ARTICLE

Influence of Nutritional Status on Carbon Dioxide Tolerance and Avoidance Behavior in a Freshwater Teleost

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Abstract

   Challenging environmental conditions can induce voluntary behavioral avoidance in animals. Dissolved carbon dioxide (CO_2) is an environmental stressor that was previously shown to upregulate the stress axis in fish and also causes voluntary avoidance. Variation in individual state or context, such as whether an animal is fasted or fed, can alter animal behavior, including the response to environmental challenges. In the current study, we sought to define the influence of nutritional status on the response of Largemouth Bass *Micropterus salmoides* to elevated CO_2. Two groups of Largemouth Bass—one fed group and one fasted group—were first subjected to a CO_2 shuttling protocol to define avoidance thresholds, followed by a CO_2 tolerance protocol to define the time required to lose equilibrium and recover. Data showed that although feeding and fasting had no influence on the avoidance of CO_2, fasted fish required 17% longer to lose equilibrium in elevated CO_2. Avoidance of elevated CO_2 is therefore independent of animal state, but fish in poor nutritional condition from fasting are more tolerant. Thus, managers considering elevated CO_2 as a nonphysical barrier to deter fish movements should be cognizant of food availability, as fasted animals may require increased partial pressures of CO_2 to ensure successful deterrence.

Animals regularly experience fluctuations in their environment, and specific conditions can be challenging. For fish, abiotic factors (e.g., temperature, water currents, or dissolved oxygen) fluctuate, and all can reach levels that can become challenging, potentially leading to an upregulation of the stress response. Over the short term, an upregulation of the stress axis can be an energetically costly mechanism designed to maintain homeostasis in the face of external challenges, but when engaged over extended periods of time, it can lead to a host of negative consequences, such as suppressed immune function and impaired reproduction (Schreck and Tort 2016). Rather than engage in the process of upregulating the stress response, a simple behavior used by animals to avoid challenging conditions is to move away from degraded water and relocate to habitats that are less stressful or less

energetically costly (Beitinger and Freeman 1983; Beitinger 1990; Tierney 2016). For example, previous studies have shown that Largemouth Bass *Micropterus salmoides* will avoid waters with reduced dissolved oxygen, presumably to maintain metabolic scope for growth and activity (Burleson et al. 2001). Thus, voluntary avoidance is a behavioral strategy used by fish to inhabit water that is favorable, thereby avoiding the short-term and long-term costs of stress axis upregulation to maintain homeostasis.

   Another important aspect of animal biology that can mitigate the short-term and long-term costs of "stress" is the acquisition of energy through feeding (Mittelbach 2008). Feeding is a key behavior linked to energy intake, survival, and fitness for animals, and individuals are expected to engage in feeding behaviors that allow for the efficient acquisition of energy (Mittelbach 2008).

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Interestingly, the presence (or absence) of food resources and the resulting change in nutritional status have been identified as factors that lead to plastic or flexible changes in animal behavior (Killen et al. 2013; Metcalfe et al. 2016). For fishes, owing to a combination of physiological (e.g., metabolic rate and hunger state), morphological (e.g., gape size), and behavioral (e.g., hierarchical position that influences access to feeding patches) factors, gut fullness and nutritional status can vary across animals in the wild (Ringler 1983). More importantly, poor nutritional status brings about changes in physiology, which in turn can change behavior and/or performance. Reductions in food intake, for example, can induce weight loss, consumption of energy stores, and catabolism (Gingerich et al. 2010), as well as behavioral outcomes, likely related to an increased need for food (Killen et al. 2013; Metcalfe et al. 2016). For example, animals that have had restricted access to food have been shown to become more active and take more risks than satiated animals (Killen et al. 2013). Beukema (1968) showed that hungry Threespine Sticklebacks *Gasterosteus aculeatus* swam more actively than satiated individuals, likely as they sought out food, while Näslund and Johnsson (2016) reported that activity and aggression in Brown Trout *Salmo trutta* increased with food deprivation. Similarly, during a food deprivation study (Krause et al. 1998), Threespine Sticklebacks that lost the most body mass were observed to engage in more risk-taking behaviors (e.g., left a refuge sooner and spent more time outside of the refuge) relative to individuals that lost smaller amounts of body mass. Thus, access to food resources and nutritional status can have a pronounced impact on both the physiology and behavior of animals, presumably owing to individuals' need to accumulate energy resources for survival (Metcalfe et al. 2016).

Dissolved inorganic carbon is naturally occurring, and fish can encounter high levels of free carbon dioxide (CO_2; Cole and Prairie 2009; Hasler et al. 2016). Exposure to elevated free CO_2 (i.e., hypercarbia) is known to cause physiological and behavioral changes in fish as well as an upregulation of the stress axis. In freshwater environments, the amount of free CO_2 in the water can be highly variable both spatially and temporally and is influenced by factors such as primary productivity, land use, respiration, geology, and storms (Hasler et al. 2016; Crawford et al. 2017). There also has been recent interest in intentionally elevating free CO_2 for developing nonphysical barriers to deter the movement of invasive fishes (Noatch and Suski 2012). Acute exposure to elevated CO_2 can result in acid/base imbalance or ionic disequilibrium, which can translate into elevated energetic costs (Kates et al. 2012). Acute exposure to elevated CO_2 can also result in voluntary avoidance, as animals will leave areas of high CO_2, presumably to occupy habitat with improved water quality and avoid energetic costs related to reduced water quality (Kates et al. 2012). Chronic exposure to elevated CO_2 can result in an involuntary loss of equilibrium due to the anesthetic properties of CO_2 (Iwama et al. 1989). Although the behavioral response to CO_2 is consistent across species (Kates et al. 2012; Donaldson et al. 2016), there is a great deal of variation across individuals within species in their response to elevated CO_2. Kates et al. (2012), for example, showed that levels of CO_2 resulting in avoidance varied approximately threefold, while Hasler et al. (2017) observed that the partial pressure of CO_2 (pCO_2) resulting in equilibrium loss varied twofold, with some fish being more tolerant of hypercarbia than others. At present, the source of this interindividual variation to CO_2 exposure is not known (e.g., Hasler et al. 2017), but one hypothesis to explain this observation relates to food resources and nutritional status, with the nutritional state of individual fish influencing their behavioral response to the CO_2 stressor. Fasted juvenile Green Sturgeon *Acipenser medirostris* were less tolerant of elevated temperatures relative to fed individuals (Lee et al. 2016), indicating that a feedback between nutritional status and the behavioral response to environmental stressors is plausible, but this relationship has not been established for CO_2. Knowledge of how contexts such as nutritional status can impact both behavioral and physiological responses to elevated CO_2 is important, not only because fish can face high CO_2 naturally but also because such information can be used to define avoidance thresholds or tolerance limits for practitioners looking to deploy CO_2 barriers (Donaldson et al. 2016). For example, fish in a poor nutritional state could be motivated to engage in more risk-taking behaviors as they search for food, which in turn could make them more willing to swim into areas of high CO_2, reducing the efficiency of hypercarbic zones as fish barriers.

