

Glucocorticoid and behavioral variation in relation to carbon dioxide avoidance across two experiments in freshwater teleost fishes

Emily K. Tucker  · Cory D. Suski · Madison A. Philipp · Jennifer D. Jeffrey  · Caleb T. Hasler 

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Abstract Organismal responses to stressors can be influenced by several internal and external factors including physiological condition and inherent behavioral type. Carbon dioxide (CO₂), a known stressor for fish, is naturally increasing in fresh water, and has been proposed as a non-physical barrier to prevent invasive fish movement. Intraspecific differences in how fish respond to CO₂ challenges have been noted, with some individuals responding at low partial pressures of CO₂ (*p*CO₂), and others responding at higher *p*CO₂. Sensitivity to *p*CO₂ may play a role in avoidance behaviors with respect to CO₂ barriers and may predict how fish respond to naturally occurring CO₂ challenges. We sought to determine the role that both physiological condition (i.e., elevated cortisol) and personality (i.e., boldness) play in influencing

behavioral responses. To accomplish this goal, a shuttle box assay was used to determine the *p*CO₂ that elicited avoidance in cortisol-injected or non-injected largemouth bass (*Micropterus salmoides*), as well as bold or shy bluegill (*Lepomis macrochirus*). Cortisol-injected largemouth bass shuttled at 45% higher *p*CO₂ than control fish, but personality of bluegill had no effect on shuttling. It appears that an individual's cortisol level can affect CO₂ avoidance, likely mediated through the effects of cortisol on acid–base balance at the gill, or through the effects of cortisol on coping styles. Our finding has important implications for how fish respond to either natural or anthropogenically-driven changes in CO₂, as stressed fish with high cortisol would appear to be more tolerant of elevated CO₂, independent of personality type.

Keywords Boldness · Cortisol · Environmental hypercarbia · Weak acidification

E. K. Tucker (✉)
Department of Animal Sciences, University of Illinois at Urbana-Champaign, 1207 W. Gregory Drive, Urbana, IL 61801, USA
e-mail: etucker3@illinois.edu

E. K. Tucker · C. D. Suski · M. A. Philipp · J. D. Jeffrey · C. T. Hasler
Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, 1102 S. Goodwin Avenue, Urbana, IL 61801, USA

Present Address:
C. T. Hasler
Department of Biology, University of Winnipeg, 515 Portage Avenue, Winnipeg, MB R3B 2E9, Canada

Introduction

The response elicited by an organism following an external challenge can vary based on a combination of extrinsic and intrinsic factors including behavior, physiology and the magnitude of the environmental challenge. For example, in teleost fish, the severity and the duration of a stressor typically correlates positively

with the size of the stress response (i.e., the amount of cortisol produced) (Winberg et al. 2016), and circulating cortisol concentrations can follow diel and seasonal patterns (Pickering and Pottinger 1983). In addition, there is genetically driven inter-individual variation in the magnitude of the stress response (Pottinger and Pickering 2015). Physiologically, individuals that exhibit a ‘fast pace of life’ syndrome [i.e., faster growth rates, higher metabolic scope, etc.; reviewed in (Réale et al. 2010)] show a reduced stress response relative to ‘slow’ individuals (Réale et al. 2010). Behaviorally, the magnitude of the stress response can be impacted by an individual’s behavioral ‘coping style’, whereby individuals that are ‘reactive’ increase cortisol levels to a greater extent in response to external stimuli, relative to ‘proactive’ individuals, that have a more muted stress response (Koolhaas et al. 2010). Thus, organismal responses to external challenges vary widely and are controlled by several extrinsic and intrinsic factors.

Carbon dioxide (CO₂) is a naturally-occurring substance present in the environment that, at high levels, has been shown to be a challenge for fish and can initiate a stress response (Shelford and Allee 1913). Partial pressures of CO₂ (*p*CO₂) in fresh water can vary greatly both spatially and temporally (Crawford et al. 2017), and are influenced by factors such as stream order, terrestrial productivity, aquatic respiration, overland flow, land-use, and underlying geology (Butman and Raymond 2011; Hasler et al. 2016a). Fish may also experience elevated *p*CO₂ upon encountering non-physical fish barriers (Kates et al. 2012; Noakes and Jones 2016; Treanor et al. 2017). Studies have shown that once a certain threshold of *p*CO₂ has been reached (approximately 50,000–75,000 μ atm (5.07–7.60 kPa)), fish will choose to voluntarily swim away from areas of elevated *p*CO₂, presumably to minimize costs related to inhabiting degraded water quality (e.g., upregulation of the stress axis, increased metabolic rate, etc.). Avoidance responses by fish to high *p*CO₂ have been demonstrated for several species, size classes, and environments (Kates et al. 2012; Dennis et al. 2015a, 2016). Despite the clear presence of this threshold to induce avoidance, there is considerable variation around this mean, likely driven in part by inter-individual variation in avoidance of CO₂ (Kates et al. 2012; Dennis III et al. 2015a, b, 2016; Hasler et al. 2017). To date, the roles that stress hormones or

personality may play in influencing the avoidance of fish to areas of high *p*CO₂ have not been defined.

