

Elevated carbon dioxide has the potential to impact alarm cue responses in some freshwater fishes

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Abstract Freshwater fish behaviors have the potential to be impacted by acidification due to increases in dissolved carbon dioxide (CO₂). Recent work in the marine environment suggests that increased CO₂ levels due to climate change can negatively affect fishes homing to natal environments, while also hindering their ability to detect predators and perform aerobically. The potential for elevated CO₂ to have similar negative impacts on freshwater communities remains understudied. The objective of our study was to quantify the effects of elevated CO₂ 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 was significantly impaired following exposure to elevated CO₂ levels for at least 96 h, while silver carp behaviors were unaltered. However, fathead minnow behaviors did return to pre-CO₂ exposure in high-CO₂-exposed fish following 14 days of holding at ambient CO₂ levels. Overall, this study suggests there may be potential impacts to freshwater fishes alarm cue behaviors following CO₂ exposure, but these responses may be species-specific and will likely be abated should the CO₂ stressor be removed.

Keywords Acidification · Olfaction · Climate change · Behavior · Fathead minnows · Silver carp

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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 and Quinn 1996). Olfaction is particularly important for fishes within the super-order 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, 2009, 2013). First, one of the main chemical components of the alarm pheromone is hypoxan-thione-3(*N*)-oxide (H_3NO), and the structure of this molecule can be altered in water at a pH of 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 (Dixson et al. 2010; Munday et al. 2010). In the marine environment, the partial pressure of CO_2 ($p\text{CO}_2$) in water can increase due to many factors (e.g., atmospheric levels of CO_2 , seeps, upwellings, etc.), and $p\text{CO}_2$ 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; Regan et al. 2016). More specifically, exposure to elevated $p\text{CO}_2$ 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 $p\text{CO}_2$ for a short duration (11 days 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 days displayed impaired responses to conspecific alarm cues (Ferrari et al. 2011). Despite these findings, behavioral changes driven by elevated $p\text{CO}_2$ 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 $p\text{CO}_2$ (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 exists for freshwater fishes.

In freshwater, $p\text{CO}_2$ 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 $p\text{CO}_2$ in freshwater are naturally variable, and ranged from 107 to 4128 μatm . across 62 lakes measured globally (Cole et al. 1994), and, within a lake, free CO_2 may increase sevenfold above atmospheric concentrations in Fall, Winter and early Spring (Maberly 1996). Furthermore, freshwater fishes may experience elevated $p\text{CO}_2$ due to a number of mechanisms including a rise in atmospheric CO_2 (Phillips et al. 2015), increased terrestrial primary productivity (Arneith 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 the mechanism behind potential increases in $p\text{CO}_2$, coupled with a reduction in pH, is essential for predicting consequences of elevated

$p\text{CO}_2$ 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 $p\text{CO}_2$ would change olfactory predator avoidance behaviors of fathead minnows (*Pimephales promelas*) and silver carp (*Hypophthalmichthys molitrix*) and (2) if impaired olfaction behavior occurred, determine whether ‘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 days and were then exposed to conspecific skin extracts. Naïve fish in holding tanks were returned to ambient $p\text{CO}_2$ for at least 11 days prior to undergoing the same behavioral trials.

Materials and methods

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 ≈ 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 2 days of laboratory acclimation to further reduce stress and promote fish health (Swann and Fitzgerald 1991). Fathead minnows were given a total of 5 days 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 m with a SenTix 41 probe, Germany); the pH probe was calibrated daily during this study (Moran 2014). In addition, $p\text{CO}_2$ 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) (Supplementary Table S1). During fathead minnow trials, $p\text{CO}_2$ 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.

