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Shelter-seeking behavior of crayfish, *Procambarus clarkii,* in elevated carbon dioxide

M. D. Robertson · M. F. Hernandez · S. R. Midway · C. T. Hasler · C. D. Suski

Abstract Carbon dioxide has been found to produce various negative consequences for a number of aquatic species and is projected to rise in the future for many aquatic ecosystems. Crayfish act as indicators of water quality and function as keystone species in aquatic food webs; however, there is a paucity of research on how crayfish may respond to elevated CO_2. This study quantified how shelter-seeking behavior in freshwater crayfish (e.g., family Cambaridae), a behavior critical for survival and fitness, may change following exposure to elevated pCO_2. Red Swamp crayfish (*Procambarus clarkii*, Girard, 1852*)* were exposed to one of three potential levels of dissolved CO_2 that could be found in freshwater basins currently or under future climate change conditions: Control (< 100 μatm), Low (6853 ± 1206 μatm), and High (19,086 ± 2043 μatm) for 8 days, and were then transferred to a treatment aquarium with the same CO_2 levels as the respective initial exposure. The treatment aquarium contained a shelter and was divided into three equal sections based on proximity to that shelter. Crayfish proximity to the shelter (defined by the tank sections) in the treatment aquarium was monitored every 5 s for a 2-min trial. Crayfish spent differing amounts of time in differing zones of the experiment and had different levels of activity, depending on their pCO_2 exposure; crayfish acclimated to High pCO_2 increased their time spent hiding and decreased their overall activity when compared to the Low pCO_2 and Control treatments. Augmented shelter-seeking behavior may affect crayfish social hierarchies, feeding, mating, and mortality, which could generate cascading effects on the ecology of many freshwater ecosystems.

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Introduction

Atmospheric carbon dioxide (measured as partial pressure of CO_2 or pCO_2) has been increasing for decades, and this increase in pCO_2 will almost certainly continue into the future (IPCC 2014). More

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importantly, elevated atmospheric CO_2 has had a number of negative consequences for many biological systems (IPCC 2014). A growing number of studies on the environmental impacts of elevated pCO_2 have been completed in marine environments where rising atmospheric CO_2 has not only altered water temperature, but also has resulted in acidification (Harvey et al. 2013). Contrastingly, whether or not a rise in atmospheric CO_2 may result in acidification of freshwater environments has received relatively little attention (Hasler et al. 2018), though there is evidence that some freshwater systems have experienced elevated CO_2 (Phillips et al. 2015; Weiss et al. 2018). Unlike many marine systems, which have relatively constant levels of pCO_2, freshwater systems tend to have greater variability in pCO_2 (Balmer and Downing 2011; Hasler et al. 2016a), with partial pressures being influenced by variables such as geology, rainfall, and land use (Jacinthe et al. 2004; Ritchie et al. 2007); therefore, predicting how pCO_2 might change in freshwater systems in the future is a challenge. If future levels of pCO_2 in freshwater increase to a point beyond a species' capacity to respond, whole-organism changes (e.g., physiological, behavioral) may occur, which may further result in population declines in keystone species, and potentially larger ecosystem modifications.

To date, there have been relatively few studies on the effects of elevated pCO_2 on freshwater fishes and invertebrates (Hasler et al. 2016a). The studies that have been completed have shown that the response of freshwater organisms to elevated CO_2 appears to be dependent on the species and metrics examined (Hasler et al. 2017a; Midway et al. 2017; Tix et al. 2017a, b). For example, a study on fish alarm cue behavior following exposure to elevated CO_2 identified species-specific impairments related to predator avoidance in fathead minnows (*Pimephales promelas*, Refineseque, 1820) and silver carp (*Hypophthalmichthys molitrix,* Valenciennes, 1844) (Tix et al. 2017a), potentially linked to the same alteration in the GABA$_a$ receptor function that has been observed in marine fishes exposed to High CO_2 vents (Fuiman and Magurran 1994; Nilsson et al. 2012). In contrast, Midway et al. (2017) found that pCO_2 had no effect on the ability of Largemouth bass (*Micropterus salmoides*, Lacépède, 1802) to prey on Fathead minnow (*Pimephales promelas*, Refineseque, 1820), while Tix et al. (2017b) reported that exposure to High