Knowledge of how inter-individual differences may influence the behavioral response to high *p*CO₂ is critical information for managers and conservation practitioners seeking to design CO₂ barriers and understand fish responses to naturally occurring elevated *p*CO₂. For example, fish that are in a ‘stressed’ state (high cortisol) may have compromised ability to sense high *p*CO₂ (Schreck et al. 1997) and require a higher *p*CO₂ threshold to achieve avoidance relative to non-stressed fish (low cortisol). As a result, stressed fish may spend more time in naturally occurring high CO₂ environments, potentially leading to negative outcomes, such as increased mortality and behavioral changes (Tix et al. 2017a). With respect to personality, a ‘bold’ fish may ignore the potential risks of high *p*CO₂ for potential benefits, while a ‘shy’ fish may choose to avoid the new environment created by high *p*CO₂. Knowledge of how stress hormones and personality can influence the response of fish to high *p*CO₂ can therefore help managers predict if the effectiveness of a CO₂ barrier can fluctuate depending on the characteristics of a population, and may help provide a mechanism for the inter-individual variation in CO₂ avoidance seen previously (Kates et al. 2012; Dennis III et al. 2015a, 2016; Hasler et al. 2017). Similarly, how populations respond to naturally rising *p*CO₂ may also be predicted if stress and personality relate to responses to high *p*CO₂.

Based on this background, the goal of this study was to examine how physiology (i.e., cortisol) and behavior (i.e., boldness) mediate the response to elevated *p*CO₂. To accomplish this goal, we performed two separate but complementary experiments and compared CO₂ avoidance responses between fish with a) high cortisol versus low cortisol, and b) bold versus shy personality-types. In the first experiment, CO₂ avoidance was examined in largemouth bass (*Micropterus salmoides*) with plasma cortisol levels that were elevated exogenously. We hypothesized that largemouth bass with experimentally-elevated cortisol would adjust their response to CO₂ due to (1) the effects of cortisol on CO₂-sensing mechanisms, such that a reduced ability to sense CO₂ would lead to delayed CO₂ avoidance; (2) the potential for cortisol to increase H⁺ excretion at the gills, which would induce higher CO₂ tolerance; or, (3) a shift in behavioral coping styles from proactive to reactive in response to

cortisol. In a second experiment, CO₂ avoidance and tolerance were examined in bluegill (*Lepomis macrochirus*) following an assessment of their personality. We hypothesized that bold and shy bluegill might differ in their response to CO₂ due to differences in coping styles, with bold fish being more proactive and shy fish being more reactive. Results from the present study will not only help to define how cortisol and boldness influence responses to an environmental stimulus, but also will help to define individuals in a population that may be more (or less) susceptible to fish barriers generated by areas of high pCO₂.

Materials and methods

Experimental animals

Largemouth bass ($n = 63$ total length [TL] 225 ± 4 mm [mean \pm standard error of the mean, S.E.M.]) and bluegill ($n = 34$, 144 ± 14 mm) were transported from local fish hatcheries (largemouth bass: Keystone Hatcheries, Richmond, IL; bluegill: Logan Hollow Fish Farm, Murphysboro, IL) to the University of Illinois Aquatic Research Facility, Champaign-Urbana, Illinois. Largemouth bass were housed in an indoor facility in 1280 L plastic tanks supplied with water from a 0.04 ha earthen-bottom pond. Indoor tanks were also equipped with an ultraviolet water sterilizer (Vecton-6, American Aquarium Products, Grants Pass, OR, USA), canister filters to remove nitrogenous waste (FX6 High Performance, Fluval, Mansfield, MA, USA), and an aquarium chiller (Tank TK-1000, Teco, Ravenna, Italy) to maintain a constant temperature of 14.5 °C. Bluegill were housed outdoors in a single 1280 L plastic tank connected to a 0.04 ha, earthen-bottom pond. Water was continuously supplied to the outdoor tank from the pond and then drained back into the pond for oxygenation and removal of nitrogenous waste. Both the indoor and outdoor tanks received supplemental aeration from a blower and were covered with mesh netting to prevent escapement. Largemouth bass were fed pelleted food (Dense Culture Fish Food 5L, Pentair, Apopka, FL) and bluegill were fed frozen bloodworms (Brine Shrimp Direct, Ogden, UT) until satiation every day. Largemouth bass were held for 15 days and given a minimum of 48 h to recover after transport prior to starting experiments (Milligan 1996;