$p\text{CO}_2$ 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), low $p\text{CO}_2$ (≈ 1500) and high $p\text{CO}_2$ ($\approx 7500 \mu\text{atm.}$; Supplementary Table S1). These treatment levels were chosen because (a) Kates et al. (2012) found that short-term exposure to 70 mg/l of CO_2 ($\approx 150,000 \mu\text{atm.}$) 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 $\mu\text{atm.}$ would prevent such consequences, (b) many marine acidification studies that have demonstrated an impact of CO_2 exposure on olfactory responses targeted $\approx 1500 \mu\text{atm.}$ for high CO_2 exposure, which is a future projection of $p\text{CO}_2$ in marine ecosystems (Forsgren et al. 2013; Jutfelt et al. 2013) and (c) Heuer and Grosell (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 $p\text{CO}_2$ levels as high as 7000 $\mu\text{atm.}$ as an experimental treatment, $p\text{CO}_2$ in freshwater ecosystems fluctuates widely and can experience higher levels of $p\text{CO}_2$ than marine ecosystems (Leduc et al. 2013; Hasler et al. 2016), making 7000 $\mu\text{atm.}$ a

relevant and valuable level for holding. Target $p\text{CO}_2$ levels were held constant using a Pinpoint pH Regulator Kit (American Marine Inc., Ridgefield, CT, USA) (Munday et al. 2012; Allan et al. 2013) adjusted to add CO_2 to the water when water pH rose above a set level (The pH set level to target the corresponding $p\text{CO}_2$ levels for fathead minnows was low =8.20, high =7.25 and for silver carp was low =7.35, high =7.05; 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 days, while silver carp were held for 4–10 days 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 $p\text{CO}_2$ (Munday et al. 2010; Ferrari et al. 2011). Following the behavioral tests (described below), all holding tanks were returned to ambient $p\text{CO}_2$ for 11–14 days for fathead minnows and 14–17 days for silver carp by replacing CO_2 -rich water in the tank with water at ambient levels. Ambient $p\text{CO}_2$ levels varied and were higher than many marine studies, as $p\text{CO}_2$ levels in freshwater typically experience both daily and seasonal fluctuations (Maberly 1996). For example, $p\text{CO}_2$ in a productive lake was shown to be depleted to almost zero during the day and replenished during the night causing an overall shift in pH of 1.8 units (Maberly 1996).

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. Breeding-conditioned males and females, identified by the presence of gametes, were not used as breeding males prohibit the production of 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 were measured, and the total area of skin collected was ≈ 274.4 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 (≈ 5 °C) and homogenized with a disperser (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 1800 ml of ultra-pure water (total volume was 2400 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) as effects of CO_2 on fish behavior is not altered by different experimental test water (Munday et al. 2016).

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 (verified with a flow meter; 807 series Rotameter, Georg Fischer, Schaffhausen, Switzerland). The out-flowing 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 response not influenced by conspecifics (Lawrence and 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). 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) (Supplementary Table S2). 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. Gender was unknown for both species. 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 11–14 days in water at ambient $p\text{CO}_2$ ($\approx 400 \mu\text{atm.}$) 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 14–17 days in

ambient $p\text{CO}_2$ water ($\approx 950 \mu\text{atm.}$), 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 $p\text{CO}_2$ 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 (1994), 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 whether elevated $p\text{CO}_2$ 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 $p\text{CO}_2$ 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) and 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 across treatments (i.e., due to inherent inter-individual differences, some fish may be more active or freeze more, than others, which

needs to be considered across treatments) (Laird and Ware 1982; Lindstrom and Bates 1990).

For silver carp, data were not parsed by $p\text{CO}_2$ treatment due to obtaining normality in residuals, and 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 $p\text{CO}_2$ 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 and 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 was used to assess normality (Anscombe and Tukey 1963), and visual inspection of the distribution of residuals was used to assess homogeneity of variance. 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 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 was 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 mean \pm standard error, SE, where appropriate. Treatment duration for each individual was initially included as a covariate in all analytical models, but was not significant for both fathead minnows and silver carp and was therefore excluded in final models (Engqvist 2005).

