Research Article

Laboratory trials to evaluate carbon dioxide as a potential behavioral control method for invasive red swamp (*Procambarus clarkii*) and rusty crayfish (*Faxonius rusticus*)

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Abstract

Few effective strategies are available to control invasive crayfishes. Carbon dioxide (CO_2) acts as a behavioral deterrent for invasive fishes and could be a useful crayfish control tool. The objective of this laboratory study was to quantify CO_2 concentrations that caused red swamp crayfish (RSC; *Procambarus clarkii*) and rusty crayfish (RYC; *Faxonius rusticus*) avoidance behavior, altered emergence behavior, and caused loss of equilibrium. Behavioral endpoints were quantified under light and dark conditions and at 10 and 24 °C. Avoidance responses from both species varied widely. Under light conditions, 35 mg/L CO_2 was needed to induce the first avoidance shuttle in both crayfish species at 10 °C. CO_2 concentrations of 42 mg/L for RYC and 46 mg/L for RSC were required for first shuttle at 24 °C. The first avoidance shuttle was induced at 37 mg/L CO_2 for RYC and 54 mg/L CO_2 for RSC at 10 °C in the dark. At 24 °C, 44 mg/L CO_2 was required for first shuttle for both species. Less CO_2 was needed to cause the last avoidance shuttle in RYC compared to RSC at both temperatures and under both lighting conditions. RSC emergence occurred at 418 ± 77 mg/L CO_2, and loss of equilibrium occurred for both species at 1,231 ± 201 mg/L CO_2. RYC appeared to be more sensitive than RSC to CO_2, but behavior did not differ among light and water temperature treatments. These results demonstrate that CO_2 alters crayfish behavior. The CO_2 concentrations identified during this study may inform field testing to develop CO_2 as a potential control tool for invasive crayfishes.

Key words: invasive crayfish, behavioral deterrent, invasive species control

Introduction

Introductions of invasive crayfishes into novel habitats potentially disrupt the receiving ecosystem and cause economic damage by decreasing species diversity and abundance of macrophytes, invertebrates, amphibians, native crayfish, and fish species (Twardochleb et al. 2013). Unfortunately, the desire for economic gain is the predominate mechanism for the spread of invasive crayfishes across the world (Loureiro et al. 2015). Humans have introduced crayfish into non-native habitats through aquaculture, aquarium trade, bait industries, and as a biological control of disease hosts. Red swamp crayfish (RSC) (*Procambarus clarkii* Girard, 1852) are one example of a crayfish species introduced outside their native range as ornamental pets or as a human food source (Lodge et al. 2012; Loureiro et al. 2015). Subsequent escapes and releases contributed to their widespread presence in once novel habitats.

Two common invasive crayfish species in North America are the RSC and the rusty crayfish (RYC) (*Faxonius rusticus* Girard, 1852), formerly *Orconectes rusticus* (Crandall and De Grave 2017). The native range of RSC in the U.S. is along the Gulf coast from the Florida panhandle to Mexico extending north along the Lower Mississippi River drainage to Illinois. As of 2018, RSC are considered invasive in at least 17 U.S. states along the Upper Mississippi River drainage (Loureiro et al. 2015) and have continually expanded across the U.S. states from the 1920s to the present (Nagy et al. 2018). RSC burrowing behavior appears to be related to reproduction and rearing of young, and as a way to avoid predation and environmental stressors (Correia and Ferreira 1995; Souty-Grosset et al. 2014). Their burrowing behavior is often destructive and can cause bank collapse, damage to levees and water control structures (Hobbs et al. 1989), and increased erosion and suspend solids (Lodge et al. 2012). The native range of RYC in the U.S. is likely within the Ohio River drainage (Hobbs et al. 1989) with expanded invaded ranges in over 20 states and into Ontario, Canada (Lodge et al. 2012). RYC have been expanding their range since the 1930s and within the first 20 years of their invasion in Wisconsin they increased from 7% to 36% of all crayfish occurrences (Olden et al. 2006). RYC are so prevalent that within 5 years of discovering them in the John Day River in Oregon their range had doubled to 145 river km (Sorenson et al. 2012). Prevention and control methods are needed to reduce or eliminate range expansion and reduce ecosystem impacts caused by these species.

Potential control tools for invasive crayfish include chemical toxicants, mechanical removal, physical removal, biological controls, and legislative action (Manfrin et al. 2019). As of 2019, there are no registered toxicants for invasive crayfish. Pyrethroids have shown promise as a chemical control tool (Morolli et al. 2006; Cecchinelli et al. 2012) but are toxic to other desirable aquatic invertebrates (Loureiro et al. 2015). Mechanical removal includes trapping, netting, and electrofishing. Traps are effective at reducing the population of invasive crayfish (Hein et al. 2007), but requires expenditure of human capital and may have a bias toward size and sex (Ulikowski et al. 2017; Loureiro et al. 2018). Additionally, continuous removal efforts may result in increased growth rates and an increase in population size. (Loureiro et al. 2018). Physical removal through pond desiccation or draining is one approach that could enhance removal from isolated areas, but extended survival of RSC for up to 3 months out of water makes this extremely challenging (Hobbs et al. 1989). Biological control can be achieved by stocking predators such as the European eel (*Anguilla anguilla* Linnaeus, 1758) (Aquiloni et al. 2010) or sportfish such as largemouth bass (*Micropterus salmoides* Lacepède, 1802) and channel catfish (*Ictalurus punctatus* Rafinesque, 1818) into invaded ponds (Gherardi et al. 2011; Musseau et al. 2015). However, biological control may not be practical in all areas. Legislation has been passed in many locations to prevent the introduction, sale, or possession of invasive crayfish. This method is most effective when used proactively (Dresser and Swanson 2013). None of these methods would result in eradication of an established population, thus an integrated pest management plan would likely be the most effective mechanism of control.

Carbon dioxide (CO_2) has shown promise in controlling invasive and nuisance fishes (Wu and Bridges 2014; Cupp et al. 2017b, c) and could be an effective tool in an integrated pest management program to control invasive crayfish. Laboratory and pond studies using telemetered fish conclusively demonstrate that fish avoid waters with elevated CO_2 (Kates et al. 2012; Dennis et al. 2016; Cupp et al. 2017a). Additionally, CO_2, in the form of dry ice, has been used in controlled pond studies with invasive silver carp (*Hypophthalmichthys molitrix* Valenciennes, 1844) and bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) to determine the efficacy of under-ice treatments as a control method (Cupp et al. 2018). Bierbower and Cooper (2010) observed RSC avoided CO_2-enriched water but they did not report effective concentrations. Rather, they demonstrated that CO_2 more effectively altered RSC behavior than hypoxia or acid-adjusted water equivalent to the pH at which CO_2 altered behavior. Altering RSC behavior with CO_2 could be useful for control purposes to enhance physiological disturbances, reduce feeding, or increase vulnerability to trapping.