Suski et al. 2006). Bluegill were held for a maximum of 30 days prior to starting experiments. Water quality measurements in the holding tanks were taken daily and included temperature (largemouth bass: 14.5 ± 0.2 °C; bluegill: 20.5 ± 0.10 °C), dissolved oxygen (largemouth bass: 9.70 ± 0.10 mg/L; bluegill: 9.80 ± 0.04 mg/L; YSI, 550A Yellow Springs Instruments, Yellow Springs, OH), ammonia (largemouth bass: 0.89 ± 0.20 mg/L; bluegill: 0.08 ± 0.04 mg/L; Ammonia Nitrogen kit No. 3351-02, LaMotte Company, Chestertown, MD), and pH (largemouth bass: 8.07 ± 0.30 ; bluegill: 8.49 ± 0.10 ; WTW pH 3310 m with a SenTix 41 probe; Weilheim, Germany). Total alkalinity, water temperature, atmospheric air pressure, and pH were combined to calculate pCO₂ using CO₂Calc (Robbins et al. 2010). Atmospheric air pressure on each day was recorded from the National Weather Service Forecast Office Daily Climate Report (University of Illinois–Willard (KCMU), w2.weather.gov/climate). A modified infrared CO₂ probe (GMT221, 0–20%, Vaisala, Vantaa, Finland; (Johnson et al. 2009)) was used to validate pCO₂ values generated by CO₂Calc. Largemouth bass were tagged with injectable Passive Integrated Transponder (PIT) tags (10 mm [length] \times 2 mm [diameter], BioMark Tags, Boise, ID) after anesthetization with 250 mg/L Tricaine methanesulfonate (MS222, Western Chemical, Ferndale, WA). Bluegill were individually marked with a unique dorsal spine clip (Guy et al. 1996). All protocols were approved by the University of Illinois Institutional Animal Care and Use Committee (protocol #16126).

Cortisol injections and plasma cortisol measurements in largemouth bass

To quantify the influence of cortisol on the avoidance of elevated pCO₂, circulating cortisol levels in a subset of largemouth bass ($n = 31$, 153 ± 46 g) were elevated exogenously using a 5 mL/kg intraperitoneal injection of 10 mg/mL hydrocortisone 21-hemisuccinate (Sigma H4881; Sigma Aldrich, St. Louis, MO, USA) emulsified in melted cocoa butter, a technique that has been used previously in largemouth bass (O'Connor et al. 2010a; Redfern et al. 2017). Control largemouth bass ($n = 32$, 147 ± 45 g) were handled in a manner identical to cortisol-injected largemouth bass, but did not receive an injection. Because the

purpose of the study was to compare individuals with low (i.e., control) versus high cortisol levels on CO₂ avoidance, a sham-injected group (i.e., non-cortisol containing cocoa butter implant) was not used, as this may have raised cortisol levels unintentionally in that group (Redfern et al. 2017). Furthermore, previous studies have shown that sham-injected fish tend to have intermediate cortisol levels between control fish and cortisol-injected fish, so a sham group is often not included when assessing artificially elevated cortisol levels (O'Connor et al. 2009, 2010, 2011, 2013; Dey et al. 2010). The two treatment groups did not differ in size (*t* test comparing TL, $t_{58} = 0.37$, $p = 0.71$). Both the cortisol-injected largemouth bass and control largemouth bass were kept in two separate holding tanks after injections to prevent control fish from being exposed to cortisol excretions from treated fish (Vermeirssen and Scott 1996). Experimental assays commenced 4 days after fish were injected with cortisol to allow time for fish to recover from handling and for the cortisol treatment to manifest in the injected fish (Wang et al. 2005).

To quantify plasma cortisol concentrations in both cortisol-injected and control largemouth bass without causing further stress to fish used in the avoidance test, a second subset of largemouth bass (control: $n = 17$, 149 ± 11 g; cortisol-injected: $n = 16$, 136 ± 10 g) were used exclusively for monitoring the dynamics of cortisol in plasma, and did not undergo the CO₂ avoidance test. Fish were handled and held as described above. The two treatment groups did not differ in total length (*t*-test comparing TL, $t_{32} = 1.53$, $p = 0.14$). For 5 days following the initial treatment, 3–5 fish from each treatment group were randomly sampled to quantify plasma cortisol concentrations. Individual largemouth bass were quickly dip-netted and terminally anaesthetized in a solution of MS222 (250 mg/L, Western Chemical, Ferndale, WA). Approximately 1–2 mL of blood was drawn from the caudal vessel of the fish with a heparinized syringe and 23-gauge needle within 3 min of dip-netting (Lawrence et al. 2018), and immediately centrifuged at 6000 RPM for 120 s to extract plasma. Plasma samples were flash frozen in liquid nitrogen and stored at -80 °C. Plasma cortisol concentrations were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY), previously validated for use in fishes (Sink et al. 2008).

Behavioral assessments in bluegill

To define the role of personality on the avoidance of high $p\text{CO}_2$, a standardized novel object test in a closed arena, used previously for behavioral assessments in fishes, was employed (Ou et al. 2015). Bluegill were used in this experiment because this species exhibits a repeatable boldness behavioral syndrome (Wilson and Godin 2009). For the behavioral assay, an opaque, non-reflective plastic arena (61 cm diameter) was filled with 10 cm of water taken from the bluegill holding tank. A novel object (3 cm Rubik's cube) was glued to the center of the arena and covered with an opaque PVC cap connected to clear fishing line, which allowed for the removal of the PVC cap during the trial. The arena was surrounded with a tall black curtain to prevent external disturbance during the trial, and an overhead-mounted video camera (iDS uEye 1480-C camera, iDS, Obersulm, Germany) was used to monitor fish position. An ultra violet light array was placed under the arena to allow for the use of automated fish tracking software (see methods below). Prior to the start of each trial, a bluegill was carefully netted from the common holding tank, identified using the unique spine clip pattern, and gently placed in the arena with the covered novel object. Each fish was given 30 min to acclimate to the arena, as fish reduced exploratory behaviors after this amount of time in pre-trials, and 30 min is similar to acclimation times used in other studies (Kates et al. 2012; Tix et al. 2017b). After the acclimation time, the novel object was revealed to the fish by lifting the cap using the fishing line, and video of fish position and activity was collected for an additional 10 min. After each trial, the fish was collected from the arena, measured to the nearest mm (TL). Water in the arena was drained and replaced between each trial.