Results

Both fathead minnows and silver carp responded to the skin extracts of conspecifics. Specifically, for fathead

minnows held at ambient $p\text{CO}_2$, the number of irregular activities (e.g., darts and freezes) increased 4.5- and 2.4-fold during the treatment and recovery periods after being exposed to skin extracts, respectively (Fig. 1a; Supplementary Table S3). 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 (Fig. 2; Supplementary Table S3). In addition to responding with increased irregular activities, fathead minnows held at ambient $p\text{CO}_2$ also had faster swimming velocity and greater distance travelled, as fish swam 0.34 ± 0.11 BL/s faster (Fig. 5a; Supplementary Table S4) and travelled 138 ± 46 BL more (Table 1; Fig. 4a; Supplementary Table S4), respectively, following exposure to skin extracts.

Exposure to elevated $p\text{CO}_2$ resulted in changes to the responses of fathead minnows to skin extracts, but not silver carp. More specifically, unlike fish held at control conditions (600 $\mu\text{atm.}$), fathead minnows treated with high $p\text{CO}_2$ (7000 $\mu\text{atm.}$) displayed no irregular responses to skin extract exposure (Fig. 1c; Supplementary Table S3). Fathead minnows exposed to low $p\text{CO}_2$ (800 $\mu\text{atm.}$) 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 (Fig. 1b; Supplementary Table S3). Furthermore, the changes in distance travelled (Table 1; Fig. 4b, c; Supplementary Table S4) and swimming velocity (Fig. 4b, c; Supplementary Table S4) 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 (800 and 7000 $\mu\text{atm.}$). For silver carp, all behavior metrics did not differ relative to the acclimation period following exposure to skin extracts, regardless of $p\text{CO}_2$ treatment (Table 2; Supplementary Tables S3, S4).

Returning fathead minnows to water at ambient $p\text{CO}_2$ 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 $p\text{CO}_2$ demonstrated a 2.7-fold increase in irregular activities after exposure to skin extract (Fig. 1; Supplementary Table S3). Fathead minnows

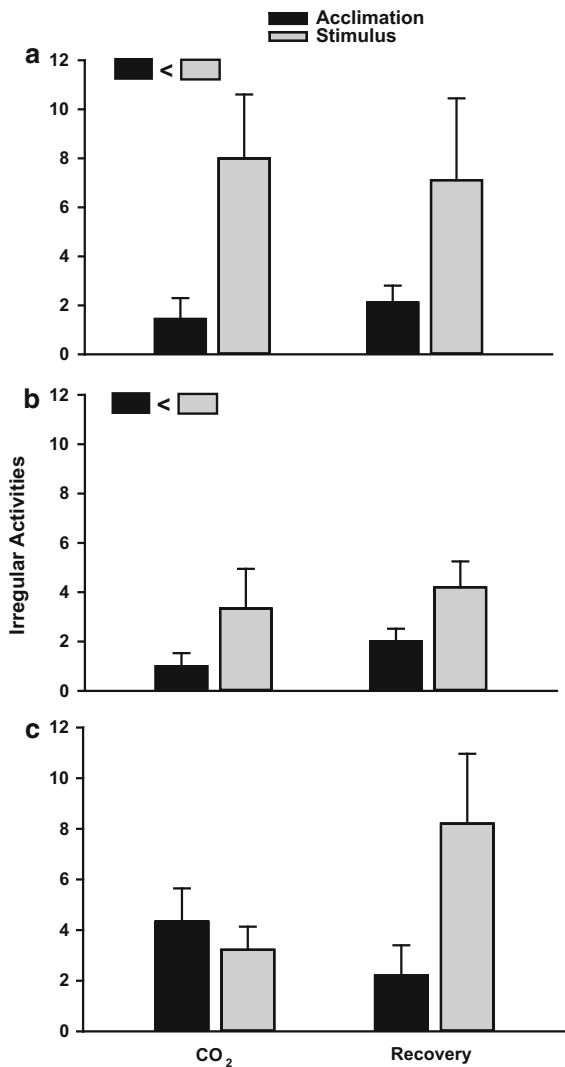


Fig. 1 Number of irregular activities (i.e., darts and freezes) for fathead minnows (*P. promelas*) during the acclimation and stimulus periods following CO₂ exposure and for separate fathead minnows during recovery following CO₂ exposure. Fathead minnows were exposed to either **a** control (600 µatm.), **b** low (800 µatm.) or **c** high (7000 µatm.) CO₂ levels for 4–12 days and then held for an additional 11–14 days at ambient (750 µatm. CO₂) conditions. Data are presented as mean ± SE (*N* = 9–10). For **a**, **b** the gray and black boxes with a less than symbol represent a significant effect of monitoring period between the acclimation and stimulus period (GLMM, see supplementary Table S3). For **c**, a significant interactive effect of stimulus and recovery was detected, but multiple comparisons did not reveal the source of the significant difference (GLMM, see supplementary Table S3)

