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The Effects of Acute Carbon Dioxide on Behavior and Physiology in *Procambarus clarkii*

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ABSTRACT Vertebrates and invertebrates show a similar response of rapid anesthesia with high levels of carbon dioxide. In this study, we use crayfish to examine both behavioral and physiological responses to increasing [CO_2] to explain the rapid unresponsiveness and cessation of an autonomic response. Hypoxic and low pH environments that are induced by CO_2 exposure were also examined, although neither produced the identified CO_2 effects. In insects, low concentrations play a vital role in providing information for task performance such as food location through attraction cues, whereas high concentrations produce avoidance responses. We found behavioral responses in crayfish that demonstrate a strong repellent effect to high [CO_2] and that the avoidance behavior decreases with lower [CO_2]. There was not a preference and/or repellent behavioral response with 5% CO_2, hypoxic or low pH environments. Mechanosensory stimulation showed that only at high [CO_2] there is an unresponsiveness to stimuli within a 30 min time period. Additionally, the autonomic bioindex of heart and ventilatory rates showed a complete cessation with high acute exposure within in the same time period for unresponsiveness to mechanosensory stimulation. *J. Exp. Zool. 313A:484–497, 2010.* © 2010 Wiley-Liss, Inc.

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For an organism to survive it must be able to constantly monitor its environment through sensory stimulation and respond though behavioral modification. All animals use the nervous system to sense their external and internal environments. Most organisms show diversity in the type and amount of peripheral sensors, even within a single sensory modality (Derby et al., 2001). The world is chemical, and chemical senses are among the most basic tools evolved for locating resources and avoiding danger (Devine and Atema, '82; Atema, '95). The sensory system of chemoreception is generally divided into the senses of smell (olfaction) and taste (gustatory) and is typically associated with specific receptors for detection in a particular medium (i.e., air, water; Carr, '88).

Chemosensors are used to detect external environmental chemical cues; therefore, sensors are highly vulnerable to physical, chemical and biological damage which can affect sensory function (Hamilton and Case, '83; Derby et al., 2001). Even though Putnam and Filosa (2004) examined the acidsensitive cellular mechanisms of chemosensitive neurons, there are still many unsolved mechanistic cellular actions to carbon dioxide (CO_2). CO_2 is an important constituent of the chemical environment, and insects possess specialized receptor cells that can detect and measure environmental CO_2. Past studies have furthered the understanding of chemosensitivity by showing the capability of non-neuronal cells to be sensitive to either increased levels of CO_2/H^+ or acid alone. CO_2 affects physiology of invertebrates in the same manner as vertebrates through the well-known reaction: $CO_2+H_2O \leftrightarrow HCO_3^-+H^+$ catalyzed by carbonic anhydrase (Lindskog and Coleman, '73; Stone and Koopowitz, '74; Baker and Honerjiger, '78; Henry, '96; Lindskog, '97).

CO_2 is universally present and impacts all organisms throughout their lifetime. On average, the atmosphere today contains approximately 0.038% CO_2 or 385 ppmv (parts per million by volume (Nicolas and Sillans, '89; http://cdiac.ornl.gov/ftp/trends/co2/maunaloa.co2) and is continuing to rise as recorded from prolonged data collections from the Mauna Loa, Hawaii,

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observatory. Many organisms live in environments with a CO_2 concentration [CO_2] much higher than the atmosphere, such as insects located under the bark of trees or stumps (Pasche and Zachariassen, '73), bee hives (Buhler et al., '83), termite mounds (Luscher, '61) and caves (Howarth, '83). Increased levels of CO_2 can be attributed to many causes and yet animal respiration, decrement of organic matter and decomposition of minerals are the major sources that result in the change of acidity in unpolluted waters (APHA Standard Methods, American Public Health Association, 2007). Tidal pools also show daily changes in [CO_2] with temperature and salinity to which animals are exposed (Truchot, '86).

In air-equilibrated surface waters, the [CO_2] would be expected to be approximately the same as in the atmosphere (~380 ppmv, or 1.3 mg/L) as the CO_2 solubility coefficient is roughly the same in the two media (depending on the temperature). However, Cole et al. ('94) reported that lake surface waters are often supersaturated with CO_2, by an average factor of 3 (~1,140 ppmv or 3.9 mg/L). If the water column is well mixed, and/or oligotrophic, there may be little or no increase in [CO_2] with depth. In stratified, hypoxic waters, [CO_2] at the bottom may reach 10–12 mg/L (~2,900–3,500 ppmv), but only rarely will it be higher (Cole et al., '94). The CO_2 produced by volcanic hot springs can rise to 75% overnight when sunlight is not present. This level is high enough to kill insects and animals. There are also rare occurrences when deep lake waters, saturated with CO_2, were disturbed and the gas escaped, which resulted in human deaths (37 died at Lake Monoun, Cameroon in 1984 and 1,700 casualties at Lake Nyos, Cameroon in 1986; Baxter et al., '89; Martini, '97). We have yet to find reports on the type of animal life present in these Cameroon lakes. Also, liquid CO_2, which is known to be present at geologic vents, significantly affects local ecology (Konno et al., 2006).

Early studies suggested that some vertebrates may be able to detect CO_2 and it is now known to be detected by the olfactory system of mammals, insects and worms (Youngentob et al., '91). Current literature, primarily in insects, shows that low levels of CO_2 acts as an attractant (chemotaxic properties; host–plant interactions, Jones and Coaker, '77; blood-sucking insects, Allan et al., '87). Some insects show behavioral responsiveness to increases in [CO_2] as small as 0.002–0.003% (wireworms, Doane et al., '75; Lehane, 2005). The role of CO_2 in foraging was identified many years ago both in the field and in the laboratory. It has been shown that CO_2 alone stimulates and modulates host-seeking behavior of mosquitoes (Gillies, '80; Bowen, '91; Takken, '91; Takken and Knols, '99). In many insects, electroantennogram recordings show that the antennules are sensitive to CO_2 in a dose-dependent manner (Schneider, '57; Roelofs and Comeau, '71). It is thought that other arthropods (e.g., centipedes, ticks, terrestrial crabs) also possess CO_2 receptor cells (Yamana et al., '86; Steullet and Guerin, '92; Kleineidam et al., 2000; Stensmyr et al., 2003).

In moths, mosquitoes, biting midges, the tsetse fly and many other insects, CO_2 receptors provide information such as specificity, sensitivity with a broad concentration response and the ability to sense fluctuations in addition to the continuous background CO_2 levels through receptor activation (Guerenstein and Hildebrand, 2008). One might ask how an organism may respond both behaviorally and physiologically to increasing levels of CO_2. Higher concentrations of CO_2 induce paralytic effects in vertebrates and invertebrates alike. Recently, it was proposed that honeybees can defend themselves as a group by producing CO_2 and increasing temperature, which results in killing hornets (Sugahara and Sakamoto, 2009). Interestingly, early human surgeries used CO_2 as an anesthetic (Eisele et al., '67). Although invertebrates and vertebrates are very different at a system level, the effects of CO_2 at a cellular level are not. Thus, studies that examine both behavioral and physiological effects with high levels of CO_2 will provide critical information on an ecosystem level.