To quantify fish behavior during the behavioral tests described above, tracking software [Lolitrak, Loligo Systems; www.loligosystems.com; (Poulsen et al. 2014)] was used. The arena was divided into three equal-sized, concentric zones at least equal to or larger than the body length (BL) of bluegill used in the trials (Schnörr et al. 2012). The three zones consisted of zone 1 (outer or near the wall; useful for determining “thigmotaxis” or “wall hugging”), zone 2 (middle), and zone 3 (center or near the object) (Ou et al. 2015). Maximum velocity (BL/second), activity (the proportion of time spent moving), and distance

travelled in each zone (BL) were calculated. Fish were considered to be ‘bold’ if they spent more time in zone 3 near the novel object, while ‘shy’ fish spent more time in zone 1 (Jutfelt et al. 2013; Ou et al. 2015).

Acute hypercarbia avoidance

To quantify the level at which both largemouth bass and bluegill avoided the zone of elevated $p\text{CO}_2$, a “shuttle box” (Loligo Inco, Hobro, Denmark) assessment was used (Kates et al. 2012). The shuttle box consisted of two circular tanks (1.5 m diameter, 0.5 m deep) connected by a narrow tunnel (20 cm wide, 0.5 m deep) filled with water to a depth of 20 cm. The entire arena was surrounded by a dark curtain to prevent external visual stimuli, and an overhead video camera (iDS uEye 1480-C camera, iDS, Obersulm, Germany) was used to remotely monitor fish position in the arena. To start this assay, fish (either cortisol-injected largemouth bass, control largemouth bass, or bluegill that had received behavioral testing) were carefully netted from their holding tank, identified using either the PIT tag or unique spine clip, and placed in one side of the shuttle box, with the side determined by a coin flip. Preliminary trials indicated that an acclimation time of 30 min was sufficient for fish to reduce exploratory movements and become stationary on either side of the shuttle box. After this acclimation period, the external chamber connected to the side of the shuttle box where the fish resided started receiving a continuous flow of water injected with CO_2 gas, while the opposite chamber was continuously supplied with water injected with compressed air. The resulting effect was a continuous increase in $p\text{CO}_2$ in the side of the shuttle box holding the fish, while the opposite tank remained at ambient $p\text{CO}_2$ (see Kates et al. 2012). The flow of CO_2 -rich water was continued until the fish moved (i.e., “shuttled”) to the other side of the arena. At the point when the fish shuttled, which ranged between 0 min 27 s and 11 min 26 min and averaged 3 min 4 s, the pH in the side of the shuttle box receiving CO_2 was noted using a pH probe (pH 150 Meter, Oakton Instruments, Vernon Hills, IL), and temperature and alkalinity measurements were also collected as described above to allow for the calculation of $p\text{CO}_2$ using CO_2Calc . Each fish was allowed to shuttle only once to avoid potential influences from learning or habituation (Clingerman et al. 2007), and the shuttle box was

drained and re-filled with fresh water after each trial. At the end of the avoidance assay, largemouth bass were euthanized by MS222 and sex was determined by gonadal inspection.

Acute hypercarbia tolerance

Following the hypercarbia avoidance assay, bluegill were also assessed for CO_2 tolerance using an acute hypercarbia challenge (Hasler et al. 2017) administered a minimum of 24 h after the shuttle box trial. For this, $p\text{CO}_2$ pond water in a small experimental tank ($0.36 \times 0.35 \times 0.66$ m) was raised to a level of approximately 123,000 μatm (12.46 kPa) using an air stone connected to a compressed CO_2 tank, verified with the infrared CO_2 probe. Individual bluegill were carefully netted from the common holding tank, identified using their unique spine clip, and quickly added to the experimental tank where they were monitored continuously. When the bluegill lost equilibrium (i.e., loss of motor function and turned upside down) and remained that way for at least 3 s, the time was recorded and the fish was put into ambient water supplied with aeration. The time taken for the fish to regain equilibrium and respond positively to a tail pinch was also recorded. At the end of the tolerance assay, bluegill were euthanized by decapitation and sex was determined by gonadal inspection.

Statistical analyses

Plasma cortisol concentrations for largemouth bass were compared using a two-way analysis of variance (ANOVA) using treatment (control or cortisol-injected), day of monitoring, and the interaction of treatment and day as main effects. Differences across treatment groups were determined using a Tukey’s post hoc test (Whitlock and Schluter 2015).