The objective of this study was to define the impact of nutritional status on voluntary avoidance of elevated CO_2 as well as involuntary tolerance and subsequent recovery from exposure to elevated CO_2. To accomplish this goal, one subset of Largemouth Bass was fed ad libitum, and a second group was deprived of food (i.e., was fasted) for 14 d to simulate a real-life situation in which fish are denied access to food (e.g., increased competition for limited food resources; or a stochastic event negatively impacting prey resources). Hypercarbia avoidance for fed and food-deprived fish was quantified using a "shuttle box" avoidance assay, while hypercarbia tolerance was quantified with an acute exposure assay. Results will be valuable not only for defining how context and physiological status impact performance and behavior when fish face environmental challenges but also for identifying characteristics that make a fish more (or less) tolerant to elevated CO_2, which can inform management actions related to CO_2 barriers.

METHODS

Experimental animals.— Largemouth Bass were transported from Jake Wolf Fish Hatchery (Topeka, Illinois) to the Aquatic Research Facility at the University of Illinois, Urbana–Champaign; all fish came from the same hatchery cohort and were approximately 1.5 years of age (age data not generated). After arriving at the research facility, fish were housed indoors in a single, 300-L plastic tank supplied with water from a 0.04-ha, earthen-bottom pond (Figure 1). The indoor holding tank was equipped with an ultraviolet water sterilizer (Vecton-6; American Aquarium Products, Grants Pass, Oregon), a canister filter to remove nitrogenous waste (FX6 High Performance; Fluval, Mansfield, Massachusetts), an air stone connected to a blower for aeration, and an aquarium chiller to maintain a constant temperature (18°C; Tank TK-1000; Teco, Ravenna, Italy). Twice per day, approximately 40% of the water in the holding tank was replaced with fresh pond water to help maintain appropriate water quality. Water changes involved lowering an external standpipe while concurrently pumping water into the tank via a hose. Water quality measurements were taken daily (Table 1) and included temperature, dissolved oxygen (YSI 550A; Yellow Springs Instruments, Yellow Springs, Ohio), ammonia nitrogen
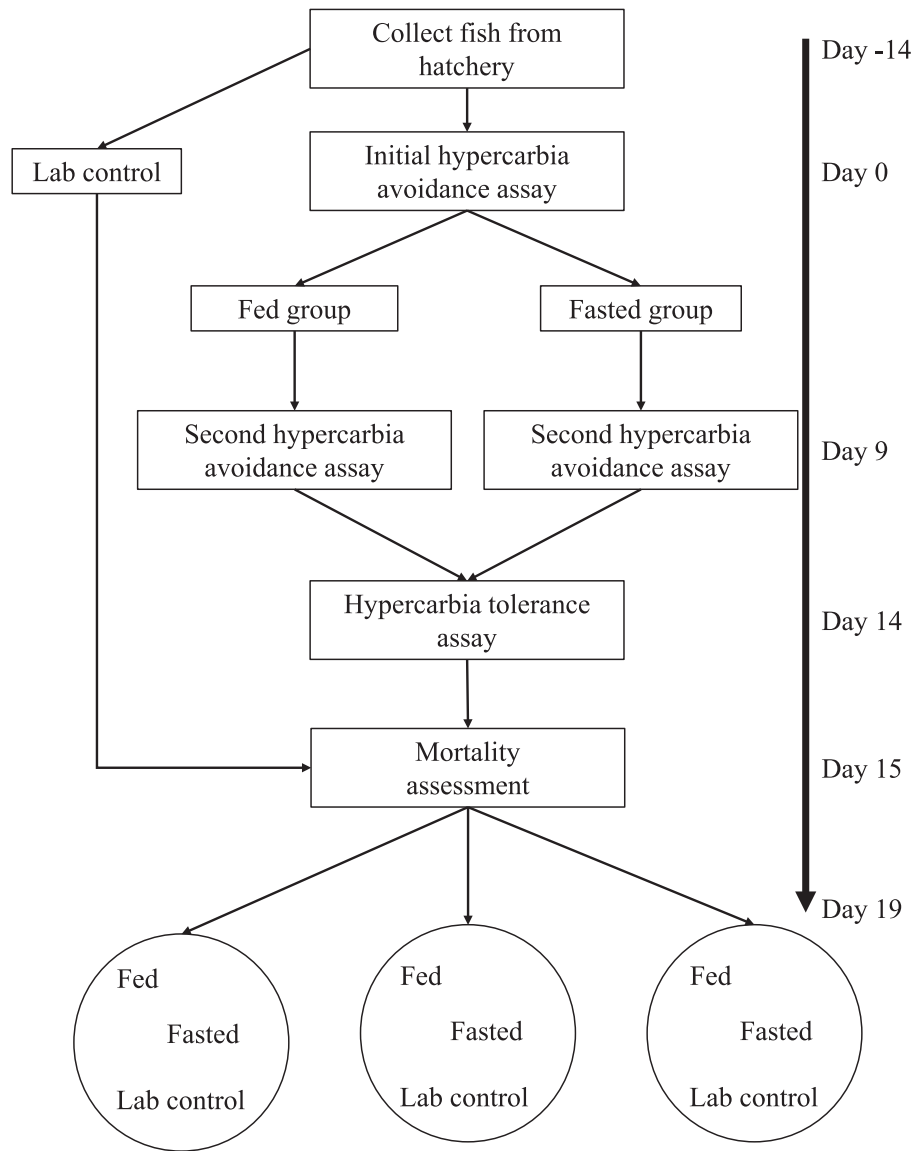

