Effect of Social Status on the Behavior and Neurophysiology of Crayfish

Fadi Aziz Issa

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EFFECT OF SOCIAL STATUS ON THE BEHAVIOR AND NEUROPHYSIOLOGY OF CRAYFISH

By

FADI AZIZ ISSA

Under the direction of Dr. Donald H. Edwards

ABSTRACT

Adaptation to changing social conditions is important for many social animals. Here, the effects of social experience on the behavior and neurophysiology of the red swamp crayfish, *Procambarus clarkii*, were studied. Evidence is presented that shows juvenile crayfish interact and form social order, and their behavior patterns shift in accordance to social status. Dominant animals maintain a high level of aggressive behavior, while subordinates shift their behavior pattern from aggressive to submissive behavior. Adult male crayfish show similar behavior pattern during dominance formation. However, this work demonstrates that male crayfish adopt a unique strategy to signify the formation of a social order expressed in the form of pseudocopulation. Pseudocopulation between male crayfish signifies the acceptance of the social status and leads to the reduction of aggression of dominants and enhances the survival of subordinate animals.

I investigated the long-term effects of social status on the behavioral and physiological responses of crayfish to unexpected sensory touch. I discovered that animals of different social experience display different orienting responses that correlate with *in vivo* electromyographic recordings from the legs’ depressor muscle. The status-
dependent response patterns observed \textit{in vivo} are retained in a reduced, \textit{in vitro}, preparation that lacks descending input from the brain. The role of serotonin (5-HT) was investigated in mediating the motor output patterns of the depressor nerve. Putative serotonergic innervations of the depressor nerve were identified that originate from serotonergic neurons located in the first abdominal ganglion. Selective stimulation of the ipsilateral 5-HT neuron enhances the response of the depressor nerve to sensory stimulation. Application of 5-HT modestly increased the tonic firing activity of the depressor nerve in social isolates and subordinates but significantly decreased the activity in dominants. This work illustrates that the formation of a dominance relationship significantly and immediately alters the behavior of the participants. As the social relationship matures, the social experience that develops affects the underlying neurophysiology that mediates the behavior. It will be of great interest in future studies to identify not only the effects rather the mechanisms of how social experience induces physiological changes.

\textbf{Index Words:} Aggression, Behavior, Crayfish, Dominance, Invertebrate, Motor Control, Neuromodulation, Serotonin, Physiology, Pseudocopulation.
EFFECT OF SOCIAL EXPERIENCE ON THE BEHAVIOR AND NEUROPHYSIOLOGY OF CRAYFISH

by

FADI AZIZ ISSA

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EFFECT OF SOCIAL EXPERIENCE ON THE BEHAVIOR
AND NEUROPHYSIOLOGY OF CRAYFISH

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It is said that the social company is what defines a person. I believe that this cannot be more true. I owe a great deal of gratitude to so many people that shared my life and contributed significantly to my scientific and personal development during my graduate studies. In many respects, they defined the person that I am.

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<tr>
<td>CBCO</td>
<td>Coxo-Basipodite Chordotonal Organ</td>
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<td>LG</td>
<td>Lateral Giant</td>
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<td>MG</td>
<td>Medial Giant</td>
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<td>MN</td>
<td>Motor neuron</td>
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<td>NG</td>
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<td>Offensive Tailflip</td>
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<td>P-c</td>
<td>Pseudocopulation</td>
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<td>Tailflip</td>
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<td>Serotonin</td>
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CHAPTER I

GENERAL INTRODUCTION

Social interactions that lead to hierarchy formation are a major driving force in shaping animal behavior and much work is conducted documenting their effects. One behavioral element adopted by many social species is aggressive behavior. Despite the obvious drawbacks aggressive behavior poses on group members, its continued and widespread presence attests to its usefulness as an organizing mechanism in shaping animal societies. The degree of agonistic interactions varies among species but its underlying objective of establishing dominance and social cohesion is universal. The social interactions and the ritualized behaviors evolved to form dominance hierarchies have been studied extensively. What emerged is a more comprehensive understanding of how different species adopt different behavioral strategies and social hierarchies in order to achieve optimal and peaceful coexistence among group members.

In recent years it has become apparent that social dominance not only plays a major part in shaping animal behavior but greatly influences nervous system function as well. The dynamic interactions and feedback loops among gene expression, cellular functions and behavior, the neural and neuroendocrine mechanisms that underlie social dominance are not well understood. Despite these complexities, some neurochemicals that play a significant role during dominance interactions have been identified. Among the most studied of these is serotonin (5-HT), whose functional role during social dominance formation will occupy the primary focus of the dissertation (Edwards and Kravitz, 1997; Miczek et al., 2002). In vertebrates, serotonin levels negatively correlates with social status (Manuck et al., 2006; Miczek and Fish, 2006). In fish and monkeys,
the levels of 5-HT or its metabolites, 5-HIAA, are lower in dominant animals than in subordinates (Winberg et al., 1997; Fairbanks et al., 2004). Conversely, serotonin levels and social status positively correlates with social status in crustaceans as reported in shore crabs (Sneddon et al., 2000). In crabs, normal levels of 5-HT in the blood are higher in winners as compared to losers, and they increase after agonistic interactions in winners but not in losers. In crayfish, however, CNS levels of 5-HT do not differ between new dominants and subordinates shortly after dominance formation (Panksepp et al., 2003).

Various animal model systems such as rats (Kozorovitskiy and Gould, 2004; ), crayfish (Yeh et al., 1996), lobsters (Sullivan et al., 2007) and marmosets (Kozorovitskiy et al., 2005) have been developed to study the effects of social interactions and social dominance on the function of the nervous. Each of these animal models provides distinct empirical advantage for addressing the particular question of interest. The work presented here was conducted on crayfish because of the three distinct advantages they provide to address the influence of social dominance on changes in behavior and of neural circuits function. First, crayfish are social animals that frequently engage in agonistic interactions that lead to the formation of stable social hierarchies (Bovbjerg, 1953; Lowe, 1956; Issa et al., 1999). Their social interactions are easily reproduced, measured and quantified in a controlled laboratory setting. Second, the neural networks that mediate many of the behaviors have been characterized anatomically and physiologically (Edwards et al., 1999; Cattaert & Le Ray, 2001; Clarac et al., 2000; Wine & Krasne, 1982). Specifically, the neural controls of leg movements during posture and walking have been described (Cattaert & Le Ray, 2001). Finally, the role of the
neuromodulator serotonin (5-HT) on the neural circuit controlling leg movement has been extensively studied. Serotonin is shown to modulate synaptic transmission (Pearlstein et al., 1998; Wang and Zucker, 1998; Southard et al., 2000) and cellular properties of the neurons that control leg movements (Rossi-Durand, 1993; Alvarado-Alvarez et al., 2000; Nagayama, 2002). Serotonin is also shown to influence posture, walking patterns (Tierney and Mangiamele, 2001), aggressiveness (Huber et al., 1997a,b), and social interactions of freely behaving crayfish (Huber et al., 1997b, 2001). The extent of our knowledge of the behavior and physiology of crayfish and the accessibility of their nervous system make them a great model to study the neural basis of social behavior.

In this chapter, I will provide a general background on the social behavior of crayfish and other crustaceans that will be relevant to the topics presented in subsequent chapters. My focus will be primarily on four well-studied topics (Social interactions, tailflip escape, posture, and walking) and the underlying neural networks mediating the later three behaviors. I will present these topics in the context of how dominance formation influences their patterns of activation. The intent of this introduction is to provide relevant but rather general background information to which the reader can refer. Finally, I will conclude with a brief summary of the main findings of my dissertation work.

**Crayfish social behavior**

Crayfish are fresh water animals with a wide range of habitat: from the great lakes to the Louisiana swamps. Over two hundred species of crayfish have been identified. They can be found along the banks of streams, in lakes, in caves and in
burrows. Crayfish populations can be rather dense up to seventy crayfish per square meter. Burrows are often clustered together that naturally lead to large gathering of animals at burrow openings that compete for food, burrows and access to mates. Competition for these resources is in the form of agonistic interactions. As with many social animals there are advantages to being socially dominant. Large dominant animals attack and chase smaller individuals from burrows. These interactions can be frequent because crayfish repeatedly return to the same burrows, and they reside in the same area for an extended time and may form groups that interact frequently (Davis & Huber, 2007).

Most of our knowledge of crayfish social behavior comes from observations in the laboratory where animals are usually matched in size and each animal has little opportunity to avoid others in the same aquarium. Thus, the levels and duration of agonistic interactions are most likely higher and longer than in the wild where there are more frequent opportunities to escape (Goessman et al., 2001; Peeke et al., 2000).

The first laboratory studies on crayfish agonistic behavior showed that small groups of crayfish form linear dominance hierarchies (Bovbjerg, 1953; Lowe, 1956). These dominance hierarchies are usually transitive, with an alpha animal that is dominant over all group members, followed by a beta animal that is dominant to all others but alpha, and a gamma that is subordinate to all. Size is the predominant factor that determines dominance order (Pavey and Fielder, 1996); however, more recent work demonstrated that individual aggressiveness (Issa et al., 1999) and experience can be important factors as well (Lowe, 1956).
During dyadic interactions, dominance is also determined primarily by the relative sizes of the two animals, although other factors such as residence status, sex, and molting state are important (Figler et al., 1995, 1999). A significant difference in size between animals usually leads to brief agonistic interactions where the smaller animal avoids the larger animal by either retreating or tailflipping away. However, evenly matched animals engage in an increasingly more aggressive series of interactions that begin with assessing each other’s size, strengths and weaknesses. Both animals begin by extending their claws forward and laterally toward their opponent, thus, forming a mirrored image as the tips of their claws touch (Bruski and Dunham, 1987; Huber and Kravitz, 1995). This “meral spread” is identical to the defense posture that is evoked by visual looming stimuli (Atwood and Wiersma, 1967; Kelly and Chapple, 1990). If this size comparison fails to resolve the dominance relationship, the two animals perform a dance very similar to that seen in lobsters (Huber and Kravitz, 1995), in which one animal advances and the other retreats, followed by a reversal of direction (Goessmann et al., 2001). As each animal moves forward, it lashes the other with its antennae while ejecting urine from the antennal glands directly toward the opponent (Breithaupt and Eger, 2001; Bushmann and Atema, 1994). This stage is followed by one in which the animals grasp each other with their claws and wrestle, each trying to turn the other over and move it about. If neither animal retreats then fighting escalates during which time one animal will finally decide to escape by either retreating away or performing one of the tailflip escapes to rapidly move away from its opponent.
**Tailflip escape behavior in crayfish**

Crayfish can produce one of three different types of tailflip escape responses, each is produced by a different set of neural circuits. The three different tailflips are called the lateral giant tailflip (LG), medial giant tailflip (MG) and non-giant mediated tailflip (non-G). Two of the tailflip types are mediated by giant interneurons. The lateral giant (LG) interneurons respond to a phasic tactile stimulus on the abdomen with a single spike that activates pre-motor interneurons and motor neurons in a highly stereotyped manner to produce an equally stereotyped tailflip escape response (Edwards et al., 1999; Wine and Krasne, 1982). The tailflip results from a strong, rapid flexion of the anterior abdominal joints and simultaneous promotion of the uropods. This causes the animal to “Jack-knife” upward and forward, away from the attack. The pair of medial giant (MG) interneurons responds to similar strong, phasic tactile stimuli to the front of the animal, or to rapidly looming visual stimuli. A single spike in the MGs moves caudally along both sides of the nerve cord and excites the same pre-motor interneurons and motor neurons in a different segmental pattern to produce a pattern of rapid flexion at each abdominal joint. This pattern thrusts the animals backward and away from the frontal attack. The non-giant circuit is much less understood, but is known to consist of a set of non-giant interneurons that excite sets of abdominal fast flexor motor neurons in a pattern that will carry the animal away from the point of attack. Non-giant tailflips are evoked by more gradually developing noxious stimuli such as pinching a limb, but can also be produced “voluntarily,” in which the animal tailflips in response to no obvious stimulus, and “swimming,” a repetitive series of flexions and extensions that propels the animal backward rapidly through the water.
The influence of social status on the lateral giant tailflip (Yeh et al., 1996) and to a much lesser extent the medial giant tailflip (Herberholz et al., in preparation) has received some attention in the past few years. The effect of the neuromodulator serotonin on the response of the lateral giant neuron to sensory stimulation was found to depend on the social experience of the animals (Yeh et al., 1996). When applied, serotonin enhanced the LG’s response to sensory stimulation in dominant animals but depressed it in subordinates. Using various serotonergic agonists to tease out possible differences leading to the differences in the effects, it was determined that these status-dependent responses were attributed to changes in the pattern and levels of the serotonergic receptors expressed by the LG neuron, although further experiments are needed to confirm this hypothesis. More recent evidence shows that the lateral giant neuron expresses the 5-HT$_{2B}$ receptor on the initial segment leading to the cell body (Johnstone et al., personal communication). However, it is still not known whether the level and expression pattern of this receptor and others is regulated by social status.