Crayfish avoidance to CO_2-enriched water could be used in a control program to push crayfish into a smaller section of a pond to reduce the water volume of a chemical treatment, to provide a smaller area for trapping, or to aid in hand collection in water or after emergence. CO_2 may be useful to control invasive species in small urban ponds such as those found on golf courses or retention ponds with limited native species (Treanor et al. 2017). Under-ice treatments may be effective as a lethal control to reset an entire pond that could be restocked with desired or native species.

Several environmental factors could influence the behavioral responses of crayfish to elevated CO_2. For instance, RSC are more active in low light compared to daytime light levels (Page and Larimer 1972). Water temperature may also alter avoidance behavior in crayfish, similar to that seen in invasive round goby (*Neogobius melanostomus* Pallas, 1814) and bigheaded carps (*Hypophthalmichthys* spp.) (Cupp et al. 2017b; Tix et al. 2018). Therefore, understanding the responses of invasive crayfish to increasing CO_2 concentrations under varying temperature and light conditions is needed to optimize CO_2 as a control method. Additional research is needed to define the range of CO_2 concentrations that induce avoidance and loss of equilibrium (LOE) in invasive crayfish for managers to effectively use CO_2 for control.

The objectives of our study were to (1) determine avoidance responses of RSC and RYC to elevated CO_2 concentrations at two water temperatures and two light conditions and (2) determine the CO_2 concentration that causes LOE. Ideally, the concentration that alters behavior but does not result in sedation could be identified and further developed as a control tool in field tests before transferring the technology to resource managers.

Materials and methods

Study Animals

RSC (70.8 ± 7.3 mm; mean total length ± standard deviation, n = 89) were obtained from Carolina Biological Supply (Burlington, North Carolina, USA). Wild-caught RYC (44.2 ± 6.2 mm total length, n = 80) were collected from the North Fork Bad Axe River in Vernon County, Wisconsin, USA. Both species were held at the U.S. Geological Survey (USGS) Upper Midwest Environmental Sciences Center (UMESC), La Crosse, Wisconsin, USA in accordance with Wisconsin Department of Natural Resources (WDNR) NR 40 permit. Crayfish were held in two 378-L flow-through tanks covered with wire mesh to prevent escape and separated into halves to isolate species. All crayfish were held for at least 1 week before being used in the trials. Artificial habitat was made of polyvinyl chloride (PVC) pipe (7.6 cm length × of 3.2 cm diameter) and scattered throughout the tanks. The number of PVC habitats per tank were always more than the number of crayfish to reduce competition and cannibalism within species. Crayfish were fed 25 g of ground rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) daily per half tank. Leftover feed was removed by siphoning before fresh feed was added. Molted exoskeletons were periodically left in the holding tanks to be fed upon by the crayfish to improve health (Lodge and Hill 1994). Holding tank temperature was either 12 °C or 24 °C. Crayfish were acclimated to 24 °C by changing water temperature no more than 3 °C per day. Two 10.5 W LED white light lamps (130–300 lux; 75W incandescent equivalent) were hung over the holding tank for a 12:12 light:dark cycle. A third 10.5 W lamp placed in the corner of the room pointing away from the holding tanks was on during the 12 h dark cycle to simulate natural night light conditions (0–2 lux).

Water Chemistry and CO_2 Standard Curve

Temperature (Thermapen Mk4 thermometer, ThermoWorks, American Fork, Utah, USA), dissolved oxygen (DO) and pH (HACH model: HQ40d meter, HACH Inc., Loveland, Colorado, USA), light intensity at the water's surface (Light meter MW700, Milwaukee, Rocky Mount, North Carolina, USA), and flow rates were measured daily in each holding tank. Temperature, pH, and DO were measured before and after each trial and lighting (lux) at the water's surface in each tank was measured from the shuttle

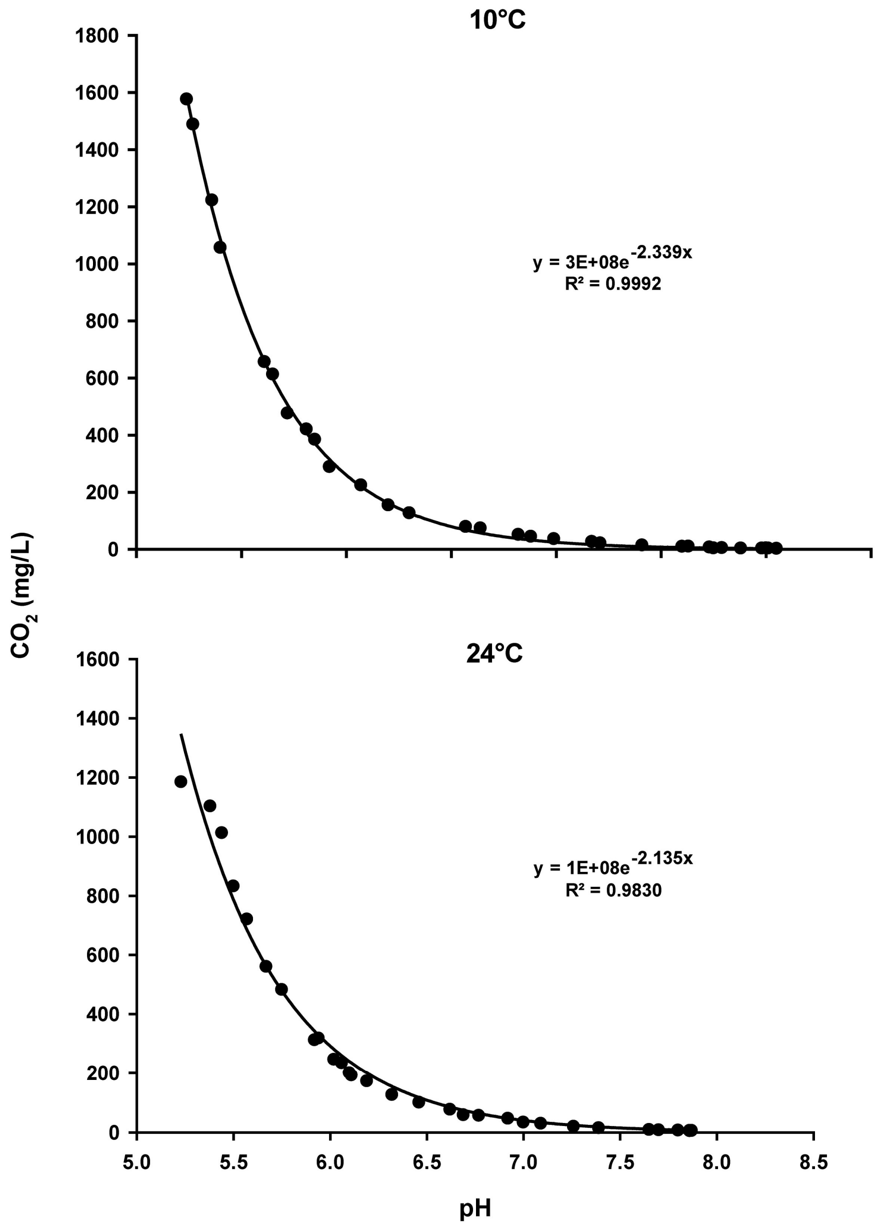

Figure 1. Relationship between pH and free carbon dioxide (CO_2) concentration (mg/L) completed before testing. These were used to estimate the free CO_2 concentration from the pH measurements taken in the shuttle box tanks during trials.

