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Elevated carbon dioxide has limited acute effects on *Lepomis macrochirus* behaviour

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The current study investigated the behavioural response of *Lepomis macrochirus* following exposures to elevated carbon dioxide (CO_2). For this, *L. macrochirus* were held at ambient pCO_2 (160 μatm pCO_2) for 7 days, then exposed to elevated pCO_2 (8300 μatm pCO_2) for 5 days, and then returned to ambient conditions for a further 5 days to recover. At the end of each exposure period, several behavioural metrics were quantified (boldness, lateralization and activity). Data showed no change in lateralization and most metrics associated with performance and boldness. During the boldness test, however, average velocity, velocity in the thigmotaxis (outer) zone and proportion of activity in the thigmotaxis zone increased with pCO_2 exposure. During post-exposure, average velocity of *L. macrochirus* decreased. In addition, individual rank was repeatable during the pre-exposure and post-exposure 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), but not during the CO_2 exposure period, suggesting that elevated pCO_2 disrupted some behavioural performances. Overall, this study found elevated pCO_2 caused disruption to behaviours of freshwater fishes such as *L. macrochirus* and effects do not appear to be as serious as has been shown for marine fishes.

Key words: acidification; bluegill; boldness; climate change; fresh water; lateralization.

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 and metabolism) can also be impacted by environmental variables (Fry, 1971) and can directly influence fish behaviour (Kramer, 1987; Beitinger, 1990; Wendelaar 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, and disruptions in behaviours due to environmental stimuli can influence fitness and mortality in fishes (Cantalupo *et al.*, 1995; Toms *et al.*, 2010; Chapman *et al.*, 2011).

Behaviours important for fitness and survival in fishes include: behaviours that place individuals on the bold-shy continuum (boldness or bold or anxiety behaviours), lateralization and swimming performance. Studies have shown that these behaviour metrics are altered by environmental change (Tuomainen & Candolin, 2011; Wong &

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Candolin, 2015). Bold behaviours in fishes, such as willingness to take risk (*e.g.* inspect a novel object), are repeatable across time and contexts (Coleman & Wilson, 1998) and may result in increased access to food resources, however, they may also be costly in high predation areas (Wilson & Godin, 2009). Conversely, shyer or anxiety-related behaviours may be beneficial for fishes to avoid predation but may result in fewer reproductive opportunities in social groupings (Wilson *et al.*, 1993). Other behaviours, such as lateralization (*i.e.* turning preference), have been well studied in fishes to assess brain function and asymmetry (Bisazza & Dadda, 2005; Dadda & Bisazza, 2006; Dadda *et al.*, 2010). These turning preferences are also thought to be important for predator avoidance (Dadda *et al.*, 2010), and play an important role in cognitive tasks (Dadda & Bisazza, 2006) and shoaling behaviours (Bisazza & Dadda, 2005). Lastly, performance metrics, such as activity (*e.g.* time spent moving), may provide a direct link to fitness in animals (Irschick, 2003) and have been shown to be consistent across large temporal periods (Hanson *et al.*, 2010). Should environmental changes influence boldness, lateralization and swimming performances, freshwater fishes may be prone to a variety of negative outcomes, including predation, reduced growth and altered activity patterns.

One potential human-induced environmental stimulus that may impact freshwater fishes is an increase in carbon dioxide (CO_2) levels (Hasler *et al.*, 2016). Research from the marine environment has shown that CO_2 partial pressure (pCO_2) in sea water increases as atmospheric pCO_2 rises (Sabine *et al.*, 2004), however, precisely how pCO_2 in fresh water will change remains unclear (Hasler *et al.*, 2016). It is possible that freshwater pCO_2 will rise, specifically in large water bodies such as the Laurentian Great Lakes (Phillips *et al.*, 2015). High pCO_2 can also occur in fish hatcheries (Colt & Orwicz, 1991) and high pCO_2 plumes may be used as a barrier to fish movement in natural systems (Noatch & Suski, 2012). Regardless of the reason for elevated pCO_2, increasing water pCO_2 causes a concomitant decrease in water pH, which combined with higher pCO_2, causes respiratory acidosis in fishes (Heuer & Grosell, 2014). The decrease in the blood pH of fishes results in a disruption of cellular ionic gradients, and, in marine fishes, it has been established that this disruption alters the function (switch from inhibitory to excitatory) of the neural gamma-aminobutyric acid type A ($GABA_A$) receptor, which has consequences for fish behaviour, specifically anxiety behaviours due to its role in controlling anxiety (Nilsson *et al.*, 2012; Hamilton *et al.*, 2014). For freshwater species, Leduc *et al.* (2013) suggested that behavioural changes in freshwater fishes exposed to acidification may be more likely to be a result of the olfactory cue being altered and reduced olfactory sensitivity in fishes, rather than a disruption of neurotransmitter pathways in the brain. A recent study on anadromous fish during juvenile freshwater life stages [*i.e.* pink salmon *Oncorhynchus nerka* (Walbaum 1792) embryos], however, demonstrated a link between $GABA_A$ and behaviour, specifically anti-predator and anxiety-linked behaviours (Ou *et al.*, 2015). Research by Ou *et al.* (2015) showed juvenile *O. nerka* became bolder (spent more time in a centre zone) in a novel object arena test after elevated pCO_2 exposure. Another recent study by Jutfelt *et al.* (2013) also suggested that CO_2 exposure can impact brain function as temperate three-spined stickleback *Gasterosteus aculeatus* L. 1758 became less bold as they decreased time spent investigating an object in a novel object arena, and had a reduction in relative and absolute lateralization, after elevated pCO_2 exposure. CO_2-induced behavioural changes, however, have been found to be variable across fish species, as behaviours (*e.g.* boldness, lateralization and swimming performance) of some marine fish species were unchanged after exposure to elevated pCO_2 (Jutfelt & Hedgärde,

2013; Maneja *et al.*, 2013; Sundin & Jutfelt, 2015). Clearly understanding the possible effects of elevated CO_2 levels to influence freshwater fish behaviours and their potential to recover from a CO_2-stressor is important for predicting ecological responses to future environmental change and understanding contextual exposure to elevated pCO_2.

