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ENVIRONMENTAL EFFECTS ON BEHAVIOR AND PHYSIOLOGY IN CRAYFISH

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences at the
University of Kentucky

By
Sonya M. Bierbower

Lexington, Kentucky

Director: Dr. Robin Lewis Cooper, Associate Professor of Biology

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Despite dramatic morphological differences between animals from different taxa, several important features in organization and sensory system processing are similar across animals. Because of this similarity, a number of different organisms including mammals, insects, and decapod crustaceans serve as valuable model systems for understanding general principles of environmental effects. This research examines intrinsic and extrinsic factors by behaviorally and physiologically means to identify the impact of environmental conditions on two distinct crayfish species- *Procambarus clarkii* (surface) and *Orconectes australis packardi* (cave).

The research identified behavioral and physiological responses in these two morphological and genetically distinct species. The studies also examined multiple levels of complexity including social behavior, an autonomic response, chemosensory capabilities and neuronal communication, identified comparative similarities/differences, addressed learning and environmental influences on learning and examined behavioral and cellular responses to high levels of carbon dioxide. I found environmental factors directly influence crayfish behavior of social interactions. Interactions were more aggressive, more intense and more likely to end with a physical confrontation when they took place 'in water' than 'out of water'. The modified social interaction resulted in a altered fighting strategy.

A study on motor task learning was undertaken which showed similar learning trends among these crayfish species despite their reliance on different sensory modalities. I also demonstrated learning was dependent on perceived stress by the organism. Previously trained crayfish inhibited from completing a task showed significant increase in an autonomic stress response.

Studies on the behavioral and physiological responses to CO_2 revealed that high [CO_2] is a repellent in a concentration dependent manner. The autonomic responses in heart rate and an escape tailflip reflex shows complete cessation with high [CO_2]. A mechanistic effect of CO_2 is by blocking glutamate receptors at the neuromuscular junction and through inhibition of the motor nerve within the CNS.

KEYWORDS: Physiological Compensation, Hypoxia, Hypercapnia, Cardiovascular and respiratory systems, Invertebrates

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ENVIRONMENTAL EFFECTS ON BEHAVIOR AND PHYSIOLOGY IN CRAYFISH

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To my Mother. She was person who never gave me any idea that I couldn't do whatever I wanted to do or be whomever I wanted to be. As she guided me through these incredible years, I don't know if she ever realized that the person I most wanted to be… was her.

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Chapter One
General Background and Significance

Over the last 30 years or so, human thinking has begun to center on the relationship between humans and their environment. It is through this change that scientists are able to understand the broader issues that humans face with sustaining their environment. By understanding how nature works and the impacts on all life forms, we can enhance our understanding of the impacts the environment has on humans. Thus, by looking at ecological systems, science can benefit by a greater understanding of function, as well as causation in ecosystems.

A famous American animal ecologist, Victor Shelford, brought about the fundamental idea that the law of tolerance gives the best understanding for distribution of species in nature (Krebs, 2001). In summary, the law states that the environmental factor which has the narrowest range will be the determining factor for distribution. Therefore, studies which understand the effects of specific factors will identify areas that are the most detrimental to populations and/or species while also delineating the basic requirements in which a species can survive. This idea of examining the reactions of organisms to physical and chemical factors is termed physiological ecology (Krebs, 2001). Today, many researchers study these factors to understand the many aspects influencing an organism's ability to survive in varying environments.

Most animals constantly monitor their environment and alter their behavior based upon sensory information according to various stimuli. These animals possess the complex ability to integrate sensory information and in turn relay the information into motor output to target tissues. Thus, identifying quantifiable measures of internal and external changes will provide insights into environmental and physiological impacts on organisms. Much of the previous research underlying an organism's reaction to its environment, has been through behavioral observational studies conducted in the field. Although this gives useful insights into typical natural behaviors, it can give a false conclusion that an

organism/species is not affected by environmental changes. Specifically, behavior is not always indicative of an animal's awareness to the environment. A physiological assessment (autonomic response) of an animal's state of being**,** allows greater insights into the functional aspect of the animal. Thus, the physiological assessment, in combination with behavioral observations (such as feeding, locomotion, inactivity, social interactions, and communication) will give a complete understanding of an environmental impact on an organism/population/species.

Autonomic Response as Bioindex

While the physiological response can theoretically be measured in a variety of ways, the fear, flight or fight response has been widely studied in vertebrates and serves as the fundamental physiological basis for examining an organism's awareness of its environment under an impending predator attack (Carpenter, 1976; Nicholls et al., 2001). One possible avenue for quantification is through the cardiac and respiratory systems. It is well known that autonomic control of the respiratory and cardiovascular systems can regulate oxygen availability and nutrients to specific target tissues needed for an impending behavioral response.

Arthropods (largest phylum of organisms including insects, arachnids, crustaceans, etc.) are one of the primary invertebrate components of the intermediate level in aquatic ecosystems. More specifically, many studies have examined the importance of crustaceans in maintaining a balanced ecosystem: amphipods (Cooper, 1965; Hall et al., 1964; Mathias, 1971), isopods (Berglund, 1968) and crayfish (Momot et al., 1978). Crayfish are the largest and longest-lived organism of freshwater crustaceans in North America (Momot et al., 1978). The role of crayfish is not restricted to a single trophic level and they are often an important component of multiple trophic levels in aquatic ecosystems. While playing a major role in the benthos feeding, they also have a significant role in converting primary production (leaf material) into animal flesh (Mason, 1963; Momot et al., 1978). It is most likely that they release major sources of energy

available to higher trophic levels at a faster rate than other organisms (Flint and Goldman, 1975; Momot et al, 1978) and have been proposed to maintain stability in aquatic ecosystems.

In 1888, T.H. Huxley (known most often as “Darwin’s Bulldog) published an introduction to Zoology using crayfish (Huxley, 1888). Huxley points out a significant statement made by Roesel von Rosenhof that still holds true to this day which says, “’ Common and lowly as most may think the crayfish, it is yet still full of wonders that the greatest naturalist may be puzzled to give a clear account of it.’” The crayfish has served as a foundation in vast areas of research. An area of particular importance such as vision has greatly benefited from research done with crayfish. Nobel Laureate in Medicine (1967) George Wald, discovered primary physiological and chemical visual processes in the eye and elucidated of the role of vitamin A in vision (Wald, 1967, 1968). The simplicity of the organism allows for many research questions to be addressed, while the complexity of the organism also suits this purpose. While this may sound like a contradiction, it is in fact true when describing typical behaviors, as well as physiological processes more easily studied in the crayfish than other organisms. Crayfish are also a great model stream organism in which to assess different stream systems for conservation priorities (Crandall, 1998; Whiting, 2000), and to understand phylogenetic diversity and relationships to generalize diversity in other stream organisms as well (Perez-Losada *et al.*, 2002).

Even though vertebrates and invertebrates are very different systematically; a ‘sympathetic-like’ response can also be quantified as a physiological response in invertebrates. Dating back to 1927, many of the very early studies have been conducted using arthropods in general (Alexandrowicz, 1932; Orlov, 1927; Zavarzin, 1941). Despite such early studies, there are relatively few investigations into whole animal autonomic function in invertebrates. Specifically, by using the autonomic response, one can directly measure ventilation rate (VR) and heart rate (HR) as a direct measure of a physiological response (McMahon, 1995; Schapker et al., 2002). Interestingly, there are several studies which specifically use HR and VR as a bioindex for correlating autonomic function with

social interactions or environmental changes in crustaceans (Burmistrov and Shuranova, 1996; Cuadras, 1979, 1980; Li et al., 2000; Listerman et al., 2000; McMahon and Wilkens, 1983; Schapker et al., 2002; Wilkens 1976), thus providing a foundation in which to further this area of research. In general, crayfish are a well-studied species commonly used at multiple levels of organismal complexity.

Caves as a natural laboratory

Crayfish have been well-studied in many areas from behavior to synaptic transmission. In examining the area of behavior, it is interesting to note that while current literature describes typical behaviors in normal and laboratory environments, most of the studies are referring to surface sighted (epigean) crayfish and relatively few address the less common cave blind (hypogean) crayfish. It has only been in recent years that the cave environment has become a focus for study since it acts like a natural laboratory. Specifically, the cave environment climate is stable as well as easily defined (Poulson and White, 1969). While the climate is stable, the microenvironments can differ significantly to include running streams, pools maintained by dripping water and/or pools resulting from periodic sources of incoming water. Pools of water are typically characterized by high concentration of dissolved carbonates, low food sources, high density of conspecifics and/or low oxygen levels (Howarth, 1983). Thus, it has been shown that organisms living in these environments have evolved distinct characteristics from their counterpart surface species. Due to these distinct behavioral, anatomical, biochemical, morphological and/or physiological adaptations, these organisms are of great fascination and interest in understanding how they are able to adapt and survive in extreme environments.

Cave crayfish been shown to fit the general characteristics of anatomical and morphological adaptations of most cave organisms. Specifically when compared to surface crayfish, cave crayfish are smaller in size, have longer/thinner appendages, possess highly developed non-visual sensory capabilities, lack

pigment and eyes (Gannon et al., 1999). In addition, behavioral, physiological and biochemical adaptations have been identified in cave crayfish such as, a decrease in locomotion and oxygen consumption, as well as a decrease in metabolic rates (Caine, 1978) all mostly likely directly correlated to a reduction in energy from limited food sources and/or oxygen availability (Hochachka, 1980; Culver, 1982; Hüppop, 1985). It is in these environments that scientist can study the impact of the light-free cave environment on the molecular evolution of the opsin molecule (function is to absorb light) and study the distinct lineages of crayfish that have independently evolved cave adaptations (Hobbs and Barr, 1972). Thus, these distinct evolutionary adaptations allows for studies discerning behavioral and physiological differences in two species of crayfish. One can study learning at the behavior, physiological and neuronal level rather easily in crayfish.

Here I extend this field of investigation by examining the effects on behavior and physiology during environmental changes using crustaceans, specifically two closely related species of crayfish, *Procambarus clarkii* and *Orconectes packardi australis*. Crayfish are well documented to establish social hierarchies resulting from aggressive interactions. Chapter 2 investigates the influence of sensory cues and/or environmental conditions on crayfish behavior during social interactions and how changes in environmental conditions impact fighting probability and/or intensity. Additionally, since interactions are typically mimicking natural environments (aquatic), little is known of interaction dynamics in the absence of water eliminating typical escape response (i.e. tail flip) allowing for a fast retreat from conspecifics. The absences of water results in other factors such as a higher energy demand (no buoyancy), greater injury probability (due to slower response) and lack of chemical cues for assessment during social interactions.

Chapter 3 investigates learning of an operant motor task in crayfish. Learning is thought to be the fundamental basis for species adaptation through acquisition of new information/behaviors. Organisms will adapt in a species-specific manner shaped by evolution. The capacity to learn and retain the memory may allow an

individual to adapt into an environment that was previously hostile and/or resource deficient by out-competiting others through possession of resources. Thus, individuals able to learn (i.e., successful foraging/survival techniques) would increase the likelihood of reproducing (i.e., increased fitness). A powerful tool to study learning is the analysis of an animal's ability to learn to complete a task. For invertebrates, there have been limited studies examining this area of interest. Operant conditioning, which is behavior controlled by its' consequences, is not as well studied as other areas such as classical conditioning or habituation and sensitization. A key component of operant conditioning is that some aspect of the motor response is involved with learning and the organism makes a connection through its behavioral response between a neutral stimulus and a second stimulus which is either a reward or punishment. Thus, an organism set to solve a problem, learns to solve the problem and get the reward; therefore operating on its environment. Here I used crayfish to study the ability to complete a specific motor task which consists of, manipulating a cheliped (large claw) through a cheliped-sized access point in a plexiglas divider and remove a mosquito larvae bloodworm tethered above the access point.

Chapter 4 investigates physiological and behavioral impacts in crayfish when exposed to an excess of dissolved gas. Environmental stressors can have significant effects on organisms both behaviorally and physiologically. In many cases, adaptation to a changing environment causes changes in physiological processes (i.e., metabolic, neuroendocrine, behavioral) that when complexed together elicit responses often associated with stress. One such area of interest is in excess dissolved gas in water supplies which can act as a stressor. Carbon dioxide (CO_2) is universally found and impacts all organisms throughout their lifetime. Interestingly, invertebrates and vertebrates are very different systemically, yet the effect of CO_2 is not. It is through motor output (i.e., locomotor activity, heart and ventilatory measures) that we can assess the internal state of the organism and understand behavioral (i.e., whole animal) responses due to exposure. Chapter 5 examines mechanistic cellular actions of carbon dioxide at the skeletal neuromuscular junction and on a neural 'sensory

root – ganglion – motor root' circuit to understand previously identified paralytic effects relating back to the whole animal change in behavior with acute exposure.

Specific aims of the dissertation research

1. To advance previous studies of environmental impacts on behavior and physiology in the crayfish, as well as further understand environmental impacts on populations/species.

2. To establish if crayfish are capable of learning a motor task, examine long-lasting memory, environmental influences on learning, determine learning differences between two species that rely on different primary sensory modalities and examine whether crayfish show a physiological stress response when inhibited from completing a learned motor task.

3. To examine the role of CO_2, as an environmental cue for a potentially toxic environment, to further understand innate behavioral responses to possible toxic environments and to further understand the behavioral and physiological effects associated with increasing levels of CO_2.

4. To identify mechanistic action of CO_2 on neuronal communication and function of neuromuscular junctions (NMJs) on skeletal muscles, as well as the effect on the central nervous system (identified neural circuit).

Chapter Two

Environmental Modulation of Intrinsic Behavior during Social Interactions

INTRODUCTION

Social relationships may take many forms when organisms live in a group and often times, individuals must determine their status within the social structure (Allee, 1936; Rowell, 1974; Drews, 1993). Social dominance is a form of a social relationship in which individuals aggressively interact repeatedly. The interaction between individuals is a sequential series of interactions, with each individual having the option of terminating or continuing the interaction/contest at any time. The consequence of these interactions most likely results in a dominant individual who repeatedly wins encounters against a subordinate (Drews, 1993). Agonistic encounters will ultimately establish social hierarchies between individuals in a population (Bovbjerg, 1953, 1956; Huber et al., 1997; Li et al., 2000; Issa et al., 1999; Goessmann et al., 2000). The dominance hierarchies have been shown to decrease aggressive interactions between individuals based upon social status, therefore stabilizing the population over time (Lomnicki, 1988).

One can hypothesize why some individuals become dominant and other maintain a more subordinate role. Maynard Smith (1982) suggests that rank may be a strategy individuals adopt to maximize fitness in the population based upon the role of other individuals. This correlates with the Barnard and Sibly (1981) producer-scrounger game in which mixes of strategies works better than all one or the other of a specific strategy.

There are obvious ecological benefits to being the dominant individual and little point in interacting if there is an absence of benefits with aggressive interactions. The benefit of interactions must account not only the resource but the cost in obtaining the resource. The dominate individuals often have increased access to resources such as mates, food, and shelters (Collias, 1943; Barrette and Vandal, 1986). However, this may not always be the case since

many other factors play a role such as the value of the resource (Frank, 1986), the inability to monopolize a resource (Stahl et al., 2001), and the loss of resources' due stealing of stores/caches by other individuals (Sklepkovych, 1997). Furthermore, females with young often rise in the social ranks to better provide for their young (Figler et al., 1995), as well as hungry subordinate individuals often win encounters against dominants for access to food (Rohwer et al, 1981; Hansen, 1986).

There are many factors involved in the establishment of social dominance and it is well-documented that environmental cues play a major role in the outcome of social interactions whether through chemosensory (odors, Rutherford et al., 1996; Zulandt-Schneider et al., 1999), visual (opponent posturing, Bruski and Dunham, 1987; Smith and Dunham, 1990) and/or tactile cues (physical combat, Bovbjerg, 1953, 1956; Issa et al., 1999; Goessmann et al., 2000).

A great model system to study social interactions is in crayfish since typical interactions have been well documented for decades. It is from the many studies on social interactions that crayfish are known to form social hierarchies after very aggressive interactions (Bovbjerg, 1953, 1956, 1970; Kravitz et al., 1980; Huber et al., 1997; Li et al., 2000; Issa et al., 1999; Goessmann et al., 2000). Typically, the encounters escalate from visual threats of defense posturing to actual physical confrontations that include cheliped grasping and more aggressive behaviors where one will try to dismember or even kill another individual.

Currently, most studies observing social interactions occur in a natural field site or a location that mimics the environment. While this gives insights into typical behaviors, little is known of the interaction dynamics when an organism leaves the aquatic environment or when sensory systems are diminished. Crayfish leave the water for various reasons such as to find food, mates or excessive competition. The environmental change with the absence of water would eliminate the typical escape response (i.e., tail flip) which allows for a fast retreat from conspecifics. In addition, the absence of water results in other factors influencing social interactions, such as a higher energy demand for movement mainly due to the lack of buoyancy, a greater probability of injury due

to a slower response in movement, as well as retreat and also the lack of assessment through chemical cues of not only conspecifics, but the environment in general. Thus, an organism is at greater risk since they lack the ability to assess their opponent and/or locate a safe place for retreat. This is especially true for a species evolutionarily lacking a sensory modality. It is of interest to also examine the effect of diminished visual and olfactory/chemosensory sensory system. This is possible through studies in the absence of white light and the removal of the primary sensory appendage, antennules.

Although vision and tactile have been suggested to be very important in social interactions for mediating the transfer of information, the full understanding on the ways these two sensory cues are used in agonistic communication remains unclear. It has been well-studied and shown that vision is important for agonistic communication in other decapod species, such as fiddler crabs (Crane, 1966; Wright, 1968; Hyatt and Salmon, 1979), hermit crabs (Hazlett and Bossert, 1965; Hazlett, 1972; Dunham, 1978a, b; Dunham and Tierney, 1983), lobsters (Scrivener, 1971) and mantis shrimp (Dingle, 1969). Due to this obvious factor in so many other decapod crustaceans, I assumed that the visual sensory cue would also be very important for information exchange among crayfish. I chose to separate the roles of vision and chemosensory in the agonistic communication of *P. clarkii* by conducting experiments in red light (not visible to *P. clarkii*) as well as removing the antennules, both independently and additively. The study of vision in this species is particularly appropriate, given that *P. clarkii* is normally active under a wide range of environmental light levels.

Blind cave crayfish (*Orconectes australis packardi*) lack visual capabilities and therefore they provide the opportunity to examine whether behavioral, morphological, and/or physiological evolutionary adaptations may have evolved unique to their species based upon the cave environment. Since cave crayfish have a reduced optic system they have been shown to have more olfactory projection neurons than surface crayfish, suggesting they have more neural processing related to olfaction (Cooper et al., 2001). Thus, cave crayfish rely primarily on olfactory and tactile modalities while surface crayfish rely primarily

on visual and olfactory to monitor their surroundings. In accordance with the above information, it is logical that cave crayfish do not show the typical postural behaviors (visual display) identified in social encounters within their natural cave environment. It can be hypothesized that such displays would not be beneficial since conspecifics are not able to observe the visual display. While studies have addressed the neural systems of optic systems (Cooper et al., 2001) and the effects of light on social interactions (Li and Cooper, 2002), the typical behaviors of cave crayfish have not been as thoroughly studied as surface crayfish. Currently, little is known of interaction dynamics in the absence of water which eliminates the typical escape response (i.e. tail flip) and/or with diminished chemosensory systems will introduces other factors previously outlined.

Chemical cues are known to be involved in the establishment of social hierarchies and are known to impact behavior (Ameyaw-Akumfi and Hazlett, 1975; Thorpe and Ammerman, 1978). Bovbjerg (1956) first suggested that both vision and tactile are involved in establishment social hierarchies and he also demonstrated that antennae are important for tactile orientation. The antennule is considered the organ most specialized for chemosensory detection and plays a leading role in tracking odor plumes (Devine and Atema, 1982) and individual recognition (Karavanich and Atema, 1998b). One way to address the influence of sensory cues is to remove important sensory systems individually and simultaneously. Specifically, by removing the antennules and vision through the absence of white light (in red light), one can understand the reliance on sensory cues. Surface crayfish rely primarily on visual and olfactory modalities for monitoring surroundings and one can only assume that cave crayfish rely primarily on chemosensory cues to evaluate the environment and opponents.

Chemical signals are important sources of information. This is especially true for aquatic environments were visibility in water is often limited when compared to terrestrial environments (Eisner and Meinwald, 1995). The transmission of chemical signals involves information by the sender to be received by anther individual which will most likely elicit a behavioral response (Bradbury and Vehrencamp, 2009). Crayfish are known to have a well developed olfactory

system and studies have shown that chemical signals play an important role in many aspects of their life (Tierney and Dunham, 1982; Zulandt Schneider and Moore, 2000; Zulandt Schneider et al., 1999). Specifically in agonistic encounters, chemical signals appear to be more important than other offensive displays and signals for settling a fight (Breithaupt and Petra, 2002). Interestingly, research has shown that some species are able to recognize individuals that they have encountered in the recent past such as two species of hermit crabs (Gherardi and Atema, 2005; Gherardi and Tiedemann, 2004; Hazlett, 1969), a crab (Vannini and Gherardi, 1981), a mantis shrimp *Gonodactylus festae* (Caldwell, 1979; Caldwell, 1985), the lobster *Homarus americanus* (Karavanich and Atema, 1998a) and the crayfish (Lowe, 1956). It has been shown that the individual recognition is based upon chemical signals that are emitted during social interactions (e.g. Hurst 1990a,b,c), including crustaceans (Atema and Steinbach, 2007). The chemical signals are important in maintaining the stable dominance hierarchies.

In the matter of visual cues, previous experiments examining social interactions in white light will be compared to experiments in red light to understand the photoreceptors influence on social interactions. Comparisons will allow generalizations concerning communication as the relationship between environment and type of display pattern. It is obvious that behavioral analyses require extensive quantitative data, yet too detailed extensive quantitative analysis may generalize the results too much and therefore lose the behaviors that are not common in the literature. Thus, much of the analysis in this chapter is presented graphically to understand species differences.

In some theoretical models, interactions were assumed to occur at random (Theraulaz, et al., 1991; Bonabeau et al., 1995) while others examined the relationship between particular interaction probabilities and social and spatial characteristics (Theraulaz et al., 1995, Bonabeau et al, 1996; Hemerlrijk, 1998, 2000). Presumably, the information derived from the environment could modify baseline perception of cost based on cues that would be used by naïve individuals at the onset of interactions, including visual, chemical or the lack there

of. Information about perceived cost, which reflects fighting ability, in a future contest might come from one or more sensory systems. Numerous sensory cues are relevant to a general model of integration of sensory information which ultimately influences perception of potential costs with interactions.

Early work on social organization in animals assumed that a hierarchy was simply a combination of dyadic relationships, the outcomes of which were based only on factors intrinsic to the individuals (size, age, physical prowess; Ginsburg and Allee, 1942; Collias, 1943; Allee et al., 1955). However, since contests often terminate early before escalation, a variety of information is utilized in addition to the intrinsic factors. Thus, given that individuals terminate early, it suggests a more complex relationship between environmental effects, contest initiation and sensory cues. It is apparent that many factors determine social interactions, specifically the role of environmental cues. Whether environment effects alter social interactions in an extreme manner has yet to be empirically tested. With the assumption that all group members begin with equal fighting abilities, environmental effects or additive diminished sensory cues will mostly likely disrupt typical intrinsic behavior. Furthermore, when multiple cues are diminished, the influence additively affects intrinsic behavior. Thus, by examining reliance on single sensory systems on well-defined social behavior; we can begin to understand environmental impacts on populations/species.

Past research has examined many extrinsic factors that influence intraspecific aggression. Such areas studied are shelter acquisition (Capelli and Hamilton, 1984; Peek et al., 1995; Figler et al., 1999), chemical communication (Bovbjerg, 1956; Zulandt Schneider et al., 1999, 2001), mating (Hill and Lodge, 1999), food preferences (Capelli and Munjal, 1982), and starvation (Hazlett et al., 1975; Stocker and Huber, 2001). An area not yet addressed is the extrinsic factor of ‘out of water’ for crayfish social interactions and it is unclear whether the hydrodynamics of natural habitats allows for the successful use of chemical signals and typical behavior during social interactions in nature. Thus, the purpose of this chapter is to present quantitative analysis of environmental

influence on social interactions in two species of crayfish with special reference to reliance of different primary sensory modalities.

Intrinsic and extrinsic factors affect intraspecific aggression in many ways and both should be examined for the impact on agonistic behavior. Here, a simple additive model for this integration of multiple sensory systems as well as multiple environmental factors in individual's expected fighting ability determined the impact of additive effects. Examination of environmental influence on behavior was through the measure of fighting strategy and intensity of *Procambarus clarkii* (sighted) and *Orconectes australis packardi* (blind). Social interactions understand whether environmental conditions (i.e. in and out of water) and/or sensory cues (i.e., olfaction, vision/photoreceptors) influence fighting strategy and/or intensity. The two main study focuses are, 1) sighted crayfish behavior compared to blind behavior in varying environmental conditions and 2) in water social behavior compared to out of water behavior.

Specific Aims:

Examine environmental modulation of intrinsic behavior during social interactions in and out of water among dyads:

a. To determine the significant reliance on olfaction and environmental influence, which impacts the ability to assess the opponent (urine plumes) and to escape (i.e. tail flip response) in cave crayfish.

b. To understand the role of vision and olfaction during social interactions of surface crayfish resulting from changes in environmental conditions (i.e. presence/absence of water, presence/absence of vision).

c. To identify species-specific behaviors through comparison of cave and surface species, as well as determining the environmental and olfactory influence on intrinsic behaviors.

METHODS

Animals

Crayfish, *Procambarus clarkii* (sighted), measuring 5.08-6.25 cm in body length were obtained commercially (Atchafalaya Biological Supply Co., Raceland, LA). Crayfish, *Orconectes australis packardi* (Rhoades) (the blind crayfish), measuring 4.6-6.4 cm were obtained from the Sloan's Valley Cave System near Somerset, KY (collecting permits were obtained for this study; Figure 2.1). A total of 25 sighted and 15 blind crayfish were used in the study. Only male crayfish of approximate length were used in this study. Animals were housed individually in rectangular plastic containers and cared for in the same manner, except *O. a. packardi* were covered with black plastic to omit light 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 before and throughout the experiment. Crayfish handling was conducted by using a glass beaker to transfer crayfish from one container to the other. Due to 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.

Figure 2.1. Blind cave crayfish, *Orconectes packardi australis*, engaged in an agonistic encounter.

Social Interactions

Initial experiments (i.e., low light) were focused on characterizing the general behavior interactions for both species of crayfish. Crayfish were randomly distributed into fourteen treatment groups (N = 5 pairs per group) discussed below. Social interactions were staged in sized matched males. A social interaction behavioral scoring index was developed (Table 2.1 A) for species comparison of *Procambarus clarkii* and *Orconectes australis packardi.* Observational pre-experimental trials identified typical crayfish behavior to establish a quantifiable scale for interactions based on both aggressiveness, as well as intensity (time duration of the interaction, Table 2.1B). Crayfish male pairs of approximate equal size were staged in a glass aquarium and videotaped for one hour, allowing for interaction without outside interference. The crayfish were monitored indirectly through a TV monitor so that the observer could interject to ensure that the crayfish do not seriously harm one another. If interjection occurred, the trial was stopped and the pair was removed. Trials conducted in low light in the water act as controls for the sighted crayfish, while trials conducted in red light and in water act as controls for blind crayfish. The scale was then used to quantify each of the trials for an across condition and species comparison. Behavioral scores were assigned to pairs of crayfish (not individual scoring) for every interaction occurring during the 60 minute time period.

Behavioral Analysis

Previous research and prior observation of aggressive interactions between individuals indicate that the behavior could be classified into several rather distinct categories. These categories, with the exception of walking around before and interactions, represent behavior patterns in what are relatively stereotyped and which are known to be typical behaviors of crayfish. Briefly the behavioral acts established are as follows (also see Table 2.1A):

No Interaction – 0 – No encounter without any evidence of awareness of other individual.

Territory Invasion/Approach/Retreat – 1 – Deliberate movement towards other individual and a direct, initiation into conspecifics space and/or movement away.

Intentional touching – 2 – A short rapid movement forward directed at individual.

Acknowledgement/Standoff – 3 – Facing one another without visual threat display.

Meral Spread/Threat Display – 4 – Outward raising and spreading of the chelipeds.

Chase – 5 – Pursuit after the individual.

Grasp/Strike – 6 – A blow to or seizing of other individual.

Dismemberment – 7- Very aggressive action to individual in which dismemberment or likelihood of killing is apparent.

Most of the general characteristics are previously described in Dingle and Caldwell (1969). Interactions typically began with an invasion of territory or an acknowledgment/standoff. Termination of the interaction is when the observer thought that individuals no longer appear to be directing behavior at each other. Communication may be occurring, but since the purpose of this study was to concentrate on aggressive interactions, no attempt was made to analyze other possible communicative behaviors. Quantification of behavior was based upon

total number of interactions in and out of the water as well as the length of each interaction.

Every trial for each condition was analyzed for individual crayfish behavior, as well as general behavioral trends within and across species. For each experimental set, five trials (N = 5) were run in the water and out of water. All trials were digitally recorded and analyzed through video analysis to record behavioral scores and intensity. To understand behavioral trends, 3-D graphs combined all trials together for comparison of the type of behavior as well and intensity of each encounter. ANOVA statistical analysis based upon probability $p<0.05$ and Holm-Sidak post hoc analysis were used to determine significance in specific conditions. A Student's t-test with probability of $p < 0.05$ was used to determine significance between conditional groups. Antennule flicking was observed in the control experiment as a further measure of olfactory cues.

The duration of trials was used as a measure of interaction intensity. Since interactions are known to be relatively short (Bergman and Moore, 2003), a time scale was used (Table 2.1B).

Table 2.1. Social interactions behavioral scoring bioindex. (A) Indicates the behavioral scoring bioindex used to quantify behavior during each trial in experimental conditions. (B) Indicates the intensity scale based upon time duration in which the pairs engaged in a specific behavior.

A

0	No Interaction
1	**Territory Invasion**
2	**Intentional Touching**
3	**Acknowledgement**
4	**Threat Display**
5	**Chase**
6	**Grasp/Strike**
7	**Dismemberment**

B

0.1	1-15 seconds
0.2	**16-30 seconds**
0.3	**31-45 seconds**
0.4	**> 45 seconds**

Environmental Conditions

Several environmental conditions were observed and study conditions are outline in Table 2.2. Social interactions were examined in and out of water in low light (25 lux). 'In water' studies used a glass aquarium (20 cm x 10 cm x 12 cm) filled 4 cm from the top with aerated water. 'Out of water' studies were conducted using the same aquarium but without water and still providing wet sand for the animals to walk on. 'Out of water' studies were typically conducted first followed by rinsing the container, filling with water and conducting the 'in water' studies with a second pair of crayfish. Each pair of crayfish (N = 5) was observed in and out of water on different days to avoid individual recognition and therefore changing the outcome of interactions. 'In water' studies (control) for both sighted and blind crayfish will be compared to other environmental conditions to determine changes in intrinsic behaviors. Thus, study results examine 1) sighted 'in water', 2) sighted 'out of water', 3) blind 'in water', 4) blind 'out of water'.

Social interactions were also observed in red light. Red light conditions used a filtered red light (2.5 Lx) to remove the visual sensory stimulation for the sighted crayfish. The red light (Kodak Adjustable Safeway Lamp, 15 watts), was previously noted to be a wavelength not detected by crayfish (Li et al. 2000) thus providing no visual sensory stimulation. The purpose is to examine the reliance of visual cues for sighted crayfish out of water when chemosensory cues are

diminished. Furthermore, blind crayfish in red light will determine if low light induces a stress response that influences social behavior. Thus, study results examine 1) sighted/in water/red light, 2) sighted/out of water/red light, 3) blind/in water/red light, 4) blind/out of water/red light.

The removal of olfactory cues was conducted by removing the antennules (primarily sensory system for chemical detection) at the base of the antennules on the head of the crayfish. 'In water' and 'out of water' studies were again conducted for both species of crayfish. The purpose of removing the antennules was to further understand the reliance of visual cues for sighted crayfish and to understand impacts on social behavior for blind crayfish if there was a complete lack of environmental cue information. Thus, study results examine 1) sighted/in water/no antennules, 2) sighted/out of water/no antennules, 3) blind/in water/no antennules, 4) blind/out of water/no antennules.

To determine the reliance on environmental cues during social interactions for sighted crayfish, both chemosensory and visual cues were removed. Social interactions were examined for sighted crayfish only in red light and with the removal of antennules. Thus, study results examine 1) sighted/in water/red light/no antennules, 2) sighted/out of water/red light/no antennules.

Table 2.2. Social interaction study conditions for both species of crayfish. Social interactions were observed both in and out of the water for sighted and blind crayfish. Assessment of different sensory modalities impact on intrinsic behavior was examined through methodical removal of one or many sensory cues.

SIGHTED		BLIND	
In Water	**Out of Water**	**In Water**	**Out of Water**
Low Light	Low Light	Low Light	Low Light
Red Light	Red Light	Red Light	Red Light
Low Light/ No Antennules	Low Light/ No Antennules	—	—
Red Light/ No Antennules	Red Light/ No Antennules	Red Light/ No Antennules	Red Light/ No Antennules

Recording ECGs

The autonomic response was examined when sighted crayfish (N = 5) were placed into the experimental aquarium. Crayfish pairs were randomly chosen from the naïve population stock for 'in water' and 'out of water' trials. Order of which trials occurred first was random. There were multiple days in between running of the trials to ensure that social recognition was unlikely. Crayfish were wired to record electrocardiograms (ECGs) for heart rate (HR) (Listerman et al. 2000; Schapker et al. 2002; see JoVE, Bierbower & Cooper 2009). In brief, to obtain ECGs, two insulated stainless steel wires (diameter 0.005 inches and with the coating 0.008 inches; A-M Systems, Carlsburg, WA) were placed under the dorsal carapace directly over the heart 3 days prior to experimentation. Wires were inserted through holes drilled in the carapace and cemented in place with instant adhesive (Eastman, 5-min drying epoxy). These two wires were placed to span the heart in a rostral-caudal arrangement to insure an accurate impedance measure during each heart contraction as shown in Figure 2.2. A lid was used to prevent the crayfish from exiting the chamber but left a small section uncovered

for the wires to exit the chamber and did not prohibit the crayfish from moving freely (Figure 2.3). 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). All events were measured and calibrated with the PowerLab Chart software version 5.5.6 (AD Instruments, Australia). Previous studies showed that 3 days was enough time for the animals to return to physiological measurements similar to levels prior to handling (Wilkens et al., 1985). Cave crayfish typically have a thinner, more brittle exoskeleton resulting in more delicate handling during wiring. Although wiring is more difficult, I was successful in wiring cave crayfish for heart and ventilatory rates.

Analysis of the autonomic response used BPM changes between environmental conditions. HR was monitored in and out of water under control conditions to determine physiological responses during social interactions. This provided an internal measure to external cues. The experimental procedure consisted HR recordings before, during and after social interactions. HR was analyzed to provide a beats per minute (BPM) to note changes in the internal response based upon interactions, as well as environmental conditions.

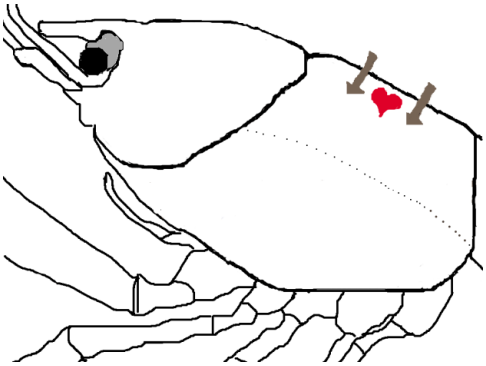


Figure 2.2. Schematic representation for the placement of the recording wires for monitoring the heart and ventilatory rates from a crayfish *(Procambarus clarkii*.). On the dorsal carapace, large arrows represent the two wires which

span the rostral-caudal axis of the heart to monitor any change in the dynamic resistance, which is used as a measure of heart rate.

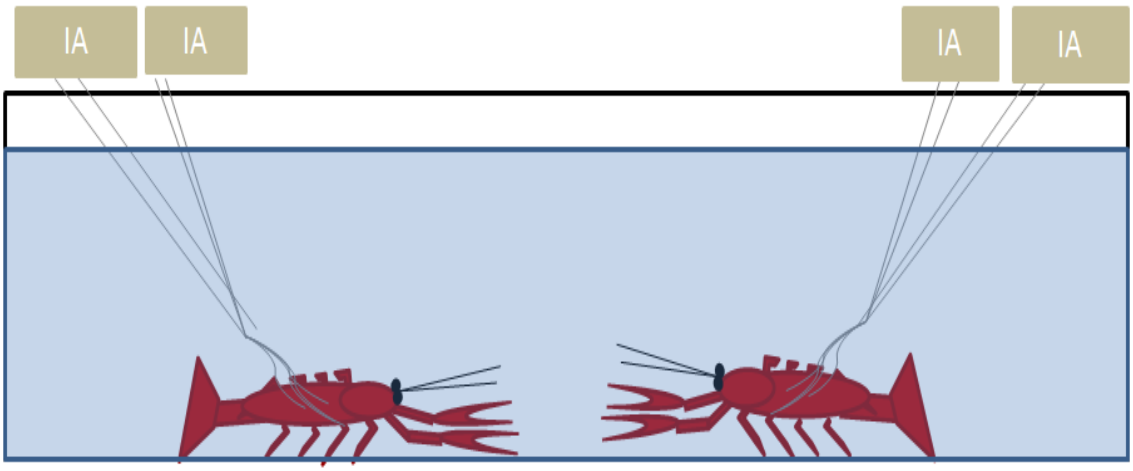


Figure 2.3. Schematic representation of social interactions. Crayfish were placed into the experimental aquarium and attached to the impedance amplifier. All behavioral interactions were recorded and later scored on behavioral score and interaction intensity.

RESULTS

Social Behavior in low white light

Five pairs of sighted and five pairs of blind crayfish were allowed to separately interact for 60 minutes in low white light (25 lux) to determine typical behavioral interactions. For sighted crayfish, the behavior of these pairs of animals was scored. For sighted crayfish in water, they were shown to interact regularly within the time period, as well as escalate the interactions to high levels of aggression indicated by the total interactions for the higher behavioral scores (i.e., 6 and 7; Figure 2.4 A). Interactions of sighted crayfish out of the water were

shown to occur less often and the interactions were shown to be less aggressive due to the few higher behavioral scores (Figure 2.4 B). Specifically, there are a relatively high number of interactions across all behavioral scores. Although there are few interactions overall, blind crayfish show the same trend for in and out of water (Figure 2.5 A, B). Total interaction comparisons shows a dramatic difference in that crayfish out of water are less likely interact with conspecifics (Figure 2.6) and that of those interactions, they are less likely to escalate to high levels of aggression. While total interactions show the trend for both environmental conditions it does not account for individual pair variation across treatments; therefore mean values were used for statistical analyses.

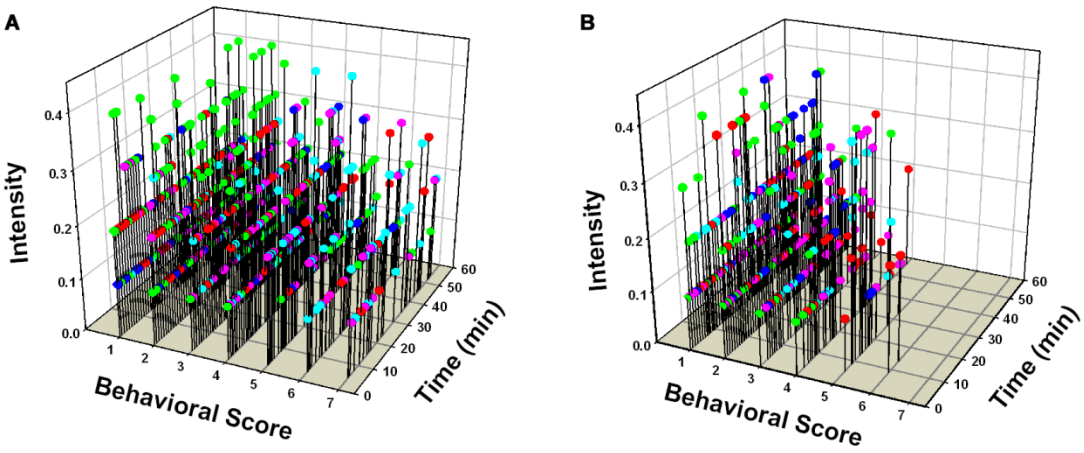


Figure 2.4. Comprehensive representation of social interactions for sighted crayfish in low white light (25 lux). (A) In water. (B) Out of water. A single vertical line indicates a given behavior at a specific point in time as well as the intensity of the behavior. The different colored points represent individual pairs in the trials (N = 5).

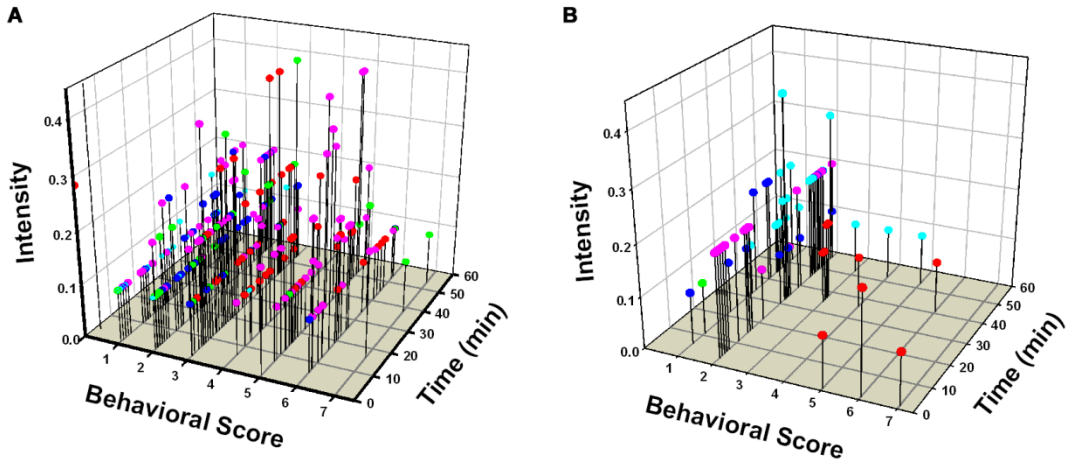


Figure 2.5. Comprehensive representation of social interactions for blind crayfish in low white light (25 lux). (A) In water. (B) Out of water. A single vertical line indicates a given behavior at a specific point in time as well as the intensity of the behavior. The different colored points represent individual pairs in the trials (N = 5).

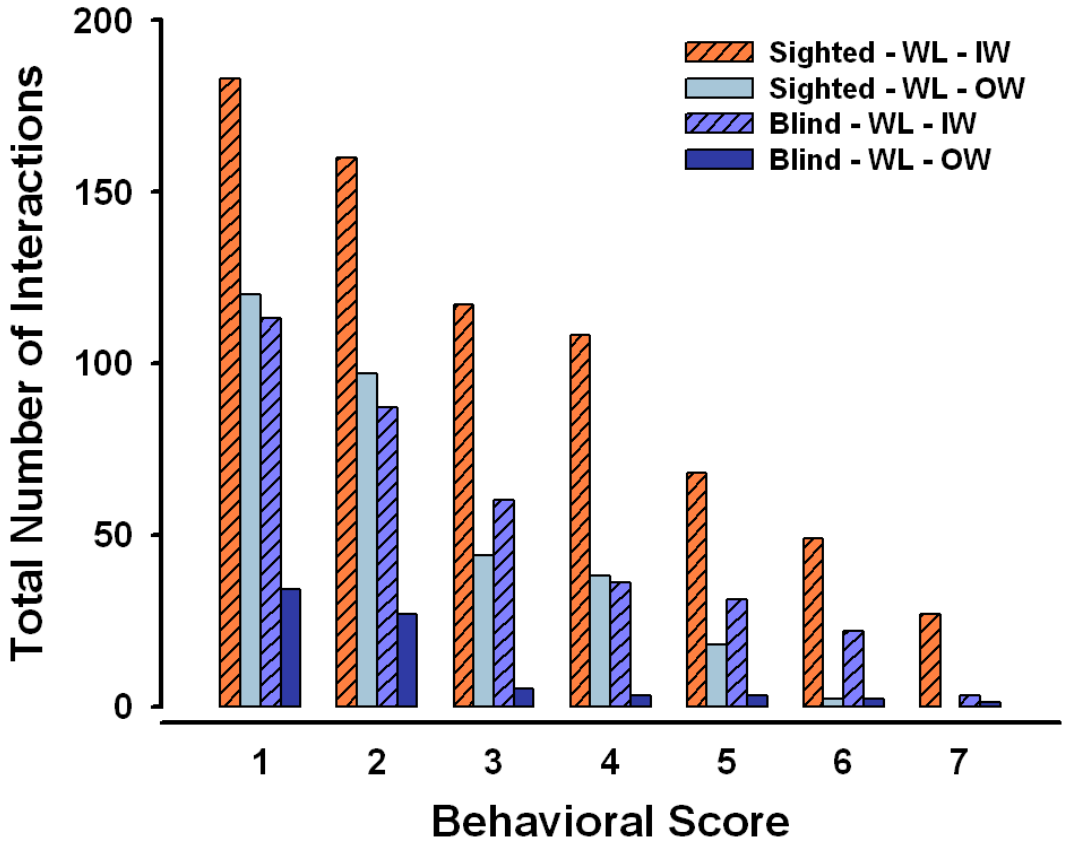


Figure 2.6. Comprehensive representation of the average number of social interactions for sighted crayfish in low white light. A single vertical bar indicates a given behavioral score. Black bars indicate sighted crayfish behavior in water and grey bars indicates out of water (N = 5). The mean number (± SEM) of behavioral score interactions over the 60 minute trial period was assessed for each condition.

'In water' versus 'out of water': statistical analysis

The pairs of animals were examined for variability within each behavioral score. Pairs were normally distributed for sighted and blind crayfish for both 'in water' and 'out of water' shown by behavioral score, sample size, mean ± SEM. Sighted 'in water', 1(N = 5, 37±4.1), 2(N = 5, 32±4.2), 3 (N = 5, 23±3.5), 4 (N = 5, 22±2.5), 5 (N = 5, 14±3.3), 6 (N = 5, 10±3.4), 7 (N = 5, 5±2.1). Sighted 'out of

water', 1(N = 5, 24±4.2), 2(N = 5, 19±2.9), 3 (N = 5, 9±1.8), 4 (N = 5, 8±0.9), 5 (N = 5, 4±0.6), 6 (N = 5, 0.4±0.4), 7 (N = 5, 0.2±0.3). Pairs were also normally distributed for blind crayfish. Blind 'in water', 1(N = 5, 23±2.6), 2(N = 5, 17±3.2), 3 (N = 5, 12±2.5), 4 (N = 5, 7±2.1), 5 (N = 5, 6±1.6), 6 (N = 5, 4±1.9), 7 (N = 5, 1±0.4). Blind 'out of water', 1(N = 5, 7±1.8), 2(N = 5, 5±1.7), 3 (N = 5, 1±0.8), 4 (N = 5, 1±0.4), 5 (N = 5, 1±0.4), 6 (N = 5, 0.4±0.4), 7 (N = 5, 0.2±0.2).

Across groups statistical analysis for sighted crayfish in Figure 2.7 shows there is a significant decrease in low light of 'in water' compared to 'out of water' for each behavioral score (ANOVA, Holm-Sidak post-hoc: $F_{13, 69} = 47.13$, $p < 0.001$). The number of interactions is significantly less when social interactions occur out of water. This is also true for blind crayfish as shown in Figure 2.8 for behavioral scores 1-5 (ANOVA, Holm-Sidak post hoc analysis: $F_{13, 69} = 37.71$, $p < 0.001$). High aggressive scores were shown to not be significant due to blind crayfish escalating so few interactions both in and out of the water. Although it has never been tested, it is possible that cave species may be less likely to establish dominance hierarchies than their surface counterparts.