box before each trial (Supplementary material Table S1). Ammonia nitrogen (NH_3-N) and alkalinity (as calcium carbonate; $CaCO_3$) were measured at the beginning and end of each week with a HACH® Model: DR3900 spectrophotometer and TNT 830 and 870 test kits, respectively (HACH Inc., Loveland, Colorado). Hardness as $CaCO_3$ was recorded at the start and end of each week using standard methods (Rice et al. 2012). CO_2 was measured in the holding tanks twice a week and concentration was determined by titration to a pH 8.3 endpoint with 0.3636 N or 3.636 N sodium hydroxide (NaOH) titrant (Rice et al. 2012).

Standard curves of the relation between pH and free CO_2 (mg/L) were developed for both temperatures (Figure 1) in SigmaPlot Version 13.0 (Systat Software, San Jose, California, USA). To construct the standard curves,

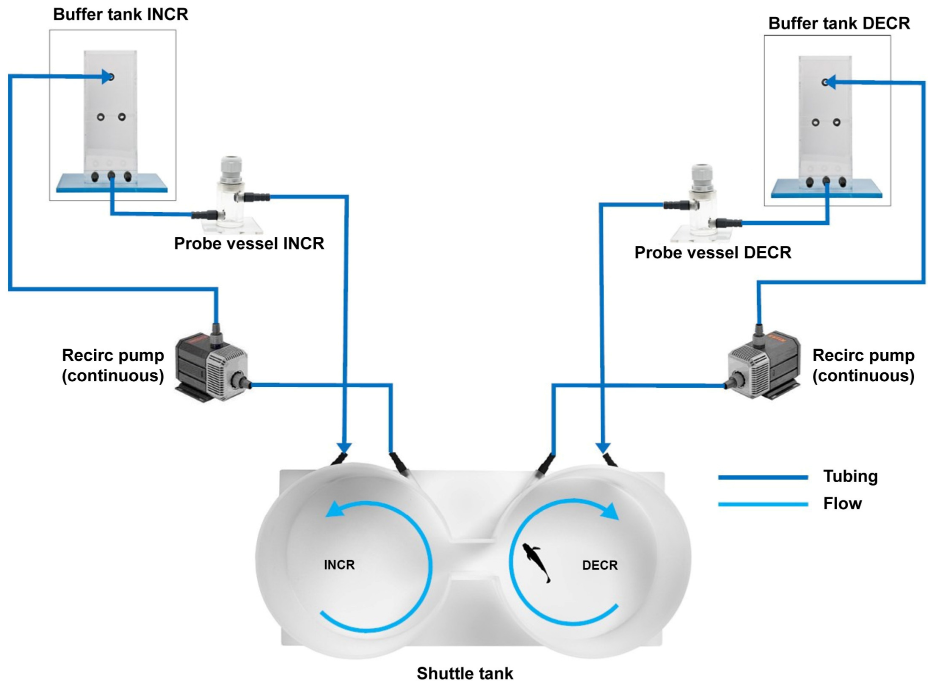

Figure 2. Schematic of the Loligo shuttle box system (www.loligosystems.com; used with permission). Two circular tanks are connected by a channel. Each tank is independently fed from a buffer column that is infused with either room ambient air or CO_2. Water recirculates continuously between the buffer column and the tank. Increase (INCR) indicates a side of the system being injected with CO_2 and decrease (DECR) indicates a side injected with room air.

the shuttle box was filled with temperature-conditioned well water and baseline water chemistry parameters mentioned above were measured. CO_2 was then injected into the shuttle box system at a rate of 1 L/min. Water samples were collected and capped at timed intervals and used to determine temperature, pH, and CO_2 concentration at each interval. The pH at each CO_2 concentration was then plotted and a regression equation generated. To extend the curve beyond the saturation achieved in the shuttle box to accommodate the CO_2 concentration attained during loss of equilibrium trials, CO_2 was injected into a smaller volume of water and water samples collected at timed intervals. This method was repeated to develop standard curves for both 10 and 24 °C water.

Avoidance Trials

CO_2 concentrations that caused avoidance in RYC and RSC were determined using a shuttle box choice arena (Figure 2; Loligo Systems Inc., Viborg, Denmark). Fifteen trials were performed for each species at two water temperatures (10 and 24 °C) and at two lighting conditions (dark 40 ± 4 lux and light 173 ± 9) for a total of 120 trials following methods described in Cupp et al. (2017b) and Tix et al. (2018). If crayfish avoid CO_2, we expected they would spend less time in the side with a higher CO_2 concentration and have fewer crossing during infusion. Briefly, the test system consisted of two circular tanks (dimensions: 0.83 m diameter × 0.5 m depth) connected by a channel (dimensions: 0.10 m width × 0.20 m length × 0.5 m depth).

Each circular tank had an outflow drawn by small electric pumps to the top of a respective buffer column (dimensions: 0.21 m width × 0.21 m length × 1.00 m depth). Water then passed through the buffer column and returned to its respective circular tank by gravitational flow (3.5–3.6 L/min). The angles of the inflow and outflow caused circular mixing within the tanks to reduce unwanted mixing of CO_2 into the adjacent untreated tank. A digital video camera (acA1300-30gc, Basler, Ahrensburg, Germany) was installed above the shuttle box to record crayfish activity. The shuttle box was behind a black liner suspended from wall to wall to separate the test system from the observer and minimize behavioral changes resulting from activity in the room (Kates et al. 2012).

The shuttle box was filled with 270 L of predetermined temperature-conditioned well water (10 or 24 °C) to a depth of 0.24 m. Test temperatures were selected based on expected water temperatures where crayfish control measures are needed. The cold temperature was selected to test the control method for use in the spring or fall and the warm temperature was selected to test the control method for use in the summer months. Two lighting systems were installed above the shuttle box to provide appropriate lighting for dark and light avoidance trials. A 100 W red incandescent light simulated low-level light periods when crayfish are most active (Page and Larimer 1972) and a 56 W white incandescent light simulated high-level light periods when crayfish become less active (Gherardi and Barbaresi 2000).