pCO_2 did not alter bluegill personality. In studies of freshwater mussels, both prolonged and acute increases in pCO_2 elicited variable outcomes for the general stress response (Jeffrey et al. 2017a; Hannan et al. 2016a, b, c), increased energy demands (Jeffrey et al. 2017a), reduced biomineralization (Jeffrey et al. 2017b), along with interspecific differences in both behavioral and physiological responses to elevated pCO_2 (Hasler et al. 2017b; Hannan et al. 2016b, c). Overall, for freshwater biota, the effects of elevated pCO_2 vary across individuals, species, and taxa.

Even though several recent studies have begun to quantify biological outcomes for freshwater fishes and invertebrates, as of yet, there is a paucity of research on the effects of increased pCO_2 in crayfish, despite their ubiquity and importance to many freshwater ecosystems. Freshwater crayfish (e.g., family Cambaridae) can act as indicators of water quality and function as keystone controllers of trophic food webs (Momot 1995; Reynolds et al. 2013). Shelter-seeking behavior in crayfish is critical for survival and fitness (Bubb et al. 2006) and plays three primary roles. First, movement into shelters allows for reduced inter- and intra-specific predation risk of crayfish, thereby reducing potential mortality (Figler et al. 1999). Second, shelter-seeking behavior in crayfish is a characteristic of lower ranking individuals in social dominance hierarchies—in fact, shelter use on its own is believed to be dependent on social context (Fero et al. 2007). And finally, crayfish may seek shelter to avoid negative environmental stimuli (Huner and Barr 1991). If crayfish behavior is affected by future environmental change, it is possible that we could see corresponding cascades in the structure and function of freshwater ecosystems and potential declines in crayfish harvest rates. For example, if rising levels of pCO_2 have a negative effect on the ability of crayfish to sense predator or conspecific cues, or if it alters anti-predator behaviors as has been shown in some freshwater fishes (Tix et al. 2017a, b), mortality rates of crayfish may increase from increased predation rates, which may alter grazing pressure on algae and detritus, leading to negative impacts on aquatic ecosystems. Alternatively, it is possible that High pCO_2 levels would lead to overall increased sheltering behavior, which could also negatively impact grazing rates in freshwater systems.

Given the importance of crayfish for controlling freshwater ecosystems, the purpose of our study was to

define the effects of elevated $p\mathrm{CO_2}$ on crayfish behavior, specifically shelter-seeking behavior. To accomplish this, we subjected Red Swamp crayfish (*Procambarus clarkii,* Girard, 1852) to varying levels of both pre-treatment and treatment $p\mathrm{CO_2}$ in order to evaluate behavioral trials of shelter use. We predicted that shelter-seeking behavior would increase with exposure to rising $p\mathrm{CO_2}$ as elevated $\mathrm{CO_2}$ may cause crayfish to react to undesirable environmental conditions (Huner and Barr 1991; Bierbower and Cooper 2010).