Due to the lack of understanding of how the behaviours of freshwater fishes are affected by elevated pCO_2, the objectives of this study were to (1) quantify the consequences of exposure to various levels of CO_2 on lateralization and boldness-linked behaviours in bluegill *Lepomis macrochirus* Rafinesque 1819, (2) determine whether affected behaviours recover after several days post-exposure to elevated pCO_2 (*i.e.* return to pre-exposure levels) and (3) determine if exposure to elevated pCO_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. Given the contrasting findings of how elevated pCO_2 influences marine fish behaviour (*i.e.* some species demonstrate significant changes, while others do not), predicting the potential for behavioural changes in *L. macrochirus* to elevated pCO_2 is difficult. There is evidence from physiological studies (Brauner *et al.*, 2004), however, to suggest that freshwater fishes may be adapted to high pCO_2 environments, which would suggest that freshwater fish behaviours may be robust against exposure to elevated pCO_2.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND HUSBANDRY

Lepomis macrochirus [$n = 16$; total length (L_T), $11{\cdot}3 \pm 1{\cdot}2$ cm (mean $\pm$ S.D.); mass, $22{\cdot}6 \pm 6{\cdot}7$ 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 outdoor single 379 l plastic flow-through holding tank supplied with compressed air (Sweetwater, Aquatic Eco-Systems; www.pentairaes.com) through an air stone, and a continuous flow of water from a 0·04 ha earthen-pond. *Lepomis macrochirus* were given 7 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.; www.nmt.us; Wilson & Godin, 2009). Tags were implanted using a syringe (29 gauge, 1 cc) and were arranged into different combinations of two colours 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 <30 s and occurred 24 h before the initial testing period. Following tagging, *L. macrochirus* were returned to the common 379 l plastic flow-through holding tank. An ultraviolet water sterilizer (Vecton-6, American Aquarium Products; www.americanaquariumproducts.com) was attached to a small pump to reduce bacterial growth in the tank, which also circulated water in the tank. Throughout the study, *L. macrochirus* were fed red worms *Eisenia fetida ad libitum* daily until 24 h before each trial period. Water quality measurements in the holding tank were taken daily (Table I) and included temperature, dissolved oxygen (YSI, 550A Yellow Springs Instruments; www.ysi.com), ammonia (LaMotte Company, Ammonia Nitrogen kit No. 3351-02; www.lamotte.com) which was always $<0{\cdot}05$ mg l^{-1}, total alkalinity (TA) (Hach Company, Titrator model 16900 and kit 94399; www.hach.com) and pH (WTW pH 3310 m with a SenTix 41 probe; www.wtw.com). 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; www.vaisala.com) wrapped in a semi-permeable polytetrafluoroethylene cover (Johnson *et al.*, 2010; Munday *et al.*, 2014).

TABLE I. Water quality measurements (temperature, dissolved oxygen, pH, dissolved CO_2, total alkalinity and pCO_2) sampled during each treatment period. Pre-exposure represents the 7 day period where *Lepomis macrochirus* were held at ambient conditions and exposure represents the 5 day period where *L. macrochirus* were held at elevated pCO_2. The post-exposure treatment was the period where *L. macrochirus* were returned to ambient pCO_2 for 5 day. Values reported as mean ± S.D.

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
Exposure	21·20 ± 1·16	8·61 ± 0·20	7·15 ± 0·09	24·83 ± 3·92	148·00 ± 4·90	8300 ± 400
Post-exposure	16·50 ± 1·48	9·46 ± 0·29	9·51 ± 0·10	1·40 ± 0·55	149·20 ± 7·40	44 ± 13

$_{P}CO_2$ EXPOSURE TREATMENTS

Over the course of the study, three pCO_2 exposure treatments were applied to the common holding tank and behavioural assays (see below) were carried out following each exposure. Prior to the first set of behavioural tests, *L. macrochirus* were held for 7 days at ambient pCO_2, which was 160 ± 43 μatm (mean ± S.D.). Following completion of the first set of tests, the pCO_2 in the common holding tank was raised to 8300 ± 400 μatm (mean ± S.D.) for 5 days. This holding duration and pCO_2 were chosen as a 4 day 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 physiological changes, but not a loss in equilibrium as fishes lose equilibrium at high levels of CO_2 (Kates *et al.*, 2012). Chosen pCO_2 levels were held constant throughout the experiment using a Pinpoint pH Regulator Kit (American Marine Inc.; www.americanmarineusa.com) (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 A air compressor (Sweetwater, Aquatic Eco-Systems), which also prevented hypoxia. Following the second set of behavioural tests, *L. macrochirus* were returned to the common tank. The pCO_2 in the common tank was then returned to ambient conditions 44 ± 13 μatm (mean ± S.D.) for 5 days, after which behavioural tests were carried out for the third time. Variation in ambient pCO_2 occurred because the earthen pond (with natural vegetation) typically experiences variation in ambient pCO_2 due to changes in variables such as temperature, photoperiod and rainfall (Maberly, 1996; Riera *et al.*, 1999). The variation in ambient pCO_2 seen in this study is within expected values for freshwater systems, is small in relation to the experimental treatments, and not a source of error for this study. The two behavioural tests described below were conducted on the same day, and which test came first, and what individual chosen, were random during the three trial periods.

LATERALIZATION TEST

Lepomis macrochirus were assessed for turning preference *via* a lateralization test immediately following each of the three experimental exposure treatments described above. To quantify lateralization, a double T-maze [15·2 cm × 45·7 cm main runway with a 15·2 cm × 45·7 cm runway at each end that were parallel to one another and attached perpendicular to the main runway; similar diagram is in Jutfelt *et al.* (2013)] 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 separated ($n = 3$ to 4) into 5·7 l containers held within the common holding tank still being exposed to the same pCO_2 concentration at that exposure period to limit exposure to repeated netting during the behavioural trials. During the lateralization trial, individual

L. macrochirus were 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 concentration in the maze was identical to that of the common holding tank during that exposure period. 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 *L. macrochirus* 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 *L. macrochirus* left the main channel and entered one of the perpendicular side channels. Each animal was tested 10 times during each sampling exposure period (Domenici *et al.*, 2012). Following completion of the 10th choice event, animals were removed from the maze and returned to their individual containers within the common holding tank for additional testing or returned to the common holding tank if both tests were completed for that individual.