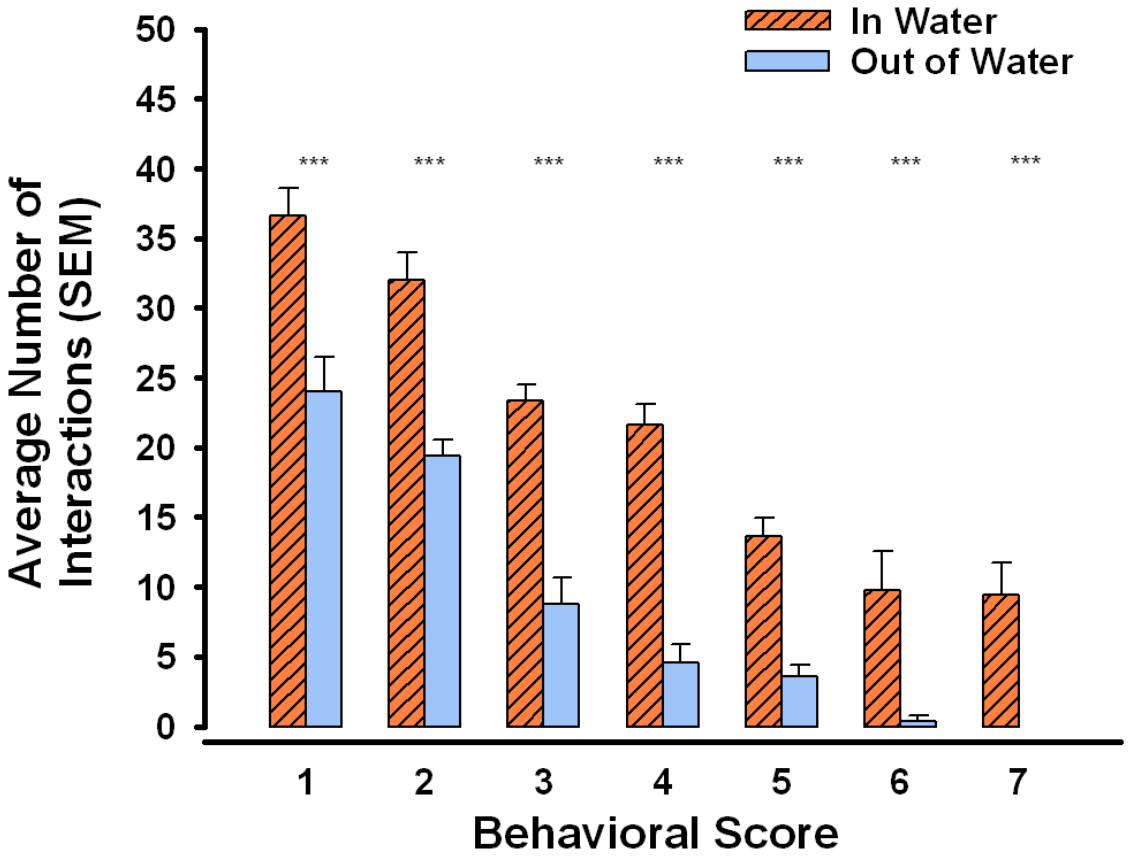


Figure 2.7. Comprehensive representation of the average number of social interactions for sighted crayfish in low white light. A single vertical bar indicates a given behavioral score. Black bars indicate sighted crayfish behavior in water and grey bars indicates out of water (N = 5). The mean number (± SEM) of behavioral score interactions over the 60 minute trial period was assessed for each condition. There is a significant decrease in the number of interactions 'out of water' as compared to 'in water' conditions. Across group ANOVA test: *** indicates $p < 0.001$.

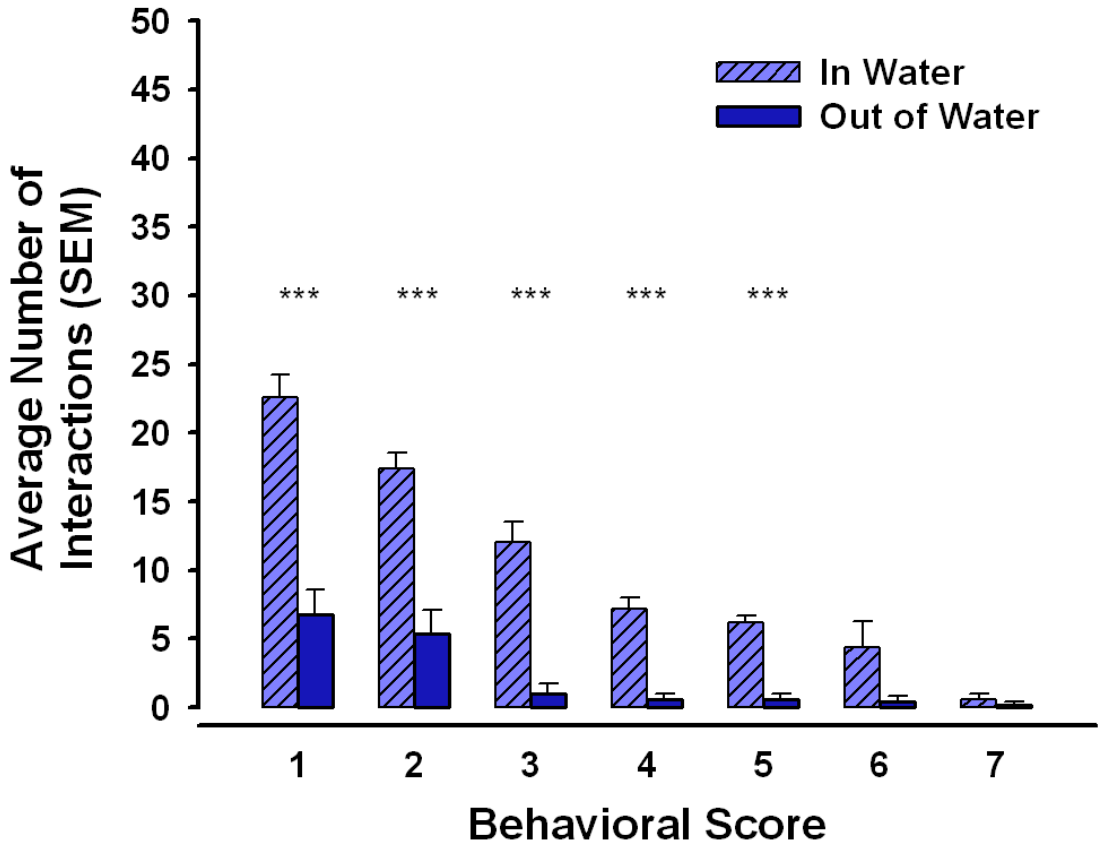


Figure 2.8. Comprehensive representation of the average number of social interactions for blind crayfish in low white light. A single vertical bar indicates a given behavioral score. Black bars indicate sighted crayfish behavior in water and grey bars indicates out of water (N = 5). The mean number (± SEM) of behavioral score interactions over the 60 minute trial period was assessed for each condition. There is a significant decrease in the number of interactions 'out of water' as compared to 'in water' conditions (Student's t-test; *** $p < 0.001$).

Analysis of varying environmental conditions

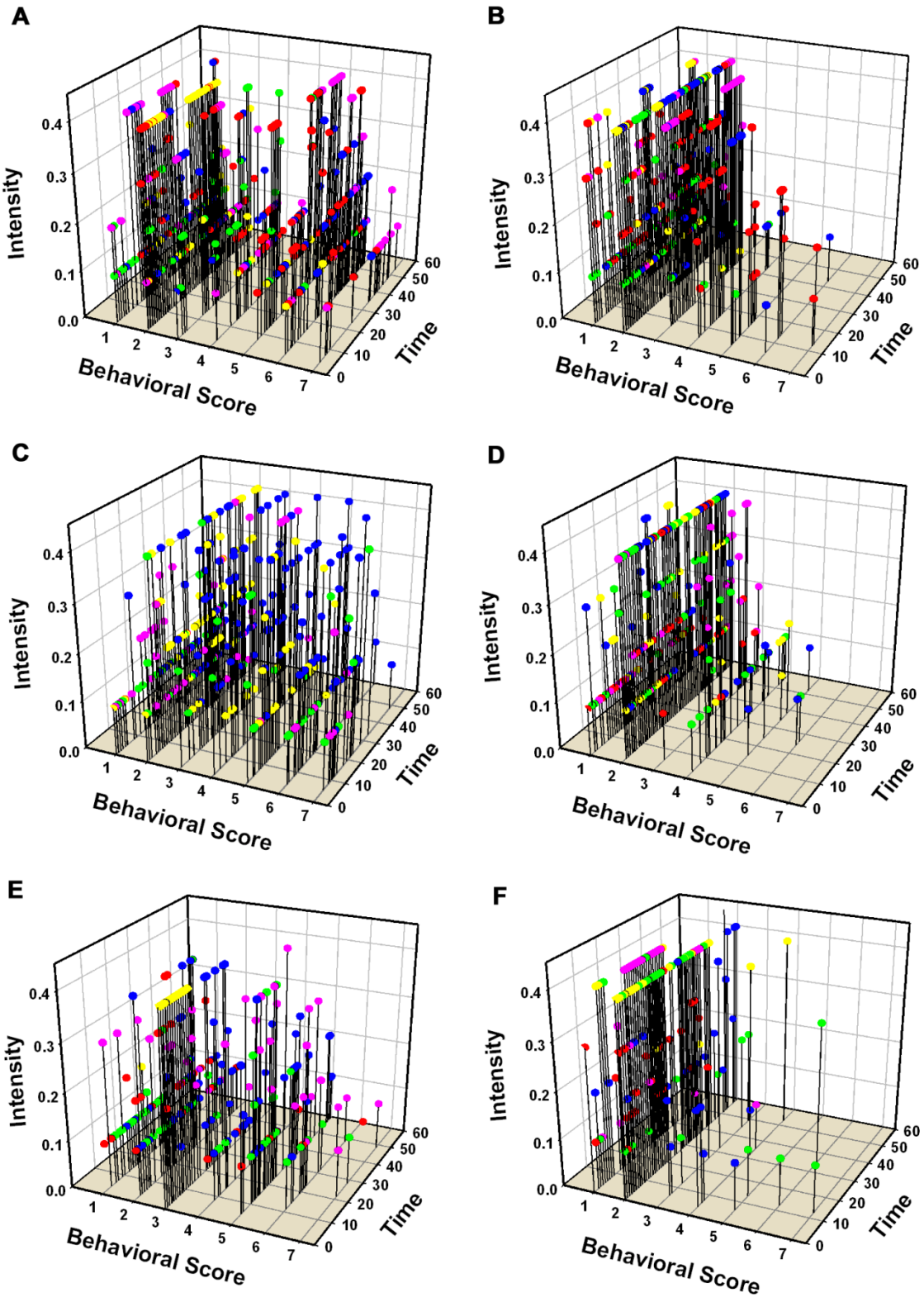
Analysis of in and out of water treatment groups showed significant changes in fighting strategy due to environmental effects. Specifically, out of water results alone or in combination with other conditions, reveal that both species do not tail flip and show less intrusion into conspecifics territory when compared to social

interactions in the water (Figures 2.9, 2.10). Blind crayfish were less responsive to the presence of conspecifics (fewer interactions) while surface crayfish showed an increase in visual displays (possible bluffing mechanism) when interacting out of the water, but failed to escalate the interaction when compared to interaction conducted in the water. Thus, for both species, out of the water has the most significant impact on intrinsic behavior and social interactions. ANOVA statistical analysis for each environmental condition shows a significant difference between in and out of water conditions as indicated in the summary tables below (Tables 2.3, 2.4). ANOVA values are as follows: sighted in red light ($F_{13, 69} = 33.7$, $p < 0.001$), blind in red light ($F_{13, 69} = 17.0$, $p < 0.001$), sighted in white light with no antennules ($F_{13, 69} = 17.8$, $P < 0.001$), sighted in red light with no antennules ($F_{13, 69} = 7.588$, $p < 0.001$), and blind in red light with no antennules ($F_{13, 69} = 19.3$, $p < 0.001$). Therefore, interactions occurring out of water showed both species of crayfish were less likely to interact and more likely to explore their environment.

Comparison across environmental conditions is not as easily assessed for significance. Due to this fact, more detailed statistical analysis is currently unavailable and this project is in collaboration with the University of Kentucky, Department of Statistics. Total number of social interactions for sighted (Figures 2.11) and blind (Figures 2.12) crayfish show that each condition For all treatment conditions, the pairs of animals were examined for variability within each behavioral score. Pairs were normally distributed for sighted and blind crayfish for both 'in water' and 'out of water' shown by behavioral score, sample size, mean ± SEM. For sighted in low white light, no antennules, 'in water', 1(N = 5, 15.6±7.8), 2(N = 5, 19.6±3.6), 3 (N = 5, 5.0±1.4), 4 (N = 5, 7.6±0.7), 5 (N = 5, 12.8±0.96), 6 (N = 5, 11.4±1.8), 7 (N = 5, 3.6±1.5). Sighted 'out of water', 1(N = 5, 10.8±2.2), 2(N = 5, 27.0±3.1), 3 (N = 5, 4.0±1.9), 4 (N = 5, 5.2±2.2), 5 (N = 5, 2.1±0.73), 6 (N = 5, 0.4±0.24), 7 (N = 5, 0.0 ±0.0). For sighted in red light, 'in water', 1(N = 5, 20.2±1.8), 2(N = 5, 25.6±1.4), 3 (N = 5, 9.2±0.4), 4 (N = 5, 7.6±1.0), 5 (N = 5, 14.2±0.8), 6 (N = 5, 17.4±1.8), 7 (N = 5, 5.2±0.8). Sighted

‘out of water’, 1(N = 5, 17.4±2.9), 2(N = 5, 29.8±2.9), 3 (N = 5, 12.6±1.3), 4 (N = 5, 12.4±0.7), 5 (N = 5, 2±1.0), 6 (N = 5, 1.0±0.6), 7 (N = 5, 0.4±0.4).

Pairs were also normally distributed for blind crayfish. For blind in red light, ‘in water’, 1(N = 5, 25.4±4.6), 2(N = 5, 32±5.2), 3 (N = 5, 15±2.2), 4 (N = 5, 1.4±0.4), 5 (N = 5, 20.6±2.6), 6 (N = 5, 7.4±1.2), 7 (N = 5, 3.8±0.8). Blind ‘out of water’, 1(N = 5, 15.8±1.4), 2(N = 5, 21.8±2.7), 3 (N = 5, 1.2±0.4), 4 (N = 5, 1.0±0.6), 5 (N = 5, 0±0.0), 6 (N = 5, 0.0±0.0), 7 (N = 5, 0.0±0.0). For sighted in red light, no antennules, ‘in water’, 1(N = 5, 10.8±2.9), 2(N = 5, 14.0±2.0), 3 (N = 5, 5±3.0), 4 (N = 5, 3.6±1.2), 5 (N = 5, 8.6±2.8), 6 (N = 5, 2.6±1.2), 7 (N = 5, 0.8±0.4). Sighted ‘out of water’, 1(N = 5, 11.6±2.9), 2(N = 5, 19.8±2.9), 3 (N = 5, 2.0±0.3), 4 (N = 5, 1.0±0.7), 5 (N = 5, 0.4±0.2), 6 (N = 5, 0.4±0.4), 7 (N = 5, 0.2±0.2). For blind in red light, no antennules, ‘in water’, 1(N = 5, 27.0±1.6), 2(N = 5, 20.8±3.1), 3 (N = 5, 18.6±3.2), 4 (N = 5, 11.0±2.3), 5 (N = 5, 7.6±2.2), 6 (N = 5, 0.8±0.4), 7 (N = 5, 0±0). Blind ‘out of water’, 1(N = 5, 15.0±1.4), 2(N = 5, 19.4±2.1), 3 (N = 5, 4.4±2.0), 4 (N = 5, 0±0.0), 5 (N = 5, 0.2±0.2), 6 (N = 5, 0.0±0.0), 7 (N = 5, 0.0±0.0).


A
B
C
D
E
F
Intensity
Behavioral Score
Time
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0.1
0.2
0.3
0.4
1
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3
4
5
6
7
0
10
20
30
40
50
60

Figure 2.9. Comprehensive representation of social interactions for sighted crayfish in varying environmental conditions. (A) Red light and in water. (B) Red light and out of water. (C) Low white light, no antennules and in water. (D) Low white light, not antennules and out of water. (E) Red light, no antennules and in water. (F) Red light, no antennules and out of water. A single vertical line indicates a given behavior at a specific point in time as well as the intensity of the behavior. The different colored points represent individual pairs in the trials (N = 5).

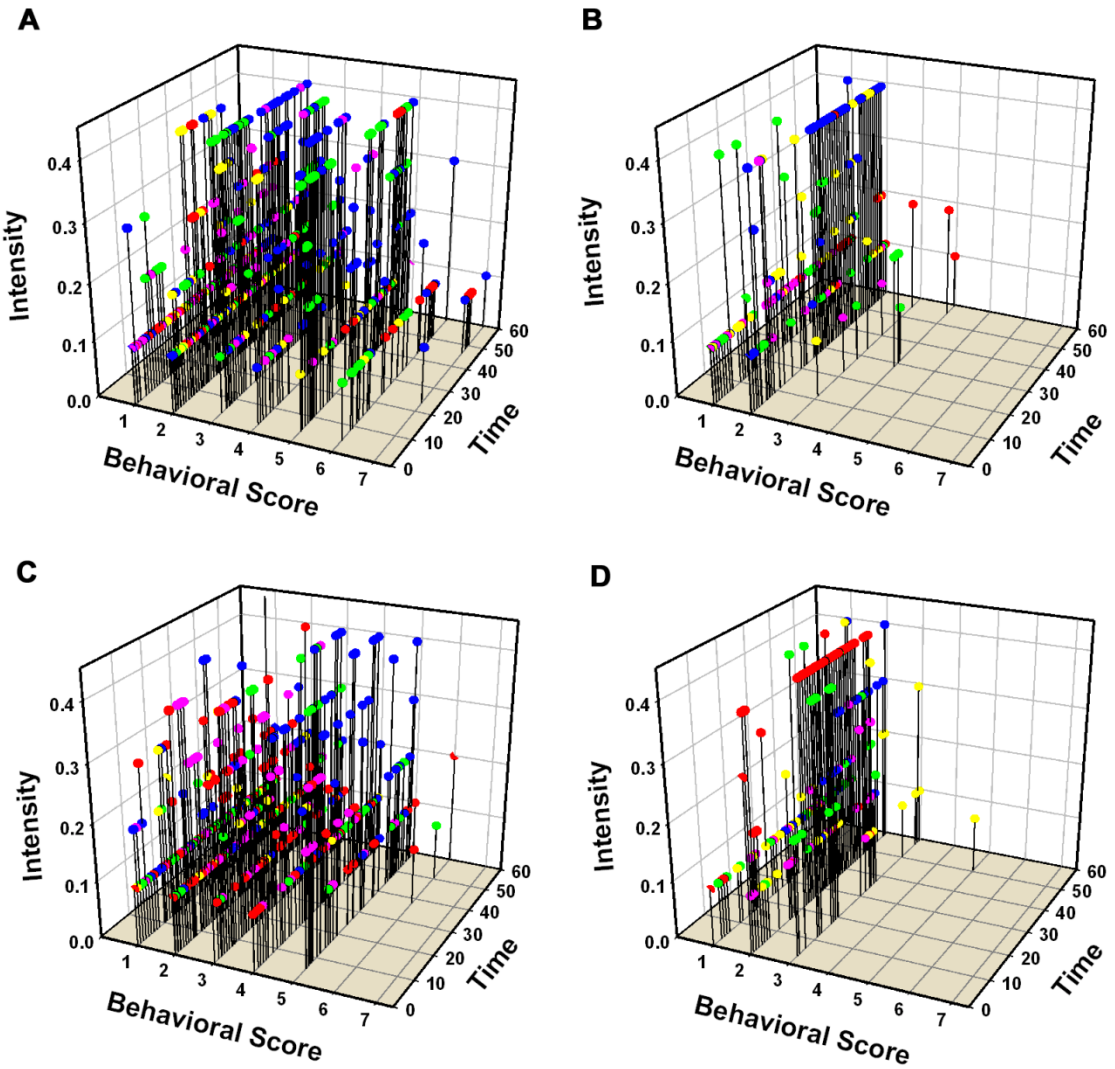


Figure 2.10. Comprehensive representation of social interactions for blind crayfish in varying environmental conditions (A) Red light and in water. (B) Red light and out of water. (C) Red light, no antennules and in water. (D) Red light, no antennules and out of water. A single vertical line indicates a given behavior at a specific point in time as well as the intensity of the behavior. The different colored points represent a total of each individual pairs of crayfish in the trials (N = 5).

Table 2.3. Total number of social interaction across study conditions for sighted crayfish. Social interactions were observed for both 'in water' and 'out of water'. Each row corresponds to the total number of interactions for a given behavioral score. Each column corresponds to an environmental condition. In this and succeeding tables, the numbers in brackets are the total 'out of water' interactions.

Behavior	Low Light	Red Light	No Antennules	Red Light / No Antennules
Invasion (1)	183 (120)***	101 (77)**	47 (54)*	54 (58) NS
Touching (2)	160 (97)***	107 (120)**	98 (135)***	70 (99)***
Acknowledgement (3)	117 (44)***	41 (33)*	43 (20)**	25 (10)**
Threat Display (4)	108 (38)***	33 (22)*	39 (26)*	43 (5)***
Chase (5)	68 (18)***	51 (10)***	34 (6)***	43 (2)***
Grasp (6)	49 (2)***	63 (5)***	31 (2)***	13 (2)***
Dismemberment (7)	27 (0)***	20 (2)***	8 (0)***	4 (1)***

Table 2.4. Total number of social interaction across study conditions for blind crayfish. Social interactions were observed both 'in water' and 'out of water'. Each row corresponds to the total number of interactions for a given behavioral score. Each column corresponds to an environmental condition.

Behavior	Low Light	Red Light	Red Light / No Antennules
Invasion (1)	113 (34)***	127 (59)***	135 (75)***
Touching (2)	87 (27)***	160 (89)***	104 (97)*
Acknowledgement (3)	60 (5)***	65 (6)***	93 (22)***
Threat Display (4)	36 (3)***	37 (5)***	55 (0)***
Chase (5)	31 (3)***	73 (0)***	38 (1)***
Grasp (6)	22 (2)**	29 (0)***	4 (0)^{NS}
Dismemberment (7)	3 (1)^{NS}	8 (0)*	0 (0)^{NS}

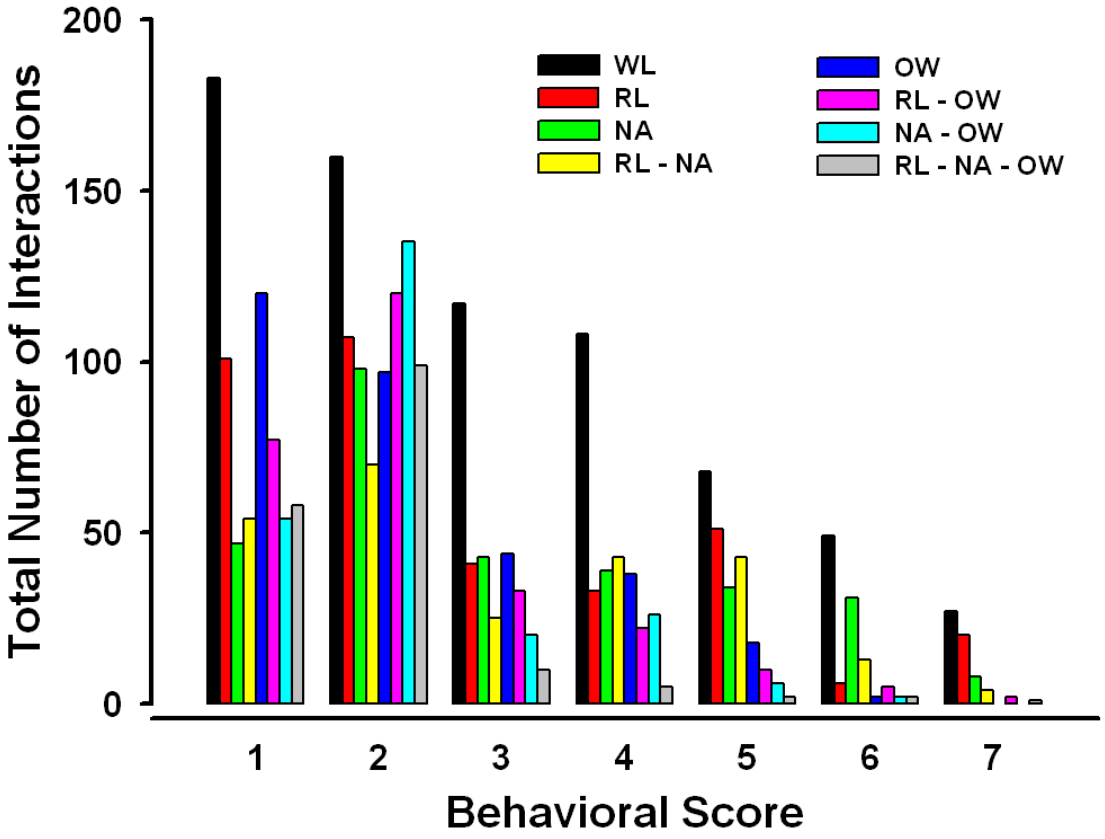


Figure 2.11. Comprehensive representation of total social interactions for sighted crayfish in all varying conditions. A single vertical line indicates a given behavior for a specific condition. (WL) White light. (RL) Red light. (NA) No antennules. (RL-NA) Red light, no antennules. (OW) Out of water. (RL-OW) Red light, out of water. (NA-OW) Low white light, no antennules, out of water. (RL-NA-OW) Red light, no antennules, out of water.

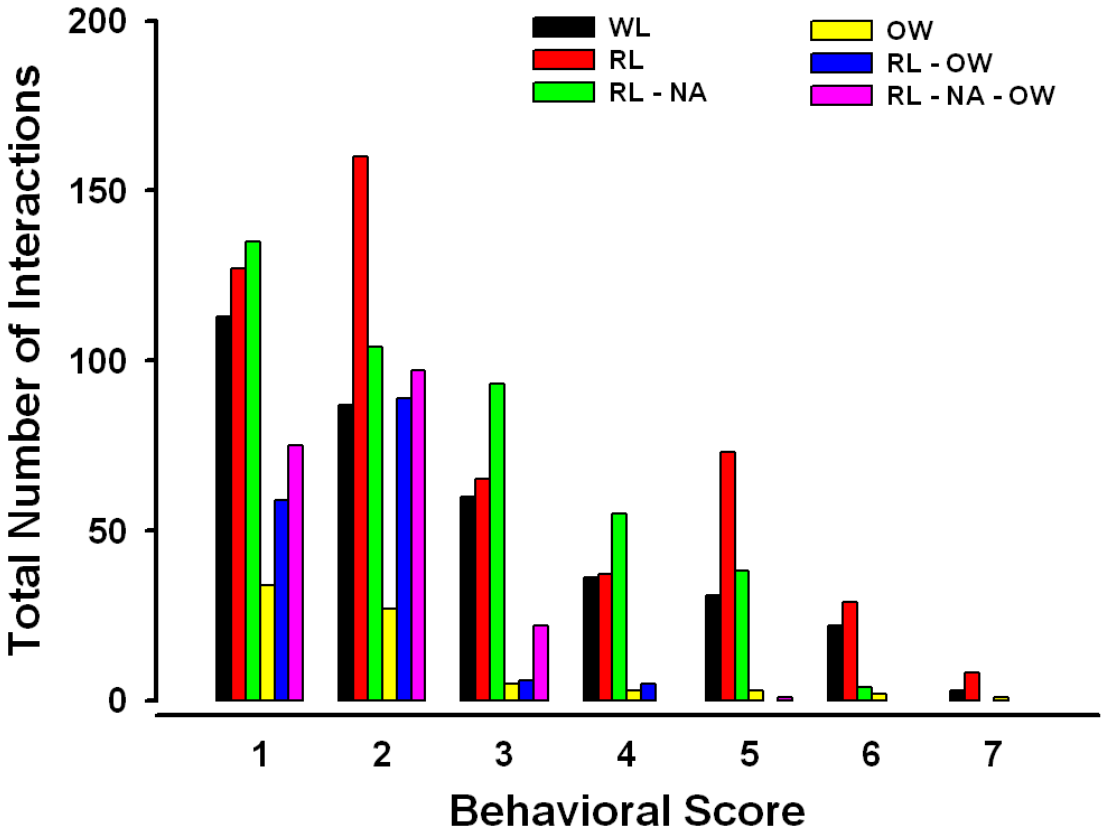
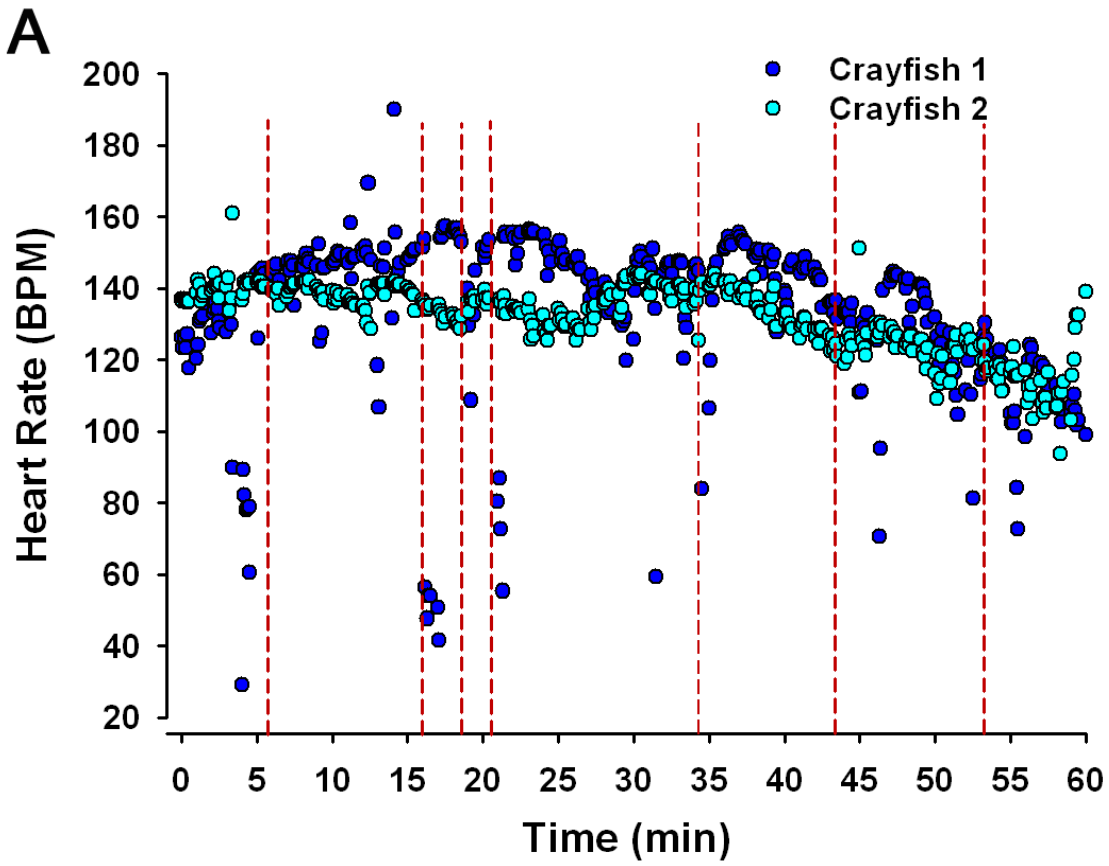


Figure 2.12. Comprehensive representation of social interactions for blind crayfish in all varying conditions. A single vertical line indicates a given behavior for a specific condition. (WL) White light. (RL) Red light. (RL-NA) Red light, no antennules. (OW) Out of water. (RL-OW) Red light, out of water. (NA-OW) Low white light, no antennules, out of water. (RL-NA-OW) Red light, no antennules, out of water.

Recording ECGs

The physiological response of crayfish was recorded to characterize the autonomic response during social interactions as well as with environmental change. Heart rate (HR) was recorded before, during and after confrontation, plotted for each crayfish during the entire duration of the trial. A frequency plot of

the raw traces shows dramatic changes in HR during interactions when comparing ‘in water’ to ‘out of water’ conditions (Figure 2.13). Specifically, there is a greater fluctuation for one individual (most likely the subordinate) during and after interactions. As consistent with previously described experiments, it is also shown that the ‘out of water’ condition has fewer interactions. The raw traces show a rapid response during interactions, especially for one individual, as well as the continued response after the interaction is over. This suggests that ‘out of water’ conditions have a greater effect on intrinsic factors, such as HR, for the individuals. This is most apparent for the individual most likely to become the subordinate since retreat away from the conspecifics is now not as feasible and greater injury is likely.


A
Crayfish 1
Crayfish 2
Heart Rate (BPM)
200
180
160
140
120
100
80
60
40
20
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)

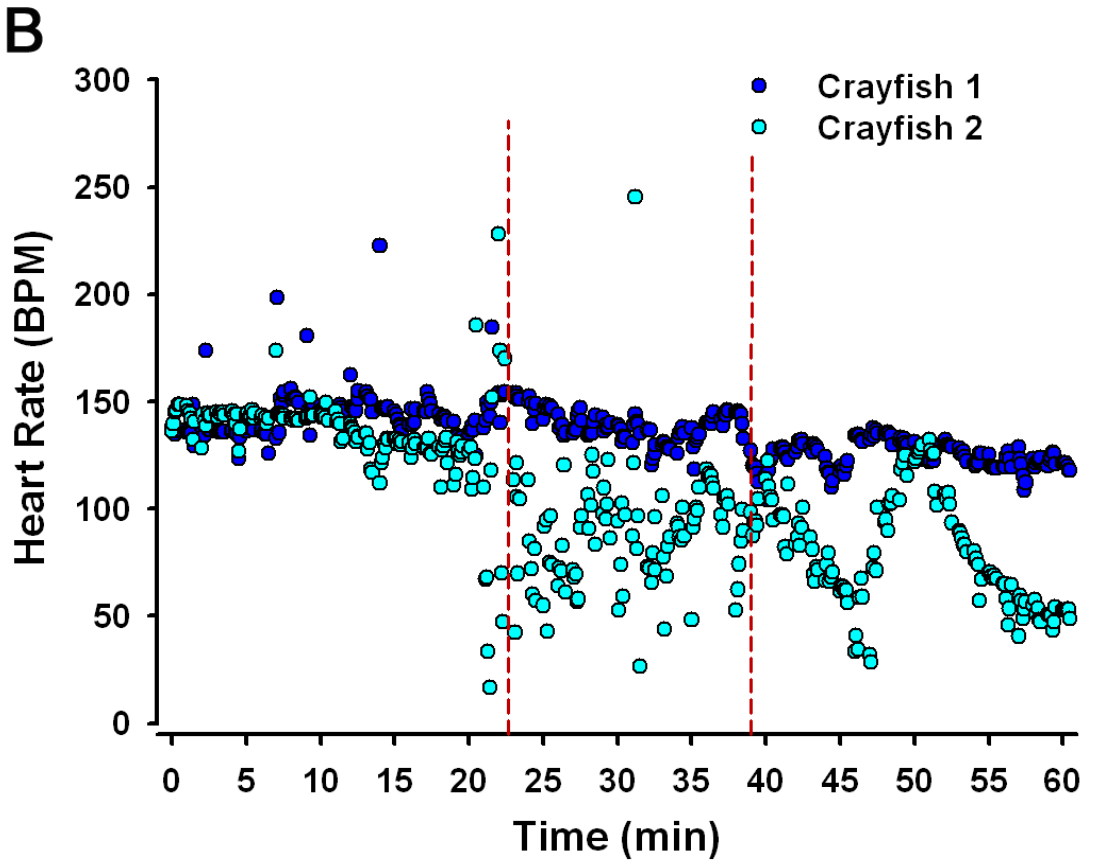

B
Crayfish 1
Crayfish 2
Heart Rate (BPM)
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)

Figure 2.13. Physiological response of a single pair of crayfish. (A) 'In water'. (B) 'Out of water'. The dark blue line indicates crayfish one and light blue indicates crayfish two. Each point represents direct counts of each beat over 10-s intervals and then converted to beats per minute (BPM). The same pair was used in both conditions with multiple days in between each trial.

DISCUSSION

This study demonstrated that environmental factors directly influence crayfish social interaction behavior. Here, we show that interactions were more aggressive more intense and more likely to end with a physical confrontation when they took place 'in water' than 'out of water' for two morphologically and genetically distinct species of crayfish (Fig. 2.9 and 2.10). It is shown that altering environmental conditions induced crayfish to change their intrinsic behavior which resulted in modified social interactions and a fighting strategy. For both species in weak white light and in water there are a high numbers of interactions and those interactions were likely to escalate to higher levels of aggression (behavioural score of 5, 6 or 7). The duration of interaction was consistently longer in time (intensity of 0.3 or 0.4). When water was removed from the environment the total number of interactions as well as the aggression level and duration of each interaction dramatically decreased for both species. Across all environmental conditions and removal of sensory systems (i.e., vision and chemosensory), removal of water produced the greatest and most consistent change in social interactions. For both species, results showed that when 'in water' crayfish had a higher frequency of more aggressive interactions, with higher levels of intensity than crayfish 'out of water'. For 'out of water' trials, both species were shown not to tail flip (typical escape response), showed less intrusion into the conspecifics territory and are less likely to engage in social interactions. While sighted crayfish did show an increase in visual displays out of

the water, a possible bluffing mechanism, they failed to escalate in social interactions.

Interactions in red light for sighted crayfish did not appear to decrease aggression levels. This is most likely due chemical cues providing enough information about the environment and the conspecific. The removal of antennules along with red light showed a reduction in the interactions numbers but did not diminished the aggression levels since many of the interactions escalated to behavioral score of 5 (chase) and 6 (grasp/strike). When these crayfish were taken out of water in combination with the diminished sensory cues, there was a dramatic decrease in aggression of social interaction. This pattern was similar for blind crayfish in red light and the lack of antennules. There were very few interactions and the aggression levels were dramatically decreased. Heart rate measures during social interactions for a single pair of crayfish showed that out of water interactions has a greater effect on the organism. It is likely that the dramatic effect on one of the individuals in the pair (most likely the subordinate) is due to an increased probability of injury will occur in the absence of water. Although heart rate remained relatively unchanged when the crayfish were placed into the chamber, heart rate was shown to immediately decrease for one individual upon interaction with the conspecific as well as remained lower for out of water trial. This same type of trend upon interaction was shown for in water trials but the decrease returned to previous levels rapidly.

Agonistic behavior is a fundamental factor of ecological nature and aggression has been studied extensively in many invertebrate species such as bees (Halling and Oldroyd, 2001; Paxton et al., 1999), ants (Beye et al., 1998; Brown et al., 2003; Nowbahari et al., 1999), termites (Polizzi and Forschler, 1999), wasps (Ruther et al., 2002), lobsters (Doernberg et al., 2001; Antonsen and Paul, 1997; Livingston et al., 1980; Peeke et al, 2000), crabs (Sneddon et al, 2000) and crayfish (Huber et al, 2001; Panksepp and Huber, 2002; Schroeder and Huber, 2001). Ritualized displays and cues that are predicative of agonistic success enable the assessment a rivals' relative fight ability (Kravitz and Huber,

2003). Fights occurring in nature are shown to be shorter, less intense and less likely to end with a tailflip but do show the fundamental fight dynamics seen in laboratory studies (Bergman and Moore, 2003). Although studies are often conducted in the lab, fighting is potentially costly to each contestant for a variety for factors including time and energy (Haller and Wittenberger, 1988; Haller, 1991; Thorpe, et al., 1995: Halperin et al., 1998; Neat et al, 1998), physical injuring (Austad, 1983; Gottfried, et al., 1985; Roberston, 1986; McPeck and Crowley, 1987; Crowley et al., 1988; Marler and Moore, 1988; Neat et al., 1998). A limited number of studies integrate multiple factors that can influence current contest behavior. Details of multiple factor integration for any one species are virtually unknown.

The types of behavioral repertories we described are similar to those indexed by Huber and Kravitz (1995) in the American lobster *Homarus americanus* and Bergman and Moore (2003) in two species of crayfish *Orconectes rusticus* and *Orconectes virilis*. However, we used a scale of 0-7 where Bergman and Moore used (-2)-5. While the general descriptions were similar for each behavioral level, there were modified classifications in areas described in holding an opponent as a 'do-see-do', which relates to a dance term, where we considered this behavior as a dismemberment grasp since they would try to twist the others cheliped off. We also indexed the time of interaction along with the aggression score and duration so that we could assess over time, the complexity of the repetitive interactions. As expected, behavioral scores incrementally decreased with increasing aggression levels and duration of interaction, as the hierarchy is likely established. Observational data from video as well as graph summaries document that the interactions do occur throughout the entire hour of the observation period. Specifically, interactions are just as likely to occur in the last ten minutes as they are in the first ten minutes. So even though a social status is being determined within the early interactions there are continuous bouts to confirm or test the opponent within this initial hour of being introduced. Previous work on the crayfish *Astacus astacus* showed that the number of agonistic challenges, mean duration and maximum intensity of encounters, were also

initially high but then decreased steadily as the hierarchy developed (Goessmann et al., 2000). Thus, the fact that interactions are still common after 50 minutes suggest that development of dominance relationships is incomplete. However, it should be noted that a limitation to laboratory studies is the restriction of escape from an opponent. This would be less of an issue in natural ecosystems; however, small interaction arenas in the laboratory may lead to more aggressive interactions (Hediger, 1950; Bergman and Moore, 2003).

If one were to document the sensory cues necessary for social dominance and maintenance of social hierarchy a more in-depth study is required. In this study the type of interactions and the effect of environment on these general levels of interactions was the focus. Many observations of crayfish behavior have been made to examine specific factors influencing intraspecific aggression such as in shelter acquisition (Capelli and Hamilton, 1984; Peek et al., 1995; Figler et al., 1999), chemical communication (Bovbjerg, 1956; Zulandt Schneider et al., 1999, 2001), mating (Hill and Lodge, 1999), food preferences (Capelli and Munjal, 1982), and starvation (Hazlett et al., 1975; Stocker and Huber, 2001). These studies and mine provide valuable information to determine intrinsic and extrinsic factors that affect agonistic interactions.

There are other extrinsic factors that influence intraspecific interactions such as previous history in agonistic encounters (Rubenstein and Hazlett, 1974; Daws et al, 2002; Bergman and Moore, 2003), different fighting strategies (Guiasu and Dunham, 1997), and prior residence (Peeke et al., 1995, 1998). These can all significantly impact the outcome of social interactions. While we cannot control for all these factors due to these organisms not being raised exclusively in the lab, we can use crayfish that have never been before placed together into a new environment not previously occupied by either in the past. Crayfish housed individually have been shown to be more aggressive (Dunham, 1972) and that previous agonistic encounters with the same individuals can change the outcome of encounters (Rubenstein and Hazlett, 1974; Burk, 1979).

While the use of a new environment will eliminate a prior residence variable, it does still pose other variables that need to be considered. The use of the new

environment introduces the problem of the animals wanting to explore the new surroundings and thus could take away an interested in the opponent. Searching/exploring behavior for both species of crayfish is likely a major drive; however, previous studies of cave crayfish showed this was especially true (Kellie et al., 2001; Shuranova et al., 2005). Therefore, animals might be in an anxious state in the conditions of pairing in this study (new environment) and upon meeting an opponent they could be hesitant to interact as compared to an intruder invading one's space when an opponent is introduced to a resident's tank.

Work examining short-term changes in behavior, specifically social interaction outcomes, has shown physiological changes occur in both learning and the neuroendocrine system. The changes in either of these are associated with effects of experience on the neuroendocrine system of the individuals. Encounter behavior is modified as a result of learning (Oliveira et al., 1998; Dugatkin, 2001). Learning itself is a physiological change in synaptic transmission rate in specific neuronal pathways. Whether the changes a pre-synaptic response of releasing neurotransmitters or a post-synaptic response to released neurotransmitter is not the issue, but only that physiological changes occur through experience (Hsu et al., 2006). Neuroendocrine changes such as in corticosteroid and androgen as a relation to fighting strategy has been well studied in invertebrates (Hannes et al., 1984; Huhman et al., 1991, 1992; Schuett et al., 1996; Sakakura et al., 1998; Schuett and Grober, 2000; Overli et al., 2004). The relationship between dominance status and corticosteroid levels is less clear since in many cases hormone levels can correlate positively, negatively or not all with social rank as there appears to more of a species specific response (Overli et al., 2004; Sloman et al., 2000; Muller and Wrangham, 2004; Sand and Creel, 2004). Serotonin (5-HT) has been associated with aggressive behavior (Livingston et al., 1980; Sandoueet al., 1994; Cases et al., 1995; Wieger, 1997; Edwards and Kravitz, 1997). In invertebrates, increased serotonin shows an increase aggression on aggression (Weiger, 1997) since infusion of 5-HT in the hemocoel cavity of the crayfish

Astacus astacus caused the animal to fight longer in an encounter (Huber et al., 1997; Huber and Delago, 1998). It is most likely that after aggressive interactions, further physiological changes associate with energy metabolism may occur with the modification to the neuroendocrine system due to energy depletion.

An intrinsic index is more reliable than a visual assessment of the animal's responsiveness and basal status. The autonomic control of the cardiovascular and respiratory systems can regulate the availability of oxygen and other nutrients needed for a behavioral response without causing any outward behavioral change (Schapker et al., 2002). Due to this fact, observational data alone would incorrectly assess environmental factors effecting organisms. Previous work in crayfish showed that visual and/or chemical cues from other crayfish altered HR without any real apparent behavioral changes (Listerman, et al, 2000; Li et al., 2000). Thus, HR has been shown to be a good index in crayfish to use of environmental disturbances to fully understand if the animals can detect a change. Schapker et al., (2002) showed that crayfish rapidly alter HR and VR with changes in the environment and that HR and VR indicators were far more sensitive than behavioral data alone. Our assessment of HR showed rapid and prolonged response to social interactions when out of water. This seems to be more prominent for one individual in the dyad since one will become the subordinate. This may also account for a stress response when one individual can not retreat. Out of water conditions remove typical rapid escape responses; thus, individuals are more likely to sustain injuries during intense interactions. The disruption of social behavior 'out of water' was consistently demonstrated with each environmental or physiological modification.

It is important to highlight some limitations in the methods used in this analysis of interactions. With regard to the statistical tests across conditions, it is important to note that future development of analysis is in progress. Currently work is being done on new techniques to show significant difference between conditions. These will be used in the future publication of this work.

There are many distinct experimental advantages to using crayfish in behavioral and physiological studies. In particular, the present experiments have shown that crayfish are ideally suited to bring invertebrates studies of environmental effects on behavior and physiology to a level of more complex behavior phenomena. This also provides a rich and vast foundation in which to study much broader evolutionary representations among taxa. The present study built upon a wealth of existing research that has explored the social hierarchies in crayfish.

ACKNOWLEDGEMENTS

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Chapter 3

Comparative Study of Environmental Factors Influencing Motor Task Learning and Memory Retention in Sighted and Blind Crayfish

INTRODUCTION

There are many key scientists in the history of animal learning and cognition as early as 1888. One of the first scientists noted for interests in animal cognition was George Romanes in his book *Animal Intelligence* in which he states his overall objective to be determining the upper limit of animal intelligence in different classes, order and species. Here, he discusses anecdotes from other people to describe intelligent behaviors. While this was a beginning to discussing animal behavior, it raised many debates to the validity of the behaviors. One scientist that opposes Romanes was C. Lloyd Morgan, an English psychologist who became known for his trial and error learning which is termed "instrumental" learning today in his 1894 writing of *Introduction to Comparative Psychology*. Morgan was an evolutionist that became interested in animal behavior because he was tired of all the anecdotal exaggerations and lack of experimental analysis. Morgan marks the beginning of association learning as response to the consequences of actions.

Many years later, the work of Romanes was still being discussed by a scientist name Edward Thorndike. In 1911, Thorndike wrote *Animal Intelligence: Experimental Studies* in which he criticizes the anecdotal stories because they provide abnormal or super-normal qualities of intelligence to the animals. Another criticism is that this type of observation excludes normal or stupid animals, therefore making generalizations based upon very few with only a single case being studied not necessarily true to all. Furthermore, the observations do not account for past history or repeatedness of the behavioral studies. To counter these criticisms Thorndike developed the puzzle box in which he showed association learning over time. Thorndike's contribution is the gradual acquisition of skill through initial trial and error learning behavior and showing the stimulus

and reward relationship. In 1924, Wolfgang Kohler introduced a book, *The Mentality of Apes*, in which he criticizes Thorndike for making the experiments so difficult that they were destined to fail and thus the capability generalizations resulting from the failed outcomes were incorrect. Kohler suggests the importance of designing the correct type of intelligence tests for the animal. He is known for his contribution to going beyond simple association learning and more into the learning through insight. It is here that he suggests the importance of understanding the capabilities of different animals and designing the experiments in a way that can test the intelligence.

As shown, Ivan Pavlov was not the first scientist to discuss animal learning but he is thought to be the first that studied it systematically and using a standard set of terminology. Pavlov is noted to establish classical conditioning in relation to a physiological process of learning. In 1927, Pavlov wrote Conditioned Reflexes in which he distinctly describes the difference between unconditioned (instinctive) and conditioned (environmental or learned) reflex response due to a stimulus. Pavlov discussed the salivary conditioning in relation to a physiological response that basically states when two stimuli are given together, then the two are treated the same which overall suggests learning. Although the scientists discussed are not a comprehensive list of all contributors, it is a foundation to the beginnings of animal learning and cognition.

Human and animal behavior was previously thought to be regulated only through genetic information. Today, we understand that there are many factors that can influence behavior such as the nervous system and chemical factors resulting from environmental influences. If genes were the only factor controlling behavior, an organism could not adapt to a changing environment and modify actions based upon conditions.

The underlying goal of every individual is to reproduce and pass on its' genes to successive generations. In order for this goal to be accomplished, one must overcome environmental conditions and survival obstacles to reproduce. The fundamentals of adaptation encompass variation, differential reproduction, natural selection favoring specific trait(s) and ultimately, leading to evolution of a

population (Darwin, 1859). Learning is thought to be the fundamental basis for species adaptation through acquisition of new information/behaviors and organisms will adapt in a species-specific manner shaped by evolution. As in humans, the learning capacity of the brain enables recognition and understanding of different tasks in a wide array of environmental challenges. The capacity to learn and retain may allow an individual to adapt into an environment that was previously hostile and/or resource deficient by out-competing others through possession of resources. Since food is often unevenly distributed in the environment, it would be an advantage to learn and retain the memory of areas containing the high quality food sources, as well as cues that predict the availability. Thus, individuals able to learn (i.e., successful foraging/survival techniques) would increase the likelihood of reproducing (i.e., increased fitness). A given behavior of an individual may be influenced by obtaining processes such as learning. It is important to note that differences occur across individuals, as well as, species; therefore, the goal is not to make specific statements but to understand basic principles that apply to learning which may be applicable in a general context. In many cases, learning in animals is a gateway to understanding more complex learning in humans. It provides a foundation to understanding the human mind and it is well known that humans and animals share many similarities in physiological processes.