Methods

Animal husbandry

Red Swamp crayfish were collected by dipnet from artificial ponds at the Louisiana State University Agricultural Center in Baton Rouge, Louisiana, USA, in March and April 2017. At the time of capture, crayfish were weighed and then measured with digital calipers from their rostrum to the end of their carapace. A total of 45 crayfish were captured over two separate events; mean carapace length was 29.36 mm (SD 4.58 mm, range 18.60–38.10 mm) with a mean mass of 5.48 g (SD 2.27 g, range 1.63–9.66 g). While crayfish sex and reproductive status are known to affect shelter defense in crayfish (Figler et al. 2005), crayfish were tested singly in treatment tanks and no previous literature had described a differential response to elevated $p\mathrm{CO_2}$ between sexes. As a result, we did not collect data on crayfish sex. The crayfish from each collection event were divided into three, 37.85-L holding tanks ($0.5 \times 0.25 \times 0.25$ m) per acclimation/treatment (nine tanks in total) resulting in a maximum of 5 crayfish per tank. Each tank included one terracotta pot (15.2 cm height $\times$ 13.3 cm diameter) placed on its side on the bottom of the tank, with the top opening facing toward the center of the tank. The tanks did not have any form of substrate. An Aqueon 06080 QuietFlow filter (Central Aquatics, Franklin, Wisconsin, USA) was run continuously to remove solid wastes from the holding tank. Weekly water changes, filter cleanings, and water quality measurements (Ammonia, pH, and Nitrite) were conducted using a Freshwater Master Test Kit (API®, item #34, Chalfont, Pennsylvania, USA). The tanks sides were covered in polyurethane, and tops were covered in blackout lids to minimize potential environmental stressors. Blackout lids were removed at 9:00 every day and placed on top of tanks at 16:00 to simulate a typical diel cycle in Louisiana, USA. Holding tanks each received approximately 10 shrimp pellets on a regular schedule every other day to both minimize cannibalism and to ensure minimal variation between experimental treatments due to hunger or growth in individual crayfish. Crayfish were housed in holding tanks for a 1-week acclimation period prior to the start of experiments.

$p\mathrm{CO_2}$ exposure

Crayfish were exposed for a period of 8 days to one of three levels of dissolved $\mathrm{CO_2}$: Control (ambient; < 100 μatm), Low (6853 ± 1206 μatm), and High ($19{,}086 \pm 2043$ μatm) (Table 1). We selected ~ 7000 μatm as a Low $p\mathrm{CO_2}$ exposure level because the current modeled estimate of median global freshwater stream and river $p\mathrm{CO_2}$ is ~ 3100 μatm (Raymond et al. 2013) and is expected to increase under future climate change (e.g., increase commensurate with atmospheric $\mathrm{CO_2}$ (Phillips et al. 2015) and predicted increase of 16.03 ± 5.69 μatm per year based on lentic systems [Weiss et al. 2018]). We selected $\sim 19{,}000$ μatm as a High $p\mathrm{CO_2}$ exposure level because we wanted to test a relatively extreme scenario. However, freshwater $p\mathrm{CO_2}$ is known to vary dramatically (Hasler et al. 2016a) and eutrophic systems have been measured with $p\mathrm{CO_2}$ values as High as 40,390 μatm (Balmer and Downing 2011), so our value of 19,000 μatm, while relatively extreme, is not unrealistic. An 8-day exposure period was chosen as previous studies on the impacts of $\mathrm{CO_2}$ have demonstrated behavioral modifications after only four or 5 days of acclimation (Munday et al. 2010; Hasler et al. 2016b; Tix et al. 2017b). Elevation in $p\mathrm{CO_2}$ was

Table 1 Partial pressure of carbon dioxide to which crayfish were acclimated prior to behavioral trials

| Treatment	$p\mathrm{CO_2}$ (μatm)	No. of trials
Control	Ambient (< 100)	17
Low $\mathrm{CO_2}$	6853 ± 1206	14
High $\mathrm{CO_2}$	$19{,}086 \pm 2043$	14

Data are shown as mean $\pm$ SD

achieved through the common method of bubbling compressed CO_2 into each holding tank through an airstone (Brauner et al. 2000). Dissolved CO_2 targets were maintained within holding tanks by monitoring pCO_2 with a modified Vaisala infrared carbon dioxide meter (GMT221, 0–20%, Vaisala, Vantaa, Finland; Johnson et al. 2010) and bubbling compressed CO_2 into the holding tanks as needed; CO_2 additions began 8 days prior to the beginning of a trial. Partial pressures of CO_2 were monitored every 6 h (6:00, 12:00, 18:00, 0:00) for the duration of the study period, and, when off gassing occurred (i.e., a fall in $pCO_2 \geq 1000$ µatm below target levels, as read on the Vaisala meter for either Low or High treatments), additional CO_2 was added to return the tanks to the target pCO_2. Throughout the entire study, both ammonia and nitrite levels remained ≤ 1 ppm. The pH ranged 7.4–8.2 with changes commensurate with pCO_2 level. After 8 days of CO_2 exposure, crayfish were challenged in a behavioral assay.

