000 μatm pCO$_2$, but returned to values equal to ambient in the post-exposure period (Fig. 5).

Discussion

A single exposure to elevated pCO$_2$ in flowing water resulted in significant changes in behavior for both largemouth bass and bluegill, even during 1 min of exposure. Largemouth bass movement and speed significantly increased when exposed to 100,000 μatm pCO$_2$, and while not statistically significant, bluegill movement and speed both increased by 100% when fish were exposed to 25,000 μatm pCO$_2$ (Figs. 2, 3). When exposed to external stimuli, short-term behavioral modifications are one of the first responses of fish (Atchison et al. 1987), and these responses may occur to increase fitness or survival (Scott and Sloman 2004). For example, fathead minnows (*Pimephales promelas*) typically display irregular activities such as darts and freezes when exposed to conspecific alarm cues (to reduce the likelihood of predation), but these behavioral responses occur less often when fathead minnows are exposed to elevated pCO$_2$ (Chivers and Smith 1994; Tix et al. 2016b). Another example of fish behaviorally responding to elevated pCO$_2$ is in largemouth bass, which were found to avoid areas of

Table 4 Results of linear mixed-effects models used to analyze effects of a single CO_2 exposure (CO_2 pressure: ambient, 25,000, 50,000 or 100,000 µatm) and exposure period (exposure: pre-exposure, CO_2 exposure, or post-exposure) on fish behaviors (distance moved and velocity)

Species	Measured variable	Main effects	df	F	p
Bluegill	Distance moved (cm)	CO_2 pressure	3, 16	0.32	0.808
		Exposure	**2, 17**	**15.77**	**< 0.0001**
		CO_2 pressure × exposure	6, 13	1.07	0.3901
	Velocity (cm s^{-1})	CO_2 pressure	3, 16	0.22	0.8816
		Exposure	**2, 17**	**22.14**	**< 0.0001**
		CO_2 pressure × exposure	6, 13	1.39	0.2306
Largemouth bass	Distance moved (cm)	CO_2 pressure	3, 17	2.02	0.1258
		Exposure	**2, 18**	**16.77**	**< 0.0001**
		CO_2 pressure × exposure	**6, 14**	**6.52**	**< 0.0001**
	Velocity (cm s^{-1})	**CO_2 pressure**	3, 17	**4.95**	**0.0051**
		Exposure	**2, 18**	**15.22**	**< 0.0001**
		CO_2 pressure × exposure	**6, 14**	**7.91**	**< 0.0001**

Significant results are highlighted in bold

elevated pCO_2, likely to maintain homeostasis (Dennis et al 2016a). Following exposure to elevated pCO_2 in the current study, largemouth bass and bluegill are likely displaying behavioral changes in response to a noxious level of CO_2, and responses such as an increase in movement and speed likely represent attempts at seeking a suitable environment. Similar findings were reported in Kates et al. (2012), who showed largemouth bass, bluegill and silver carp displayed agitated behaviors in approximately 30,000 µatm pCO_2, and also of Dennis et al. (2016a) who showed largemouth bass increase agitation behavior when exposed to elevated pCO_2 (40,000 µatm) even after long-term acclimation to hypercarbia. While these previous studies were performed in static water, the present study shows that these increased behavioral metrics also occur in flowing water, even though fish have different sensory and behavioral interactions with their environment in flowing water versus static water (Montgomery and Coombs 2011). Together, these findings indicate that behavioral changes occur in bluegill and largemouth bass when exposed to elevated pCO_2 over short time scales in flowing water.