A single crayfish was indiscriminately removed from a holding tank and randomly placed (determined by coin flip) in the center of one of the circular shuttle box tanks and allowed 30 min to acclimate. During acclimation the crayfish was free to move between the tanks. Water pumps for each buffer column ran continuously while crayfish were in the shuttle box. After the 30-min acclimation, the time and location of each shuttle was recorded for 30 min to establish baseline movement. A shuttle was defined as movement from one tank to the other when the longitudinal center of the crayfish crossed into the opposite tank. During acclimation and baseline behavioral observations, both buffer columns were supplied with room air via airstones at a rate of 1 L/min (Sweetwater, SL94A, Pentair, Apopka, Florida). After the 30-min baseline behavior observations, CO_2 gas (Airgas Inc., La Crosse, Wisconsin, USA) infusion began into the buffer column that supplied the side of the shuttle box occupied by the crayfish at a rate of 1 L/min. Room air was supplied to the other buffer column at a rate of 1 L/min. The time and location of each shuttle was recorded during CO_2 for 30 min. Total time a crayfish spent in the shuttle box was 90 min. A pH probe recording at 10-sec intervals was placed in each tank between the inflow and outflow at mid-depth to record pH during the trial.

Morphometric data (weight, total length and carapace length [rostrum to end of carapace]) and sex were recorded after each trial (Table S2). After morphometric measurements were recorded, crayfish were euthanized in accordance with UMESC internal animal care and standard operating procedures.

Emergence Trials

Emergence trials were conducted to determine if altered environmental conditions would force crayfish from their environment if they had no other option to escape. Due to limited numbers of RYC, only RSC were tested for emergence. We expected RSC would remain submerged during baseline observations and that they would climb the incline during infusion to avoid CO_2-enriched water. A total of nine trials were conducted with RSC to determine the concentration of CO_2 that caused emergence from the shuttle box. The shuttle box used in avoidance trials had the channel blocked on one end with a piece of clear acrylic to isolate one tank. A piece of perforated (0.6-cm hole size for crayfish to grip) grey PVC sheet was cut to 113 cm long and 8.9 cm wide. The length of perforated PVC was heated to allow for bending and modified to provide a 20.3-cm long flat area on the bottom of the tank that led to an incline that led to a 21.6 cm long plateau positioned 1 cm below the water surface on each side of the plateau. The two 35° inclines were each 25.4 cm long. The plateau height was 14.6 cm. Two 3.2-cm diameter PVC T's were placed at each side of the tank perpendicular to the incline to act as artificial habitats for crayfish during testing. These T-PVC habitats had adapters added to the upright hole that could accommodate a pH probe. O-rings were placed around the pH probe to regulate depth and prevent obstruction of crayfish movement.

The tank was filled with 85 L of temperature-conditioned well water to a depth of 15.1 cm. An electric pump was turned on to supply the buffer column with water. Temperature, pH, and DO were measured before and after each trial and lighting (lux) at the water's surface in each tank was measured before each trial. Ammonia, hardness, and alkalinity were measured at the beginning and end of each week (Table S1). All trials were performed in 24 °C water and in low-light conditions (about 38 lux) to simulate light conditions when RSC are most active (Page and Larimer 1972). A single RSC was netted from the holding tank, placed on the plateau of the incline, and given 30 min to acclimate. Baseline movement (time and location) was recorded for an additional 30 min. Locations of crayfish were recorded as free movement around the tank, inside PVC habitat, on incline, or out of water. The latter was defined as emergence of the thorax from water (Bierbower and Cooper 2010). After the 30-min baseline period, CO_2 was injected for 30 min at 2 L/min and behaviors were recorded. CO_2 was monitored by pH meters recording at 10-sec intervals in each PVC-T habitat. Morphometric data were recorded at the end of the trial as previously described (Table S2).

Loss of Equilibrium Trials

Ten LOE trials were performed for each species at both temperatures (10 and 24 °C). CO_2 concentrations required to achieve loss of equilibrium were determined following methods previously described (Kates et al. 2012; Cupp et al. 2017b; Tix et al. 2018). Briefly, a dark colored bucket was filled with 10 L of temperature-conditioned well water. A single 56 W light was hung above the testing area for all LOE trials so changes in behavior could be easily observed (81 ± 5 lux). pH, DO, and water temperature were recorded before and after each trial. Light intensity at the water's surface was recorded before each trail. Ammonia nitrogen, alkalinity, CO_2, and total hardness were recorded at the start and end of the week as previously described (Table S1). A single crayfish was placed into the bucket and allowed 15 min for acclimation. After acclimation, CO_2 was continuously injected at 1 L/min through a single airstone placed in the center of the bucket. After the crayfish lost equilibrium, time, pH, DO, and water temperature were recorded. LOE was defined as inability to remain upright but with ability to move (not narcotized). CO_2 concentration was later calculated based on pH and the standard curves for each temperature. Morphometric data were recorded as previously described (Table S2).

Data Analysis

Avoidance Trials

To compare the amount of time (sec) spent on each side of the tank (right and left) a generalized linear model (GLM) in R (Zar 2010; R Core Team 2017) was fit using a binomial error distribution with response being on either left or right side of the tank. The response variable (side of tank) was weighted by seconds while period (baseline, CO_2 injected on the right side and CO_2 injected on the left side) was included as a fixed effect.

To analyze the number of crossings from one side of the tank to the other, a GLM in R (Zar 2010; R Core Team 2017) was fit using a Poisson error distribution with the number of crossings being count data. Period (baseline and injection), species (RYC and RSC), temperature (10 and 24 °C), lighting condition (dark and light), and sex (male and female) were included as fixed effects. CO_2 concentrations that caused the first shuttle and last shuttle away from CO_2 were analyzed separately using a multiple linear regression model with species (RYC and RSC), temperature (10 and 24 °C), lighting condition (dark and light), and sex (male and female) included as fixed effects. Fitted residuals of all models were visually inspected using a normal probability plot to assess normality; assumption of homogeneity of variances was assessed across groups using a visual inspection of fitted residuals plots (Zar 2010). All avoidance data were analyzed in R (Zar 2010; R Core Team 2017) and plotted using the ggplot2 package (Wickham 2016).

Emergence Trials

Because crayfish never emerged from underwater during the baseline period, statistics on the total emerges and the CO_2 concentration that induced emergence could not be performed. Instead, basic summary statistics are provided to describe the emergence behaviors that were observed.

Loss of Equilibrium Trials

To determine the concentration of CO_2 needed to cause loss of equilibrium a two-way analysis of variance (ANOVA) was applied using species (RYC and RSC), temperature (10 and 24 °C), and their interaction as fixed effects (Sokal and Rohlf 2012). Visual inspection of fitted residuals using a normal probability plot was used to assess normality while assumption of homogeneity of variances was assessed across groups using a visual inspection of fitted residuals plots (Zar 2010). A Tukey's honest significant difference test was used to make pairwise comparisons of significant effects (Zar 2010). Loss of equilibrium data were analyzed in R (Zar 2010; R Core Team 2017) and plotted using the ggplot2 package (Wickham 2016).

Significance for all trials was defined as $p < 0.05$. Raw data supporting the analysis for all trials are available at https://doi.org/10.5066/P9R7AQVM (Fredricks et al. 2020).