Behavioral Assay

A behavioral assay was conducted to quantify the impact of the exposure to elevated CO_2 on shelter-seeking behavior in Red Swamp crayfish. To start, individual crayfish were carefully netted from their CO_2 exposure tank and transferred to an 18.9 L treatment aquarium (Fig. 1). The pCO_2 in the experimental aquarium was similar to the pCO_2 that the crayfish experienced during the exposure period, which was achieved using methods identical to those described above. Individual crayfish were placed in the center of tank to start each trial (although they were dropped into the tank and did not always land exact in the center as they often swam upon entry into the treatment tank). A small, black cup (10 cm diameter opening that tapered to 5 cm at the bottom) with a dark interior was placed on its side near one end of the treatment aquarium and acted as a shelter. The experimental aquarium was divided into three sections which described crayfish proximity to the shelter. As a result, there were four locations that the crayfish could occupy: (1) away from shelter (the crayfish was within the marked zone opposite the shelter); (2) middle of the tank (the crayfish was within the marked zone in the middle third of the tank); (3) adjacent to shelter (the crayfish was within the marked zone in the third of the tank with the shelter but was not within the shelter); and (4) inside the shelter (the crayfish had at least one part of its body within the shelter, or was under the shelter) (Fig. 1). The distances from the shelter were delineated by a marked piece of cardboard that was placed under the glass bottom of the tank and visible to the observer. Crayfish were allowed to acclimate to the treatment aquarium for 5 min (Pintor et al. 2008), after which time crayfish proximity to shelter was recorded every 5 s for 2 min. Two minutes was decided to be an appropriate trial period because crayfish used in practice trials for longer periods of time tended to stop moving considerably after 3 min of observation. Similar observation periods have been used in a past study on the effect of temperature on Red Swamp Crayfish behavior (Zhao and Feng 2015). Additionally, decreased exploration over time, especially in familiar environments, has

Fig. 1 Diagram of the treatment aquarium for quantifying shelter-seeking behavior crayfish exposed to elevated carbon dioxide. Numbers indicate the zones of the treatment aquarium described in the Behavioral Assay section of methods. The height of the tank was 25 cm, the width was 21 cm, and the length was 40 cm. Zones *1–3* were 13.33 cm wide
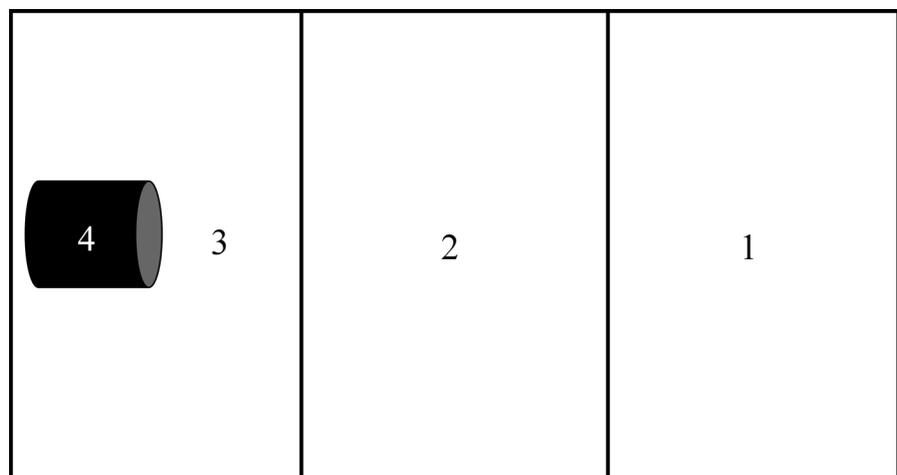
been described in crayfish (Shuranova et al. 2005). Over the course of the experiment two observers took shifts in recording data; however, because of the simplicity and impartiality of observations, it was assumed that there would not be an observer effect. Finally, to ensure that no crayfish were tested twice, the crayfish were not returned to their original exposure tank until all crayfish from that exposure tank had been tested.