NOVEL OBJECT TESTS

Lepomis macrochirus were assessed for boldness and anxiety-linked behaviours during 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 *L. macrochirus* were exposed to at that time before each trial (Hamilton *et al.*, 2014). A schematic of a similar arena can be found in Ou *et al.* (2015). A novel object was placed in the centre 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 *L. macrochirus* (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 exposure test, and a 2·5 cm diameter multi-coloured bouncy ball was used during the post-exposure 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 centre of the tank, facing in the direction of the covered novel object. Each *L. macrochirus* 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 *L. macrochirus* was recorded for 10 min using an overhead-mounted video camera (iDS uEye 1480-C camera, iDS; http://en.ids-imaging.com/) (Maximino *et al.*, 2010; Hamilton *et al.*, 2014). Assessments of boldness and anxiety variables related to *L. macrochirus* movement (*e.g.* velocity, acceleration, time spent in zones and shuttles between zones) were quantified using the programme Lolitrack (Loligo Systems; www.loligosystems.com; Poulsen *et al.*, 2014). Using the analysis software, the arena was divided into three equal-sized, concentric zones to determine time spent near the novel object [centre or near the object, middle and outer or near the wall (thigmotaxis); Ou *et al.*, 2015], as well as to understand activity levels in these zones, which may also indicate boldness or anxiety (*e.g.* greater movement near the object could be a risk taking behaviour). The zones were at least equal to or larger than the body length (B_L) of the *L. macrochirus* (Schnörr *et al.*, 2012). Following the 10 min observation period, the trial was terminated, and *L. macrochirus* were either returned to the holding tank or given the lateralization test based on if they had 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: $L_R = 100\ (T_R - T_L)\ (T_R + T_L)^{-1}$, where T_R = turn right and T_L = turn left. Mean L_R was used to evaluate turning preference of the *L. macrochirus* (*i.e.* bias in left or right turns). Values of between −100 (*i.e. L. macrochirus* that turned left during all 10 choice events) and 100 (*L. macrochirus* that turned right during all 10 choice events) were calculated. An L_R of zero represented a non-bias in turning preference (*i.e. L. macrochirus* 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 zero (*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 (*i.e.* wall hugging) behavioural test, variables such as active time (time in motion), total time in each of the three zones, total shuttles (when *L. macrochirus* exited one zone and entered another) and average velocity were analysed. Previous studies have used active time to quantify boldness in fishes [*e.g. G. aculeatus* (Bell, 2005) and zebrafish *Danio rerio* (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 fishes (Cachat *et al*., 2011*a*, *b*). To further quantify boldness and anxiety, the proportion of time spent in the centre 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 centre zone, divided by the test duration (Hamilton *et al.*, 2014). Thigmotaxis was defined as the 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 centre, middle and outer and thigmotaxis zones) and multiplied by 100. The proportion of total activity corrects for potential individual differences in activity (Bouwknecht & 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 B_L to standardize distance, velocity and acceleration measurements.

STATISTICAL ANALYSIS

To determine if elevated pCO_2 had an effect on lateralization, generalized linear mixed models (GLMMs) were performed, with appropriate error and distributions and link-functions (Quinn & Keough, 2002), where the L_R and L_A were the response variables, treatment (pre-exposure, exposure and post-exposure) was included as a fixed effect, and *L. macrochirus* 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 & Ware, 1982; Lindstrom & Bates, 1990). These GLMMs were fitted with linear mixed effects regression models (LMER) (glmer from the lme4 library in R; www.r-project.org; 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 (*i.e.* wall hugging) or boldness behaviours, GLMMs were again used. Similar to above, treatment was included as a fixed factor and *L. macrochirus* ID was included as a random effect. For shuttle variables (movement between zones), a GLMM using Penalized Quasi-Likelihood (PQL) [glmmPQL from the MASS library in R (Venables & 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 variables 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 *L. macrochirus* performance 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 exposure, and pre-exposure and post-exposure to specifically assess the potential for influence of the CO_2 exposure on rank order.

For all statistical tests, analyses were performed in R Studio (www.R-project.org), and differences were considered significant if α was $<0{\cdot}05$. *Lepomis macrochirus* L_T was initially included

TABLE II. Statistical outputs (value, S.E., d.f., *z*-value and *P* value) for each output (intercept, pre-exposure × exposure and pre-exposure × post-exposure) of generalized linear mixed effects regression models from R for relative lateralization (L_R) and absolute lateralization (L_A). L_R was analysed using a binomial distribution while L_A was analysed using a Poisson distribution. The intercept value represents the pre-exposure values. For pre-exposure *v*. exposure and pre-exposure *v*. post-exposure each parameter value represents the change in the model intercept associated with that response. Significant parameters are highlighted in bold

Response	Model	Parameter	Estimate	S.E.	d.f.	*z*-value	*P* value
L_R	Binomial GLMER	**Intercept**	**−0·687**	**0·183**	**30**	**−3·748**	**<0·001**
		Pre-exposure *v.* exposure	−0·431	0·25	30	−1·728	0·084
		Exposure *v.* post-exposure	−0·116	0·241	30	−0·482	0·63
L_A	Poisson GLMER	**Intercept**	**1·964**	**0·094**	**30**	**20·966**	**<0·001**
		Pre-exposure *v.* exposure	0·084	0·13	30	0·648	0·517
		Exposure *v.* post-exposure	0·017	0·132	30	0·132	0·895

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 II and Fig. 1(a)]. 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 II and Fig. 1(b)].

Following the initial acclimation period and exposure to the novel object, *L. macrochirus* explored the arena and approached the object. Many of the variables measured, however, were not significantly different after exposure to elevated pCO_2 or following the subsequent post-exposure (recovery) period (Table III and Figs 1–3). 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 III and Fig. 2). Similarly, the level of exploration (movement) 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 III and Fig. 3(a)–(c)]. The proportion of activity in the middle zone of the tank, and near the novel object, during the entire monitoring period also did not differ significantly across treatments (Table III). In addition, the average velocity of each *L. macrochirus* within each zone was not significantly different after exposure to elevated pCO_2 (Table III).