exposed only to control values of *p*CO₂ were also monitored and showed a response to skin extracts during the recovery period too, as activity decreased

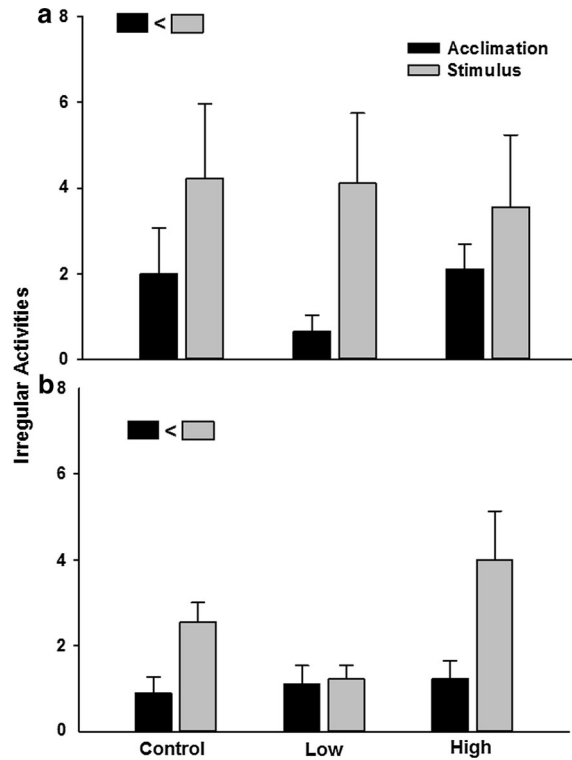


Fig. 2 Number of irregular activities (darts, freezes, and jumps) for silver carp (*H. molitrix*) during acclimation and stimulus periods following CO₂ exposure and recovery. Silver carp were **a** exposed to either ambient (1000 µatm.), low (3000 µatm.) or high (8000 µatm.) CO₂ levels for 4–10 days and then **b** held for an additional 11–14 days at ambient (750 µatm. CO₂) conditions. Data are presented as mean ± SE (*N* = 9). The gray and black boxes with the less than symbol represent a significant effect of monitoring period between acclimation and stimulus (GLMM, see supplementary Table S3)

Table 1 Changes in the intercept estimate for response variables with a significant interactive effect (see Supplementary Table S4) for control fathead minnows during the CO₂ exposure and recovery period

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)	CO₂	1.11	0.21, 2.06
	Recovery	−0.71	−1.58, 0.17

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

Table 2 Total activity (s), total distance travelled in body lengths (BL) and velocity (BL/s) for silver carp during acclimation and stimulus periods following CO₂ exposure and recovery

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

Silver carp were exposed to either ambient (1000), low (3000) or high (8000 µatm.) CO₂ levels for 4–10 days and then held for an additional 11–14 days at ambient (750 µatm. CO₂) conditions. Data are presented as mean ± SE ($N = 9$). No significant effect of monitoring period or treatment were detected within the CO₂ or recovery treatment periods (GLMM, see Supplementary Tables S3, S4)

by about double during the stimulus period (Table 1; Fig. 3; Supplementary Table S4).