**Posture**

Body posture of crayfish depends on the behavioral context. Crayfish may rest on their ventral side, with the abdomen flexed or extended, or hold themselves more erect, often while walking. Abdominal postural motor neurons, which are always tonically active in dissected preparations, are silent in freely behaving resting animals, suggesting that the overall level of nervous excitability is low in living animals (Edwards, 1984). When the animal is aroused, the body posture can be elevated or flat against the substrate. Depending on the context: the posture of dominant animals, and animals engaged in a dominance contest tends to be elevated, whereas that of subordinate
animals is more prone and close to the substrate (Livingstone et al., 1980). The control of body posture is produced by a balance between central commands and local reflex systems (Cattaert and Le Ray, 2001; Fields, 1966). Abdominal posture is controlled by a command network of interneurons, subset of which is activated to produce specific flexed or extended postures (Jones and Page, 1986; Kennedy et al., 1966; Miall and Larimer, 1982a, b). Overall control of body posture may be held by interneurons in the circumeosophageal connectives that activate different body postures, usually in the context of a specific behavior such as walking or defense (Bowerman and Larimer, 1974). Local control systems include sets of proprioceptors in the limbs, abdominal segments, and ventral nerve cord that mediate both resistance and assistance reflexes, and a set of photoreceptors in each abdominal ganglion that excites postural flexion motor neurons locally and in all of the more caudal abdominal ganglia (Clarac et al., 2000; Edwards, 1984).

**Walking in Decapods**

Walking in decapods is the primary means of locomotion, although some species, like crayfish, lobsters, and sand crabs can swim either forward or backward using their swimmeret or tailflip escape systems, respectively. A considerable effort has been exerted studying walking in crustaceans at the behavioral, network, and cellular levels (Newland and Neil, 1987; Paul, 1971).

The walking pattern of crayfish is characteristic of many decapods. Crayfish possess the remarkable agility that allows them to walk in any direction using only a subset or all of their legs. Each of the legs performs periodic stepping movements, where each step consists of two principal phases: the stance and the swing phases.
During the stance phase, the leg is in contact with the substrate, the leg is partially supporting body weight and produces resistive force to counter the weight to maintain an elevated body posture. During forward walking, the leg gradually moves backward in relation to the body, from an anterior position to a posterior position. In the swing phase, the leg does not support the body. Rather, the leg is swings above the ground and moves forward and lands at the anterior extreme position. During backward walking, the reverse leg movement occurs whereby the leg is on the ground when it moves anteriorly, and off the ground when it moves posteriorly (Clarac and Chasserat, 1986).

Distal joints of a leg also perform periodic flexion-extension movements during walking; these movements are phase linked to the movements in the proximal joints (Clarac 1977).

Approximately 20 muscles control leg movements by flexing and extending the joints connecting the various leg segments. Forward and backward walking is mainly controlled by two pairs of antagonistic muscles that flex and extend the thoracocoxal and coxobasal joints. The first pair of muscles is the remoter and promoter muscles. The remoter muscle moves the whole leg backward, and the promoter muscle moves it forward. The second pair of muscles is the levator and depressor muscles. The levator muscle moves the leg upward, and the depressor muscle moves it downward. The mutual relationship between the EMGs of the four muscles control leg movement horizontally during forward walking. The remoter is active in the stance phase while the promoter is active in the swing phase. Similarly the levator and depressor muscles are responsible for the movement of the leg in the vertical plane. However, phase relationships between the two pairs of muscles are different for forward and backward
Figure 1-1: Electromyographic recordings from the 4 proximal muscles of the fourth walking leg during forward (A) and backward walking (B) of the rock lobster. (A) During forward walking the Depressor (Dep) and Remotor (Rem) muscles are active in phase during power stroke while the Promotor (Prom) and Levator (Lev) muscles are active during the return swing phase. (B) During backward walking the Depressor and Promotor are active in phase during the power stroke phase while the Levator and Remotor are active in phase during the return swing phase. *Figure Modified from Chrachri & Clarac (1990).*
walking (Chrachri and Clarac 1990). During forward walking, both the remoter and depressor are jointly active in the stance phase, whereas the promoter and levator are active in the swing phase together (Figure 1-1 A). When the animal walks backward the remoter and levator are active during the swing phase, while the promoter and depressor are active during the stance phase (Figure 1-1 B).

When a crayfish walks forward, the two homologous legs on either side move anti-phase to each other with a phase shift of approximately one-quarter of the cycle (Clarac and Chasserat 1983, 1986). Due to these phase shifts, all legs perform stepping in a precise order, thus forming a specific gait. Although leg movements and their phase relationships are regular, they can change spontaneously or in response to environmental factors (i.e. when an animal walks over a complicated terrain). Adaptations of leg movements are mediated by the mechanosensory organs located in the legs. These mechanosensory organs can modify both the stepping movements of individual legs and interlimb coordination (Clarac and Chasserat 1979; Barnes 1975; Ayers and Davis 1977; Barnes et al., 1972). When walking conditions for different legs differ considerably, their stepping movements become independent. This suggests that each leg has its own driving mechanism or (controller). The controller generates the rhythmical movement and possesses a relatively high degree of autonomy bypassing the interlimb coordination mechanism that is based on the interaction between the controllers of individual legs. Further evidence underscoring this notion of separate controllers for each of the legs is provided by physiological recordings of isolated thoracic ganglia showing the continued presence of rhythmic pattern generation of an individual ganglion or even hemi-ganglion. The rhythm of stepping in individual legs is
produced by a special group of interneurons, some of which have been identified (Chrachri and Clarac, 1989), and that these interneurons supply a rhythmical drive to the motor neurons that innervate the muscles described earlier.

The interactions among the legs’ CPGs allow movement coordination of the legs during walking. These reciprocal interactions among the CPGs were illustrated in experiments where the movement of one leg was perturbed while recording the activity of either ipsilateral or a homologous leg (Cruse and Müller, 1986; Cruse, 1990). It was found that leg parameters during walking of a single leg depend on the positions of the neighboring legs. This relationship applies for the timing of the transition from stance to swing phase and for the strengths of the motor outputs during the two phases. Between ipsilateral legs, there are both rostrally and caudally directed influences, so that each leg can affect its neighbors while simultaneously being influenced by them. The cellular interactions that coordinate leg movements are poorly understood. On one hand, the continued presence of a common rhythm in all thoracic ganglia during fictive locomotion of an isolated nerve cord suggests that the controllers for individual legs have common central connections (Chrachri and Clarac 1990; Sillar and Skorupski, 1986; Sillar et al., 1986). Conversely, leg proprioceptors are known to affect controllers of other legs either through their powerful action on the controller of the same leg or via specific intersegmental paths.

In addition to common central connections that coordinate legs movements there is also a set of command neurons that plays a measure role in controlling walking. Electrical stimulation of axons in the circum-oesophageal connectives of an isolated nerve cord activates approximately five command neurons that initiate forward walking,
and four command neurons that induce backward walking (Bowerman and Larimer, 1974a, b). These command neurons also activate other motor systems such as the superficial flexor motor neurons that control abdomen flexion, abdominal extension motor units, and swimmeret beating. Thus, it seems that these command neurons and possibly others yet unidentified can induce different forms of behavior that involve locomotion as the primary behavior (Larimer, 1976).
Dissertation outline

The dissertation is divided into two main parts. The first part consists of chapters two and three, which explore the short-term effects of social interactions during hierarchy formation on the behavior pattern and neural network activation of tailflip escape circuits. The second part consists of chapter four, which explores the long-term effects of social experience on orienting behavioral responses to sensory stimulation and the underlying neural networks driving the behavior.

In chapter two, I investigated the patterns of neural circuit activation underlying the tailflip escape responses in juvenile crayfish during dominance formation. I used electric field potentials produced in the fresh water surrounding a pair of crayfish to monitor activation of neural circuits for four different tailflip behaviors. These include the three well-studied types of escape tailflip and a newly described offensive tailflip. I found that the sequence of tailflip circuit excitation during agonistic interactions traces the development of dominance formation. During the early phases of social interactions both combatants show similar behavior patterns. However, once the dominance is formed it marks a sudden shift and divergence in the aggressive and defensive behavior pattern between the two animals. The results of this chapter were previously published in The Journal of Neuroscience. This particular publication was co-authored with my colleague Jens Herberholz. Therefore, I will be more specific of my own contribution to this work in my discussion.

Although the behavior pattern of adult crayfish is similar to that of juveniles, in chapter three I discuss how agonistic interactions between adult male crayfish develop into more ritualized behavior. I found that males engaged in pseudo-copulatory behavior
to signify their dominance relationship. This was followed by a reduction in aggression and an increased likelihood of the subordinate’s survival. This differential mortality indicates that the reduction of aggression induced by the pseudo-copulatory ritual directly enhances the differential survival of male crayfish that engage in this behavior. The results of this chapter were previously published in *Current Biology*.

Chapter four explores the long-term effects of social experience on the behavior and neurophysiology underlying the orienting response of crayfish to an unexpected sensory stimulus. A question that is often addressed is: how does the social experience of an animal affect its behavior and neurophysiology? In this chapter I investigated the effect of social experience on the orienting response to an unexpected sensory stimulus. I show that animals of different social experience display different behavioral and physiological response patterns. These response patterns are evident *in vivo* neural recordings and are retained *in vitro*. I monitored, *in vivo*, the leg motor nerve involved in orienting the animals when stimulated. I show that socially isolated and dominant animals turn toward the startle stimulus while subordinate animals avoid it by lowering their posture or by stepping backward. These behavioral responses are reflected accurately in the *in vivo* neural recordings. I investigated the neural basis of these different response patterns *in vitro*. I show that the same social status-dependent responses are retained in the reduced, *in vitro, preparation*. Endogenous release of serotonin is also found to be social status dependent, and it modulates the response patterns of the leg motor nerves. I postulate that the social status-dependent responses displayed *in vivo* indicate a reconfiguration of the underlying neural network driving the
behavior. The results of this chapter will be submitted for publication in The Journal of Neuroscience.

In summary, the social transitions that animals undergo during dominance formation are reflected in immediate differences in behavior patterns reflecting the social order. As the dominance order matures the behavioral differences are translated into long-term differences in behavioral responses that are retained in the nervous system as illustrated in the reconfiguration of the neural network underlying behavior. My results point to a possible imprinting mechanism of the social experience that enables the reconfiguration of the central nervous system to produce the desired behavioral outputs. The mechanism of how social information induces such dramatic network reconfiguration will be discussed in chapter five.
CHAPTER II
PATTERNS OF NEURAL CIRCUIT ACTIVATION AND BEHAVIOR DURING DOMINANCE FORMATION

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Fadi Issa solely developed the technique of recording field potentials of neuromuscular activity. Fadi Issa and Jens Herberholz jointly conducted and analyzed the experiments. Jens Herberholz conducted the statistical tests. The first draft of the manuscript was jointly written by Fadi Issa and Jens Herberholz and was revised and edited by Donald Edwards.

Introduction

Social dominance hierarchies are an organizing mechanism for most animal societies (Wilson, 1975), and are readily observable because of the different patterns of behavior displayed by animals of different social rank. Although the formation of social hierarchies has been studied in many species, the neural bases of the status-related differences in behavior are unknown, as are the neural mechanisms for the usually sudden change in social behavior that marks the initial formation of a hierarchical relationship.
Decapod crustaceans, especially lobsters and crayfish, provide a useful model for the study of the neural mechanisms of hierarchy formation (Kravitz, 1988). Social dominance hierarchies form readily among crayfish following a brief period of fighting (Bovbjerg, 1953; Lowe, 1956; Figler et al., 1995), and are stable over many days if the dominant animal is the largest of the group (Pavey and Fielder, 1996; Issa et al., 1999). Fighting between pairs of sized-matched crayfish and lobsters is marked by an escalating series of behaviors leading to grappling and wrestling with the heavy claws (Huber and Kravitz, 1995; Bruski and Dunham, 1987; Krasne et al., 1997). The fighting decreases when one animal (the new subordinate) breaks off contact with its opponent (the new dominant) by escaping or retreating (Issa et al., 1999).