Lepomis macrochirus did display some altered behaviours after being exposed to elevated pCO_2. Compared to the pre-exposure period, average velocity significantly increased from $0{\cdot}23 \pm 0{\cdot}07$ to $0{\cdot}30 \pm 0{\cdot}10\, B_L\, s^{-1}$ (mean ± S.E.) after elevated pCO_2

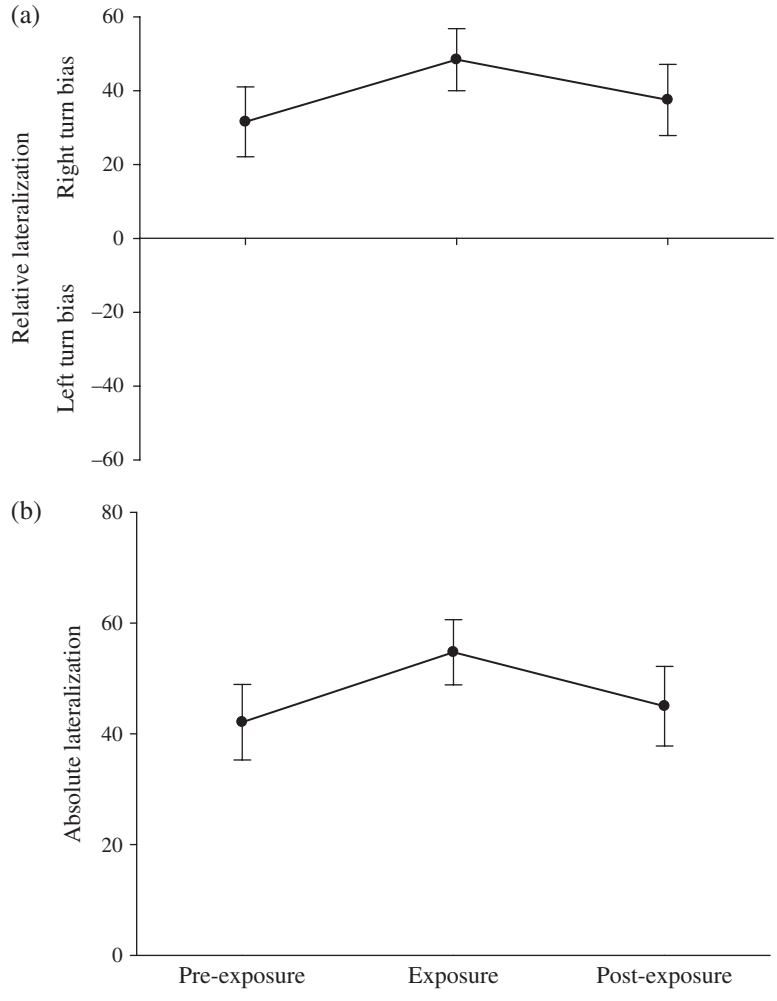

FIG. 1. Lateralization index for *Lepomis macrochirus* held at ambient pCO_2 (156 μatm, pre-exposure), at the end of a 5 day period at 8300 μatm (post-exposure), or after a return to ambient pCO_2 for 5 days (44 μatm, recovery). Individual *L. macrochirus* ($n = 15$) were tested in a double T-maze over the three treatment periods and each *L. macrochirus* 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 zero 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 ± S.E. Treatment groups were not significantly different from one another (see Table II).

exposure, and then decreased to $0{\cdot}24 \pm 0{\cdot}07\, B_L\, s^{-1}$ during the post-exposure period and was significantly different than the exposure period [Table III and Fig. 4(a)]. Within the thigmotaxis zone, the mean ± S.D. velocity during the pre-exposure period significantly increased from $0{\cdot}22 \pm 0{\cdot}09$ to $0{\cdot}30 \pm 0{\cdot}10\, B_L\, s^{-1}$ during the exposure period and decreased to $0{\cdot}24 \pm 0{\cdot}07\, B_L\, s^{-1}$ after *L. macrochirus* were moved to ambient conditions, although this change was not significantly different from the exposure period [Table III and Fig. 4(b)]. The proportion of time spent being active

TABLE III. Statistical outputs (value, S.E., d.f., *t*-value and *P*-value) for each output (intercept, pre-exposure *v*. exposure and exposure *v*. post-exposure) of generalized linear mixed effects models from R using penalized quasi-likelihood for each response. The intercept value represents the exposure values. For pre-exposure *v*. exposure and exposure *v*. post-exposure each parameter value represents the change in the model intercept associated with that response. Significant parameters are highlighted in bold

Response	Model	Parameter	Value	S.E.	d.f.	*t*-value	*P*-value
Proportion of time active during trial	Quasi GLM	Intercept	0·367	0·04	28	9·14	0
		Pre-exposure *v*. exposure	−0·071	0·049	28	−1·465	0·154
		Exposure *v*. post-exposure	−0·039	0·048	28	−0·799	0·4311
Proportion of time active in thigmotaxis zone during trial		Intercept	0·284	0·028	28	10·21	0
		Pre-exposure *v*. exposure	**−0·082**	**0·036**	28	**−2·278**	**0·0305**
		Exposure *v*. post-exposure	−0·024	0·036	28	−0·661	0·514
Proportion of time active in middle zone during trial		Intercept	0·055	0·024	28	2·282	0·03
		Pre-exposure *v*. exposure	0·041	0·032	28	1·295	0·206
		Exposure *v*. post-exposure	−0·015	0·032	28	−0·462	0·647
Proportion of time active by object during trial		Intercept	0·028	0·011	28	2·546	0·017
		Pre-exposure *v*. exposure	0	0·013	28	−0·38	0·707
		Exposure *v*. post-exposure	0	0·013	28	−0·016	0·988
Average velocity		Intercept	0·303	0·021	28	14·504	0
		Pre-exposure *v*. exposure	**−0·077**	**0·028**	**28**	**−2·776**	**0·01**
		Exposure *v*. post-exposure	**−0·059**	**0·028**	**28**	**−2·113**	**0·044**
Average velocity thigmotaxis zone		Intercept	0·301	0·023	28	13·144	0
		Pre-exposure *v*. exposure	**−0·084**	**0·032**	**28**	**−2·63**	**0·014**
		Exposure *v*. post-exposure	−0·058	0·032	28	−1·806	0·082
Average velocity middle zone		Intercept	0·253	0·041	28	6·221	0
		Pre-exposure *v*. exposure	−0·085	0·047	28	−1·803	0·082
		Exposure *v*. post-exposure	−0·072	0·047	28	−1·518	0·14
Average velocity by object		Intercept	0·214	0·049	28	4·4	0
		Pre-exposure *v*. exposure	−0·1	0·051	28	−1·95	0·061
		Exposure *v*. post-exposure	−0·063	0·051	28	−1·232	0·228