The threshold for bluegill sensing and reacting to elevated pCO_2 is likely less than 25,000 µatm, whereas the threshold for largemouth bass is closer to 100,000 µatm. Despite the lack of a pCO_2 or interaction effect, distinct trends in activity levels can

be seen that were to be expected based on previous work. Movement in bluegill increased one to three-fold when fish were exposed to 25,000 µatm pCO_2, while largemouth bass movement and speed significantly increased at 100,000 µatm. Animals can exhibit a large range of potential behavioral responses to external stimuli, but often these responses are displayed only after a threshold level is reached (Hara 1994; Tierney 2016). For example, fish respond to olfactory reception of amino acids, steroid hormones and salts in their environment, but the point at which the response occurs may differ depending on the ambient levels experienced before dosage (Hara 1994). However, the thresholds for CO_2 to elicit responses has not been well documented. Our results indicate that the threshold for bluegill to sense CO_2 is likely lower than the 60 mg L^{-1} previously identified (likely around 50,000 µatm, from Dennis et al. 2016a), as we show similar trends in movement and speed at 25,000 µatm in the behavioral choice tank. Furthermore, the pCO_2 at which we saw this behavioral response is lower than the level of 50,000 µatm, which has previously been recommended as a target for a movement barrier (Noatch and Suski 2012; Cupp et al. 2016). While testing at more CO_2 pressures can establish an even more accurate threshold, our results not only validate other lab-based work but also identified pressures that are too low to produce a
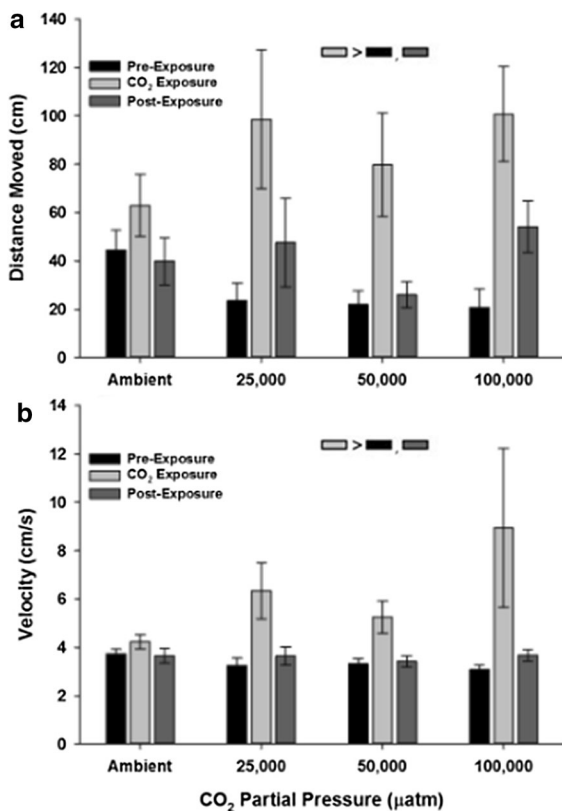
Fig. 2 Behavioral metrics for bluegill (*L. macrochirus*) during exposure to varying pCO$_2$ ranging from ambient (< 200 μatm) to 100,000 μatm shown as mean ± 1 SE. Panels represent **a** distance moved (cm) and **b** average velocity (cm s^{-1}) before, during and after CO$_2$ exposure. For each panel, the boxes with a greater than symbol (>) indicate that *CO$_2$ exposure* period is significantly greater than both *pre-* and *post-exposure* periods (per a Tukey test)

Fig. 3 Behavioral metrics for largemouth bass (*M. salmoides*) during exposure to varying pCO$_2$ ranging from ambient (< 200 μatm) to 100,000 μatm shown as mean ± 1 SE. Panels show **a** distance moved (cm) and **b** velocity (cm s^{-1}). For each panel, an asterisk indicates a significant increase in a behavior relative to the *pre-exposure* period within an individual partial pressure (Tukey test). In all four panels, the numbers 1 and 2 indicate differences in a behavior between the *CO$_2$ exposure* periods (Tukey test)

response, thus influencing barrier development. Together, these findings suggest that thresholds to elicit behavioral responses may vary depending on setting, and it is important to understand the levels at which behavioral responses occur in flowing water.