The often sudden change in the behavior of one animal from fighting to escaping marks the decision point at which the social hierarchy is determined. The neural mechanisms in crayfish and lobsters that underlie this decision are unknown, but may involve changes in the threshold of neural circuits that mediate discrete behavior patterns displayed during agonistic interactions. Three well-known circuits for tailflip escape are obvious candidates. The Lateral Giant (LG) and Medial Giant (MG) circuits are each organized around a set of giant interneurons that function as command neurons to trigger stereotyped tailflip escapes in response to massive phasic sensory stimulation of the abdomen or cephalothorax, respectively (Edwards et al., 1999). A set of non-giant (NG) interneurons excites a variable form of tailflip escape either voluntarily, during swimming, or in response to more gradual stimuli (Wine and Krasne, 1972; Reichert and Wine, 1982).
Changes in LG threshold can occur through the imposition or removal of ‘tonic inhibition’ or by application of the neuromodulator serotonin (Vu et al., 1993; Vu and Krasne, 1993; Glanzman and Krasne, 1983), which also promotes a dominant posture and aggressive behavior in freely behaving crayfish (Livingstone et al., 1980; Huber and Delago, 1998). The modulatory effects of serotonin on LG depend on the animal’s social history and status: superfused serotonin enhances LG excitability in social isolates, in new and experienced dominant crayfish, and in new subordinate crayfish, but reduces LG excitability in experienced subordinates (Yeh et al., 1996; Yeh et al., 1997). Fighting also reduces LG excitability in experienced subordinates, but produces only a slight reduction of LG excitability in experienced dominants (Krasne et al., 1997). It is not known whether tonic inhibition is removed or serotonin is released in crayfish during fighting. If they are, the excitability of the LG circuit (and perhaps that of the MG and NG circuits as well) might be increased in isolates, in new dominants and in new subordinates as they fight to determine a dominance hierarchy. Here we determine whether changes in the excitability of these circuits occur by recording their patterns of activation in freely behaving pairs of juvenile crayfish (Procambarus clarkii) as they interact to form a dominance hierarchy. By tracking the occurrence of each tailflip behavior in two crayfish as one becomes dominant and the other subordinate, we can gain insight into how shifts in the thresholds of an ensemble of circuits can produce coherent new patterns of social behavior.

We have recorded the activation of each escape circuit in freely behaving juvenile crayfish by recording the tailflip behavior and the associated change in the electric field around the animal. Activation of each tailflip circuit creates a distinct
electrical field potential pattern in the water surrounding the animal (Fricke, 1984; Fricke, 1986; Beall et al., 1990; Featherstone et al., 1991; Macmillan, 2000). We used the tailflip behaviors and corresponding field potentials to identify activation of each tailflip circuit in either member of a pair of freely behaving crayfish as they fought to determine their relative social status. During the study, we identified a fourth type of tailflip behavior that occurs during agonistic interactions between crayfish, and we describe it here for the first time. This behavior, labeled ‘offensive’ tailflipping (OT), plays a prominent role in deciding relative social status.

**Methods and Material**

Sixteen juvenile crayfish (Procambarus clarkii; 2.2 – 3.0 cm) of both sexes that had been raised individually in isolation since becoming free-swimming (> 4 mos) were used throughout. Extracellular nerve and muscle potentials were recorded with a bipolar pair of electrodes implanted on the abdominal ventral nerve cord. Wire leads from the ventral cord electrodes were fixed to the carapace by superglue and connected to a differential amplifier (A-M Systems). Amplified (1000X) signals were displayed on an oscilloscope, digitized at 6.7KHz and recorded in a PC with Axoscope (Axon Instruments). Field potentials from the aquarium bath were recorded with a second pair of copper wire electrodes (1mm OD, insulated except at the tips) placed at either end of the 9.5 cm (L) by 1.5 cm (W) by 5.5 cm (H) aquarium (Figure 2-1A). These potentials were similarly amplified, displayed and recorded. The aquarium was filled with de-ionized water to a depth of 5.5 cm and the bottom was covered with gravel to facilitate walking. Sharp taps delivered by a hand-held probe to the abdomen evoked an escape
Figure 2-1 Experimental arrangement and the escape circuitry of crayfish.  A. Experimental setup. Neural and muscular activity of one (control) or two (fighting) juvenile crayfish placed in a small test aquarium were recorded with implanted and bath electrodes or with bath electrodes alone. The reflected image of the signals were recorded in the top half of a high-speed video that also captured the tailflip behavior of the animal(s) at 5ms/frame. B. Escape tailflip circuitry of the crayfish. Red: Primary mechanosensory afferents on the abdomen (top row red circles) excite the LG neurons directly and through an intervening layer of mechanosensory interneurons (second row red circles). The LG neurons in anterior segments excite motor giant (MoG) motor neurons in the anterior part of the abdomen, but not in the posterior part. The LGs also excite the segmental giant (SG) interneurons (green circles) in each segment, and the SGs then excited pre-motor interneurons and the set of fast flexor (FF) motor neurons in the anterior abdominal segments. These two sets of motor neurons excite fast flexor muscle in the anterior abdomen. Their contraction produces a rapid flexion around the thoracic-abdominal joint, which pitches the animal up and forward (bottom). The MG neurons (blue) are excited by phasic visual and mechanosensory input to the cephalothorax, and produce a rearward tailflip (blue, below) by exciting MoG and FF motor neurons in all the abdominal segments. The NG neurons (clear box) are excited by less phasic stimuli delivered anywhere on the body surface. At much longer latency, they excite FF motor neurons in several segments to produce a pattern of abdominal flexion that will carry the animal away from the stimulus source.
tailflip mediated by the lateral giant (LG) interneurons, whereas taps delivered to the cephalothorax evoked a tailflip triggered by the medial giant (MG) interneurons (Wine and Krasne, 1972). Gentle pushes of the probe on the carapace evoked tailflips triggered by non-giant (NG) circuitry (Figure 2-1B). A pair of bipolar electrodes at the tip of the probe established the time of contact with the animal by the sharp change in impedance between the electrodes. The animals' behavior was recorded simultaneously with the field potentials by high-speed videography (5ms/frame, JC Labs, San Mateo, CA). A mirror reflection of the oscilloscope traces in the upper half of each video frame was used to align the temporal sequence of field potentials with the behavior.

Video recordings were used alone to identify three behavior patterns, attack, approach, and retreat (Issa et al., 1999), whereas field potentials and video recordings were used to identify and distinguish four different forms of tailflip, LG, MG, non-giant (NG) and offensive (OT). NG tailflips were further divided into those that initiate an escape (NG), and those that constitute swimming (Swim), by occurring repetitively following an initial LG, MG, or NG tailflip. Attacks were defined (after Issa et al., 1999) as sudden movements of one animal toward another that led to physical contact and a response (one of the defined behavior patterns or tailflips) by the other animal. Approaches were defined as movements of one animal toward the other that failed to lead to contact but did provoke a response by the other animal. Retreats were locomotor movements of one animal away from the other in response to an attack or approach. Escapes are LG, MG, or NG tailflips that carry one animal rapidly away from the other, usually in response to an attack or approach. OTs are defined below. The
animal that initiated most of the aggressive behaviors (attacks, approaches, or OTs) during the 30 min period of interaction was identified as the dominant, whereas the animal that initiated most of the defensive behaviors (retreat, escape) was the subordinate (Issa et al., 1999).

Results

Comparison of recordings from the aquarium electrodes and implanted electrodes

Comparison of the amplified signals from the aquarium electrodes and the implanted electrodes revealed very similar electrical potential waveforms (Beall et al., 1990; Figure 2-2). In both the implanted and bath recordings, action potentials produced in the LG and MG neurons were immediately followed (after 1.4 ± 0.1 ms) by a much larger phasic potential that is attributable to the synchronous excitation of the segmental motor giant (MoG) motor neurons and the fast flexor (FF) muscle fibers they excite (Figure 2-2A, 2-2B; (Wine and Krasne, 1982). The phasic MoG potential was followed by a series of lower amplitude oscillations that result from excitation of the FF muscle by the non-giant FF motor neurons (Heitler and Edwards, 1998). The NG tailflip potentials lacked both the giant neuron action potential and the MoG potential, but did display the lower amplitude potential oscillations indicative of FF motor neuron excitation (Figure 2-2C).

To determine whether the field potentials recorded from unimplanted, freely behaving animals were the same as those recorded from implanted animals, potentials evoked during each of the three types of tailflip were recorded five times in each of 16 unimplanted crayfish. There were no qualitative differences between the field potentials
**Figure 2-2** Digitized recordings from the implanted (upper traces) and bath electrodes (lower traces) with simultaneously recorded video frames of the animal's tailflip behavior. Each video frame also displays the reflected oscilloscope trace of the bath recording (at top; the traces are reversed in the figure so that increasing time is from left to right), the animal, and the stimulus probe (white diagonally vertical line). The bracketed periods of each trace correspond to the period of the frame displayed below. (A) MG tailflip response due to a phasic probe stimulus to the front of the animal. The muscle potential is preceded by the MG giant spike potential (*) and inset. (B) LG tailflip response due to a phasic probe stimulus to the abdomen. The muscle potential is preceded by the LG spike potential (*) and inset. (C) NG tailflip response to a non-phasic probe stimulus to the thorax. No giant spike potential was recorded.
recorded from unimplanted animals and the corresponding potentials from implanted animals.

Each type of field potential in the unimplanted animals had a distinct amplitude and duration. The mean peak-to-peak amplitude of the MoG potential (3.4 ± 1.2 mV, m ± SD) evoked by an MG spike was significantly greater (p < 0.05, Friedman test of repeated measurements on ranks) than the MoG potential evoked by LG (2.3 ± 0.8 mV) or the largest potential evoked by NG (1.4 ± 0.7 mV). However, the duration of the MoG potential evoked by LG (1.9 ± 0.4 ms, m ± SD) was significantly longer than that evoked by MG (1.4 ± 0.1 ms). The duration of the largest NG potential (2.6 ± 0.5 ms) was significantly longer than either of the MoG potentials (p < 0.01, Friedman test of repeated measurements on ranks). The mean durations (± SD) of the entire LG- and MG-related field potentials were similar (18.1 ± 1.8 ms and 15.0 ± 1.0 ms, respectively), and both were shorter than the NG field potential (23.8 ± 2.4 ms). Although the mean values of each measurement of the different potentials were significantly different, their ranges overlapped.

**Categorization of tailflips during interactions between crayfish**

Categorization of a tailflip depends on the correlation between the high-speed video recording of the tailflip and the simultaneously recorded field potential. The field potentials were evoked only by tailflips, and animals were never seen to tailflip simultaneously, so that attribution of the field potential to a tailflipping animal is unambiguous.
NG tailflips can take any behavioral form, including those typical of LG and MG tailflips, but they have a much longer response latency than LG or MG tailflips in response to an applied mechanical stimulus (Wine and Krasne, 1972). This measure was lacking in experiments where two crayfish interact, and so identification of NG tailflips depended on the field potential. The NG field potential lacked both the initial MoG potential and the immediately preceding giant neuron action potential that are characteristic of an MG or LG tailflip (Figure 2-2), and so was readily identified.

Although both LG and MG tailflips produce an initial MoG field potential, their tailflip trajectories are readily distinguishable. Crayfish bend only the first three abdominal segments during LG tailflips, but bend all abdominal segments during MG tailflips, and so produce distinct trajectory angles for each type of giant tailflip (Wine and Krasne, 1972). These differences were apparent in the high-speed video recordings of the animals, which allowed a precise analysis of the animals’ movements during tailflipping. The tailflip angles were measured in the sagittal plane of the animal, from the direction in which the animal faced and 25ms after the first movement in each control experiment. Angles produced by LG (98.9° ± 7.7°, m ± SD) and MG (155.3° +/- 7.6°) tailflips in the control experiments were significantly different from each other (Wilcoxon paired test on ranks, p < 0.01), and were similar to the angles produced by the MG and LG tailflips reported previously (Wine and Krasne, 1972). The differences were maintained in giant-evoked tailflips produced during agonistic encounters between crayfish (LG: 98° ± 0°; MG: 150.1° ± 12.2°), and helped us to identify MG and LG tailflips produced during those encounters. The differences between the amplitudes and durations of the MoG potentials evoked by the LG and MG circuits described above
provided additional criteria for distinguishing the tailflips produced by each circuit during agonistic encounters.

**Field potentials and behavior during hierarchy formation**

Following a one day period of rest, the 16 juvenile crayfish used in the control experiments were paired for 30 min with another animal from the group of the same size and opposite sex. The two animals began a series of mutual agonistic encounters that soon resulted in one animal escaping and retreating in response to the other’s attacks and approaches. The relative dominant and subordinate status of each pair was determined from counts of the numbers of attacks, approaches, escapes and retreats that occurred throughout the interaction (Issa et al., 1999). The dominant animals were female in 3 of the 8 pairs of juvenile crayfish, and male in the others. No differences related to sex were observed in the behavior or recorded potentials.