TABLE III. Continued

Response	Model	Parameter	Value	S.E.	d.f.	*t*-value	*P*-value
Total shuttles	Quasi Poisson GLMM	Intercept	2·593	0·276	28	9·409	0
		Pre-exposure *v.* exposure	−0·177	0·272	28	−0·652	0·52
		Exposure *v.* post-exposure	−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 *v.* exposure	−0·06	0·244	28	−0·246	0·808
		Exposure *v.* post-exposure	−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 *v.* exposure	−0·202	0·294	28	−0·685	0·499
		Exposure *v.* post-exposure	−0·363	0·308	28	−1·177	0·249
Total shuttles to object zone		Intercept	0·528	0·39	28	1·353	0·187
		Pre-exposure *v.* exposure	−0·405	0·323	28	−1·254	0·22
		Exposure *v.* post-exposure	−0·331	0·316	28	−1·047	0·304
Proportion of time spent thigmotaxis zone		Intercept	−0·222	0·078	28	−2·83	0·008
		Pre-exposure *v.* exposure	−0·076	0·102	28	−0·743	0·464
		Exposure *v.* post-exposure	0·074	0·098	28	0·753	0·458
Proportion of time spent middle zone		Intercept	−2·055	0·317	28	−6·475	0
		Pre-exposure *v.* exposure	0·449	0·345	28	1·301	0·204
		Exposure *v.* post-exposure	−0·525	0·44	28	−1·193	0·243
Proportion of time spent object zone		Intercept	−3·476	0·437	28	−7·951	0
		Pre-exposure *v.* exposure	−0·471	0·371	28	−1·269	0·215
		Exposure *v.* post-exposure	−0·064	0·331	28	−0·193	0·848

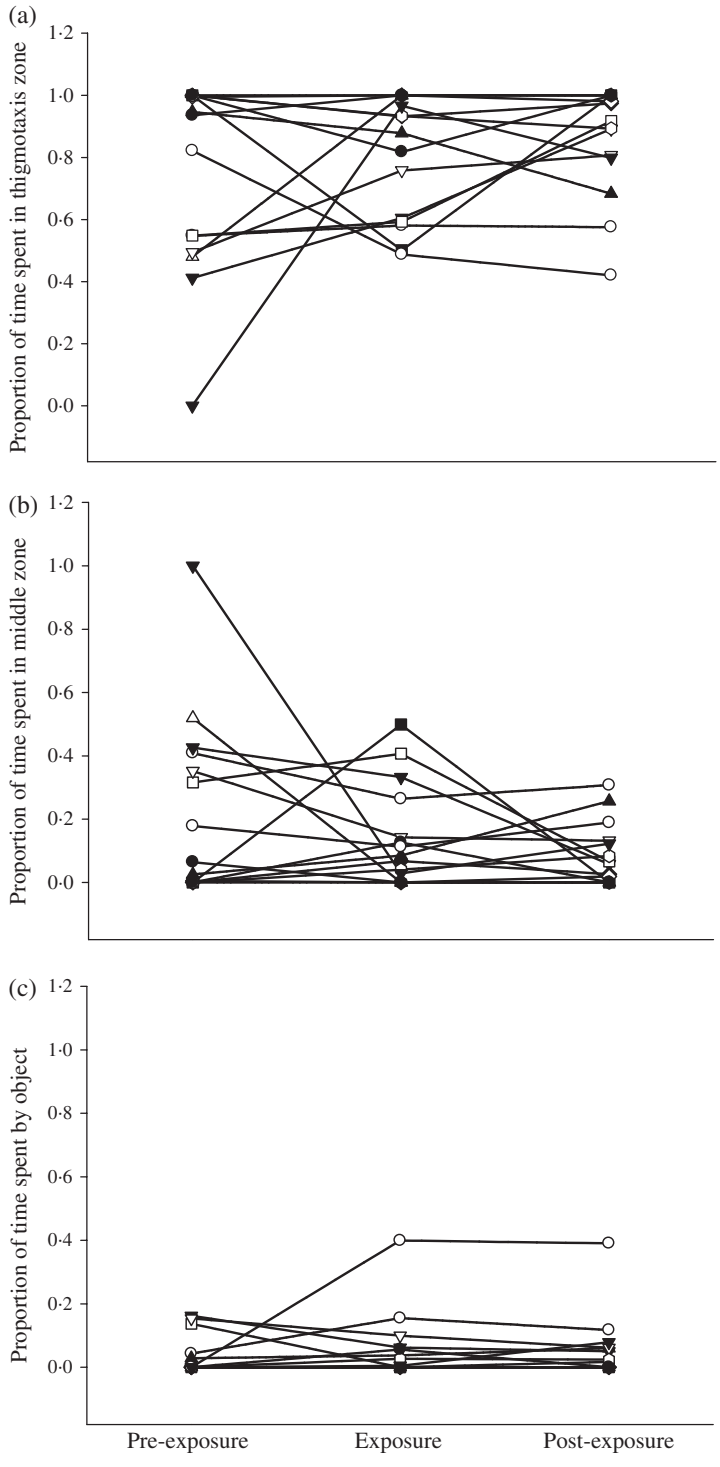

FIG. 2. Legend on next page.

in the thigmotaxis zone during the trial also increased from $0{\cdot}20 \pm 0{\cdot}10$ during the pre-exposure to $0{\cdot}28 \pm 0{\cdot}10$ s in the exposure period [Table III and Fig. 4(c)]. During the post-exposure period, time spent active in the thigmotaxis zone was $0{\cdot}26 \pm 0{\cdot}12$ s, which did not differ significantly from the exposure period [Table III and Fig. 4(c)].

For each behavioural metric, the performance of each individual across the three treatment periods was used to assess intra-individual variability across treatments. Rank orders for only three of the 17 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 post-exposure period (return to ambient conditions following elevated pCO_2 treatment), but not during the period of elevated CO_2 exposure (Table IV). In all but the average velocity near the object, however, the rank coefficients (r_s) were $<0{\cdot}50$, indicating that only a strong association was found for one of the three behavioural metrics.