Discussion

Some alarm cue behaviors in fathead minnows exposed to conspecific skin extracts were altered when fish were held in water with elevated $p\text{CO}_2$ for 4–12 days. Specifically, fathead minnows exposed to high $p\text{CO}_2$ (7000 µatm.) did not display changes in irregular activities, velocity or distance travelled when exposed to skin extracts (Table 1; Figs. 1, 4, 5; Supplementary Tables S3, S4). No changes in velocity or distance travelled were observed for low (800 µatm.) $p\text{CO}_2$ -treated fathead minnows (Table 1; Figs. 1, 4, 5; Supplementary Tables S3, S4) following exposure skin extracts as well. 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 $p\text{CO}_2$ (Munday et al. 2009; Dixon 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 days at 850 µatm. $p\text{CO}_2$. One possible explanation to why fish exposed to elevated $p\text{CO}_2$ have a reduced alarm cue response may

be due to reduced sensitivity of the olfactory receptors. Specifically, with respect to acidification, mucus can increase on the olfactory epithelium in low pH and can disrupt olfaction capabilities (Lemly and Smith 1987; Klaprat et al. 1988). Olfactory receptor alterations and increased mucus production may have caused the decrease in fathead minnow's ability to respond to the alarm cue. Previous research has also shown that rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and fathead minnows exposed to a pH of 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). However, with some of these studies, the cause of no response was most likely due degradation of the alarm cue (Leduc et al. 2013). Our testing was completed in ambient freshwater and not acidified water as the previous stated research has been conducted. Similar results to ours were found where alarm cue testing in pH 7.54, similar to ours, Pacific salmon could no longer avoid conspecific skin extracts in a two-choice flume, and anxiety behaviors were reversed with the treatment of gabazine suggesting GABA_A receptors are being impacted by $p\text{CO}_2$ exposure (Ou et al. 2015). Nilsson et al. (2012) and Regan et al. (2016) showed function of the GABA_A

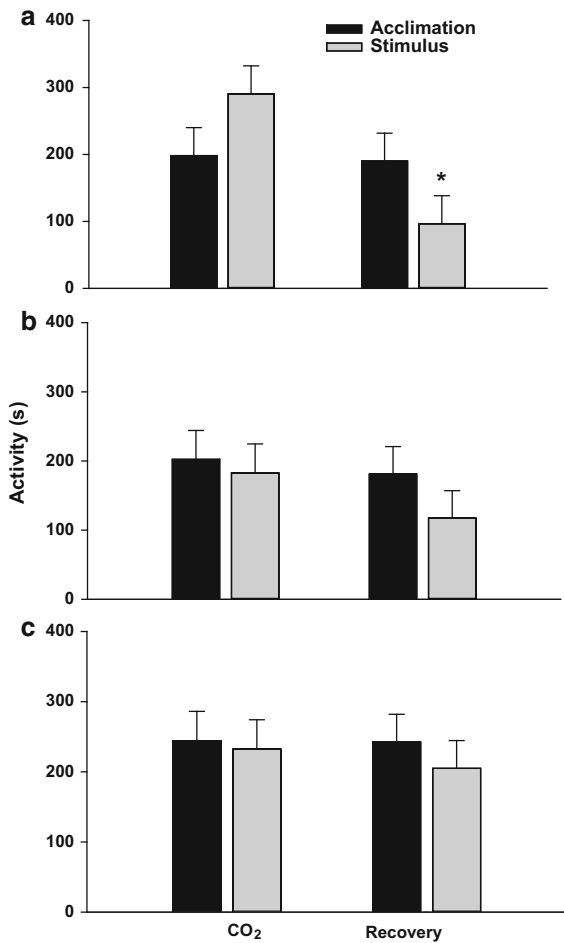


Fig. 3 Mean activity (s) for fathead minnows (*P. promelas*) during the acclimation and stimulus periods following CO₂ exposure and recovery. Fathead minnow were exposed to either **a** ambient (600 μatm.), **b** low (800 μatm.) or **c** high (7000 μatm.) CO₂ levels for 4–12 days and then held for an additional 11–14 days at ambient (400 μatm. CO₂) conditions. Data are presented as the mean ± SE. An *asterisk* represents a significant interaction between acclimation and stimulus during the recovery period (GLMM, see Tables 1, S4)

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 CO₂ exposure especially with no degradation of the alarm cue. Together these results clearly demonstrate that in fathead minnows, some conspecific alarm cue responses are affected by elevations in pCO₂.