Bluegill and largemouth bass displayed behavioral responses at different treatment levels of CO$_2$, showing varying levels of sensitivity to this stimulus. For example, bluegill showed a 100% increase in movement and speed at 25,000 μatm, whereas largemouth bass did not respond with elevated movement and speed until 100,000 μatm pCO$_2$. Interspecific differences in response behaviors (such as reactions to toxins or oxygen levels) have been previously shown in fish in a variety of contexts (Ferrari et al. 2011), and sometimes these behavioral differences result from
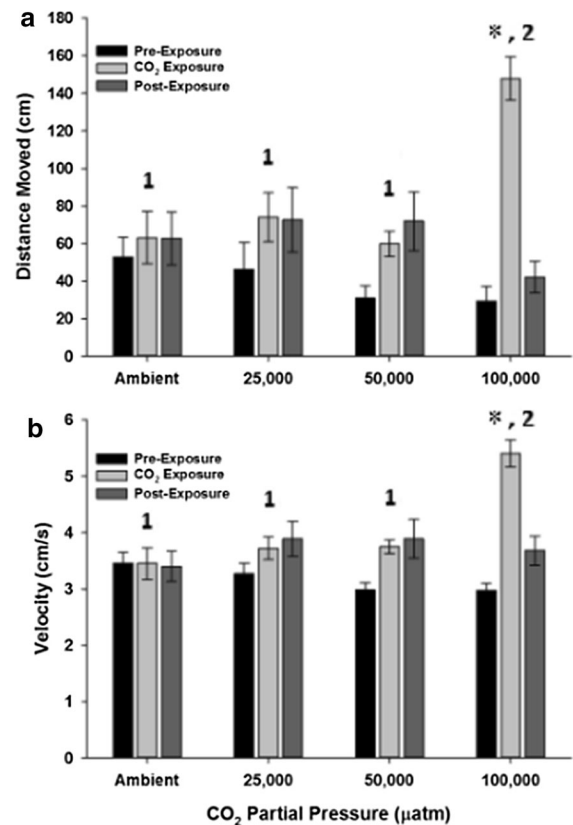
varying degrees of sensitivity to a stimulus (Cummins et al. 2014). However, previous research has shown that fish generally respond the same to elevated pCO$_2$, with minimal interspecific variation. For example, Kates et al. (2012) showed that, after a 3 h exposure to 70 mg L^{-1} CO$_2$, 100% of bluegill, silver carp and bighead carp, and 70% of largemouth bass showed irregular activities such as irregular ventilation, twitching or loss of equilibrium. Additionally, all six freshwater species of fish tested showed little difference in avoidance when CO$_2$ was injected into a pond (Donaldson et al. 2016). It is possible that these interspecific differences seen in our study arose because fish interact differently with a flowing environment that with a static one, or because we used a

Table 5 Results of linear mixed-effects models used to analyze effects of multiple successive CO_2 exposures (5 in total) and exposure period (pre-exposure, CO_2 exposure, or post-exposure) on fish behaviors (distance moved and velocity)

Species	Measured variable	Main effects	df	F	p
Bluegill	Distance moved (cm)	**Exposure**	**2, 17**	**23.72**	**< 0.0001**
		Number	4, 15	1.62	0.1728
		Exposure × number	**8, 11**	**3.61**	**0.0008**
	Velocity (cm s^{-1})	**Exposure**	**2, 17**	**17.69**	**< 0.0001**
		Number	4, 15	1.42	0.231
		Exposure × number	**8, 11**	**4.71**	**< 0.0001**
Largemouth bass	Distance moved (cm)	Exposure	**2, 18**	1.17	0.3139
		Number	4, 16	1.65	0.1692
		Exposure × number	**8, 12**	1.07	0.3898
	Velocity (cm s^{-1})	Exposure	**2, 18**	0.96	0.3857
		Number	4, 16	1.78	0.1393
		Exposure × number	**8, 12**	1.37	0.2181

Significant results are highlighted in bold

wider breadth of CO_2 levels than in some previous studies. Because the present study saw bluegill beginning to respond at 25,000 μatm and largemouth bass responding at 100,000 μatm, the need for context-specific barrier trials becomes evident due to the differences in how fish respond to elevated CO_2 between static and flowing water conditions.