Invertebrate Learning

Relatively recently, invertebrate learning has been a focal point of interest. Invertebrate learning systems provide the ability to ask complex questions in relatively simple systems as compared to vertebrates. While learning can be widely interpreted, a broad definition is any change in behavior shaped by experience and persists over time (Krasne, 1973). An area of particular interest is the role of conditioning in learning. This area has mostly been studied in vertebrates mainly focusing on the associative capabilities of rats and pigeons. However, in the last 30 to 40 years many researchers have been investigating

associative learning using invertebrates such as work by Kandel and colleagues in *Aplysia* to understand neuroanatomical and neurochemical mechanisms, as well as work by Bitterman and colleagues which shows that much of the learning phenomena observed in rats and pigeons are also observed in honey bees (Couvillon and Bitterman, 1980; Kandel and Schwartz, 1982; Burmiester, Couvillon and Bitterman, 1995). Past research has provided the foundation to understanding innate responses to habituation and sensitization as well as mechanisms of associative learning (Bitterman, 1988; Abramson, 1994; see review Krasne and Glanzman, 1995).

Early experiments by Bethe (1898) and Yerkes (1902) have convincingly shown that crustaceans can learn. Behavior is modulated by experience through the acquisition of new information, (learning) and the retention of the experience (memory) about the environment. Several invertebrate studies show that organisms are able to modify behavior, especially avoidance behavior, as seen in mollusks with habituation of the rapid gill withdrawal reflex (Castellucci and Kandel, 1974), food aversion with electric shock (Mpitsos and Davis, 1973; Mpitsos and Collins, 1975) and CO_2 poisoning (Gelperin, 1975). Recently, a punishment scheme devised by Horridge shows that cockroaches as well as multiple species of crab and crayfish can maintain a specific appendage position to avoid aversive stimulation (Horridge, 1962; Abramson and Feinman, 1987; Dunn and Barnes, 1981; Hoyle, 1976; Punzo, 1983; Rafuse, 1973; Stafstrom and Gerstein, 1977).

Operant Motor Task Learning

A powerful tool to study learning is the analysis of an animal's ability to learn to complete a task. For invertebrates, there have been limited studies examining this area of interest. While aversive stimulation studies can result in associative learning, a more powerful technique to demonstrate learning is through studies of operant condition learning. Operant conditioning, which is behavior controlled by its' consequences, is not as well studied as other areas such as classical

conditioning or habituation and sensitization. A key feature of motor operant conditioning is that some aspect of the motor response must be part of the learning and that changes through experimentation are related to the experience of learning. In operant learning, the organism makes a connection through its behavioral response between a neutral stimulus and a second stimulus which is either a reward or punishment. Thus, an organism set to solve a problem, learns to solve the problem and get the reward; therefore operating on its environment.

For invertebrates, there have been limited studies on task experiments even though early work has demonstrated that arthropods, more specifically crustaceans, could learn from the consequences of their actions (see review Krasne, 1972). A key task study showed green crab *Carcinus maenas* is able to demonstrate a lever-press task and this increased when given food reinforcement for every bar press. Performance increased over time and high response rates were observed after 2 days of training (Abramson and Feinman, 1990). This is also especially interesting given our developing knowledge of neural circuitry and neuronal control in decapods such as crayfish and lobster (Kennedy et al., 1969; Davis, 1970: Larimer et al., 1971).

Currently, an interest of operant conditioning in crustaceans (i.e., multiple species of crab) involves an appendage maintained in a specific position to avoid a negative stimulus. Precise manipulation of appendages is a powerful technique to study learning abilities since this tests the degree in which manipulative and motor behaviors are a part of the learning paradigm. Crustaceans are a likely group of organisms for motor operant learning due to the ability to do complex manipulations with their limbs in a natural environment. A current advantage to research on limb manipulation is the well-documented foundation on physiology of appendages in crustaceans. Furthermore, crustacean neural systems have been well-studied and provide a foundation to examine the underlying mechanisms of learning.

The Autonomic Response

Crustacean nervous systems are valuable due to the many facets on which to develop physiological studies. In addition to observing changes in behavior, a physiological response can be measured and can serve as a biological index for stress responses directly related to learning. The physiological response is so well evolved in vertebrates; it would seem likely that complex invertebrates would similarly possess a developed response system (Schapker et al., 2002; Zavarzin, 1941). Even though vertebrates and invertebrates are very different in their body systems; highly developed invertebrates require a rapid cardiovascular and respiratory response to respond in a 'fight or flight' manner. For many invertebrates, a 'sympathetic-like' physiological response can be quantified. Dating back to 1927, many of the very early studies have been conducted in invertebrates, specifically using arthropods in general (Alexandrowicz, 1932; Orlov, 1927; Zavarzin, 1941).

Crayfish are known to exhibit a wide range of rapid behaviors as well as the ability to assess and respond to environmental stimuli. Very early studies have noted a 'sympathetic-like' response of an immediate and rapid response of defense posturing (Bethe, 1897; Huxley, 1880; Shuranova et al., 2006; Wiersma, 1961). The adult crayfish heart is known to be neurogenic since the beat and rhythm are controlled by the central nervous system (Alexandrowicz, 1932; Yamagishi and Hirose, 1997; Yamagishi et al., 1997; Wilkens, 1999). The measurement of heart rate (HR) provides a measure of the animal's excitability and readiness of the internal environment. Previous research shows an increase in HR during defense posturing when a crayfish is presented with a perceived threatening stimulus (Listerman et al., 2000). In addition to heart rate, the ventilatory system is also neuronally controlled with a ventilatory central pattern generator (VPG) responsible for oxygen uptake across the gills.

The movement of water across the gills is produced by the pumping action of two specialized appendages termed scaphognathites (Mendelson, 1971). A single scaphognathite is found in each branchial chamber at the anterior ends

and draws water across the gills by a rhythmic movement (Pasztor, 1968). Previous work in the crab, *Cancer magister* shows cardiac and ventilatory rhythm can be altered by central control through command neurons (Wilkens et al., 1974). In addition, activity of the VPG is known to change with changes in the internal response and with social interactions or environmental changes in crustaceans (Burmistrov and Shuranova, 1996; Cuadras, 1979, 1980; Li et al., 2000; Listerman et al., 2000; McMahon and Wilkens, 1983; Schapker et al., 2002; Shuranova et al., 2002; Wilkens 1976). As seen in the crayfish, ventilatory activity can vary depending on the internal state and changes in VR can be recorded during unexpected external stimuli (Shuranova et al., 1993; Shuranova et al., 2002).

All animals have evolved a set of characteristics of anatomical, morphological, biochemical, physiological and behavioral adaptations to cope with environmental stress and change (Howarth, 1983). Freshwater crayfish provide a dramatic model of evolutionary adaptation in the contrast of sighted, *Procambarus clarkii* (surface) and blind, *Orconectes australis packardi* (cave) species. *O. a. packardi* show typical cave-dwelling characteristics such as elongated appendages, lack of pigmentation and non-functional eyes. Whereas the sighted crayfish have ommatidia and known visual capabilities both in and out of water, blind crayfish are known to lack ommatidia and not respond to visual cues (Cooper et al., 2001). To compensate for the lack of visual cues, some cave organisms have increased or heightened sensitivity to non-visual environmental cues through tactile and chemosensory systems (Barr, 1967; Barr and Holsinger, 1985; Poulson, 1963). Thus, the freshwater crayfish provides an excellent model to examine whether similar species of crustaceans using different primary sensory modalities would differ in the rate of learning a motor task.

Crayfish are a great model organism to study motor learning due to the feasibility of working with the animals as well as the multiple levels of complexity that can be addressed. One can study learning at the behavior, physiological and neuronal level rather easily in crayfish. In addition, crayfish are especially

good in finding their way and exploring the environment. The primary regulatory factor for animal behavior is based on the motivation of the organism. The second regulatory factor is learning and memory which is built upon the primary influencing factor. Thus, simple curiosity will motivate the animal even more to explore an unknown environment and will be a strong motivator in the ability to learn.

This study investigates learning in an operant chamber in which crayfish complete a task to obtain food. The purpose of the study was to: 1) establish if crayfish are capable of learning a motor task, 2) examine long-lasting memory, 3) examine environmental influences on learning, 4) determine learning differences between two species that rely on different primary sensory modalities and 5) demonstrate if crayfish show a physiological stress response when inhibiting them from completing a learned motor task. To our current knowledge, no other study in invertebrates examines whether environmental factors directly influence learning and task completion, as well if a stress is associated with inhibition of a learned motor task.

METHODS

Animals

Crayfish, *Procambarus clarkii* (sighted), measuring 5.08-6.25 cm in body length were obtained commercially (Atchafalaya Biological Supply Co., Raceland, LA). Crayfish, *Orconectes australis packardi* (Rhoades) (the blind crayfish), measuring 1.8-2.5 inches were obtained from the Sloan's Valley Cave System near Somerset, KY (collecting permits were obtained for this study). A total of 24 sighted and 24 blind crayfish were used in the study. Both sexes of crayfish were in this study but learning differences between the sexes were not analyzed. Animals were housed individually in rectangular plastic containers and cared for in the same manner, except *O. a. packardi* were covered with black plastic to omit light 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 until two weeks prior to experimentation. During experimentation, food was restricted to 30% of normal feeding amounts. Crayfish handling was conducted by using a glass beaker to transfer crayfish from one container to the other. Due to 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.

Chamber Design

The experimental chamber is shown in Figure 3.1 (angled view, A; side view, B). Four experimental chambers were used and were rectangular in shape constructed from plexiglass (18 × 8 x 8cm) with an 8 cm plexiglass divider one-third distance in the container. The crayfish was placed in the larger portion of the chamber and the food reward in the smaller portion of the chamber. Sand was permanently glued to the bottom surface for traction. The smaller portion of the chamber contained a vertical platform approximately 1cm distance from the divider. The platform was a square shaped plastic object (5.5 cm^2) with mesh material on the surface. The access point was oval in shape, located on the bottom at mid-length of the plexiglass divider. This allowed only a single cheliped to enter into the smaller portion of the chamber. The food reward were thawed bloodworms (mosquito larvae, N = 5; PetCo, Lexington, KY) attached one-third through the mesh material and placed into the chamber before the crayfish were added. The worms were placed so that they are center to the access point but 3 cm above which required the animal to reach in and up to obtain the food source (Figure 3.1 A, B). Each chamber was filled to approximately 2.35 cm from the top of the chamber with carbon-filtered water which was aerated for at least 12 hours prior to the running of all experimental trials. All four experimental chambers were simultaneously recorded by a digital video camera by placing them in a stair step

placement with two chambers on each level. The video camera was started prior to addition of the animals to ensure complete monitoring of the crayfish's action. Animals were placed into the chamber and a Plexiglass lid was placed over the top of the chamber to prohibit any crayfish from escaping the chamber. The animals were free to move within the chamber during the experimental trial.

Experimental Procedure

I examined learning processes in common sighted (surface) and blind (cave) crayfish. A three week training period exposed all animals to the experimental chamber every other day (i.e., 3-4 times a week) starting at 08:00 in during the months of May-December. There were four main studies: Operant learning in low white light 25 Lux (Lx), *P. clarkii,* N = 16; red light 2.5 Lx, *P. clarkii,* N = 8; low white light 25 Lx, *O. a. packardi,* N = 8; red light 2.5 Lx, *O. a. packardi*, N = 16. After the training period, a four day delay in time was introduced to examine task retention. After the four day delay in time, all animals were placed into the chambers for a one week reminder training (i.e., 4 trials). Once the reminder training was completed, a seven day delay in time was introduced. (Operant learning trials first examined whether crayfish can learn a motor task and if so, learning differences between the two species. Ultimately, the comparison was learning trends and whether visual sensory stimulation (sighted) aided in learning the motor task. This also examined if low white light had any effect on learning in blind crayfish. The 25 Lx illumination is a low level of illumination to mimic periods of the day (dusk and dawn) when the crayfish are known to be most active.

Motor task learning examined in red light used a filtered red light (2.5 Lx) to remove the visual sensory stimulation for the sighted crayfish. The red light (Kodak Adjustable Safeway Lamp, 15 watts), was previously noted to be a wavelength not detected by crayfish (Li et al. 2000) thus providing no visual sensory stimulation.

To examine long-lasting memory formation, a subset of previously trained sighted crayfish (N=7) was placed into the chambers after a 92 day delay in time in low white light, 25Lx. During the time delay, these crayfish were not exposed to the experimental chamber and were housed the same as all other crayfish.

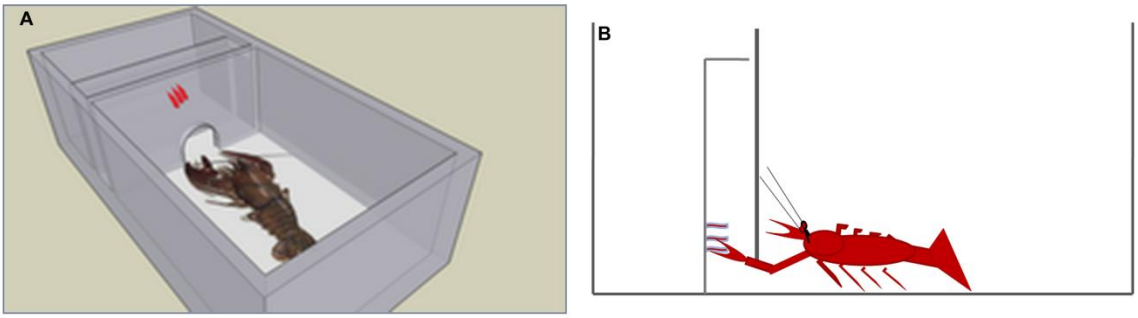

Figure 3.1. Schematic representation of motor operant chamber. The operant chamber is shown to be separated into two compartments, one which houses the animal (larger) and the other which contains a mesh platform containing the food reward (smaller). Not shown is a roof which was placed over the chamber. The food was attached to the mesh screen for easy removal by grasping. See method section for further details. (A) A stylized angled view representation of the two compartments including the mesh screen with the worms attached from an above angle to show access point location. (B) Side view schematic representation of the two compartments including the mesh platform to show the manipulative task of reaching in and up to obtain the food reward.

Statistical Quantification of Learning

All trials were digitally recorded and analyzed through video analysis to record the time in which the first worm was pulled. Trial success was based upon successful removal of the first bloodworm. Quantification of learning was based upon the time to pull the first worm on subsequent days. Each experiment was analyzed for individual crayfish learning, as well as average learning trends

within and across species. Furthermore, to account for variability in individual rates of learning, each crayfish was analyzed for a percent change in learning over time. Percent change values were determined by taking the absolute value of the first day of learning, minus subsequent days and dividing by the first day (see formula below). This value was then multiplied by 100 to get a percent change from the first day of learning, designated as performance index (i.e., % change from the first day). To understand trends, % change values were averaged together to achieve an average percent change for each experiment. Quantification of memory was based upon changes in task efficiency over repeated access in the experimental chamber after delays in time were introduced (i.e., 4 and 7 day delays). ANOVA statistical analysis based upon probability $p<0.05$ and Holm-Sidak post hoc analysis were used to determine significance in subsequent days. A t-test with probability of $p<0.05$ was used to determine significance of a single day of learning compared to the first day.

$$\left(\frac{\left| \text{First day of learning} - \text{Subsequent day of learning} \right|}{\text{First day of learning}} \right) \times 100 = \textbf{Performance Index}$$

Recording of ECGs and ESGs

The autonomic response was examined when previously trained crayfish (N=7) were inhibited from completing the learned motor task. The task inhibition operant chamber utilizes the previous motor chambers except that two out of four chambers had the access point physically blocked with a piece of plexiglass. Crayfish were wired to record electrocardiograms (ECGs) for heart rate (HR) and electroscaphognathites (ESGs) for ventilation rate (VR; Listerman et al. 2001; Schapker et al. 2002). For details refer to Chapter 2 materials and methods as well as video experimentation as showed in Bierbower and Cooper (2009). The experimental procedure consisted of 30 minutes of baseline HR and VR

recordings in which the crayfish was left undisturbed without a food reward. The absence of the food reward was to gain a HR and VR recording before chemosensory cues elicit an autonomic response and the access point was left open. After 30 minutes, for the control group (N = 7), the access point remained unblocked and the crayfish was provided the food reward. For the experimental group (N = 7), the food reward was provided but the access point was blocked and the crayfish were inhibited from completing the motor task. In addition to baseline recording, each trial recorded HR and VR for one hour after food reward was provided.

RESULTS

Operant Learning

I compared learning processes in common sighted (surface) and blind (cave) crayfish. I first established the crayfish's ability to learn a motor task of manipulating their very large and cumbersome cheliped through a single-cheliped sized access point for a food reward. Individual performance over time to pull the first worm is shown for sighted and blind crayfish for each day of the experimental trial in low while light (sighted, Figure 3.2A; blind, Figure 3.2B). Data are plotted as the time it took each crayfish to complete the motor task. As expected, learning was shown to vary among individuals in both sighted and blind crayfish in both initial task completion and task efficiency over time. Specifically, the blind crayfish were not consistent in completing the task on subsequent days (Figure 3.3B). The crayfish that did not perform the task on an experimental day were removed from subsequent trials and trial analysis.

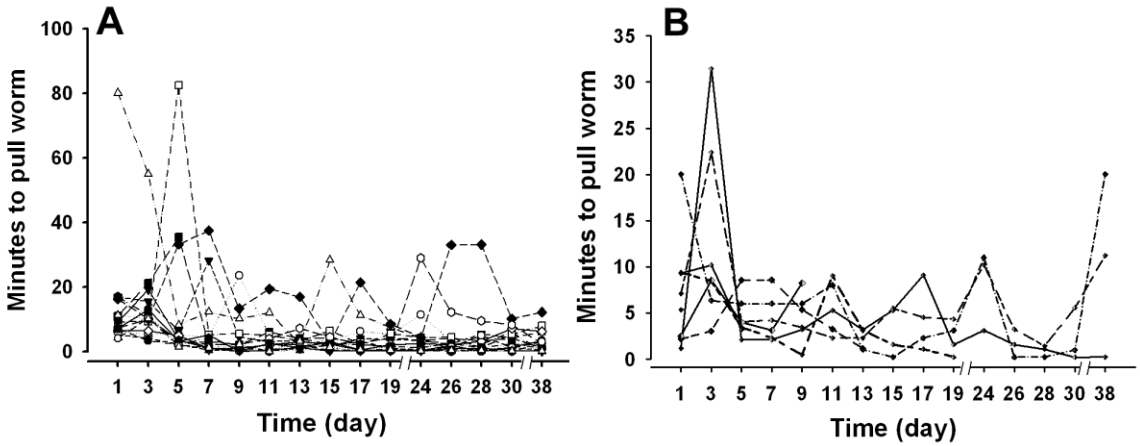

Figure 3.2. Graphs of individual motor task learning responses in crayfish over the duration of the experimental days. Each graph shows the performance of individual crayfish with each line representing a single crayfish. (A) Sighted crayfish time to pull the first worm. (B) Blind crayfish time to pull the first worm. The experimental procedure consisted of exposure to the chamber every other day continually for 3 weeks followed by a delay in time of 4 days and 7 days (indicated by breaks in x-axis).

Individuals in the groups showed a systematic performance and signs of learning. To account for learning differences, a percent change was used (see methods for details) to relate individual differences in learning in both sighted and blind crayfish relative to their own first day of learning (sighted, Figure 3.3A; blind, Figure 3.3B). As shown even with normalized data, there is large variation in learning within the populations.

If crayfish can learn to complete a motor task, they should show a general trend in task efficiency with subsequent exposure to the experimental chamber. The statistical analysis of performance index for the two species confirmed the prediction for the sighted crayfish in which the animals showed a significant increase in task efficiency over time (ANOVA; $F_{14, 225} = 8.09$, $p < 0.001$). Based on the Holm-Sidak post hoc test there is a significant increase between day 1 and all days starting after day 5 ($p < 0.001$; Figure 3.4). In comparison between sighted and blind crayfish, the analysis indicates that a learning trend is not

observed in blind crayfish since there is no significant difference for any of the days compared to the first day of learning due to high variation among the group (ANOVA: $F_{14,48} = 1.23$, $p = 0.29$).

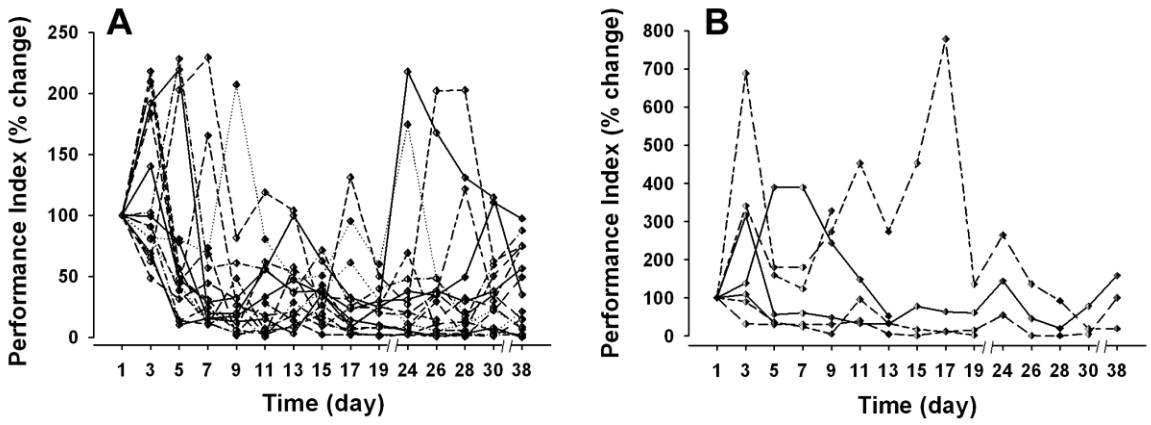

Figure 3.3. Graph of normalized (% change) motor task learning responses in crayfish over the duration of the experimental days. Each graph shows the performance index as the percent change from the first day of learning of individual crayfish with each line represents a single crayfish. (A) Index for individual sighted crayfish to pull first worm. (B) Index for individual blind crayfish to pull the first worm. Delays in time are indicated by breaks in the x-axis.

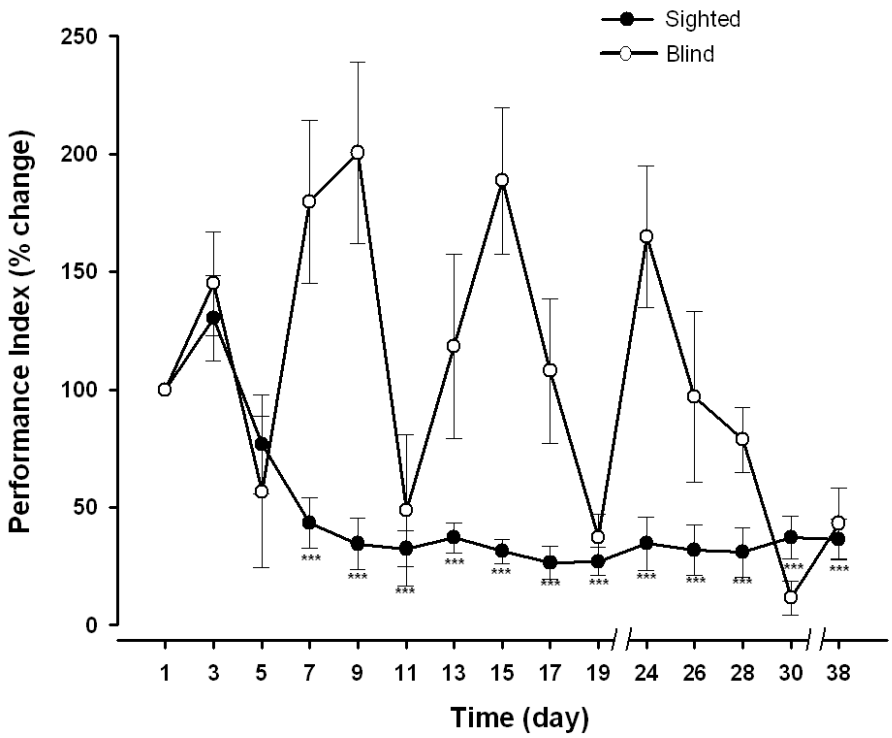

Figure 3.4. Cumulative graph of motor task performance in both sighted and blind crayfish over all the experimental days. The graph depicts the performance index as the percent change from the first day of learning as an average. The closed dark circles represent sighted crayfish and the open circles represent blind crayfish. Breaks in the testing series are indicated by the gaps on the x-axis.

Long-Lasting Task Memory

Sighted crayfish previously trained and showed successful task completion were used to examine long-lasting memory formation. While task efficiency remained significant after 4 and 7 day delays (Figure 3.5B), a further comparison of the average minutes of the first day of the trial and after a 92 day delay in time (day 130) was shown to be significantly different (t_{12} = 2.4, p = 0.333; Figure 3.5A). The normalized performance index indicated a statistical significance of task efficiency (ANOVA: $F_{15,\ 96}$ = 13.18, p < 0.001; Figure 3.5B). There is a

significant differences between day 1 and all days starting after day 7 including the 92 day delay ($p < 0.001$; Holm-Sidak post hoc analysis). From the results I suggest that a long-lasting memory is present due to a significant difference in task efficiency when the crayfish is not exposed to the task chamber for 92 consecutive days. Although task efficiency remains statistically different after 92 days, the trend in suggests a longer period without chamber exposure would result in task performance similar to the first day.

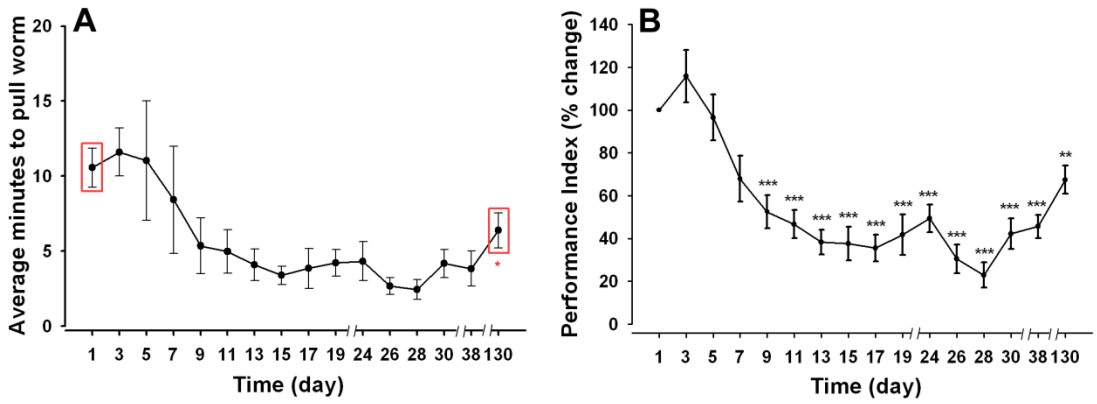

Figure 3.5. Long-lasting memory of operant task for sighted crayfish on all experimental days. (A) The average minutes for crayfish to complete the motor task including the 92 delay in time. (B) The performance index including the 92 day delay in time. Breaks in the testing series are indicated by the gaps on the x-axis.

Visual Sensory Stimulation in Operant Learning

In sighted crayfish, comparison of task efficiency in two different environmental factors, illumination of the experimental chamber with white (visible) or red (invisible) light, showed no difference for each day of chamber exposure between the two groups (ANOVA: $F_{29,\ 315} = 6.66$, $p < 0.05$; Figure 3.6A). The motor task experiment was also conducted with blind crayfish,

illumination of the experimental chamber with white light (visible), as a way to test the use of the vision for the sighted crayfish under the same conditions. Unexpectedly, blind crayfish experiments conducted with illumination of the experimental chamber with white (visible) or red (invisible to crayfish) light were not as clear and showed significant differences between the two groups (ANOVA: $F_{29,373} = 3.33$, $p < 0.001$; Figure 3.6B). Specifically with a Holm-Sidak post hoc analysis there is a significant differences between day 1 and on day comparisons 7, 9, 15 and 24($P<0.001$). Also the variation throughout the experiment was more pronounced as compared to the sighted crayfish with white light and red light. One explanation is that although this species of blind crayfish do not have ommatidia (Cooper et al. 2001), they do have a caudal photoreceptor similar to that in sighted crayfish. This receptor can drive phototactic behavior to or way from white light (Larimer 1966; Li & Cooper 2002). Thus, even though both species have a caudal photoreceptor, blind crayfish exposed to white light may have presented a stress response.

Further analysis of species comparisons show similar learning trends between the two species. Specifically, statistical comparison of both sighted crayfish conditions (i.e., white and red light) to that of blind in red light have no significant differences (ANOVA: $F_{44, 435} = 4.56$, $p < 0.05$; Figures 3.6C, 3.6D). Examination of the two groups (i.e., sighted and blind) in both experimental conditions (i.e., light and no light) shows only that blind crayfish in low white light impacts task efficiency when compared to any of the other treatment groups (Figure 3.6C). This suggests to us that similar learning trends despite reliance on different primary sensory modalities. Furthermore, environmental influences on learning may be dependent on whether there is a stress response in the organism. It was shown in an earlier study that exposure to light did increase heart rate in cave crayfish (Li & Cooper 2002).

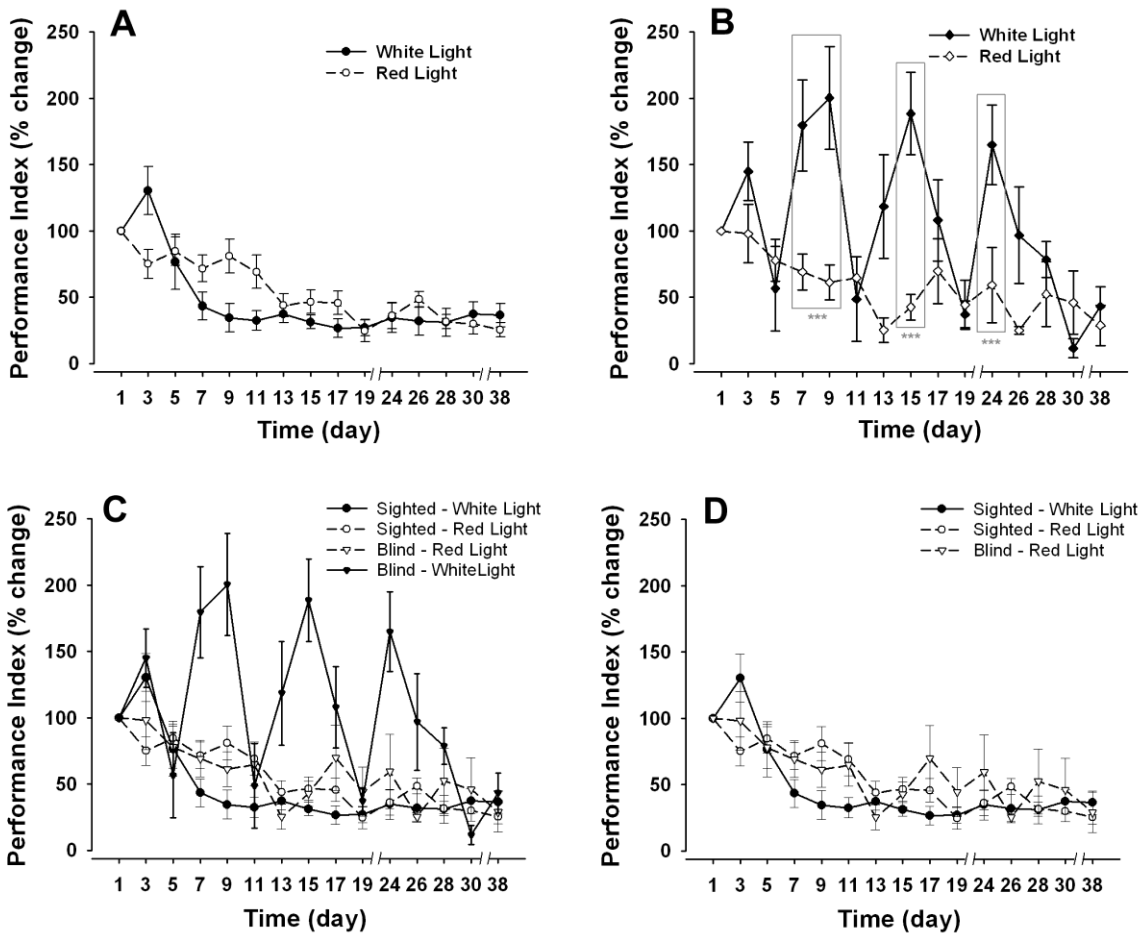

Figure 3.6. Species comparison of motor task efficiency (performance index) in white and red light on experimental days. (A) Sighted crayfish. (B) Blind crayfish. (C) Cumulative results for both sighted and blind crayfish in white and red light. (D) Cumulative results excluding blind crayfish in white light.

Autonomic Response Recording During Task Inhibition

Routinely, measures of an animal's heart rate (HR) are used to assess the internal excitability and response to a given stimuli. Crayfish show a rapid increase in HR during fight or flight situations such as defense posturing (Listerman et al. 2000; Schapker et al. 2002). The HR was plotted for each crayfish during the entire duration of the experiment to demonstrate the changes

from baseline recordings. Crayfish that previously learned a motor task but were inhibited from completing the task are shown to respond with an increase in the autonomic response. ECG measurements in the experimental group show that the frequency of the heart rate began to increase almost immediately after the food source was provided and the access point was blocked (Figure 3.7A). Heart rate measures for the first five minutes (0-300 sec) and last five minutes (1500-1800 sec) reveals a consistent increase in the animals HR when denied access to the food source and are significantly different from the control group (ANOVA: $F_{47, 240} = 261.16$, $p < 0.001$). Holm-Sidak post hoc analysis showed a significant difference between the groups for all points (25 second averages) starting at time 0 ($P<0.001$). Heart rate comparison of experimental and control animals suggests an anxious/stress response since both groups of animals are moving consistently to obtain or try to obtain the food source (as noted through observations from the recorded video). Analysis of standard error of the mean for control group shows non-significant consistent stable recordings in heart rate throughout the duration of the trial although continuous movement was occurring (ANOVA: $F_{23, 96} = 2.52$, $p < 0.05$).

It is known that significant changes in VR can occur in the absence of significant HR changes as well as rapidly change to a greater degree than HR (Larimer 1964). For crayfish denied access to the food reward, ESG measurements of VR in the experimental group shows an immediate significant increases in VR and the VR remains elevated for the entire duration of the experimental trial (ANOVA: $F_{47, 240} = 114.60$, $p < 0.001$; Figure 3.7B). Holm-Sidak post hoc analysis showed a significant difference between the groups for all points (25 second averages) starting at time 0 ($P<0.001$). Furthermore, VR in the control group was shown to fluctuate similar to that of the experimental group but remains significantly lower, suggesting continuous movement was not the cause of the increase in both HR and VR for the experimental group.

It is known from earlier studies that a water drop into the water environment of a previously undisturbed crayfish will cause an increase in both HR and VR above the resting state. The increased response of both HR and VR

rates were shown to return to near resting levels after approximately 5-10 minutes (Schapker et al. 2002). The initial responses for crayfish with the closed access point are illustrated in Figure 3.7. The rate of change for HR and VR are shown as a running mean of percent change of beats per minute over time as compared to resting levels. The alterations in the response shows an immediate increase in both HR and VR which remains elevated for the entire duration of the trial. Furthermore, the increased levels do not return to the resting levels quickly which would be assumed if the crayfish were habituated to the environment. The extent to which HR and VR increase due to walking and movement can not explain the experimental group's immediate elevation of HR and VR, as well as the fact both remain elevated the entire duration of the 30 minute trial. Instead, this alteration in the autonomic response suggests a stress response due to inability to complete the learned motor task.

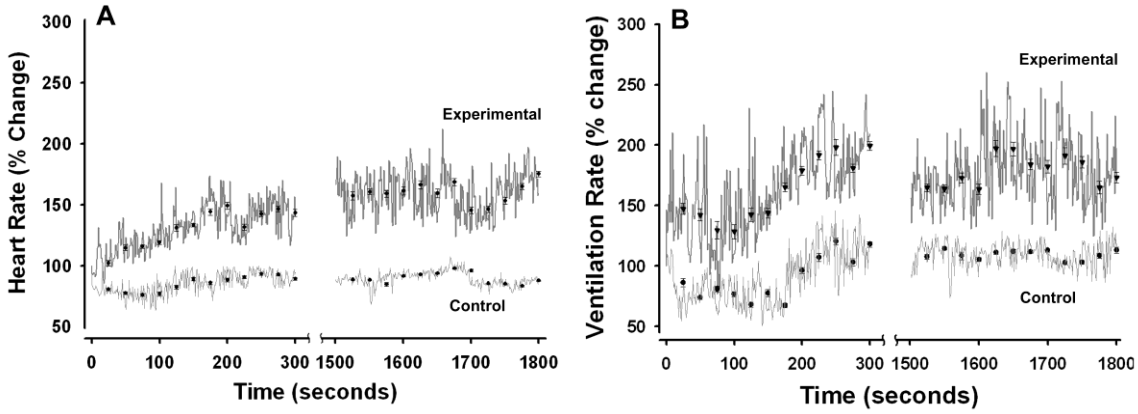

Figure 3.7. Autonomic response recordings during operant task. (A) Heart rate measures for the first (0-300 sec) and last (1500-1800 sec) five minutes after the food reward was provided. The recording reveals a consistent increase in the experimental animals HR when denied access to the food reward. The significant increase in HR when compared to the stable control animals suggests an anxious and/or stressed response. (B) Ventilation rate measures for the first (0-300 sec) and last (1500-1800 sec) five minutes after food source was provided.

The recording reveals a consistent increase in the experimental animals VR when denied access to the food reward. The significant increase in VR when compared to the stable control animals suggests a stress response.

DISCUSSION

I compared learning processes in sighted and blind crayfish and provide the first study on cave crayfish learning. In this, I quantified the ability to complete an operant task, how rapid the acquisition occurred, how efficient the performance was, how well the animals retained the learned task and what was the physiological response when the task was inhibited. I established that crayfish have an ability to learn a motor task by placing them in an operant motor task chamber in which they had to manipulate the large cumbersome cheliped through a small access point to retrieve a food reward. To complete a motor task, sighted crayfish could be assumed to rely heavily on the visual cues for task efficiency. When visual sensory information was removed, by conducting the experiment in red light, it was concluded that the visual cue is not essential for searching the worm and task completion. This was further demonstrated by comparing the learning trend seen in sighted crayfish to that of a blind species which relies on tactile and chemosensory modalities instead of visual sensory information. I demonstrated that there was no difference in learning between two species of crayfish which rely on different primary sensory modalities, suggesting integration of multiple pathways in the process of learning with removal of sensory systems. I also showed introduction of a novel stimulus (i.e., low white light in blind crayfish) could negatively impact learning. In addition, I demonstrated that previously trained crayfish inhibited from completing the task showed significant increase in both heart and ventilatory rates when compared to control groups which indicates a stress response.

Study results suggest similar learning trends in two morphological distinct species despite reliance on different primary sensory modalities. Furthermore,

environmental influences on learning may be dependent on whether there is a stress response perceived by the organism. Interestingly, both species quickly learned to complete the task (i.e., 5-7 days) which suggests they both easily habituated to the task chamber. This behavioral task is indicative of a behavior possibly utilized in the natural environment. While sighted crayfish are known to rely on visual sensory information about the environment (Bruski & Dunham 1987; Smith & Dunham 1990), it is also known they use sensory integration of tactile (Bovbjerg 1953, 1956; Issa et al. 1999; Goessmann et al. 2000) and chemosensory systems (Rutherford et al. 1996; Zulandt-Schneider et al. 1999) for refinement of sensory information**.** Blind crayfish provide a comparison for isolation of a sensory system (visual) and identification of reliance on other primary sensory modalities during learning. Learning is a thought to be a collection of sensory systems and since removing one modality does not impact learning suggests sensory integration ultimately leading to task completion.

It can be suggested that this type of motor task is not true motor learning (i.e., development of a motor habit) and only an increase in the approach of the food source. However, observation of the trial videos show that although both species of crayfish are approaching the access point in a relatively shorter amount of time, they are able to find the access point and manipulate their chelipeds in a shorter amount of time as well. Specifically, the placement into the access point and the turning of the cheliped in and up is more refined than the initial trial days. It is this manipulation that results in the motor task and a decrease in the latency of taking the worm, thus suggesting learning how to manipulate the cheliped into the small space and then rotating up to reach the food is evidence of true motor learning. However, many trials are needed to concretely state motor learning.

The impact of this study is that it further provides a foundation for complex behaviors since much of the operant learning in Crustacea has been simple position habits in which the organism is given a choice in a two way chamber (i.e., Y- or T-mazes) or punishment schemes developed by Horridge (Yerkes 1902; Yerkes & Huggins 1903; Schwartz & Safir 1915; Gilhousen 1927; Datta et

al. 1960; Schone 1961; Horridge 1962; Morrow 1966; Harless 1967; McMahon et al. 2005). In these earlier studies, learning to escape has often been away from something that is a negative result such dryness (Yerkes & Huggins 1903; Gilhousen 1927; Schone 1961) or electric shock (Agar 1927).

Long-lasting memory was shown due to a significant difference in task efficiency remaining when the crayfish were not exposed to the task chamber after 4, 7 and 92 consecutive days. Although task efficiency remains statistically different after 92 days, the results suggest that a longer period without chamber exposure would result in task performance similar to the first day and possible extinction of the learned behavior over time. Learning and memory formation is important in the natural environment and especially true for social animals since many social hierarchies depend on recognition through experience. As seen with many crustaceans, agonistic outcomes between conspecifics create a history of social experience that can influence future behavior (Goessmann et al. 2000; Daws et al. 2002; Bergman et al. 2003). Although the exact mechanism has yet to be understood, learning and long-term memory formation is suggested to begin with long-term potentiation (LTP) due to consistent and prolonged strengthening of synapses to targets by the Hebbs postulate (Lynch 2004). Previous work in *Aplysia* first looking at short-term sensitization and then short-term behavior provided the foundation into cellular mechanism behind long term memory formation. Early work suggested that neuronal plasticity and short-term behavior depended on second messenger pathways such as cAMP (Cedar et al. 1972). Further work showed stimulation of modulatory pathways increased cAMP in the abdominal ganglion (Cedar & Schwartz 1972) and that serotonin (5-HT) and dopamine could increase levels of cAMP due to serotonergic interneurons (Brunelli et al. 1976; Glanzman, et al. 1989; Mackey et al., 1989).

Blind crayfish did not show an operant motor task learning trend in low white light. One explanation is that although this species of blind crayfish do not have ommatidia (Cooper et al. 2001) they do have a caudal photoreceptor similar to that in sighted crayfish. This receptor can drive phototactic behavior to white light (Larimer 1966; Li & Cooper 2002). Even though both species have a caudal

photoreceptor, blind crayfish exposed to white light may have resulted in a stress response not allowing the animal to adapt to the environment. This is further supported by Li & Cooper (2002) where cave crayfish will leave a white light but not a red light environment. How the animal acts during a stress response provides insight into the internal physiological state of the organism. Cooper et al. (2001) showed that *O. a. packardi* cannot perceive light via the eyes but due respond to light on the abdomen (the caudal photoreceptor). Light exposure in the blind crayfish has been shown to cause an increase in heart rate which remains elevated for a relatively long period of time (Li et al. 2000). One would expect that processing of sensory stimuli would be compromised or less efficient in stressed individuals when compared to non-stressed individuals (Basso 2001).

Stress is a strong biological modulator of learning and memory formation. Early studies in vertebrates show stress and stress hormones (i.e., corticosteroids) negatively impact LTP (Foy et al 1987; Meaney et al. 1987). Extensive studies in humans and rodents shows stress has long been considered to negatively impact cognitive abilities (Selye 1936; McEwen & Sapolsky 1995; Lupien & Lepage 2001; Sandi 2004; Fuchs et al. 2006) with a wide array of physiological consequences (Sapolsky 1992; Selye 1973). Low levels of stress hormones were associated with low levels of serotonin (5-HT), little calcium influx and efficient LTP, whereas increasing levels of stress increased the responsiveness to serotonin (Meany et al. 2004), increased calcium (Mitchell et al. 1990a) and impaired LTP (Mitchell et al. 1990b). This is interesting because it provides a foundation to examine the role of 5-HT in learning as well as the impact on the stress response by possibly blocking 5-HT receptors.

Routinely, measures of an animal's heart rate (HR) are used to assess the internal excitability and response to a given stimuli. Study results show HR and VR for crayfish with the closed access point immediately elevate and remain elevated through the entire trial duration (Figure 3.7). The rates of change for HR and VR are shown as a running mean in percent change of beats per minute over time as compared to resting levels. The increased levels do not return to the

resting levels quickly which would be assumed if the crayfish were habituated to the environment. The extent to which HR and VR increase due to walking and movement can not explain the experimental group's immediate elevation of HR and VR, as well as the fact that both remain elevated the entire duration of the 30 minute trial. Instead, this alteration in the autonomic response suggests a stress response due to inability to complete the learned motor task. This alteration in the autonomic response relates to other studies examining the intrinsic state of the organism with environmental disturbances. Crayfish are known to rapidly increase HR during fight or flight situations such as defense posturing and it is known from earlier studies that a water drop into the water environment of a previously undisturbed crayfish will cause an increase in both HR and VR above the resting state (Listerman et al. 2000; Schapker et al. 2002). The increased response of both HR and VR rates were shown to return to near resting levels after approximately 5-10 minutes (Schapker et al. 2002).

A study which examined the physiological stressed response demonstrated that male crayfish exposed to novel environmental stress for 4 days have an altered responsiveness to neuromodulators at synaptic sites and altered behaviors to defined sensory stimuli (Page & Cooper 2004). The concept of altered responsiveness of neuromodulators, such as 5-HT, directly relates to previously mentioned studies in vertebrates showing impaired cognitive abilities. This further emphasizes the presence of a stress response in the blind crayfish exposed to light as well as the crayfish inhibited from completing the learned motor task.

Crustaceans, particularly crayfish, provide well defined characteristics to examine the role stress plays on the responsiveness of induced behaviors. Such well characterized behaviors include the tail flip response since the circuitry and muscle used are known (Edwards et al. 1999) and the circuitry shows plasticity in responsiveness (Yeh et al. 1996; Krasne et al. 1997). In crayfish, and most crustaceans, 5-HT is shown to increase synaptic strength and neurotransmitter release at neuromuscular junctions (NMJs) (Dudel 1965; Kupferfann 1979; Kravitz et al. 1980; Fisher & Florey 1983; Southard et al. 2000; Sparkes &

Cooper 2004) and alter the input resistance directly of muscle fibers (Dudel 1965; Crider &Cooper 1999; Strawn et al. 1999; Southard et al. 2000). Thus, circulating levels of neuromodulators, such as 5-HT, can impact sensory input, integration and motor output (Sneddon et al. 2000) resulting in the noted changes in behavior.

5-HT is suggested to modulate aggressiveness of lobsters and crayfish since defensive posturing is seen with 5-HT injections (Livingston et al. 1980; however see Strawn et al. 2000). Furthermore, 5-HT is known to increase heart rate (Florey & Rathmayer 1978; Wilkens & McMahon 1992) although the neural circuitry to regulate HR and VR is likely to be influenced by neuromodulators as well as the cardiac ganglion directly. The neural circuitry that drives the scaphognathite and the cardiac ganglion is not fully elucidated yet (Shuranova et al. 2006). Modification of the neural circuit is possible through CNS regulation as well as neuromodulators, particularly 5-HT (Page et al. 2007). The tail flip response is shown to habituate rapidly indicating a hormonal effect and direct relationship with neural synaptic regulation of the sensory-motor reflex (Listerman et al. 2000; Strawn et al. 2000; Kellie et al. 2001).

The cellular mechanisms for motor task learning as well as the stress response could be addressed possibly by pharmacological agents that block 5-HT receptors after the motor task has been successfully learned. As to how memory formation occurs, it is still being studied to this day in vertebrates as well as in invertebrates. Groundbreaking studies examining long term memory formation have opened new doors but have yet to provide a single general mechanism. It has been suggested that many routine motor commands may use short-term plastic characteristics of neurons to some extent. This is suggested because it is well known that the neuromuscular junction in crustaceans show short-term facilitation processes in which closely spaced action potentials cause an increase in the neurotransmitter release (Dudel & Kuffler 1961; Wiersma 1970). Thus, temporal codes are formulated by common use pathways possibly leading to more precise motor movements (Wilson & Davies 1965).

Importantly, to our knowledge this is the first study to address cave crayfish learning. I have shown that environmental factors that induce a stress response significantly impact learning. Future directions can be directed to determine the regions of the crayfish CNS responsible for learning like the mushroom bodies in *Drosophila* (de Belle & Heisenberg 1994) or the cerebellum suggested for mammals in motor learning (Eccles & Ito 1967; Marr 1969; Ito 2006). Also, it would be interesting to understand which hormones or peptide might be associated with the stress response in crayfish to further understand the impact on sensory input.