Statistical analyses

The proportion of time that crayfish spent in each section of the tank for the entire 2-min monitoring period was compared within and across treatments using beta regression. Beta regression can be used when response data assumes values within the interval (0,1) (Cribari-Neto and Zeileis 2010; e.g., White et al. 2017), and the beta regression models examined the null-hypothesis that proportion of time spent in each section of the tank did not change between or within treatments. Beta distributions tend to describe more variation near the mean with less variation near its limits and as a result are more appropriate for proportion data than a Gaussian distribution, which assumes normally distributed variation. Our observed data (y_i) included zeros and ones and as a result were transformed by

$$y = \frac{[y_i(n-1) + 0.5]}{n}$$

which modifies each proportion of time to allow for the inclusion of these extreme values without affecting model outcomes (Smithson and Verkuilen 2006). In addition to the beta regression, we compared the number of times that each crayfish crossed the boundary between sections (also known as activity) using a zero-inflated Poisson (ZIP) model (Jackson 2017). A ZIP model was used because the response data were positive integers (counts) and because a number of crayfish did not cross a boundary during the experiment; therefore, there were a large number of zeros in all treatments. Finally, we tested for the differences in the mean carapace length between treatments using an ANOVA and we also tested for an interacting effect of crayfish size within and between treatments in all models run. All analyses were completed in R (R Core Team 2013).

Results

Throughout the entire study period, four crayfish died in High pCO_2 holding tanks, one crayfish died in the Low pCO_2 holding tanks, and none died in the control holding tanks. Water quality was examined after each mortality; however, no measure was greater than ambient (NH_4 and $NO_2^- \leq 1$ ppm, pH = 7.4–8.2). Crayfish size differed between treatments ($F_{3,42}=168.9$, $p < 0.001$); however, there was no significant interaction effect of crayfish carapace length on location ($p > 0.5$), which allowed us to focus analyses on the main effects of pCO_2 treatment and crayfish location.

Crayfish spent variable amounts of time at a different proximity to the shelter in the treatment aquarium depending on the pCO_2 to which they had been acclimated. Crayfish acclimated to ambient pCO_2 for 8 days spent more time close to the shelter, but not in it ($z = 3.477$, $p < 0.001$; Fig. 2), and, when compared across treatments, crayfish held at ambient pCO_2 spent more time close to the shelter than the other treatments ($z = 3.683$, $p < 0.001$). When crayfish were acclimated to 19,000 μatm pCO_2 for 8 days,
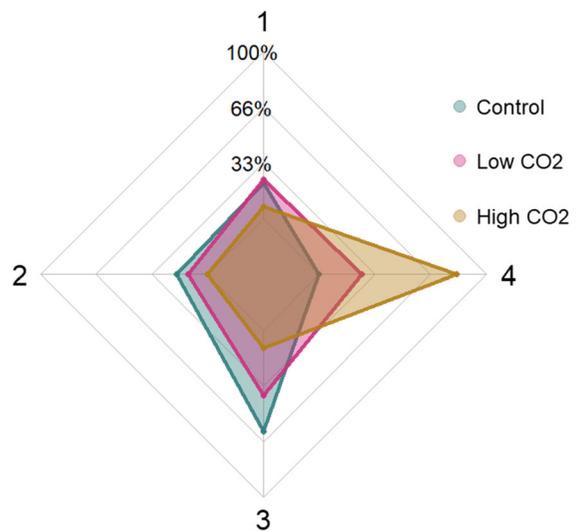