A previous study from our lab identified behavioral and physiological responses in *Drosophila* larvae exposed to acute high levels of CO_2. Such levels are commonly used in laboratories to anesthetize insects and as noted above high levels can occur in natural environments that can be toxic. The identified behaviors in *Drosophila* larvae include behavioral unresponsiveness characterized by immobilization and lack of movement when mechanosensory stimulation is applied, as well as rapid cardiac arrest and cessation of body wall movements (Badre et al., 2005). In this study, crayfish were used because they are arthropods, like insects, and can be used for comparative purposes to address commonalities in cellular responses. Also, many species of crayfish are invasive species. Effort has been expanded world wide to bait, by attraction, for removal as well as repelling crayfish from locations. Thus, it is of economic interest to know if CO_2 can serve in these dual approaches. The levels of CO_2 used in this study were high compared with most natural conditions, but examining these extreme levels allows one to understand characteristic behavioral and physiological consequences. Future studies can examine more subtle changes on the noted effects with lower concentrations. In addition, one approach being proposed to reduce atmospheric CO_2 is to pump CO_2 into lakes, oceans and deep underground geologic layers. These approaches will undoubtedly have an effect on the aquatic vertebrate and invertebrates alike. Crayfish, which are a major component in the ecological food web, serve as a good sentinel of water quality and as a model for assessing biological effects of environmental alterations for other animals.

Here, we used both behavioral and physiological measures in crayfish to understand if the identified behaviors seen in *Drosophila* are species-specific or possibly a general characteristic of CO_2 exposure. Both behavioral and physiological effects observed will provide insights into physiological stressors and how an organism might compensate in toxic environments. Four different experimental studies were conducted to understand previously identified behavioral effects with acute CO_2 exposure: (1) CO_2 as an attractant and/or repellent in crayfish, (2) environmentally driven behavior, (3) tail touch as a bioindex of behavioral responsiveness and (4) physiological measurements of the autonomic response in varying environmental conditions.

Crayfish possess the complex ability to integrate sensory information, relay the information into motor output to target tissues and allow the "sympathetic-like" autonomic response to be easily studied (Shuranova et al., 2006). Behaviorally, the measurement of tail flip responsiveness in crustaceans, due to a stimulus applied to the dorsal aspect of the telson, has been used as a measure of its responsiveness in many past studies (Lang et al., '77; Copp, '86; Fricke, '86; Bruski and Dunham, '87; Pavey and Fielder, '96; Guiasu and Dunham, '97; Kellie et al., 2001). Thus, the tail flip provides a well-documented bioindex as responsiveness in varying environmental conditions. Importantly, crayfish provide multiple levels of measurements to address questions pertaining to CO_2 exposure and in combination with results using *Drosophila* larvae will delineate between species-specific effects of CO_2. It is known that crabs and crayfish alter their ventilation in response to low levels of CO_2 (Massabuau et al., '84; Gannon and Henry, 2004). Here we also assessed the effects of low pH that can be induced by CO_2, as well as anoxia in addition to exposure of CO_2 on not only heart and ventilatory rates but whole animal behaviors.

MATERIALS AND METHODS

Animals

Crayfish, *Procambarus clarkii* measuring 5.0–6.4 cm in body length were obtained commercially (Atchafalaya Biological Supply Co., Raceland, LA). A total of 171 crayfish were used in the study. Both sexes of crayfish were used in this study but differences between the sexes were not analyzed. Animals were housed individually in rectangular plastic containers and cared for in the same manner in an aquatic facility within our regulated-temperature laboratory (17–20°C). All animals were on a 12-hour period light–dark cycle. They were fed dried fish pellets weekly and handled by using a glass beaker to transfer crayfish from one container to the other. As housed containers being cleaned weekly, crayfish are handled often; the limited handling during experimentation is assumed to have little to no effect on the internal status of the crayfish. Only crayfish in their intermolt stage, possessing all walking legs and both chelipeds were used.

CO_2 as an Attractant and/or Repellent

An aquatic Y-maze was constructed from plexiglass sheets (Fig. 1). A total of 33 crayfish were used in this behavioral study to examine arm choice in different conditional water treatments compared with aerated water for a trial period of 5 min. Crayfish were randomly divided into six groups: (1) Eight crayfish to test arm choice of 100% CO_2 and aerated water; (2) Five crayfish to test arm choice of 50% CO_2 and aerated water; (3) Five crayfish to test arm choice of 5% CO_2 and aerated water; (4) Five crayfish to test arm choice of 100% N_2 and aerated water; (5) Five crayfish to test arm choice of low pH of 4.85 and aerated water; (6) Control experiment using five crayfish to test arm choice of aerated water and aerated water. Conditional water treatment was alternated on every other trial in each treatment group to eliminate preference for an arm independent of treatment condition. A pH of 4.85 was used as experimental trials showed this to be the pH for a CO_2-saturated environment (established through continuous bubbling to a minimum pH value). A nitrogen-saturated environment was used to show that the effects are CO_2 mediated and not the result of a hypoxic environment.

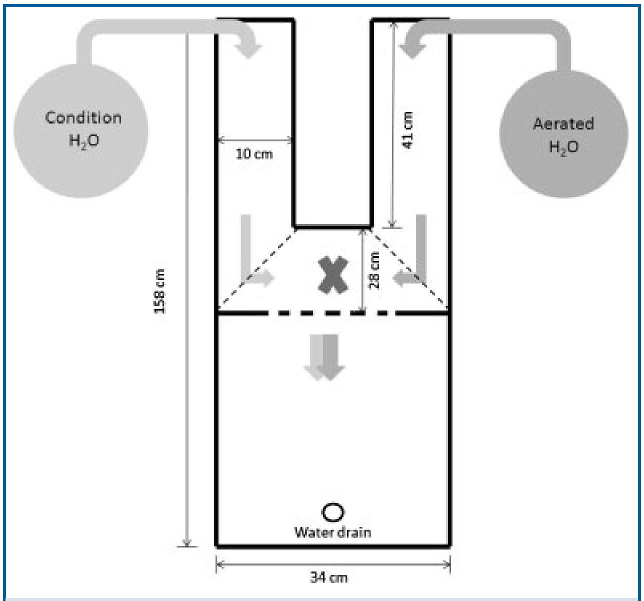

Figure 1. Schematic representation of Y-maze apparatus. Crayfish were placed in a water flow-through system with one side delivering one of the varying treatment conditions and the other side delivering aerated water as the control. The "X" indicates the point of crayfish placement in the water integration zone. Arm choice was recorded when the thorax of the crayfish crossed into one of the arms, (indicated by the black dotted lines shown in the figure).