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Chapter Four

The Effects of CO_2 on Behavior and Physiology in *Procambarus clarkii*

INTRODUCTION

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 environment. 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, 1982). The sensory system of chemoreception is generally divided into the senses of smell (olfaction) and taste (gustatory) and is typically based on the anatomical location of sense organs and associated receptors for detection or transport medium of the stimuli (i.e., atmospheric, aquatic; Carr, 1988). These distinctions are further classified with classification of non-vertebrate and/or aquatic animals. The integration and processing of sensory information, such as that gathered from chemoreceptors, aids in navigation and overall survival (Atema, 1995).

Chemosensors are used to detect external environmental chemical cues; therefore sensors are highly venerable to physical, chemical and biological damage which can affect sensory function (Hamilton and Case, 1983; Derby et al., 2001). Much research examines the acid sensing capability of organisms, specifically cellular mechanisms of chemosensitive neurons (Putnam et al., 2004). Carbon dioxide (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, $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$ (Stone and Koopowitz, 1974). A characteristic of CO_2 exposure is a change

in pH due to the increase in protons since carbonic anhydrase rapidly catalyzing CO_2 and is likely the primary reason that intracellular pH quickly drops upon exposure of CO_2 (Baker and Honerjager, 1978).

CO_2 is universally found and impacts all organisms throughout their lifetime. On average the atmosphere contains approximately 0.035% CO_2 or 350 parts per million (ppm; Nicolas and Sillans, 1989). Many organisms live in environments with a CO_2 concentration much higher than the atmosphere, such as insects located under the bark of trees or stumps (Pasche and Zachariassen, 1973), bee hives (Buhler et al., 1983), termite mounds (Luscher, 1961) and caves (Howarth, 1983). Increased levels of CO_2 can be attributed to many causes with animal respiration, decrement of organic matter and decomposition of minerals to be the major sources resulting in the change of acidity in unpolluted waters (APHA Standard Methods). On average, normal levels of CO_2 in surface waters typically contain less than 10 ppm (mg/L) of dissolved CO_2 and will increase considerably based upon depth. In addition, consistently high, non-fluctuating levels of CO_2 in surface waters may act as an indicator of abnormal organic or mineral decomposition (APHA Standard Methods).

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., 1991). Recently, it was discovered that CO_2 is odorless to humans (Shusterman and Avila, 2003) due to a psuedogene (Torrents, 2003; Young et al., 2007). It is well known that CO_2 plays roles in the biology of insects despite being a ubiquitous sensory cue. Recently, those basic roles have been extended to an increasing number of insects, and similarities in its use appear to arise even among insects with rather different habitats. After years of work, the mechanisms underlying transduction of CO_2 stimuli at the level of receptor-cells are still being discovered, and processing of CO_2 cues in insect brains is just beginning to be understood.

Current literature, primarily in insects, shows that low levels of CO_2 acts as an attractant (chemotaxic properties; host-plant interactions, Jones and Coaker, 1977; blood-sucking insects, Allan et al., 1987). Some insects show behavioral

responsiveness to increases in CO_2 concentration as small as 0.002-0.003% (wireworms, Doane et al., 1975). Evidence shows that CO_2 emitted from vertebrates aids foraging for bloodsucking (hematophagous) insects to detect and orientate towards a host (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, 1980; Bowen, 1991; Takken, 1991, 1999). The fine-scale structure of the CO_2 plume strongly influences the behavioral responses of mosquitoes source-finding are observed mainly when flying mosquitoes are exposed to intermittent increases in concentration. Such a plume is what mosquitoes would encounter naturally at a relatively long distance from a host, and it was proposed that CO_2 plays an important role as a long-range orientation cue (Zwiebel and Takken, 2004). It is important to note that CO_2 also acts at a close range (Eiras and Jepson, 1991; Stange, 1996).

In many insects, electroantennogram recordings show that the antennules are sensitive to CO_2 in a dose dependent manner (Schnieder, 1957; Roelofs and Comeau, 1971). A detailed understanding of the physiology of the CO_2 receptor cells (RCs) has mostly been done in many different species of insects. Studies suggest that CO_2 RCs are not typical odorant receptor cells (ORCs) due to the cells high sensitivity to CO_2 (Stange and Stowe, 1999). In insects, CO_2 RCs are typically housed in thin-walled sensilla on insect mouthparts or antennae (Shanbhag et al., 1999; Stange and Stowe, 1999; Kleineidam et al., 2000) and also found to be densely populated to form a specialized sensory organ (Stange and Stowe, 1999). It is thought that other arthropods (e.g., centipedes, ticks, terrestrial crabs) also possess CO_2 RCs (Yamana et al., 1986; Steullet and Guerin, 1992; Stensmyr et al., 2005).

A subset of chemosensory receptors and neurons may provide information on chemicals that represent environmental factors of immediate ecological importance, such as the presence of food or danger. In *Drosophila*, at least three genes from the Gr family, Gr10a/b, Gr21a and Gr63a, are expressed in the antennae and have been suggested to have a role in olfaction (Scott et al.,

2001). Interestingly, the Gr21a gene is expressed in ab1C neurons that mediate avoidance of carbon dioxide in *Drosophila*; however, results do not indicate whether Gr21a is involved in the detection of CO_2 (de Bruyne et al., 2001; Larsson et al., 2004; Suh et al., 2004). The class of ORNs, Gr21a-expressing ab1C neurons, mediates an innate avoidance response to a specific stimulus. The ab1C neurons avoidance response to CO_2 is similar to the way that Gr5a- and Gr66a-expressing GRNs mediate attraction or avoidance to sugars and bitter compounds (Larsson et al., 2004; Suh et al., 2004).

Detection of CO_2 occurs through sensilla that are shown to resemble the sensilla used in olfaction; however the CO_2 sensilla are thought to function differently. Specifically, CO_2 RCs function as concentration detectors which are capable of delineating between concentrations of CO_2 and changes in air speed (Kaissling, 1998). This is thought to be possible because the RCs can reversibly absorb CO_2 to sensory structures even with a continuous equilibrium with the external CO_2 environment. This is significantly different from typical ORCs which are thought to act as flux detectors and are unable to detect changes in speed from changes in concentration, the rate of odorant molecules are irreversibly adsorbed by the sensory structures and the absorption is dependent on the external odorant environment. Furthermore, physiologically, CO_2 RCs are similar to RCs which respond to changes in ambient temperature and humidity (Waldow, 1970; Yokohari and Tateda, 1976; Kleineidam et al., 2000). Thus, current literature suggests that CO_2 RCs could be not only considered ORCs but also classified with another group of RCs that monitor environmental factors such as temperature and humidity (Stange and Stowe, 1999). Thus, CO_2 RCs are not typical ORCs.

In moths, mosquitoes, biting midges, the tsetse fly and many other insects, CO_2 RCs provide information such as specificity, sensitivity with a broad concentration response curve and the ability to signal continuously in the CO_2 background levels based on receptor activation and CO_2 signal. Although there are many studies to understand the environmental role of CO_2, it is of particular interest that there are relatively few studies available that examine the

physiological repercussions of increasing CO_2 levels in the environment. 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. Interestingly, early human surgeries used CO_2 as an anesthetic (Eisele et al., 1967). Although invertebrates and vertebrates are very different at a system level, the effects of CO_2 at a cellular level are not. Thus, studies which 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. The identified behaviors in larvae include behavioral unresponsiveness characterized by immobilization and lack of movement when mechanosensory stimulation is applied, as well as cardiac arrest characterized by the cessation of body wall movements (Badre et al., 2005). In this study, crayfish are used because they 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. Behaviorally, the measurement of tail flip responsiveness of crayfish and lobsters, 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, 1977; Copp, 1986; Fricke, 1986; Bruski and Dunham, 1987; Pavey and Fielder, 1996; Guiasu and Dunham, 1997; 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 study results, in combination with results using *Drosophila* larvae, will delineate between species-specific effects of CO_2 and the probability of a common mechanism of action with acute CO_2 exposure.

Here, I use 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 in crayfish, 2) forced movement, 3) tail touch as a bioindex of behavioral responsiveness, and 4) physiological measurements of the autonomic response in varying environmental conditions.

METHODS

Animals

Crayfish, *Procambarus clarkii* (sighted), measuring 5.0-6.4 cm in body length were obtained commercially (Atchafalaya Biological Supply Co., Raceland, LA). A total of 171 sighted 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 handling was conducted by using a glass beaker to transfer crayfish from one container to the other. Due to 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

Apparatus and experimental design

An aquatic Y-maze was constructed from plexiglass sheets (Figure 4.1). A total of 33 crayfish were used in this behavioral study to examine arm choice in

different conditional water treatments compared to aerated water for a trial period of 5 minutes. Crayfish were randomly divided into 6 groups: (1) 8 crayfish to test arm choice of 100% CO_2 and aerated water; (2) 5 crayfish to test arm choice of 50% CO_2 and aerated water; (3) 5 crayfish to test arm choice of 5% CO_2 and aerated water; (4) 5 crayfish to test arm choice of 100% N_2 and aerated water; (5) 5 crayfish to test arm choice of low pH of 4.85 and aerated water. (6) Control experiment using 5 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 since experimental trials showed that 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.

Initial placement of the crayfish was at the point of water integration (indicated by 'X' on Figure 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 white dotted line in Figure 4.1. 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 minutes 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 Larimer 1972).

Analysis of general exploratory behavior

Arm choice which was scored as time spent in either the experimental condition or control arm for a five minute trial duration. Statistical analysis used a Student's t-test for each treatment group. Time spent in the integration of water zone was not scored as an arm preference and this time was eliminated from analysis. An average % time spent in each experimental condition arm generated compared to control arm.

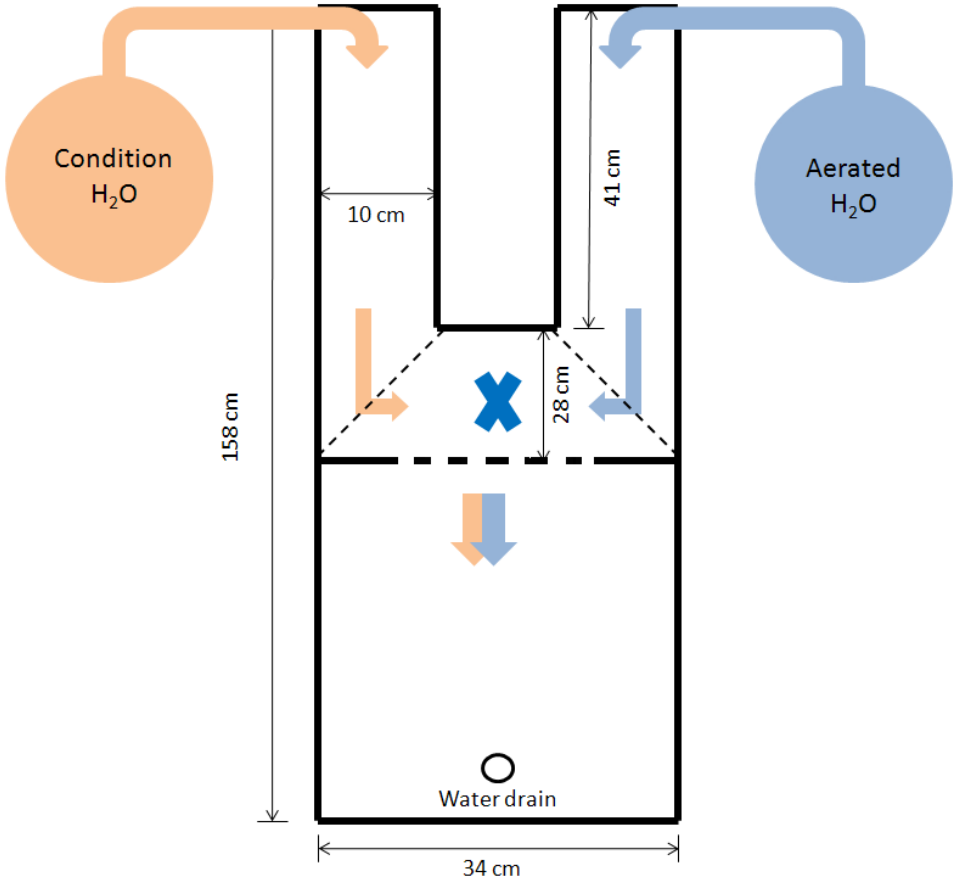

Figure 4.1. Schematic representation of Y-maze apparatus. Crayfish were placed in a water flow-through system with one side delivering one of the varying treatment conditions and the other side delivering aerated water as the control

ultimately to give the crayfish choice of environmental preference. 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 white dotted lines shown in the figure).

Environmental Forced movement

Apparatus and experimental design

A large plexiglass chamber was used to examine whether environmental conditions could force an organism to leave their environment. The rectangular chamber (234 cm x 23 cm x 32 cm) was divided into two separate environments using an incline (~ 25 degrees) covered in rocks leading to another pool (Figure 4.2). 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 to aerated water for a trial periods no more than 60 minutes. Crayfish were randomly divided into 6 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 treatment condition.

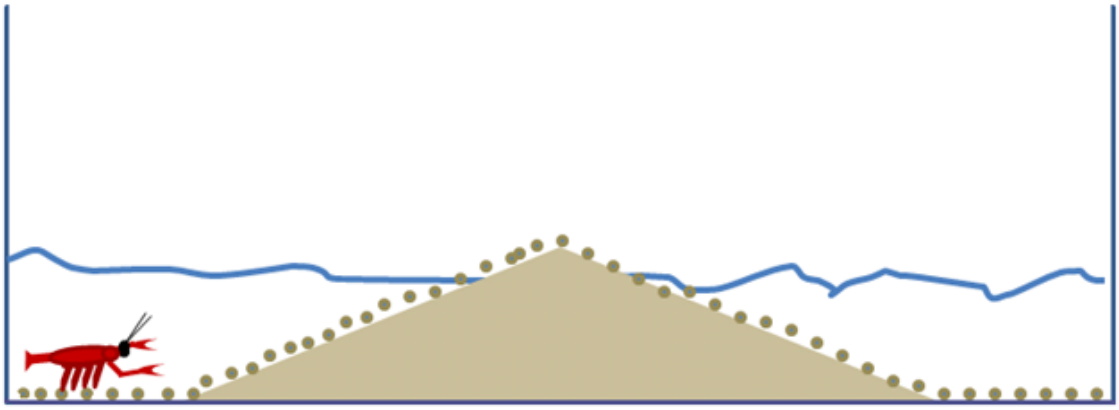

Figure 4.2. Schematic representation of forced movement chamber. Crayfish were placed in a rectangular chamber with one side having a experimental treatment environment with either 100% CO_2, 50% CO_2, 5% CO_2, 100% N2 or low pH of 4.85 and the other chamber acts as a control of an aerated environment. Chamber movement was recorded when the thorax of the crayfish crossed out of one environment.

Crayfish were introduced to the treatment side after 1 hour 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 Larimer, 1972). 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 to not impede the crayfish from exiting the water. In addition, a large plexiglass sheet

covered entire chamber to retard the gaseous environment from exchanging with the atmosphere.

Statistical analysis used a Student's a t-test for each treatment group. Time spent on the incline was scored as a forced out of the conditional environment. An average % forced from each experimental environment was generated and compared to aerated control environment.

Tail touch as a bioindex

Apparatus and experimental design

To measure responsiveness in the varying environmental conditions, a touch was applied to the tail of a crayfish to elicit a 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 forceful stimulus (touch using a metal probe) 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 minutes) which then the crayfish was removed from the treatment environment.

Experimental conditions were prepared by bubbling the treatment gas into a closed container for 45 minutes. Crayfish were randomly chosen for 6 treatment conditions: (1) 5 crayfish in 100% CO_2; (2) 5 crayfish in 50% CO_2; (3) 5 crayfish in 5% CO_2; (4) 5 crayfish in 100% N_2; (5) 5 crayfish in pH 4.85 and (6) Control experiment using 5 crayfish in aerated water.

Recording ECGs and ESGs

Crayfish were wired to record electrocardiograms (ECGs) for heart rate (HR) and electroscaphognathites (ESGs) for ventilation rate (VR; Listerman et al. 2001; Schapker et al. 2002). Experimental details are shown in video format

(Bierbower and Cooper, 2009). A lid was used to prevent the crayfish from exiting the chamber but left a small section uncovered for the wires to exit the chamber and did not prohibit the crayfish from moving freely. The placements of the recording wires are shown in Figure 4.3. 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). All events were measured and calibrated with the PowerLab Chart software version 5.5.6 (AD Instruments, Australia). Previous studies showed that 3 days was enough time for the animals to return to baseline physiological values prior to handling (Wilkens et al., 1985).

Statistical analysis

To determine study significance, 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 for a percent change from initial baseline recordings (i.e., first 20 minutes). Percent change values were determined by taking the absolute value of the baseline, minus each subsequent beats per minute and dividing by the baseline and 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.

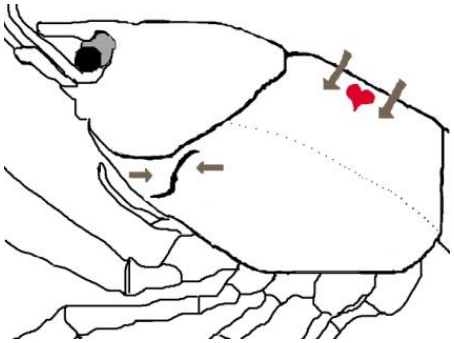

Figure 4.3. Schematic representation for the placement of the recording wires for monitoring the heart and ventilatory rates from a crayfish *(Procambarus clarkii*.). On the dorsal carapace, large arrows represent the two wires which span the rostral-caudal axis of the heart to monitor heart rate. On the lateral side, two smaller arrows represent the two wires which span the scaphognathite (SG) (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.

A physiological trial began when a crayfish was placed into an experimental container of normal aerated water; the other end of the wire was connected to the impedance detectors and the crayfish was left for 15 minutes to acclimate to the new surroundings. After the initial 15 minutes, recording baseline HR and VR measures commenced. Each experimental trial initially began with a 30-minute recording of normal baseline rate for HR and VR rates which was used as a reference for changes in an autonomic response to environmental conditions. After the initial 30 minutes, 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 time point when the HR and VR dropped out completely for 1 minute. Once the autonomic response dropped out completely, the crayfish was removed from the experimental conditioning chamber and returned to the aerated chamber with continued measures of HR and VR for 2 hours. The trial was terminated at the end of the two hour recovery period.

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

RESULTS

CO_2 as an attractant

When placed into the Y-maze, crayfish showed exploratory behavior by walking around in the maze. Typically, they walked up the middle of an arm with both antennae held out in front with swaying them back and forth touching the walls on either side of the arm to guide themselves (thigmotaxis). Often times, 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 not being significantly different ($t_8 = 0.207$, $p = 0.841$).

To identify the influence of CO_2 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 4.4. There is a significant reduction in time spent with CO_2 exposure which directly correlates to increasing levels of CO_2 gas. Specifically, crayfish spend significantly less time in 100% CO_2 ($t_{14} = 40.557$, $p < 0.001$) when compared to 50% CO_2 ($t_{14} = 2.232$, $p = 0.042$). Furthermore, crayfish did not significantly show arm preference when exposed to only 5% CO_2 ($t_{30} = 0.134$, $p = 0.895$). The lack of significant difference in mean time for 5 % CO_2 is mostly suggests 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. Thus, results show a behavioral repellent effect with increasing levels of CO_2. In addition, crayfish do not show an arm preference for N_2 in which they spend approximately equal time in both arms (t_{12} = 0.109, p = 0.915). 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 since there was not a significant difference in arm preference during low pH exposure (t_{10} = 1.060, p = 0.314).

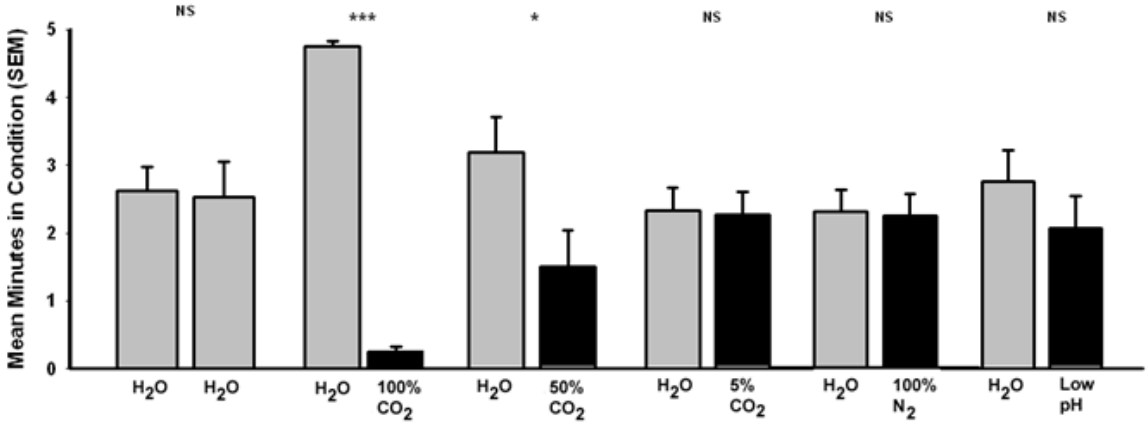

Figure 4.4. The influence of CO_2 on arm preference in a Y-maze. To identify if the effects were due to CO_2 we also examined behavioral responses of general arm choice (H_2O only), the effects of hypoxia (N_2) and also effects of low pH (4.85). Crayfish were placed in a water flow-through system with one arm delivering one conditional water (black bars) and the arm delivering aerated water as the control (grey bars). The mean rate (± SEM) of time over a 5 minute periods was assessed for the conditions. There is a significant decrease in the amount of time spent for CO_2 as compared to N_2 and low pH (Student's t-test; * p

< 0.05 and *** p < 0.001. NS indicates no significant difference between variable and aerated control).

Environmental forced movement

To identify the influence of carbon dioxide on forcing movement out of an environment, crayfish were tested in a forced movement chamber. The degrees of movement for specific conditions of CO_2, N_2 or pH 4.85 are shown in Figure 4.5. There is a significant difference in the movement out of CO_2 environments compared to the control aerated. Specifically, crayfish were significantly shown to leave 100% CO_2 (t_{38}= -24.582, $p < 0.001$), 50% CO_2 (t_{20} = -28.741, $p < 0.001$), and 5% CO_2 (t_{20} = -4.276, p = <0.001). Results also indicate that crayfish were significantly shown to move out of N_2 when exposed (t_{18} = -0.452, p = 0.657) 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 since there was not a significant difference in movement out of a low pH environment (t_{18} = 0.000, p = 1.000).

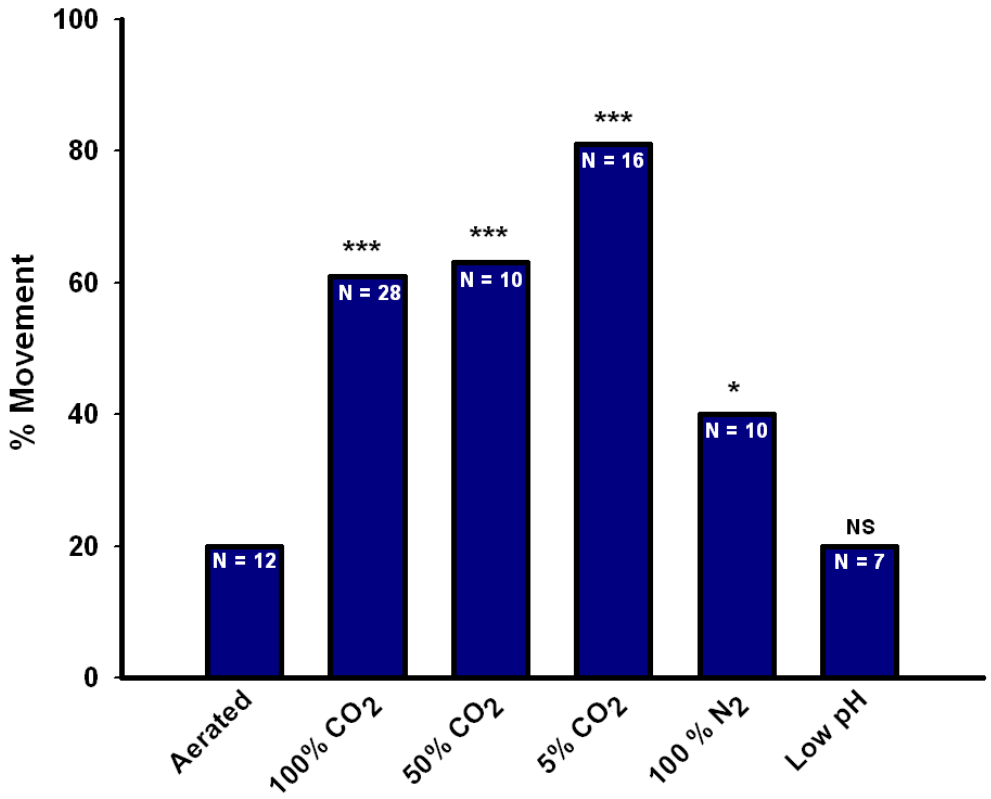

Figure 4.5. Assessing behavioral effects of carbon dioxide exposure on crayfish movement. When crayfish are place in CO_2 environmental conditions, the animals moved out of the possible toxic environment. A rectangular chamber was divided into two separate aquatic environments by a rock embankment which allowed access to either environment. Crayfish were placed in the treated water for a maximum of 30 minutes or until paralysis. Data is represented as the percentage of individuals who moved to the aerated water side from treated side. N = sample size. (Student's t-test; * $p < 0.05$ and *** $p < 0.001$. NS indicates no significant difference between variable and aerated control).

Tail touch as a bioindex

Here, 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. Animals that tail flipped were marked as having a response and those that did not were recorded nonresponsive. In this study, no differentiation was made between giant mediated and non-giant mediated tail flip (Krasne and Wine, 1975) 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 4.1). Specifically, 100% CO_2 produced unresponsiveness within 20 minutes in 5 out of 5 preparations ($p < 0.05$; Wilcox non-parametric 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 response times for cessation of locomotor activity varied with each crayfish with an average of 13 minutes before complete unresponsiveness to stimuli occurred. Unresponsiveness to stimuli was seen with 50% CO_2 after approximately 30 minutes in 5 out of 5 preparations ($p < 0.05$; Wilcox non-parametric test). It should be noted that response times for cessation of locomotor activity varied with each crayfish with an average of 27 minutes before complete unresponsiveness to stimuli occurred. 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 with 100% CO_2. Thus, only 100% CO_2 induced the unresponsive behavioral effect within 15 minutes and it was shown to be dependent on the concentration since the same effect will result with 50% CO_2 over a longer period of time.

Table 4.1. Assessment of carbon dioxide's impact on responsiveness to stimuli. A tail touch was given once per minute up to 30 minutes or until the crayfish no longer responds for two consecutive touches. If the crayfish no longer responds, it was removed from the experimental condition. Yes = response to tail touch stimuli by tail flip, No = non-responsive to tail touch stimuli.

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	10 Minutes	20 Minutes	30 Minutes
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

Recording ECGs and ESGs

The physiological response of crayfish was recorded to characterize the autonomic response with carbon dioxide exposure. Heart (HR) and ventilation (VR) rates 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-s intervals and then converted to beats per minute (BPM).

The handling and initial response to conditions usually resulted in an immediate but brief elevation in HR and VR.

In 100% CO_2, cessation of heart (HR) and ventilation (VR) rates was significant (5 out of 5 crayfish, $p < 0.05$, Wilcoxon non-parametric analysis) observed during the experimental trial was a cessation of heart (HR) and ventilation (VR) rates. Raw traces show a rapid and complete lack of both HR and VR with CO_2 exposure (Figure 4.6). Although cessation times of HR and VR activity varied slightly with each crayfish, an average time for HR was 9 minutes and VR was 11 minutes (Figure 4.7). A complete cessation of both HR and VR are shown to occur before the 15 minute time point with 100% CO_2 exposure; thus the 15 minute time point will be used for sampling other treatment conditions for time point comparison.

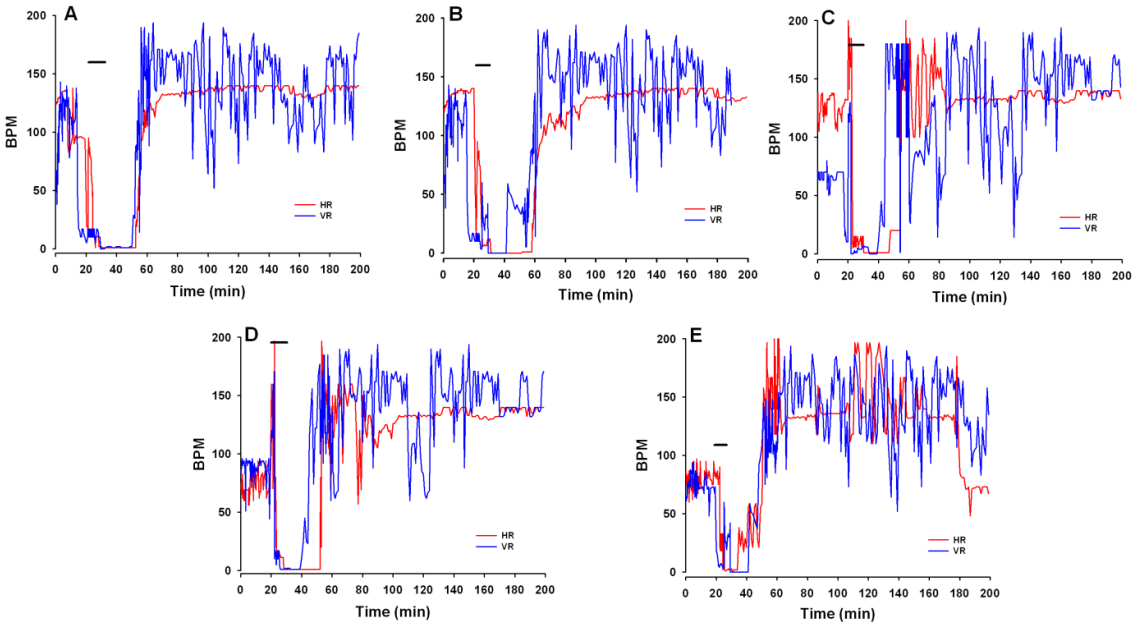

Figure 4.6. Physiological response of crayfish exposed to saturation of (100%) carbon dioxide over time. Graphs A-E represent each crayfish (N=5) exposed to 100% CO_2. Traces represent the physiological response of heart rate (red line) and ventilatory rates (blue line) during CO_2 exposure. The dark black lines on the

top of each graph indicate the period of time each crayfish was exposed to the CO_2 saturated environment.

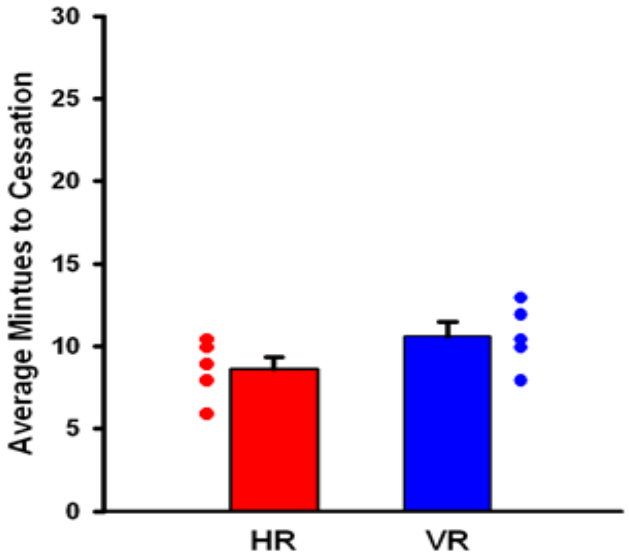

Figure 4.7. The average cessation time of heart and ventilation rates for crayfish exposed to 100% CO_2. All crayfish (N=5) exposed to 100% CO_2 exhibited a complete drop out in both heart (HR) and ventilation rate (VR) with an average of 9 minutes for HR and an average of 11 minutes for VR. The average time to cessation is indicated by the bars, while the scatter plot indicates each individual crayfish time of HR and VR cessation.

To determine the effect that CO_2 has on HR and VR, various amounts of CO_2 exposure were used with crayfish. To further understand if the rapid effects noted with 100% CO_2 (complete cessation of HR and VR) is concentration dependent, I exposed crayfish to 50% CO_2. The relatively lower concentration of CO_2 did not result in the rapid cessation in HR and VR (Figure 4.8). Although there was not a complete dropout within the 30 minute exposure time, it is important to note that there was a decrease in both HR and VR for 50% CO_2

exposure. Furthermore as expected, 5% CO_2 did not induce the cessation in the autonomic response (Figure 4.9).

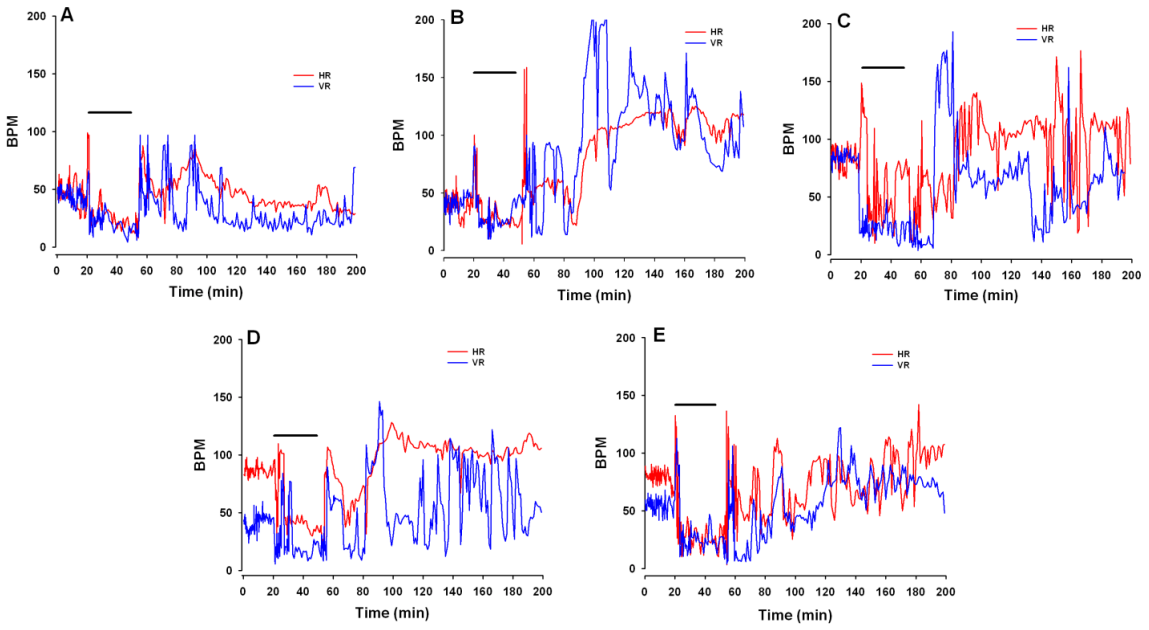

Figure 4.8. Physiological response of crayfish exposed to 50% carbon dioxide. Lines on the top of the graph indicate periods of time the crayfish was exposed to oxygenated and CO_2 saturated environments. Traces represent the physiological response of heart rate (red line) and ventilatory rates (blue line) before, during and after CO_2 exposure.

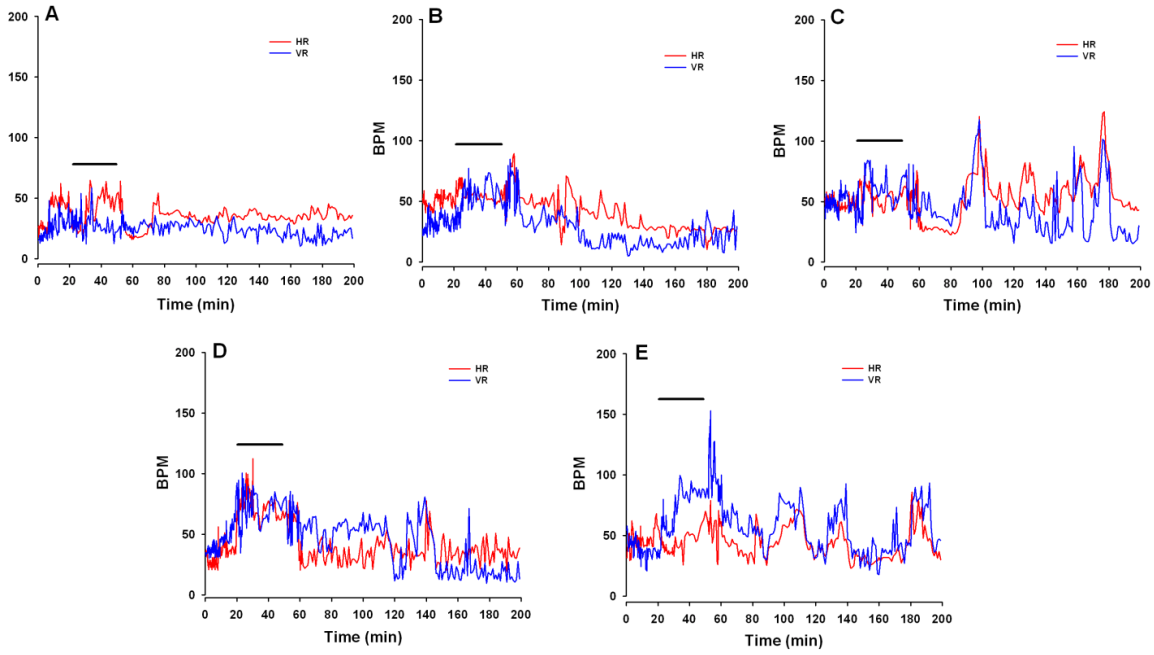

Figure 4.9. Physiological response of crayfish exposed to 5% carbon dioxide. Lines on the top of the graph indicate periods of time the crayfish was exposed to oxygenated and CO_2 saturated environments. Traces represent the physiological response of heart rate (red line) and ventilatory rates (blue line) before, during and after CO_2 exposure.

To assay the effects of a hypoxic environment on HR and VR, the crayfish was exposed to 100% N_2 environment. In 5 out of 5 crayfish, an opposite response in both HR and VR was noted when compared to 100% CO_2 (Figure 4.10). Specifically, both HR and VR were shown to increase 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, the crayfish were exposed to a low pH of 4.85 environments. A pH of 4.85 was used since this is the lowest pH observed with bubbling of 100% CO_2 into a closed aquatic environment. After a 30 minute exposure time period, both HR and VR were shown to increase (Figure 4.11). 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.

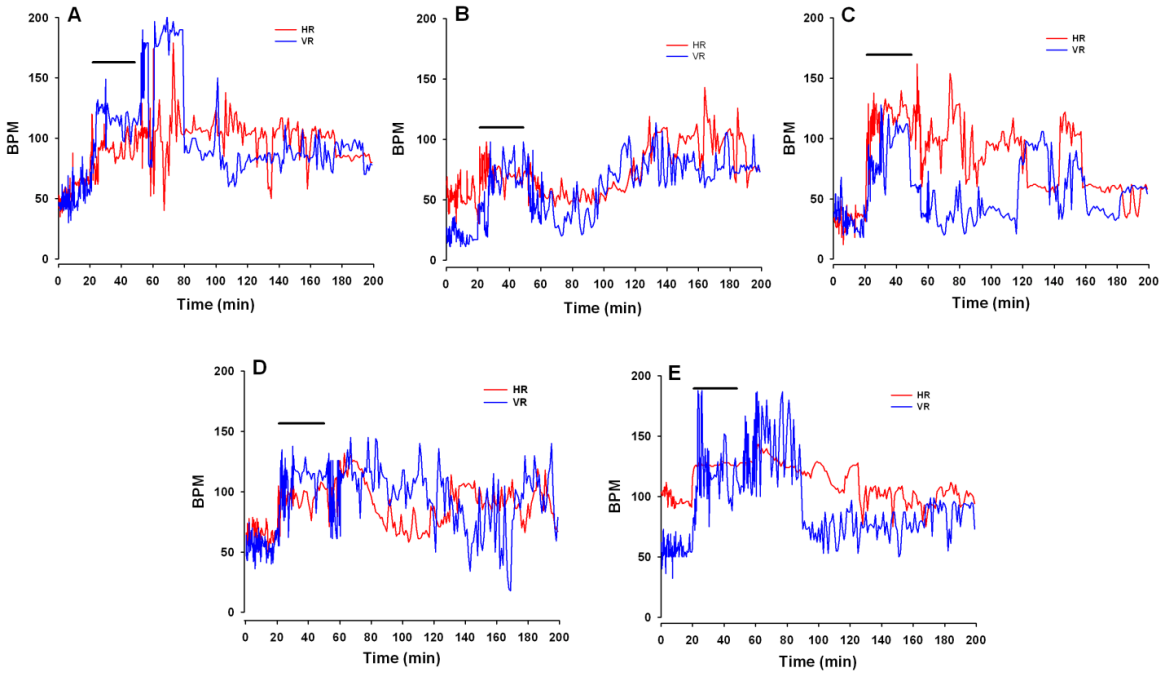

Figure 4.10. Physiological response of crayfish exposed to saturation of (100%) nitrogen. Lines on the top of the graph indicate periods of time the crayfish was exposed to oxygenated and CO_2 saturated environments. Traces represent the physiological response of heart rate (red line) and ventilatory rates (blue line) before, during and after CO_2 exposure.

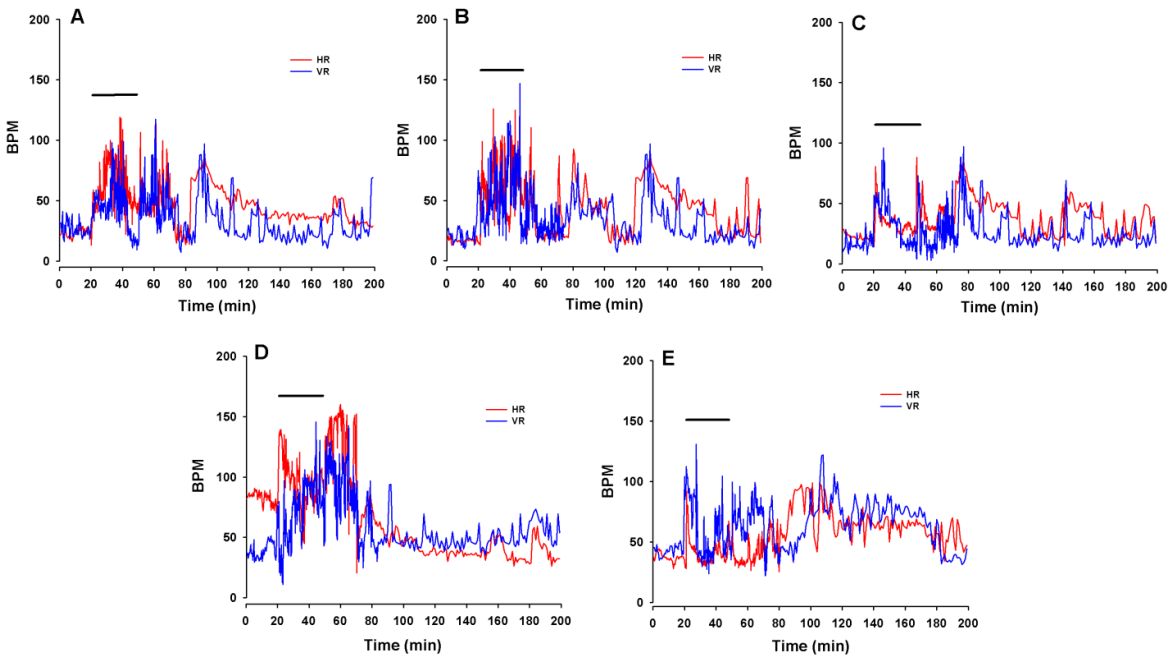

Figure 4.11. Physiological response of crayfish exposed to low pH 4.85. Lines on the top of the graph indicate periods of time the crayfish was exposed to aerated and CO_2 saturated environments. Traces represent the physiological response of heart rate (red line) and ventilatory rates (blue line) before, during and after CO_2 exposure.

Given that the cessation of the autonomic response occurred in all crayfish by the 15 minute time point (Figure 4.7), this was used as a comparison time point for all other treatment conditions for statistical analysis. Thus, at the 15 minute time point for 100% CO_2, percent change from baseline recordings becomes a 100% significant decrease change (HR, $t_8 = -1000$, $p < 0.001$; VR , $t_8 = -1000$, $p < 0.001$; Figure 4.12). Across groups statistical analysis compared the decrease in 100% CO_2 to 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 to baseline recordings (HR, $t_8 = 5.37$, $p < 0.001$; VR, $t_8 = 14.76$, $p < 0.001$) and results show that after 15 minutes of 50% CO_2 exposure, HR is still significantly different to the HR of 100% CO_2 (ANOVA, $t_8 = 17.85$, $p < 0.001$). However, the decrease in 5 out of 5

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 significantly decrease from baseline recordings and also to be significantly different from the 100% CO_2 VR rate (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, $t_8 = 0.19$, $p = 0.853$). Results show that after 15 minutes of 5% CO2 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 minutes for both 100% N_2 and low pH 4.85, HR is still significantly different to the 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 minute time point but did not cause a complete dropout. 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 that amount of time.

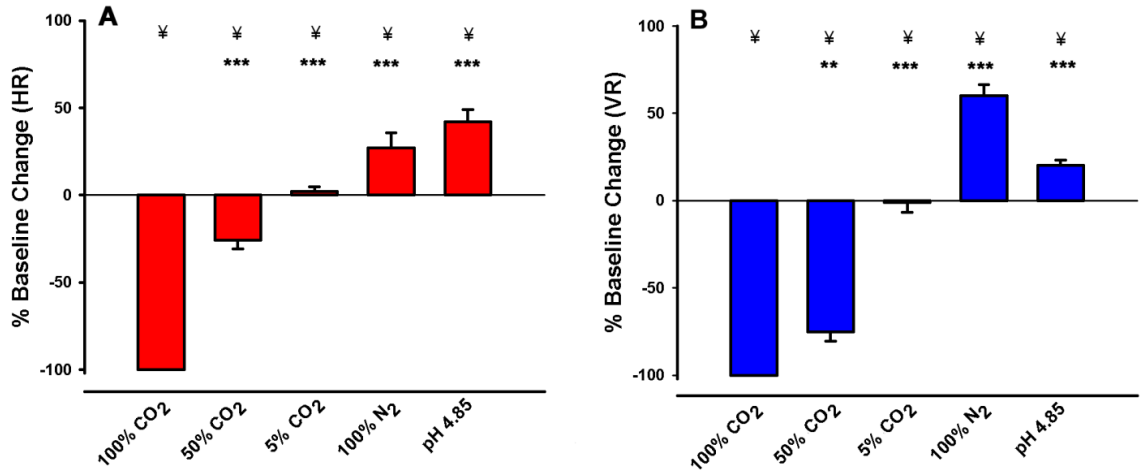

Figure 4.12. Autonomic response of crayfish exposed to varying environmental conditions. (A) Graph represents HR in varying environmental conditions (red bars), (B) Graph represents VR in varying environmental conditions (blue bars). Across group ANOVA test: ** indicates $p < 0.02$ and *** indicated $p < 0.001$. Students t-test: ¥ indicates 5 out of 5 crayfish within group using a Wilcoxon non-parametric analysis.

DISCUSSION

In this study, it was demonstrated that crayfish rapidly respond to high levels of CO_2 in their environment. Specifically, crayfish show a strong repellent behavioral response to high levels of 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%). Since 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 of 4.85 were also assessed. Crayfish do not show a preference and/or a repellent behavioral response to hypoxic (induced by N_2 displacement of O_2) or low pH (4.85) environments.

In further examining the repellent behavioral effect, it was shown that crayfish will rapidly leave a potentially toxic environment as seen with crayfish exposed to 100% and 50% CO_2. Movement was also significantly shown 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. The effect on the tail touch response appears to be directly mediated by CO_2. By the 15 minute time point, crayfish exposed to 100% CO_2 no longer responded to mechanosensory stimulation. All other treatment conditions (including lower levels of CO_2) showed responsiveness to tail touch up to 30 minutes.

The autonomic response with CO_2 exposure shows a complete cessation of both HR and VR with exposure to 100% CO_2 within in the time period shown for unresponsiveness to tail touch. Raw traces of BPM data indicate that HR remains consistent during the baseline recordings and significantly decreases very quickly upon CO_2 exposure. In addition, 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. Also, 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 decrease in both HR and VR after 30 minutes of exposure, activity persists. 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.

Previously identified effects seen in *Drosophila melanogaster* larvae were also shown in the crayfish with acute CO_2 exposure (Badre et al., 2005). The study showed behaviorally that 3rd instar larvae became unresponsive to mechanosensory stimulation with acute CO_2 exposure as well as cessation of body wall movements and heart rate.

Synaptic examination in the larvae showed effects at the neuromuscular junction that were also examined in the crayfish and is discussed in detail in Chapter 5.