Fig. 2 Radar plot showing the proportion of a 2-min trial that Red Swamp crayfish (*Procambarus clarkii*) resided in each zone of the treatment aquarium following placement in CO_2 treatments shown in Table 1. Each treatment is described by a different color polygon where the position of the nodes to the polygon represents the proportion of time spent in each location of the treatment aquarium (numeric locations references in Fig. 1 and the Behavioral Assay section of methods)

they spent more time within the shelter itself ($z = 5.667$, $p < 0.001$) relative to other areas of the treatment aquarium and also spent more time within the shelter relative to the other treatments ($z = 4.565$, $p < 0.001$). Crayfish acclimated to 7000 µatm (Low) pCO_2 for 8 days spent equal amounts of time throughout the aquarium ($z = 1.065$, $p > 0.25$) and did not show differences in the proximity to shelter when compared to the other treatments ($z = 1.445$, $p > 0.1$). Finally, although crayfish size differed between treatments, there was no effect of carapace length on crayfish location within the tank within or between treatments ($p > 0.5$).

The number of boundaries that crayfish exposed to High CO_2 crossed between sections was not significantly different from zero ($z = -0.086$, $p = 0.88$), while those exposed to ambient CO_2 ($z = 5.525$, $p < 0.001$) and Low CO_2 ($z = 6.378$, $p < 0.001$) were significantly greater than zero. Once again, carapace length had no effect on the number of boundaries crossed within or between treatments ($p > 0.5$).

Discussion

Our study sought to quantify the effects of elevated pCO_2 on shelter-seeking behavior in Red Swamp crayfish. Following an 8-day acclimation period to elevated pCO_2 (19,000 µatm), crayfish increased their time in hiding and decreased their overall activity when compared to Low pCO_2 (7000 µatm) and Control (ambient pCO_2) treatments. It is worth noting that the elevated CO_2 treatment represents a relatively extreme scenario, although it is still well within the range of pCO_2 experienced in freshwater systems (Balmer and Downing 2011). Behavioral assays have been used in the past to examine hiding behavior in various species including crayfish and crickets (Biro and Sampson 2015; Hedrick and Kortet 2006). Additionally, the novel environment assays, similar to those used in our study, are relatively common in behavioral research. For example, similar methods have been used to examine fathead minnow responses to predator chemical cues (Chivers and Smith 1995) and to link standard metabolic rates of common lizards (*Zootoca vivipara*, Lichtenstein, 1823) with activity, aggressiveness, and risk-taking behaviors (Mell et al. 2016). Our results complement those of previous research by Bierbower and Cooper (2010), who

showed that Red Swamp crayfish will avoid elevated concentrations of carbon dioxide, a behavior hypothesized to be related to either escaping degraded water quality, or avoiding negative physiological consequences from environmental CO_2 (or both). In addition, shelter-seeking behavior for crayfish has previously been shown to occur in response to predation risks (Englund and Krupa 2000) and to attract mates (Bergman and Moore 2003), but also in response to changes in the environment, which helps them to avoid negative environmental stimuli (e.g., increasing temperature; Huner and Barr 1991). During certain conditions such as droughts, Red Swamp crayfish have even demonstrated burrowing behavior in an effort to avoid the environmental stressor, effectively creating their own shelter (Kouba et al. 2016). Crayfish are also commonly involved in intraspecific competition for space, which can induce conflict and end in cannibalism if the quantity of suitable shelters is limiting (Figler et al. 1999). This intra-specific competition also defines social hierarchical structures (Figler et al. 1999; Fero et al. 2007).