Repeated exposures to elevated pCO_2 resulted in decreases in behavioral responses across successive exposures in both bluegill and largemouth bass. Bluegill showed a significant increase in movement during the first and second exposures to 50,000 μatm pCO_2, however this then remained unchanged from the pre-exposure to the CO_2 exposure periods during the third, fourth, and fifth exposures. Largemouth bass showed a similar pattern (albeit nonsignificant), exhibiting an increase in movement relative to control during the first exposure, followed by negligible changes of the same during successive exposures. It has been previously shown that animals may respond differently to repeated exposures of a stimulus. For example, individuals may be rendered more or less tolerant to a stimulus throughout successive exposures, and this may affect fitness either directly or indirectly through negative physiological responses or behavioral changes (Reinert et al. 2002). Similarly, if a stimulus is presented frequently, physiological responses to the stimulus may be sustained if individuals cannot recover between exposures, therefore producing a continual response (Hannan et al. 2016). Alternatively, if mechanisms such as detoxification enzymes are induced, successive exposures may not affect an animal (Reinert et al. 2002). In the present

study, fish repeatedly exposed to elevated carbon dioxide may be experiencing habituation, defined as a diminished response to a repeated stimulus, shown by activity levels after several CO_2 exposures equal to those before the first CO_2 exposure. Alternatively, fish could also be learning that the stimulus in the experiment was not associated with any adverse outcomes. Tierney (2016) showed that CO_2 is a compound that normally elicits unconditioned avoidance in fish, and several studies have shown that fish will avoid elevated CO_2 following multiple exposures (Kates et al. 2012; Dennis et al. 2016b), so the lack of response after successive exposures could be a result of some degree of non-associative learning (Best et al. 2008). In the past, CO_2 has been used as a fish anesthetic (Gilderhus and Marking 1987), thus prolonged exposure could result in a decrease in activity. Our results suggest that fish are likely not becoming anesthetized from repeated exposures to the chemical because pre-exposure levels of activity over the successive exposures to CO_2 do not decrease significantly. We show that repeated exposure to 50,000 μatm CO_2 resulted in a significant decrease in behavioral responses in bluegill, which may be indicative of habituation. Additional research, ideally in a field setting, should be performed to corroborate this finding in the context of avoidance.

Zones of elevated carbon dioxide have shown promise as an effective non-physical barrier to prevent the movement of invasive fishes (Cupp et al. 2016; Donaldson et al. 2016). As the need for methods of invasive species containment increases, the current results have several implications for the design and
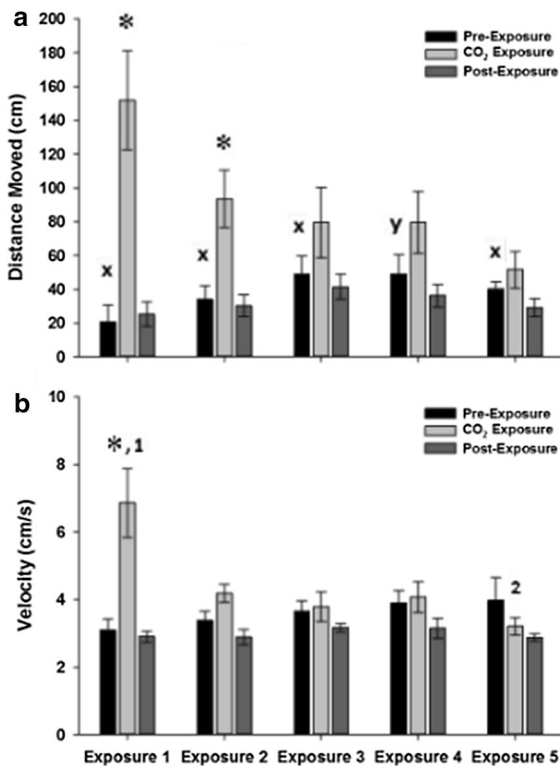
Fig. 4 Behavioral metrics for bluegill (*L. macrochirus*) during repeated exposures to 50,000 µatm CO_2 shown as mean $\pm$ 1 SE. Panels represent **a** distance moved (cm) and **b** average velocity (cm s^{-1}). For each panel, an asterisk indicates a significant increase in a measured behavior from the *pre-exposure* period to the *CO₂ exposure* period. In panel **a** lower-case x and y indicate differences in the measured behavior between the *pre-exposure* periods across successive exposure numbers where the letters differ. In panel **b** the numbers 1 and 2 indicate differences in the measured behavior between the *CO₂ exposure* periods across successive exposure numbers (all symbols per a Tukey test)