It is important to understand the impact CO_2 may have on behavior of organisms since nature functions as a food web. 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, 1978; Nicolas and Sillans, 1989). The larvae of the beetle *Diabrotica virgifera virgifera* uses only volatile CO_2 to orient themselves toward corn roots (Bernklau and Bjostad, 1998). Furthermore, field foraging behavior of the larvae of the noctuid moth *Helicoverpa amrmigera* show the of CO_2 in foraging because the larvae feed on plant tissues that do not use CO_2 but that are sources of CO_2 such as fruits and flowers. The foraging behavioral responses were shown to be dose dependent (Rasch and Rembold, 1994).

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., 1967), and has known physiological effects (fish, Mitsuda et al., 1982; Iwama et al., 1989; rat, Gautier and Marariu, 1998; St.-John and Rybak, 2002; termite, Shelton and Appel, 2000; Drosophila, Badre et al., 2005). In the well-studied fish system, as CO_2 increases, the anesthetic affect is seen at concentration of 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, 1959). In addition to the anesthetic effect, the increase in CO_2 also has the aversive effect resulting in a decrease in oxygen availability (hypoxia). Basu (1959) conducted a study using 3 species of fish which showed the rate of oxygen consumption in all 3 active species decreased significantly as CO_2 levels increased. Previous research examining moderate CO_2 exposure and acclimation shows that oxygen uptake reduced with CO_2 exposure recovers after a few hours in continued CO_2 exposure (Saunders 1962). This acclimation is most likely due to a change in blood bicarbonate in response to the CO_2 levels, as seen in the rainbow trout (Lloyd and Jordan, 1964). Although, this buffering with bicarbonate contributes to the blood acid-base balance through increased

ventilation; this is not an effective method to sustain long-term effects of CO_2 exposure.

The concerning high levels of CO_2 are a pertinent issue for the state of Kentucky due to mining processes. Mining results in acid deposition which causes significant effects on aquatic resources in North America (Watten et al., 2005). Acid mine drainage (AMD) has a significant effect on the water quality of surface waters and many of the regions of the Appalachia and the Ohio River basin have already been significantly affected due to dissolution of pyrite and oxidation to sulfuric acid. Thus especially for this area, high levels are prevalent and are known to be detrimental to the environment significantly effecting populations and/or species dependent on environmental conditions. It can be concluded that behavioral and physiological studies of cave species in a karst region with cave endangered and threatened organisms for extinction, water quality and CO2 content is of concern.

Many studies examine this effect on vertebrates, mainly fish, to understand chronic and long-term exposure of fish reared in hatcheries (Krise, 1993; van Raaij et al., 1996; Ross et al., 2001). It has been shown that excess dissolved gas (i.e., O_2) results in stress and often leads to a disease state with super-saturation characterized by formation of gas bubbles on the external surface, within the circulatory system or in the tissues of the fish (Krise, 1993). This is an important issue since it is known to reduce growth and survival due to long-term stress on physiological processes over time (Mazeaud et al. 1977; Schrack 1981; Wedemeyer et al., 1984, Colt et al., 1985).

Crayfish play an important role in aquatic ecosystems (ponds, lakes, streams, marches, etc.) by serving as a favored food item for a large diverse number of aquatic and terrestrial animals (Momot et al, 1978). Many fish (trout, bass and larger sunfishes), birds (ducks, geese, herons, kingfishers, egrets), amphibians (bullfrogs, salamanders), reptiles (turtle, water snakes) and mammals (otters, raccoons, mink) consume large amounts of crayfish. The role of crayfish in the ecosystem is not specific since they will eat both living and dead plant/animal material. Crayfish are known to help reduce the amount of decaying

matter and thereby improve the water quality. Furthermore, most crayfish are not active predators since they have difficulty capturing fast moving animals.

Organisms have been shown to use low levels of CO_2 to locate food sources and gain valuable information about their environment as well as evidence suggesting high levels also provide valuable information and signal a toxic environment. Much of the work examining high levels of CO_2 and changes in behavior has been conducted in insects due to the feasibility of experimental studies. Insect social behavior has been shown to control the environmental variable within the nests. It has been shown that higher than normal temperatures or levels of CO_2 can have detrimental and catastrophic effects on colonies. 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, 1974; Southwick and Moritz, 1987). It has been shown that bumble bees, *Bombus terrestris*, also collectively fan and that the number of fanning bees increases as CO_2 levels increase (Weidenmuller et al., 2002). Other behavioral response to temperature and CO_2 concentrations is seen with nest structure. For ants and termites, the structure of the nest utilizes surface wind for ventilation and keeps internal CO_2 concentrations relatively low (Stange, 1996; Kleineidam and Roces, 2000; Kleineidam et al., 2001). Both species are known to 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).

Whereas it has been known that CO_2 cues play an important role in the foraging behavior of many insect larvae, it was not clear whether other organisms may also use those cues. It would seem very likely that given their role in the ecosystem, crayfish would use CO_2 cues in finding decaying matter and other food sources.

Crayfish placed into the Y-maze for condition preference did not show a preference for low levels of CO_2 (i.e., 5%). However, the lack of response could be due more to the fact that the level was too low to elicit a response since these crayfish come from a swamp environment which is most likely to be high in CO_2.

Thus, a future direction should examine dose response curves both below and above this level to more concretely state attraction to CO_2 levels or the use of CO_2 to find food sources. More interestingly, there is a definite and obvious repellent effect with higher CO_2 levels (i.e., 50%, 100%). The repellent effect was seen in the Y-maze in which the crayfish avoided the higher levels of CO_2 concentrations but did not show a repellent effect for low levels of CO_2, hypoxic or low pH environments. Thus, this strongly suggests that CO_2 is a strong predictor of toxic environments and a cue for organism to vacate.

This type of repellent behavioral response was further demonstrated when crayfish were placed into varying concentrations of CO_2, hypoxic and pH environments with the ability to leave the environment in search of other water sources. The results revealed that a higher percentage of crayfish leave CO_2 environments when compared to the hypoxic or low pH environments. Again, this suggests that CO_2 acts as a strong indicator of toxic environmental conditions.

A common behavioral effect with acute CO_2 exposure is the lack of a behavioral response over time as seen in 3rd instar *Drosophila* larvae (Badre et al., 2005). The lack of behavioral response with acute CO_2 exposure was also shown in crayfish. Crayfish acutely exposed to CO_2 were given a tail touch once every minute until a lack of behavioral response (i.e., tail flip). Interestingly, the onset of responsiveness was shown to be CO_2 concentration specific since crayfish exposed to 100% CO_2 were shown to be unresponsive after approximately 10 minutes whereas crayfish exposed to 50% were shown to be unresponsive after approximately 30 minutes. The unresponsiveness behavioral effect within 30 minutes was not shown to occur with crayfish exposed to 5% CO_2, 100% N_2 or low pH environments. Thus, effects are once again shown to be CO_2 mediated.

The crustacean circulatory system is able to react to several ecological demands interfering with normal function such as air exposure (Taylor and Wheatly, 1981; Airries and McMahon, 1996), exercise (Herried et al., 1983; Hamilton and Houlihan, 1992; Hokkanen and DeMont, 1992; Reiber, 1994, 1997)

or hypoxia (Butler et al., 1978; Wheatly and Taylor, 1981; McMahon, 1992; Reiber et al., 1992; Airries and McMahon, 1994) though various physiological adaptations. While the exact mechanisms for CO_2 detection and effects on physiological processes are not fully understood, much work has been done mostly in insects to characterize specific pathways. To further understand physiological effects in other arthropods, the autonomic response was examined during acute exposure in crayfish. Effects on physiology in combination with effects in behavior further characterized acute exposure effects.

The effect of acute CO_2 exposure on the autonomic response is shown to be CO2 mediated and concentration dependent. With 100% CO_2, there was rapid and complete cessation of both HR and VR. In the relatively lower concentration of 50% CO_2, there was not a rapid and complete cessation in HR and VR. However, there was a significant decrease within the 30 minute exposure time period that would most likely have resulted in complete cessation of the autonomic response 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 since the crayfish can most likely physiological compensate to this environmental condition. The observed effect shown with 100% CO_2 is not due to a hypoxic environment induced by the bubbling of a gas into a closed environment. In 100% N_2 and pH 4.85, both HR and VR were shown to increase during acute exposure. These results suggest that neither a hypoxic or low pH environment can explain the behavioral and physiological effects seen with acute exposure to 100% CO_2.

High levels of CO_2 are shown to have detrimental effects on organisms. As shown in the this study, higher than normal levels can signal toxic environments in which organisms will vacate the environment in search of new aquatic sources. While new aquatic environments might not be available and certain death may be likely, it is also likely that the organism will die if remaining in a CO_2 environment due to organ failure. These experiments show behaviorally that an organism will leave the environment based upon the environmental cue of CO_2 levels and

physiologically that there is a CO_2 mediated effect on both the cardiac and ventilatory systems.

On an ecosystem level, crayfish provide such a critical and fundamental role in ecosystem dynamics that the loss could produce staggering consequences. 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 insecticide 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 organisms.

A particular interest is to understand the mechanisms of action of CO_2 resulting in the unresponsive 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.

ACKNOWLEDGEMENTS

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Chapter Five

The mechanistic action of carbon dioxide on neural communication

INTRODUCTION

As discussed in Chapter 4, carbon dioxide (CO_2) plays a pivotal role through the complex interactions between many organisms and their environment. CO_2 is known to be an attractant for many invertebrates at low levels (orientation response), an important cue for host location, and acts as an anesthetic at higher concentrations.

Chemicals are often used to convey a wide variety of information through many sensory channels from a diverse amount of sources. Currently, much of the research on chemical sensory and neural processing has been conducted in Insects. Insects are favorable model organisms for examining underlying neural mechanisms in olfaction because they possess olfactory controlled behaviors and have the accessibility to sensory organs (Rospars and Hildebrand, 1992). Specifically, the olfactory systems of many insects have been extensively studied to understand neural mechanisms and the orderly array of glomeruli that allow for primary processing of olfactory information. Thus, recent research has provided detailed information into the behavior, as well as, the underlying neural mechanisms including chemical composition and CNS processing and coding. The applications of CO_2 is unlimited from alternatives to chemical fumigation, controlling insect vectors of diseases and infestations in stored food to the most important of understanding atmospheric effects on plant productivity and impacts on herbivores. The study of chemical sensing within invertebrate models, through both contrast and analogy, can produce information that can relate to a wide range of subjects including vertebrates, mammals, and even humans.

The chemosensory system of decapod crustaceans is organized into multiple anatomically distinct neuronal pathways as in other organisms, including both vertebrates and insects. The two main pathways of chemosensory sensing are the aesthetasc-olfactory lobe pathway and non-aesthetasc-lateral antennular

neuropil pathway. These two pathways originate in different populations of antennular sensilla and project to different neuropils in the brain. Interestingly, even though this organization is well defined, the functional significance is not well understood in crustaceans or in many other species.

Some organisms detect environmental CO_2 by external sensory receptors (Guerenstein and Hildebrand, 2008) while others monitor environmental levels and internal concentrations through intra-receptors (Baker and Honerjager, 1978). Although internal CO_2 levels are constantly monitored and regulated, exact mechanism of detecting the partial pressure of CO_2 (pCO_2) is not fully understood. It is known that there are many cell types capable of sensing CO_2. Carotid and aortic bodies within the brainstem are primary sites in detection in mammals (Ainslie and Duffin, 2009). CO_2 can diffuse across the lipid bilayer and is driven by concentration gradients due to the rapid enzymatic reaction of carbonic anhydrase ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) (Stone an Koopowitz, 1974). Carbonic anhydrase is one of the fastest enzymes in mammals (Breton, 2001) and is known to be crucial in pH balance.

The mode of action in which CO_2 operates as an anesthetic has been discussed, but still little is known on the underlying mechanism of the induced effects seen with acute exposure. Much of the speculation has discussed whether the action of CO_2 results from a specific effect of CO_2 itself, anoxia and/or due to changes in pH. The impact of CO_2 can be indirect through rapid changes in pH, effects on cellular processes and/or organelles. Currently, empirical evidence of a general mechanism of action of all anesthetics involves physical changes in permeability of the cell membrane (Mullins, 1975).

CNS processing of information

Animals of almost all levels of complexity have been shown to integrate raw sensory information into electrical signals. In many cases this results in the generation of action potentials (AP) which are the basic units of neural communication in most central nervous systems (CNS). An AP is a large,

localized change in membrane potential (V_m) that travels along a plasma membrane and produces the same change in V_m along the way. Changes in V_m are the result of increased conductance of Na^+ (influx) leading to depolarization (rising phase) of the membrane (Overton, 1902, Hodgkin, 1939). Soon thereafter, there is a decrease in Na+ conductance due channel inactivation as well as rise in outward conductance of K^+ (falling phase) which causes repolarization of the cell.

The integration of the sensory signals is then processed in the CNS and often leads to a behavioral change in response to the sensory stimulation. Neurons communicate these signals to each other as well as to non-neuronal targets such as muscles and endocrine cells. The communication is essential as it allows the animal to respond to its' environment and regulate bodily functions. Thus, sensory neuroscience is a complex system of neural integration and processing which provides critical information for an organism to survive in a changing environment and is a major area of current research interest.

Sensory information is transduced from specialized sensory structures and transferred to the CNS by way of electrical activity. For proper function of nervous systems at a multitude of levels with both excitation and inhibition, communication at synapses is finely regulated and able to adjust in response to changing environments. Although there are many kinds of synapses within the CNS, communication between neurons and neurons to targets can be either electrical or chemical (Purves et al., 2004). Electrical synapses permit direct, passive flow of electrical current from one neuron to another.

In electrical communication, the membranes separating two communicating neurons the pre- and post-synaptic cells are in extremely close proximity at the synapse and direct cell-to-cell communication is mediated by gap junction channels made of two hemichannels (vertebrates, connexin proteins; invertebrates, innexin proteins; Purves et al., 2004). Each connexon is composed of six connexins which are shown to be radially arranged around a relatively large pore. This pore allows an ionic current to flow passively as well as ATP and other important intracellular components such as second

messengers to be transferred. The more general purpose of electrical communication junction coupling appears to play a role in a variety of cellular tasks involving direct electrical and/or metabolic (i.e. biochemical) cell-to-cell communication quickly among populations of neurons. This same type of function is seen with electrical synapses in invertebrates.

Chemical communication occurs at synapses between targets. The chemical signal transduction is due to transmitter release from stores within vesicles contained in the presynaptic nerve terminal (Purvis et al., 2004). Transmission from one neuron to another at chemical synapses occurs through a sequence of steps. The process begins with an action potential traveling to the terminal of the presynaptic neuron. The changes in membrane potential cause the opening of voltage-gated calcium channels which allows a rapid influx of Ca^2 into the terminal. For motor neurons, the increase in intracellular Ca^2 causes vesicle to fuse with the membrane of the presynaptic neuron and neurotransmitter is released into the synaptic cleft (del Castillo & Stark, 1952; Dodge & Rahamimoff, 1967). Neurotransmitter diffuses across the synaptic cleft to receptors on the post-synaptic cell.

The post-synaptic receptors determine if the chemical signal is excitatory or inhibitory. If the postsynaptic cell depolarizes (becomes more positive) then we refer to the signal being excitatory. If the response results in a hyperpolarization (becomes more negative) then we generally refer to it as inhibitory. However, second messenger cascades can result in excitatory or inhibitory responses without necessarily causing the membrane potential to change.

Agents affecting neuronal communication

There are numerous mechanisms in which the internal and external environment can result in alterations of ion channels, cells to uncouple and communication between populations of neurons to be lost (Bennett et al., 1991; Beyer, 1993; Peracchia et al., 1994). It is well known that CO_2 has an effect on electrical communication by uncoupling gap junctions (Arellano et al, 1990).

Some of the earliest known work was conducted in crayfish lateral giant (LG) interneurons since these were among the first identified neurons (Johnson, 1924) and the LG escape tail flip circuit was one of the first neural circuits to be described in detail of individually identifiable neuronal components and physiological properties (Kennedy et al., 1969; Antonsen and Edwards, 2003). The LGs consist of a bilateral pair of tight electrically coupled neurons in each abdominal ganglion that is also tightly coupled to their homologs in adjacent anterior and posterior segments by gap junction channels (Watanabe and Grundfest, 1961). This allows for a ladder neural network along the abdominal nerve cord that functions as a single neuron.

The spike produced in any LG neuron was shown to be inhibited in the presence of high levels of CO_2. CO_2 mediated uncoupling of gap junctions has been well-studied in crayfish axons (Peracchia and Peracchia, 2004; Campos et al., 1986), frog trial cells (Mazet et al., 2004) and midges salivatory glands (Zimmerman and Rose, 1985). The speed of closing of gap junctions has been shown to increase with increases in CO_2 concentration (Peracchia and Peracchia, 2004). Early studies related reversible cell uncoupling with an increase in intracellular [Ca^{2+}] (Délèze, 1965; De Mello, 1975; Loewenstein, 1966; Rose and Loewenstein, 1975) and/or [H^{+}] [(Spray et al., 1981; Turin and Warner, 1977; Turin and Warner, 1980). More recent data suggest that cytosolic acidification combined with an increase in $[Ca^{2+}]_i$ to high nanomolar concentrations is what primarily uncouples gap junctions (Lazrak and Peracchia, 1993; Peracchia, 1990). It is suggested that calcium ions regulate cell communication through direct interaction with the channel protein and that phosphorylation of channel proteins and low pH do not alter junctional conductance directly, but most likely modulate the affinity of calcium to the channel. However, reversible closure of junction channels is also shown to occur by factors such as alcohols (heptanol and octanol; Johnston et al., 1980; Spray et al., 1984) or application of calmodulin inhibitors (Peracchia, 1984, 1987) or second messengers such as cAMP (Hax et al., 1974; Flagg-Newton et al., 1981; Lasater and Dowling, 1985; Saez et al., 1986).

Central nervous system of crayfish

In crustaceans, the neuronal network controlling abdominal posture has been extensively studied to understand the neural basis of movement (Kennedy et al. 1966a, b; Evoy and Kennedy 1967; Kennedy et al. 1967; Kennedy and Davis 1977). Control of movements has been shown in both crayfish and lobster when flexion and extension movements of the abdomen are elicited through single command fibers and then stimulated (Kennedy et al. 1966a; Thompson and Page 1982). Precise control over movement and posture is important since the decapod abdomen assumes a variety of diverse postures related to defense, walking, burrowing, grooming and resting (Page 1982).

The crayfish nervous system presents several features which feasibly enable the study of a multitude of questions. Regions of interest in the CNS and well-defined musculature are readily exposed in relatively intact preparations. Flexor systems of the crayfish abdomen producing fast twitches and those producing slow, tonic contractions are physically separated for clear distinction between muscle systems (Wiersma, 1947; Takeda & Kennedy, 1964). The superficial muscles control abdominal posture by acting across each of the five intersegmental joints (Kennedy and Takeda, 1965*b*; Fields *et al.* 1967). The antagonistic superficial extensor muscles (SEMs) and superficial flexor muscles (SFMs) determine the angle of each joint. An area of particular interest involves a superficial flexor muscle circuit comprised of a ‘sensory nerve root – ganglia – motor nerve root’. The superficial muscles are histologically and functionally different from the underlying twitch muscles. The massive deep flexor twitch muscles are primarily used in the rapid response that occurs during the swimming reflex and innervated by ten large diameter axons that do not produce visible tonic muscle contractions. The superficial flexor muscle is a thin planar sheet of approximately 40 fibers of a generally slow type (Atwood, 1974) and is innervated by a bundle of six small motor axons known to produce smooth graded responses (Kennedy and Takeda, 1965). They have long sarcomere

lengths (10-12 µm) with typical graded tension changes through summation or facilitation of excitatory junctional potentials (Kennedy and Takeda, 1974).

The motor root (3rd root) that innervates the superficial flexor muscle contains five excitor motor neurons and one inhibitor motor neuron (Velez and Wyman, 1978). Although all the motor neurons are in the tonic slow range, the five excitor axons innervating the muscle differ in their properties. Since this nerve has a small number of axons, individual motor neurons can be discriminated electrophysiologically by the amplitudes of their extracellularly recorded action potentials (Kennedy and Takeda, 1965). The amplitude of an extracellular action potential is a function of the diameter of the axon in which it travels. The 3rd root has a wide range of axon diameters, so most of the axons produce action potentials of different amplitudes when recorded with an extracellular electrode. This nerve root contains only motor axons innervating the superficial flexor. Action potentials of the different neurons show different patterns of activity. These patterns can be changed by stimulating reflex circuits that activate the superficial flexors. The motor nerves to the abdominal superficial flexor muscles are spontaneously active.

The sensory nerve (2nd root) is comprised of the well-known very large primary afferent axons. The second root also contains muscle receptor organ (MRO) afferents and the "shared" efferents as well as other extensor motor neurons (Fields and Kennedy, 1965). The sensory axons are numerous and very small (1 – 10 µm). The primary mechanosensory neurons have direct connections, by electrical synapses with the LG (Krasne 1969; Zucker 1972). In addition, mechanosensory are known to excite interneurons via chemical synapse.

Glutamate is a major excitatory transmitter in the vertebrate central nervous system (Curtis and Johnston, 1974), but it is also the transmitter at excitatory neuromuscular junctions in crustaceans, such as the superficial abdominal slow flexor muscle (Kawagoe et al, 1981) and the opener muscle of the crayfish claw or walking legs (van Harreveld and Wiersma, 1937; Kennedy and Takeda, 1965). The excitatory post-synaptic potential (EPSP) of the slow

flexor muscle and its sensitivity to L-glutamate were similar to those observed in the opener muscle in the walking leg or claw of the crayfish (Kennedy and Takeda, 1965). It was found that the amount of glutamate release by stimulation at 5Hz for 15 min was 7 X 10^{-11} mole which was found to be similar to the amount of GABA released by inhibitory nerve stimulation. While this amount shows that glutamate is the neurotransmitter, exact measures are not known since the structure of the terminal nerve distribution is complicated and it is likely that only part of the released transmitter may be recovered in the fluid (Kawagoe et al, 1981).

Neuromuscular mechanisms of appendages are well known. The opener muscle is controlled by two efferent axons, one inhibitor and one excitor (van Harreveld and Wiersma, 1937). The excitatory motor neuron, which branches to the separate muscle fibers, is identifiable from preparation to preparation. The majority of synapses occur at varicosities (series of swellings) in motor nerve terminals. These motor terminals also produce graded post-synaptic potentials that are non-spiking. Low frequency stimulation of the excitatory motor neuron produces a low occurrence of vesicular fusion with the presynaptic membrane. Due to this, individual vesicular events can be monitored as well as identified effects of when compounds are applied.

The aims of this study were to examine the mode of action of CO_2 on each component within a crayfish 'sensory-ganglia-motor nerve-muscle' circuit consisting of specific, identified cells within second or third abdominal segments as well as synaptic communication at the neuromuscular junction. Experiments were designed to assess the effect of CO_2 on (1) EPSP activity and axon conductance for the opener neuromuscular junction in the walking leg; (2) activity of the sensory neurons which drive motor neurons and (3) the activity of superficial flexor motor neurons driven by the central nervous system.

METHODS

Animals

Mid-sized crayfish, *Procambarus clarkii,* measuring 6-10 cm in body length were obtained from Atchafalaya Biological Supply Co. (Raceland, LA). The animals were housed individually in an aquatic facility and fed dried fish food. All animals were in the laboratory at least 2 weeks before any experimentation. A total 48 sighted crayfish were used in the study. Both sexes of crayfish were in this study but differences between the sexes were not analyzed. All dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2.2H_2O$; 2.45 $MgCl_2.6H_2O$; 5 HEPES adjusted to pH 7.4; Sparks & Cooper, 2004).

Opener Muscle - Neuromuscular Junction Physiology

Crayfish were induced to autotomize the first or second walking leg by forcefully pinching at the merus segment. Recording of evoked responses were conducted by first placing a branch of the leg excitatory nerve (from the merus segment) of the opener muscle into a suction electrode connected to a Grass stimulator (Dudel & Kuffler, 1961). The opener muscle is divided in three general regions: distal, central and proximal. While these three general regions of the opener muscle are innervated by a single motor neuron, NMJs are known to be structurally different and have regional specific differences in synaptic efficacy (Cooper *et al*. 1995a, b). The muscle fiber phenotype type has also been shown to be different in these regions (Mykles *et al.* 2002). For these reasons, I used the most distal fibers for these studies (Figure 5.1A).

The short term facilitation (STF) was induced by providing a train of ten pulses at 40Hz, at five second intervals, to the excitatory nerve. Intracellular excitatory post-synaptic potentials (EPSPs) recordings were performed by standard procedures (Crider & Cooper, 2000; Cooper *et al.* 1995b; Dudel *et al*.

1983; Sparks & Cooper 2004). Intracellular recordings in muscles were made with 30-60MΩ resistance, 3M KCl-filled microelectrodes. The stimulator (S-88, Grass) output was passed through a stimulus isolation unit in order to alter polarity and gain (SIU5, Grass). Electrical signals were recorded on-line to a PowerLab/4s interface (ADInstruments, Australia) and calibrated with the Powerlab Scope software 3.5.4 version. All chemicals were obtained from Sigma chemical company (St. Louis, MO) and all experiments were performed at room temperature (21-22^{o}C).

Two conditions were tested and analyzed for comparison. The conditions were: (1) normal saline at pH 7.2 switched to a saline saturated with 100% CO_2, and (2) normal saline at pH 7.2 switched to a saline of pH 5.0. Recording was continuous before, during and after exposure to treatment conditions.

To test if CO_2 was directly altering sensitivity of the postsynaptic muscle to the motor nerve neurotransmitter, glutamate, the NMJs were exposed to exogenously applied glutamate (1mM) while recording EPSPs and membrane potential. The experimental paradigm for CO_2 was normal saline, then to expose the preparation to CO_2-saturated saline, followed by CO_2-saturated saline with glutamate and then finally a normal saline washout to ensure the preparation was not damaged during experimentation. The paradigm for a saline at a pH 5.0 consisted of normal saline, then to expose the preparation to saline at a pH 5.0, followed by saline at pH 5.0 with glutamate and then finally normal saline washout. Exogenous glutamate was used to determine postsynaptic sensitivity to transmitter release.

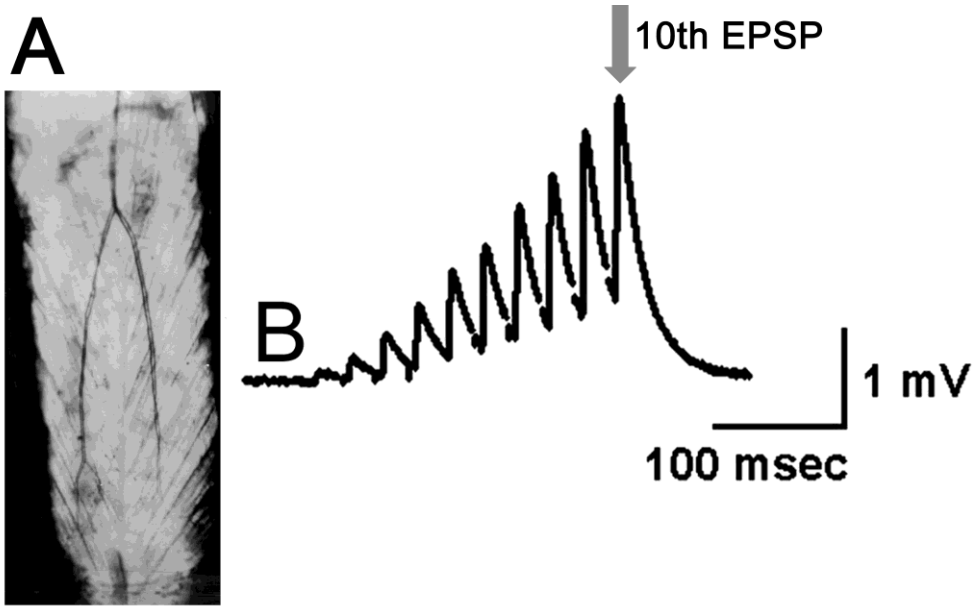

Figure 5.1. Crayfish walking leg opener muscle preparation: (A) A picture of the opener muscle (propus segment) in the crayfish walking leg (distal is directed downward). (B) The EPSP responses recorded intracellular from the distal muscle fibers and the response shows a marked facilitation that occurs throughout the stimulation train at 40 Hz. Peaks indicate each pulse stimulation in the 10 pulse train with the 10^{th} EPSP indicated by the arrow. The 10^{th} EPSP amplitude was used for analysis.

Recording Action Potentials in the Motor Axon

To measure the electrical signal propagation capability with CO_2 exposure, the motor nerve (merus segment) was stimulated by placing it into a suction electrode which was connected to a stimulator (Dudel & Kuffler, 1961). In order to examine if CO_2 had an effect on the shape of the presynaptic action potential the pre-terminal of the excitatory axon, of the walking leg opener muscle, was impaled with sharp intracellular electrode filled with 3 M KCl. The neuromuscular junctions was exposed in situ and bathed in a physiological saline. First, stimulations were given at a frequency of 1 Hz in normal saline. The amplitudes

of the action potentials produced were measured from digital records obtained at a 20 KHz acquisition rate. Initially the preparation was maintained in normal crayfish saline and stimulated. After 200 stimulations (delivered at 0.5Hz) the external saline was replaced by saline saturated with CO_2. After 200 stimulations, the CO_2 saline was removed and replaced by normal saline.

Analysis- Opener Muscle

Neuromuscular junction analysis used evoked post-synaptic potentials (EPSPs). The frequency of stimulation within the train was 40 Hz at 10 s intervals. Analysis of response used the 10^{th} EPSP of the short-term facilitations train of pulses as determined by procedures previously described (Crider & Cooper, 2000). Statistics employed the use of a Wilcoxon non-parametric test. The amplitudes of the EPSPs were measured using the Powerlab program Scope. An average of every 100 sec was used for graphical representation. Analysis of presynaptic action potential propagation used Wilcoxon nonparametric analysis

Abdominal Neural Circuit Physiology

All animals were sacrificed in less than 5 s by rapid decapitation followed by removal of the abdomen. As detailed in Strawn et al., (2000) the ventral nerve cord (VNC) was cut between T5 and A1, the abdomen was separated from the thorax and pinned ventral side up in a sylgard dish. The nerve roots of the VNC, segmental ganglia and superficial flexor muscles were exposed and bathed in physiological saline solution (schematic, Figure 5.2; picture, Figure 5.3).

Activity recordings were obtained from the 2^{nd} or 3^{rd} abdominal segment. Extracellular recordings of nerve activity in the 2^{nd} sensory nerve root and 3^{rd} motor nerve root were obtained by suction electrodes. For intracellular recordings used during cadmium exposure, a microelectrode (2 M potassium acetate, 20-30 MΩ) was inserted into a medial muscle fiber of the superficial

flexor. All data were recorded by a computer via PowerLab/4s A/D converter (ADInstruments).

For stimulation of the cuticle, a stiff-bristled paintbrush was mounted on a micromanipulator to control pressure and movement. The brush was positioned along the lateral side of the recording segment and moved an approximate distance of 2.54 cm in a forward and then backward motion.

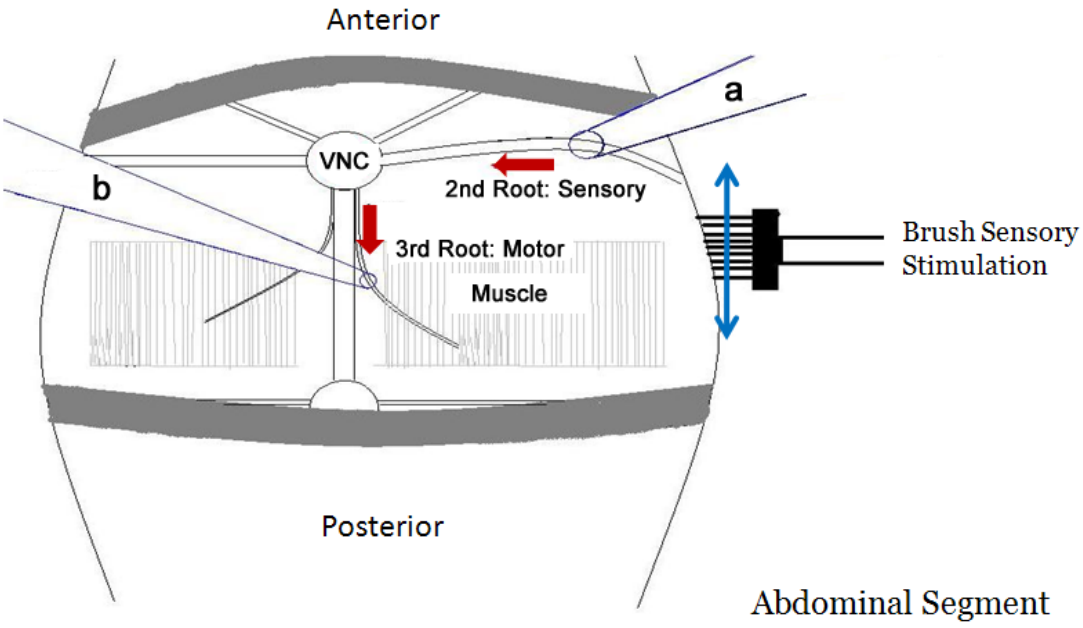

Figure 5.2. Schematic of ventral view in a dissected abdominal segment of the crayfish abdomen. Stimulation of cuticle occurred along the lateral side of the recording segment. Suction electrodes are labeled ‘a’ and ‘b’ above.

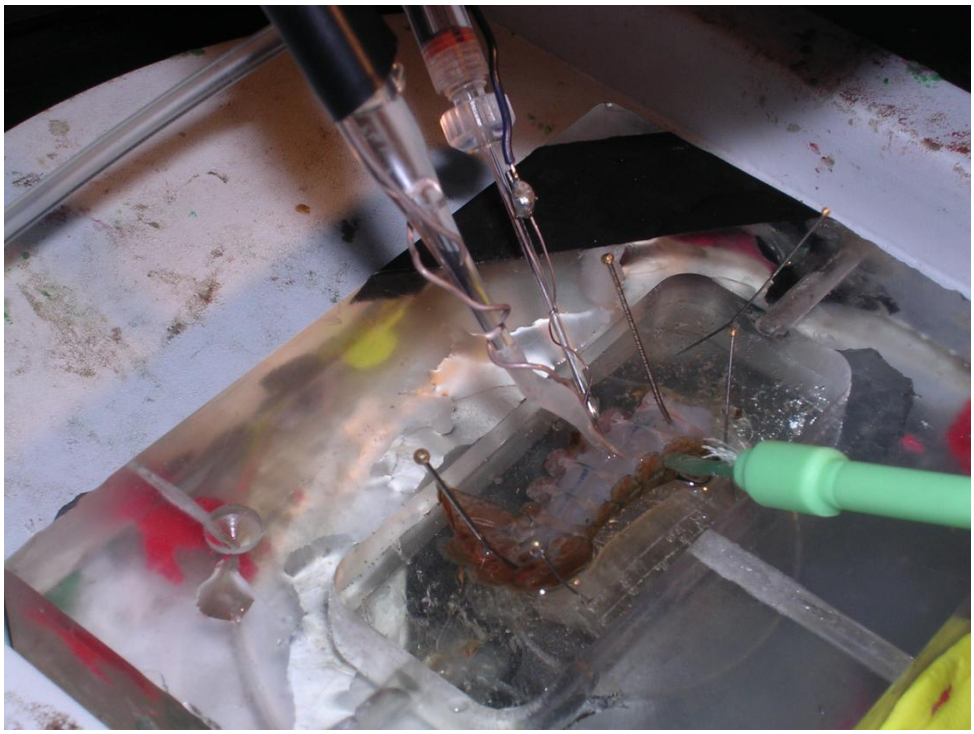

Figure 5.3. Ventral view in a dissected abdominal segment of the crayfish abdomen. Stimulation of cuticle occurred along the lateral side of the recording segment.

Analysis – Abdominal Superficial Flexor Circuit

Neural circuit analysis used direct counts of the evoked spike frequency recordings. Frequency counts were done by directly counting. A 30 msec time period prior to bush stimulation and a 30 msec time period during the stimulation was used for analysis of spike frequency. This was repeated 5 times and the average percent change in frequency was the index used for the effect of stimulating the circuit. The average activity prior to stimulation and the average activity during the stimulation were also used to assess the effect of exogenously applied compounds on the activity without and with stimulation in addition to the percent change in activity due to stimulation. Direct counts were then used to get

an average value for each condition on every individual trial. These individual values were then used to obtain spike frequency percent changes.

Chemicals

All chemicals were obtained from Sigma chemical company (St. Louis, MO).

Nicotine

Acetylcholine (Ach) is a major excitatory neurotransmitter in the crustacean and insect nervous system, in which many of its actions involve nicotinic receptors (Tsunoyama and Gojobori, 1998). To test for nicotinic cholinergic receptors influencing the sensory or motor roots, a 10µM nicotine containing saline was bathed on the preparation.

Glutamate

Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system and known to be a major excitatory neurotransmitter in the peripheral nervous system of most invertebrates (Monoghan et al, 1989; Watkins, et al., 1990). To induce spike activity in the sensory root, glutamatergic interneurons and/or the motor nerve root a 1mM glutamate containing saline was used.

Domoic Acid

A kainate receptor agonist, domoic acid, was used to examine if a quisqualate-type glutamate receptor may have a role in the sensory-CNS-motor neuron circuit. Since it was shown at the NMJ in *Drosophila melanogaster* that quisqualate-type glutamate receptors at the NMJ are blocked by CO2 as well as domoic acid (Badre et al., 2005; Lee et al., 2009) it was of interest to know if domoic acid would have an effect in crayfish. Since it was shown that 1mM is sufficient to inhibit quisqualate-type receptors at the NMJ in *Drosophila melanogaster* the same concentration was used in this study.

Cadmium

Cadmium is known to block chemical transmission, as seen in the crayfish *Pacifastacus leniusculus* (Heitler et al., 1991). In order to understand the role of chemical transmission in the CNS circuit of interest, a saline with cadmium (1mM) was used.

Heptanol

The uncoupling effects of heptanol was first described in crayfish septate axons by Johnston et al. (1980). This effect was soon confirmed in all vertebrate and invertebrate systems tested with electrical communication (Bernardini et al., 1984; Meda et al., 1986). To understand the role of electrical communication in this circuit, a saline with (1mM) 1-heptanol was used to uncouple gap junctions.

RESULTS

Opener Muscle - NMJ

To address mechanisms as to why crayfish show a rapid behavioral unresponsiveness with high CO_2 exposure, the leg opener muscle was examined for changes in synaptic responses at the NMJ. Since CO_2 has been shown to reduce sensitivity to glutamate at the skeletal NMJs in *D. melanogaster* and that the glutamatergic receptors at the NMJ in crayfish are of a quisqualate subtype which demonstrate a quick recovery from desensitization (Dudel et al., 1993) just as in *D. melanogaster* I expected similar effects. The induced depolarization of this muscle is graded and non-spiking (Katz & Kuffler 1946; Katz, 1949; Wiersma, 1949). To determine, by physiological means, if changes in synaptic responses in this preparation are occurring as a result of fewer vesicles being released or alterations of the function of postsynaptic receptors, exogenous glutamate was applied to the preparation through the saline bath. Another possibility tested was whether or not the presynaptic motor nerve remains excitable during CO_2 exposure. Since dissolving CO_2 in saline causes the

external pH (pH_o) to drop, the possibility that low pH_o is mediating any of the identified effects needed to be addressed independently of CO_2 exposure.

The EPSP response amplitude was measured over time during exposure to normal saline, saline saturated with CO_2, saline saturated with CO_2 and glutamate and followed by normal saline to examine recovery of the preparation. In the normal physiological saline, the EPSP responses obtained by intracellular recording show a marked facilitation at 40Hz stimulation within the 10 pulse stimulus train without a change in resting membrane potential (Figure 5.4A, Figure 5.5). Facilitation is consistently shown to occur in all preparations although there are differences in the degree of facilitation. In this study, the terminals from distal muscle fibers were used as there are differences in facilitation depending on the region of the opener muscle that is being monitored (Cooper *et al.* 1995a, Mykles *et al.* 2002). It has been established that the majority of the facilitation during the STF is due to presynaptic components, primarily by enhanced calcium build up (Katz & Miledi, 1968; Rahamimoff, 1968; Zucker & Lara-Estrella, 1983; Winslow *et al.* 1994). This build up of residual intracellular [Ca2+] enhances vesicular fusion (Sparks & Cooper, 2004).

When the preparation was exposed to CO_2 saturated saline, EPSPs rapidly became smaller and faded out all together. In 5 out of 5 individual preparations, there was a consistent attenuation of the EPSP within 1 minute resulting in a significant effect with CO_2 exposure (5 out of 5 animals; $P<0.05$, non-parametric analysis sign test for paired-sampled data). In addition, the resting membrane potential did not change with CO_2 exposure. A typical response for one preparation is shown in Figure 5.4B. Furthermore, the membrane potential did not depolarize upon exposure to exogenous glutamate (Figure 5.5). With the removal of CO_2 saturated saline the EPSPs returned (Figure 5.4C).

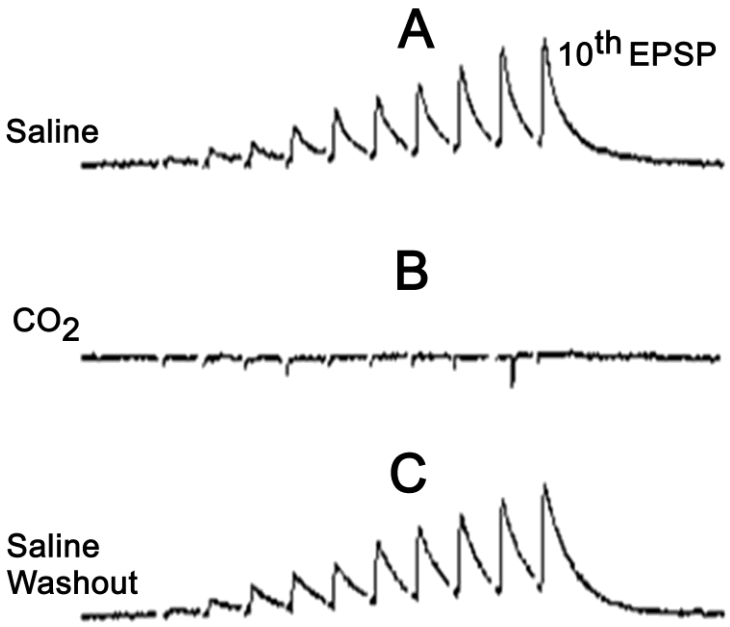

Figure 5.4. Intracellular recordings of junction potentials. (A) EPSPs in normal physiological saline before CO_2 exposure, (B) EPSPs during CO_2 exposure, (C) EPSPs after returning to physiological saline without CO_2. Junction potentials show significant reductions with CO_2 exposure (5 out of 5 preparations, $P<0.05$, Wilcoxon non-parametric analysis).

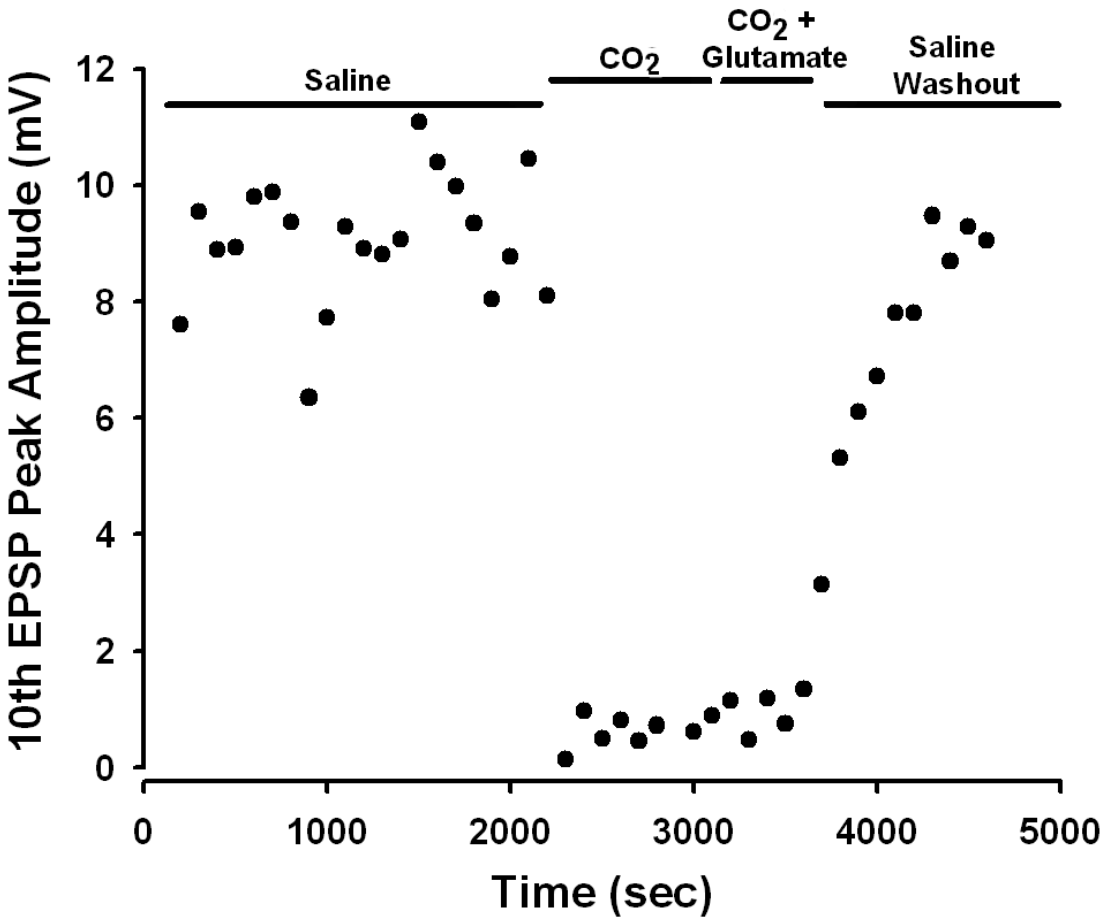

Figure 5.5. The effect of CO_2 on EPSP amplitude in opener muscle. Exposure to CO2 resulted in a complete attenuation of the EPSP in five out of five preparations within 1 min without any change in resting membrane potential ($p < 0.05$, non-parametric analysis). Scatter dots indicate 100 second averages of EPSP amplitudes. The black lines at the top indicate treatment conditions.

The effect of the acidic environment was also tested by using saline adjusted to pH 5.0. In each preparation, EPSP amplitudes remained unchanged with exposure of the acidic saline (Figure 5.6; 5 out of 5 animals; $p < 0.05$, Wilcoxon non-parametric analysis). Interestingly, the resting membrane potential of the muscle fiber was shown to become more negative (hyperpolarization) with exposure. Application of exogenous glutamate caused the resting membrane potential to quickly depolarize to approximately 0 mV within 1 min and quickly

returned to resting membrane potential, indicating a quick depolarization and then receptor desensitization. EPSPs amplitude attenuates during exposure and then quickly attenuates to the baseline, indicating depolarization of the muscle with application of glutamate (Figure 5.7; 5 out of 5 animals; $p < 0.05$, Wilcoxon non-parametric analysis).

Glutamate application showed no effect on the CO_2 saturated NMJ; whereas, NMJs without CO_2 exposure did respond to glutamate as did the for exposure to pH 5.0 saline. The reduction in the resting membrane potential (RMP) was used as an assay for sensitivity to exogenous glutamate. Due to glutamatergic insensitivity, the site of action was identified as postsynaptic. Furthermore, the reduced EPSP amplitude and attenuation could not be accounted for by the low pH but rather a direct effect of CO_2 exposure. A summary of EPSP and resting membrane responses are summarized in Table 5.1. These results clearly indicate that CO_2 blocks the responsiveness of the muscle to exogenously applied glutamate. This is also likely to explain the reduced EPSP amplitude with endogenously evoked glutamate release as well as unresponsiveness to exogenously applied glutamate.