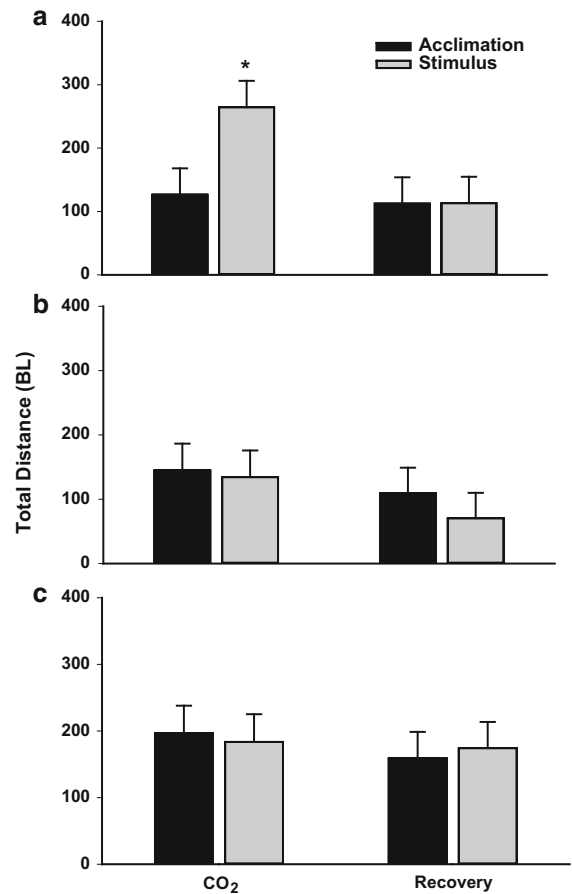


Fig. 4 Mean total distance (BL) for fathead minnows (*P. promelas*) during the acclimation and stimulus periods following CO₂ exposure and recovery. Fathead minnow were exposed to either **a** ambient (600 μatm.), **b** low (800 μatm.) or **c** high (7000 μatm.) CO₂ levels for 4–12 days and then held for an additional 11–14 days at ambient (400 μatm. CO₂) conditions. Data are presented as the mean ± SE. An *asterisk* represents a significant interaction between acclimation and stimulus during the recovery period (GLMM, see Tables 1, S4)

Fathead minnows may be able to recover and respond to conspecific skin extracts after pCO₂ exposure and subsequent return to ambient freshwater. Fathead minnows that showed behavioral impairments after exposure to ≈7000 μatm. displayed a threefold increase in irregular activities during the recovery period (Fig. 1c; Supplementary Table S3). 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.