Initial placement of the crayfish was at the point of water integration (indicated by "X" on Fig. 1) to allow assessment of both water treatment conditions. The trial was recorded using a digital video camera and all behavior was monitored on a TV screen. Crayfish movement was scored by watching the TV monitor and/or from the recorded videos. The crayfish moved at the junction and proceeded to enter into one of the two side arms. A crayfish was deemed to have entered the arm when its thorax crossed a line diagonal from the arm to the corner, which is shown by a black dotted line in Figure 1. When an animal exited the arm, it was recorded as having returned to the initial zone or entered into the other experimental arm of the maze. After each trial, the water was allowed to run for 2 min to remove any chemical cues from a previous crayfish. Trials were conducted in a low light environment (25 Lux, Licor Model LI-185A) to mimic periods of dusk and dawn when the crayfish are known to be most active (Page and Latimer, '72).

Analysis of general exploratory behavior examined arm choice which was scored as time spent in either the experimental condition or control arm for the 5-minute trial duration. Statistical analysis used a Student's *t*-test for each treatment group. The amount of time spent in the zone of water integration was not scored as an arm preference and this time was eliminated from analysis. The average percent of time spent in each arm of the experimental conditions was used for comparisons to the arm used for the control aerated water.

Environmentally Driven Behavior

A large plexiglass chamber was used to examine whether environmental conditions could force an organism to leave its' environment. The rectangular chamber (234 cm × 23 cm × 32 cm) was divided into two separate environments using an incline (~25°) covered in rocks leading to another pool (Fig. 2). The incline was constructed from a plexiglass divider and covered with rocks to mimic a pond/pool bank (to a height of 14 cm). A total of 83 crayfish were used in this behavioral study to force movement in different conditional water treatments compared with aerated water for a trial periods no more than 60 min. Crayfish were randomly divided into six groups: (1) 28 crayfish to test forced movement out of 100% CO_2 and into aerated water; (2) 10 crayfish to test forced movement out of 50% CO_2 and into aerated water; (3) 16 crayfish to test forced movement out of 5% CO_2 and into aerated water; (4) 10 crayfish to test forced movement out of 100% N_2 and into aerated water; (5) 7 crayfish to test forced movement out of low pH 4.85 and into aerated water. (6) Control experiment using 12 crayfish to test forced movement out of aerated water and into aerated water. Conditional water treatment side of the chamber was alternated on every other trial in each treatment group to eliminate preference for a side independent of the treatment condition.

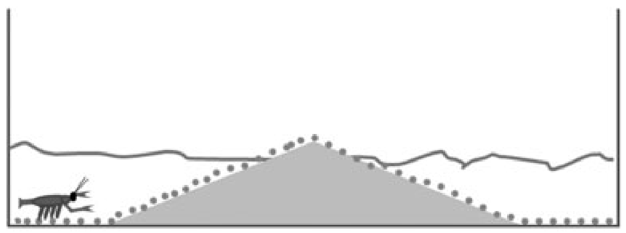

Figure 2. Schematic representation of forced movement chamber. Crayfish were placed in a rectangular chamber with one side having an experimental treatment environment with either 100% CO_2, 50% CO_2, 5% CO_2, 100% N_2 or low pH 4.85 and the other chamber acts as a control of an aerated environment. Chamber movement was recorded when the thorax of the crayfish crossed out of one environment.

Crayfish were introduced to the treatment side after 1 hr of bubbling of the gas (previously shown to be saturated). All trials were recorded using a digital video camera and all behavior was monitored on a TV screen. Crayfish movement was scored by watching the TV monitor and/or recorded videos to note the times of movement within and out of the water conditions. A crayfish was deemed as being forced out of the environment if its thorax crossed out of the water. After each trial, the water was drained, chamber washed and refilled to remove any chemical cues from a previous crayfish. Trials were conducted in a low-light environment (25 Lux) to mimic periods of dusk and dawn when the crayfish are known to be most active (Page and Latimer, '72). A layer of plastic wrap was used to seal the water environment by floating it on the water surface and gluing it to the side except for 5.1 cm from the rock slope so as not to impede the crayfish from exiting the water. In addition, a large plexiglass sheet covered entire chamber to retard the experimental gas from exchanging with the atmosphere.

Statistical analysis used a Student's *t*-test for each treatment group. Total number of animals that left each experimental environment was determined and compared with aerated control environment. A percentage of crayfish that moved from one side to another was calculated for each experimental condition.

Tail Touch as a Bioindex

To measure responsiveness in the varying environmental conditions, a touch was applied to the tail of a crayfish to elicit a tail flip away from the stimulus. Behavioral trials were conducted by placing a crayfish into each of the experimental conditions and applying a tail touch stimulus. The stimulus (touch using a glass rod) was applied to the tail of the crayfish. Responses were recorded as (Y) yes, a behavioral response was observed (a tail flip away from stimulus) or (N) no, a behavioral response was not observed. The touch was induced every minute until the crayfish became unresponsive to the tail touch for two consecutive touches (i.e., 2 min) which then the crayfish was removed from the treatment environment. The possibility of habituation in the tail flip response can be compared with previously published data for this size range of crayfish (Pagé et al., 2007). This size range of crayfish will not habituate as rapidly as shown with exposure to a 100% CO_2. Also the crayfish exposed to 5% CO_2 and 100% N_2 as well as low pH in the aquaria still responded to a tail touch by moving away from the stimulus for a prolonged period of time as compared with the 100% CO_2 exposure.

Experimental conditions were prepared by bubbling the treatment gas into a closed container for 45 min. Crayfish were randomly chosen for six treatment conditions: (1) Five crayfish in 100% CO_2; (2) Five crayfish in 50% CO_2; (3) Five crayfish in 5%

CO_2; (4) Five crayfish in 100% N_2; (5) Five crayfish in pH 4.85 and (6) Control experiment using five crayfish in aerated water.

Recording HR and VR

Crayfish were wired to record heart rate (HR) and ventilation rate (VR; Listerman et al., 2001; Schapker et al., 2002). Experimental details are shown in video format (Bierbower and Cooper, 2009a,b). A lid was used to prevent the crayfish from exiting the chamber. A small section of the wires exits the chamber and is clamped with the lid. This did not prohibit the crayfish from moving freely. The placements of the recording wires are shown in Figure 3. All physiological measures were recorded though an impedance detector, which measured dynamic resistance between the stainless steel wires and recorded on-line to a PowerLab via a PowerLab/4SP interface (AD Instruments, Bella Vista, Australia). All events were measured and calibrated with the PowerLab Chart software version 5.5.6 (AD Instruments). Previous studies showed that 3 days was enough time for the animals to return to baseline physiological values before handling (Wilkens et al., '85).

A physiological trial began when a crayfish was placed into an experimental container of normal aerated water and the crayfish was left for 15 min to acclimate to the new surroundings. After the initial 15 min, recording baseline HR and VR measures commenced. Each experimental trial began with a 30-minute recording of normal baseline rates, which were used as a reference for changes in an autonomic response to environmental conditions. After the initial 30 min, the crayfish was removed from the aerated water chamber and placed into the experimental condition chamber. At this point, the crayfish was monitored constantly to identify the point in time when the HR and VR stopped for 1 min. Once the autonomic response ceased, the crayfish was removed from the experimental condition chamber and returned to the aerated chamber with continued measures of HR and VR for 2 hr. The trial was terminated at the end of the 2-hr recovery period.