The LG, MG, and NG tailflips that occurred during fighting were readily recognizable. They and the accompanying voltage records (Fig. 2-3A, B) were indistinguishable from the corresponding records obtained earlier from the same animals when isolated, and from the implanted animals (Figure 2-2). The peak-to-peak amplitudes and durations of the electrical field potentials were measured before and after pairing. There were no differences in the measurements for each tailflip between dominant and subordinate animals, or within one animal before and after pairing. NG tailflips occurred both as the initial response to attack (“NG” tailflips), and during swimming movements that followed each of the three types of escape (“Swim” tailflips).
Figure 2-3 Recordings from the bath electrodes and tailflip behavior during fighting. (A) MG circuit activation and tailflip signal. The MG spike potential (*) and the large, phasic MoG potential can be seen, followed by the lower amplitude response of the FF muscle to the FF motor neurons. (B) NG tailflip signal. The signal lacks the giant spike potential and the large MoG potential, and consists only of FF muscle potentials in response to the FF motor neurons. (C) Offensive tailflip signal (above) and behavior (below). The signal is identified by its small amplitude and extremely long signal duration, and results from a prolonged activation of the FF muscle by the FF motor neurons. No giant spike potential is recorded. Bracketed periods of the trace correspond in time with the sequence of frames. Time bar, A and B: 5ms; C: 14ms.
A fourth, previously undescribed form of tailflip ("offensive") occurred only when the tailflipping animal had a secure grip on its opponent (Figure 2-3). These offensive tailflips (OTs) began with an abdominal extension followed by several \((2.9 \pm 1.4, \text{m} \pm \text{SD})\) abdominal flexions and re-extensions. The duration of the entire offensive potential \((74.1 \text{ ms} \pm 5.3)\) exceeded all other tailflip potentials and correlated with a longer-duration abdominal movement (Figure 2-3). The abdominal extensions were accompanied by a spread of the tailfan that was maintained during the abdominal flexion, which occurred largely around the anterior abdominal segmental joints, while the posterior segments remained extended. This configuration helped to throw the animal up into the water column, above the opponent held in the tailflipping animal's grasp.

**Tailflip circuit activation and behavior during dominance hierarchy formation**

Dominance hierarchy formation between two crayfish began with fighting that differed in intensity and duration among eight pairs of animals, from a pair of one-sided interactions with little fighting to a prolonged, intense fight that lasted nearly 4.5 min before it was interrupted by the withdrawal of one animal. In six of eight pairs of animals, initial bouts of fighting included attacks, approaches and OTs by both animals, and few defensive behaviors such as retreats or escapes (Figure 2-4). OTs usually occurred in alternating bouts, in which several tailflips made by one animal were followed by a series of OTs by the other. The future dominant animal always displayed the final bout of OTs before dominance status was decided, and it displayed more OTs than the future subordinate. The status decision was apparent when a sudden change
Figure 2-4 Sequence of tailflip circuits activated and behavior patterns displayed by two of the eight pairs of animals during the 30 min period of dominance hierarchy formation. Events are presented according to their time of occurrence (top panels), and in their order of occurrence (bottom panels). Xs and Os mark the behavior of the new dominant and subordinate animals, respectively. The approximate time of the dominance decision is given by the dashed vertical lines. A. A pair in which the initial decision (dashed line) was decisive. B. A pair in which the new subordinate re-engaged the new dominant at intervals following the initial decision (dashed line). OT: offensive tailflip; MG: medial giant evoked tailflip; NG: non-giant evoked tailflip; Swim: swimming non-giant evoked tailflip.
in the behavior of one animal, the future subordinate, occurred. The aggressive behavior of this animal, including attacks, approaches, and offensive tailflips, ceased and defensive behavior, including escapes, retreats and swims, began (Figure 2-4). The new dominant animal maintained its aggressive behavior after the decision and persisted with attacks and approaches. In three of the pairs the initial fight was decisive, and produced a sharp switch from offensive to defensive behavior in one animal, while the other continued to behave offensively. This is illustrated by the ethogram of Figure 2-4A, in which the different behavioral events are displayed above according to their time of occurrence, and below according to their order of occurrence. The temporal display shows that for this pair, most of the activity occurred within the first 10 min of interaction. The lower display shows that the decision of one animal to withdraw and cease offensive behavior was abrupt. The temporal display shows that the level of agonistic activity by both animals declined over the remaining part of the half hour. The ethograms of two other pairs of animals are similar (not shown): an abrupt change in the behavior of one animal persists through the remainder of the 30 min interaction. In four other pairs the initial decision was incomplete: having switched from offensive to defensive behavior once, the new subordinate animal re-engaged the new dominant with brief bouts of offensive behavior. An example is seen in Figure 2-4B, where after an initial decision following 5 min of interaction, the new subordinate re-engaged the new dominant with periodic approaches, offensive tailflips and attacks. These were isolated events in all the pairs and did not change the balance of behavior between the animals.
The changes in behavior over time can be seen in the plots of Figure 2-5, where the frequencies of each behavior are expressed as the total number of occurrences in all animals in 5 min periods over the 30 min period of interaction. The period between their introduction and the onset of vigorous fighting differed among the pairs. This onset was marked by the first tailflip, whether an escape or an OT, which enabled the time-series of responses from all 8 pairs to be compared by aligning them along the time-axis with the time of the first tailflip at time zero. Dominant and subordinate animals made similar numbers of attacks, approaches and retreats during the period before that first tailflip (Figure 2-5A). The greatest activity occurred during the first five minutes after the initial tailflip, when large numbers of attacks (45), approaches (20) and offensive tailflips (85) by the dominant animal evoked correspondingly high frequencies of retreats (21) and escape tailflips of all types by the subordinate (210), the vast majority of which were NG and Swim tailflips (194). Dominant animals in all pairs produced few retreats (2) or escape (LG, MG, or NG) tailflips (n = 24) within the first five minutes of vigorous interaction, while the subordinate produced few attacks (7), approaches (1), or offensive tailflips (36) during this period (Figure 2-5). Both types of animals produced a small number of MG tailflips, with the greater number being produced by the subordinates (Dom: 11; Sub: 16).

Both dominants and subordinates performed many fewer offensive tailflips (14 and 5, respectively) during the second 5 min period (Figure 2-5B), after the decision had been made for most pairs. The drop in OTs reflects the absence of fighting in which the animals grapple each other. The dominant animals persisted in approaching (22) and attacking (27) the subordinates, which responded by retreating (21) and escaping (127).
Figure 2-5: Patterns of offensive and defensive behavior during 30 min of interaction between eight pairs of unacquainted crayfish. Total numbers of events are shown for dominant (top) and subordinate (bottom) animals in all eight pairs in sequential 5 min periods. The first tailflip of each pair marks the beginning of the first 5 min period (i.e., time 0) for that pair; the sequence of periods of each pair is aligned with that mark. A, Attacks, approaches, and retreats. Before indicates behavioral events that occurred before the first tail flip. B, Different types of tail-flip behavior. The 5 min periods correspond to the similarly labeled periods in A.
Only 5 of these escapes were of the more forceful MG type, reflecting the reduced level of aggressiveness of the dominant animals.

The level of aggressiveness of the dominant animals fell nearly linearly to about 15% of its initial level after 30 min, as reflected in the decline in the numbers of attacks and approaches (Figure 2-5). An increase in the number of attacks by the dominant animal in two pairs occurred in the fourth five min period (15-20min), and accounts for the deviation from the downward trend in summed agonistic activity. The dominant member of one of those pairs also produced the increase in number of OTs reported during that period (Figure 2-5). The frequency of retreats and NG escapes (including Swim) by the subordinate fell in parallel with the decline in attacks and approaches by the dominant. MG tailflips occurred primarily during the first period of intense interaction, primarily in the subordinate animal. MG tailflips continued at a low level in both animals throughout their interaction. The only LG tailflip recorded in all pairs occurred when a dominant animal struck a subordinate on the abdomen during the second 5 min period.

The differences in the behavior of dominant and subordinate animals are made clear in Figure 2-6. Dominant animals made more attacks than subordinates ($P < 0.01$, Wilcoxon signed rank test), more approaches ($P < 0.02$), more OTs ($P < 0.04$), fewer retreats ($P < 0.02$), and fewer NG tailflips ($P < 0.01$) than subordinate animals. No significant differences occurred in the number of MG tailflips made by dominant and subordinate animals.
Figure 2-6: The average numbers of different agonistic behavior patterns (± SD) performed by dominant and subordinate animals in eight pairs during 30 min of interaction (**, significantly different with p < 0.01; *, significantly different with p < 0.05; Wilcoxon signed rank test).
**Discussion**

*Field potential measurements and tailflip circuit activation*

Field potential measurements have provided a technique for distinguishing between giant-neuron-evoked and NG-mediated tailflips in freely behaving crayfish (Beall et al., 1990). Together with an analysis of the form and trajectory of the tailflip escape, this technique has enabled us to distinguish clearly the separate activation of three circuits, LG, MG, and NG, that govern tailflip escape behavior in Procambarus clarkii. A similar application of the technique with the Australian crayfish, Cherax destructor, has led to the same conclusion (L. Finley and D. Macmillan, personal communication). The technique has enabled us to determine the natural patterns of activation of these tailflip circuits during agonistic interactions between freely behaving animals that led to formation of a social dominance hierarchy. It has also helped us to identify a new tailflipping behavior used offensively during agonistic interactions.

The use of field potential recordings to identify patterns of tailflip circuit activation takes advantage of the large size of the giant fibers and the specific circuit arrangement through which they trigger a tailflip. The large phasic potential that follows the LG or MG spike (Fig. 2-2A, B) results from the near synchronous excitation of the set of abdominal motor giant motor neurons (MoGs), and the subsequent near-synchronous excitation of the segmental fast flexor (FF) muscles (Beall et al., 1990; Heitler and Edwards, 1998; Fraser and Heitler, 1991; Heitler and Darrig, 1986; Heitler and Fraser, 1993; Edwards et al., 1999). The phasic potential evoked by MG excitation is larger than that produced by LG because MG excites MoG motor neurons and FF muscles in all abdominal segments, whereas LG excites MoGs and FF muscles only in the most rostral segments.
In experiments with the Australian crayfish Cherax destructor, the initial phasic potentials produced by LG and MG circuits had opposite polarity (L. Finley and D. Macmillan, personal communication). This was not the case with Procambarus clarkii, perhaps because of differences between the animals or between the experimental arrangements.

No large impulse appears during NG activation in either Procambarus or Cherax (L. Finley and D. Macmillan, personal communication) because the NG circuit makes no use of the MoG motor neurons. Instead, the NG circuit excites a set of non-giant fast flexor (FF) motor neurons in each abdominal segment according to the needed pattern of abdominal flexion. The FF motor neurons excite subsets of FF muscles, and this pattern of muscle excitation accounts for the longer, lower-amplitude field potentials recorded during an NG tailflip. The similar pattern of low-amplitude field potentials that follows the initial phasic MoG response when LG or MG is activated also results from excitation of the FF motor neurons and their excitation of the FF muscle. Whereas the MoG motor neurons are excited by en passant synapses made directly by the LG and MG axons (Furshpan and Potter, 1959), the FF motor neurons are excited indirectly by LG and MG through a disynaptic pathway that produces a delay in the response (Edwards et al., 1999). The subsequent patterns of FF muscle excitation account for the prolonged field potential that follows each large MoG-related response.

Offensive tailflips may be a variant of the NG escape tailflip, but are more likely to be produced by a circuit that is distinct from the three escape circuits. The hallmark of the OT is the slow abdominal flexion performed as the animal grasps its opponent. The NG tailflip is much faster, but might conceivably be slowed if the animal were to perform
it while dragging a heavy load. The animal drags its opponent during an OT, but the
direction of the tailflip is initially upward and perpendicular to the axis of the connection
between the animals. The perpendicular direction of the tailflip relative to the direction
of the inertial force of the load (i.e., the opponent) suggests that the force developed by
the abdominal flexion should initially be largely unaffected by the opponent’s load. As a
result, one would expect that the initial flexion of an NG tailflip would be rapid until the
load slows it down. This is not what happens. An OT begins with an extension that is
immediately followed by a slow flexion that throws the animal upwards. This result
suggests that the OT differs categorically from the NG tailflip.

**Sequences of behavior that lead to hierarchy formation**

Earlier studies have shown how the expression of agonistic behaviors, including
approaches, attacks, retreats, and escapes, changes over two weeks following the
formation of a dominance hierarchy (Issa et al., 1999). An initial period of vigorous
attacks and approaches by the new dominant animal, and retreats and escapes by the
new subordinates, was followed by a significant reduction in agonistic activity as each of
the animals became used to their new status. Here we have tracked the sequence of
behavior displayed by two animals during initial encounters that led to formation of a
dominance hierarchy, and determined how the different patterns of tailflip behavior
contribute to hierarchy formation.

Changes in the pattern of tailflip circuit activation underlie part of the behavioral
change that occurs as one animal becomes dominant and the other subordinate. These
changes hinge on a decision point when the prospective subordinate switches from
offensive tailflipping and fighting to initiating repeated MG and NG escapes. The change in circuit activation appears to result from corresponding changes in the thresholds for excitation of the different circuits (Krasne et al., 1997). Prior to the decision point, both animals made attacks and approaches, and performed offensive tailflips when grappling with the opponent, although the prospective dominant was the more active. During this time, both animals performed very few retreats or escapes. Following the decision point, the new dominant continued to behave aggressively, whereas the subordinate switched from aggressive to defensive behavior. The dominant maintained the frequency of attacks during the initial period after the decision, but performed fewer approaches and OTs. The subordinate ceased offensive behavior and began performing repeated tailflip escapes, primarily those mediated by NG circuits, but also those mediated by MG circuitry. The LG circuit was excited only once, by a direct attack on a subordinate’s abdomen.