Freshwater ecosystems have high variability in pCO_2 (236–9894 µatm as reported in Crawford et al. 2017), and recent studies suggest elevations in pCO_2 are possible (Moss et al. 2011; Hasler et al. 2016a; Weiss et al. 2018). Should aquatic pCO_2 rise in the future to levels that approach 19,000 µatm or higher, an increase in shelter-seeking behavior and decrease in activity level may be expected. This increase may be particularly true for some freshwater basins in Louisiana, which experience a high amount of eutrophication—a driver of some extreme pCO_2 levels (Balmer and Downing 2011; Hopkinson and Day 1980). In turn, this change in behavior may translate into a number of potential outcomes for aquatic ecosystems. For example, dominant (larger) crayfish tend to spend less time in shelters than subordinate crayfish (Fero et al. 2007); however, if this hierarchy shifts and competition for shelter increases, it may leave the smaller crayfish more exposed to predation. In addition, $GABA_a$ receptors have previously been shown to influence multiple processes for crayfish, including tail-flip behavior (Vu and Krasne 1993; Edwards et al. 1999) and anxiety (Fossat et al. 2014); if increased shelter-seeking behavior observed in this study is related to $GABA_a$ receptor impairment as has been shown in fishes (Fuiman and Magurran 1994; Nilsson et al. 2012), crayfish in waterbodies with

rising pCO$_2$ may be more at risk of predation due to limited ability to escape through altered tail-flip propulsion strength (Edwards et al. 1999). Additionally, some native crayfish have shown lower resilience to environmental stressors and limited abilities to compete with invasive crayfish, such as Red Swamp Crayfish (Antonelli et al. 1999; Gherardi and Daniels 2004; Kouba et al. 2016), and competition may be further limited by an inability to seek shelter. If crayfish are more likely to seek shelter in environments with higher pCO$_2$, they may also encounter increased rates of conflict and cannibalism. An environmental stressor such as elevated pCO$_2$ may also increase the energetic demands required to maintain biochemical homeostasis of individual crayfish (Jeffrey et al. 2017a), which could potentially reduce foraging efficiency and growth (Edwards et al. 2013; Whitledge and Rabeni 2002). Finally, couched in terms of acidification, increasing pCO$_2$ may also impair molting, growth, and reproduction, by shunting energy toward homeostatic maintenance, ultimately leading to crayfish extirpation (Beaune et al. 2018). Hence, our findings suggest that augmented shelter-seeking behavior in elevated pCO$_2$ environments may have far-reaching effects on crayfish populations and aquatic ecosystems.

Our results add to previous work in both the freshwater and marine environments that have shown that exposure to elevated carbon dioxide can result in altered behavior for many species of both vertebrates and invertebrates (de la Haye et al. 2012; Fuiman and Magurran 1994; Hasler et al. 2017b, Hannan et al. 2016b, c; Nilsson et al. 2012; Tix et al. 2017a). For example, Nilsson et al. (2012) detected increased boldness and activity in larval fish (*Amphiprion percula,* Lacepède, 1802 & *Neopomacentrus azysron*, Bleeker, 1877*)* in High pCO$_2$ environments due to impairment of GABA$_a$ receptor functionality. Additionally, de la Haye et al. (2012) found decreased activity levels and impaired information gathering and decision making in hermit crabs (*Pagurus bernhardus*, Linnaeus, 1758*)* when exposed to increased pCO$_2$. Clearly, elevated pCO$_2$ effects species differently and is likely dependent on interactions between physiology and environment. However, it is important to note that our results might not be limited to behavioral modifications as seen in the above studies, as other studies on aquatic invertebrates in raised pCO$_2$ environments have demonstrated impaired development at larval stages (Walther et al. 2010; Arnold et al. 2009). Overall, our finding that elevated pCO$_2$ changed shelter-seeking behavior and decreased activity levels in crayfish is consistent with the findings of several studies that have shown aquatic organisms are impacted by elevated pCO$_2$, and there are potentially other impacts not quantified here.

Our study demonstrated a pronounced increase in shelter-seeking behavior and decrease in activity level of Red Swamp crayfish in High pCO$_2$ environments when compared to crayfish in ambient and low levels of pCO$_2$. It is possible that the observed behavioral change with increased pCO$_2$ could increase mortality rates of crayfish via competition or predation. However, additional work is needed to define other behaviors that may change with increased CO$_2$—such as feeding rates, tail-flip escape behaviors, reproduction, and hierarchy formation, as well as how differing partial pressures and exposure durations interact to drive responses. Changes in behavior, physiology, or development could have deleterious effects on crayfish populations and, subsequently, the ecology of many freshwater systems.

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