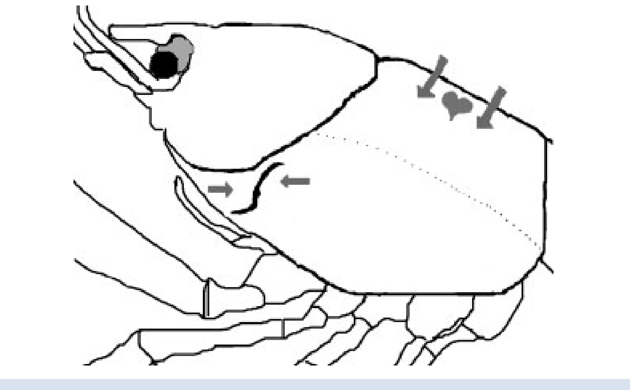

Figure 3. Schematic representation for the placement of the recording wires for monitoring the heart and ventilatory rates from a crayfish (*Procambarus clarkii*). On the dorsal carapace, large arrows represent the two wires which span the rostral–caudal axis of the heart to monitor heart rate. On the lateral side, two smaller arrows represent the two wires which span the scaphognathite (i.e. prebranchial chamber—outlined by the dotted line) to monitor any change in the dynamic resistance, which is used as a measure of ventilation.

Experimental conditions were prepared by bubbling the treatment gas into a closed container for 45 min. Crayfish were randomly chosen for six treatment conditions: (1) Five crayfish in 100% CO_2; (2) Five crayfish in 50% CO_2; (3) Five crayfish in 5% CO_2; (4) Five crayfish in 100% N_2; (5) Five crayfish in pH of 4.85 and (6) Control experiment using five crayfish in aerated water. After the baseline recording, crayfish were handled using a glass beaker to move them from one container to another.

Statistical Analysis

Analysis of variance (ANOVA) statistical analysis based upon probability $P<0.05$ and Holm–Sidak post hoc analysis were used to determine significance in environmental conditions. A Student's *t*-test with probability of $P<0.05$ was used to determine significance between conditions. In recording an autonomic response, to account for variability in individual heart and ventilatory rates, each crayfish was analyzed as a percent change from initial baseline recordings (i.e., first 20 min). Percent change values were determined by taking the absolute value of the baseline, minus each subsequent beats per minute (BPM) and dividing by the baseline and the result multiplied by 100 to get a percent change. The value is designated on the graphs as percent baseline change. To understand trends, % change values were averaged together to achieve an average percent change for each experimental condition.

RESULTS

CO_2 as an Attractant and/or Repellent

When placed into the Y-maze, each of the crayfish typically walked up the middle of an arm with both antennae held out in front while swaying them back and forth touching the walls on either side of the arm to guide themselves (thigmotaxis). In many cases, once they reached the end of an arm, they turned around and went back down the arm to the junction in which they were originally placed and then moved into the other arm of the maze. Animals entered the side arms without preference for a particular direction: 18 animals first turned right and 15 first turned left (Fisher Exact Test, $n = 33$, $df = 31$, $P = 1.0$). As a control, general behavior without an experimental condition showed no preference for a specific arm and mean time in each arm was not significantly different ($t_8 = 0.21$, $P = 0.84$).

To identify the influence of carbon dioxide on arm preference, crayfish were tested in the maze in varying environmental conditions. The degree of preference for specific conditions of CO_2, N_2 or pH 4.85 is shown in Figure 4. There is a significant

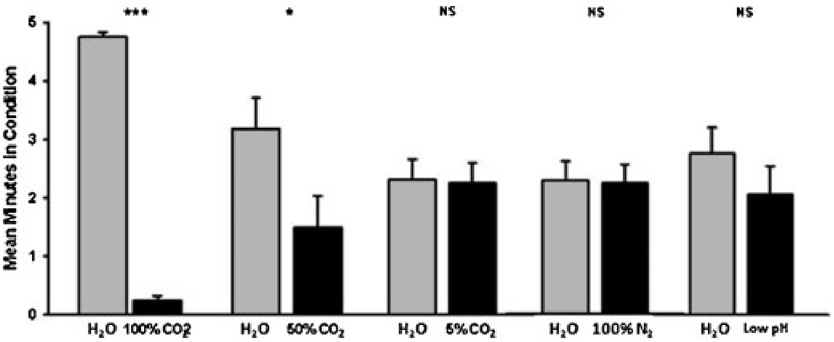

Figure 4. The influence of CO_2 on arm preference in a Y-maze. To identify if the effects were due to CO_2 the behavioral responses of arm choice were also examined to H_2O only, the effects of hypoxia (N_2) and also effects of low pH (4.85). Crayfish were placed in a water flow-through system with one arm delivering one conditional water (black bars) and the arm delivering aerated water as the control (grey bars). The mean time (minutes; ±SEM) for a response in 5-minute periods was assessed for each condition. There is a significant decrease in the amount of time required for a response to CO_2 as compared with N_2 and low pH (Student's *t*-test; $^{*}P<0.05$, $^{**}P<0.02$ and $^{***}P<0.001$. NS indicates no significant difference between variable and aerated control).

reduction in time spent in CO_2 exposure which directly correlates to increasing levels of the concentration of CO_2 dissolved gas. Specifically, crayfish spend significantly less time in 100% CO_2 ($t_{14} = 40.56$, $P<0.001$) when compared with 50% CO_2 bubbled water ($t_{14} = 2.23$, $P = 0.04$). Furthermore, crayfish did not have a significant difference in arm preference when exposed to only 5% CO_2 ($t_{30} = 0.13$, $P = 0.90$). The lack of significant difference in mean time for 5% CO_2 is suggestive that crayfish do not use CO_2 as an attractant. However, it is also likely that 5% CO_2 is within a normal range of environmental levels or even a level in which the animal is easily able to compensate and does not signify a toxic environment. However, results indicate a behavioral repellent effect with increasing levels of CO_2. In addition, crayfish do not show an arm preference for N_2 as they spend approximately equal time in both arms ($t_{12} = 0.11$, $P = 0.92$). Thus, the hypoxic environment during CO_2 exposure does not explain the rapid repellent behavioral effect seen in the high CO_2 environment. Also, the acidic environment resulting from CO_2 exposure does not explain the behavioral repellent effect as there was not a significant difference in arm preference during low pH exposure ($t_{10} = 1.06$, $P = 0.31$).