For behavioral assays with bluegill, highly correlated behavioral variables (i.e., velocity in zones 1 and 3) were removed using the Kaiser–Meyer–Olkin (KMO) test until a KMO value greater than 0.6 was obtained, as this suggests suitability for factor analysis (Budaev 2010). Independence of the remaining variables was validated with Bartlett’s Test for sphericity (Fouladi and Steiger 1993). With the uncorrelated dataset, a principal components analysis (PCA) was used to transform the correlation matrix into a series of linear combinations of variables. Components with an

eigenvalue greater than 1 were extracted and rotated using a varimax rotation (Grossman et al. 1991). After rotation, factors with loadings greater than or equal to an absolute value of 0.5 were considered to be primary factors for the associated principal component (PC) (Budaev 2010). Behavioral scores for each individual fish were calculated based on the rotated loadings. The $p\text{CO}_2$ of the water at the time of shuttling, time to loss of equilibrium during acute tolerance challenge, and subsequent time to regain equilibrium were analyzed with respect to PC behavioral scores for bluegill using multiple linear regression analyses. Total length was initially included as a co-variate but removed from all models when no effect was found (Engqvist 2005).

For the hypercarbia avoidance assay using cortisol-injected and control largemouth bass, the $p\text{CO}_2$ of the water that the fish was in when it shuttled was compared between treatment groups using a two-sample t -test. Sex and total length were initially included as co-variables in a general linearized model, but were removed because no significant effect was found (Engqvist 2005).

Prior to completing statistical tests, all data were assessed for normality using a Shapiro–Wilk test, and non-normal variables ($p < 0.05$) were normalized using the Box–Cox procedure (Box and Cox 1964). Models generated from ANOVAs and linear regression analyses were assessed for appropriate fit using residual by predicted plots, and Quantile–Quantile plots (Zurr et al. 2010). For all statistical tests, the null hypothesis was rejected if the p -value was less than 0.05. Results are presented as mean \pm S.E.M. unless otherwise noted. All analyses were performed in R 3.2.3 (R Core Team 2017), with KMO tests conducted using the “psych” package (Revelle 2017), and all other statistical tests were completed using the R base package. Data were visualized using “ggplot2” (Wickham 2009).

Results

Impact of cortisol-treatment on CO_2 avoidance in largemouth bass

Plasma cortisol concentrations of cortisol-injected largemouth bass were significantly higher than control fish and did not vary over the 5-day monitoring period (Fig. 1, Table 2). Largemouth bass that received a

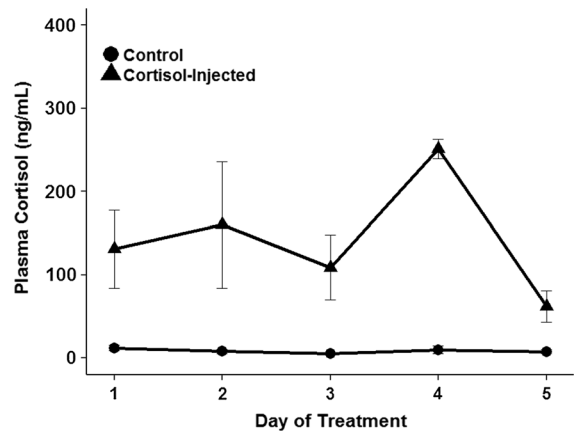


Fig. 1 Plasma cortisol concentrations (ng/mL) for control and cortisol-injected largemouth bass (*Micropterus salmoides*) over a 5-day period. Injections were given on day 0, and plasma cortisol was measured on days 1–5. Data are presented as means \pm SEM ($n = 3$ –5 largemouth bass per sampling time). Cortisol-injected fish had a significantly higher concentration of cortisol (143 ± 23 ng/mL) relative to control fish (8.49 ± 1.26 ng/mL; $p < 0.001$). The effect of day was not statistically significant ($p = 0.0516$) and the interaction effect between day and cortisol treatment was not statistically significant ($p = 0.2530$)

cortisol injection shuttled at $p\text{CO}_2$ 45% higher than the control group (two-sample t -test, $t = -3.34$, $df = 15.46$, $p < 0.01$, Fig. 2).

Impact of personality on CO_2 avoidance and tolerance in bluegill

Values from the nine parameters measured during the novel object assay are listed in Table 1. After removal of correlated variables with a KMO test, seven variables, including maximum velocity in zone 2, proportion of trial spent moving in all three zones, and distance travelled in all three zones remained, with a KMO value of 0.63. These remaining seven variables were factorable (Bartlett’s Test for sphericity; $X^2 = 145.3$; $df = 421$; $p < 0.0001$).

Two factors with eigenvalues > 1 were subsequently generated and these factors explained 74% of the total variance in behavior (Table 2). PC1 explained 50% of the total variation and was associated with the proportion of the trial spent moving and distance traveled in zone 1 (i.e., zone nearest the wall). PC2 was characterized by the proportion of trial spent moving and the distance traveled in zone 3 (i.e., zone closest to the novel object), and explained 24% of the total variance. Therefore, high PC1 scores were

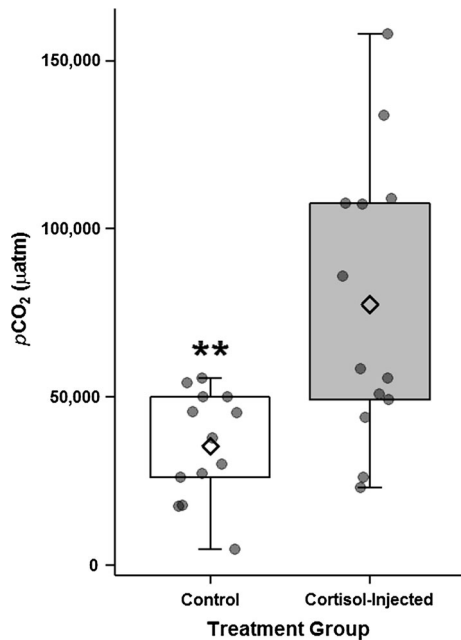


Fig. 2 Comparison of $p\text{CO}_2$ at time of shuttling for cortisol-injected and control largemouth bass (*Micropterus salmoides*). Data are presented as means \pm SEM ($n = 13$). **Indicates a statistically significant difference between treatments (t -test, see text for details). The diamond represents the mean and the dots represent data for an individual fish