*The use of tailflip circuits during hierarchy formation*

These experiments have changed our view of the three different escape circuits, and the ways the animal uses them. The LG and MG have been seen as escape command neurons that trigger rapid, reflexive escapes upward or backward in response to rearward or frontward attacks, respectively (Wiersma, 1947; Wine and Krasne, 1972). LG and MG tailflips require strong, phasic stimuli when the animal is at rest (Wine and Krasne, 1982), although the precise stimulus threshold of an LG tailflip can be affected by descending inputs, applied serotonin, reafference, and by on-going behaviors, including fighting (Beall et al., 1990; Krasne et al., 1990; Kennedy et al., 1980; Krasne
et al., 1997; Glanzman and Krasne, 1983; Yeh et al., 1997; Yeh et al., 1996). The NG circuits were seen to trigger longer latency tailflips in response to more gradual stimuli, to control swimming movements following an initial tailflip, and to trigger escape tailflips in long-term subordinates (Krasne and Wine, 1984).

The current experiments make clear that stimulus conditions necessary for the MG and NG tailflips change significantly in new subordinate animals, whereas the adequate stimulus for LG appears not to change. Both the MG and NG circuits appear to become more excitable once dominance has been decided. Many of these MG tailflips cannot be readily attributed to any stimulus other than the near presence of the dominant animal, suggesting that MG threshold decreases significantly during fighting, and may even become ‘voluntary’. Subordinates performed 20 of the 29 ‘voluntary’ tailflips seen in all 8 pairs of animals, and they performed more of them than dominants in 6 pairs, suggesting that the threshold for activating an MG tailflip is lower in subordinates than in dominants. The greatest change in apparent threshold was experienced by NG escape behavior, which was a rare event before the status decision was made and quickly became the predominant behavior of the subordinate animal afterwards.

Earlier studies suggested that serotonin may be released during fighting between crayfish, where it promotes an aggressive posture, reduces the motivation of a subordinate to retreat (Livingstone et al., 1980; Kravitz, 1988; Huber and Delago, 1998), and facilitates the excitability of LG in new dominant and subordinate crayfish (Yeh et al., 1996; Yeh et al., 1997). Contrary to our expectation, however, the LG escape circuit did not appear to become more excitable during a confrontation and fighting between
two isolate crayfish, or between new dominant or subordinate crayfish following the
decision of relative social status. Indeed, in the present experiments, LG-mediated
escape occurred only once, in response to the same sort of phasic abdominal stimulus
that triggers an LG escape in a quietly resting animal. However, the lack of LG tailflips
may be an artifact of the confined space of the small, narrow aquarium used in these
experiments, in which the animals normally occupied positions facing each other.
Informal observation of crayfish in a larger aquarium suggests that the body orientation,
position and behavior of crayfish are governed by the direction and distance of possible
threats, including larger dominant animals. LG tailflips may occur more frequently
during interactions in a larger, object-rich arena, in which potential threats are more
numerous and the direction of possible attack is less certain. A similar, context-
sensitive change in LG threshold is seen in crayfish that acquire a small portable piece
of food (the threshold drops) or a large, immovable piece of food (threshold rises)
(Bellman and Krasne, 1983; Krasne and Lee, 1988). Such changes in threshold may
reflect the imposition or removal of ‘tonic inhibition’, which has been shown to produce
momentary variation in the excitability of the LG neuron (Vu and Krasne, 1993; Vu et al.,
1993).

Offensive tailflipping has not been described previously, but appears to play an
important role in dominance hierarchy formation between size-matched crayfish. During
initial fighting, crayfish appear to use bouts of repeated OTs to drag their opponent and
gain a position above it. The alternation in bouts of OTs between two animals as they
grapple suggests that each is trying to demonstrate its size and strength to the other.
The slow rate of abdominal flexion relative to escape movements is consistent with this
suggestion in that OTs are not used to injure or dismember an opponent. The future dominant displays more OTs in each bout, more bouts, and the last bout before the status decision. The decision often followed the last bout quickly, as the subordinate displayed a series of NG tailflips that signaled its defeat. We conclude that the OTs appear to provide a means for each crayfish to assess its strength relative to its opponent and to reach a status decision without suffering injury.
CHAPTER III
RITUALIZED SUBMISSION AND THE REDUCTION
OF AGGRESSION IN AN INVERTEBRATE

Introduction

Aggression is a social tool used in human and animal societies to determine social dominance and access to desirable resources (Huxley, 1966; Moynihan, 1998; Wingfield, 2006). However, aggression brings the risk of injury or death to rivals and increased stress to the entire community, so that it is often avoided and used briefly when necessary. In many social species, including dogs (Darwin, 1872), chimpanzees (de Waal, 2000), and humans (Koziol, 1992), aggression that results from a dominance competition is avoided or reduced by a ritualized display of submission of the subordinate to the dominant. Although invertebrates display dominant postures, more complex dominance rituals are unknown.

Crayfish are aggressive social invertebrates that form and maintain dominance hierarchies through aggressive interactions (Bovbjerg, 1953; Lowe, 1956; Guiasu, 1997; Issa, 1999; Goessmann, 2000). We report that dominance contests were resolved sooner and levels of aggression fell faster between pairs of adult male crayfish that engaged in bouts of pseudo-copulation during the period of dominance hierarchy formation. In these bouts, the new dominant displayed sexual behavior typical of males.
in male-female sexual interactions, while the new subordinate displayed female behavior. Subordinates that engaged in bouts of pseudo-copulation were much more likely to survive the first 24 hrs of interaction than subordinates that did not.

**Methods and Material**

*Animal maintenance*

Adult crayfish (*Procambarus clarkii*, 8-10cm, Form I) with intact limbs, antennae, and antennules were bought from a commercial supplier (Atchafalaya Biological Supply, Raceland, Louisiana) and housed individually in 20 L tanks each containing de-chlorinated water, a filter (Duetto 100 submersible power filters) and air-stone for oxygenation. The animals were isolated for a minimum of three months on a 12/12 hr light/dark cycle and were fed shrimp pellets once a week.

*Pair formation*

2 days prior to each experiment fresh water was made. The water was made from distilled water then fresh water aquarium salt was added to the water in accordance to the supplier’s recommendations. This enabled us to ensure that the water would not contain contaminants and chlorine that might harm the animals. For 2 days air was pumped into the water to ensure ambient oxygen levels before introducing the animals into the tanks. Each tank contained its own filtration and oxygen system. We used Duetto 100 submersible power filters designed to handle 20 gallon tanks exceeding the requirements of the tanks used in this study of 5 gallons.
Between 8 and 10AM, pairs of individually marked crayfish were placed on either side of a transparent divider in the testing aquarium (20 L, 20x40x27 cm). The divider was removed after 15 min to allow the animals to interact. The animals were free to interact at all times during the 5 day pairing period. Two shelters were provided for each pair after the first 6 hours of interaction. A digital camcorder (Panasonic Corp.) recorded the agonistic interactions (attacks, approaches, defensive and offensive tailflips, and retreats) for each pair during the first hour of interaction. A separate time-lapse video system (Panasonic Corp. Time-Lapse AG-6730) recorded the first 24 hr of interactions. For the remaining 4 days the pairs were observed for 30 minutes in the morning (crayfish peak activity time) and the number and type of aggressive and submissive behaviors were noted.

**Dominance indices and formation**

**Data analysis:** Dominance between the two animals was determined based on the fraction of aggressive and submissive behavior each animal performed as described elsewhere (Issa et al., 1999). The videotaped behavior was analyzed at each second and tabulated in an Excel spreadsheet. The dominance relationship between the two animals was accurately tracked at this time resolution.

The agonistic interactions of the animals were termed *encounters*. All the encounters were recorded chronologically for the five agonistic behaviors (Attack, Approach, Offensive tailflips, Retreat, and Escape tailflips). Each behavior was defined as follows: an “attack” is an *aggressive physical contact* initiated by one animal towards another. An “approach” is the movement of one animal towards another with no physical contact but does evoke a response from the other. “Offensive tailflips” are a series of
rapid flexion of the abdomen while the animals are interlocked (used to test each others
strength). A “retreat” is an ambulatory movement away from an Approaching or
Attacking animal. An “escape” is a rapid movement away from an aggressor produced
by one or more tailflips (i.e., rapid flexions of the abdomen).

Each aggressive behavior (an attack, an approach, or an offensive tailflip) was
assigned a value of +1, and each defensive behavior (an escape or a retreat) was
assigned a value of -1. The dominance index for each animal was calculated as the
sum of its values over each two min period of the first hour of pairing. A dominance
transition was assumed to occur when one animal’s index became positive and the
other’s index became negative for 5 min or longer.

Results

Male crayfish have been seen to display pseudo-copulatory behavior in the wild and in
the laboratory, and to leave a spermatophore deposited on the subordinate (Chidester,
1912; Mason, 1970). However, the social significance of this behavior is not known.
Here we have observed pseudo-copulation by pairs of adult male crayfish as each pair
formed an initial dominance relationship in a laboratory aquarium. In 80% of the pairs
tested (16 out of 20 pairs), the emerging dominant attempted to mount the emerging
subordinate in a manner similar to a male sexually mounting a female (Ameyaw-Akumfi,
1981). In 60% of pairs (12 pairs), the attempt led to a behavior characteristic of male-
female mating (‘pseudo-copulation’, Figure 3-1). In 20% (4 pairs) the subordinates
Figure 3-1 Pseudocopulation between male crayfish resembles copulation between female and male crayfish.

Male-female copulation (A) and male-male pseudocopulation (B) in crayfish (Procambarus clarkii). The scale bar represents 2cm.
refused all of the dominants’ attempts and pseudo-copulation failed to occur, and in the remaining 20%, no attempts were made.

Prior to a mounting attempt, the dominant often displayed typical male courtship behavior by approaching the subordinate from behind with a lowered posture and chelipeds (claws) held close to the body, and lashing the subordinate with its antennae (Mason, 1970; Rubenstein and Hazlett, 1974). The dominant then climbed up on the subordinate and used its chelipeds and walking legs to turn the subordinate over, ventral side up. When the subordinate accepted the dominant’s approach and mount, it extended its abdomen and promoted its chelipeds and walking legs forward and parallel to its body in a supine posture characteristic of female mating behavior (Figure 3-1B). The subordinate became very passive as the dominant grasped the subordinate’s chelipeds with its own, clasped the subordinate’s cephalothorax with its walking legs, and extended its abdomen parallel to and facing the subordinate’s extended abdomen. The dominant erected its gonopodia and thrust them rhythmically towards the subordinate’s gonopodia, which remained motionless. Bouts of pseudo-copulation lasted from 7 sec to 9 min, 3 sec (m ± SD: 1 min, 17 sec ± 2 min, 9 sec) in the 12 pairs observed, a significantly shorter period than the 30 min to 90 min durations reported for male-female copulation (Ameyaw-Akumfi, 1981). Pseudo-copulation ended in the same manner that females end bouts of copulation, when the subordinate slowly flexed its abdomen, dissociated from the dominant’s grasp, and retreated.

The dominance relationship within each pair became apparent when the balance of one animal’s behavior suddenly became aggressive (Attacks, Approaches, Offensive tailflips) and the other became defensive (Escape tailflips, Retreats) (Figure 3-2).
Figure 3-2  Sequence of behavior patterns (top panels) and dominance indices (bottom panels) displayed by two of the twenty pairs of animals during the first hour of interaction (See Methods). The time of the dominance decision is given by the dashed vertical lines. Red and blued ‘O’ symbols mark occurrences of the behaviors of the new dominant and subordinate animals, respectively. Dashed lines mark the dominance transitions. (A) A pair in which pseudo-copulation occurred. Red bars indicate the time and duration of the pseudo-copulatory bouts in which the red animal was dominant. (B) A pair in which pseudo-copulation did not occur and the subordinate was eventually killed by its dominant counterpart. Note the reversal in dominance hierarchy 39 minutes into the pairing indicated by the second dashed line. The double-headed arrow indicates the time period when the dominance hierarchy was being re-established. (Off. T.F.= offensive tailflips).
Dominance relationships in pairs that pseudo-copulated were established early (m ± SD: 8.8 min ± 10 min) and remained stable over 5 days for 11 of 12 pairs (Figure 3-2). Relationships in 3 out of the 8 pairs that did not pseudo-copulate or failed to pseudo-copulate were reversed during the first hour of interactions, and the dominance relationship for one pair was never established. The mean time of final formation of a dominance relationship for these animals was 23 min ± 20 min (m ± SD). Although larger animals tended to dominate, in 6 out of the 20 pairs the smaller animals dominated the larger ones (maximum body size difference did not exceed 6%)

Bouts of pseudo-copulation occurred both before (in 4 of 12 pairs) and after (in 11 of 12 pairs) the time of dominance formation (Figure 3-3). In 6 of 12 pairs, the first bout of pseudo-copulation occurred during the first 15 minutes of interactions when the dominance relationship was being established and agonistic interactions were most intense (Issa et al., 1999). The average percent time that pairs spent pseudo-copulating remained constant over the first one-half hour of pairing and declined to low values over the second half hour (Figure 3-4A). All but one bout (n=20) were observed during the first hour of interaction; the exception occurred 10 minutes into the second hour of pairing.