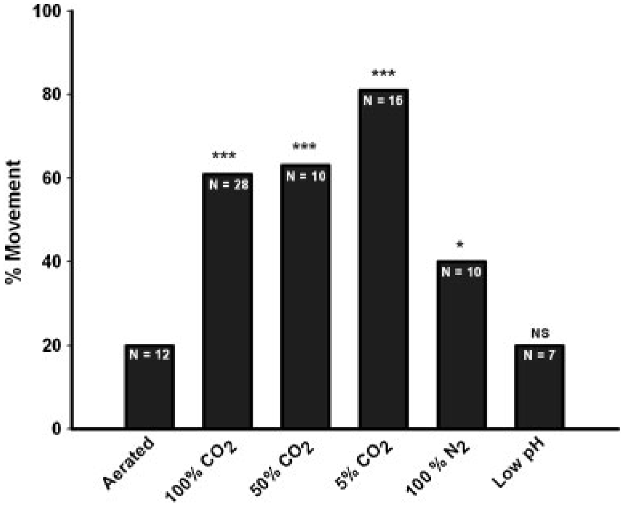

Figure 5. Assessing behavioral effects of CO_2 exposure on crayfish movement. When crayfish are place in CO_2 environmental conditions, the animals moved out of the potential toxic environment. A rectangular chamber was divided into two separate aquatic environments by a rock embankment which allowed access to either environment. Crayfish were placed in the treated water for a maximum of 30 min or until paralysis. Data are represented as the percentage of individuals that moved to the aerated water side from treated side. N = sample size. (Student's *t*-test; $^{*}P<0.05$, $^{**}P<0.02$ and $^{***}P<0.001$. NS indicates no significant difference between variable and aerated control).

Environmentally Driven Behavior

To identify the influence of carbon dioxide on forcing movement out of an environment, crayfish were tested in a static chamber. The degrees of movement for specific conditions of CO_2, N_2 or pH 4.85 are shown in Figure 5. There is a significant difference in the movement out of CO_2 environments compared with the control aerated side. Specifically, crayfish showed a significant difference with exposures to 100% CO_2 ($t_{38} = -24.58$, $P<0.001$), 50% CO_2 ($t_{20} = -28.74$, $P<0.001$), as well as 5% CO_2 ($t_{20} = -4.28$, $P = <0.001$). Results also indicate that crayfish were significantly shown to move out of N_2 when exposed ($t_{18} = -0.45$, $P = 0.66$) although behaviorally this occurred after a longer period of time than the movement out of 100% or 50% CO_2. Thus, it is possible that a hypoxic environment could eventually cause a crayfish to leave their environment during CO_2 exposure, but this alone does not explain the rapid repellent behavioral effect seen in the high CO_2 environment. Also, the acidic environment resulting from CO_2 exposure does not explain the behavioral repellent effect as there was not a significant difference in movement out of a low pH environment ($t_{18} = 0.00$, $P = 1.00$).

Tail Touch as a Bioindex

The tail flip response was used as a bioindex of the whole animal status to exposure in environmental stressors of CO_2, N_2 and low pH 4.85. A tail touch was given once per minute and tail flip responses away from the stimulus were noted. In this study, no differentiation was made between giant-mediated and nongiant-mediated tail flip (Krasne and Wine, '75) as the interest here was only whether the animal responded to the forceful touch on the telson.

Behavioral analyses of tail touches indicate that high levels of CO_2 produced unresponsiveness (Table 1). Specifically, 100% CO_2 produced an unresponsiveness within 20 min in five out of five preparations ($P > 0.05$; Wilcoxon nonparametric test). Recovery of locomotion from CO_2 exposure is fairly rapid, taking several minutes for complete locomotor activity to resume. It should be noted that time for cessation of locomotor activity varied with each crayfish. On average it took 13 min before complete unresponsiveness to stimuli occurred. Unresponsiveness to stimuli was seen with 50% CO_2 after approximately 30 min in five out of five preparations ($P > 0.05$; Wilcoxon nonparametric test). Likewise, the time to cessation of locomotor activity varied for 50% CO_2 exposure with an average of 27 min. Also, it is important to note that there was no change in responsiveness to tail touch with 50% CO_2, 5% CO_2, 100% N_2, low pH 4.85 or aerated exposure conditions within the same time period that occurred for 100% CO_2. Thus, only 100% CO_2 induced the unresponsive behavioral effect within 15 min and it was shown to be dependent on the concentration as the same effect will result with 50% CO_2 over a longer period of time.

Table 1. Assessment of carbon dioxide's impact on responsiveness to stimuli.

	10 min	20 min	31 min
100% CO_2	Yes	–	–
50% CO_2	Yes	Yes	Yes
5% CO_2	Yes	Yes	Yes
100% N_2	Yes	Yes	Yes
Low pH	Yes	Yes	Yes
Aerated	Yes	Yes	Yes

A tail touch was given once per minute up to 30 min or until the crayfish no longer responded for two consecutive touches in each of the environmental conditions. If the crayfish no longer responded it was removed from the experimental condition. Yes = response to tail touch stimuli by tail flip, No = nonresponsive to tail touch stimuli. An $N = 5$ crayfish were used in each experimental condition.

Recording HR and VR

The physiological responses of crayfish were recorded to characterize the autonomic response to carbon dioxide exposure. HR and VR were recorded before placement into experimental condition (baseline), during condition exposure and after exposure (recovery). The autonomic response was plotted for each crayfish during the entire duration of the trial to demonstrate the fluctuation in HR and VR during exposure to environmental conditions. A frequency plot of the raw traces shows dramatic changes in the autonomic response with HR and VR determined by direct counts of each beat over 10-sec intervals and then converted to BPM. The handling and initial response to conditions usually resulted in an immediate but brief elevation in HR and VR.

In 100% CO_2, a typical response (five out of five crayfish, Wilcoxon nonparametric analysis) observed during the experimental trial was a cessation of HR and VR. Raw traces for individual crayfish show a rapid cessation of both HR and VR with 100% CO_2 exposure (A, Fig. 6). Although cessation times of HR and VR activity varied slightly with each crayfish, an average time for HR was 9 min and VR was 11 min (Fig. 7). To further understand the effect of CO_2 on HR and VR, various levels of CO_2 exposure were used. To further understand whether the rapid effects noted with 100% CO_2 (cessation of HR and VR) is concentration dependent, crayfish were exposed to 50% CO_2. The relatively lower $[CO_2]$ did not result in the rapid cessation in HR and VR (B, Fig. 6). Although there was not a complete arrest of HR and VR within the 30-minute exposure time, it is important to note that there was a significant decrease in both HR and VR for 50% CO_2 exposure. Furthermore, as expected due to lack of behavioral unresponsiveness, 5% CO_2 did not induce the cessation in the autonomic response (C, Fig. 6).

To assay the effects of a hypoxic environment on HR and VR, crayfish were exposed to 100% N_2 environment. In five out of five crayfish, an opposite response in both HR and VR was noted when compared with 100% CO_2 (D, Fig. 6). Specifically, both HR and VR increased during exposure to 100% N_2. Thus, the observed effect shown with 100% CO_2 is not due to a hypoxic environment, which is induced by the bubbling of a gas into a closed environment. Furthermore, to quantify the effects of low pH on HR and VR, crayfish were exposed to low pH 4.85 environments. After a 30-minute exposure time period, both HR and VR were shown to increase (E, Fig. 6). Thus, the observed effect of cessation of the autonomic response in 100% CO_2 was not observed during exposure to a low pH environment. These results suggest that neither a hypoxic or low pH environment can explain the effect seen with 100% CO_2.