FIGURE 1. Timeline of key project events ranging from when Largemouth Bass were acquired (14 d prior to the commencement of the study) to the last day of mortality assessments (day 19). Day 0 is when experimentation started. The circles at the bottom of the figure represent three outdoor tanks where fish were held for 4 d after the experimental portion of the study to quantify mortality.

(NH$_3$-N; Ammonia Nitrogen Kit 3351-02; LaMotte Company, Chestertown, Maryland), total alkalinity (Hach Titrator Model 16900 and Kit 94399; Hach Company, Loveland, Colorado), and pH (WTW pH 3310 meter with a SenTix 41 probe; WTW, Weilheim, Germany). The holding tank was covered with mesh netting to prevent escapement, and artificial habitat was provided in the form of cement blocks and cut polyvinyl chloride pipes. Fish were given a total of 7 d to recover from any physiological stress they may have endured during transport (Milligan 1996); each fish was then implanted with a uniquely coded PIT tag (10-mm length × 2-mm diameter; Biomark HPT12 tags; Biomark, Boise, Idaho). Fish were then allowed to recover in the holding tank for a further 7 d before experimentation started; during this 7-d period, fish were given a pelleted food to satiation daily (BioVita Fry; Bio-Oregon, Inc., Longview, Washington). Of the Largemouth Bass acquired for this study, 31 fish were used for hypercarbia experiments described below, and a separate group of 9 fish (tagged in a manner identical to the methods described above; mean TL ± SE = 161.1 ± 3.1 mm) was not used in hypercarbia experiments and remained in the common holding tank throughout the study. These nine Largemouth Bass continued to be fed to satiation daily during holding, did not participate in any of the CO$_2$ experiments, and acted as a laboratory control for the mortality assessments described below (Figure 1). All activities were approved by the Institutional Animal Care and Use Committee at the University of Illinois (Protocol 16126).

Initial hypercarbia avoidance.— The pCO$_2$ that induced behavioral avoidance (i.e., baseline avoidance) was quantified prior to feeding manipulations for all Largemouth Bass by using a shuttle box (Loligo, Inc., Hobro, Denmark; e.g., Kates et al. 2012; Figure 1). 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) and was filled to a water depth of 20 cm. During the experiment, the entire arena was surrounded by a frame with a black 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 shuttle box to prevent observer influence. During each trial, a fish was carefully netted from the common holding tank, identified using the unique PIT tag, and placed in a randomly determined side of the shuttle box. The fish was left in the shuttle box to acclimate for 30–40 min, a time consistent with acclimation periods in previous studies using shuttle boxes (e.g., Kates et al. 2012; Tix et al. 2017). After the acclimation period, when the fish had ceased moving between the two sides, CO$_2$ gas was injected into the water of the external chamber connected to the side of the shuttle box where the fish was located. The external chamber connected to the opposite side of the shuttle box received a continuous injection of compressed air. The resulting effect was a continuous reduction in the monitored pH (WTW pH 3310 meter with SenTix 41 probe), which can be inferred as a continuous increase of pCO$_2$ in the side of the shuttle box holding the fish (Kates et al. 2012). The opposite side remained at ambient pH and, thus, ambient dissolved CO$_2$. The flow of CO$_2$-saturated water continued until the fish moved to the other side of the arena (i.e., "shuttled"), and this was deemed to be behavioral avoidance (Kates et al. 2012). Immediately after the shuttle, three water samples were collected from the CO$_2$-saturated side of the shuttle box and were used to measure the pH and alkalinity of the arena water. The pCO$_2$ at the time of the shuttle was calculated by combining the pH, total alkalinity, water temperature, and atmospheric air pressure using CO$_2$Calc (Robbins et al. 2010). Each fish was only allowed to shuttle once per trial to avoid potential confounding factors, such as acclimation, learning, or habituation. Furthermore, it is difficult to fully strip CO$_2$ from water to maintain heterogeneity during subsequent shuttles. After this initial shuttle, the fish was removed, and the shuttle box was completely drained and re-filled with fresh water in preparation for the next trial. Thirty-one Largemouth Bass were assayed as part of this protocol over 4 d.

After the initial hypercarbia avoidance assessment, fish were randomly assigned to either the fed or fasted

TABLE 1. Water quality parameters (mean ± SE) for the laboratory control holding tank, two treatment holding tanks, and the outdoor tanks, measured daily over periods of 28 d (holding), 14 d (experimental), and 5 d (outdoor). Data that were not collected are represented as "N/A" (pCO$_2$ = partial pressure of carbon dioxide, expressed in microatmospheres [µatm]).