Results

Water Chemistry

Water chemistry of UMESC well water that supplied the holding tanks and shuttle box was stable during the study. Water temperature for cold water trials was 12.4 ± 0.5 °C (mean ± standard deviation) and holding tank warm water temperature was 23.9 ± 0.5 °C. Holding tank pH was 7.91 ± 0.1, DO was 8.24 ± 1.1 mg/L, and lux during the 12 h light phase was 192.9 ± 45.0. Flow rates in the holding tanks were 2.7 ± 0.5 L/min. Water chemistry for the shuttle box is summarized in Table S1.

Avoidance Trials

CO_2 deterred both crayfish species from occupying water enriched with CO_2. Significantly less time was spent on the side of the shuttle box infused with CO_2. Crayfish spent 56% less time on the CO_2-injected side of the shuttle box compared to the 30-min baseline (p-value < 0.05; Table 1; Figure 3). Crayfish spent 25% more time on the air-injected side compared to the baseline (p-value < 0.05; Table 1; Figure 3). CO_2 also significantly reduced the number of tank crossings in crayfish (p-value < 0.001; Table 1). Crayfish displayed 2.1 times more crossings in 24 °C water compared to 10 °C water (p-value < 0.05; Table 1) and RSC crossed the tank 1.5 times more often than RYC (p-value < 0.05; Table 1). Crayfish also crossed fewer times in the dark compared to light (p-value < 0.05; Table 1). The number of crossings

Table 1. Statistical outputs (estimate standard error [S.E.], degrees of freedom [d.f.], *z* value and *p*-value) from the generalized linear models. A binomial error distribution was used for the amount of time rusty crayfish and red swamp crayfish spent on the left side while a Poisson distribution was used for the number of crossings during baseline and CO_2 infusion. Significant ($p < 0.05$) parameters are bolded.

Measured Variable	Model	Parameter	Estimate	S.E.	d.f.	*z* value	*p*-value
Time on left side	Binomial GLM	**(Intercept)**	**−0.08**	**0.00**	**467**	**−19.55**	**< 0.001**
		CO_2 on left side	**0.96**	**0.01**	**467**	**126.69**	**< 0.001**
		CO_2 on right side	**−0.61**	**0.01**	**467**	**−75.15**	**< 0.001**
Crossings	Poisson GLM	**(Intercept)**	**1.02**	**0.09**	**234**	**10.97**	**< 0.001**
		Injection*	**−0.18**	**0.05**	**234**	**−3.51**	**< 0.001**
		Species	**−0.38**	**0.06**	**234**	**−6.78**	**< 0.001**
		Temperature	**0.05**	**0.004**	**234**	**13.26**	**< 0.001**
		Lighting	**0.13**	**0.05**	**234**	**2.40**	**0.016**
		Sex	0.01	0.06	234	0.22	0.827

*Injection is defined as when CO_2 was infused on the side the crayfish occupied at the end of the baseline observations. During baseline observations both buffer columns received continuous infusion of room air.

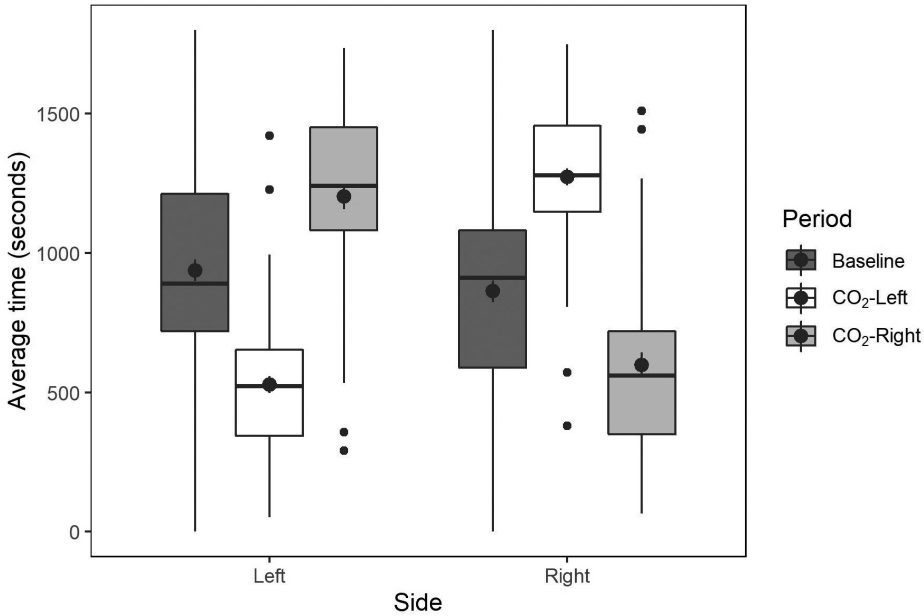

Figure 3. Average time (seconds) red swamp crayfish (RSC) and rusty crayfish (RYC) spent during the baseline (before CO_2 was added to either side) and during CO_2 injection (CO_2-Left or CO_2-Right) for all test temperatures and lighting conditions. The boxes represent the first and third quartiles with the interquartile range and the median (black line inside the box) between them. The whiskers represent the minimum and maximum values excluding outliers (black circles). The bolded circles within the boxes represent the mean and the lines coming from them represent the standard error. Fifteen crayfish (120 total) from each treatment combination were tested.

did not differ between sexes at either the baseline or CO_2-injection (p-value > 0.05; Table 1). Total crossing data are summarized in Table S3.

CO_2 concentration (35–54 mg/L) required to induce the first shuttle away from CO_2 were not significantly different among temperature and light conditions, or species and sex and light condition (p-value > 0.05; Table 2). However, it took a 63% higher CO_2 concentration to cause the crayfish to shuttle away from CO_2 for the final time at 24 °C (190–204 mg/L) compared to 10 °C (52–119 mg/L) (p-value < 0.05; Table 2; Figure 4). RSC also required a 45% higher CO_2 concentration to shuttle away from CO_2 for the final time compared to RYC (p-value < 0.05; Table 2; Figure 4). Lighting

Table 2. Statistical outputs (Estimate standard error [S.E.], degrees of freedom [d.f.], *t* value and *p*-value) from the multiple linear regression models (CO_2 concentration at first shuttle and CO_2 concentration at last shuttle for red swamp and rusty crayfish). Significant ($p < 0.05$) parameters are bolded.