DISCUSSION

Five days of exposure to elevated pCO_2 (*c.* 8300 μatm) resulted in minor changes to *L. macrochirus* behaviours. Specifically, three metrics out of the 17 assessed differed following exposure to elevated pCO_2, and included increases in average velocity in the whole arena, average velocity in the thigmotaxis zone, and the proportion of time active in the outer zone. These increases in velocity and activity may point to a shift to more active behaviours, which can indicate boldness (Sih *et al.*, 2004). Increased activity and potentially being bolder can result in fishes having greater risk of being consumed by predators and may shift their habitat-use (Coleman & Wilson, 1998). While the current study is one of the first to examine CO_2-induced behavioural changes for an obligate freshwater fish species (Hasler *et al.*, in press), previous work on marine, temperate and anadromous species have also suggested that exposure to fishes to elevated pCO_2, even for short durations, can alter behaviour. For example, Munday *et al.* (2010) found that post-settlement stage Ward's damsel *Pomacentrus wardi* Whitley 1927 exposed to 850 μatm CO_2 for 4 days displayed bolder and more active behaviour by venturing further away from a shelter. Similarly, several studies have shown that fishes 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 related to the function of the $GABA_A$ neural receptor which becomes 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 by dosing fish with gabazine (a $GABA_A$ receptor inhibitor) and it has shown to prevent the CO_2-induced changes in behaviour (Nilsson *et al.*, 2012; Lai *et al.*, 2015; Ou *et al.*, 2015).

FIG. 2. The proportion of time (a) spent in the thigmotaxis (outer) zone, (b) in the middle zone and (c) by the novel object for *Lepomis macrochirus* held at ambient pCO_2 (156 μatm, pre-exposure), at the end of a 5 day period at 8300 μatm (post-exposure) or after a return to ambient pCO_2 for 5 days (44 μatm, recovery). The figure shows responses for each individual ($n = 15$), with each individual *L. macrochirus* represented by a single line and a common symbol across all three treatments. No significant effects of treatment were detected (see Table III).

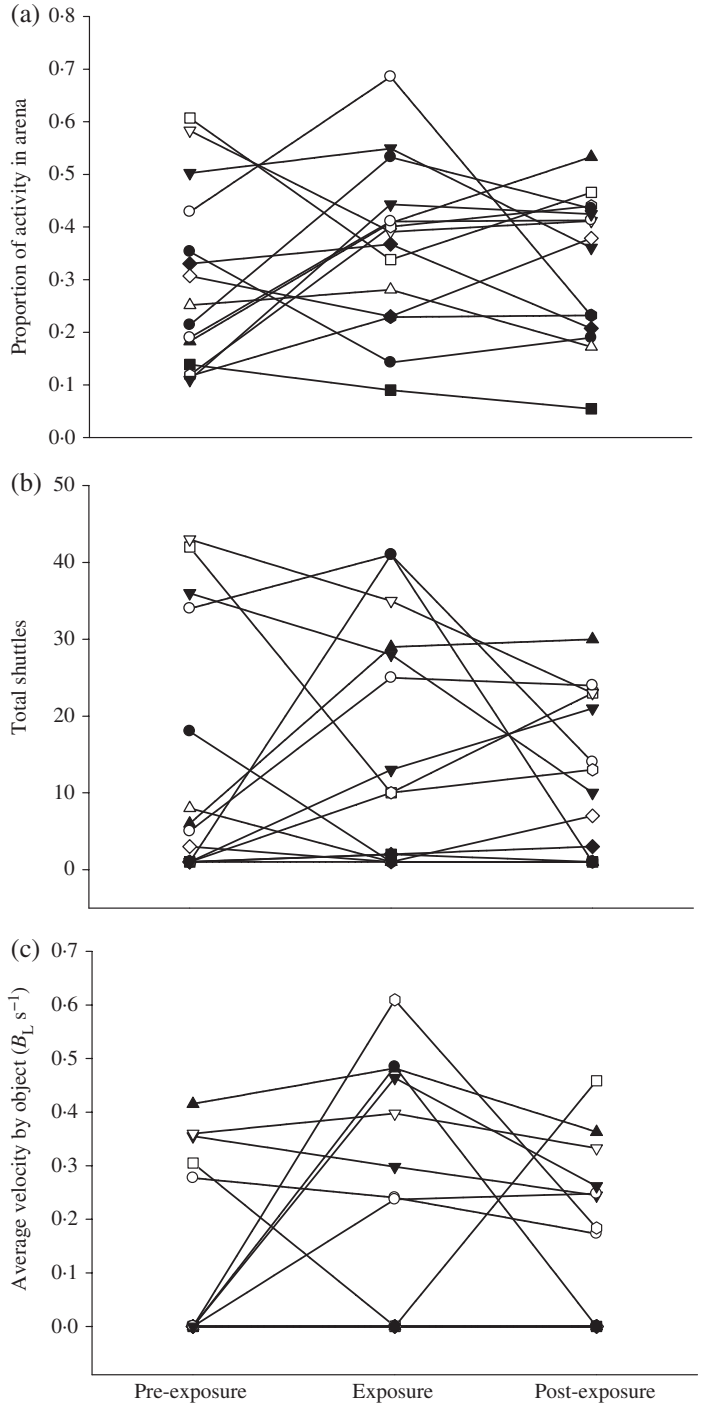

FIG. 3. Legend on next page.

Although the role of $GABA_A$ receptors as a mediator of CO_2-induced behavioural effects was not tested in the present study, 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 have consequences for fitness or survival in fishes exposed to high pCO_2.

Of the three behavioural metrics that changed following exposure to high pCO_2, only average velocity in the whole arena returned to similar levels observed during the initial test following 5 days of post-exposure at ambient pCO_2 (44 μatm). The other two metrics (average velocity and the proportion of time active in the outer zone) remained 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 sea water for 2 days (Munday *et al.*, 2010), and anxiety behaviours in juvenile splitnose rockfish *Sebastes diploproa* (Gilbert 1890) affected by CO_2 exposure returned to normal after 12 days in ambient conditions (Hamilton *et al.*, 2014), and movement patterns between CO_2-exposed and non-exposed adult largemouth bass *Micropterus salmoides* (Lacépède 1802) were no longer different after 11 days (Hasler *et al.*, in press) probably because previously disrupted cellular ion gradients re-established. In *L. macrochirus*, it is possible that additional recovery time at ambient conditions (*i.e.* beyond 5 days) may be necessary to reverse the physiological effects of CO_2 exposure, and that all measured behavioural metrics may return to pre-high CO_2 exposure levels following a longer post-exposure period.