Given that the cessation of the autonomic response occurred in all crayfish by the 15-min time point (Fig. 7), this was used as a

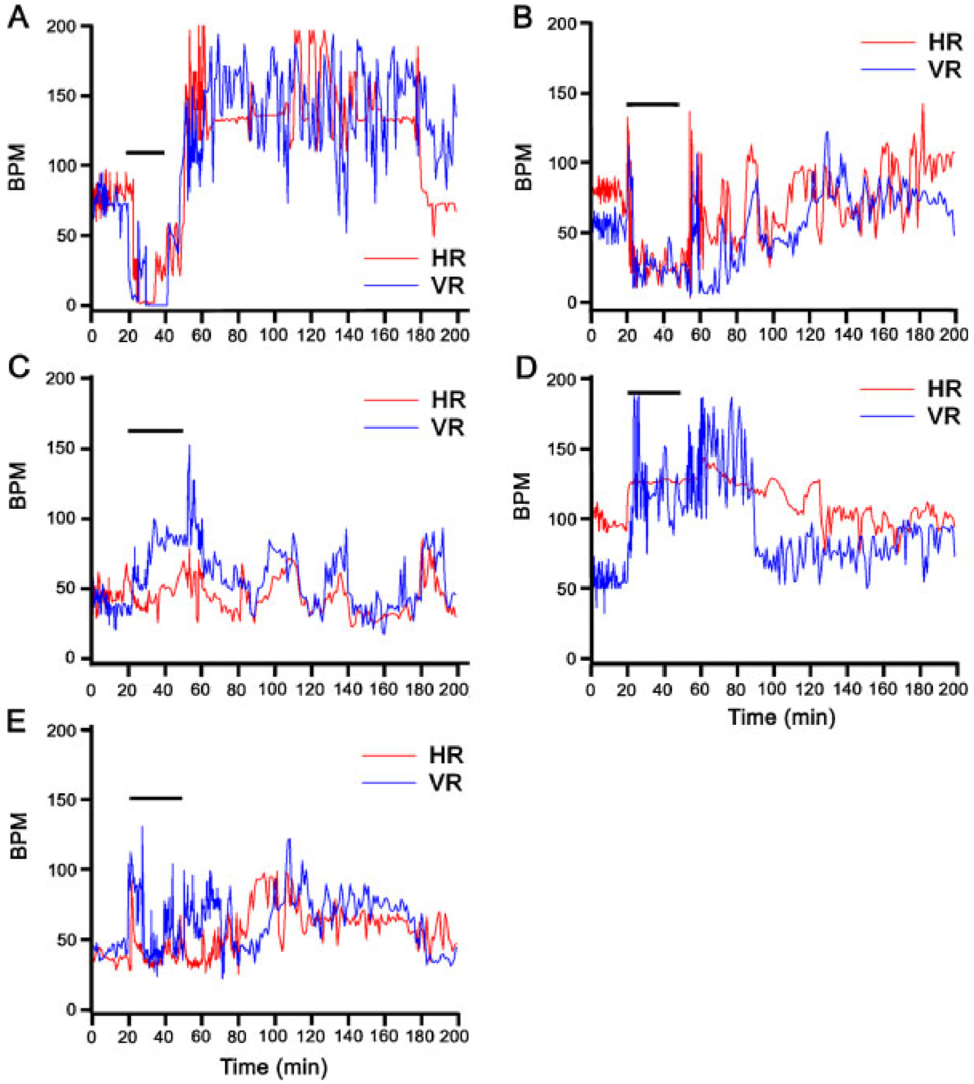

Figure 6. Physiological response of heart and ventilitory rates in crayfish exposed to all environmental conditions. Graphs **A–E** represent each crayfish exposed to an environmental condition. (A) 100% CO_2. (B) 50% CO_2. (C) 5% CO_2. (D) 100% N2. (E) Low pH 4.85. Traces represent the physiological response of heart rate (red line) and ventilatory rate (blue line) during CO_2 exposure. The dark black line on the top of each graph indicates the period of time the crayfish was exposed to the environmental condition.

comparison time point for all other treatment conditions for statistical analysis. Thus, at the 15-min time point for 100% CO_2, percent change from baseline recordings becomes 100% significant (HR, $t_8 = -100$, $P<0.001$; VR, $t_8 = -100$, $P<0.001$; Fig. 8). Across groups statistical analysis compared the decrease in 100% CO_2 with other treatment groups to show the direct effects of CO_2 exposure and a concentration effect. For 50% CO_2, there was a significant decrease in both HR and VR when compared with baseline recordings (HR, $t_8 = 5.37$, $P<0.001$; VR, $t_8 = 14.76$, $P<0.001$) and results show that after 15 min of 50% CO_2 exposure, HR is still significantly different from the HR of 100% CO_2 (ANOVA, $t_8 = 17.85$, $P<0.001$). However, the decrease in five out of five preparations indicates that over a longer time period the same type of response is likely to occur. Interestingly, VR is shown to have a highly significant decrease from baseline recordings and also to be significantly different from the VR rate at 100% CO_2 (ANOVA, $t_8 = 3.78$, $P<0.001$). Also, note that VR in 50% CO_2 is close to that of 100% CO_2. This also indicates that a longer time period in 50% CO_2 may result in complete cessation of VR.

For 5% CO_2, there was not a significant difference for HR or VR recordings from baseline for 5% CO_2 (HR, $t_8 = -0.75$,

$P = 0.48$; VR, $t_8 = 0.19$, $P = 0.85$). After 15 min of 5% CO_2 exposure, HR and VR are both significantly different to that of 100% CO_2 (ANOVA; HR, $t_8 = 12.83$, $P < 0.001$; VR, $t = 15.14$, $P < 0.001$). For HR in both 100% N_2 ($t_8 = -3.10$, $P = 0.015$) and low pH 4.85 ($t_8 = -5.85$, $P < 0.001$), there was a significant increase from baseline recordings. The same significant increase was seen in VR from baseline recording for 100% N_2 ($t_8 = -9.42$, $P < 0.001$) and low pH 4.85 ($t_8 = -6.55$, $P < 0.001$) as well. Results show that after 15 min for both 100% N_2 and low pH 4.85, HR is still significantly different to that of HR of 100% CO_2 (ANOVA; N_2, $t_8 = 15.96$, $P < 0.001$; pH 4.85, $t = 17.85$, $P < 0.001$). This is also true for VR for both 100% N_2 ($t_8 = 24.48$, $P < 0.001$) and low pH 4.85 ($t_8 = 18.39$, $P < 0.001$). Thus, only 100% CO_2 resulted in a complete cessation in both HR and VR. A lower concentration (i.e., 50% CO_2) resulted in a decrease in the autonomic response at the 15-min time point but did not cause a complete cessation. Furthermore, there was not a significant decrease with 5% CO_2 and both 100% N_2 and low pH 4.85 caused an increase in both HR and VR. Therefore, effects seen with carbon dioxide are due only to CO_2 and not a hypoxic or low pH environment and are shown to be concentration specific in the amount of time examined.