The frequency of aggressive and submissive behavior acts came to differ between pairs that did not pseudo-copulate or failed to pseudo-copulate and those that did pseudo-copulate (Figure 3-4). During the first 15 min of pairing, there was little difference in the agonistic behavior of the three groups. However after the first 15 minutes, the pattern of fighting changed markedly (Figure 3-4B, C). For pairs that did not pseudo-copulate or failed to pseudo-copulate, the time spent fighting, the number of
Figure 3-3 Times of dominance-relationship formation and pseudocopulation.

Filled circles indicate the times of dominance formation of pairs that failed to pseudocopulate (top panel) and of pairs that pseudocopulated (bottom panel). The circle at time I (Indefinite) in the top panel denotes the pair that failed to form a stable relationship. Filled gray squares in the bottom panel indicate the times of pseudocopulation of these pairs. The vertical dashed lines indicate the average time for the formation of the dominance relationship for all pairs. The right panel denotes the time when the subordinates were killed (hr = hours, D = Day).
Figure 3-4 Changes in the patterns of pseudo-copulation and agonistic behavior of pseudo-copulating and non-pseudo-copulating crayfish pairs during the first hour of pairing. (A) The total percent time (and SD) spent pseudo-copulating in each quarter hour. The numbers next to each square give the total number of pseudo-copulation bouts during that period. (B) The percent time spent fighting. Pairs that pseudo-copulated (red squares) significantly decreased their time fighting in the second 15 min period (asterisks), while pairs that did not (open triangles) or failed to pseudo-copulate (closed triangles) spent about half their time fighting throughout the hour. Pound signs indicate significant differences between the pseudo-copulating compared to the non-pseudo-copulating pairs and ones that failed to pseudo-copulate for each time period. (C) Frequencies of aggressive behaviors per encounter for dominant animals. The number of aggressive behaviors (attacks, approaches, and offensive tailflips) were counted for each 15 min period and averaged over the pseudo-copulating and non-pseudo-copulating animals. Dominant animals in pseudo-copulating pairs significantly decreased their aggression over the hour. The frequency of aggressive behavior was significantly different between the groups at the 2nd and 4th 15 min time periods. (D) The frequencies of retreats by pseudo-copulating subordinates fell continuously over the first hour, and came to differ significantly from those of the non-pseudo-copulators in the 3rd and 4th 15 min periods. Significance levels (*) or (#) = P<0.05; (**) = P<0.005; *** = P< 0.0005.
Attacks and Approaches per encounter by dominants, and the frequency of subordinate Retreats remained unchanged over the first hour of pairing. However, for pseudo-copulating pairs, the time spent fighting decreased significantly (Wilcoxon matched pairs test, two tailed; \( P = 0.0005 \)), as did the number of Attacks and Approaches per encounter made by dominants (Wilcoxon matched pairs test, two tailed; \( P = 0.0342 \)) (Figure 3-4B, C). Subordinates that pseudo-copulated decreased the frequency of their Retreats significantly over the course of the hour (Wilcoxon matched pairs test, two tailed; \( P = 0.0068 \)) (Figure 3-4D). By the end of the first hour, each of these measures in the pseudo-copulating animals had fallen significantly below the corresponding values recorded for both groups that did not pseudo-copulate (Kruskal-Wallis One-way ANOVA test, two tailed \( P< 0.05 \)).

The difference in the frequency of aggressive behavior after the first hour led to significant differences in the mortality between pairs that pseudo-copulated and those that did not or failed to pseudo-copulate during the first 24 hrs of interactions (Figure 3-5). Subordinates of pairs that did not or failed to pseudo-copulate experienced much higher mortality (4 of 8 killed, or 50%) than subordinates that pseudo-copulated (0 of 12 killed, or 0%; total pairs=20; \( n=12 \) pseudo-copulation, \( n=8 \) no pseudo-copulation; Fisher's exact test, two-tailed, \( P=0.0144 \)).

Analysis of the time-lapse recordings indicated that the dominants in pairs which did not or failed to pseudo-copulate persisted in their aggressive behavior towards the subordinates, which repeatedly tried to Retreat or Escape. Four of these subordinates eventually slowed their movements, stopped Escaping, and were killed, dismembered and partially eaten during the first 24 hrs of pairing. Two were killed towards the
Figure 3-5 The effect of pseudocopulatory behavior on the survival of subordinate animals.

All (12/12) pseudocopulating subordinates (P-c) survived 24 hr of continuous pairing, whereas half (4/8) of nonpseudocopulating subordinates were killed by their dominant partners during that time (two subordinates that did not pseudocopulate, No P-c; two subordinates that failed to pseudocopulate, Un. P-c). The differences in survival rate among the three groups is significantly different and is maintained after 5 days of pairing.
beginning of the first 24 hrs together, and two others at the end (Figure 3-3). The frequency of aggressive behavior was much less for pseudo-copulating pairs. Although at times a dominant left its own shelter and approached and chased the subordinate out of its shelter, the lower aggressiveness of these dominants reduced the intensity of their social interactions.

The difference in aggression and mortality among the three groups diminished rapidly after the first day (Figures 3-4, 3-5). One subordinate that pseudo-copulated and one that did not pseudo-copulate were killed on the fifth day of pairing (Figure 3-3), so that 6 of the 20 animals were killed. This 30% mortality over the 5 day experiment fell within the range of 20% to 69.9% reported from various laboratory and field studies of crayfish population dynamics (Abrahamsson, 1966; Capelli, 1980; Nystroem, 1994; Savolainen et al., 2003).

**Effect of feminisation on social interactions**

Intersexuality: the presence of both male and female sexual characteristics is a well-documented phenomenon in many crayfish species (Rudolph, 1995; Sagi et al., 1996; Parnes et al., 2003). Feminisation is one form of intersexuality in which a male possesses the characteristics of both male and female external morphology and/or gonadal characteristics. In such a case, the gonads of a male consist of an ovary with a truncated oviduct on one side and ovotestis consisting of an ovary with an oviduct and testis with a vas deferens on the other side (Rudolph, 1995). Intersexuality was shown to be a stable form in that there is no change of sexual morphology and the animals are considered functionally males (Parnes et al., 2003). A number of population surveys
show that only about 1-4% of animals in a population are considered intersexual (Medley and Rouse, 1993); (Parnes et al., 2003). Although many studies reported on this phenomenon and the endocrine influence on its manifestation (Taketomi and Nishikawa, 1996); (Parnes et al., 2003), no information is available regarding the effect of feminisation on the social behavior of crayfish.

At the end of each pairing period we determined the sexual form and gonadal characteristics of each crayfish tested. We found that one individual crayfish tested was in fact a feminized male.

During the first hour of interactions the feminized subordinate pseudo-copulated with the dominant once during the 3rd 15 min. period. The agonistic and defensive behavior pattern of the pair followed the same pattern observed in other pairs in which pseudocopulation was observed. However, following the behavior of the pair beyond the first hour of interactions we noticed that the pair formed some degree of bonding typical of heterosexual pairs after mating. The bonding of the pair was manifested in the form of sharing the same shelter even though they were provided two shelters. Remarkably, even when we provided the pair with multiple shelters they continued to stay together, and their bonding continued for two months. When we attempted to disturb the pair by removing the shelter being used, the dominant animal would attack and chase the feminized subordinate for as long as the shelter is absent. Once the shelter is replaced both animals would reoccupy it and antagonism would cease. At the end of the two months pairing, we dissected both animals and determined that the subordinate was a feminized male based on its internal gonads while the dominant had normal external and internal male sex organs. The dominant had normal external male organs.
consisting of enlarged chelipeds, hooks on the 3\textsuperscript{rd} and 4\textsuperscript{th} legs and gonopods. Its gonads were typical of a normal male consisting of three testes with long \textit{vas deferens}. The feminized subordinate had only one enlarged gonad that was significantly larger in size compared to normal testes, and it was very similar in description to the ovotestis described in feminized crayfish (Rudolph, 1995). In addition, one of the \textit{vas deferens} was significantly short and thin resembling a female oviduct. Its external sexual organs were normal in that it had enlarged chelipeds, hooks on the 3\textsuperscript{rd} and 4\textsuperscript{th} legs and normal gonopods.

**Discussion**

The slower decline in the frequency of aggressive behavior among pairs that did not or failed to pseudo-copulate follows the time-course of aggression seen among groups of juvenile crayfish as they formed a dominance hierarchy. High frequency of aggression behavior during the first day of interaction led to deaths in one-third of the 32 groups of juvenile crayfish, which were too young to copulate or to pseudo-copulate. The aggression was lower the next day, and nearly disappeared over the next week as the frequency of agonistic encounters dropped by 90%. The dominance hierarchy in each group was still evident as the subordinates moved away at the approach of the dominant, thereby avoiding an attack (Issa et al., 1999).

Pseudo-copulation in crayfish has evolved into a ritualized behavior between male crayfish that signifies establishment of a dominance relationship and a rapid reduction of aggression by the new dominant. Reduced aggression allows the subordinate to survive over the first 24 hrs within the context of this dominance relationship. These effects are similar to those of copulation between male and female crayfish, which can
also begin with an aggressive encounter and has been seen as an extension of male dominance behavior (Mason, 1970; Bovbjerg, 1953). Moreover, if the female refuses the male’s attempts to mate, she can be killed (Berril and Arsenault, 1982).

Pseudo-copulation appears to facilitate a stable dominance relationship formation and to reduce aggression during agonistic interactions between male crayfish. When one animal assumes the dominant male role and the other assumes a female submissive role, pseudo-copulation functions as a mutual honest signal of the opponents’ relative social rank. Consequently, social dominance conflicts are resolved sooner leading to a reduction in both the time spent fighting and energy costs.

Although our results demonstrate the benefits of pseudo-copulation between two male crayfish, its effects on the dynamics of social interactions of larger groups remains unknown. Pseudo-copulation is observed to occur among groups of wild (Chidester, 1912; Mason, 1970) and captive crayfish of approximately equal ratios of males and females (Issa, personal observation). However, its frequency and effects remain to be studied. Pseudo-copulation within large groups may serve as a victory display for third party observers as commonly found among many animal species (known as “eavesdropping”). This behavioral strategy allows group members to assess the strength and weakness of individuals through observation of their agonistic interactions, thus, minimizing direct social conflicts and associated energy costs (Johnstone, 2001; Bower, 2004).

Pseudo-copulation and copulation can be seen as two uses of the same behavior by crayfish to resolve dominance disputes with a minimum of aggression and to mate. Both uses appear to enhance the fitness of both members of each pair: Pseudo-
copulation increases the dominant’s access to resources while it reduces the chances of injury and death for both subordinate and dominant, and copulation contributes directly to the reproductive success of both the male and female.

Although ritualized courtship displays are common among invertebrates (Huxley, 1966), similarly complex ritualized dominance displays are not common. Ritualized dominant and submissive postures, but not more complex behaviors like pseudo-copulation, have been observed in lobsters (Huber and Kravitz, 1995), crickets (Adamo and Hoy, 1995; Hofmann and Schildberger, 2001), ants (Hölldobler and Taylor, 1983) and wasps (Hölldobler and Wilson, 1990).

Among mammals, ritualized dominance displays, like pseudo-copulation, occur among animals that form social groups. Pseudo-copulation is one element in a set of ritualized submission behaviors used by various primate species to affirm a dominant/subordinate relationship and reduce aggression between rivals (de Waal, 2000; de Waal, 1986).

The similarity in the form and function of pseudo-copulation and copulation in crayfish and mammals is striking, given the very different body forms, brain structures, and lineages of these animals. This similarity allows these behaviors to provide an example of the convergent evolution of social and sexual behavior in animals across the vertebrate/invertebrate divide.
CHAPTER IV

SOCIAL STATUS-DEPENDENT RECONFIGURATION
OF THE NEURAL NETWORK CONTROLLING LEG MOVEMENT

Introduction

Social interactions that lead to hierarchy formation play an integral part in shaping the behavior of many social animals. A considerable effort has been exerted to determine the extent to which social interactions influence the function of the nervous system (Yeh et al., 1996; Drummond et al., 2001; Song et al., 2007; Gould et al., 1997; Sullivan et al., 2007). Numerous studies elegantly demonstrated that social conditions (Sandeman et al., 2000), social stress (Gould et al., 1997, 1998), or social experience (Yeh et al., 1996) affect neuromodulation (Yeh et al., 1996), gene expression (Burmeister et al., 2005, 2007) or neurogenesis (Song et al., 2007). However, how social interactions influence neural circuit function and dynamics has not been addressed previously. This is in part due to the difficulties of accessing the neural elements involved in any particular behavior or the lack of technical tools that would enable the monitoring of neural circuits of complex nervous systems.