Many of the behaviours monitored in this study did not change following 5 days 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. Previous studies using similar behavioural metrics have found high pCO_2 changes fish behaviour (detailed above), however, CO_2-mediated behavioural changes are not ubiquitous among all fishes (Heuer & Grosell, 2014). For example, L_R and activity in temperate wrasse *Ctenolabrus rupestris* (L. 1758) were unaffected after 28 days of exposure to 995 μatm pCO_2 (Sundin & Jutfelt, 2015). Similarly, juvenile Atlantic cod *Gadus morhua* L. 1758 displayed no change in L_R, L_A or emergence from a shelter (an indicator of boldness) despite exposure to 995 μatm pCO_2 for 28 days (Jutfelt & Hedgärde, 2015). In addition, swimming speed of larval cobia *Rachycentron canadum* (L. 1766) was unaltered after exposure to 2100 μatm for 3 weeks (Bignami,

FIG. 3. The (a) proportion of activity in the novel object arena, (b) total amount of shuttles between the thigmotaxis, middle and near the object zones and (c) average velocity (body lengths, $B_L\ s^{-1}$) in the novel object zone for *Lepomis macrochirus* held at ambient pCO_2 (156 μatm, pre-exposure), at the end of a 5 day period at 8300 μatm (post-exposure), or after a return to ambient pCO_2 for 5 days (44 μatm, recovery). The figure shows responses for each individual ($n = 15$), with each individual *L. macrochirus* represented by a single line and a common symbol across all three treatments. No significant effects of treatment were detected (see Table III).

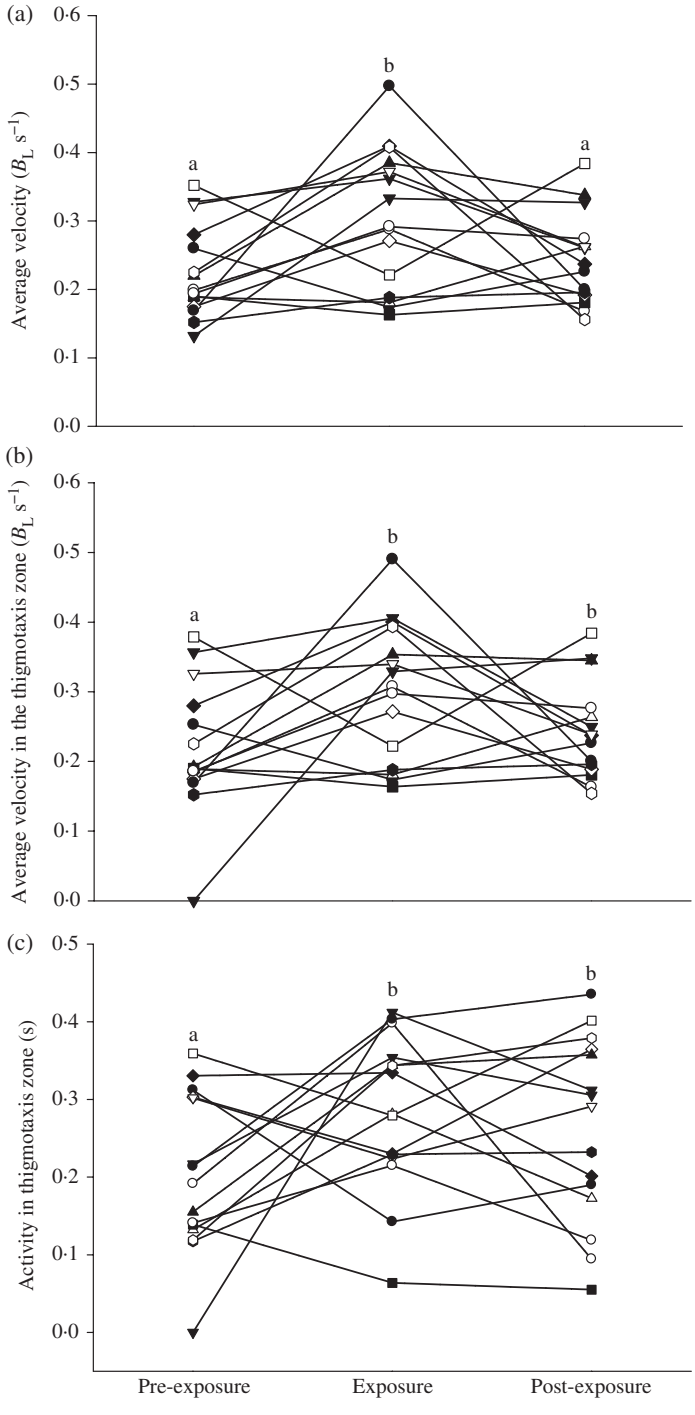

FIG. 4. Legend on next page.

2013). Though untested in this study, the mechanisms that control boldness, lateralization and swimming performance may be less controlled by CO_2 in *L. macrochirus* when compared to marine species. Potentially, *L. macrochirus* may acclimatize to changes in the environment (*i.e.* plastic response), or possibly have some behaviours affected following exposure to elevated pCO_2 (Näslund *et al.*, 2015). While the quantity and variability of CO_2 in sea water is well defined (Sabine *et al.*, 2004; Nilsson *et al.*, 2012; Hamilton *et al.*, 2014), CO_2 in freshwater systems is extremely variable (Cole *et al.*, 1994). As such, freshwater fishes have probably been exposed to fluctuations in pCO_2 over extended, evolutionary time periods, potentially making their behaviours more robust against CO_2-induced 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. *Lepomis macrochirus* may have also become accustomed to the arena and behavioural test masking behavioural changes due to repetition regardless of the novel object being changed. This was evident in previous work where the novel object was not changed after CO_2-exposed individuals were repeatedly tested and displayed no interest in the object during successive trials (Jutfelt *et al.*, 2013). In addition, the novel object used was not randomized across the three sampling periods, and this may represent a bias if *L. macrochirus* differentially reacted to the three objects used, though the authors are unsure of the influence that this potential design flaw may have had on the findings. 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 fishes 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 order in behaviours was only similar across treatments for three of the 17 metrics, and only when comparing the pre-exposure ranks to the post-exposure ranks. This indicates that, for most behaviours measured, exposure to elevated pCO_2 affected individuals in different ways, and *L. macrochirus* 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 similar during trials when *L. macrochirus* 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 thigmotaxis zone, as well as the average velocity in the whole arena 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 that were similar during ambient holding conditions but not during the high CO_2 exposure period were measures of velocity (*i.e.*