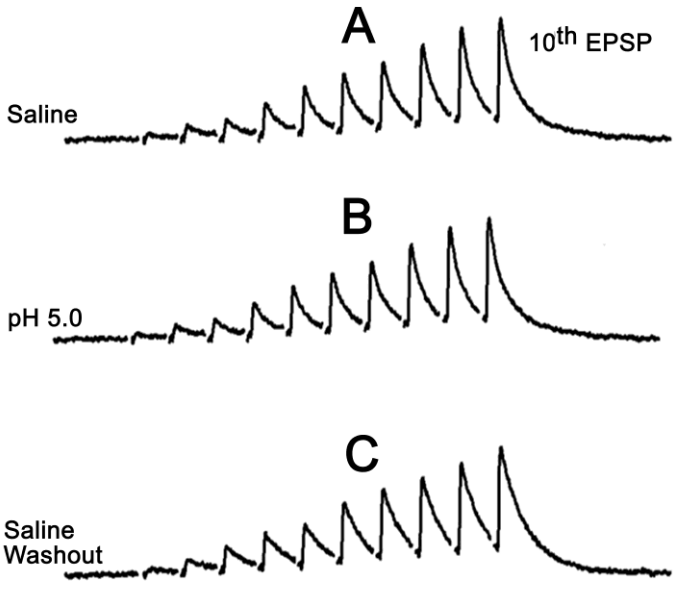

Figure 5.6. Intracellular recordings of junction potentials. (A) EPSPs in normal physiological saline before pH 5.0 saline exposure, (B) EPSPs during low pH exposure, (C) EPSPs in physiological saline after washing out the acidic saline. Recordings of junction potentials does not show significant change in EPSP attenuation with pH 5.0 exposure in 5 out of 5 preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

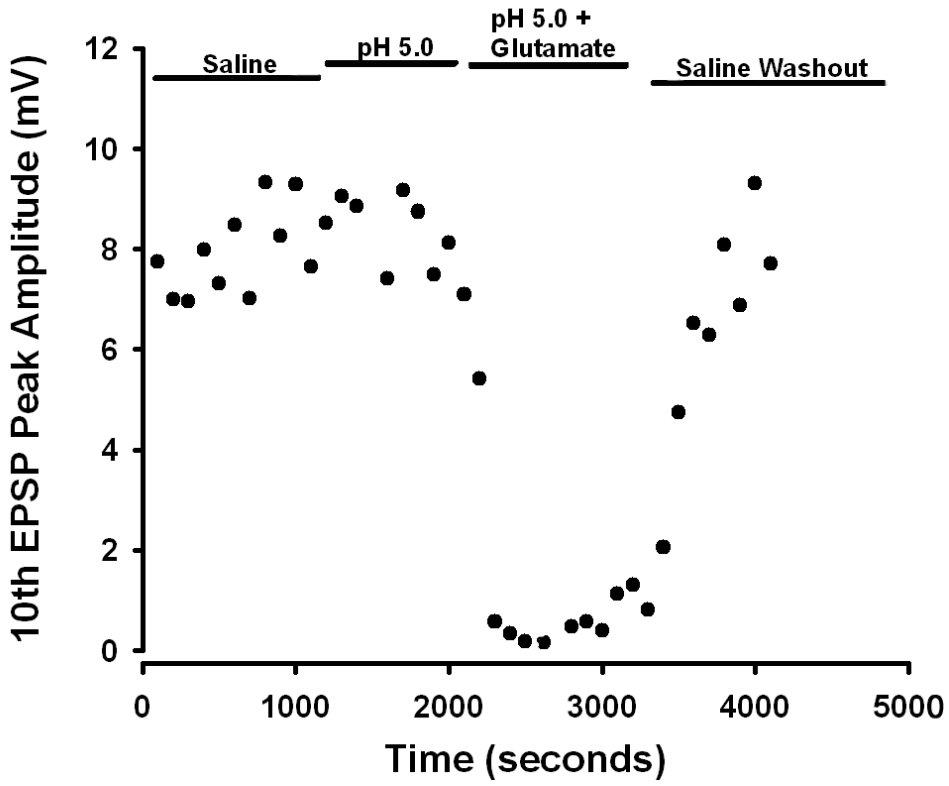

Figure 5.7. The effect of low pH 5.0 on EPSP amplitude in opener muscle. Exposure to pH 5.0 did not result in a complete attenuation of the EPSP in 5 out of 5 preparations as seen with CO_2 exposure. The resting membrane potential depolarized in acidic saline when exposed to glutamate ($p < 0.05$, non-parametric analysis). Scatter dots indicate 100 second averages of EPSP amplitudes. The black lines at the top indicate treatment conditions.

Table 5.1. Responses at the NMJ during exposure of CO_2 or pH 5. The response is characteristic for both CO2 and pH 5.0 exposure (5 out of 5 animals, $p < 0.05$, non-parametric analysis). The main point is the CO_2 blocks the responsiveness of the muscle to exogenously applied glutamate.

	RMP (Saline)	Exposure	(+) Glutamate	Saline Wash
CO_2	~ (-75 mV)	No EPSPs ~(-75mV)	No Depolarization	EPSPs slow to return
pH 5.0	~ (-75 mV)	EPSPs ~(-85mV)	Quick Depolarization & Desensitization	EPSPs

Recording Action Potentials in the Motor Axon

In order to study the influence of CO_2 on the size and shape of the action potential in the presynaptic (pre-terminal) of the excitatory axon, intracellular recordings were carried out on the crayfish tonic motor neuron which innervates the opener muscle in the first walking leg (Figure 5.8A). The electrode impaled axon showed that the action potential was typically in the range of 60 to 80 mV in amplitude with approximately -65 mV for a resting membrane potential for normal physiological saline. For all preparations, normal saline was replaced with CO_2 saturated saline. No change in action potential amplitude or shape is noted for CO_2 exposure (3 out of 3 preparations, Figure 5.8B).

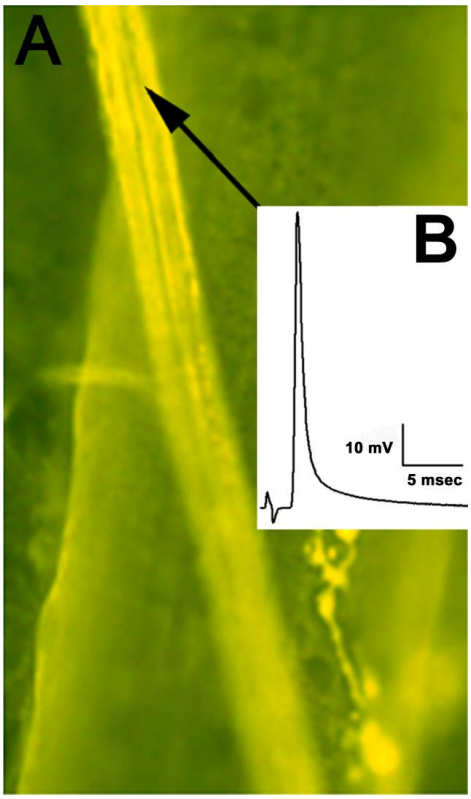

Figure 5.8. Intracellular recording of an action potential in excitatory crayfish opener motor neuron. (A) Presynaptic (pre-terminal) motor axon. (B) Schematic of amplitude and shape of action potential with CO_2 exposure. There was no noticeable change in either amplitude or shape of the action potential from normal saline to CO_2 saturated saline (3 out of 3 preps).

The effect of carbon dioxide and low pH 5.0 on the spike activity in a superficial flexor motor circuit

To record from a 'sensory root – ganglia - motor root' neural circuit, the ventral side of the abdomen was exposed. The sensory nerve root coming from the cuticle and going into the VNC was left intact as means of stimulating the circuit. The motor nerve root from the VNC to the superficial flexor muscle was typically cut. En passant recordings of the intrinsic activity as well as evoked

(through cuticular stimulation) activity were recorded from the sensory and motor roots.

Stimulation of cuticular sensory neurons increased the firing frequency in both the sensory root and motor root which is indicated by increased firing activity when compared to basal activity (boxes, Figure 5.9) The heightened effects of cuticular stimulation was observed in all preparations examined to ensure that the 'sensory root – ganglia - motor root' circuit was activated. The effect on firing frequency is shown in the representative single traces in the presence of saline, CO_2 and low pH 5.0. In each of the five preparations, application of CO_2-satuared saline to the abdominal preparation resulted in a continuous firing in the sensory root but showed a rapid decrease to the point of cessation of spike activity in the motor root (Figure 5.9A). The rate of change in the sequence of motor nerve root spikes was determined by counting the number of events for 30 msec before and during periods of cuticular stimulation. For low pH 5.0, the firing frequency persists in both the sensory and motor roots (Figure 5.9B).

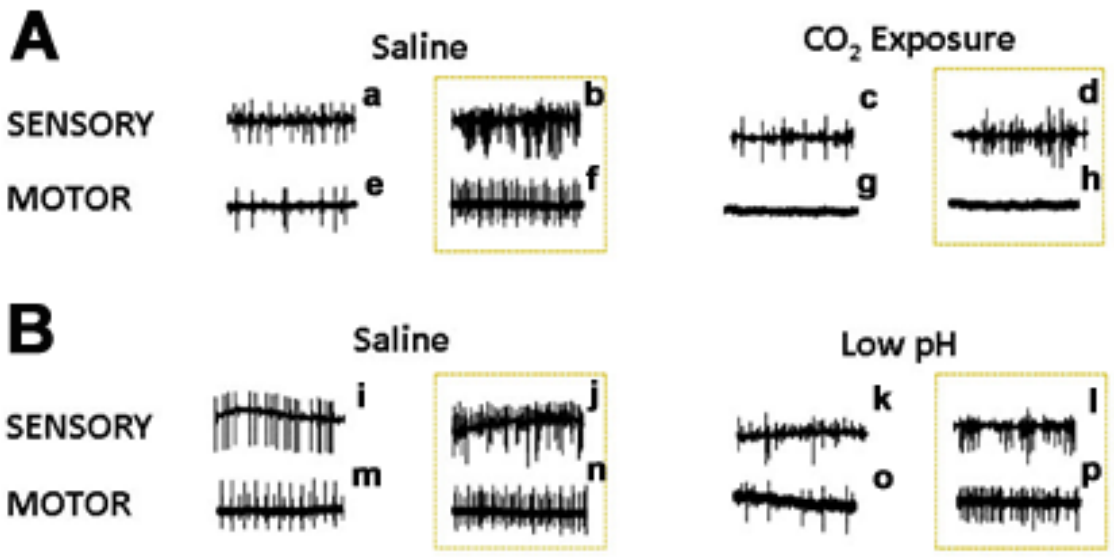

Figure 5.9. Effects of carbon dioxide on spike firing frequency in a 'sensory root – ganglia - motor root' neural circuit. (A) Spike frequency of the sensory and motor nerve roots during CO2 exposure. (B) Spike frequency of the sensory and

motor nerve roots during low pH 5.0 exposure. Boxes indicate cuticular stimulation and letters represent statistical analysis comparisons.

With the stimulation, the associated increases in activity were quantified by taking the response of 30 msec of basal rates (N = 5) and the response during cuticle stimulation (n=5) in 5 preparations to determine quantifiable measures on spike frequency with exposure to CO_2 and low pH 5.0 (Table 5.2). These enhancements of the stimulated-associated drive of the superficial flexor neural circuit were observed in five out of five preparations (Wilcoxon non-parametric test, $p < 0.05$). The mean percentage values reported are an average of 5 subsequent trials in saline and with exposure. The saline bath was exchanged with one containing saline saturated with CO_2. In the sensory root, the average change with cuticle stimulation (300%) was shown to be significantly decreased (-25%, ANOVA; * $p < 0.001$, Holm-Sidak post hoc analysis) upon exposure to CO_2 (Table 5.2A, Figure 5.11A). For the motor root, the average change with cuticle stimulation (50%) was also shown to be significantly decreased (-100%, ANOVA; * $p < 0.001$, Holm-Sidak post hoc analysis) upon exposure (Table 5.2A, Figure 5.11B). These effects on both sensory (and motor with CO_2 exposure were shown to be significant from saline control frequency measures (Figure. 5.10).

Exposure to low pH 5.0 saline did not show the effects seen with CO_2 (Figure 5.10). Specifically, cuticle stimulation during pH 5.0 significantly increased activity in the sensory root (75%, ANOVA; * $p < 0.001$, Holm-Sidak post hoc analysis) and most importantly the motor root increased (50%, ANOVA; * $p < 0.001$, Holm-Sidak post hoc analysis) upon exposure (Table 5.2B, Figure 5.11). Thus, effects on the motor root seen with CO_2 exposure are unlikely to be the result of low pH.

Table 5.2. Activity of the 2nd and 3rd roots before and during cuticular stimulation in saline and with CO2 exposure. The time during cuticle stimulation is indicated by the box. Comprehensive statistical analysis of cross comparisons with (A) carbon dioxide and (B) low pH 5.0 exposure frequency counts.

A

			CO_2 Exposure	
Sensory	0%	300%	%0	130% (c-d)
			-50% (a-c)	-25 % (b-d)
Motor	0%	50%	0%	0% (g-h)
			-100% (e-g)	-100% (f-h)

B

			Low pH 5.0	
Sensory	0%	210%	%0	250% (k-l)
			60% (i-k)	75 % (j-l)
Motor	0%	155%	0%	50% (o-p)
			40% (m-o)	90% (n-p)

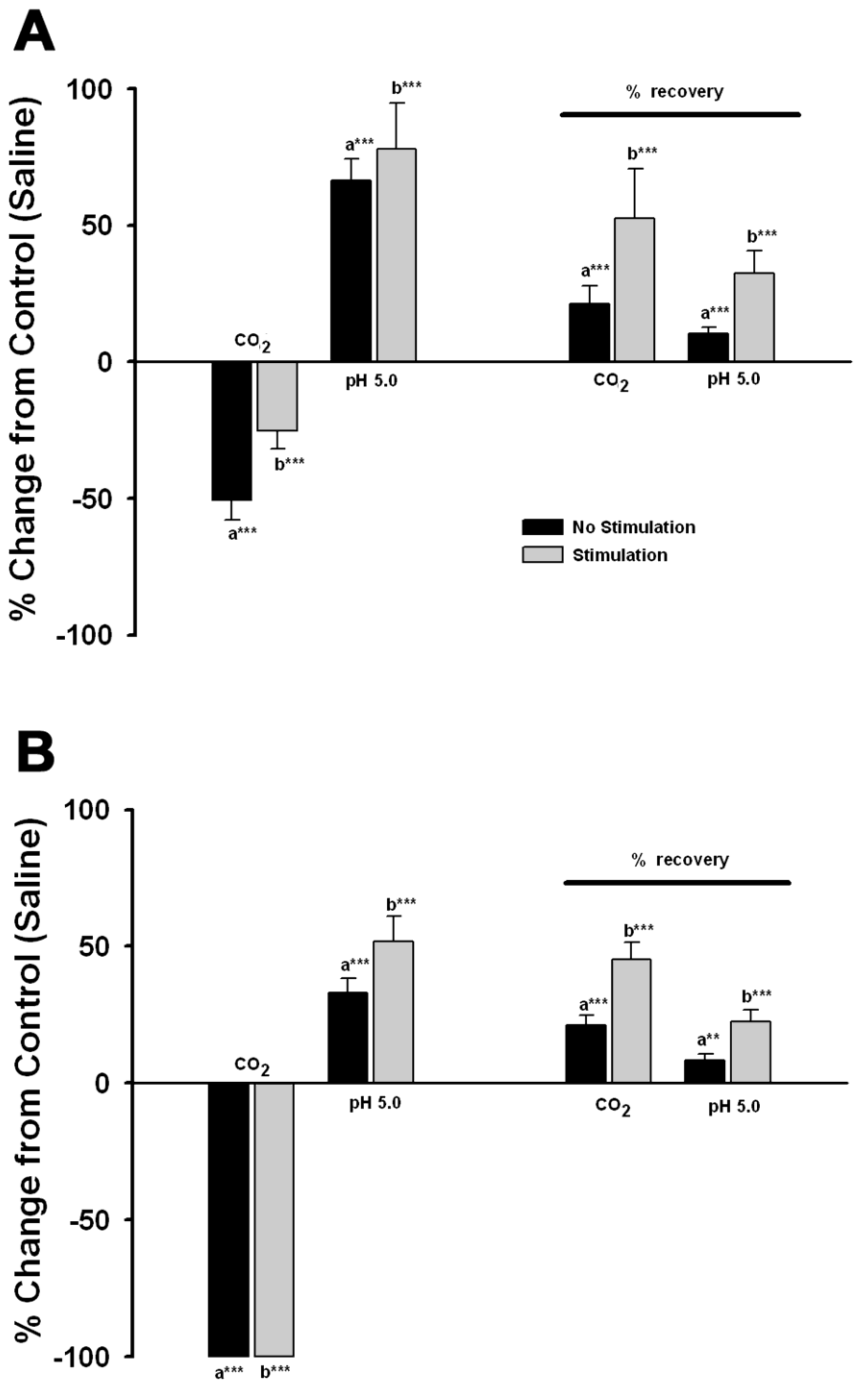

Figure 5.10. Mean activity in the spikes of the motor and sensory roots before and during cuticular stimulation in CO_2 and pH 5.0. Percent change with cuticular stimulation for (A) sensory nerve root and (B) the 3rd motor nerve root. It is important to note that there are very small deviations in the mean values (±

SEM) within the treatment groups but no significant differences among preparations. Before (a, black bars) and during cuticular stimulation (b, grey bars) were compared across conditions. The mean rate (± SEM) of spike frequency over multiple 30 msec time periods were assessed for the conditions. There is a significant decrease in spike frequency for CO_2 as compared to pH 5.0 (ANOVA; **$p < 0.02$ and *** $p < 0.001$). There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

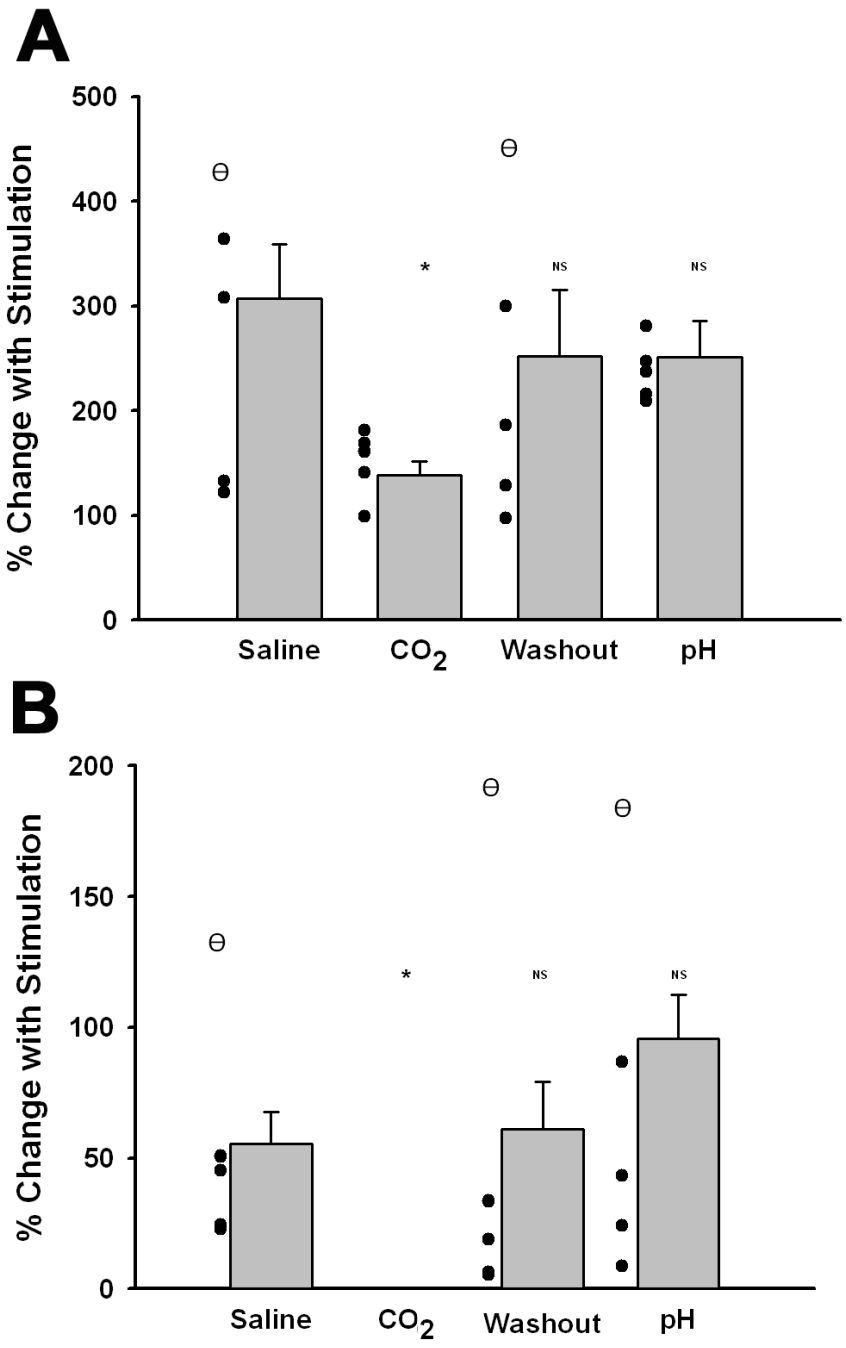

Figure 5.11. Mean spike activity for cuticular stimulation in CO_2 and pH 5.0 conditions. Percent change with cuticular stimulation for (A) sensory nerve root and (B) motor nerve root. Scatter dots represent variations in individual preparations and average mean (± SEM) frequency is represented by the gray bars for each condition. It is important to note that a single individual was responsible for most of the variation among the treatments and this individual is

shown by the symbol (Θ). There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis). There is a significant decrease in spike frequency only for CO_2 as compared to the control saline (ANOVA; * $p < 0.05$).

Pharmacological characterization of a 'sensory root – ganglia - motor root' circuit using selective agonists and antagonists, reveals the complexity of the superficial flexor motor circuit

A range of selective agonists and antagonists was used to attempt a pharmacological classification of the interneuron receptors causing activation of superficial flexor abdominal muscles. Ligand-gated ion channels normally mediate rapid chemical synaptic transmission.

Application of nicotine to the preparation showed significant increased spike frequency on both sensory and the motor roots (ANOVA; *** $p < 0.001$, Holm-Sidak post hoc analysis, Figure 5.12A, B). There was activation of the neural circuit during application for both roots indicated by further spike activity during cuticle stimulation (Figure 5.13A, B). Nicotine in combination with CO_2 caused significant inhibition of the activity of the motor root (ANOVA; *** $p < 0.001$, Holm-Sidak post hoc analysis) but did not significantly inhibit activity in the sensory nerve root (Figure 5.12). Results suggest some cholinergic drive through nicotinic receptors in the central nervous system. Exact influence or role is still undetermined.

Glutamate application showed significant increased spike frequency on both sensory and the motor root (ANOVA; * $p < 0.001$, Holm-Sidak post hoc analysis, Figure 5.14A, B). There was activation of the neural circuit during application for both roots indicated by further spike activity during cuticle stimulation (Figure. 5.15A, B). As shown in Figure 5.14, glutamate in combination with CO_2 caused significant inhibition of the activity of the motor root (ANOVA; *** $p < 0.001$, Holm-Sidak post hoc analysis) as well as activity in the sensory nerve root (ANOVA; * p

< 0.05, Holm-Sidak post hoc analysis). There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis). Results suggest some glutamatergic drive through glutamate receptors in the central nervous system. It is plausible that many of the interneuronal synaptic connections are glutamatergic and that this would explain the increase with glutamate application. Exact influence or role on the drive of the motor root is still undetermined.

Domoic acid showed no effect on spike activity from saline controls for either sensory or the motor root (Figure 5.16A, B). As shown in Figure 5.17, there was activation of the neural circuit during application for both roots indicated by further spike activity during cuticle stimulation. Glutamate application after domoic acid application and washout showed a significant increase in spike activity on the motor root (ANOVA; *** $p < 0.001$, Holm-Sidak post hoc analysis) indicating domoic acid acted as an antagonist and not an agonist. There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis). Results suggest that domoic acid is not able to perfuse readily into the tissue and thus the action is diminished or that the quisqualate type receptors at the NMJ are of a different subtype not sensitive to domoic acid. It is also likely that receptors blocked by domoic acid are easily compensated for by other interneurons or circuits. It is more likely that domoic acid is working on the receptor subtypes found at the NMJ but that some of the circuit is not chemically driven. This would explain the continued activity with application of domoic acid. The components of the neural circuit are shown to be very complex.

Cadmium is known to block chemical synapses in the crayfish system and was used to understand chemical versus electrical communication in this superficial flexor circuit. Cadmium showed no effect on spike activity from saline controls for the sensory root for all time points (Figure 5.18A). There is a significant decrease in motor root activity after 10 minutes in time (ANOVA; ** $p < 0.02$, Holm-Sidak post hoc analysis, Figure 5.18B). As shown in Figure 5.19, there was activation of the neural circuit during exposure for both roots indicated

by further spike activity during cuticle stimulation. In each preparation with exposure to cadmium, the resting membrane potential of the muscle fiber remained relatively unchanged (~ 45 mV); although in 2 out of 5 preps the RMP fluctuated to a more negative value. The effect of cadmium on firing frequency of the motor root was recorded with an intercellular electrode within a medial muscle fiber to monitor excitatory postsynaptic potentials (EPSPs). Cadmium exposure effect showed that the EPSP gradually became smaller and faded out all together and after 15 minutes the amplitude is not high enough to accurately distinguish peaks (Figure 5.20A, B, C). Representative traces of EPSP activity in saline, 1 mM cadmium after 5 minutes and 1 mM cadmium after 15 minutes showed that evoked peaks gradually become smaller. This is suggesting a presynaptic response due to cadmium block presynaptic Ca^{2+} entry and thus decreased vesicle fusion. The EPSP peak amplitude was measured over time during exposure to normal saline, followed by saline with 1mM cadmium after 15 minutes (Figure 5.20D). Exposure to cadmium resulted in a complete attenuation of the EPSP in five out of five preparations within 15 minutes without any change in resting membrane potential. ($P<0.05$, non-parametric analysis). Average EPSP amplitude showed a significant difference between saline control and 15 minutes of cadmium exposure ($t = -7.090$, $df = 34$, $p = < 0.001$).

To understand if there is a postsynaptic response to 1 mM cadmium, the postsynaptic receptor sensitivity to spontaneous quantal responses and the frequency of spontaneous events were assessed. Spontaneous events are random vesicular fusion (not due to evoked stimulation) with the presynaptic membrane that can be recorded in the postsynaptic cell as mEPSPs. It was readily apparent that cadmium at 1mM reduced the amplitude of spontaneous mEPSP quantal events over time as indicated gradual reduction in amplitude from 1 minute to that of after 15 minutes (Figure 5.21). After 20 minutes, further peaks were not discernable from noise in the baseline and thus are not detected to monitor their frequency. Thus, since mEPSPs decreased in amplitude slowly this provided evidence for a postsynaptic effect as well.

Heptanol is widely known to decrease membrane ionic currents in electrically coupled cells reversibly (Burt and Spray, 1988, Rudisuli and Weingart, 1989). Percent changes with application of heptanol on the sensory root did not show any significant changes from saline before or during cuticular stimulation (Figure 5.22A). While the sensory root did not show any significant differences from saline control with cuticle stimulation, there was an increase similar to saline indicating continued activity in the sensory root after 1 and 10 minutes of heptanol exposure (Figure 5.23A). Interestingly, the motor root showed a significant decrease (-35%) in spike frequency with stimulation after 10 minutes of heptanol exposure (ANOVA; *** $p < 0.001$, Holm-Sidak post hoc analysis Figure 5.22B). Stimulation on the cuticle and monitoring on the motor root showed there was a significant decrease in activity indicating the inhibition of the neural circuit (ANOVA; *** $p < 0.001$, Holm-Sidak post hoc analysis; Figure 5.23B). Results indicate no statistical difference from basal rates with stimulation after 10 minutes in heptanol. There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

As a composite, results are presented in Figure 5.24 and in Table 5.3 to summarize the significance of each component discussed and presented.

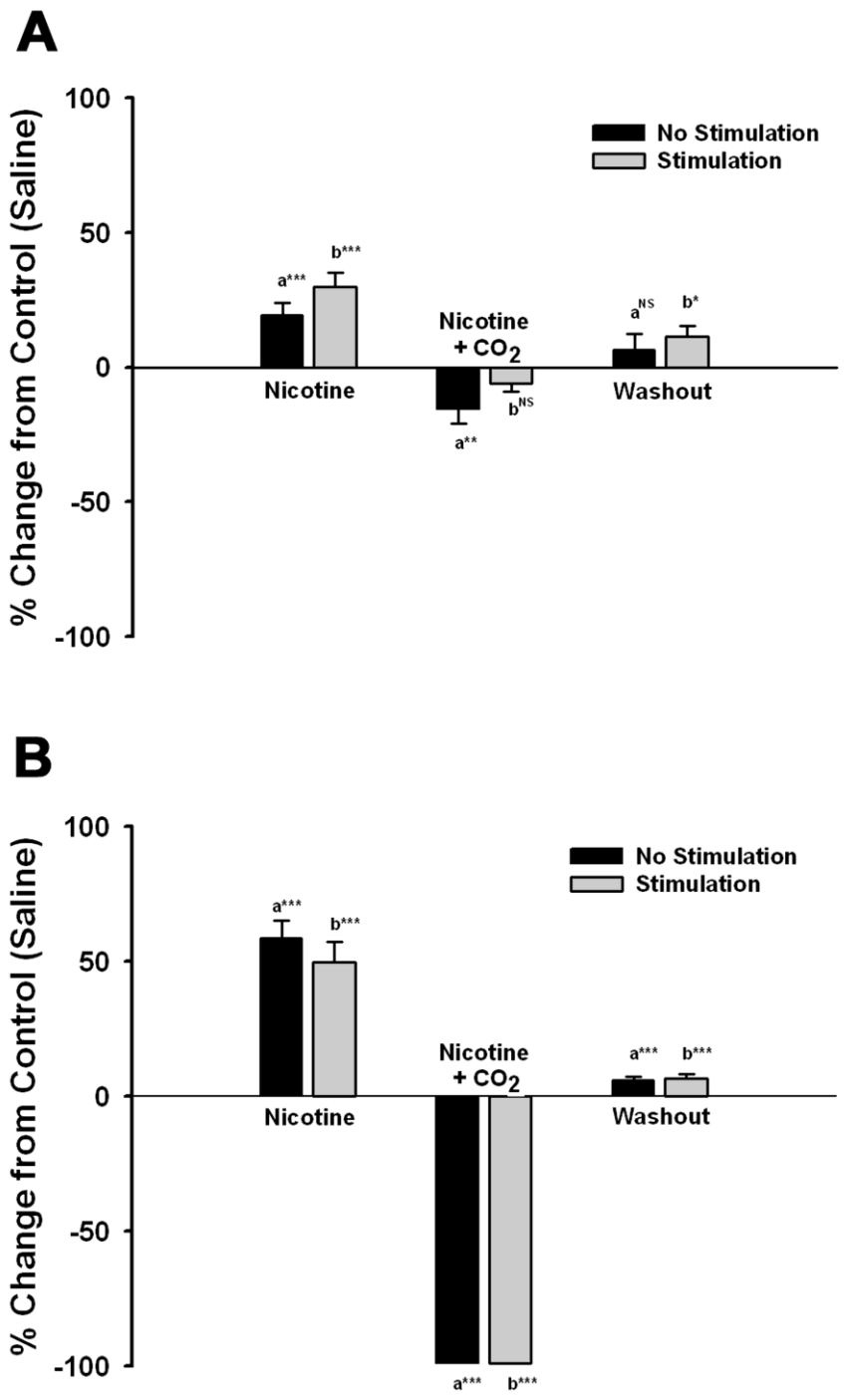

Figure 5.12. Mean spike activity before and during cuticular stimulation with nicotine and nicotine plus CO_2 exposure. Percent change in frequency with cuticular stimulation for (A) sensory nerve root and (B) motor nerve root. Before

(a, black bars) and during cuticular stimulation (b, grey bars) were compared across conditions. The mean rate (± SEM) of spike frequency over multiple 30 msec time periods were assessed for the conditions. There is a significant increase in spike frequency for nicotine and a significant decrease for nicotine plus CO2 compared to the saline control (ANOVA; * $p < 0.05$, **$p < 0.02$ and *** $p < 0.001$). There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

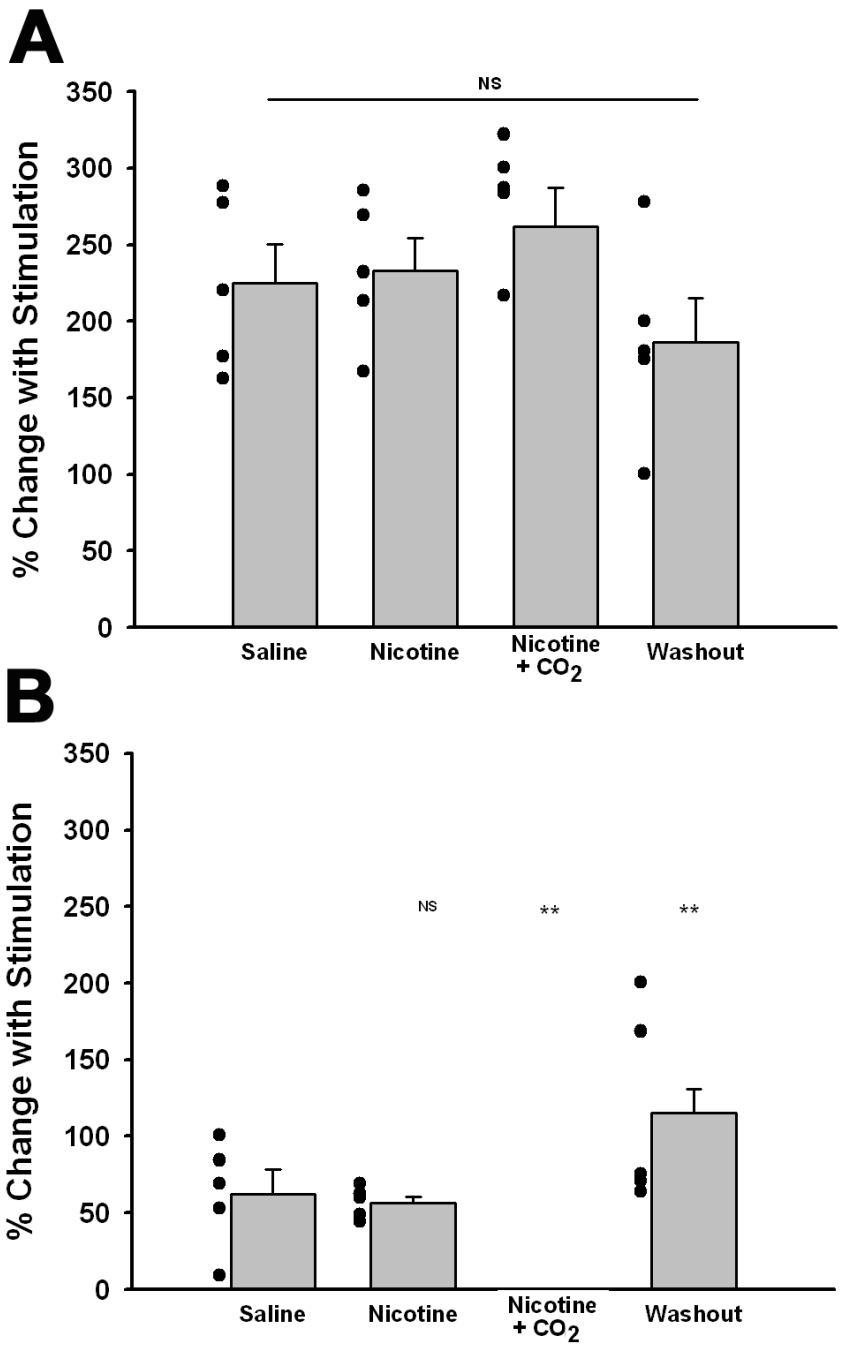

Figure 5.13. Mean spike activity of cuticular stimulation with nicotine and nicotine plus CO_2 exposure conditions. Percent change with cuticular stimulation for (A) sensory nerve root and (B) motor nerve root. Scatter dots represent variations in individual preparations and average mean (± SEM) frequency is represented by the gray bars for each condition. There was a consistent

significant effect in five out of five preparations (P< 0.05, Wilcoxon non-parametric analysis). There is a significant decrease in spike frequency for nicotine + CO_2 as compared to the control as well a significant increase in spike frequency for washout with normal saline possibly due to residual levels of nicotine in the tissue but with CO2 being rapidly cleared (ANOVA; * p < 0.05, **p < 0.02 and NS is not significant).

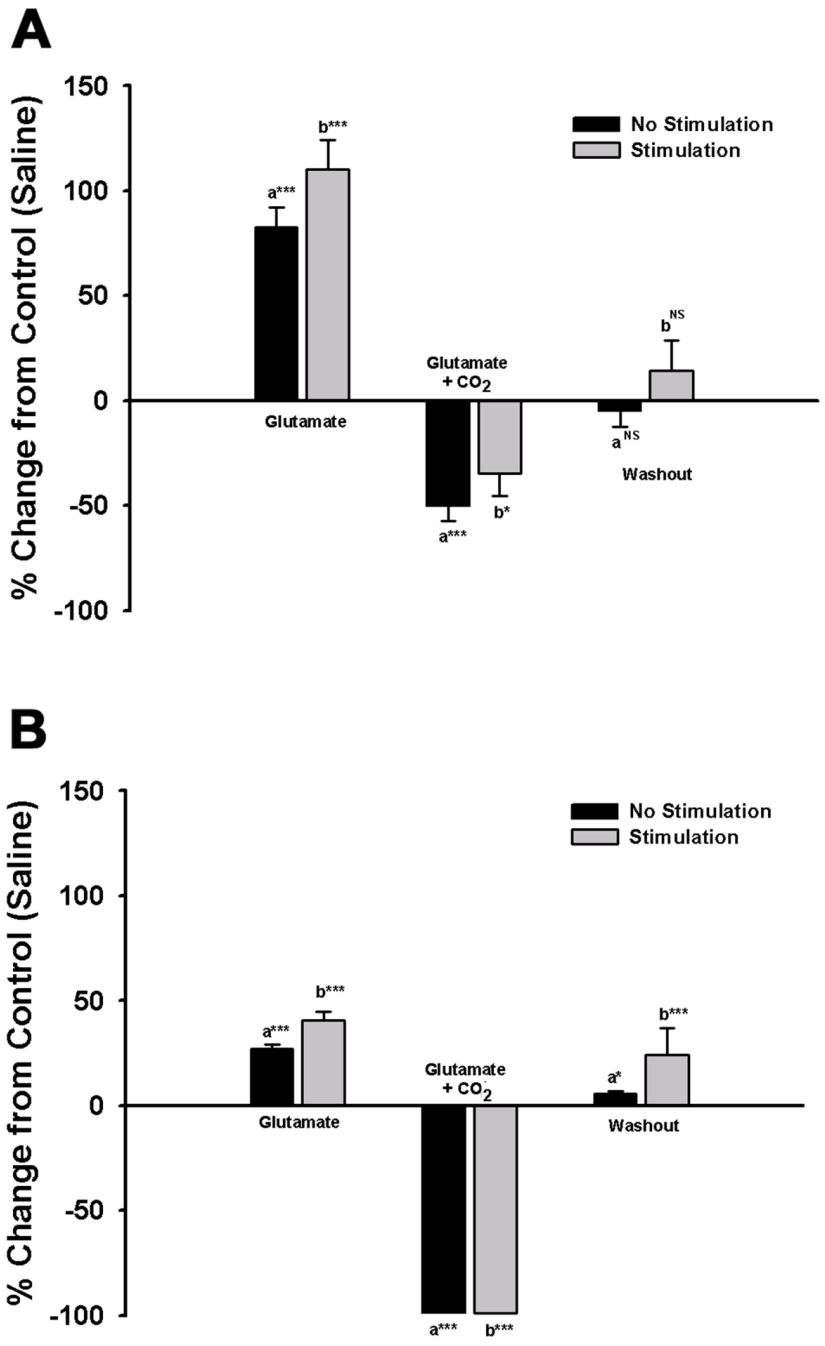

Figure 5.14. Mean spike activity before and during cuticular stimulation with glutamate and glutamate plus CO_2 exposure. Percent change with cuticular stimulation for (A) sensory nerve root and (B) motor nerve root. Before (a, black bars) and during cuticular stimulation (b, grey bars) were compared across conditions. The mean rate (± SEM) of spike frequency over multiple 30 msec

time periods were assessed for the conditions. There is a significant increase in spike frequency for both sensory and motor roots with glutamate as compared a significant decrease for both in spike frequency for glutamate plus CO_2 as compared to saline control (ANOVA; * $p < 0.05$, ** $p < 0.02$ and *** $p < 0.001$). There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

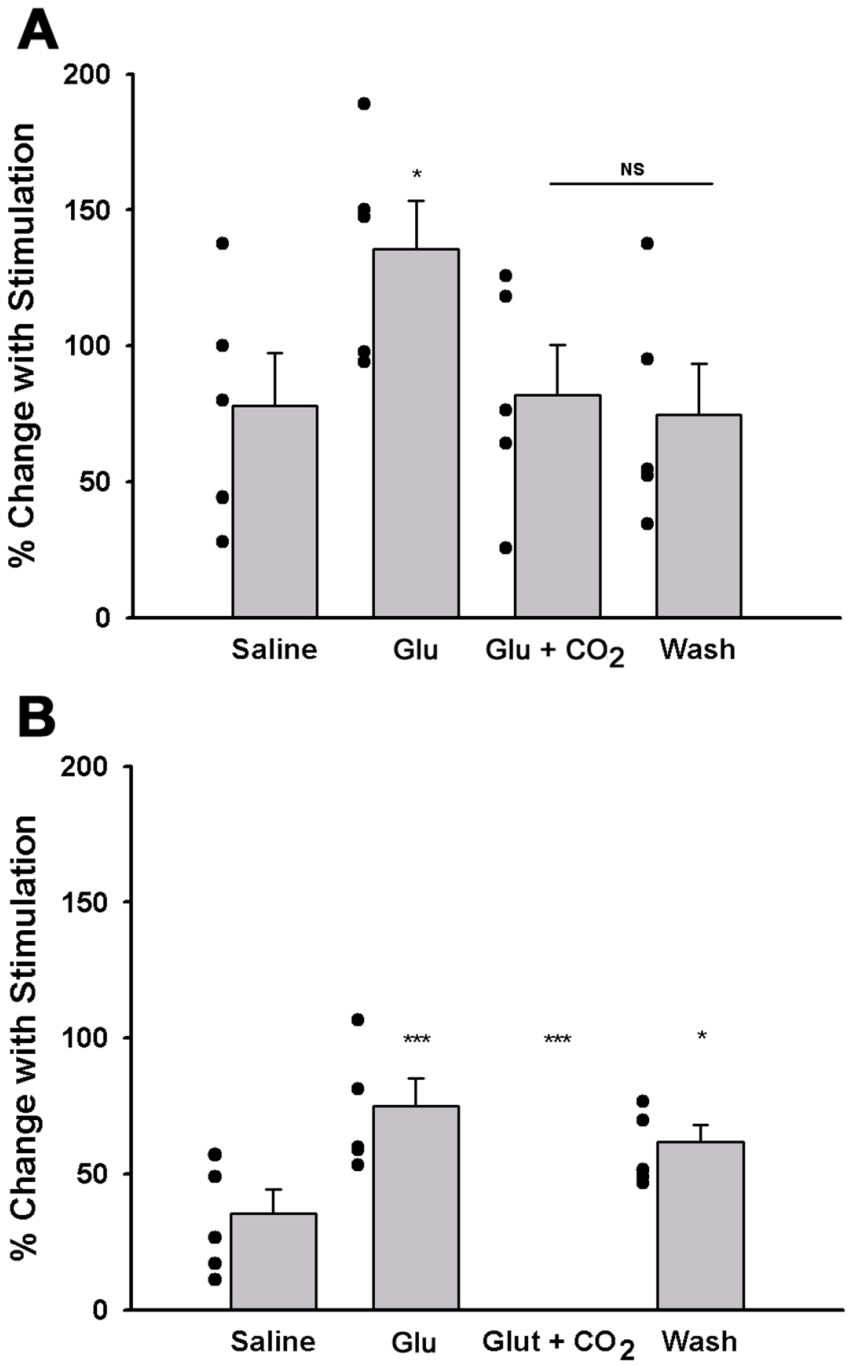

Figure 5.15. Mean spike activity of cuticular stimulation with glutamate and glutamate plus CO_2 exposure conditions. Percent change with cuticular stimulation for (A) sensory nerve root and (B) motor nerve root. Scatter dots represent variations in individual preparations. Average mean (± SEM) frequency is represented by the gray bars for each condition. There was a

consistent significant effect in five out of five preparations ($P < 0.05$, Wilcoxon non-parametric analysis). For the sensory nerve root, a significant increase in spike frequency for glutamate is shown when compared to saline control (ANOVA; * $p < 0.05$). For the motor nerve root, there is a significant increase in spike frequency for glutamate as well as a significant decrease in spike frequency for glutamate + CO_2 as compared to the control saline (ANOVA; * $p < 0.05$, *** $p < 0.001$ and NS is not significant).

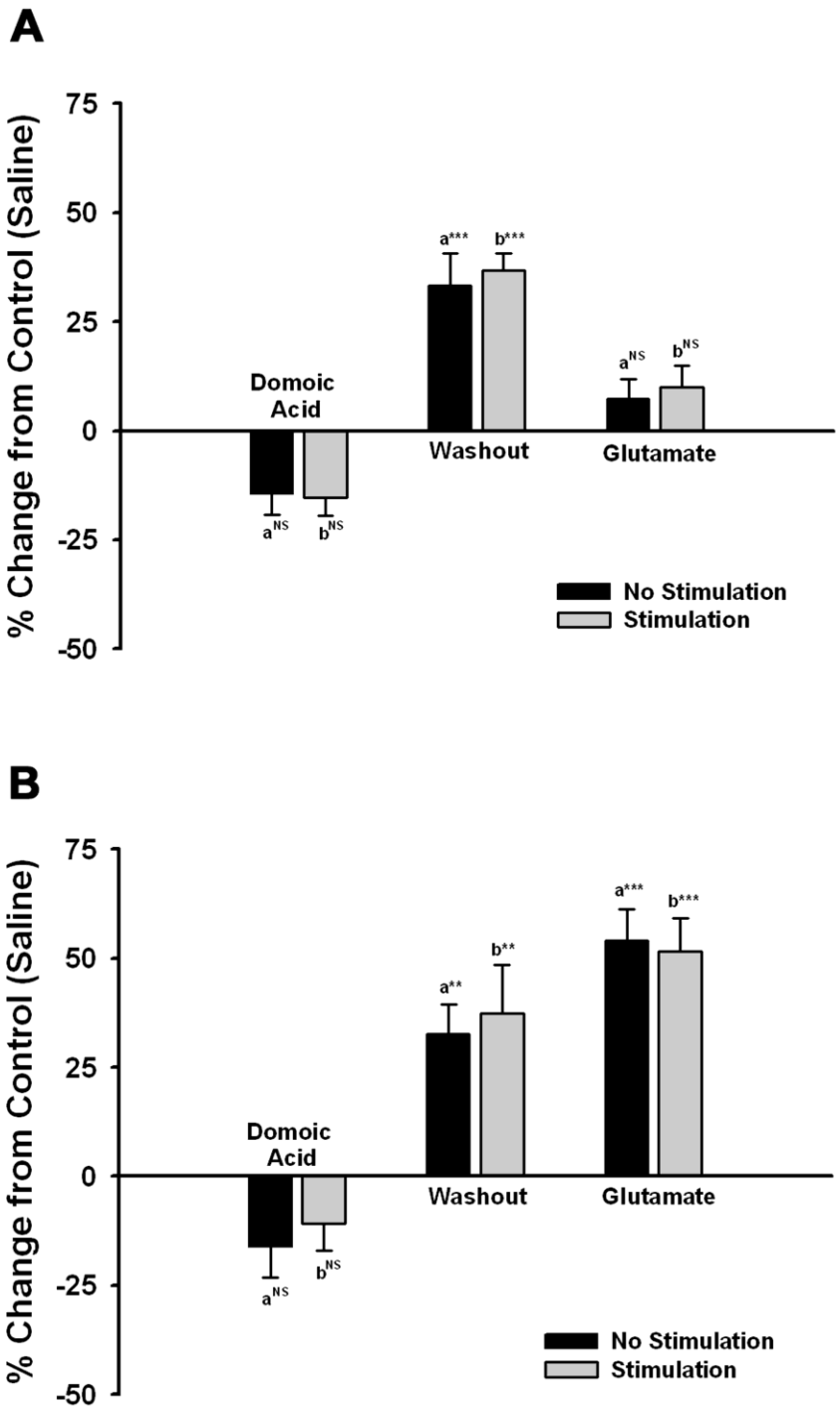

Figure 5.16. Mean spike activity before and during cuticular stimulation with domoic acid or glutamate present. Percent change with cuticular stimulation for (A) sensory nerve root and (B) motor nerve root. Before (a, black bars) and

during cuticular stimulation (b, grey bars) were compared across conditions. The mean rate (± SEM) of spike frequency over multiple 30 msec time periods were assessed for the conditions. There was no significant change in spike frequency for either sensory or motor roots for domoic acid. When exposed to glutamate there was a significant increase on the motor root compared to saline control (ANOVA; **$p < 0.02$ and *** $p < 0.001$). This was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

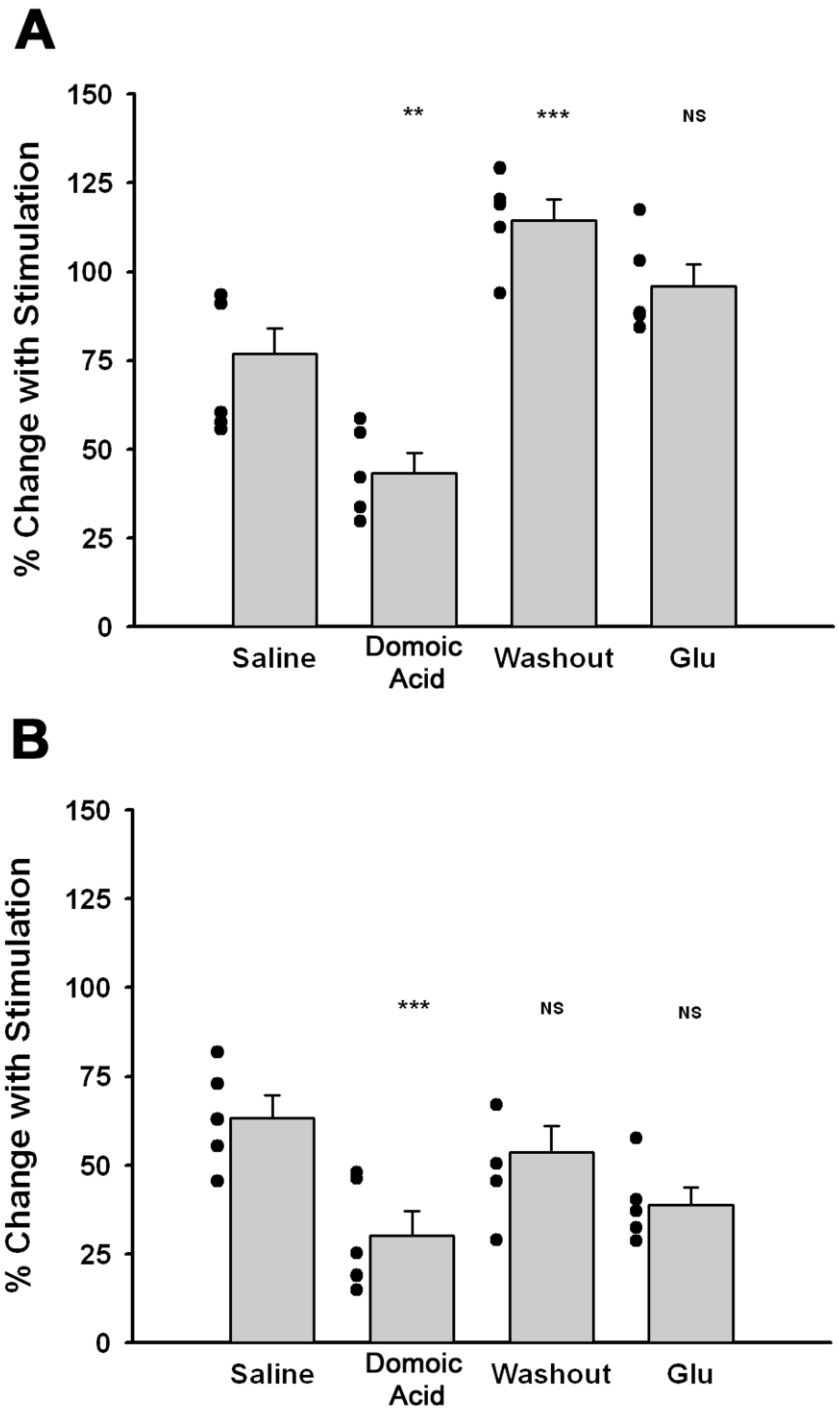

Figure 5.17. Mean spike activity for cuticular stimulation with domoic acid or glutamate present. Percent change with cuticular stimulation for (A) sensory nerve root and (B) motor nerve root. Scatter dots represent variations in individual preparations. Average mean (± SEM) frequency is represented by the

gray bars for each condition. There was a consistent significant effect in five out of five preparations for the domoic acid reducing sensory and motor activity ($p < 0.05$, Wilcoxon non-parametric analysis). For both the sensory and motor nerve roots, there was a significant decrease in spike frequency for domoic acid as compared to the saline control. Glutamate did not have a significant effect from saline control (ANOVA; $^{**}p < 0.02$, $^{***} p < 0.001$ and NS is not significant).