Measured Variable	Parameter	Estimate	S.E.	d.f.	*t*-value	*p*-value
CO_2 concentration at first shuttle	**(Intercept)**	**45.96**	**11.91**	**115**	**3.86**	**< 0.001**
	Species	−2.93	8.39	115	−0.35	0.727
	Temperature	0.36	0.57	115	0.64	0.526
	Lighting	−7.15	7.94	115	−0.90	0.370
	Sex	−13.68	9.02	115	−1.52	0.132
CO_2 concentration at last shuttle	**(Intercept)**	**63.57**	**15.00**	**115**	**4.24**	**< 0.001**
	Species	**−56.63**	**10.55**	**115**	**−5.37**	**< 0.001**
	Temperature	**5.82**	**0.71**	**115**	**8.16**	**< 0.001**
	Lighting	−4.47	11.35	115	−0.39	0.694
	Sex	−8.84	10.00	115	−0.88	0.378

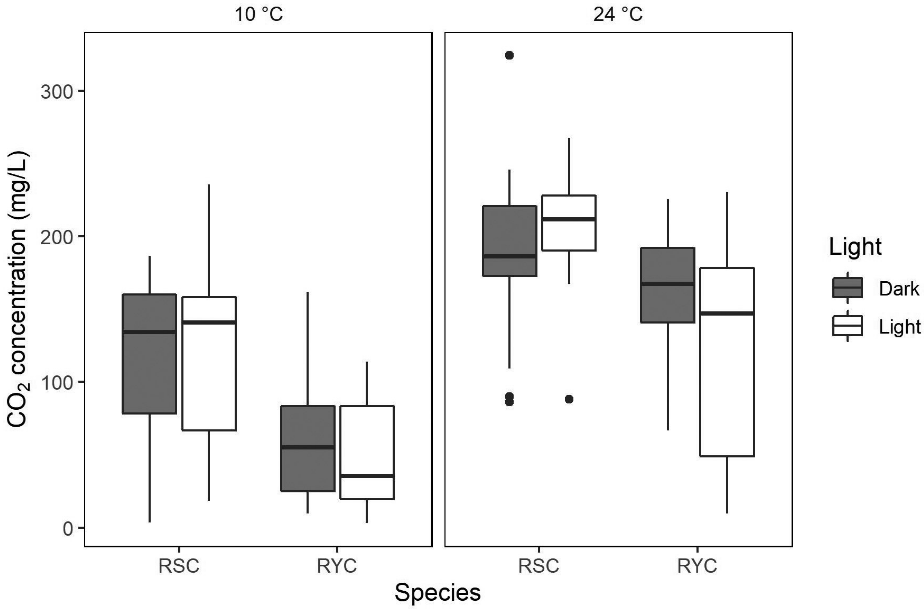

Figure 4. Carbon dioxide concentrations (mg/L) that induced the final shuttle away from the CO_2 injection. Red swamp crayfish (RSC) and rusty crayfish (RYC) were at two temperatures (10 and 24 °C) and lighting conditions. The boxes represent the first and third quartiles with the interquartile range and the median (black line inside the box) between them. The whiskers represent the minimum and maximum values excluding outliers (black circles). A total of 15 crayfish from each treatment combination were tested.

conditions and sex did not influence the concentration of CO_2 required for the final shuttle (*p*-value > 0.05; Table 2; Figure 4). Crayfish size across temperature and light did not contribute to differences in crossings within species (one-way ANOVA *F* values < 4.0, *p*-values > 0.05).

Emergence Trials

Because of limited numbers of RYC, only RSC were tested during the emergence trials. As expected, during baseline observations, crayfish remained submerged. Elevated CO_2 concentrations during injection caused RSC to emerge from the water a total of 43 times compared to no emergence during the 30-min baseline (Figure 5), with one exception. A single crayfish remained submerged throughout one trial. The average CO_2 concentration that caused RSC to climb out of water was 418 ± 77 mg/L.

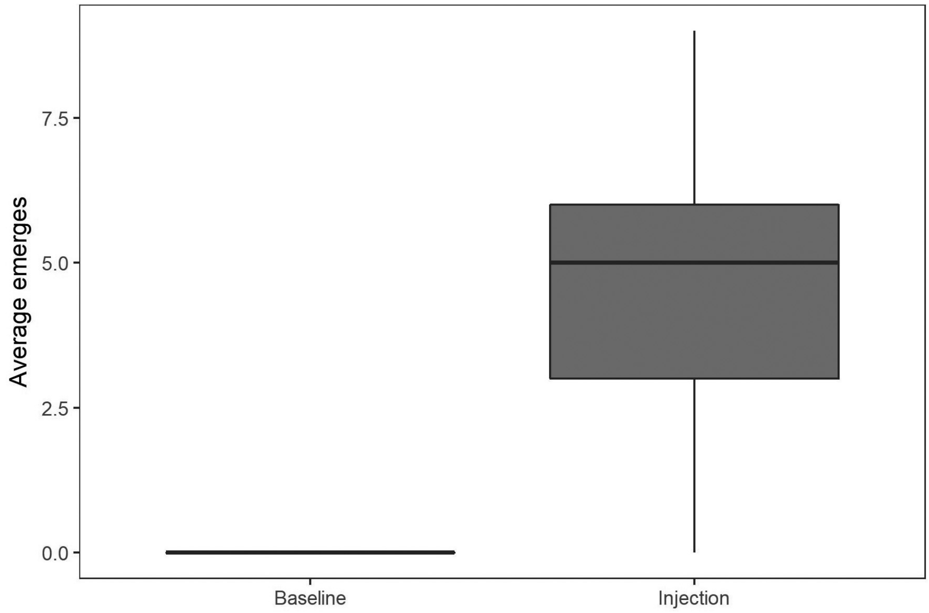

Figure 5. Average emergences from water for red swamp crayfish RSC (n = 9) at 24 °C during baseline and CO_2 injection. No crayfish emerged during baseline. The box represents the first and third quartiles with the interquartile range and the median (black line inside the box) between them. The whiskers represent the minimum and maximum values.

Loss of equilibrium Trials

The interaction of temperature and species was a significant predictor of CO_2 concentrations that resulted in crayfish loss of equilibrium (*p*-value = 0.005; Table 3; Figure 6). Although LOE was broadly induced at 1,231 ± 201 mg/L for both species, we observed LOE at 14% and 33% higher CO_2 concentration at 10 °C compared to 24 °C water temperatures for RSC and RYC, respectively (Tukey's HSD, *p*-value < 0.05; Table 3; Figure 6). Similar results were found between species acclimated to different temperatures. More specifically, higher CO_2 concentrations were needed to cause LOE for RSC acclimated to 10 °C relative to RYC acclimated to 24 °C, and RYC acclimated to 10 °C relative to RSC acclimated to 24 °C (Tukey's HSD, *p*-value < 0.05; Table 3; Figure 6). However, there was no difference between species at the same temperature (Tukey's HSD, *p*-value > 0.05; Table 3; Figure 6). No differences were found between sexes in the concentration of CO_2 that caused LOE (*p*-value > 0.05; Table 3).