FIG. 4. (a) Average velocity (body lengths, $B_L\ s^{-1}$) in the novel object arena, (b) average velocity ($B_L\ s^{-1}$) in the thigmotaxis zone and (c) proportion of activity in the thigmotaxis zone for *Lepomis macrochirus* held at ambient pCO_2 (156 μatm, pre-exposure), at the end of a 5 day period at 8300 μatm (post-exposure), or after a return to ambient pCO_2 for 5 days (44 μatm, recovery). The figure shows responses for each individual ($n = 15$), with each individual *L. macrochirus* represented by a single line and a common symbol across all three treatments. Factors with different lower case letters represent significant differences (see Table III).

TABLE IV. Statistical outputs (r_s, F-ratio and P-value) for each output (pre-exposure *v*. exposure and pre-exposure *v*. post-exposure) of the Spearman's rank correlation for each behavioural metric

Response	Parameter	r_s	F-ratio	P value
L_R	Pre-exposure *v.* exposure	0·042	0·609	0·448
	Pre-exposure *v.* post-exposure	0·003	0·043	0·839
L_A	Pre-exposure *v.* exposure	0·025	0·354	0·562
	Pre-exposure *v.* post-exposure	0·017	0·246	0·627
Proportion of time active during trial	Pre-exposure *v.* exposure	0·1	0·131	0·723
	Pre-exposure *v.* post-exposure	0·009	0·122	0·732
Proportion of time active in thigmotaxis zone during trial	Pre-exposure *v.* exposure	0·032	0·428	0·524
	Pre-exposure *v.* post-exposure	0·033	0·446	0·516
Proportion of time active in middle zone during trial	Pre-exposure *v.* exposure	0·157	2·429	0·143
	Pre-exposure *v.* post-exposure	0·21	3·464	0·085
Proportion of time active by object during trial	Pre-exposure *v.* exposure	0·147	2·238	0·159
	Pre-exposure *v.* post-exposure	0·209	3·436	0·087
Average velocity	Pre-exposure *v.* exposure	0·026	0·345	0·567
	Pre-exposure *v.* post-exposure	0·046	0·626	0·443
Average velocity thigmotaxis zone	Pre-exposure *v.* exposure	0·016	0·206	0·657
	Pre-exposure *v.* post-exposure	0·051	0·693	0·42
Average velocity middle zone	Pre-exposure *v.* exposure	0·001	0·013	0·912
	Pre-exposure *v.* post-exposure	**0·281**	**5·068**	**0·042**
Average velocity by object	Pre-exposure *v.* exposure	0·092	1·31	0·273
	Pre-exposure *v.* post-exposure	**0·514**	**13·769**	**0·003**
Total shuttles	Pre-exposure *v.* exposure	0·07	0·974	0·342
	Pre-exposure *v.* post-exposure	0·165	2·567	0·133
Total shuttles to thigmotaxis zone	Pre-exposure *v.* exposure	0·128	1·917	0·19
	Pre-exposure *v.* post-exposure	0·053	0·723	0·411
Total shuttles to middle zone	Pre-exposure *v.* exposure	0·081	1·152	0·303
	Pre-exposure *v.* post-exposure	0·216	3·58	0·081
Total shuttles to object zone	Pre-exposure *v.* exposure	0·211	3·48	0·085
	Pre-exposure *v.* post-exposure	**0·371**	**7·664**	**0·016**
Proportion of time spent thigmotaxis zone	Pre-exposure *v.* exposure	0·009	0·119	0·735
	Pre-exposure *v.* post-exposure	0·218	3·619	0·079
Proportion of time spent middle zone	Pre-exposure *v.* exposure	0·004	0·054	0·82
	Pre-exposure *v.* post-exposure	0·154	2·369	0·148
Proportion of time spent object zone	Pre-exposure *v.* exposure	0·203	3·31	0·092
	Pre-exposure *v.* post-exposure	0·237	4·028	0·066

L_R, relative lateralization.

L_A, absolute lateralization.

laboratory measured locomotory performance), and these metrics potentially relate to field locomotory performance (Irschick, 2003; Irschick *et al.*, 2008). In wild freshwater fishes 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 fishes (Domenici & Blake, 1997). Studies should continue to account for intra-individual differences

in behavioural responses, and specifically performance among conspecifics, as these differences may mask important responses among individuals.

In the context of exposure to elevated pCO_2 for freshwater fishes, results of the present study have a number of implications for fisheries management and ecology. Freshwater fishes have potential to be exposed to elevated pCO_2 due to natural environmental variation (Hasler *et al.*, 2016), climate change (Phillips *et al.*, 2015), hatchery rearing (Colt & Orwicz, 1991) and zones of elevated pCO_2 deployed as fish barriers (Kates *et al.*, 2012; Noatch & Suski, 2012). If a freshwater species such as *L. macrochirus* is exposed to sufficiently high pCO_2 for extended periods of time, data from the current study indicate that lateralization and boldness may not be altered, but there may be an increase in activity and velocity of up to 30%, resulting in additional swimming activity and higher energetic costs (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 & Burger, 1985; Werner & Anholt, 1993). Should the CO_2 stimulus be removed, however, behaviours like velocity would probably return to normal. In addition, the limited amount of significant findings shown here contrasts with a large amount of research from the marine environment (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 in fresh water. Most importantly, mechanisms underlying possible acclimatization (and perhaps adaptation) of freshwater fishes to changes in pCO_2 needs to be further explored. Together, the current data set, coupled with additional studies related to mechanisms and consequences, can help better define the responses of freshwater fishes to a common environmental stimulus such as CO_2.

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