Tank	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Ammonia nitrogen (mg/L)	Total alkalinity (mg/L as CaCO$_3$)	pCO$_2$ (µatm)
Holding tank	19.6 ± 0.3	8.3 ± 0.1	N/A	1.6 ± 0.3	207 ± 15.4	N/A
Fed tank	20.6 ± 0.9	7.4 ± 0.6	8.2 ± 0.05	2.6 ± 0.4	170 ± 3.0	1,025.8 ± 9.1
Fasted tank	20.2 ± 0.5	8.2 ± 0.3	8.4 ± 0.08	1.4 ± 0.4	178.5 ± 0.5	1,078.1 ± 3.03
Outdoor	15.8 ± 0.3	9.6 ± 0.03	N/A	N/A	N/A	N/A

treatment and were placed in perforated plastic totes ($43 \times 33 \times 36$ cm) submerged in two larger holding tanks (~426 L), with one tank for the fed treatment and a separate tank for the fasted treatment (Figure 1). The separation of fed and fasted treatments into different holding tanks is common in these kinds of studies and serves to ensure that there is no cross-contamination of food across treatments, which could occur if treatments are comingled in common tanks (Folkvord 1991; Blasco et al. 1992). Each holding tank contained three floating totes with 7–8 fish/tote, and fish were separated into the totes based on the day of their initial hypercarbia avoidance assay to easily identify individuals and ensure consistent holding times (Godin and Crossman 1994). Initially, fish were separated across totes based on the 4 d of their trials; however, over the course of fasting, three Largemouth Bass from the fasted treatment assayed on day 3 died unexpectedly. Previous work suggested that the single exposure to elevated CO_2 in the shuttle box should not be sufficient to result in mortality (Dennis et al. 2016a); as such, we considered this mortality to be the result of random chance. To maintain balance within the study design, all Largemouth Bass assayed on day 3 were removed from the study (a total of 8 fish removed: the 3 that died and 5 additional living fish). This resulted in a final sample size of 23 Largemouth Bass ($n = 10$ for the fasted treatment; $n = 13$ for the fed treatment) that completed the hypercarbia avoidance trials (mean TL $\pm$ SE $= 165.3 \pm 2.0$ mm; mean total weight $= 68.7 \pm 3.0$ g). Tanks were equipped with air stones connected to blowers for aeration, an ultraviolet water sterilizer (Vecton-6), and a canister filter for biological/mechanical filtration (FX6 High Performance). Water quality measurements were taken daily for these tanks as described above (Table 1). After fish were assigned to their respective treatment and placed into totes, they were either fed daily in a manner identical to the method described above (fed treatment) or food was withheld (fasted treatment) for a total of 9 d (Figure 1). Previous work has shown that for Largemouth Bass, 14 d of food deprivation resulted in a significant reduction in body mass, liver mass, and metabolic rate relative to individuals that had been fed (Gingerich et al. 2010). In addition, many previous studies of feeding and fasting with fish typically involve reduced rations rather than complete withholding of food (Godin and Crossman 1994), and Midway et al. (2017) showed that Largemouth Bass experiencing food deprivation for 1 week in a laboratory setting consumed almost 90% of live prey presented to them in under 15 min, indicating that the 9-d period of food withholding in the current study should be sufficient to induce hunger in Largemouth Bass. After the 9-d period of either feeding or fasting, a second round of hypercarbia avoidance trials was performed using methods identical to those described above (Figure 1). The only deviation from

the aforementioned protocol was that during the second round of avoidance assays, trials alternated between fed and fasted individuals. After the second hypercarbia avoidance assay, fish were placed back into their respective totes, and feeding and fasting continued for another 4 d (Figure 1).

Acute hypercarbia tolerance.— Four days after the second hypercarbia avoidance assay, all 23 Largemouth Bass were assessed for CO_2 tolerance by using an acute hypercarbia challenge (Hasler et al. 2017); a period of 4 d was chosen because fish should have fully recovered from handling stressors or disturbances from the avoidance assay within 24–48 h (Milligan 1996). For this, pCO_2 in a small experimental tank ($36 \times 35 \times 66$ cm) was raised to approximately 150,000 microatmospheres (µatm; SD $= 15,000$) using the common technique of bubbling compressed CO_2 gas through an air stone until the target pCO_2 was reached. The pCO_2 in the container was verified with a modified infrared CO_2 probe (GMT221, 0–20%; Vaisala, Vantaa, Finland; Johnson et al. 2010); the infrared probe used was slow to equalize and therefore was not conducive for targeting an acute change in pCO_2. For this challenge, once the target pH in the experimental tank had been reached and the pCO_2 had been verified, a single fish from either the fed or fasted treatment was netted from its holding tote (alternating between the two nutritional groups), identified using the PIT tag, and placed into the experimental tank with elevated CO_2. Fish were monitored continuously by using a mirror to avoid influence of the observer. Time was recorded until the fish lost equilibrium (i.e., lost motor function and was not upright for at least 3 s; Hasler et al. 2017). Once equilibrium loss occurred, the fish was immediately removed from the elevated CO_2 and placed into a second container of water at ambient pCO_2 that was outfitted with an air stone for aeration. Fish were monitored until they recovered (i.e., the fish could turn itself upright and actively tried to escape from a tail grab). Times for both equilibrium loss and recovery were rounded to the nearest whole second to account for potential measurement error.

Mortality assessments.— After the acute hypercarbia trial, 31 Largemouth Bass (23 that completed both hypercarbia avoidance assays and 9 laboratory controls) were transferred to one of three outdoor holding tanks (180-cm diameter $\times$ 61-cm high) to be monitored for mortality (Figure 1). Tanks were supplied with a continuous flow-through of water from the same small earthen-bottom pond described above. Fish size (TL, mm) did not differ across the fed, fasted, or control individuals (one-way ANOVA adjusted for type III sums of squares: $F_2 = 0.248$, $P = 0.782$). Fish from both the fed and fasted treatments, as well as the nine laboratory control fish, were equally mixed and dispersed across the three replicate tanks (Pollock and Pine 2007; Figure 1). Each tank

was checked daily for mortality over a 5-d period, during which time all fish were fed daily to satiation as described above.