Discussion

This laboratory study was designed to determine if invasive crayfish would avoid CO_2 and to establish concentrations that could be field tested to develop CO_2 as a potential invasive crayfish control tool. Both species of crayfish responded to a wide range of CO_2 concentrations during the avoidance trials. In 10 °C water, 35–54 mg/L CO_2 was needed to induce the first shuttle away from CO_2-enriched water. Similar levels of CO_2 (42–46 mg/L) induced the first shuttle in 24 °C water. During the 30-min baseline, crayfish explored both sides of the shuttle box and had more total crossings

Table 3. Results of a two-way analysis of variance (ANOVA; sum of squares [S.S.], degrees freedom [d.f.], *F*-value and *p*-value) examining the concentrations of CO_2 (mg/L) needed to induce loss of equilibrium (LOE) at two temperatures (10 and 24 °C) in two crayfish species (red swamp crayfish and rusty crayfish). Bold text indicates statistical significance ($p < 0.05$) for a main effect within the measured variable.

Measured Variable	Main effects	S.S.	d.f.	*F*-value	*p*-value
CO_2 concentration at LOE (mg/L)	**Temperature**	**848996**	**1**	**53.35**	**< 0.001**
	Species	390	1	0.02	0.877
	Temperature X Species	**139241**	**1**	**8.75**	**0.005**
	Residuals	572856	36		

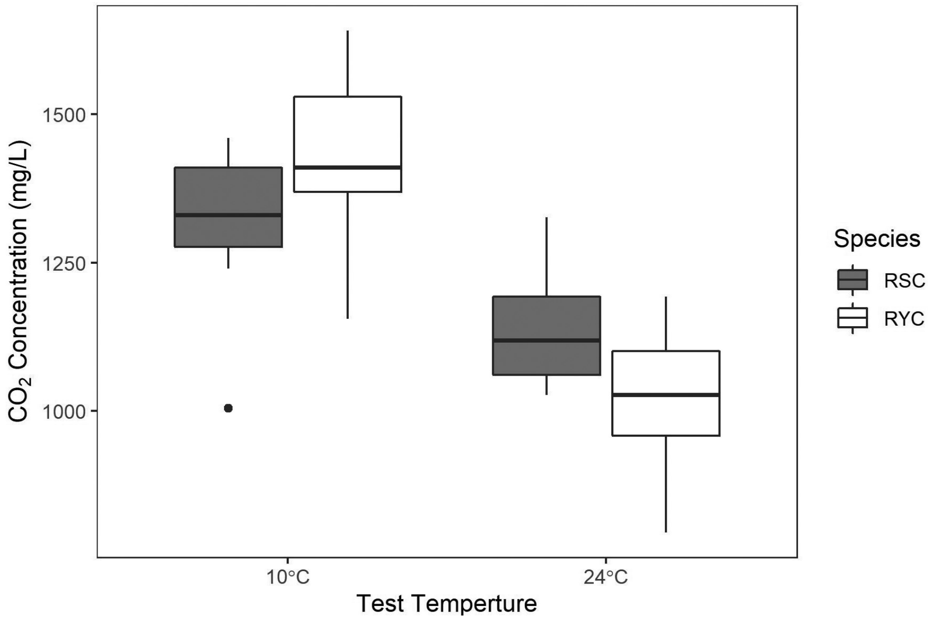

Figure 6. Carbon dioxide concentrations (mg/L) that induced loss of equilibrium (LOE) in red swamp crayfish (RSC) and rusty crayfish (RYC) at each test temperature (10 and 24 °C). The boxes represent the first and third quartiles with the interquartile range and the median (black line inside the box) between them. The whiskers represent the minimum and maximum values excluding outliers (black circles). Loss of equilibrium occurred at higher CO_2 concentrations for both species at 10 °C relative to 24 °C (*p*-value < 0.05. A total of 10 crayfish from each treatment combination were tested.

compared to the 30-min CO_2 infusion (Table S3). Although time between shuttles was not quantified, crayfish spent less time between shuttles as CO_2 concentrations increased on the infused side. Exploring behavior during infusion was expected if crayfish were attempting to avoid the CO_2-enriched water. This behavioral response could be used by resource managers if similar responses could be obtained during field trials. Increasing CO_2 at one end of a pond could potentially push crayfish to a more confined area where they could be trapped, removed by hand, or be exposed to a chemical control agent. Additional research would be needed in more natural settings to determine concentrations that effectively induce avoidance behavior. It would also be important to test native crayfish species to determine their responses to elevated CO_2.

Tail flipping (tucking end of tail under body and propelling backwards) and forward movement (walking) in crayfish are the primary avoidance responses that can be learned through external stimuli (Kawai et al. 2004). During avoidance trials both forward movement and tail flipping were observed when crayfish tested the CO_2 gradient between the two tanks. Crayfish can become habituated to external stimuli and reduction in avoidance responses can occur over time (Krasne 1969). While it is unlikely that crayfish would habituate to CO_2, prolonged exposure to CO_2 could result in reduction of quick tail flip movements and slower forward movement due to the sedative effect. This may inhibit their ability to escape treatment or capture zones.

As expected, warmer water increased activity in both species during the avoidance trials and there were a greater number of crossings at 24 °C compared to 10 °C water (Table S3). Surprisingly, a higher CO_2 concentration was required to cause complete avoidance of the CO_2-infused chamber at 24 °C than at 10 °C. A possible explanation is that because crayfish were more active at 24 °C, they explored and probed the opposite tank more often to find an escape route. Another possible explanation is that as the crayfish were more active at the higher temperature, low levels of CO_2 could have acted as an attractant and helped to draw the crayfish across the shuttle box, rather than the exploratory behavior alone resulting in a crossing. Low CO_2 levels are a known attractant to some insects (Bierbower and Cooper 2010). A low level of CO_2, slightly above the normal environmental range, may act as an attractant signaling a potential food source, which could partly explain the early exploring behavior of the crayfishes. No reports on CO_2 being a feeding attractant for crayfish were found in the literature and further research is needed to determine the influence of CO_2 on feeding behavior in freshwater crayfish.

During CO_2 infusion all but one RSC climbed the incline when CO_2 exceeded 400 mg/L, indicating that if given an escape route, crayfish will leave the water to avoid CO_2. No RSC emerged from underwater during baseline, though most climbed onto the incline at some point. Emergent behavior of RSC was similarly observed by Bierbower and Cooper (2010) during periods of hypoxia and altered water quality. Bierbower and Cooper (2010) speculated the emergence was a coping and survival mechanism. In this study, emergence was likely due to the elevated CO_2 and not hypoxia because DO during the trial was ≥ 5 mg/L (Table S1), which should not result in hypoxia. Emergent behaviors could play a role in establishing CO_2 as a possible crayfish control. CO_2 concentrations in a pond could be elevated to induce emergent behavior to increase capture efficiency of crayfish. Crayfish could be guided into pit traps as they leave water to enhance removal. However, RSC can traverse overland to seek new water bodies and survive for extended periods out of water. Care must be taken to prevent accidental spread during emergence in field applications of CO_2. Fencing or other containment methods warrant consideration around treated areas.