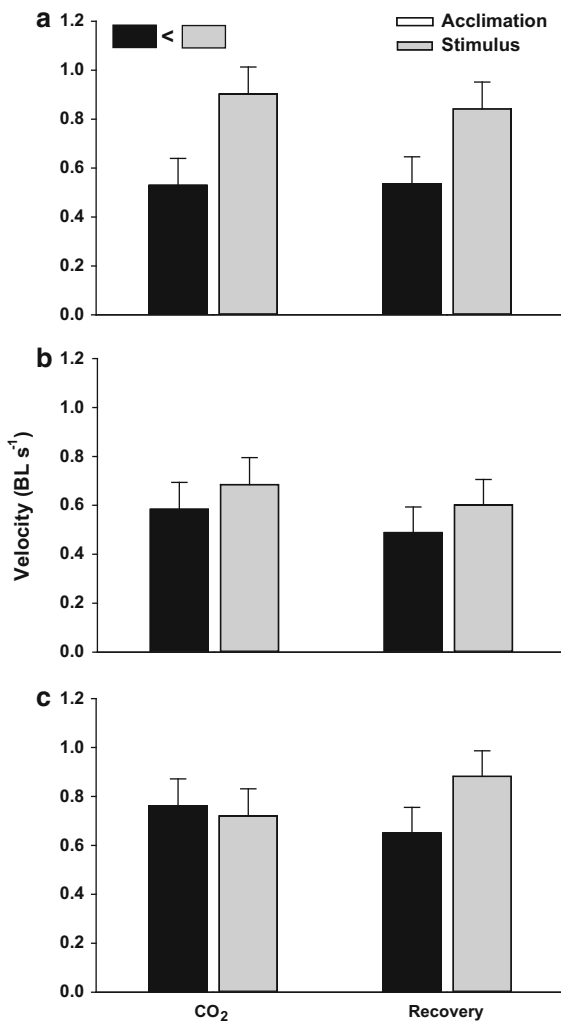


Fig. 5 Mean swimming velocity (BL/s) for fathead minnows (*P. promelas*) during the acclimation and stimulus periods following CO₂ exposure and recovery. Fathead minnows were exposed to either **a** ambient (600 μatm.), **b** low (800 μatm.) or **c** high (7000 μatm.) CO₂ levels for 4–12 days and then held for an additional 11–14 days at ambient (400 μatm. CO₂) conditions. Data are presented as the mean ± SE. The gray and black boxes with the less than symbol represent a significant effect of monitoring period between acclimation and stimulus (GLMM, see supplementary Table S4)

2006) and ammonia (Suski et al. 2007). Previous work has also shown that behaviors altered by exposure to elevated *p*CO₂ can be reversed following removal of the CO₂ 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 days

following exposure to elevated *p*CO₂. 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 *p*CO₂ 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 *p*CO₂ 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 *p*CO₂ as independent contributors to altered responses.

Interestingly, silver carp showed no impairment to alarm cue responses despite 4–10 days of exposure to low or high *p*CO₂ (3000 and 8000 μatm., respectively; Fig. 2; Supplementary Tables S3, S4). Other fish species have demonstrated conservative responses to alarm cues following exposure to environmental stressors. For example, juvenile Atlantic cod (*Gadus morhua*) were still able to avoid a predator odor following 6 weeks of exposure to 1000 μatm. (Jutfelt and Hedgärde 2013). Species like silver carp, which may live in environments where variations in pH and *p*CO₂ occur regularly, may be more adapted to elevated *p*CO₂ 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 and Dunson (1987) showed *Enneacanthus obesus* had no change in body sodium concentration after 5 weeks in a pH of 4.0. Another possible explanation of why silver carp still responded to the skin extracts following elevated *p*CO₂ exposure may have been due to either a higher concentration of alarm cue used relative to fathead minnows, or possibly a higher concentration of alarm pheromone relative to other previous studies with silver carp (i.e., over-stimulation of process related to sensing pheromones in the water) (Little et al. 2011). Species-specific thresholds that result in responses to skin extracts have not been identified in silver carp, and we did not quantify the concentration of alarm

pheromone used in this study, precluding our ability to quantitatively compare concentrations across species. However, because the purported mechanism for the impaired response to olfactory cues is based on changes to GABA_A receptors (Nilsson et al. 2012; Regan et al. 2016), it is likely that the continued response of silver carp to the skin extracts may indicate that the GABA_A receptors were likely not depolarized following CO₂ exposure, as has been shown for other species (Jutfelt and Hedgärde 2013; Maneja et al. 2013; Jutfelt and Hedgärde 2015). Some freshwater species, like silver carp, may be able to adapt to elevated pCO₂ 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 pCO₂.

A reduced alarm cue response after exposure to elevated CO₂ has many implications for management and the ecology of freshwater fish species. Freshwater fishes may be exposed to elevated pCO₂ due to natural environmental variation (reviewed by Hasler et al. 2016), climate change (Phillips et al. 2015), hatchery rearing (Colt and Orwicz 1991) and zones of elevated pCO₂ deployed as fish barriers (Kates et al. 2012; Noatch and Suski 2012). If fathead minnows were subjected to an increase in pCO₂ 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 2015). However, if the CO₂ 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 pCO₂, and elevations in pCO₂ 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 pCO₂. Together, these results provide information about the possible consequences and responses of freshwater fish to environmental changes such as elevations in pCO₂ and acidification.

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