Statistical analyses.— All statistical analyses were performed using R version 3.3.3 (R Core Team 2017). A generalized linear mixed-effects model with a Gaussian error distribution and rank-transformed data (Conover and Iman 1981; Iman et al. 1984; Potvin and Roff 1993) was used to compare the pCO_2 present in the water when Largemouth Bass performed their initial and final shuttles (main effects: feeding treatment [fed, fasted], shuttle replicate [initial, final], and an interaction term [feeding treatment × shuttle replicate]). Because Largemouth Bass were tested twice, meaning that trials were not independent, PIT tag number was nested within treatment and entered into the mixed-effects model as a random effect with random intercepts (Bates et al. 2015). After the completion of this mixed-effects model, Wald-type credible intervals were calculated to test hypotheses among fixed effects (Bolker et al. 2009), and degrees of freedom for both the numerator and denominator were generated using the Kenward–Roger method (Schaalje et al. 2002). Student's *t*-tests were used to compare the time required to lose equilibrium and the time to recover from hypercarbia exposure for fed and fasted treatments. Owing to overdispersion (defined as having residual deviance greater than the residual degrees of freedom; Crawley 2012), the number of seconds to the loss of equilibrium and the number of seconds to recover from acute CO_2 exposure across the fed and fasted treatments were compared using a negative binomial regression (Zuur et al. 2009; Lindén and Mäntyniemi 2011). Mixed-effects models were fitted using the "lmer" function from the package lme4 (Bates et al. 2015), credible intervals were calculated using "coefplot" (Lander 2013), and numerator and denominator degrees of freedom were calculated using "lmerTest" (Kuznetsova et al. 2017). Student's *t*-tests were fitted with the R base package (R Core Team 2017). The generalized linear model with negative binomial distribution was run with glmmADMB (Fournier et al. 2012). Model fit and assumptions were verified by inspection of residuals via fitted plots, along with quantile–quantile plots (Zuur et al. 2009). The level of significance (α) for all tests was set at 0.05.

RESULTS

During the shuttle box trial, Largemouth Bass voluntarily shuttled away from elevated CO_2 to water with ambient CO_2 once pCO_2 reached approximately 60,000 µatm. The pCO_2 that caused Largemouth Bass to shuttle did not vary across the fed or fasted treatment and did not differ between the initial and final shuttles (Figure 2; Table 2).

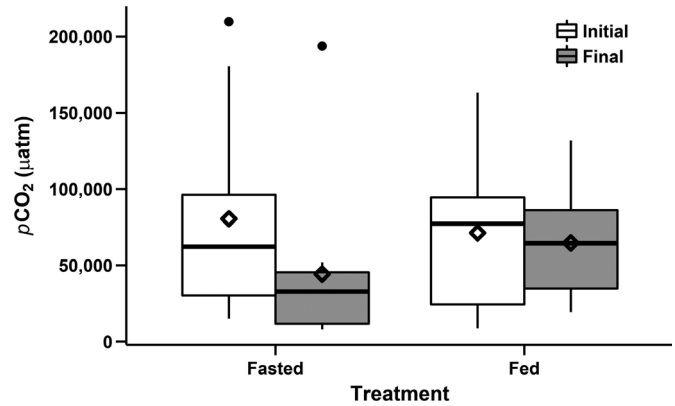Fasted Largemouth Bass took 17% longer to lose equilibrium when exposed to high pCO_2 relative to

FIGURE 2. Partial pressure of carbon dioxide (pCO_2; microatmospheres [µatm]) required to induce shuttling behavior in Largemouth Bass (i.e., shuttling away from elevated carbon dioxide). All animals were tested twice: an initial test prior to the feeding and food deprivation treatments as a baseline (white boxes), followed by a final test after 9 d of either being fed or food deprived (shaded boxes). Additional details are shown in Figure 1; sample sizes were 10 fish in the fasted treatment and 13 fish in the fed treatment. The horizontal line in the middle of the box plot denotes the median of a treatment; the diamond represents the mean; the upper and lower horizontal lines represent the 75th and 25th percentiles, respectively; the vertical lines represent data that are 1.5× the interquartile range above or below the 75th or 25th percentiles, respectively; and dots represent data that are greater than the 75th percentile plus 1.5× the interquartile range. There were no statistical differences across either main effects or the interaction (statistical outputs are shown in Table 2).

Largemouth Bass that were fed ($t_{20.964} = 2.3$, $P = 0.03$; Figure 3A). There was no difference between treatments for the length of time required for Largemouth Bass to recover after losing equilibrium following exposure to elevated pCO_2 ($t_{20.079} = -0.28$, $P = 0.78$; Figure 3B). Across both treatment groups, there was a negative relationship between the amount of time required for Largemouth Bass to lose equilibrium and the amount of time required to recover. More specifically, fish that lost equilibrium quickly required a longer time to recover from hypercarbia, while Largemouth Bass that took a long to time lose equilibrium recovered quickly (Table 2; Figure 3B). In addition, when the time to equilibrium loss and time to recover were considered concurrently in a linear regression model, the responses of the fed and fasted treatment groups to elevated pCO_2 exposure were different: both the time to equilibrium loss and the time to recover were significantly longer for the fasted group than for the fed group (Table 3; Figure 3C).

All fish that were monitored during the 5-d period after the experimental tests survived; no mortality was observed for any fish in any of the three monitoring tanks. It should be noted, however, that due to seasonal environmental change, water temperatures during the holding period for mortality assessments were approximately 4°C cooler than those during avoidance or tolerance testing.

TABLE 2. Mean (estimate), Wald-type credible intervals, and numerator and denominator degrees of freedom (df; Kenward–Roger method) for a linear mixed-effects model comparing the partial pressure of CO_2 that induced behavioral avoidance in fed or fasted (food-deprived) Largemouth Bass. All animals were tested twice: an initial test prior to the feeding/food deprivation treatment as a baseline, followed by a final test after 9 d of either being fed or deprived of food. All credible intervals, with the exception of the intercept term (bold italics), contained zero in their estimation, indicating that the terms were not significant. Data are depicted in Figure 2.