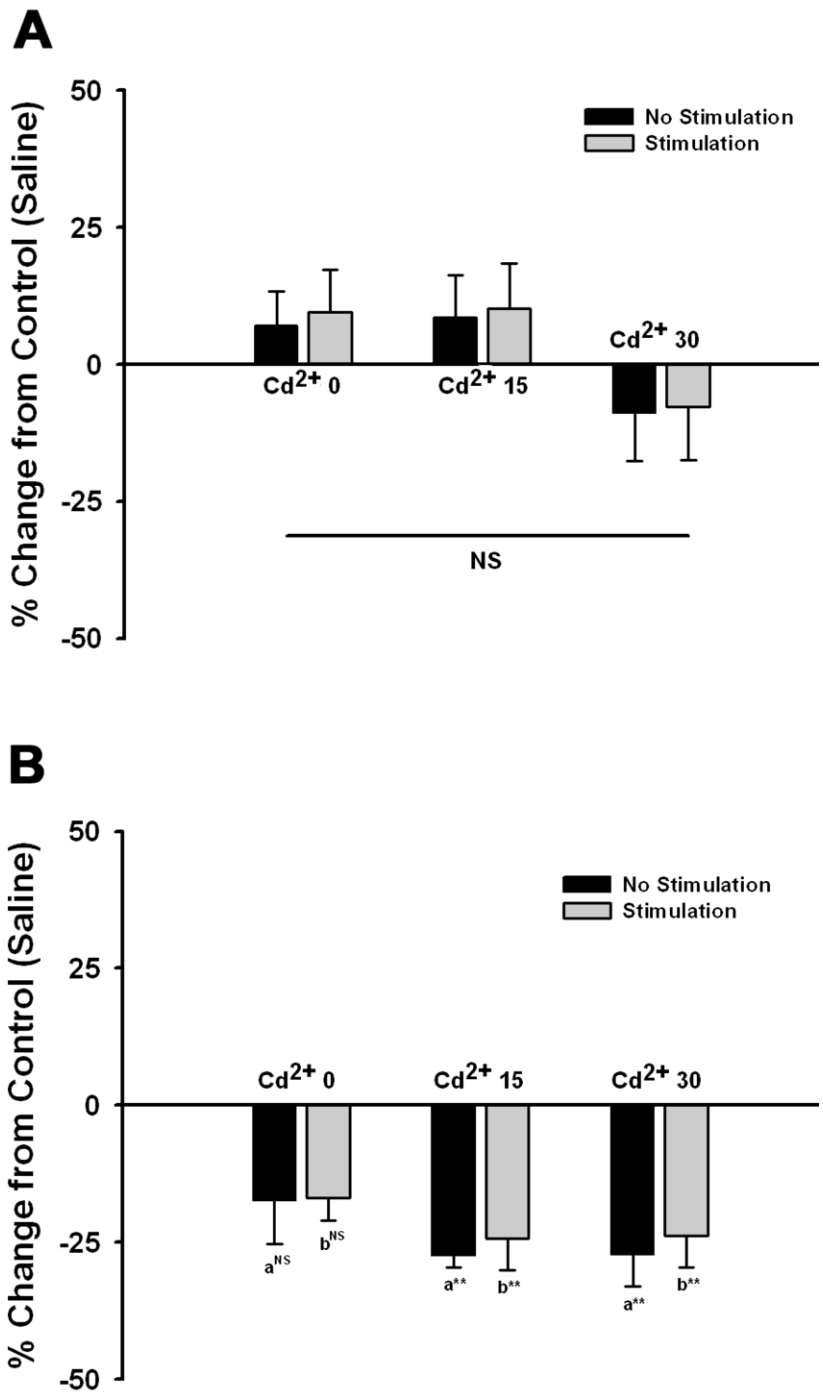

Figure 5.18. Mean spike activity before and during cuticular stimulation with cadmium at time 0, 15 and 30 minutes. Data is represented as percent change from saline for (A) sensory nerve root and (B) motor nerve root. Before (a, black bars) and during cuticular stimulation (b, grey bars) were compared across

conditions. The mean rate (± SEM) of spike frequency over multiple 30 msec time periods were assessed for the conditions. In the sensory nerve root, there was no significant change in spike frequency for all time points with cadmium exposure. The motor nerve root showed a significant decrease after in spike frequency after 15 minutes (ANOVA; **$p < 0.02$). There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

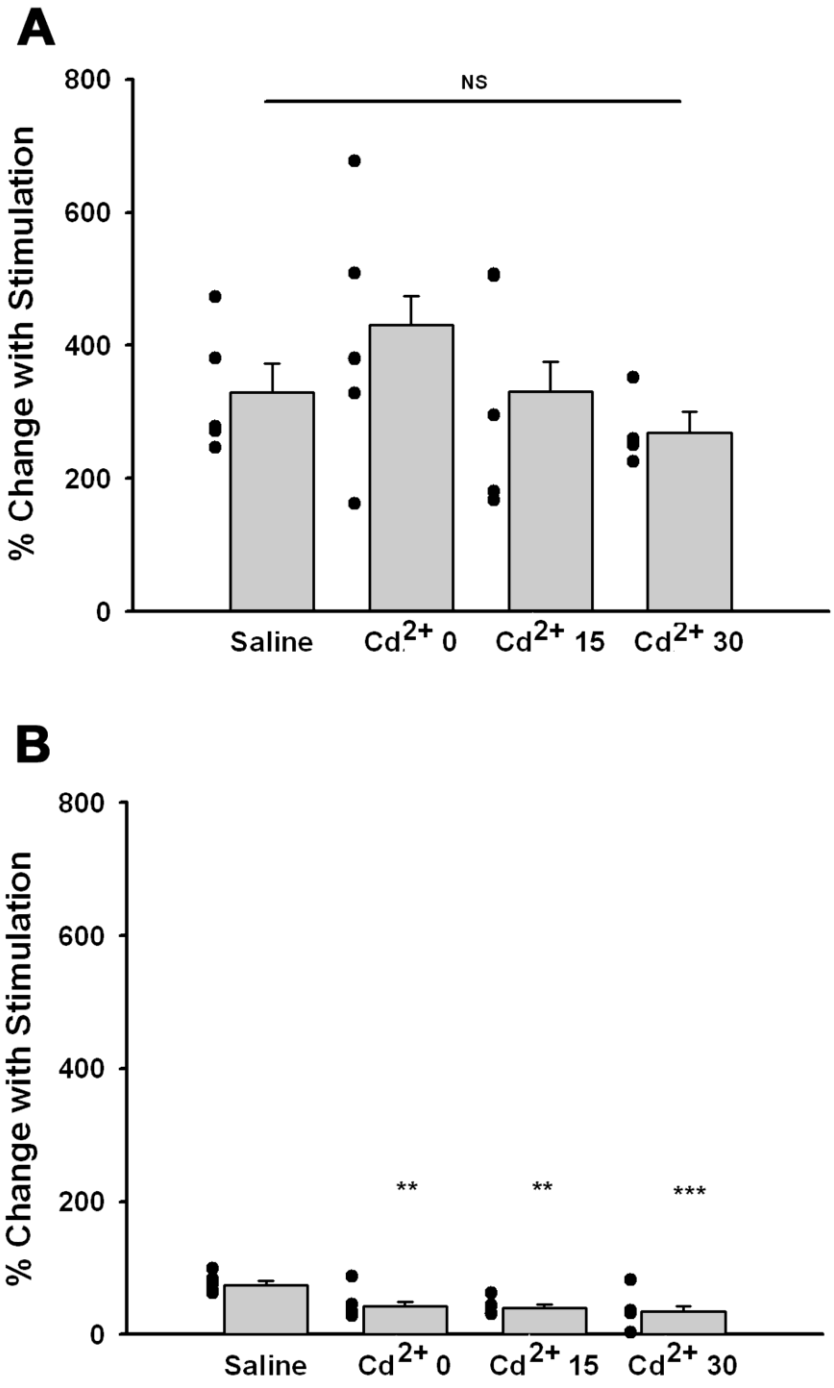

Figure 5.19. Mean spike activity of cuticular stimulation with cadmium at time 0, 15 and 30 minutes. Data is represented as percent change from saline for (A) sensory nerve root and (B) motor nerve root. Scatter dots represent variations in individual preparations. Average mean (± SEM) frequency is represented by the gray bars for each condition. There was a consistent response in five out of five

preparations (5 out of 5 preparations, P< 0.05, Wilcoxon non-parametric analysis). For the sensory nerve root, there was not a significant change in spike frequency with cadmium exposure. The motor root showed a significant decrease in spike frequency with stimulation for all time points (ANOVA; **p < 0.02, *** p < 0.001 and NS is not significant).

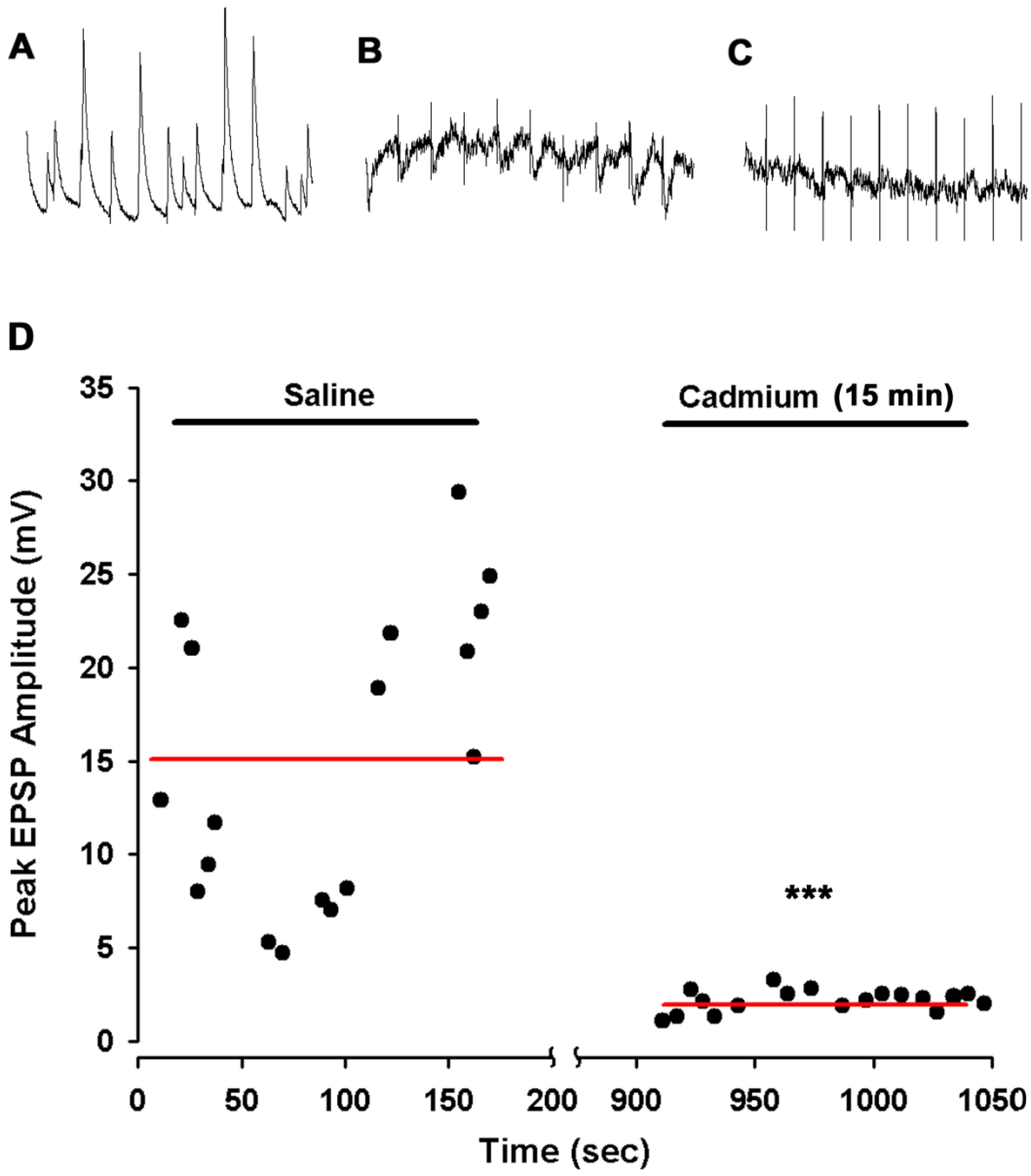

Figure 5.20. The effect of cadmium on firing frequency of the motor root as recorded with an intercellular electrode within a medial fiber of the superficial flexor muscle to monitor excitatory postsynaptic potentials (EPSPs). Representative trace of EPSP activity in (A) saline, (B) 1 mM cadmium after 5 minutes and (C) 1 mM cadmium after 15 minutes. (D) The EPSP amplitude was measured over time during exposure to normal saline, followed by saline with 1mM cadmium after 15 minutes. Exposure to cadmium resulted in a complete attenuation of the EPSP in five out of five preparations within 15 minutes without any change in resting membrane potential. ($p < 0.05$, non-parametric analysis). Average EPSP amplitude showed a significant difference ($t = -7.090$, $df = 34$, $p = < 0.001$).

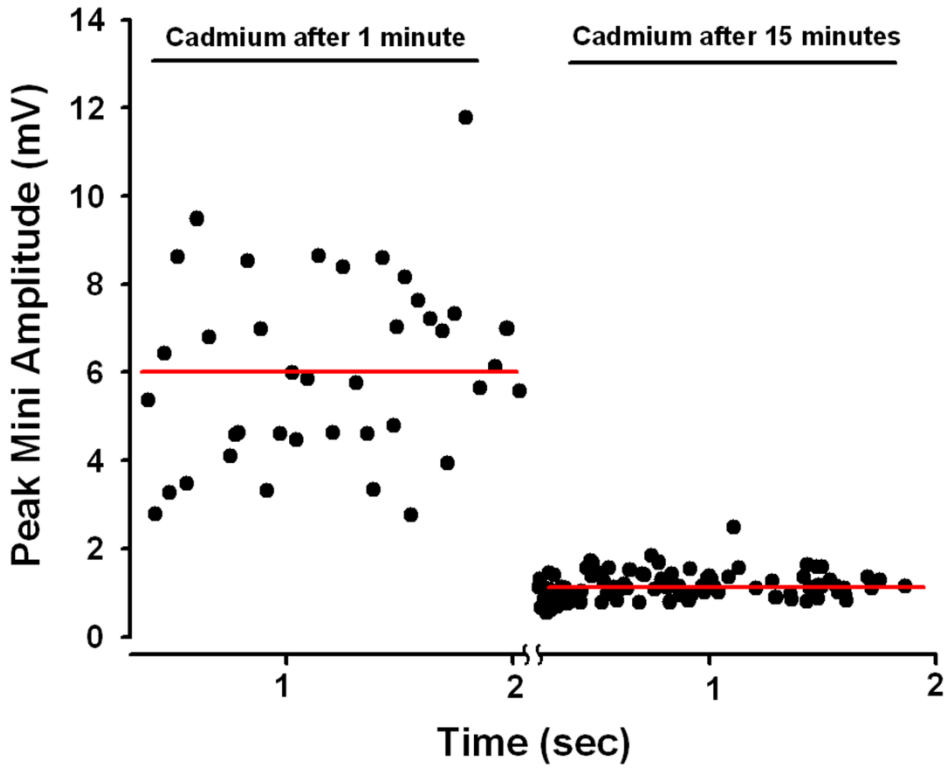

Figure 5.21. The effect of cadmium on fusion of spontaneous events as recorded with an intercellular electrode within a medial fiber of the superficial

flexor muscle to monitor mEPSPs. Representative plot of peak amplitudes show mEPSP activity after 1 minute and 15 minutes of 1 mM cadmium exposure. The EPSP amplitude was measured over time with 2 second intervals shown for both conditions. Exposure to cadmium resulted in a gradual decrease in mEPSP amplitude over time in five out of five preparations ($p < 0.05$, non-parametric analysis).

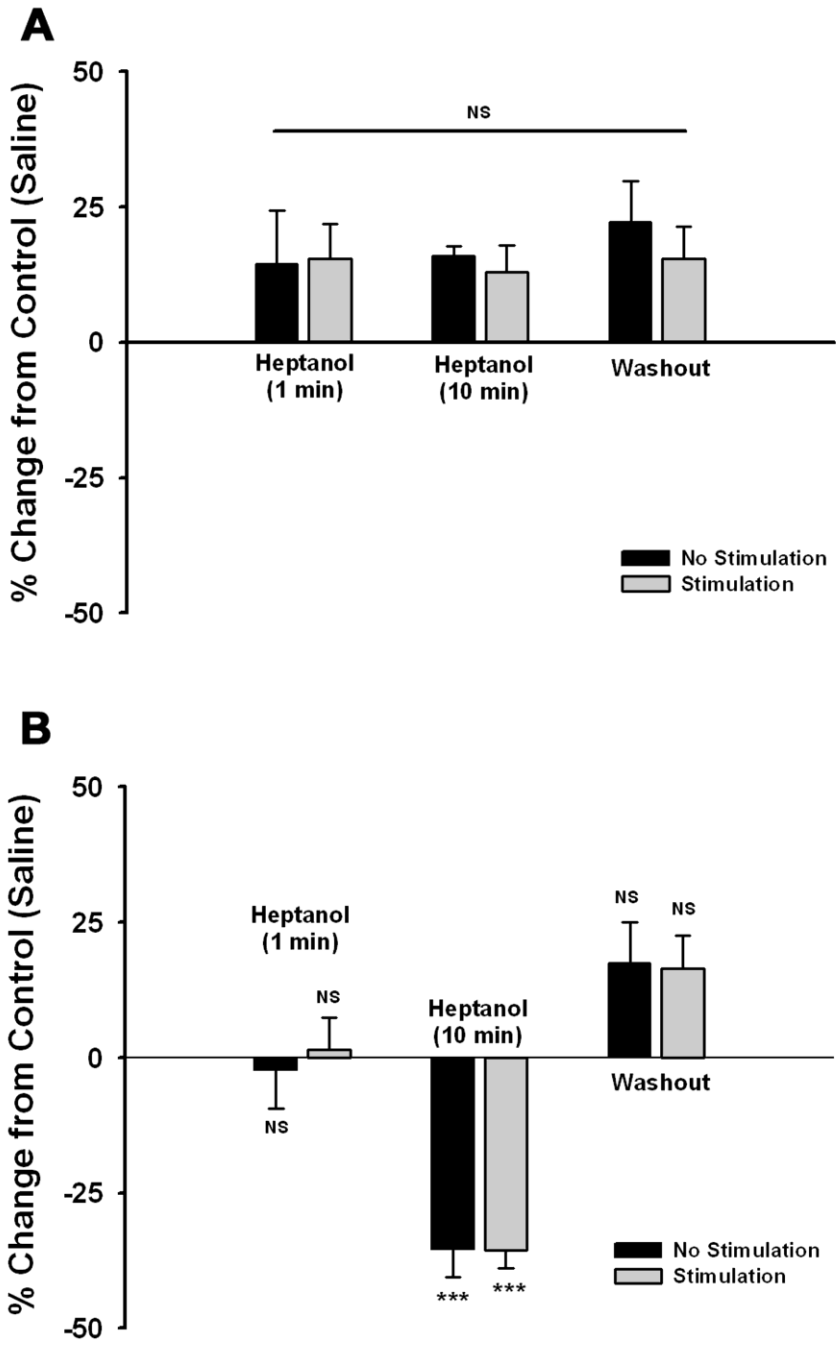

Figure 5.22. Mean spike activity before and during cuticular stimulation with heptanol. Data is represented as percent change from saline for (A) sensory nerve root and (B) motor nerve root. Before (a, black bars) and during cuticular stimulation (b, grey bars) were compared across conditions. The mean rate (± SEM) of spike frequency over multiple 30 msec time periods was assessed for

the conditions. In the sensory nerve root, there was no significant change in spike frequency for all time points with heptanol exposure. The motor nerve root showed a significant decrease in spike frequency after 10 minutes of exposure (ANOVA; *** $p < 0.001$, and NS is not significant). There was a consistent significant effect in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis).

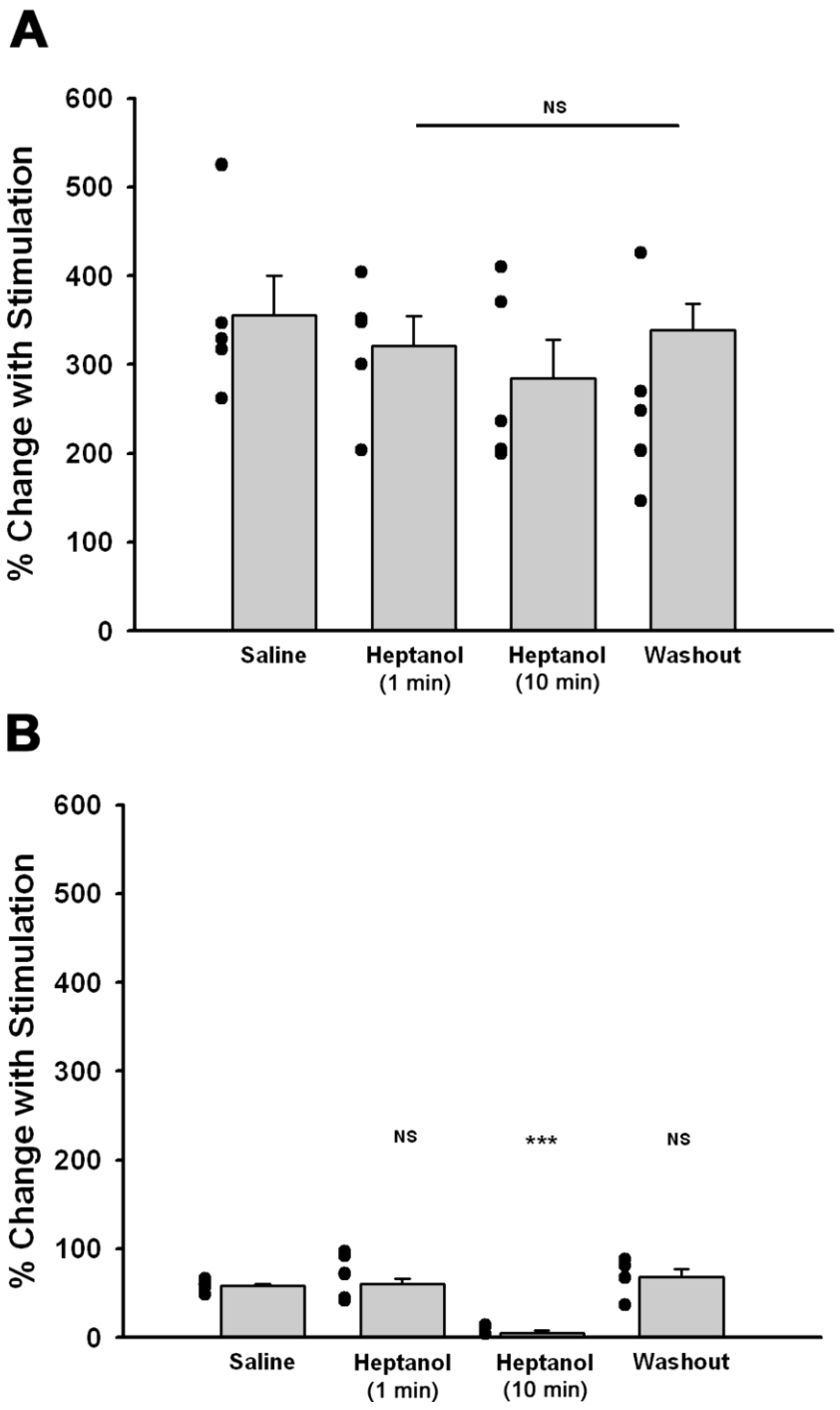

Figure 5.23. Mean spike activity of cuticular stimulation with heptanol after time points 1 and 10 minutes. Data is represented as percent change from no cuticular stimulation to stimulation for the (A) sensory nerve root and (B) motor nerve root. Scatter dots represent variations in individual preparations. Average

mean (± SEM) frequency is represented by the gray bars for each condition. There was a consistent response in five out of five preparations ($p < 0.05$, Wilcoxon non-parametric analysis). For the sensory nerve root, there was not a significant change in spike frequency with heptanol exposure. The motor root showed a significant decrease in spike frequency with stimulation after 10 minutes of exposure (ANOVA; *** $p < 0.001$ and NS is not significant).

Comprehensive Review of Agonists and Antagonists in determination of the superficial flexor circuit

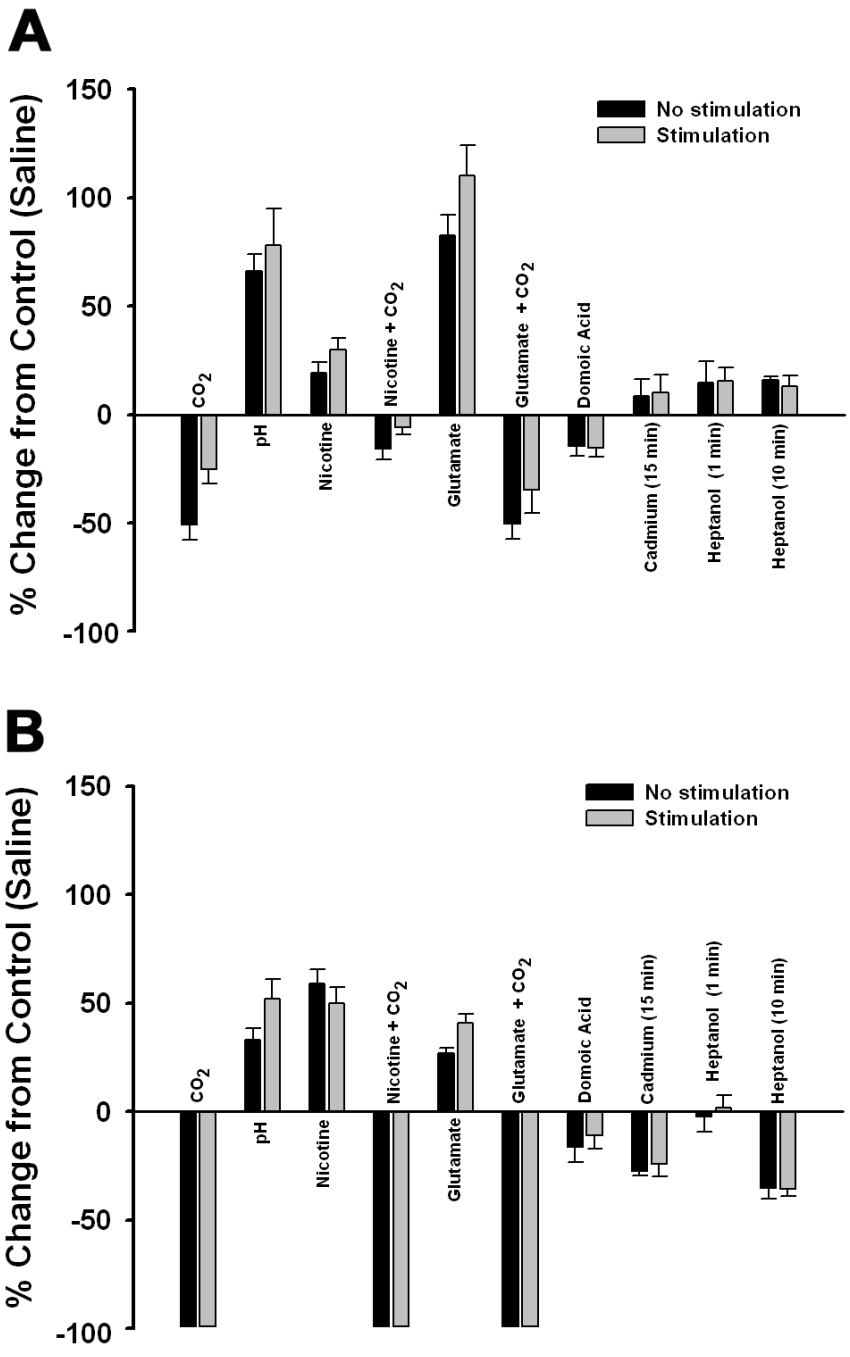

Figure 5.24. Influence of CO_2 and other compounds on a 'sensory root – ganglia - motor root' neural circuit. Average percent change from saline control is shown for the (A) sensory root and (B) the motor root. Carbon dioxide exposure consistently shows a significant decrease in spike frequency in both the sensory and motor roots. The most dramatic effect is seen in the motor root where CO_2

causes complete cessation in the motor root even when combined with excitatory neurotransmitters. Before (a, black bars) and during cuticular stimulation (b, grey bars) were compared across conditions. The mean rate (± SEM) of spike frequency over multiple 30 msec time periods were assessed for the conditions.

Table 5.3. The composite of responses on sensory and motor roots due to the various agents which were exposed to the neuronal circuit. The responses are reported for the change in activity during sensory stimulation. Arrows indicate direction of statistical significance from basal activity. NS indicates non significant effect.

Statistical Significance in the Superficial Flexor Neural Circuit		
Chemical	**Sensory**	**Motor**
CO_2	⬇	⬇
pH	⬆	⬆
Nicotine	⬆	⬆
Nicotine + CO_2	NS	⬇
Glutamate	⬆	⬆
Glutamate + CO_2	⬇	⬇
Domoic Acid	NS	NS
Cadmium (15 min)	NS	⬇
Heptanol (10 min)	NS	⬇

DISCUSSION

In this chapter, I attempted to demonstrate some of the mechanisms, at a neuronal circuit level, that could account for the behavioral alterations induced by high levels of environmental CO_2 (as discussed in Chapter 4). Since rising CO_2 also resulted in a reduction of pH_o, this variable needed to be addressed throughout these studies. The presence of CO_2 blocked the muscle from being receptive to evoked as well as exogenous glutamate; thus a paralytic effect is proposed for CO_2's action. However, the spontaneous and sensory evoked activity in the sensory root and motor neurons is also reduced in the presence of CO_2, so CO_2 also has an anesthetic effect. I propose that the reduction in motor neuron activity could arise due to blockage of electrical synapses as well as some of the glutamatergic central drive. Agonists and antagonists to various synaptic inputs, possible in the VNC, supported the idea that there is electrical as well as chemical drive within the circuit that can modulate intrinsic as well as sensory evoked activity in the motor neurons. The exact wiring diagram of the sensory-CNS-motor circuit is not possible at this time since there can be numerous theoretically sound possibilities but the conformation of any proposed work requires detailed anatomic mapping and neurophysiological studies of individual neurons. For the purposes of this study, I have documented that CO_2 has actions in the periphery as well as in the CNS to account for the behavior responses and that gap junctions as well as glutamatergic synapses are targets. Further studies are required to dissect the circuit to know where precisely these synapses are occurring within the driven circuit used herein as well as what drives the spontaneous activity of the superficial flexor motor neurons.

Neuromuscular junction

The ability to record quantal events provides many experimental advantages in understanding regulation of pre- or post-synaptic contributions by pharmacological compounds, such as CO_2 and Cd^{2+} (Cooper et al., 1995a; Viele

et al., 2006). A reduction in the frequency of spontaneous quantal events would suggest a presynaptic action of an agent; whereas a decrease in the receptivity to glutamate (as measured by the quantal amplitude or lack of response to exogenous glutamate) would generally indicate an action at the receptor level. The mechanism of action could be packaging of the vesicles, but receptivity in our study was independently checked by exogenous application of glutamate on the muscle in the presence of the compound of interest. The results we obtained at the crayfish opener and superficial flexor muscles mimic the ones documented earlier from our lab at the NMJ of larval *Drosophila.* Since the opener and superficial flexor muscles both showed a decrease in the amplitude of spontaneous quantal events and the evoked multiquatal events became smaller in addition to the muscle being unresponsive to the application of glutamate, a reasonable explanation is that the glutamate receptors are being blocked/inactivated by the presence of CO_2. Given that CO_2 can diffuse into the muscle and thus decreases pH rapidly by the action of carbonic anhydrase, we should consider that protons may have an action on the cytoplasmic side of glutamate ionotropic channel. The lower extracellular pH saline that we used to control for the drop in pH of saline bubbled with CO_2, can not rule out the effects of pH on the inner side of the glutamate ligand-gated ion channel. Possibly CO_2 blocks the receptor directly by being trapped within the channel pore since it can not diffuse through the protein structure as it can through the bilipid membrane. As a side note, there is a precedence that Rh proteins, commonly associated with human blood typing, may indeed serve as a CO_2 channel (Kim et al., 2005). However, no investigations I am aware of have addressed the presence of Rh proteins in crustaceans. Future studies, need to examine if the muscle shows a drop in intracellular pH and if so to induce a drop in pH independently of CO_2 to learn if the low pH by itself is the key contributor to the decreased glutamate sensitivity. The NMDA receptor of vertebrates is pH sensitive on the extracellular side due to protonation (Giffard et al., 1990; Low et al., 2003 Tang et al., 1990; Tombaugh and Sapolsky, 1993; Traynelis and Cull-Candy, 1990). However at the crayfish and *Drosophila* NMJ, an extracellular pH of 5.0 has no effect on

sensitivity of the glutamate receptors as shown in this study and previous studies from our lab.

Ventral nerve cord: CNS

Since the postsynaptic glutamate receptors are blocked or made unresponsive by CO_2, either directly or indirectly, the activity of the motor neurons may seem to be irrelevant to investigate in relation to the mechanism of the paralytic behavior. However, to understand the full breath of the effects of CO_2, studying effects on the neural circuit is important. In addition, it was established that in the larval *Drosophila* the motor nerve roots from the CNS remained active in the presence of CO_2, so for comparative studies in detailing the mechanisms of action on the whole animal behavior, I wanted to know if the crayfish showed a similar lack in an anesthetic effect by CO_2. Much to my surprise a CO_2 containing saline completely blocked spontaneous motor nerve root activity in the pure motor 3rd root as well as evoked activity through sensory stimulation via 2nd nerve root. To solve the conundrum if CO_2 blocks electrical conductance along an axon or the ability of the motor neurons to generate an action potential, I turned to the large diameter excitatory motor nerve axon of the opener muscle in the walking leg. Generation as well as the shape of the action potential was not substantially altered by CO_2. Since I do not expect the motor nerve axons innervating the superficial flexor muscle to be any different in response to CO_2 from the axon to the opener muscle, the site of action of CO_2 in the VNC must be at the level of neural drive to the motor neurons within the ganglion.

The source of spontaneous activity of the 3rd motor root has been a topic of sincere investigation since the 1960's when Eckert (1961) examined if the tonic firing static muscle receptor organ (MRO) within the same or neighboring segment could account for the spontaneous motor drive. In these earlier studies it became apparent that the activity was driven within the ventral nerve cord (VNC) possibly from higher centers (Eckert, 1961; Kennedy and Takeda, 1965a,

b; Strawn et al., 2000). Since the presence of CO_2 stopped the spontaneous activity, one can assume somewhere in the drive to the motor neurons there might be a glutamatergic excitatory drive where the receptors are similar in properties to those at the NMJ which are blocked/decreased sensitivity to glutamate in the presence of CO_2. Another possibility is the presence of gap junctions directly on these motor neurons and that CO_2/pH_i blocks these causing the reduced activity. The presence of CO_2 has been shown to block gap junction in the lateral giant axons of the VNC in crayfish (Arellano et al., 1990; Peracchia, 1990). It has not been established yet if the lateral or medial giant axons play a role in the spontaneous activity of the 3rd motor nerve root. As for the evoked drive of the motor neurons through sensory stimulation (i.e., brushing of the cuticle), this enhances the nerve activity but when CO_2 is present the drive is significantly depressed to the point of no activity present. Thus, the paralytic effect at the NMJ appears to be insignificant as it can not be driven anyways by this particular circuit since it is blocked centrally. By definition this could be referred to as an anesthetic action of CO_2 since the sensory to motor activity is stopped within the CNS. The possibilities to explain the potential sites of action for CO_2 blocking the evoked drive are numerous given that we do not know the extant of interneuronal connections via chemical and/or electrical synapses. Comparative studies in the command of the motor roots of the larval Drosophila brain indicate that the drive on the motor neurons is different as the spontaneous as well as the evoked drive was not blocked by CO_2 (Badre et al., 2005).

Anatomical dissection of neuronal circuits is a tedious undertaking and even after knowing the anatomical pathways one still needs to understand the physiology of the connections to explain the various contributions (Kennedy, D., Takeda, K., 1965a, b). The application of antagonist or agonist to potentially drive or reduce activity in the 3rd root can help one to understand if particular receptor subtypes are used within this system. In this regard, I tried application of nicotine to drive a nicotinic receptor subtype as the sensory input to the CNS of many invertebrates are cholinergic (olfaction input in Drosophila, Silbering et al., 2008; mechanosensory afferents in cricket - Yono and Aonuma, 2008; escape circuit in

Drosophila - Fayyazuddin et al., 2006; Aplysia- Susswein et al., 1996). The findings indicate that nicotine enhanced the motor drive, which suggest a nicotinic Ach receptor somewhere in the circuit.

To examine if quisqualate subtype of glutamate receptors had a role in this motor circuit, I tested the effects of domoic acid. I knew this compound blocks the quisqualate glutamate receptor subtype in *Drosophila* (Lee et al., 2009) and that crayfish have these same pharmacological profiled receptors at their NMJ (Shinozaki and Ishida, 1981; Shinozaki and Shibuya, 1974). In this crayfish circuit, I found that domoic acid decreased the spontaneous drive which suggests that indeed there is likely a glutamatergic input and that there are quisqualate receptors present within the synapses that drive the motor neurons. However, further stimulation of the sensory root did not result in any further decrease. Thus, the sensory to motor path is using the receptors normally but they are already blocked by domoic acid when I stimulate the sensory paths. This scenario accounts for the lack of enhanced activity during sensory stimulation in the presence of domoic acid. Thus, it would seem reasonable if glutamate itself is applied that at least a transient alteration in activity in the motor root would occur. One might expect a rapid enhanced effect and then potentially a reduced rate below basal activity in the motor root as glutamate receptors in *Drosophila* and crayfish are known to desensitize rapidly to glutamate (Dudel et al., 1992). I observed a substantial increase in the activity in the motor root without sensory stimulation upon exogenous application of glutamate. This suggests that indeed there is a glutamatergic drive in the system and supports the results obtained for domoic acid application. Upon sensory stimulation a slight increase in the activity is observed so likely there might be a non-glutamatergic drive present as I would expect the exogenously applied glutamate to swamp out any additional sensory driven glutamate contribution. It is curious to note that the activity did not increase and then decrease quickly due to desensitization of the activated glutamate receptors. Interestingly, the activity of motor neurons in the segmental roots of the larval *Drosophila* were not silenced by application of domoic acid (10µM). I conducted these studies (N = 5) for a comparison to crayfish to address

if there would also be a substantial reduction in motor neuron activity in the presence of domoic acid, but surprisingly it appears that these receptor subtypes for domoic acid may not be present in the larval CNS for motor command. This stresses the importance for comparative studies and that indeed there are pharmacological differences among what would appear to be similar types of neuronal circuits even within Arthropods.

As for the contributions of gap junctions being used in the spontaneous drive as well as the evoked sensory to motor neuron, I used heptanol, a well established blocker of gap junctions (Johnston et al., 1980). It was surprising to observe that heptanol decreased the spontaneous drive on the motor neurons. This suggests that possible background input from the lateral or medial giants might be driving the motor neuron in the 3rd root as they do have electrical input on other motor neurons, but it could also be likely that the drive is through other interneurons communicating via gap junctions. At least I now know that sensory drive to the motor neurons is not exclusively via gap junctions but some direct input could be possible. Just as I observed with the presence of Cd^{2+} in which the motor root activity is not completely blocked, thus suggesting that electrical input is also a contributing factor in regulating the 3rd root. The mechanisms of CO_2 blocking gap junctions are discussed in the Introduction of this chapter. When it becomes known which neurons specifically are contributing to the electrical junctional input on these motor neurons, then similar detailed studies into pH altered sensitivity of Ca^{2+} ions on gap junctional proteins could be studied for this circuit. However, the detailed studies of the lateral giant axons of the crayfish VNC (Arellano et al., 1990; Peracchia, 1990) serve as a good model of the likely mechanistic explanation of how CO_2 blocks gap junctions in other neuronal types. To determine if gap junctions occur directly on the superficial flexor motor neurons, back filling of the 3rd motor root with Lucifer yellow or other compounds permeable through gap junctions (Payton et al., 1969) would address this question.

In constructing a model to explain the observed results, I do know that the presence of CO_2 completely attenuates the spontaneous activity in the 3rd root as

well as evoked sensory activity. Also, I established that Cd^{2+} or heptanol by themselves do not entirely block the spontaneous or evoked activity of these motor neurons, although there is a strong reduction for each individually. Thus, it would appear there are parallel inputs and not series inputs of chemical and electrical synapses in the circuitry from the sensory drive as well as the yet unknown drive responsible for the spontaneous activity. It would appear that CO_2 is able to block both the chemical, likely glutamatergic, and electrical synapses.

Sensory input

The 2nd root is primarily composed of 100's of afferent sensory inputs which monitor stimuli from the cuticular surface in the corresponding segment; however, there are a few motor neurons to the superficial and deep extensor muscles as well as to the efferent control of the muscle receptor organs (MRO's) (Sohn et al., 2000). In addition, the MRO sensory afferents are present in this nerve root. In the experiments with exposure to glutamate, Cd^{2+}, heptanol and domoic acid it is not known what contribution they had on specific primary sensory neurons as compared to the few motor neurons in this same root for the basal activity. However, during brush stimulation of the cuticle the pronounced increase in the number and frequency of small sized spikes within the extracellular recording would indicate that primarily sensory neurons were recruited. A reduction in spike activity only occurred during exposure to CO_2 and only nicotine and glutamate significantly increased activity in the 2nd root. The other agents (domoic acid, low pH, Cd^{2+}, heptanol) did not produce a significant effect on the activity. The mechanistic action of CO_2 on these primary sensory neurons has not been established. We do not know if the transduction process itself or if the biophysical properties of the axons in their ability to conduct action potentials is altered. Since these primary cuticular sensory axons are very small in diameter and difficult to isolate individual axons, I could not directly assess if the action potential in these neurons is impacted by CO_2. However, I did address this possibility by examining the amplitude and shape of the action potential of

the excitatory opener motor neuron in the walking leg since it is easily assessable and intracellular recordings are attainable (Cooper and Cooper, 2009). I assume the fundamental biophysical properties of the opener axon are similar enough to the primary sensory axons that CO_2 did not affect the action potential characteristics or conduction in the motor neurons either in this regard. It is possible that CO_2 is targeting the transduction process. This remains to be examined in more detail. Possibly examining the large sensory endings and somata of the MROs would shed some light on this issue. The large cell bodies of the MROs can be impaled with microelectrodes such that the membrane resistance, threshold and graded potentials can be examined before and during exposure to saline containing CO_2 as has been accomplished for addressing the action of 5-HT on these primary sensory neurons (Cooper et al., 2003). As to why nicotine and glutamate increase activity in the 2nd root, there might be some action on primary sensory neurons, but mechanisms remain to be elucidated. There is no precedence that I am aware of for these agents to have a direct action on sensory neurons. Most likely, these compounds are activating the efferent motor neurons within the VNC that have their axons present in this 2nd root.

Behavior

Blocking motor neuron activity within the VNC as well as directly blocking responsiveness of glutamate at the NMJ explains the paralytic and anesthetic whole animal behavior when crayfish are exposed to an environment containing high CO_2. The use of the defined *in situ* sensory-CNS-muscle preparation allowed me to address the sites of primary action that likely account for CO_2's action on other neuronal circuits that make up the tail flip and visual cued responses which are also altered in crayfish exposed to CO_2 (Bierbower and Cooper, 2009).

Model

A wiring circuit diagram of the potential neuronal pathways may appear to be premature given that the identify of only a few neurons in the circuit are known; however, it provides a framework to visualize where future research can be specifically directed to indentify each component within the sensory-ganglion-motor path. In addition, the results in this study indicate that both chemical and electrical synapse are driving the 3rd root to the superficial muscles. It is apparent that there is some input on the 3rd superficial flexor motor root due an intrinsic spontaneous activity (Evoy et al., 1967). It may be possible that these motor neurons are coupled with the lateral giant axons as there are motor neurons coupled with the lateral giant (LG) in the 3rd root which project to the deep flexor muscles for the rapid tail flips (Furshpan and Potter, 1959). The superficial muscles would likely contract in synchrony, albeit slower, with the physic flexors to help coordinate the articulating membrane between the segments. There is noted background activity in lateral giant axons, but as far as I am aware no one has correlated it with the spontaneous activity in the superficial flexor motor neurons. Since heptanol blocks all the evoked activity in the motor root, one postulation is that the electrical connections from the LG or other interneurons to the motor neurons are blocked (Figure 5.25A). In a minimalistic view of the circuit, a sensory input could proceed directly onto the motor neuron by a mono-synaptic chemical input and some interneuron via an electrical input (Figure 5.25A). This over simplistic view would account for the responses by Cd^{2+} and heptanol blocking of chemical and electrical inputs respectively, but not the actions observed for glutamate and nicotine. Building postulated complex models allows one to design an Occam's razor approach to the circuit until otherwise disproven (Figure 5.25B). It is known that the LG does contain vesicles along with gap junctions at synapses onto the fast flexor motor neurons (Leitch et al., 1992), thus a possibility for a similar type of input on the slow flexor motor neurons is not too radical of a postulation. Given the few pharmacological trials and that it is likely the sensory input is Ach, I postulate a simple circuit with

interneurons that would account for the glutamatergic system I measured along with potential electrical connections. The electrical input is likely not a series element with the sensory as even with heptanol present, the sensory to motor root can still be driven although with a reduced efficacy (Figure 5.25C).