The avoidance behaviors to elevated CO_2 are not surprising because all fish species tested thus far have responded to increased CO_2 by avoidance (Kates et al. 2012; Donaldson et al. 2016; Cupp et al. 2017a, b; Tix et al.

2018). CO_2 (250–350 mg/L) successfully cleared predatory fish from the Tracy Fish Collection Facility in Byron, California (Wu and Bridges 2014). In laboratory trials largemouth bass and bluegill (*Lepomis macrochirus* Rafinesque, 1819) showed avoidance behavior to CO_2 at approximately 100 mg/L (Kates et al. 2012). Round goby avoided CO_2 concentrations of 99–169 mg/L in shuttle box behavior trials and lost equilibrium at 197–280 mg/L (Cupp et al. 2017b). In shuttle box behavior trials evaluating temperature dependent effects of CO_2, silver carp and bighead carp exhibited avoidance behavior from 110–141 mg/L and 117–162 mg/L and became narcotized from 115–206 mg/L and 188–278 mg/L, respectively (Tix et al. 2018).

Interestingly, we saw differences in crayfish species sensitivity to CO_2. RYC appeared to be more sensitive than RSC at 24 °C and less sensitive at 10 °C to concentrations of CO_2 that induced LOE (Figure 6). RSC exhibited more shuttling behavior than RYC during CO_2 injection (Table S3). The responses of native crayfish to CO_2 also would need to be established to determine nontarget effects. Species sensitivity may be important if CO_2 were to be implemented as part of an integrated pest management plan.

One difference between these invertebrate species and the vertebrates tested thus far is that a higher CO_2 concentration was required to induce complete avoidance in crayfish compared to fish species tested. This may be due to the behavioral adaptations of crayfish exposed to elevated CO_2. Shelter seeking behavior of RSC increased and activity level decreased when CO_2 in their water increased (Robertson et al. 2018). The lower activity level would be expected to reduce metabolic processes, such as respiration, to minimize oxygen demand. Thus, a greater CO_2 concentration may be required to saturate body fluids and cause the avoidance behavior. Further testing on the CO_2 levels in hemolymph required to induce avoidance would help define the mechanism for avoidance. Further research is also needed to determine the concentrations required for sedation in native fishes before CO_2 is used as a crayfish control tool.

A limitation of the equipment used to determine the first shuttle is that CO_2 from the infused side of the shuttle box slowly bled over into the non-CO_2 injected side causing the concentration to slowly rise to concentrations similar those that induced the first shuttle. The system will always maintain a substantial gradient of higher and lower CO_2 concentration between the sides but some of the behavioral responses, including number of crossing could have been affected by this because during the avoidance trials, crayfish had no options to avoid CO_2 other than by remaining on the side that had the least amount of CO_2. In a more natural setting, there may be areas of refugia (e.g. burrows, small inflows) that could alter behavioral responses to CO_2. These would need to be determined before this technology could be fully integrated into a control program.

We also identified CO_2 concentrations (> 1200 mg/L) that produced LOE in both crayfish species (Figure 6). The mechanism of action of CO_2 that results in lack of movement and LOE in crayfish appears to be both peripheral and centrally mediated. Peripherally, CO_2 blocks the glutamate response at the neuromuscular junction, which results in a paralytic effect (Bierbower and Cooper 2013). Centrally, the evoked response in motor neurons is reduced by CO_2, resulting in an anesthetic effect (Bierbower and Cooper 2013). For management actions, it would be important for CO_2 concentrations to be less than those that impair movement (the paralytic effect) and cause narcosis (the anesthetic effect). CO_2 concentrations that exceed those that cause LOE would inhibit crayfish from leaving a pond. Alternatively, if water clarity was reasonable, sedating crayfish may enhance manual removal, though the effort would be labor intensive.

Before CO_2 can be an effective management tool, field research is needed to determine range of CO_2 concentrations that could be effective in different naturally occurring water chemistries, as they may be different from those found in a laboratory trial. Effects of CO_2 on native species would also need to be considered. Reported CO_2 concentrations that induced LOE in fish ranged from 158–216 mg/L and were temperature-dependent (Cupp et al. 2017b; Tix et al. 2018). We determined that 335–488 mg/L CO_2 was needed to induce emergence by crayfish so it is likely that some fish species may lose equilibrium or become narcotized during a field application to control invasive crayfish. Levels of CO_2 that produce mortality in fish have not been thoroughly established and affected fish could be relocated to fresh water to promote recovery from sedation. There are limited data on the effects of CO_2 on other freshwater species. The 24-h lethal concentration 50 (LC50) value of 371 mg/L CO_2 for American bullfrog (*Lithobates catesbeianus* Shaw, 1802) larvae (Abbey-Lambertz et al. 2014) is higher than the CO_2 concentration needed to induce crayfish avoidance, but less than that needed for LOE. Waller et al. (2016) reported CO_2 toxicity values for native mussels but they were subject to continuous exposure for 28 days, which may not be comparable to CO_2 use in crayfish control. Mayfly larvae populations decreased after rotenone treatment but within 1 year, all species found before treatment were found in high numbers (Arnekleiv et al. 2001). Because CO_2 would not leave residues and would rapidly clear from a treated site, we would expect that aquatic insects adversely affected by crayfish control would rapidly similarly recolonize.

The effects of temperature and lighting condition on behavior are important considerations for planning management actions at times that optimize responses of crayfish, such as late summer and early fall. Because warmer water holds less dissolved gas, longer infusion times may be needed to reach concentrations that result in avoidance behavior. Treatments during warmer times of the year could also require more CO_2 because warmer water may increase plant growth and more CO_2 may be removed by aquatic vegetation during the photosynthetic process in the treatment pond.

In conclusion, RSC and RYC displayed avoidance behavior when exposed to CO_2-enriched water. Low-level CO_2 exposure could be useful to push crayfish to a confined area of the pond where manual removal or chemical treatments would be more efficient. Higher CO_2 concentrations could cause escapement from the water. This avoidance behavior may be useful in an integrated pest management plan to drive invasive crayfish into specific areas for trapping or toxicant application. Applying the toxic chemical terrestrially would help target the crayfish and avoid non-target mortality of other aquatic arthropods. Further research is needed in a more natural setting before this technology can be integrated into a pest management plan. Nontarget effects would need to be identified and responses of native crayfishes warrant consideration. There may be locations where CO_2 could be field tested (for example retention ponds that have limited aquatic life) to help inform how CO_2 can be incorporated into crayfish control protocols.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Baseline water chemistry and lighting condition before each carbon dioxide (CO_2) behavioral test for each species during each parameter condition tested.

Table S2. Morphometric measurements and weight after each carbon dioxide (CO_2) behavioral test for each species during each parameter condition tested (water temperature and lighting).

Table S3. Total number of crossing during avoidance trials for red swamp crayfish and rusty crayfish at each water temperature and lighting condition.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2020/Supplements/MBI_2020_Fredricks_etal_SupplementaryMaterials.xlsx