Table 2 Principal component analysis (PCA) results from novel object assay of Bluegill (*Lepomis macrochirus*) following varimax rotation

Variable	PC1	PC2	Communalities
Activity Zone 1	- 0.587	0.101	0.355
Distance Zone 1	- 0.555		0.308
Velocity Zone 2	- 0.458	- 0.105	0.221
Activity Zone 2	- 0.238	- 0.266	0.127
Distance Zone 2	- 0.182	- 0.412	0.433
Activity Zone 3	0.218	- 0.621	0.433
Distance Zone 3		- 0.593	0.352
Variance explained	49.62	24.33	
Eigen value	3.47	1.70	

Maximum velocity, activity (proportion of trial spent moving), and distance travelled by individual fish were analyzed in three zones relative to a novel object (Zone 1: Outermost zone; Zone 2: Middle zone; Zone 3: Innermost Zone). Highly correlated variables were removed by a Kaiser–Meyer–Olkin (KMO) test until the overall KMO value was > 0.6 . The remaining seven variables were analyzed by PCA, and PCs with eigenvalues > 1 (PC1 and PC2) were rotated by varimax rotation ($n = 34$). Bolded PC values indicate significant loadings

Table 1 Maximum velocity, activity (proportion of trial spent moving), and distance traveled in each of 3 zones in a novel object assay in bluegill

	Maximum velocity (BL/second)			Activity (percent of time spent moving)			Distance traveled (BL)		
	Zone 1	Zone 2	Zone 3	Zone 1	Zone 2	Zone 3	Zone 1	Zone 2	Zone 3
Mean	0.015	0.020	0.03	21.44	3.23	0.55	1.98	0.32	0.05
SEM	0.002	0.002	0.01	3.54	0.68	0.12	0.43	0.08	0.01
Range	0.010–0.070	0.00–0.060	0.00–0.01	1.88–84.05	0.00–21.06	0.00–2.76	0.05–10.87	0.00–1.95	0.00–0.24

Bluegill (*Lepomis macrochirus*) were monitored over a 10 min period where zone 1 was the area closest to the wall of the behavioral arena, zone 2 was the middle, and zone 3 was the area closest to the novel object. Values were measured in pixels with Lolitrak tracking software and converted to body lengths (BL) for each fish (34.7 pixels/mm, average body length 144 ± 4 mm)

associated with high activity and large distance covered in the zone farthest from the novel object, or the “thigmotaxis” zone. High PC2 scores were associated with high activity and low distance covered in the zone closest to the novel object.

Behavioral PC scores were not associated with $p\text{CO}_2$ at the time of shuttling in bluegill ($66,902 \pm 8983 \mu\text{atm}$ (6.78 ± 0.91 kPa), Table 3). Behavioral PC scores were also not associated with

time to equilibrium loss (198 ± 13 s), or time to regain equilibrium for bluegill (82 ± 6 s, Table 3).

Discussion

Cortisol-injected largemouth bass required higher $p\text{CO}_2$ than the control treatment before shuttling occurred, suggesting that ‘stressed’ largemouth bass

Table 3 Linear regression models of bluegill behavioral principal components from the novel object assay in relation to the acute hypercarbia avoidance assay and acute hypercarbia tolerance assay ($n = 34$)

Response variable	Estimate	Standard error	<i>t</i> statistic	<i>p</i> -value
<i>p</i> CO ₂ at time of shuttle				
Intercept	3.82	0.18	20.72	< 0.001***
PC1	0.03	0.19	0.18	0.858
PC2	- 0.06	0.19	- 0.35	0.732
Time to equilibrium loss				
Intercept	5.23	0.06	88.19	< 0.001***
PC1	0.07	0.06	1.10	0.280
PC2	0.06	0.06	1.07	0.292
Time to recovery				
Intercept	4.34	0.07	65.85	< 0.001***
PC1	0.03	0.07	0.52	0.612
PC2	- 0.03	0.07	- 0.431	0.670

PC1 was associated with activity and distance in zone 3 (closest to the novel object) and PC2 was associated with activity and distance in zone 1 (farthest from the object). None of the principal component scores significantly predicted any of the 3 response variables

were less avoidant of CO₂. The exogenous cortisol treatment elevated plasma cortisol concentrations to levels measured previously in largemouth bass following acute stressors such as angling, exercise, and air exposure (Suski et al. 2003; Gingerich et al. 2010). Furthermore, cortisol levels remained elevated for 5 days following treatment, effectively mimicking the cortisol-dependent physiological effects of chronic stress in teleost fishes (Wendelaar Bonga 1997; Algera et al. 2017). Because the rate and extent of the increase in cortisol that is induced by stress is highly variable between individuals, exogenous cortisol elevation bypasses the initial inter-individual differences in sensitivity to external stimuli and allows for examination of elevated cortisol directly on physiology, behavior, and fitness (Crossin et al. 2016; O'Connor et al. 2010b; Sopinka et al. 2015; Redfern et al. 2017). While stress can have varying impacts on individual behavior depending on the context, by isolating the effects of cortisol we have shown that cortisol levels have a direct impact on CO₂ avoidance.