In this study we investigated the effects of social experience on the underlying neural network that mediates the orienting behavioral response of crayfish to unexpected sensory touch. Previously, it was demonstrated that crayfish of known social status display different orienting responses. Socially dominant animals turn toward the sensory touch, while social subordinates walk either backward or forward away from the touch (Song et al., 2006). But how these two behavioral responses are
mediated was not determined. Here, we monitored the behavioral responses, leg kinematics, and neural activities of the leg motor neurons involved in the orienting responses in freely behaving dominant and subordinate animals. In addition, we mimicked the behavioral experiments in a reduced, in vitro, preparation in order to monitor the activation of the neural elements involved in mediating the orienting responses.

Crayfish provide two distinct advantages to address the influence of social interactions on the function of neural circuits. First, crayfish are highly social animals that engage in stereotypical agonistic interactions and form long lasting and stable social hierarchies (Bovbjerg, 1953; Lowe, 1956; Issa et al., 1999). Their social interactions are robustly reproduced, measured and quantified in the laboratory. Second, the functions of the underlying neural circuits of various behaviors are well characterized anatomically and physiologically (Edwards et al., 1999; Cattaert & Le Ray, 2001; Clarac et al., 2000; Wine & Krasne, 1982). Of particular interest, the neural circuits that control each leg’s movements during walking and posture are well described and many of their behavioral and neural characteristics are shared with other decapods and hexapods (Cattaert & Le Ray, 2001; Buschges, 2005).

Walking and postural control in crayfish are two highly coordinated and tightly coupled behaviors, in part, because they are mediated by common muscular and neural elements. Walking and posture are mainly regulated by the activity of the coxopodite segment. The coxopodite segment contains two pairs of antagonistic muscles that flex and extend the thoracocoxal and coxobasal joints. The first pair of muscles is the remoter and promoter muscles. The remoter moves the leg backward, and the promoter
moves it forward. The second pair of muscles is the levator and depressor muscles. The levator moves the leg upward, and the depressor moves it downward (Figure 4-1). During static posture, the level of co-activation of the levator and depressor muscles allows the regulation of body posture in the vertical plain. When the depressor muscle is more active than the levator then the leg depresses against the substrate; thus, providing support to body weight allowing the animal to adopt a high body posture. If the levator muscle is more active than the depressor then the leg is not providing as much of a support of body weight, and so the animal’s body posture is lowered. The level of activation of the two muscles depends on sensory feedback from the segment’s chordotonal organ (Coxopodite-Basipodite Chordotonal Organ or CBCO) and internal inputs from the postural command centers. During walking in crayfish each leg performs periodic stepping movements that consist of two principal phases: the stance and the swing phases. These phase movements are mediated by the same motor neurons and muscles that regulate static body. During the stance phase, the leg is in contact with the substrate, the leg is supporting body weight and produces resistive force to counter the weight to maintain an elevated body posture. During forward walking, the leg gradually moves backward in relation to the body, from an anterior position to a posterior position. In the swing phase, the leg does not support the body. Rather, the leg swings above the ground and moves forward and lands at the anterior extreme position. During backward walking, the reverse leg movement occurs whereby the leg is on the ground when it moves anteriorly, and off the ground when it moves posteriorly (Clarac and Chasserat, 1986).
Here we sought to determine the effect of social experience on the orienting behavioral response to unexpected sensory touch in freely behaving crayfish. We provide evidence that the response of the leg depressor motor neurons to unexpected touch depends on the animal’s social status, and these status-dependent responses are retained in a reduced, *in vitro*, preparation without descending inputs from the brain. Our results indicate that the social experience has significantly altered the underlying neural network that mediates the orienting response. However, the mechanism of how status-dependent configuration occurs is unclear.

**Methods and Material**

*Animal maintenance* Crayfish (*Procambarus clarkii*, 8-10cm) were bought from a commercial supplier (Atchafalaya Biological Supply, Louisiana) and housed individually in 5L tanks each containing de-chlorinated water, a filter and air-stone for water oxygenation. The water was changed with fresh water once every two weeks. The animals were isolated from each other physically, visually, and chemically for a minimum period of one month and were fed shrimp pellets twice weekly.

*Pair formation* dominance was established by pairing two previously isolated crayfish. One day prior to the pairing, the animals were marked on the thorax with a permanent marker for identification. The following day the animals were removed from their isolated tanks and placed in the testing tank. The testing tank was divided into two chambers with an opaque divider preventing interaction between the two animals. The animals were given 15 minutes to acclimate to the new environment before the divider was removed freeing them to interact with each other. The agonistic interactions
(attacks, approaches, escape tailflips, offensive tailflips, and retreats) between the two animals were recorded for the first 30 minutes of interactions and on every subsequent day. The animals were free to interact at all times during the pairing period, which lasted a minimum of 2 weeks and up to 1 month. Dominance between the paired animals was usually established within the first fifteen minutes of interactions on day one and was rarely reversed. Dominance was determined based on the total number of aggressive and submissive behaviors an individual animal performed (See Issa et al, 1999 for detail).

**in vivo recordings** At the end of either the isolation or pairing periods an animal was selected randomly for electrode implantation. The animal was chilled in ice water for 10 minutes to anesthetize it and then placed ventral side up onto a Petri dish and immobilized. A small incision was made in the postero-ventral side of the coxopodite segment of the left 5th leg using a sharp heated micro-pin. One pair of silver wire electrode (0.014cm, A-M system) insulated with Teflon was inserted through the hole and placed near the depressor muscle and around the depressor nerve (Figure 4-1). The electrodes were held in place by gluing the electrodes to the leg with tissue adhesive. The reader is referred to appendix I for detailed description of the electrode implantation technique. After surgery, the animal was placed into the testing tank and kept isolated for 24 hours until it recovered. I tested its behavioral response to unexpected touch under dim red light. Fringe hairs on the pleural plate of the first abdominal segment were brushed with a modified paintbrush that had a pair of electrodes attached to its end and connected to a differential amplifier. The paintbrush electrode provided a reliable measure of the contact time of the brush with the animal
Figure 4-1: In vivo and In vitro recording experimental setup.

In vivo recording: Freely behaving crayfish were stimulated by brushing the fringe hairs that line both sides of the pleural hairs of the first abdominal segment. A pair of implanted electrodes was surgically implanted around the depressor nerve, which allowed the extracellular recording of its activity and field potentials the surrounding depressor muscle. The animal's behavior was video taped on a standard VCR along with the neural recordings displayed on an oscilloscope screen. Additionally, the behavioral responses and neural recordings were also digitally acquired synchronously onto a computer, thus, phase locking the behavioral to the neural recordings.

In vitro recordings: The animals were sacrificed and an extra-cellular electrode was placed on the depressor nerve. Stimulating electrodes were placed on both the left and right sensory second nerves (N2) on either side of the animal. The sensory N2s were electrically stimulated mimicking the brush stimulus delivered in vivo.
due to the sudden change in resistance of the electrode when contact is made. The animals were brushed randomly on either the left or the right side every ~10 minutes with brush duration lasting approximately one second.

The depressor neuro-muscular activity and behavioral response of the animal was time locked and digitized using Spike2 software (Cambridge Electronic Design). A canon digital camera (model 3CCD) was used to videotape the behavior at 30 fps. The behavior was also recorded onto a standard videotape to ensure that no video frames were dropped during digital acquisition (Figure 4-1). Spike sorting analysis was conducted on all traces to separate the tonic and phasic muscular units by using spike2 software.

In vitro electrophysiological recordings Prior to dissection an animal was chilled in iced-water for ~20 minutes. Then it was decapitated and the thorax and abdomen were pinned dorsal side-up. The nerve cord was isolated while keeping the 5th legs still attached to the nerve cord. The nerve cord was pinned ventral side up at the first abdominal ganglion (A1) for later intracellular recording from the ventrally located 5-HT cell body, while the fifth thoracic ganglion was kept dorsal side-up for intracellular recording from the leg depressor motor neurons (Figure 4-1).

Extracellular pin electrodes were placed on the second sensory nerves at A1 for sensory stimulation. All signals were amplified using A-M Systems amplifiers and then the signals were digitized by Spike2 for storage and analysis.
Results

Effect of social status on the response of the depressor nerve activity in vivo

I measured the behavioral responses of animals to unexpected touch while monitoring the 5th leg depressor motor neurons’ firing activity with a pair of implanted electrodes. The implanted electrode in the depressor muscle enabled the recording of the depressor tonic activity during stance and depressor phasic activity during depression. Spike-sorting analysis enabled the separation of the both muscular types during leg depression based on the criteria described previously (see Methods). Then comparison of the depressor muscular activity to the animals’ leg kinematics was made.

Figure 4-2 illustrates the muscular and behavioral responses to unilateral unexpected touch displayed by animals of different social status. In both isolated and dominant animals, contralateral brush to the implanted leg led to its elevation as the animal began to turn toward the stimulus (Figure 4-2A). During this response, the implanted electrode recorded a delay in the onset of depressor muscle field potential as the leg began to elevate (Figure 4-2A, 2nd frame). When the depressor muscle activates, it causes the leg to depress, thus finishing the first step cycle (Figure 4-2A, 3rd frame). Finally, the animal makes another step allowing it to make a complete turn to face the brush stimulus. (Figure 4-2A, 4th frame).

When the stimulus was delivered to the same side of the implanted leg (ipsilateral) the turning response was initiated by immediately depressing the implanted leg corresponding with the immediate activation of the depressor muscle (Figure 4-2B, 2nd frame). This was followed by another complete cycle of levation and depression of
Figure 4-2: Response of freely behaving socially isolated, dominant, and subordinate animals to an unexpected unilateral brush stimulus.

The top of each panel shows the depressor tonic and phasic muscle activity recorded by the implanted electrode before, during and after the animals were brushed on either the left or the right side as indicated below. Dots below traces correspond to the time at which video frames were captured. Brackets indicate leg position corresponding with the recordings. Vertical arrows denote approximate time when the animals were brushed. Video frames displayed below each trace illustrate the animal’s behavioral response to the brush stimulus. At the top of each video frame the oscilloscope screen was videotaped along with the behavior. The oscilloscope trace shows the depressor phasic muscle field potential when the leg is depressed. Cartoons show schematic rendering of the animals’ behavioral responses and leg motions to stimuli. Arrows on animals illustrate the rotation of body movements in response to the stimulus. Insets are examples of tonic and phasic depressor EMGs. Both types of EMGs are overlapped for comparison (right). Mirror=M; Brush=B.

(A) An isolate animal turned to face the brush after having been stimulated on the side contralateral to the implanted leg (left side of animal). Turning occurs by depressing the inner ipsilateral leg (ipsi) while lifting the implanted leg (impl. contra up).

(B) A dominant turning toward the brush. The animal was brushed on the side of the implanted leg (right side of animal). Turning is initiated by depressing the inner ipsilateral leg (imp. Ipsi, 1st step) while elevating the contralateral outer leg (Contra). The depression of the implanted leg is also indicated by the activation of the depressor muscle recorded by the implanted electrode shown on the oscilloscope trace (Dep. EMG).

(C) A subordinate responding to an ipsilateral stimulus by tilting its body posture backward then down. This was produced by a bilateral depression of both 5th legs followed by a rearward thrust as the animal reaches its final stance (Final stance, 3rd and 4th frames).

(D) A subordinate responding to an ipsilateral stimulus by directly lowering its posture. This behavioral response initially corresponds with a small activity of the depressor muscle that is followed by a decrease of tonic muscle activity as the animal reaches its final stance (Final stance, 3rd and 4th frames).
A Isolate: turn
Contralateral response

Dep. tonic activity
Dep. phasic activity

0.5 sec

1 stance
2 Levate
3 1st step
4 2nd step

Ipsi Impl. contra
Ipsi (down) Impl. contra (up)

B Dominant: turn
Ipsilateral response

Dep. tonic activity
Dep. phasic activity

0.5 sec

1 stance
2 1st step
3 Levate
4 2nd step

Imp. electrode
Dep. EMG

Contralateral response
Ipsilateral response

M B

1 2 3 4

1 2 4

3 5ms

72
C Subordinate: push
Ipsilateral response

D Subordinate: drop
Ipsilateral response
the leg as the animal made a full turn to face the brush stimulus (Figure 4-2B, frames 3 and 4).