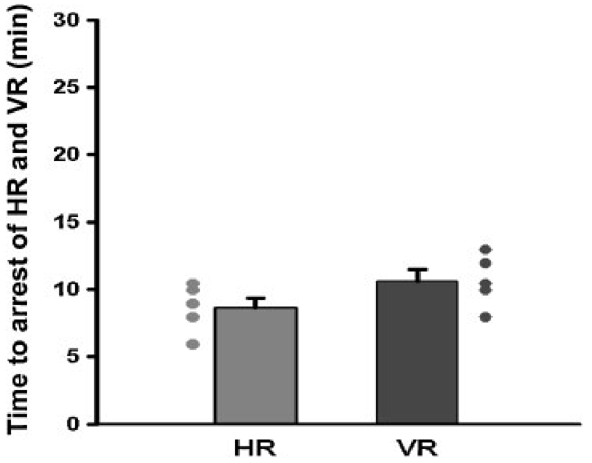

Figure 7. The average cessation time of heart and ventilation rates for crayfish exposed to 100% CO_2. All crayfish ($N = 5$) exposed to 100% CO_2 exhibited a complete arrest in both heart (HR) and ventilation rate (VR) with an average of 9 min for HR and an average of 11 min for VR. The average time to cessation is indicated by the bars, whereas the scatter plot indicates each individual crayfish time of HR and VR cessation.

DISCUSSION

This study demonstrated that crayfish rapidly respond to high levels of CO_2 in their environment. Specifically, crayfish show a strong repellent behavioral response to high [CO_2] and that the response decreases in strength with decreasing [CO_2]. Crayfish do not show a preference and/or a repellent behavioral response to low levels of CO_2 (i.e., 5%). However, the lack of response could be due to a level too low to elicit a response as these crayfish come from a swamp environment, which is most likely higher in [CO_2] as compared with freshwater streams. Thus, a future direction of investigation should be to examine dose–response curves both below and above this level in various species of crayfish for attraction to CO_2 or the use of CO_2 to find food sources (e.g., decaying organic matter) in addition to other variables such as amino acids. Furthermore, as saturating the water environment with CO_2 results in a reduction in oxygen as well as a reduction in pH, the effects of a hypoxic environment and low pH 4.85 were also assessed. Crayfish do not show a preference and/or a repellent behavioral response to hypoxic

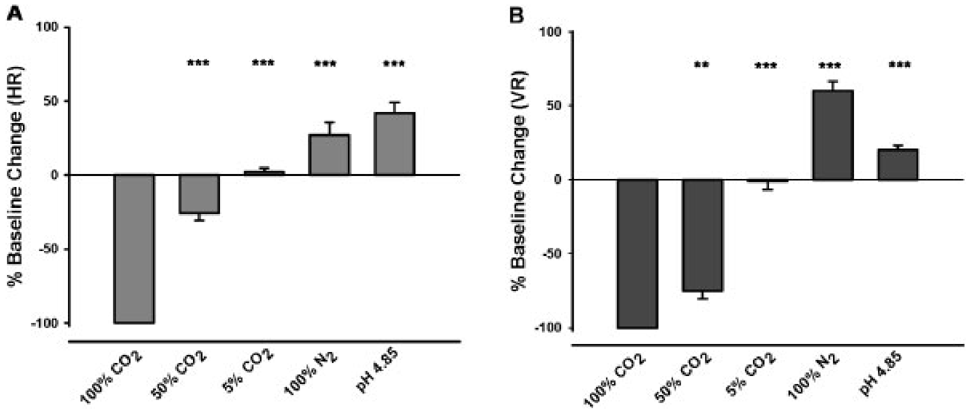

Figure 8. Autonomic response of crayfish exposed to varying environmental conditions. (A) HR in varying environmental conditions (red bars), (B) VR in varying environmental conditions (blue bars). Across group ANOVA test: *indicates $P < 0.05$, **indicates $P < 0.02$ and ***indicated $P < 0.001$. 5 out of five crayfish within each group are different by a Wilcoxon nonparametric analysis.

(induced by N_2 displacement of O_2) or low pH environments. More interestingly, there is a definite and obvious repellent effect with higher CO_2 (i.e., 50%, 100%). The repellent effect was seen in the Y-maze in which the crayfish avoided the higher CO_2 but did not show a repellent effect for low CO_2, hypoxic or low pH environments. Thus, this study strongly suggests that CO_2 is a strong predictor of toxic environments and a cue for an organism to avoid or vacate.

In further examining the attractant and/or repellent behavioral effect we observed that crayfish will rapidly leave a potentially toxic environment that is exposed to 100 and 50% CO_2. Movement was also significantly different for both 5% CO_2 and N_2 environments but this behavioral effect was over longer time durations and no effect was shown for a low pH environment. Lowering water pH by raising the CO_2 increases water bicarbonate concentration, whereas lowering water pH by acid titration lowers water bicarbonate levels. Such changes in water bicarbonate concentration may affect internal acid–base in long periods of CO_2 exposure due to changes in the CO_2 diffusion gradient across the gill and/or alterations in gill ion transport (e.g., Cl—/bicarbonate exchange), but this in unlikely to contribute substantially to account for the rapid changes in behavior observed in this study (Truchot, '84). As CO_2 can rapidly cross the biological membrane and that the crayfish avoid high CO_2, suggests that CO_2 acts as a strong indicator of toxic environmental conditions.

Crayfish acutely exposed to CO_2 were given a tail touch once every minute until a lack of behavioral response (i.e., tail flip or moving in away from stimulus by walking or turning toward the stimulus in a defense posture). Results indicate that the effect on the tail flip response is directly mediated by CO_2. Well before 30 min, crayfish exposed to 100% CO_2 no longer responded to mechanosensory stimulation. The unresponsiveness behavioral effect within the 30-min time period was not shown to occur with crayfish exposed to 5% CO_2, 100% N_2, low pH or aerated environments. Interestingly, the onset of unresponsiveness was shown to be CO_2 concentration specific as crayfish exposed to 100% CO_2 were shown to be unresponsive very quickly, whereas crayfish exposed to 50% were shown to be unresponsive after approximately 30 min. Thus, effects are once again shown to be CO_2 mediated as well as concentration specific. We cannot discriminate taxic and kinetic behaviors in relation to the cuticular sensory receptor mechanism in detecting CO_2, as the gas is also taken up into the hemolymph and can have an effect on internal physiology. To do this well we would need to know the level of CO_2 in the hemolymph or possible ventilatory rate while in the CO_2 part of the Y maze. If they ventilate faster with the scaphognathite, then CO_2 would build up quicker in the hemolymph and thus may induce a sluggish behavior quicker, but this would also impact the rate at which the animal can keep beating the scaphognathite as it also becomes paralyzed by the CO_2 action. Thus, the avoidance behavior is complicated with possible external as well as internal physiological responses, which have not yet been fully elucidated.