Fig. 5 Behavioral metrics for largemouth bass (*M. salmoides*) during repeated exposures to 50,000 µatm CO_2 shown as mean $\pm$ 1 SE. Panels represent **a** distance moved (cm) and **b** acceleration (cm s^{-2}). For each panel, no significant differences were found

implementation of a CO_2 barrier to deter fish movements. As previously discussed, one option for the deployment of a CO_2 barrier in the field would be to add CO_2-treated water into a shipping lock that would be released downstream upon lock opening, creating an intermittent plume of hypercarbic water at a chokepoint in a river. Our results suggest that fish can detect CO_2 levels of 25,000 µatm or lower, which is below levels previously identified as targets for fish barriers. Additionally, because exposures in our study were only 1 min in duration, yet we observed behavioral responses, this suggests fish do not need to be exposed to CO_2 for extended time periods for behavioral responses to occur. Fish also recover quickly
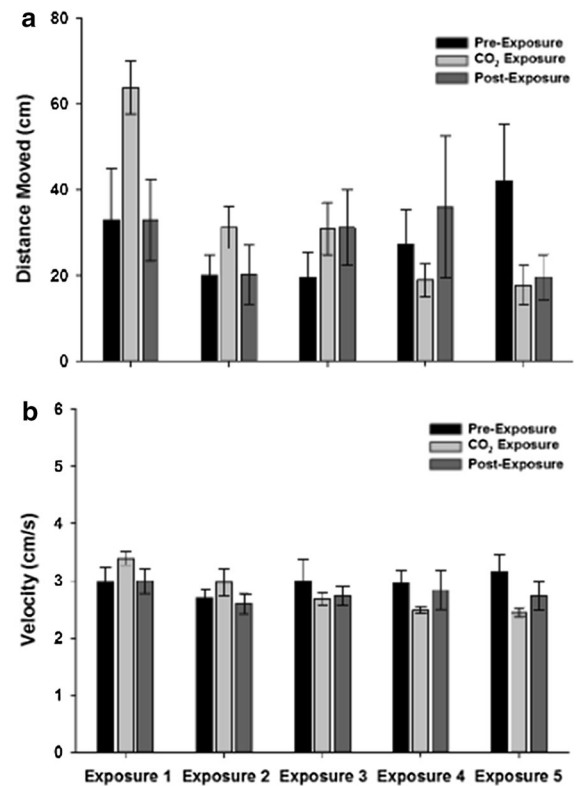
from brief exposures to elevated CO_2, as evidenced by the decrease in agitation behavior following a return to ambient CO_2 conditions. Additionally, interspecific differences in response to elevated CO_2 exist to some degree as bluegill showed an increase in activity at 25,000 µatm and largemouth bass showed the same at 100,000 µatm. It is possible that a CO_2 barrier could act synergistically with other existing technology, making it an important area of development. For example, a CO_2 barrier would not be size selective or affected by barge traffic, whereas electricity barriers may not incapacitate small fish or ones that swim directly next to a steel-hulled barge (Dettmers et al. 2005). Therefore, it is important to understand these intricacies to develop an effective barrier for a specific target species. Though further work is needed to fully understand the potential efficacy of a CO_2 barrier, this study lays the groundwork for this non-physical barrier in flowing water.

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