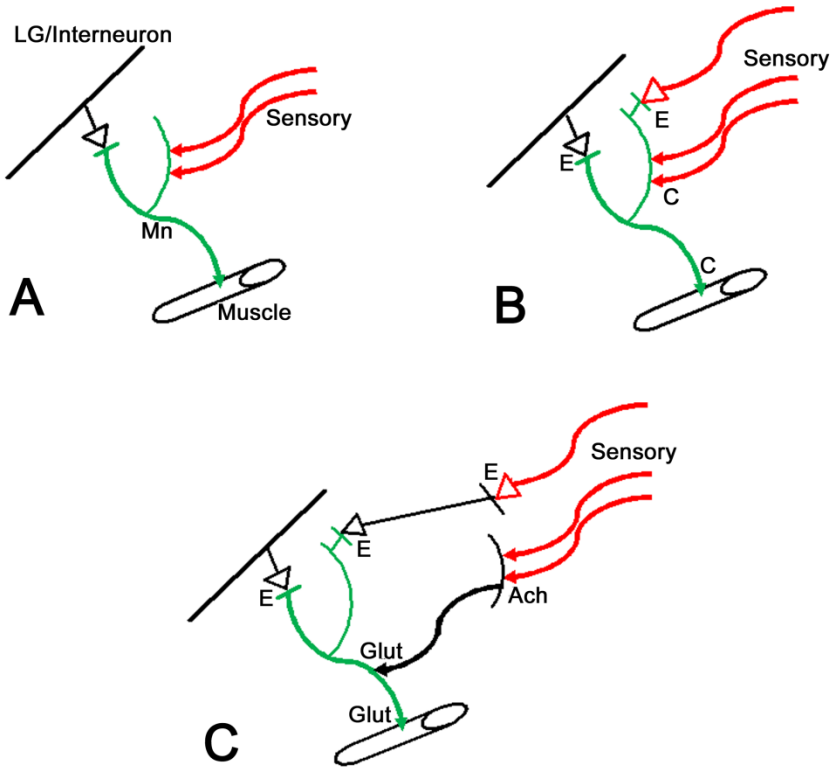

Figure 5.25. Putative neural circuit for sensory-ganglia-3rd root of the tonic superficial flexor motor neurons in the abdomen of the crayfish. (A) A simplistic model with sensory neurons directly innervating the motor neurons via chemical communication. In this model electrical drive is from the Lateral Giant (LG) or another interneuron. The spontaneous activity of the motor neurons (Mn) could be accounted for by basal activity in these inputs in a synergistic or additive fashion. (B) Sensory inputs may also contribute by direct electrical inputs in addition to chemical synapses. This could account for the reduced sensory input when gap junctions are inhibited, independent of the interneurons. (C) Given the pharmacological observations, the circuitry likely contains interneurons between

sensory and motor that are glutamatergic with cholinergic sensory drive. Gap junctions also appear to be present in the circuit at some point in the sensory to motor evoked circuit. It is important to note that no anatomic data is yet provided for these hypothetical circuits and that these are likely oversimplified (i.e., inhibitory inputs are not provided). (Gap junctions are denoted as electrical , E or as a diode Ñ ; chemical-C; Ach-acetylcholine; Glut-glutamate).

Early works addressing only a few components of the abdominal circuitry have proven to be foundational studies in describing neuronal circuits in general (Kennedy et al., 1969; Wiersma and Hughes, 1961; Watanabe and Grundfest, 1961). There has been a lot of effort, and progress has been made to understand aspects of the neuronal circuitry in the crayfish abdomen (Hanna et al., 1978; Peracchia and Dulhunty , 1976; Vu et al., 1997). I predict that progress has been limited to some extent due to small sensory axons and a complex neuropil within the ganglia. This makes it a difficult to measure physiological responses with precise established anatomic profiles. Even with anatomic mapping, the physiology can be complex with chemical and electrical connections. Leech neurons form both chemical and electrical synapses simultaneously as well as specifically electrical or chemical depending on the target neuron (Fernández-de-Miguel, et al., 1992). Such synaptic complexity has not yet been shown for the superficial slow flexor motor neurons in the crayfish abdominal ganglia, but it could exist. Great strides have been accomplished with dissecting the circuitry in the lobster STG as well as indentifying the neurotransmitters in addition to the actions of neuromodulators on the activity (Marder and Bucher, 2007). The circuits within the segmental abdomen in the crayfish have not been elucidated to this level yet; although some neuromodulation studies have been tackled (Strawn et al., 2000). Pharmacological studies are non-existent, besides this study, on the receptor subtypes within the circuit utilized in these investigations. Thus, I have just began to touch the surface in starting to integrate the various aspects of this

"defined" neuronal circuit that could serve as a model for further investigations to account for characteristic whole animal behaviors.

Future research

Knowing if the observed actions of CO_2 are due to a lower pH which then inhibits gap junctions in the crayfish giant lateral interneuron resulting in the decreased 3rd motor root activity would help to delineate if the giant axons contributions to the drive on these motor neurons and if it is through gap junctions. The effects of CO_2 reducing intracellular pH in these tissues are likely to account for the physiological observed effects in cessation of the activity and reduced sensory driven responses. Future studies, such as possible cellular adaptations to chronically raised pCO_2 and/or reduced pH, would be feasible in these readily accessible preparations. Re-examination of the spontaneous 3rd root activity after chronic low level exposure or if the sensory-CNS-muscle circuit is still sensitivity to additional application of CO_2 would be of interest for investigating the potential cellular plasticity and ecological compensation for species survival. Squid axons that were acutely and chronically exposed to CO_2 revealed that the axons had ability for buffering pH_i with various ion exchange mechanisms (Boron and DeWeer, 1976). So, it is potentially feasible that long term exposure of low CO_2 may result is some compensatory cellular mechanisms within the crayfish to allow it to survive and be responsive to additional alterations in CO_2, but this remains to be examined.

Adaptation to chronic CO_2

A phenomena not well understood in humans, particularly people inflicted with chronic obstructive pulmonary disease (COPD), 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 (Samolski et al., 2009; Zapata et al., 2009; Raurich et al., 2009). In fact, if O_2

ventilation is provided to relieve hypoxia, the respiratory rate will decrease further despite a large rise in pCO_2. This suggests evidence to the possibility, but yet unidentified, presence of O_2 receptors in humans. It is known that some species of crabs have the ability to respond to O_2 independent of CO_2 and that they likely posses O_2 sensing abilities (Batterton and Cameron, 1978; Ishii et al., 1989; Massabuau et al., 1980). This is a very interesting topic on an evolutionary perspective on the most basic physiological properties of organisms (Luo et al., 2009). Given the atmospheric history of planet earth there must have been strong selective pressures for being responsive to CO_2/O_2 in the external and internal environments of primitive organisms which has likely impacted the evolution of present day animals.

Assumptions of closely related species

Also, one does need to be cautious in assuming similarities among species for mechanistic explanations in whole behaviors. There are many similarities in structure and function of crayfish and *Drosophila* NMJs (Atwood and Cooper 1995, 1996a, b; Cooper et al., 1995b), so understanding one type allows conformation of fundamental mechanisms in the other (Stewart et al., 1996). I showed similar mechanisms of action of CO_2 at the NMJ in crayfish as shown for larval *Drosophila* NMJs; however, the action on motor neuronal drive to skeletal muscle in the CNS is not similar. Activity within the segmental roots of larval *Drosophila* is not significantly altered by CO_2 (Badre et al., 2005). Thus, it is imperative when possible, to directly examine phenomena and mechanisms of action across species in order to better understand the specific actions and reasons for the differences among species even ones that would appear to be similar in particular aspects.

It is important to note that the cells examined in this study are not chemoreceptive cells for monitoring CO_2/pH_i, but they do show substantial physiological changes due to exposure. This may add some contribution to understanding mechanism of function for the specific CO_2/pH_i sensory cells in

chemosensory neurons in crayfish, as well as other species. In birds, the intrapulmonary chemoreceptors decrease their activity with CO_2, which is thought to be due to the intracellular pH drop (Hempleman and Posner, 2004). Likewise, there are a few (~10%) neurons in the nucleus tractus solitarii of the rat brain that decrease their firing frequency when exposed to CO_2, as compared to the majority of the surrounding neurons which increase in firing frequency (Conrad et al., 2009). The mechanism of the CO_2 inhibition in the neurons of the rat brain is unknown; however, the excitation is thought to be due to modulation of the K^+ currents to promote excitation. Interestingly, in snail chemosensory neurons, CO_2 enhances a Ca^{2+} current to excite the cells (Erlichman and Leiter, 1997). Another brain stem region known to be responsive to CO_2/pH_i is the locus coeruleus. In these neurons, two types of K^+ channels and a L-type Ca^{2+} channel are altered by CO_2/pH_i which appear to contribute to the enhanced firing rate (Filosa and Putnam, 2003). Thus, a multitude of ion channels are known to be a target of CO_2/pH_i.

The actions we observed on the decreased glutamate sensitivity on muscle are likely due to effects on the ionotropic channel. However, I do not know if there is any alteration on evoked transmitter release from the nerve terminal. Despite not observing any change in the action potential shape in the motor axon, the terminal may respond differently with localized differences ion channels, transporters and pumps. Also, CO_2/pH_i may have an impact on the sensitive nature of vesicular fusion and transmitter recycling. Within the terminal, vesicles repackage neurotransmitter by a H^+ gradient within the vesicle (H^+ out for neurotransmitter in) (Chaudhry et al., 2008). I would predict the antiportor of the H^+/transmitter is impacted by a increased cytoplasmic acidification. In addition, cells are retarded in recovering from pH_i when pH_o is low (Putnam, 1995). Similar observations in that an exchange mechanism is needed to compensate for a drop in pH_i was noted for squid axons exposed to CO_2 (Boron and DeWeer, 1976). The fundamental biophysical effects of CO_2/pH_i as noted on neurons would also impact chemosensory neurons so there may well be mechanisms not yet known that shield these sensory neurons from extreme

CO_2/pH_i changes. By knowing the general effects of CO_2/pH_i in non-chemosensory neurons, one can address if the CO_2/pH_i sensitive cells have other unique functions to allow them to remain functional with changes in CO_2/pH_i.

Significance

Examination of the mechanisms that account for the neural and cellular effects of CO_2 in crayfish has direct implications for all animals. Some of the earliest and best neurophysiologists have detailed and described the central nervous system of the crayfish (Retzius, 1890; Biedermann, 1891; Freud, 1882). Historically many cellular mechanisms were uncovered in crustaceans that paved that way for more rapid investigations in the complex vertebrates. It is interesting that so much history of neuroscience has its foundation in crustaceans and often using crayfish. The nervous system structure and function became of interest in the crustacean even before general acceptance of the neural theory.

ACKNOWLEDGMENTS

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Chapter Six
General Discussion

In each chapter of this dissertation, I have addressed the purpose of the experiments that were performed and the significance of the findings to the scientific community. It is now that I will address potential future projects for each set of findings. Overall, I think long term studies on understanding the effects of environmental factors on social behavior of crayfish (Chapter 2) would provide useful information in homeostatic control and adaptations necessary for organisms to survive. Improved methods for behavioral analysis of extrinsic factors can open so many new areas for questions relating to ecosystem dynamics and preservation. I also feel that understanding how environment effects intrinsic factors will help to understand physiological compensation as well as diseased states that often arise when organisms are impacted. There is an important field, Allostasis, just to understand the concept of homeostatic compensations and the maintenance of the internal physiological environment during changes in the external environment. These changes occur through behavioral, neural, hormonal, immunological and/or other processes in which basic regulatory systems modulate in diverse situations in times of duress (Sterling and Eyer, 1988; McEwen, 1998; Schulkin, 2003). This area addresses organism's tolerable limits and has been well studied and established in such fields as medicine and physiology.

The area of adaptation with environmental changes is further expanded upon when examining learning capabilities of an organism in a variety of environmental conditions as well as during stressed situations (Chapter 3). Many areas of research, from invertebrates to humans, have shown the dramatic and often detrimental impact that stress can play on learning. It is important to understand factors involved in this process and then begin to understand possible avenues of recourse. I do think that there is a clinical significance to this aspect. Once the factors involved that impact learning have been established, one can begin to understand cascades involved and possibly understand ways to prolong or inhibit

the detrimental effects. An area of importance is heat shock proteins (HSPs) or also known as stress proteins (Christians et al., 2002). These proteins play a large role in cellular defense mechanisms and have been implicated in numerous pathological conditions in humans. Ischemic stroke often causes protein oxidative damage and HSPs have been known help in protection of organs. Recently, our knowledge of how organisms adapt to stress at the molecular and cellular levels has been limited. It is now becoming evident that even at the cellular level, there are mechanisms involved to repair and compensate to maintain homeostasis.

While I value all the research proposed and discussed in this dissertation, I feel that the carbon dioxide study has the most immediate significance for a general audience (Chapters 4 and 5). It is with awe and trepidation that I begin to understand the important and dangerous role of carbon dioxide. While the role of carbon dioxide was discussed as to why it is crucial to insect lives, it is just as important to almost every other organism on this planet. Without CO_2 we would not have photosynthesis, thus no herbivores, thus no carnivores and ultimately no humans. I am amazed the many faceted roles of CO_2 for insects and excited about the possibilities still to be discovered. However, it is in fear that I wonder about the effects with slight rises of CO_2. It has been discussed that slight increases can physiologically change the sensory systems of insects that we so vitally depend on. If small changes can cause such detrimental effects, what will happen to slightly higher concentrations after that? In this research I have shown that CO_2 has physiological mechanisms of action that are most likely common general mechanisms of action. This would explain why the fruit fly, as well as a human will become unconscious with prolonged exposure. It would seem that our heightened responses to CO_2 levels both internally and externally are there as a warning of a component that can so easily have dramatic consequences. Therefore, it is important for more fundamental research on basic mechanisms in model organisms that can be rapidly examined to test predictions. I would think future research will address CO_2 as a fumigant since it does not leave harmful residues and is relatively safe to use in that context when considering

insecticides as the alternative. A unique quality is that it is effective in killing insects in all stages of their life cycles and could be used for long-term storage of products.

So where does that leave us with rising CO_2 concentrations? Well, I believe that leaves us at a stalemate knowing that major changes need to be addressed immediately and figuring out how to clue everyone in on how to go about it. But this also begs the question, "how did we become so sensitive to CO_2?". I think this is something that will be argued for centuries to come until it is known how the world began and the early atmosphere of the gases present when life began. While I cannot begin to discuss how the earth and life formed, I can only speculate about CO_2 and O_2 sensitivity. It would seem that components present, evolution and selection pressure would be the largest factors. It is speculated that in the beginning, the earth's atmosphere was mostly hydrogen and helium that eventually escaped earth's gravity and the atmosphere became mostly carbon dioxide, ammonia, methane and water vapor (Quan et al., 2009; Palaeos). It would seem likely that is a contributing factor to the sensitivity to carbon dioxide in almost all organisms and possibly a link to how it arose.

Other projects and collaborative studies

Throughout my years here at UK, I have been involved in a number of research projects other than those discussed in this dissertation. The areas of interest are varied but are linked through environmental effects on behavior and physiology. Such other areas discussed in abbreviated detail below address, (1) hypoxic stress, (2) exercise, (3) comparative chemosensory capabilities, (4) synchronous activity during a 'sympathetic-like' response and (5) neurochemical control of parasite manipulation.

Hypoxia stress: An area of interest is the physiological response of organisms that are forced from their environment. The voluntary act of leaving would not be assumed to be as stressful for the animal as environmental changes forcing the organism to react. To study this, I used crayfish and

removed the water from the animal's environment. It is unlikely that crayfish have a diving response as some mammals and birds, but the act of leaving water and walking on land results in dramatic alterations in weight (buoyancy). In addition, the animal lacks the typical escape reflex (tail flip) to escape predation on land. This type of forced environmental change is correlative to an organism that is forced to leave the residing environment for natural reasons. A foundation study from our lab, demonstrated that VR and HR rapidly change (< 3 sec) during an environmental alteration such as a pebble drop in a small holding aquaria when the animal is quiescent (Schapker et al., 2002). The areas of focus for this study was, (1) an autonomic response (i.e., heart rate (HR) and ventilatory rate (VR)), (2) oxygen debt incurred by forcing the organism out of the environment over varying time periods and (3) the stress response most likely not seen in the freely moving organisms. Study results indicate crayfish experience high levels of stress when there is a forced environmental change. The measured autonomic response showed that both HR and VR immediately increased. Although there were elevated HR and VR for crayfish allowed to move freely between the two environments, it seems these elevations correlated more to the actual bouts of movement. Forced condition crayfish showed a continued elevated effect and a heightened ventilation rate is seen upon reintroduction of water into the tank while this is not readily seen with the freely moving individuals. The behavioral migration into the air may be a natural response, specifically seen when the animal needs to escape a hypoxic environment unsuitable for survival. However, the key difference between migration and being forced from their environment is the stress factor involved with the latter of the two scenarios. Thus, these types of studies are good measures of environmental effects on organisms, specifically on crayfish in natural environments. Analysis in this study provides insights into the stress placed upon an organism and gives an understanding to physiological compensatory mechanisms in hypoxic environments, while examining hypoxic stress simultaneously.

Exercise: The area of interest and purpose of this study was to examine the effect of intermittent periods of exercise in normal and low-oxygen environments on the HR and VR of crayfish since very few studies focus on the whole animal autonomic response. Crayfish are freshwater crustaceans that reside mostly in water, exit for various reasons (i.e. food, mates, burrowing) and do gas exchange by pulling water over the gills. During periods of stress and physical exercise, crayfish exhibit typical “fight or flight” responses since they show a sympathetic-like response which appears to increase excitability to sustain physical activity. To meet the increase in oxygen demand due to exercise, a response in both cardiac HR and VR is required. Typically, both HR and VR increase in an effort to increase oxygen delivery to target tissues, especially muscles used for locomotion. Crayfish were exercised at normal walking speeds for two, fifteen minute periods in two environments, aerated (normal) and nitrogen-saturated (oxygen-deficient). As expected, results indicate increases in both HR/VR during the first and second periods of exercise. Interestingly, while HR elevated and then returned to normal levels relatively quickly, ventilation rates increased immediately and remained elevated throughout the entire recovery time period in the oxygen-deficient crayfish. This suggests the sensitivity of the crayfish ventilatory system in regulating homeostatic balance in different environments.

Comparative chemosensory: The area of interest was to understand the ‘sympathetic-like’ response seen in invertebrates by utilizing an autonomic response (HR) heart rate and (VR) ventilation rate in crayfish, (sighted) *Procambarus clarkii* and (blind) *Orconectes australis packardi*, during chemical introduction as well as establish chemical and/or modality sensitivities by targeting multiple sensory modalities. Most organisms show diversity in the type and amount of peripheral sensors that enable detection of different sensory stimuli within and across multiple sensory modalities. Variation in sensory pathways allows organisms to monitor their environment, integrate sensory information from multiple sources, and respond accordingly, due to refined integration of information. The aesthetasc / olfactory lobe pathway is a purely chemosensory pathway that originates in the prominent aesthetasc sensilla.

Aesthetascs are a nearly universal feature of crustacean antennules and depending on the species examined can be very densely innervated (Laverack and Ardill, 1965; Sandeman and Denburg, 1976; Spencer, 1986; Grunert and Ache, 1988; Mellon *et al.*, 1989; Mellon and Munger, 1990; Schmidt and Ache, 1992, 1996b; Hallberg *et al.*, 1997; Steullet *et al.*, 2000; Derby *et al.*, 2003). The importance of aesthetascs and non-aesthetascs in food-odor mediated behaviors was evaluated through antennular ablation and subsequent observation of resulting behavioral deficits (McLeese, 1973; Reeder and Ache, 1980; Devine and Atema, 1982). Several studies showed functional roles for the aesthetascs in different aspects of food odor mediated behaviors (Reeder and Ache, 1980; Devine and Atema, 1982). Interestingly, behaviors such as food odor discrimination, food odor learning, and searching could be clearly defined (Steullet *et al.,* 2001, 2002). This is particularly important for detection and information gathering from odor plumes emanating from sources in realistic flow conditions which are spatially and temporally complex (Webster and Weissburg, 2001). Several previous studies have demonstrated that distance chemoreception in decapod crustaceans is mediated primarily by antennular chemoreceptors (Hazlett, 1971a; Reeder and Ache, 1980; Devine and Atema, 1982; Kraus-Epley and Moore, 2002).

Most invertebrates possess chemosensory neurons that permit identification of environmental chemicals and are able to behave differentially between chemical compounds based upon the sensory pathway stimulated (i.e., attractive and/or repellant). Current literature shows this is particularly true for decapod crustaceans in detecting chemical signals, especially in the cephalic and thoracic appendages. Thus for this study, crayfish were used that rely on visual and chemical cues in the environment to further build on the wealth of knowledge for chemosensory use. Behavior studies alone often exclude “fight or flight” internal readiness changes and may conclude a lack of environmental awareness. Results suggest crayfish that show no behavioral response display an internal response through changes in HR/VR. Specifically, crayfish show an increase in HR with attractant chemical introductions (i.e., cysteine) suggesting a natural

response to potential food sources, while showing more pronounced responses to toxic/warning compounds. Future research will include using chemical stimuli identified as significant to induce electrical impulses to be recorded within antennular olfaction neurons. Supplemental experimentation will entail investigating the structure of antennular sensillae and associated nerve clusters.

Synchrony: The overall goal was to monitor ventilatory patterns and behavior when the organism is moving, not moving, during an environmental disturbance (i.e., startle response) and also when the organism elicits an escape response. Since crayfish are neither constant water-dwelling (i.e., like lobsters) nor mostly terrestrial (i.e., like land crabs), it is of interest to understand the synchrony/asynchrony of the ventilatory physiological response during free movement and various environmental disturbances. Previous experiments in other crustaceans such as lobster and crab have shown that the scaphognathites are able to function together as well as independently of one another. In decapod crustaceans, a modified portion of the second maxillae called the scaphognathites are used to ventilate the gill chambers. I also recorded heart rate simultaneously since scaphognathite system and heart rhythms have been shown to be connected by many command fibers resulting in the “autonomic-like” response noted in many crustaceans including crayfish. This study could be important in providing insights into physiological mechanisms of how an organism functions normally as well as how one might respond during fight or flight response. Study results indicate that show asynchrony similar to other species during normal movement. However, due to so much variation in the experiment implications are too speculative. Currently, more work is needed to understand autonomic response recruitment and synchronization.

Parasitic neuromodulation: I had the pleasure of conducting a project in collaboration with my previous Master’s degree laboratory at DePaul University in an area new to both of us. This project allowed me to use the knowledge I gained there as well as bringing knowledge from my current research area to address something very interesting. The project examined whether modification of behavior and physiology identified with parasite infection could be mediated by

neurochemical changes. The research team measured levels of serotonin (5-HT) and dopamine (DA) in the brains and nerve cords of naturally-infected and uninfected male isopods using HPLC. To provide an abbreviated general background, the acanthocephalan parasite *Acanthocephalus dirus* infects the freshwater isopod *Caecidotea intermedius* before completing its life cycle in a fish. Infection of male *C. intermedius* correlates with changes in color pattern, behavior and energy storage. Thus, to determine whether this modification by serotonin (5-HT) and dopamine (DA), the measure of brains and nerve cords help to provide measureable differences based upon infection status. We found that infected and uninfected males did not differ in levels of 5-HT and DA either individually or in combination; however, the mass and overall size of the VNC in infected individuals was double to that of uninfected. In accounting for tissue discrepancies, the neurohormones per tissue volume (as seems to be the standard in parasite literature) show a significant increase in both 5-HT and DA. These findings suggest a component of a common mechanism that could underlie parasite-related host modification in nature. Previous studies on other acanthocephalan-host relationships have shown that parasite infection can correlate with variation in neurochemical levels (Rojas and Ojeda, 2005; Poulin et al., 2003; Tain et al., 2006). However, these studies have also revealed that the nature of these relationships may be highly variable both within host species and among different parasite-host relationships. For example in the crab *Hemigrapsus crenulatus*, acanthocephalan infection correlates positively with changes in DA in one population but not another (Poulin et al., 2003; Rojas and Ojeda, 2005). Similarly, 5-HT correlates positively with acanthocephalan presence in an amphipod (*Gammarus pulex*, Tain et al., 2006), does not correlate with parasite infection in one crab species (*Hemigrapsus crenulatus*, Poulin et al. 2003) and correlates negatively with total parasite load (acanthocephalan, trematode, nematode) in another crab species (*Macrophthalmus hirtipes*, Poulin et al., 2003). This type of variation is consistent with other studies in crustaceans, which indicate that specific neurochemical function can be highly variable between closely related species (e.g., Pasztor

and Macmillan, 1990). Thus, it may be the case that neuromodulation is a relatively general mechanism in acanthocephalan-host relationships but that the specific mechanism used is dependent on each parasite-host relationship. Although, we cannot provide specific insights into the exact mechanism of this relationship at this time, studies are currently examining the relationship between 5-HT, DA and male mating behavior in more detail.

Why multiple roles of multiple sensory pathways

It has been shown time and time again that many sensory systems can benefit an organism in several important ways (Derby and Steullet, 2001). Multiple overlapping sensors allow an animal to continue to function normally in the likelihood of loss or damage to a subset of sensors in a any one system (Derby and Steullet, 2001). The combination of inputs from two or more pathways may allow for much greater sensitivity than any pathway alone could provide, as suggested by some of the results of this study. Response summation allows for even very weak signals to be effectively detected and amplified by the central processing centers. Distribution of neurons into different sensory pathways may allow for simultaneous processing of different stimulus attributes. For instance, in chemosensory, one pathway may provide information about the quality of an odor signal, while a second pathway may provide information about the location or spatial distribution of the signal. This is further seen to be important when taking into account learning form experience and memory formation. In general, differently organized pathways could also mediate different physiological or behavioral responses to the same signal. An example is seen with environmental toxicity in which behavioral and physiological response are required. The combined activity of multiple sensory pathways in these examples would provide a more complete picture of the stimulus and could potentially allow for more complex behavioral responses as well as allowing the organism to detect and respond in changing environments.

The comparative nature taken in this dissertation examining behavior, physiological processes and synaptic transmission at the NMJs and in a neural circuit was not only extremely fun and interesting but also very helpful. It is with this enthusiasm that I will approach and challenge myself in my future scientific career.

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Bierbower, S.M., Cooper RL (2009). Measures of Heart and Ventilatory Rates in Freely Moving Crayfish. JoVE. 32. http://www.jove.com/index/details.stp?id=1594, doi: 10.3791/1594

Bierbower, S.M., Sparkes TC. (2007) Parasite-related success in an intermediate host, *Caecidotea intermedius* (Isopoda): effects of male behavior and reproductive physiology. J. Parasitol. 93 (3): 445-449.

Bierbower, S.M., Shuranova, Z. P. and Cooper, R.L. (2009) Comparative Study of Environmental Factors Influencing Motor Task Learning and Memory Retention in Blind and Sighted Crayfish. Frontiers in Neuroscience, ***(In Submission)***

Bierbower, S.M., Cooper, R.L. (2009) The Effects of Acute Carbon Dioxide on Behavior and Physiology in Procambarus clarkii. Comp. Biochem. Physiol. A. **(In Submission)**

Currently in Manuscript

Sparkes, T.C., **Bierbower, S.M.,** Mormann, K., Kopp, D. and Murphy, A.D. *(2009)* Serotonin and dopamine levels in the CNS of the intermediate host *Caecidotea intermedius (Isopoda):* Implications for

acanthocelphalan-related host modification. *Parasitology. Revising Manuscript.*

Wiggington, A., Xu, S., **Bierbower, S.M**., and Cooper, R.L. Effects of Cadmium on Behavior and Physiology in crayfish, *Procambarus clarkii*.

Xu, S., **Bierbower, S.M.,** and Cooper, R.L. The role of insecticides, Pyrethrum and Carbaryl on neural circuits in crayfish. ***In Manuscript***

Bierbower, S.M. and Cooper, R.L. CO2 Effect on neural circuitry of an identified behavior. ***In Manuscript.***

GUEST LECTURE / INVITED PRESENTATIONS

2006 Center for Ecology and Evolution, **"**Parasite-mediated sexual selection in an intermediate host, *Caecidotea intermedius* (Isopoda): effects of male mating response and sperm supplies", University of Kentucky

2006 Animal Physiology (core biology course): Ionic and Osmotic Balance, University of Kentucky

2006 General Biology (core biology course): Evolutionary Adaptations of Vampire Bats, DePaul University

2006 Non-majors General Biology course: Origins of Life, DePaul University

AWARDS/ FELLOWSHIPS/APPOINTMENTS

2009 Graduate Student Recognition of Excellence – Department Website Spotlight

2009 Gertrude Flora Ribble Graduate Fellowship (RGF) in Biology

2009 Ribble Mini Research Grant

2009 College of Arts and Science Excellence in Teaching Recognition

2009 First Place Award for Scientific Presentation – 2nd Annual Cognitive Science Symposium.

2008 Bluegrass Chapter - Society for Neuroscience Graduate Student Representative.

2008 Graduate Student Congress Biology Graduate Student Representative.

2008 Biology Graduate Student Association Faculty Meeting Representative.

2007 Tuition Fellowship, Friday Harbor Laboratory Summer Courses: Neuroethology

2007 Friday Harbor Travel aid, Biology Department, University of Kentucky

2007 Best Poster by a 1st/2nd year graduate student, University of Kentucky, Biology Department.

2006 Travel Award – Society for Integrative and Comparative Biology (SICB)

2006 Travel Award – Biology Department, DePaul University (Annual Animal Behavior Society Conference)

TEACHING EXPERIENCE

Animal Physiology, University of Kentucky

Spring 2009, Fall 2008, Summer 2008, Spring 2008, Fall 2007, Summer 2007, Spring 2007, Fall 2006, Summer 2006.

Aquatic Biology, DePaul University
Spring 2006.

General Biology, DePaul University
Winter 2005, Winter 2004.

Ecology, DePaul University
Fall 2005.

Genetics, DePaul University
Spring 2005.

MANUSCRIPTS REVIEWS

Reviewed manuscripts/chapters from the following journals or publishers since 2009

Journal of Biological Sciences 2009

MENTORING

Undergraduates

1. Jessie Simpson (Spring 2010)
2. Zach Raney (Fall 2009)
3. Logan Forsythe (Spring 2009)
4. Allison Gilberts (Spring 2009, Fall 2009)
5. Michael Baker (Fall 2008, Spring 2009, Fall 2009)
6. Yuri Boyechko (Fall 2008)
7. Raymond Geyer (Summer 2008)
8. Madison Allen (Summer 2008)
9. Jessica McQuerry (Summer 2008, Fall 2008, Spring 2009)
10. Maddy Delgado (High School Student, Summer 2008)
11. Joshua Eason (Summer 2008)
12. Joe Mando (Summer 2008, Fall 2008)
13. Becca Liberty (Summer 2008, Fall 2008, Spring 2009)
14. Vadim Galperin (Summer 2008, Fall 2008, Spring 2009)
15. Jin-Young (Summer 2008, Fall 2008)
16. Barbie Kelly (Summer 2008, Fall 2008, Spring 2009)
17. Jennifer Jackson (Summer 2008, Fall 2008)
18. Shelly Xu (*Spring, 2008, Summer 2008, Fall 2008)*
19. Martha Robinson (*Spring, Summer, Fall 2008, Spring 2009)*
20. Courtney Allen (*Spring 2008, Fall 2008)*
21. Easter Bocook (*Spring, Summer, Fall 2008, Spring 2009)*
22. Mary Catherine Wright (*Fall 2007, Spring 2008)*
23. Keith Nicholas Holmes (*Fall 2007, Spring 2008)*
24. Tori Lynn Spence (*Spring, Summer, Fall 2007, Spring 2008)*
25. Doyle Stephens (*Spring, Summer, Fall 2007, Spring 2008)*

26. Tyler McLaurine (*Spring, Summer, Fall 2007, Spring 2008)*
27. Thomas Cunningham (*Spring 2007)*
28. Geoffry Hughes (Spring 2007*)*
29. Alexandra Assalley (Winter 2005, Spring 2006)
30. Bill Toliopoulos (Winter 2005, Spring 2006)
31. Daniel Elke (Winter 2005, Spring 2006)
32. Lizbeth Rodriquez (Winter 2005, Spring 2006)
33. Darin Kopp (Spring, Fall 2005, Winter 2005, Spring 2006)

COMMUNITY SERVICE

2009 Kentucky State Science Fair
2009 University of Kentucky Regional Science Fair
2009 Glendover Elementary School Science Fair
2009 Morton Middle School Science Fair
2008 University of Kentucky Regional Science Fair
2008 Glendover Elementary School Science Fair
2008 Morton Middle School Science Fair
2007 Glendover Elementary School Science Fair
2007 Morton Middle School Science Fair
2007 University of Kentucky Science Fair

COMMUNITY SERVICE PROJECTS

1. **Two ongoing projects with Woodford High School and Wolf County High School**. *These schools have take on a thematic approach to science education. They are using the crayfish as a model organism in studying, behavior, life cycle, environmental impact on crayfish, and economic use in modeling of shrimp farming. I will be working closely with the teachers and their students on their projects over the Fall 2007 and Spring 2008 terms. See www site: http://www.as.uky.edu/Biology/faculty/cooper/TFC/crayfish.htm*
2. **Bluegrass Chapter - Society for Neuroscience**- **Brain Awareness Week**. *Participation in educating parents and children at the Children's explorium in how the brain works and functions. Individual stations were designed to teach children about sensory and the different parts of the brain, as well as reflexes and ways to help in healthy brain development.*
3. **Children's Explorium** – *Participation in educating parents and children by designing and conducting a presentation. The presentation demonstrates evolutionary traits in two differing populations of crayfish, as well as teaches about sensory adaptations.*
4. **Bluegrass Chapter - Society for Neuroscience** - Town-hall Meeting.
5. **Big Brothers/Big Sisters of the Bluegrass –** *Participation in helping a child with individual attention.*

COLLABORATIONS

1. **Motor Task Learning**
   Dr. Zhana P. Shuranova, Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow Russia
2. **Parasite-related effects on the CNS of an intermediate host**
   Dr. Timothy C. Sparkes, DePaul University, Chicago, IL
3. **Statistical Analysis of Biological Systems**
   Dr. Kert Viele, Depart. of Statistics, University of Kentucky, Lexington, KY
4. **Cadmium effects on neural circuitry in crayfish**
   Dr. Andy Wiggington, University of Kentucky, Lexington, KY

CONFERENCE PRESENTATIONS

(● indicates mentored undergraduate, * indicates presenting author)

●Robinson, M.*, Cooper, A.S.*, **Bierbower, S.M.,** Cooper, R.L. (2009) Using Heart Rate as a Bioindex to Assess Various Sensory Perceptions in Sighted and Non-sighted Crayfish: The Effects of Serotonin on Circadian Pattern and Behaviors in Drosophila. Poster's at the Capital, Frankfort, Kentucky.

●Baker, M*., Robinson, M., **Bierbower, S.M.**, Cooper, R.L. (2009) Autonomic Response to Multiple Sensory Modalities in Crayfish. Kentucky Academy of Science Annual Meeting, November 13, 2009.

●Robinson, M.*, ●Baker, M., **Bierbower, S.M.**, Cooper, R.L. (2009) Using heart rate as a bioindex to assess various sensory perceptions in non-sighted cave crayfish. Kentucky Academy of Science Annual Meeting, November 13, 2009.

Bierbower, S.M.*, Cooper, R.L. (2009) Synaptic mechanisms underlying carbon dioxide's induced paralysis. Society for Neuroscience Annual Meeting, Chicago, IL. October 17-21, 2009.

●Robinson, M.*, ●Baker, M., **Bierbower, S.M.**, Cooper, R.L. (2009) Across species comparison of the autonomic response of multiple sensory modalities in crayfish. Society for Neuroscience Annual Meeting, Chicago, IL. October 17-21, 2009.

● Liberty, B.*, ●Bocook, E., ●McQuerry, J., **Bierbower, S.M.**, and Cooper, R.L. (2009) Comparative study of quantifiable environmental factors modulating intrinsic behavior in crayfish . 4th Annual Showcase of Undergraduate Scholars, University of Kentucky, Lexington, Kentucky.

●Kelly, B.*, **Bierbower, S.M**. and Cooper, R.L. (2009) Paralytic effect of carbon dioxide on an identified behavior: Role of CNS. 4th Annual Showcase of Undergraduate Scholars, University of Kentucky, Lexington, Kentucky.

●Gilberts, A.*, **Bierbower, S.M**. and Cooper, R.L. (2009) CNS control of scaphognathite patterns during a 'sympathetic-like' response in crayfish. 4th Annual Showcase of Undergraduate Scholars, University of Kentucky, Lexington, Kentucky.

●Forsythe, L.*, **Bierbower, S.M.** and Cooper, R.L. (2009) Environmental factors influencing motor task learning and retention in crayfish. 4th Annual Showcase of Undergraduate Scholars, University of Kentucky, Lexington, Kentucky.

●Galperin, V.*, ●Kelly, B., **Bierbower, S.M**. and Cooper, R.L. (2009) Stress response due to inhibition of completing a learned motor task in crayfish. 4th Annual Showcase of Undergraduate Scholars, University of Kentucky, Lexington, Kentucky.

●Baker, M.*, ●Robinson, M., **Bierbower, S. M.,** and Cooper, R. L., (2009). Across species comparison of the autonomic response of multiple sensory modalities in crayfish. 4th Annual Showcase of Undergraduate Scholars, University of Kentucky, Lexington, Kentucky.

Bierbower, S.M.*, Cooper, R.L. (2009) Crayfish Learning and Task Retention. Center for the Integrative Study of Animal Behavior Conference on Friday April 10th at Indiana University.

●Robinson, M. *, ●McLaurine, T., ●Spence, T., **Bierbower, S.M.** and Cooper, R.L. (2009) Comparison of the autonomic response of multiple sensory modalities in crayfish. Society for Neuroscience Bluegrass chapter. March 18, 2009 Univ. of KY

Bierbower, S.M.*, Cooper, R.L. (2009) Motor Task Learning in Crayfish: Task Retention. 2nd Annual UK Cognitive Science Symposium. March 7, 2009.

●Robinson, M. *, ●Mando, J., ●Baker, M., **Bierbower, S.M.** and Cooper, R.L. (2009) Across species comparison of the autonomic response of multiple sensory modalities in crayfish. 23rd National Conference on Undergraduate Research (NCUR), University of Wisconsin-La Crosse, La Crosse, Wisconsin. April 17, 2009.

●Robinson, M.*, ●Baker, M., **Bierbower, S.M.**, Cooper, R.L. (2009) Across species comparison of the autonomic response of multiple sensory modalities in crayfish. 23rd National Conference on Undergraduate Research (NCUR), University of Wisconsin-La Crosse, La Crosse, Wisconsin. April 17, 2009

Bierbower, S.M.*, Cooper, R.L. (2009) Motor Task Learning in Crayfish: Task Retention. Cognitive Science Symposium. Annual meeting. March 7, 2009, Lexington, KY (Univ. of KY campus).

Bierbower, S.M., Cooper, R.L.* (2009) Effect of exercise and environment on the autonomic response in crayfish, Procambarus clarkia. Society for Integrative and Comparative Biology. Annual meeting. January 2-6, 2009, Boston, Mass.

Bierbower, S.M.*, Cooper, R.L. (2008) The effect of CO_2 on the neural circuitry of an identified behavior. Society for Neuroscience Annual Meeting, Washington, D.C.

●Robinson, M.*, ●McLaurine, T., ●Spence, T., **Bierbower, S.M.**, Cooper, R.L. (2008) Comparison of the autonomic response of multiple sensory modalities in crayfish. Society for Neuroscience Annual Meeting, Washington, D.C.

●Bocook, E. *, ●Liberty, B., ●Mcquerry, J., **Bierbower, S.M.**, Cooper, R.L. (2008) Social Interactions: Influence of sensory cues and environmental conditions on fighting strategy in blind crayfish. Kentucky Academy of Sciences. Annual meeting. November 1, 2008, Lexington, KY (Univ. of KY campus).

●Liberty, B. *, ●Mcquerry, J., ●Bocook, E., **Bierbower, S.M.**, Cooper, R.L. (2008) The role of sensory cues and environmental conditions on the fighting strategy in sighted crayfish. Kentucky Academy of Sciences. Annual meeting. November 1, 2008, Lexington, KY (Univ. of KY campus).

●Allen, C. *, ●Naik, S., **Bierbower, S.M.**, Cooper, R.L. (2008) Can blind crayfish learn a motor task? Kentucky Academy of Sciences. Annual meeting. November 1, 2008, Lexington, KY (Univ. of KY campus).

●Robinson, M. *, ●Mando, J., ●Baker, M., **Bierbower, S.M.**, Cooper, R.L. (2008) Across species comparison of the autonomic response of multiple sensory modalities in crayfish. Kentucky Academy of Sciences. Annual meeting. November 1, 2008, Lexington, KY (Univ. of KY campus).

●Boyechko, Y. *, ●Galperin, V., **Bierbower, S.M.**, Cooper, R.L. (2008) Long-Term Memory Retention in Crayfish. Kentucky Academy of Sciences. Annual meeting. November 1, 2008, Lexington, KY (Univ. of KY campus).

●Kelly, B. *, **Bierbower, S.M.,** Cooper, R.L. (2008) The Effects of CO2 on Behavior and Physiology in Crayfish. Kentucky Academy of Sciences. Annual meeting. November 1, 2008, Lexington, KY (Univ. of KY campus).

Bierbower, S.M.*, Cooper, R.L. (2008) Comparative study of environmental modulation of intrinsic behavior in blind and sighted crayfish. 15th Annual meeting. Center for the Integrative Study of Animal Behavior Conference. Indiana University, Bloomington, IN.

Bierbower, S.M.*, Cooper, R.L. (2008) The mechanistic effects of CO2 on physiology and behavior in Procambarus clarkii. Annual meeting of the BlueGrass Chapter of the Society for Neuroscience. University of Kentucky.

●McLaurine, T.*, ●Robinson, M., ●Spence, T., **Bierbower, S.M.,** Cooper, R.L. (2008) The role of olfactory: comparison of the autonomic response of multiple sensory modalities in crayfish. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

●Stephens, D.*, **Bierbower, S.M.,** Cooper, R.L. (2008) The effect of CO2 on behavior and physiology in crayfish. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

●Allen, C.*, ●Naik, S., **Bierbower, S.M.,** Cooper, R.L. (2008) Learning and memory in blind crayfish, Orconectes australis packardi. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

●Wright, M.C.*, ●Bocook, E., **Bierbower, S.M.,** Cooper, R.L. (2008) Effects of olfaction and environment on agonistic behavior in the crayfish, *Procambarus clarkii*. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

●Holmes, K.*, **Bierbower, S.M.,** Cooper, R.L. (2008) Effects of exercise duration and environment on the autonomic response in crayfish, *Procambarus*

clarkii. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

●Naik, S.*, **Bierbower, S.M.,** Cooper, R.L. (2008) Learning and memory in crayfish. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

●Bocook, E.*, **Bierbower, S.M**., Cooper, R.L. (2008) A quantifiable measure of interaction intensity influenced by environmental factors in blind crayfish. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

●Stephens, D.*, **Bierbower, S.M.,** Kolasa, J., Adami, M., Cooper, R.L. (2008) Heart and ventilatory measures in crayfish during altered environments. Annual meeting of the BlueGrass Chapter of the Society for Neuroscience, University of Kentucky.

Bierbower, S.M.*, Cooper, R.L. (2008) The mechanistic effects of CO2 on physiology and behavior in Procambarus clarkii. Annual meeting of the BlueGrass Chapter of the Society for Neuroscience, University of Kentucky.

●Spence, T.*, ●McLaurine, T., **Bierbower, S.M.,** Cooper, R.L. (2008) Chemosensory induced behavioral and physiological responses in crayfish. NCUR- National Council on Undergraduate Research. Salisbury University, Salisbury, MD.

●Spence, T.*, **Bierbower, S.M**., Cooper, R.L. (2007). Chemosensory: Identification of the Physiological Response during Chemical Introduction. Kentucky Academy of Sciences, University of Louisville, Louisville, KY.

Bierbower, S.M.*, Cooper, R.L. (2007) The mechanistic effects of CO2 on physiology and behavior in Procambarus clarkii. Society for Neuroscience Annual Meeting, San Diego, CA. 359.1

Chaffins, G.*, **Bierbower, S.M**., Lund, J. (2007) Identification of genes affe3cting stress resistance and lifespan in C. elegans. 16th International C. elegans Meeting, University of California, Los Angeles, CA.

Bierbower, S.M.*, Cooper, R.L. (2007) Behavior and Physiology: Now We Have A Complete Picture. University of Kentucky Department of Biology Graduate Student Association Poster Session, Lexington, Kentucky.

●Spence, T.*, ●McLaurine, T., **Bierbower, S.M.,** Cooper, R.L. (2007) Sensory: Do Some Species Do It Differently? Showcase for Undergraduate Scholars, University of Kentucky, Lexington, KY.

●Stephens, D.*, **Bierbower, S.M.,** Cooper, R.L. (2007) Crayfish: Let's Get Physical. University of Kentucky, Showcase of Scholars (2nd annual undergraduate research event).

Kolasa, J.*, **Bierbower, S.M**., Adami, M., Cooper, R.L. (2007) Physiological Acclimization in crayfish among environment alterations and social interactions. Southeastern Nerve Net, Orlando, Florida.

●McLaurine, T.*, **Bierbower, S.M.,** Cooper, R.L. (2007) CO_2: How Bad Could It Be? Showcase for Undergraduate Scholars, University of Kentucky, Lexington, KY.

Bierbower, S.M.*, Cooper, R.L. (2007) Behavior and Physiology: Now We Have A Complete Picture. University of Kentucky, Department of Biology Graduate Student Association Poster Session, Lexington, Kentucky.

Kolasa, J.*, **Bierbower, S.M**., Adami, M., Cooper, R.L. (2006) Heart and ventilatory measures in crayfish during altered environments and social interactions. Society for Neuroscience Annual Meeting, Atlanta, GA.

Badre, N.*, Hayden, B., Kolasa, J., ●Hughes, G., **Bierbower, S.M.**, Adami, M., Desai, M. (2006) Research in Neurophysiology: Calcium's role in Synaptic Transmission, Facilitation, and Behavioral Regulation. Poster's at the Capital, Frankfort, Kentucky.

●Hughes, G.*, Kolasa, J., **Bierbower, S.M.**, Adami, M., Cooper, R.L. (2006) Heart and ventilatory measures in crayfish during altered environments and social interactions. Kentucky Academy of Sciences, Moorehead, Kentucky.

Bierbower, S.M.*, Cooper, R.L. (2006) Evidence for the autonomic nervous system in decapod crustaceans: a historical perspective. Annual Meeting of Society for Neuroscience, Atlanta, Georgia

Bierbower, S.M.*, Sparkes, T.C. (2006) Parasite-mediated sexual selection in an intermediate host, *Caecidotea intermedius* (Isopoda): effects of male mating response. Annual Meeting of Society for Neuroscience, Atlanta, Georgia

Bierbower, S.M.*, Sparkes, T.C. (2006) Parasite-related changes in the mating behavior of the intermediate host, *Caecidotea intermedius* (Isopoda): is modification dependent on ecological context? Annual Meeting of the Animal Behaviour Society, Snowbird, Utah

Bierbower, S.M.*, Sparkes, T.C. (2006) Parasite-mediated sexual selection in an intermediate host, *Caecidotea intermedius* (Isopoda): male mating response, sperm viability and energetic state. Midwest Ecology and Evolution Conference, St. Louis University, St. Louis, Missouri

Bierbower, S.M.*, Sparkes, T.C. (2006) Parasite-mediated sexual selection in an intermediate host, *Caecidotea intermedius* (Isopoda): effects of male mating response, sperm production and energetic state. Annual Meeting of the Society of Integrative and Comparative Biology, Orlando, Florida

Bierbower, S.M.*, Sparkes, T.C. (2005) Parasite-related changes in male mating behavior in an intermediate host; effects of sperm supplies. Annual Meeting of the Animal Behaviour Society, Snowbird, Utah

PROFESSIONAL SOCIETIES

The *Sigma Xi* Scientific Research Society
AAAS
Society for Neuroscience
Animal Behavior Society
American Society for Parasitologists
Society for Conservation Biology

UNDERGRADUATE RESEARCH

Research Assistant: University of Georgia – Complex Carbohydrate Research Center (CCRC), Athens, GA. *The research examined the Harpin Z protein produced by the plant pathogenic bacterium* Pseudomonas syringae pv. Syringae. *We developed an efficient protocol to isolate the protein in large quantities and performed preliminary analyses to determine the cell wall component that binds Harpin Z.*

Research Assistant: University of Georgia – Psychology Department, Athens, GA. *The research examined Capuchin monkey motor task skills and the correlation with memory.*

TECHNICAL SKILLS

Electrophysiology (Intracellular, Extracellular)
Statistical Analysis Software
Data Analysis Software (SigmaPlot, Chart, Scope)
Organismal Dissections
DNA and RNA Isolation techniques
Western Blot Analysis
DNA Gel Electrophoresis
SDS-PAGE Protein Gel Electrophoresis
PCR
Gas Chromatography
HPLC
Organism Sectioning Techniques
Ability to design and conduct field-based experiments
Physiology: Autonomic Response experimentation
Learning paradigm development and experimentation

PROFESSIONAL SCIENCE APPOINTMENTS

2004 TAP Pharmaceuticals, *Research and Development*
2002 Pinnacle Priority Group, *Research and Development*

RESEARCH INTERESTS

Neurophysiology
Neuroethology
Neuropharmacology
Neuroscience
Behavioral Ecology
Animal Behavior
Parasite-Host Relationships

REFERENCES

Robin L. Cooper, Ph.D
Department of Biology,
University of Kentucky
Ph.D Advisor

Mark Prendergast, Ph.D
Department of Psychology
University of Kentucky
Committee Member

Philip Bonner, Ph.D
Department of Biology
University of Kentucky
Committee Member

Craig Sargent, Ph.D
Department of Biology
University of Kentucky
Committee Member