Term	Estimate	Credible interval	Numerator df	Denominator df
Intercept	*25*	*17.8* to *32.2*		
Shuttle	0.23	−9.9 to 10.4	1	21
Feeding treatment	1.6	−9.4 to 12.6	1	21
Shuttle × feeding treatment	−10.6	−26.1 to 4.9	1	21

DISCUSSION

Fourteen days of food deprivation resulted in an increased tolerance of Largemouth Bass exposed to elevated CO_2. More specifically, in the hypercarbia tolerance challenge, Largemouth Bass that had been fasted for 14 d demonstrated an increase in the duration of time required to induce equilibrium loss relative to fed individuals. In addition, relative to fed Largemouth Bass, fasted individuals required additional time to recover after the hypercarbia challenge relative to the time required to lose equilibrium. It is common for free-swimming fish in the wild to experience variations in food availability, resulting in periods of time with little or no feeding. For example,

up to 40% of Largemouth Bass sampled from the wild in Minnesota and Oklahoma had empty stomachs (Scalet 1977; Cochran and Adelman 1982), while Largemouth Bass collected in the winter demonstrated food ingestion rates that were greatly reduced relative to summer feeding (Keast 1968). Fish (including several studies with Largemouth Bass) experiencing acute episodes of food deprivation (e.g., 24 h to 19 d) exhibit weight loss, consumption of muscle glycogen, and reductions in liver size (Niimi and Beamish 1974; Blasco et al. 1992; Gingerich et al. 2010) relative to individuals that are feeding, as animals shift to catabolism and employ strategies that reduce energetic demands and ensure survival. A loss of equilibrium
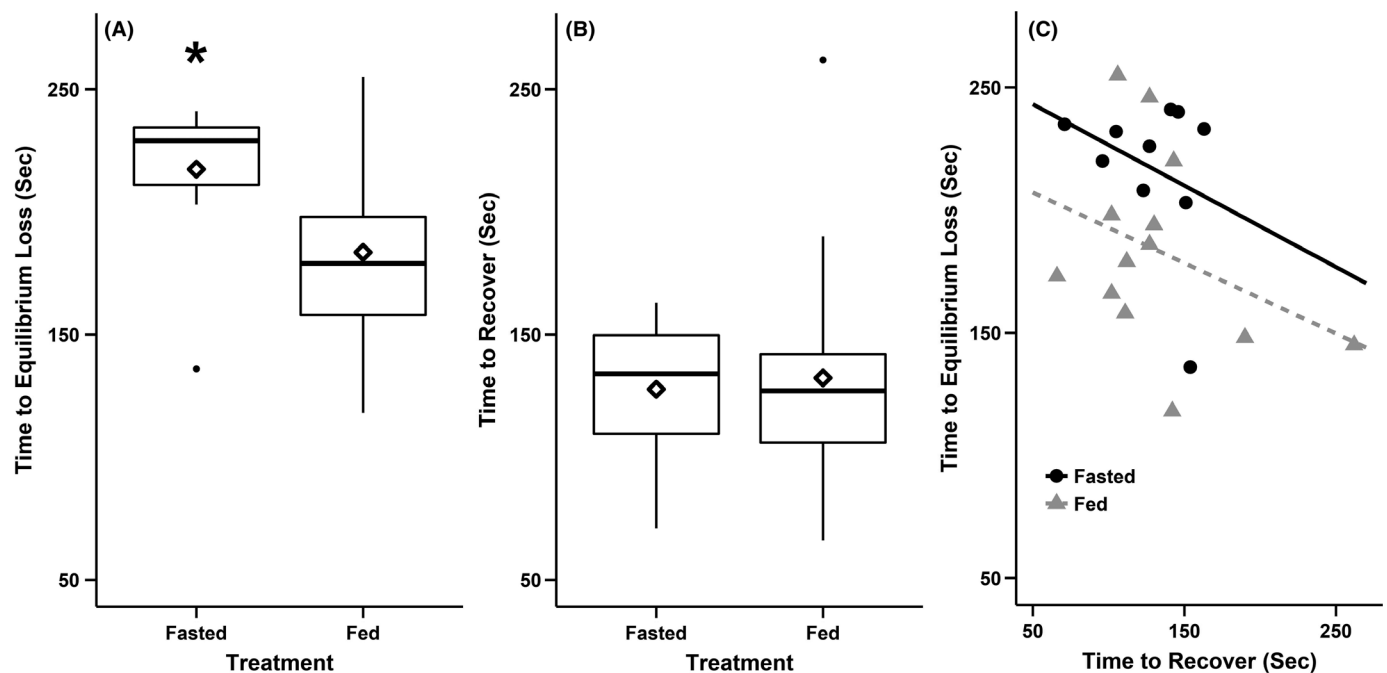

FIGURE 3. Comparison of hypercarbia tolerance between two groups of Largemouth Bass, one that was fed for 14 days and one that was fasted for 14 days. Tolerance was determined by (A) the time (s) it took for fish to lose equilibrium in high partial pressure of carbon dioxide (pCO_2) water, (B) the time (s) it took for the fish to recover from the hypercarbia stress exposure, and (C) the relationship between time to equilibrium loss and time to recover for fed and fasted treatments. Statistically significant differences ($P < 0.05$) between the two treatments are indicated by an asterisk (*). The horizontal line in the middle of the box plot shows the median, the diamond indicates the mean for a treatment, and filled circles represent data points that exceed 1.5× the interquartile range of a treatment.

TABLE 3. Negative binomial linear regression model comparing the number of seconds required for fed and fasted Largemouth Bass to lose equilibrium and to recover from hypercarbia stress in the hypercarbia tolerance assay ($n = 23$). Significant model parameters are shown in bold italics.