There are several potential mechanisms by which elevations in cortisol could translate into reduced avoidance of hypercarbia for largemouth bass. For example, upon detection of increased *p*CO₂, physiological compensation typically involves hydration of CO₂ by carbonic anhydrase, followed by exchange of

H⁺ for Na⁺ via the gills using an apical membrane Na⁺/H⁺ exchanger in conjunction with a Na⁺/K⁺-ATPase (Perry and Gilmour 2006). This mechanism is important for counteracting Na⁺ loss at the gills due to increased paracellular epithelial permeability in water at a low pH (Evans et al. 2005). Studies in zebrafish have revealed that H⁺ secretion from the gills during metabolic compensation is coupled with Na⁺ retention, which is mediated by cortisol (Kwong and Perry 2013). It is thought that cortisol acts directly on epithelial tight-junction proteins in the gill to reduce paracellular permeability and thus reduce Na⁺ loss (Chasiotis et al. 2010; Kwong and Perry 2013). Cortisol has also been found to promote the proliferation of Na⁺/K⁺-ATPase rich cells in the gill within 14 h, which increases the rate of K⁺ secretion (Cruz et al. 2013). Largemouth bass in the current study received behavioral testing following 5 days of cortisol elevation, meaning that one potential mechanism for the decreased avoidance of hypercarbia in cortisol-injected largemouth bass could be an increased propensity for H⁺ excretion, thereby maintaining acid/base balance despite CO₂ exposure, allowing cortisol-treated fish to shuttle at higher *p*CO₂ than control fish. A second potential mechanism by which stress could reduce avoidance of CO₂ is the interaction between cortisol and coping styles. Coping style refers

to the behavioral and physiological response of an individual to stress, and can be classified as either reactive or proactive (Øverli et al. 2007). Reactive individuals have been found to have higher hypothalamic-pituitary-interrenal (HPI) axis reactivity than proactive individuals, leading to higher post-stress cortisol levels after an environmental challenge (Koolhaas et al. 1999). Proactive fish are also more likely to ‘freeze’ in response to stress, as opposed to reactive fish which are predisposed to engage in ‘fight or flight’ responses (Vindas et al. 2017). Previous work has also shown that animals can transition between coping styles based on internal energy reserves and environmental conditions (Sih et al. 2015). It is therefore plausible that experimentally elevating cortisol levels leads to a shift towards a more reactive coping style, resulting in “freezing” behavior in cortisol-injected fish when experiencing an environmental stressor, meaning largemouth bass spent longer in the high $p\text{CO}_2$ side of the shuttle box before shuttling. Although the mechanistic underpinnings of the decreased CO_2 avoidance in cortisol-injected largemouth bass were not assessed in the present study, we suggest these as considerations for future studies examining CO_2 avoidance. Together, largemouth bass experiencing stress due to cortisol injections showed reduced avoidance of environmental hypercarbia, evidenced by an increase in $p\text{CO}_2$ needed to induce avoidance behavior, relative to control individuals.

By measuring the behavioral tendencies of bluegill using the novel object assay, we sought to define an interaction of CO_2 avoidance and personality. The PCA of behavioral metrics from the novel object assay using BG identified two PCs that summarized BG behavioral types-PC1 was related to increased activity and distance travelled in the zone closest to the wall and furthest from the novel object (i.e., zone 1) and PC2 was related to increased activity and decreased distance travelled in the zone nearest the novel object (i.e., zone 3). Previous studies suggest that fish that spend more time near the novel object are considered ‘bold’ and fish that remain on the perimeter of the arena (i.e., display thigmotaxis behavior) are considered ‘shy’ (Jutfelt et al. 2013; Ou et al. 2015). Accordingly, in the present study, BG were considered ‘bold’ if they tended to spend more time investigating the novel object (i.e. more time in Zone 3) and thus had high PC2 scores (and low PC1 scores), while fish that