The effect of acute CO_2 exposure on the autonomic response is further shown to be CO_2 mediated and concentration dependent. The autonomic response shows a rapid and complete cessation of both HR and VR with exposure to 100% CO_2 within in the time period shown for unresponsiveness to mechanosensory stimulation. In addition, raw traces of BPM for individual crayfish indicate that HR remains consistent during the baseline recordings and significantly decreases to cessation very quickly upon CO_2 exposure. Interestingly, HR remains suppressed for a few minutes after the crayfish is returned to the oxygenated water environment. This suggests a physical blocking or intracellular mechanism requiring a readjustment period of time before returning to normal functioning. The VR is shown to drop out almost immediately upon CO_2 exposure while also taking a period of time to return to normal function.

Although 50% CO_2 shows a significant decrease in both HR and VR after 30 min of exposure, activity persists. However, it is most likely that a complete cessation of the autonomic response would occur over a longer time period. Furthermore as expected, 5% CO_2 did not induce the cessation in the autonomic response within that time period. It is likely that 5% CO_2 would not induce cessation of the autonomic response as the crayfish can likely compensate physiologically to this environmental condition. All other treatment groups show no change or an increase in HR and VR with exposure. Thus, effects are CO_2 mediated, concentration dependent and cannot be explained by hypoxic or low pH environmental conditions. Again, these results support previously shown data that neither a hypoxic or low pH environment can explain the behavioral and physiological effects seen with acute exposure to 100% CO_2.

It is important to understand the impact CO_2 may have on behavior of organisms as natural fluctuations occur and impact complex food webs. Many organisms use CO_2 to find food and thus there is a possibility of significantly impacting foraging behavior. Many insects, as well as other organisms, that feed on living or decaying plant material use CO_2 as a source to find food (Jones and Coaker, '78; Nicolas and Sillans, '89) and foraging behavioral responses are typically dose dependent (Rasch and Rembold, '94). For example, beetle larvae *Diabrotica virgifera virgifera* use only volatile CO_2 to orient themselves toward corn roots (Bernklau and Bjostad, '98). Also, field foraging behavior of the noctuid moth larvae*Helicoverpa amrmigera* shows the use of CO_2 in foraging as the larvae feed on plant tissues that dose not consume CO_2 but emits CO_2 (Rasch and Rembold, '94). However, more importantly are the changes that occur in these behaviors with increasing $[CO_2]$.

Although low levels aid in finding food sources, high levels can create stressed or toxic environments as well as possibly cause physiological changes in organisms. Much of the work examining high levels of CO_2 and changes in behavior have been conducted in insects due to the feasibility of experimental studies (see review, Guerenstein and Hildebrand, 2008). Increased CO_2 is shown to influence oviposition in *Cactoblastis cactorum* and nectar foraging in the hawk moth *Manduca sexta* (Abrell et al., 2005). Organisms have found creative ways to control the environmental variable. Many social animals control nest conditions through collective social behaviors. Honey bees collectively ventilate a hive by wing-fanning workers near the hive entrance to drive CO_2 out of the hive (Seeley, '74; Southwick and Moritz, '87). Bumble bees, *Bombus terrestris*, also collectively fan and that the number of fanning bees increases as CO_2 increases (Weidenmuller et al., 2002). Other behavioral response to temperature and CO_2 is seen with nest structure. For ants and termites, the structure of the nest utilizes surface wind for ventilation and keeps internal CO_2 relatively low (Stange, '96; Kleineidam and Roces, 2000; Kleineidam et al., 2001). Both species alter the shape of the nest's channel openings as a long-term response of a colony to unfavorable CO_2 levels (Kleineidam et al., 2001).

In higher than what is considered normal blood gas levels, CO_2 is shown to be a common anesthetic in vertebrates and invertebrates alike (Eisele et al., '67), and has known physiological effects (fish, Mitsuda et al., '82; Iwama et al., '89; rat, Gautier and Mararui, '98; termite, Shelton and Appel, 2000; St.-John and Rybak, 2002; *Drosophila*, Badre et al., 2005). In numerous studies with fish, as CO_2 increases, the anesthetic affect is seen at a concentration of approximately 8%, while also contributing to reduced pH, elevated blood P_{CO2}, bicarbonate, cortisol, hematocrit, plasma glucose and adrenalin levels. For fish, levels of 1% CO_2 are shown to cause hypercapnia (increased levels of dissolved gas in the blood) and stress (Basu, '59). In addition to the anesthetic effect, the increase in CO_2 also has the aversive effect resulting in a decrease in oxygen availability (hypoxia). Internal buffering, such as with bicarbonate, can contribute to the blood acid–base balance but this is not an effective compensatory method to sustain long-term pH regulation (Saunders, '62).

High levels of CO_2 are shown to have detrimental effects on organisms. An organism will leave environments based upon the environmental cue of CO_2. Physiologically there is a CO_2-mediated effect on both the cardiac and ventilatory systems. Thus, understanding environmental factors and behavioral changes provide a feasible model system to understand environmental impacts. Furthermore, studies on the overall detection and processing of CO_2 cues across organisms and taxa will highly contribute to our understanding of the molecular and neural bases of CO_2-related behaviors. This knowledge will help to understand the behavioral roles of CO_2 and also may lead to the development of protocols and methods to help control harmful organisms as well as act as predictors of environmental conditions.

The behavioral and physiological responses reported here have broad implications concerning consequences of increasing levels of CO_2. Specifically, the repellent/avoidance behavior could be the result of avoiding the paralytic action resulting with CO_2 exposure. Interestingly, this study shows that instead of using toxic insecticides, which often have drastic and residual consequences on whole ecosystems, using high levels of CO_2 for short periods of time will serve the same purpose for eradication of populations of crayfish as used for euthanizing large mammals (Meyer, 2005). Possibly a CO_2 alternative might be a means to control invasive crayfish species (Corkum and Belanger, 2007).

A phenomena not well understood in humans, particularly people inflicted with chronic obstructive pulmonary disease, is the unresponsiveness to chronic increased levels of pCO_2 in the blood. Such people become more responsive to hypoxia (low pO_2) for respiratory drive than to elevated pCO_2 (Raurich et al., 2009; Zapata et al., 2009; Samolski et al., 2010). In fact, if O_2 ventilation is provided to relieve hypoxia, the respiratory rate will decrease further despite a large rise in pCO_2. Of particular interest to our study is to understand the mechanisms of action of CO_2 resulting in the unresponsiveness to mechanosensory stimulation as well as the cessation of the autonomic response. Of interest would be to identify points of action and whether the responses in crayfish are similar to the results found in *Drosophila* larvae, ultimately suggesting a common mechanism of action in other invertebrates or possibly even in vertebrates. Specifically, the next step is to identify receptor subtypes and whether the action is the result of intracellular hypoxia, low pH or if the action is directly CO_2 mediated. These studies are now underway in our research group (Bierbower and Cooper, 2009a,b).

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