Factor	Estimate	SE	z	Pr(>|t|)
Intercept	*5.595*	*0.130*	*42.83*	*<0.001*
Recovery time	−0.0017	0.001	−1.08	0.072
Group (fed)	*−0.164*	*0.072*	*−2.26*	*0.024*

for fish exposed to hypercarbia is believed to occur because external CO_2 passively diffuses down its concentration gradient into the bloodstream, where it crosses the blood–brain barrier, altering brain activity and resulting in anesthesia and an involuntary loss of equilibrium, and concurrently results in a decline in internal pH; the excretion of CO_2 in fish occurs passively at the gills and therefore either slows or becomes reversed in high-CO_2 environments (Iwama et al. 1989; Yoshikawa et al. 1991, 1994; Brauner and Randall 1996).

There are several potential hypotheses that may explain the improved tolerance to hypercarbia exposure for Largemouth Bass that were fasted in the current study. Gills have been shown to be quite plastic and labile; they can be restructured based on external stimuli, and this process can occur within a few days (Nilsson 2011). For example, Nance et al. (1987) showed that 5–10 d of starvation in Rainbow Trout *Oncorhynchus mykiss* resulted in gill restructuring and a reduction of passive ion influx from the environment; Laurent and Perry (1995) reported a considerable reduction in gill surface area after only 6–48 h of exposure to an environmental stressor (hypercapnia); and Nilsson (2011) suggested that a reduction in gill size would reduce volume in the oral cavity to facilitate feeding. Thus, it is possible that food-deprived Largemouth Bass experienced a gill restructuring and/or reduction of gill surface area that reduced the passive influx of CO_2, thereby resulting in improved tolerance. Second, Gingerich et al. (2010) reported that Largemouth Bass experiencing 16 d of food deprivation demonstrated an almost 50% decline in resting metabolic rate (oxygen consumption) relative to individuals that had been fed—a strategy seen in other animals, likely to conserve energy and reduce maintenance costs (Wen et al. 2017). Largemouth Bass that were fasted for 14 d in the current study likely experienced a decrease in metabolic rate (Gingerich et al. 2010), which in turn may have slowed the internal transport of CO_2 and increased the time required for CO_2 to enter the brain, translating to an increased duration to induce equilibrium loss relative to fed fish. Third, fasted fish typically experience protein catabolism as muscle is consumed to generate energy (peptides; Sundell and Rønnestad 2011). The uptake of peptides into cells often occurs through H^+/peptide co-transporters,

which uptake protons into cells concurrent with the transport of peptides (Terada and Inui 2012). As such, a shift to reliance on protein catabolism for energy induced by food deprivation can have the unintended consequence of removing protons from plasma, helping plasma pH to remain elevated and maintaining the quantity of oxygen being carried by hemoglobin by inhibiting offloading of oxygen from Bohr/Root effects (Fromm 1980; Brauner and Randall 1996), such that fasted fish maintained elevated oxygen transport that could have improved CO_2 tolerance. Finally, it is also possible that the improved tolerance to hypercarbia shown by fish in the fasted treatment was related to improved blood buffering capacity resulting from reduced metabolic rates, increased activity of Cl^-/HCO_3^- transporters, or increased activity of H^+ transporters (Brauner and Rummer 2011). Future work should corroborate these hypotheses to better define the mechanism of improved CO_2 tolerance in fasted Largemouth Bass relative to fed conspecifics.

Nine days of food deprivation did not influence the voluntary behavioral avoidance of elevated CO_2 in Largemouth Bass. Upon encountering water quality that has been degraded, one of the first responses that fish exhibit is to simply avoid the area and relocate to water with improved quality (provided that suitable alternate habitats exist). Presumably, this response reduces energetic costs associated with reduced water quality (e.g., increased metabolic rate, production of stress hormones, and ionic imbalance) and increases survival or fitness (Beitinger and Freeman 1983; Beitinger 1990; Tierney 2016). Past studies have used a shuttle box arena to demonstrate that several species of fish, including Largemouth Bass, display behavioral avoidance in response to elevated CO_2 once a threshold level has been achieved, and the threshold that has been previously shown to induce behavioral avoidance was similar to partial pressures reported in the current study (Kates et al. 2012; Dennis et al. 2016b). The mechanism responsible for how fish sense CO_2 in the environment is complex and not fully understood but likely occurs through a combination of internal or external sensory pathways (Gilmour 2001; Lahiri and Forster 2003; Gilmour and Perry 2006). However, environmental stressors can alter an individual's behavior, such that the presence of an environmental stressor can lead to either augmented or diminished behavioral responses, depending on a suite of factors that include individual variation, individual experience, and the stressor in question (Dingemanse and Wolf 2013; Killen et al. 2013). Carbon dioxide is a byproduct of oxidative metabolism generated by respiring cells, and internal accumulations of CO_2 can reduce homeostasis and lead to several pathological states (Cummins et al. 2014). Indeed, environmental CO_2 has been shown to be a stressor for fish, resulting in physiological and behavioral responses that include an upregulation of stress hormones, the production of heat

shock proteins, and the occurrence of erratic behaviors (e.g., twitching and surface ventilations) (Kates et al. 2012; Dennis et al. 2015). Although the pCO_2 required to induce avoidance in the current study varied across individuals, the response of individuals to external CO_2 was independent of nutritional status, indicating a consistent behavioral response despite variation in context. Behavioral traits have previously been shown to be "plastic" or "flexible," meaning that an individual's behavior can change across contexts, particularly when animals are confronted with a challenge or stressor (Killen et al. 2013; Sih et al. 2015; Stamps 2016). More specifically, behavior can be driven by different animal states, and animals can adjust their behavior in an adaptive fashion due to changes in factors such as information state, the presence of predators, elevated temperature, social rank, or reproductive state (Killen et al. 2013; Sih et al. 2015). Previous work has shown that periods of food deprivation can lead to behavioral changes for animals such that nutritionally deprived animals often engage in more risk-taking behaviors, or elevated activity, presumably to acquire food (Godin and Crossman 1994). The ability of organisms to sense CO_2 is remarkably conserved across taxa and is critical for survival and fitness (Cummins et al. 2014). Data from the current study suggest that the ability to sense CO_2 is consistent across contexts, likely to reduce the consequences of remaining present in hypercarbic water. Collectively, our results demonstrate that the pCO_2 required to induce voluntary shuttling behavior away from hypercarbic water is not altered by food deprivation in Largemouth Bass.