tended to spend more time in the thigmotaxis zone (i.e. zone 1) and thus had high PC1 scores (and low PC2 scores) were considered “shy”. Previous work relating elevated $p\text{CO}_2$ to behavior has sought to define how behavior was *influenced by* hypercarbia, and results from this work have shown high variability [i.e., (Midway et al. 2017; Jutfelt et al. 2013; Hasler et al. 2016b; Tix et al. 2017a, b; Sundin et al. 2017)]. With respect to personality, the effects of elevated $p\text{CO}_2$ on boldness and novel object investigation has also varied across species, with some fish showing increased anxiety after CO_2 exposure, and other species showing reduced anxiety (Jutfelt et al. 2013; Munday et al. 2013; Hamilton et al. 2013; Ou et al. 2015; Tix et al. 2017b). CO_2 avoidance behavior could potentially differ across personality types due to differences in coping styles. For example, ‘bold’ individuals are less responsive to external stressors (Koolhaas et al. 2010), which could result in ‘bold’ individuals remaining in elevated $p\text{CO}_2$ longer than ‘shy’ individuals. However, the fact that none of our behavioral scores influenced avoidance of CO_2 indicates that boldness does not influence avoidance of high $p\text{CO}_2$. It is plausible, therefore, that inter-individual differences in CO_2 avoidance are primarily driven by physiological differences rather than personality differences. Fish sense CO_2 through receptors on the gill as well as through olfaction (Perry and Gilmour 2002), suggesting that factors that influence detection of CO_2 are likely to have a more significant influence on CO_2 avoidance than are variations in personality. Though CO_2 exposure influences fish behavior, inherent boldness does not seem to influence hypercarbia avoidance in bluegill.

Time to equilibrium loss was not associated with PC scores in bluegill, indicating that boldness did not affect tolerance to CO_2 . Fish lose equilibrium in high $p\text{CO}_2$ environments due to reduced brain pH caused by increased dissolved CO_2 crossing the blood–brain barrier, resulting in anesthesia (Mitsuda et al. 1980; Hiromasa et al. 1994). Equilibrium loss in high $p\text{CO}_2$ is also a repeatable trait in largemouth bass (Hasler et al. 2017), suggesting that individual differences promote a consistent mechanism contributing to CO_2 tolerance. Behavior and physiology are linked (Roche et al. 2016), and previous work has shown that animals with certain personality syndromes also show differences in physiology (Costantini et al. 2008; Careau et al. 2008; Careau and Garland 2012). We predicted

that the link between physiology and personality could manifest itself through differences in coping styles and metabolism. For example, a ‘bold’, ‘proactive’ fish may have a higher metabolism than a ‘shy’ ‘reactive’ fish (Koolhaas et al. 2010), which could theoretically increase the rate of CO₂ passage to the brain (Metcalf et al. 2016), and therefore reduce equilibrium loss time. However, Hasler et al. (2017) found that metabolic rate was not related to equilibrium loss in largemouth bass, suggesting that metabolic rate, as it relates to personality type or coping style, will likely not influence equilibrium loss time. Together, tolerance to elevated carbon dioxide, evidenced by equilibrium loss time, is likely due to factors other than personality.

Results from our study have implications not only for the relationship between physiology and behavior in fishes, but also for managers working to develop non-physical CO₂ deterrent systems. CO₂ as a management tool is primarily proposed to block the movement of invasive species such as bigheaded carp (Cupp et al. 2017a, b; Donaldson et al. 2016), sea lamprey (Dennis et al. 2016), and round goby (Cupp et al. 2017b). Previous studies have demonstrated that teleost fish of different species do not vary significantly in their avoidance of CO₂ (Kates et al. 2012; Donaldson et al. 2016), allowing largemouth bass and bluegill to serve as suitable proxies for invasive fishes in a controlled laboratory environment. However, there is wide variation among individuals within the same species (i.e., round gobies and bigheaded carps) (Kates et al. 2012; Dennis et al. 2015a, b, 2016; Cupp et al. 2017b). Our results suggest that the high level of inter-individual variation in CO₂ avoidance found in previous studies may have been driven, at least in part, by inter-individual variation in circulating cortisol levels. In fact, cortisol-treatment appeared to increase the inter-individual variation in CO₂ avoidance in our study, as shown in Fig. 2. Thus, managers should consider the stress status of target invasive fishes when predicting behavioral responses to a CO₂ barrier, as individuals in a ‘stressed’ state (i.e., high cortisol levels) may require higher *p*CO₂ levels to induce deterrence relative to ‘unstressed’ individuals (i.e., low cortisol levels). It is also likely that the effects of cortisol on CO₂ avoidance could be compounded or mitigated by contexts related to the natural environment that are not present in a controlled laboratory setting (Killen et al. 2013). Therefore, the potential

effects of migratory behavior, predator–prey interactions, and other community- and population-level processes on CO₂ avoidance require further study. Stress in wild fish can occur due to a variety of reasons including high temperatures, low dissolved oxygen, or pollution (Birnir-Gauvin et al. 2017), potentially making a non-physical barrier less effective and more prone to being breached by invasive fishes in conditions such as highly contaminated water or elevated temperature. Should deterrent systems be needed in multiple water bodies, and given that fish in some landscapes are more stressed than in others (King et al. 2016), knowing typical circulating cortisol levels in wild invasive fish populations, or the likelihood of animals exhibiting a ‘stressed state’, may predict barrier efficiency. A fish’s behavior is linked with its physiological state, so measuring physiological metrics, such as plasma cortisol, can give an indication of stress levels that might drive a fish’s behavior when confronted with a challenge. Measuring physiological metrics also provides a rapid and sensitive assessment of fish performance after a large-scale disturbance, such as addition of a CO₂ barrier (Jeffrey et al. 2015; Chown and Gaston 2016). In conclusion, internal factors such as stress hormones, are more likely to influence hypercarbia avoidance than boldness, and these physiological factors must be considered in relation to fisheries management actions to help conserve and protect aquatic ecosystems.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards

of the institution or practice at which the studies were conducted.

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