No mortality was observed during the monitoring period at the conclusion of the study, despite two exposures to elevated CO_2 coupled with a period of almost 2 weeks of restricted food. In response to acute or chronic external challenges, such as exposure to CO_2 or restriction of food, fish will upregulate their stress axis in an effort to overcome a perceived challenge or to restore homeostasis (Schreck and Tort 2016). Starvation, for example, can lead to activation of the stress response and physiological disturbances—and ultimately mortality—in fish, while CO_2 exposure can upregulate the stress axis and again can end with narcosis or mortality depending on the pCO_2 and exposure duration (May 1974; Byström et al. 2006). Upon experiencing multiple stressors, the response of animals can be antagonistic (the combined effect of the stressors is less than their individual effects), additive (the response observed is equal to the cumulative effect of the stressors), or synergistic (the observed response exceeds the impacts of individual stressors; Folt et al. 1999). Results from the current study indicate that the combined impacts of food restriction and acute exposure to CO_2 did not result in mortality for Largemouth Bass, suggesting that the fish response to these combined challenges was likely not synergistic. Previous work has indicated that fish are relatively resilient to food

restriction. Gingerich et al. (2010), for example, reported no mortality in Largemouth Bass that were fasted for 16 d at 15°C. Folkvord (1991) showed that juvenile Atlantic Cod *Gadus morhua* could withstand 8 weeks of food withholding at approximately 8°C. Kiessling et al. (1990) documented that Rainbow Trout were able to survive after 3 months of food withholding at 9°C. Similarly, acute exposure to CO_2 has previously been used an anesthetic for fish undergoing surgery or hauling with typically full recovery from equilibrium loss (Iwama et al. 1989; Ross and Ross 2009). The current data do not allow inferences to be made about the potential impacts to fish over longer time frames (e.g., potential reductions in growth, likelihood of immune suppression, or potential fitness consequences; Schreck and Tort 2016), but our results indicate that acute exposure to elevated CO_2 does not translate to short-term mortality for Largemouth Bass, even when coupled with periods of extended food withholding.

Results from this study have several implications for how fish respond to either anthropogenically derived or natural occurrences of elevated CO_2 in aquatic environments. For example, studies have shown that preventing the establishment of an invasive species is a more reliable and economically favorable strategy than attempting to remove that species after its establishment (Leung et al. 2002). The prospect of deploying zones of elevated CO_2 barriers to prevent the movement and establishment of invasive fishes is growing in popularity (Treanor et al. 2017), having been successfully demonstrated for invasive Sea Lampreys *Petromyzon marinus* (Dennis et al. 2016b), bigheaded carp *Hypophthalmichthys* spp. (Donaldson et al. 2016), and Round Goby *Neogobius melanostomus* (Cupp et al. 2017). When deployed, CO_2 barriers could influence fish movements via two primary mechanisms. First, fish have been shown to actively avoid areas of elevated CO_2 once a target threshold has been achieved (typically around 30,000–80,000 μatm; Donaldson et al. 2016; Cupp et al. 2017), allowing zones of CO_2 to (1) deter passage through a choke point as fish "choose" to swim to water with improved quality or (2) deflect organisms to a target area to facilitate harvest. Second, due to the anesthetic properties of CO_2, if fish are forced to spend extended periods of time in elevated-CO_2 zones—for example, if CO_2 is deployed in a confined space, such as a shipping lock—animals can lose equilibrium, and movement can be prevented. The current study shows that for barrier deployments intended to induce avoidance or deflect fish to a target area, the nutritional status of target fishes will likely not impact barrier efficacy, as there were no differences in the pCO_2 required to induce avoidance across the fed and fasted treatments. However, if the goal of a CO_2 barrier is to cause equilibrium loss for target fishes, managers and practitioners need to be cognizant of the nutritional status of fish, as fasted Largemouth Bass in the

current study required an approximately 25% longer exposure time to lose equilibrium relative to fish that had been fed, potentially reducing the efficacy of CO_2 barriers for fasted individuals. For fish, access to food can be impacted by a host of factors that include population density, prey availability, water quality, seasonal variation, and individual competitiveness; thus, if there are periods when resources are predicted to be limiting, extra care may be needed to prevent unintentional passage of target organisms through a CO_2 barrier. Continued work to corroborate this finding in additional species would be valuable. In addition, studies have suggested that the pCO_2 in freshwater may be increasing in certain water bodies (Hasler et al. 2016; Weiss et al. 2018). Although the mechanism responsible for this increase has not been identified and the consequences for aquatic ecosystems have not been well defined, results from the current study suggest that the sensitivity of freshwater fish to future increases in aquatic CO_2 may be influenced by individual nutritional status, making this an important area for future research.

To conclude, CO_2 is a naturally occurring compound that may be increasing in freshwater and has been shown to be a stressor for fishes. In addition, the potential to use zones of elevated CO_2 as a nonphysical fish barrier is growing in popularity among managers (Donaldson et al. 2016; Treanor et al. 2017). As such, there are potential mechanisms by which wild fish could be exposed to elevated CO_2 in the future. Although previous work has shown that the presence of a stressor or periods of food deprivation can lead to behavioral changes in animals (Killen et al. 2013; Sih et al. 2015), the current study demonstrates that nutritional status and food restrictions have no impact on voluntary behavioral avoidance in fish. However, nutritional status does increase the involuntary response to the anesthetic properties of CO_2 for individuals with restricted food intake, possibly making those fish more tolerant to elevated CO_2 relative to individuals with better access to food. Future work should replicate these findings with additional fish species to better define the role of synergistic stressors at influencing fish responses.

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