Subordinates displayed a markedly different response pattern in comparison to isolated and dominant animals. Regardless of which side subordinates were brushed, they displayed one of two responses: they stepped backward and then lowered their posture (Figure 4-2 C), or they immediately lowered their posture without any steps taken (Figure 4-2 D). The recordings of the implanted leg corresponded with these responses. In the first instance, the implanted leg and its contralateral homolog were immediately and simultaneously depressed, thereby pushing against the substrate and providing the animal with the backward thrust needed to step back. This response corresponded with the activation of the depressor muscle (Figure 4-2 C, frame 2). Following the backward movement, the animal began to lower its posture, which corresponded with the inhibition in depressor tonic muscle activity (Figure 4-2C, frame 4). This behavioral response was typical of half the subordinates. The remaining fifty percent displayed an immediate decrease of body posture without any backward movement (Figure 4-2 D, frame 2). The implanted electrode recordings reflected the behavioral response showing a decrease in the depressor tonic muscular activity as the posture of the animals was lowered.

**Status-dependent response pattern of the depressor nerve to unexpected touch**

I analyzed and quantified the firing frequency of the depressor activity of all animals tested in order to determine the effect of social status on the response pattern of the depressor muscle to ipsilateral and contralateral brushes (Figure 4-3). Figure 4-3A1 shows the average firing frequency of the depressor units over 0.1 sec bins to
Figure 4-3: *In vivo* response of the depressor units to ipsi and contralateral sensory stimuli in socially isolated, dominant, and subordinate animals.

The averaged response of the depressor unit for an isolate animal that turns (A1), dominant animal that turns (B1), subordinate animal that pushes (C1), and a subordinate animal that drops (D1) to ipsilateral touches (left graph) and contralateral touches (right graph).

The bins represent the average of 5-10 touches over 0.1 second time period. Arrows denote stimulus onset. “Initial stance” bracket is the averaged firing frequencies prior to touch. “Depress” or “levate” bracket is the averaged firing frequencies in response to touch. “Final stance” the averaged firing frequencies after the animal came to a complete stop. The two Ipsilateral stimuli black bars and contralateral stimuli white bars denote the time period that was averaged across animals to calculate the percent change in firing frequencies from base-line (A2, B2, C2, D2). Final stance brackets denote the time period averaged to calculate the percent change in firing frequency when the animals came to a complete stop (A3, B3, C3, D3). Note the difference in x-axis time scale between isolated and dominant animals compared to subordinates. Statistical test for all conditions: Two-tailed, Wilcoxon signed rank test, ±SEM with 95% confidence intervals, *= P<0.05).
ipsilateral and contralateral stimuli from one isolated animal (number of stimuli averaged was 5-10, \( \pm \)SEM). Ipsilateral brushes significantly enhanced the firing frequency of the depressor while contralateral brushes led to a very small response. The asymmetry in the response pattern of the depressor that depended on the side stimulated (Ipsi versus Contra) was found to be highly consistent (Wilcoxon signed rank test, \( P<0.05 \), \( n = 4 \)) (Figure 4-3 A2). Similarly, dominants showed asymmetry in the response of the depressor to ipsilateral versus contralateral touches (Figure 4-3B). Ipsilateral touches significantly enhanced the firing frequency of the depressor while contralateral touches led to a very small response. As with social isolates, there was a statistically significant difference in the response of the depressor between ipsilateral versus contralateral sensory stimulation in dominants (Wilcoxon signed rank test, \( P<0.05 \), \( n = 7 \); \( \pm \)SEM) (Figure 4-3 B2).

In subordinates, the response of the depressor to sensory touches showed two different patterns that corresponded with the two different behavioral responses displayed (Figure 4-2 C & D, and Figure 4-3 C & D). Subordinates that stepped backward when brushed increased the depressor firing frequency to both ipsilateral and contralateral touches (Figure 4-3 C1). On average, there was no statistical difference between the two sides of stimulation (Wilcoxon signed rank test, \( P>0.05 \), \( n = 6 \); \( \pm \)SEM) (Figure 4-3 C2). Subordinates that immediately lowered their posture without any backward movement showed symmetrical decrease in the depressor firing frequency to ipsilateral and contralateral touches (Figure 4-3 D1).

In addition to differences in behavioral and physiological response patterns to unexpected touches, I found status dependent differences in the depressor firing
frequency when the animals reached their final stance after they were brushed. In isolated animals, the average depressor firing frequency increased by 30% as compared to the activity prior to brushing (Figure 4-3 A3). In dominant animals, the average depressor firing frequency decreased by 15% as the animals reached their final posture (Figure 4-3 B3). On the other hand, subordinate animals showed a decrease in both body posture (Figure 4-2 C, D), which corresponded with a decrease in firing activity of about 38% as the animals reached their final posture. (Figure 4-3 C3 and 4-3 D3).

**Effect of social experience on the response pattern of the depressor nerve in vitro**

To determine the neural basis of the different response patterns displayed by animals of different social status, I conducted *in vitro* isolated nerve cord recordings on preparations taken from these animals. I recorded the firing activity of the depressor nerve while delivering analogous sensory stimulations to the same nerves that innervate the fringe hairs brushed in the *in vivo* experiments (Figure 4-1). I found that the social status-dependent response patterns of the depressor nerve displayed *in vivo* are retained *in vitro* (Figure 4-4).

In socially isolated and dominant animals the depressor nerve displayed asymmetrical responses to sensory stimulation analogous to the responses displayed in the *in vivo* experiments (Figure 4-4 A1, 4-4 B1). The depressor nerve increased its firing frequency when the ipsilateral sensory nerve was stimulated, but it showed little or no response when the contralateral sensory nerve was stimulated. The difference in the response of the depressor nerve between ipsilateral and contralateral sensory stimulation was statistically significant and consistent with the *in vivo* response patterns
Figure 4-4: *in vitro* response of the depressor nerve to electrical sensory stimulation in socially isolated (A), dominant (B) and subordinate (C, D) animals. Top trace of each panel shows the neural recording of the depressor nerve. Bottom bar graph shows the mean firing frequency of the depressor nerve averaged over 5-10 stimuli. Electrical stimulus occurs at bin time 0.

(A1) The response of the depressor nerve of an isolated animal to ipsi and contralateral electrical stimuli of sensory N2s. (A2) Percent change in depressor firing frequency relative to baseline for all isolates. Percent change values were calculated by taking the difference in the firing frequency between the 2 bins immediately following stimulus offset (Black bins for ipsi, and white bins for contra) to the average of the bins prior to stimulation (gray bins). The difference in the percent change in firing frequency is statistically significant between ipsi and contralateral stimulations.

(B1) The response of the depressor nerve of a dominant animal to ipsi and contralateral stimuli of sensory N2s. (B2) The percent change in firing frequency relative to baseline for all dominants. The difference in the percent change in firing frequency is statistically significant between ipsi and contralateral stimulations.

(C1) The response of the depressor nerve of a subordinate animal to ipsi and contralateral stimuli of sensory N2s. Note that this is the response of the animal that displayed a “push” behavioral response to unexpected unilateral touch *in vivo*. (C2) The percent change in firing frequency relative to baseline for all subordinates that displayed symmetrical excitation in depressor response. The difference in the percent change in firing frequency between ipsi and contralateral stimulations is not significant.

(D1) The response of the depressor nerve of a subordinate animal to ipsi and contralateral stimuli of sensory N2s. Note that this is the response of the animal that displayed a “drop” behavioral response to unexpected unilateral touch *in vivo*. (D2) The percent change in firing frequency relative to baseline for all subordinates that displayed symmetrical inhibition in depressor response. The difference in the percent change in firing frequency between ipsi and contralateral stimulations is not significant.
(Figure 4-4 A2, 4-4 B2, Wilcoxon signed rank test, \(P<0.0001\), Isolates = 14; dominants = 23; ±SEM).

The depressor nerve recordings in subordinate animals showed two different response patterns, both of which were symmetrical and consistent with the \textit{in vivo} responses (Figure 4-4 C1, 4-4 D1). Regardless of which sensory nerve was stimulated, the depressor nerve either increased its firing frequency, a characteristic response of the majority of subordinates tested (Figure 4-4 C2), or the depressor decreased its firing frequency (Figure 4-4 D2). In either case, the symmetrical response pattern of subordinates was consistent with the responses recorded \textit{in vivo}. The difference in the firing frequency of the depressor nerve to ipsilateral and contralateral sensory stimulation was insignificant (Figure 4-4 C2, D2, Wilcoxon signed rank test, \(P>0.05\), subordinate 1 = 7; subordinates 2 = 3; ±SEM).

\textbf{Social status-dependent differences in the tonic firing activity of the depressor nerve}

We compared the average tonic firing frequency of the depressor neurons among animals of different social status and found that the firing frequencies differed among the three social groups (Figure 4-5, Kruskal-Wallis one-way ANOVA; Kruskal-Wallis test \(P=0.0234\); isolates=33; dominants=13; subordinates 9; ±SEM). Post test paired comparison showed that the firing frequencies of the depressor nerve of dominant animals were significantly faster than that of subordinates and isolates (Figure 4-5, Mann Whitney test post-test; dominant vs. isolates \(P=0.0461\); dominants vs. subordinates \(P=0.0155\)). The average firing frequency of the depressor in isolates was intermediate and did not significantly differ from that of subordinates (Mann Whitney test post-test, \(P=0.2161\)).
Figure 4-5: Basal tonic firing frequency of the depressor nerve among animals of different social experience.

On average, the tonic firing frequency of the depressor nerve of dominant animals is significantly faster than those of subordinates while the firing frequency of the depressor of isolates did not differ from that of either dominants or subordinates. Filled dots represent averaged frequency measurements of individual animals. Bars represent the averaged data of all the filled dots. Statistical test: two-tailed one-way ANOVA; post test Kruskal-Wallis test $P<0.05$. 
Discussion

We have shown that social status affects the behavioral and muscular responses to unexpected touch in vivo (Figure 4-2). Analysis of leg movements and the neural activity of the leg depressor muscle show status dependent differences in the pattern of the depressor activity that correspond with the three different leg movement responses (Figure 4-3). We conducted in vitro experiments in an effort to identify the neural bases that account for the status-dependent response patterns and found that the same response pattern is retained in a reduced preparation (Figure 4-4). Our results suggest that the network underlying the orienting behavioral response is reconfigured by social status providing behavioral adaptabilities to social conditions.

How can crayfish produce a repertoire of different orienting responses? The ability of crayfish and many decapods and hexapods to produce different leg coordination patterns is attributed to the many legs and joints that provide enormous adaptability. However, the presence of many legs poses a problem of coordination that is highly controlled by the central nervous system (Cattaert & Le Ray, 2001). The neural circuit driving the movement is separate for each of the legs (Cattaert & Le Ray, 2001, Büschges et al., 1995). And the coordination of these separate circuits is achieved through inter leg networks (Hess and Büschges, 1999; Akay et al., 2001). One advantage of having separate circuit for each of the legs is that it is easier to reconfigure patterns of movements as environmental contexts dictate; for example, to switch from walking to turning. In the cockroach, descending brain inputs are responsible for mediating turning behavior by allowing an animal to switch from symmetrical walking to asymmetrical turning response (Mu and Ritzmann, 2005; Ridgel et al., 2007). Two
hypotheses are presented as to how the descending input regulates and coordinates the activities of the local circuit. First, a switch in behavioral pattern can occur by having the descending input activating a completely new neural circuit unique to a particular behavior (i.e. walking, turning or dropping). Conversely, the descending input can modify one neural circuit by either recruiting or inhibiting interneuronal elements to switch from walking to turning, dropping or standing. Daily social interactions that encompass visual, olfactory and tactile social cues are likely to be integrated by the brain. These social signals would then reconfigure the thoracic local circuits according to the integrated social cues via descending inputs. Descending inhibitory inputs from the brain are known to play a major role in regulating the threshold of the lateral giant command neuron involved in the tailflip escape system of crayfish (Krasne & Wine, 1975; Krasne et al., 1990; Vu & Krasne, 1992) suggesting that similar descending inputs are likely to exist for regulating leg coordination.

What is most interesting about the current findings is that the response differences observed among the three social groups continue despite the absence of any continuous descending input from higher brains centers in the reduced preparation (Figure 4-4). This suggests that the descending inputs are likely to reconfigure local thoracic circuits during the pairing period but are not necessary for maintaining local circuit activity once the brain is separated. The duration required for reconfiguration of the orienting response circuit is not yet determined. However, there is evidence indicating that daily social interactions on the order of few days is necessary for robust manifestation of the behavioral response difference observed between dominant and subordinate crayfish (Song et al., 2006).
Our results show that status-dependent differences also extend to the general tonic firing of the depressor neurons. Tonic firing of the depressors in dominant animals was significantly faster than that of subordinates, while social isolates showed an intermediate rate of firing (Figure 4-5). In the freely behaving animals these differences may translate into differences in postural stance. Faster firing frequency of the depressors would likely to induce greater depression of the leg than slower firing, and consequently would lead to an elevated body posture. This is consistent with the previous findings that dominant and subordinate lobsters and crayfish adopt different postural stances (Livingstone et al., 1980). Dominant crayfish adopt higher body postures compared to their subordinate counterparts. However, it is yet to be determined whether the observed differences seen in isolated nerve cord also occur in freely behaving animals. The observed status-dependent differences in the tonic firing frequency were measured in an isolated nerve cord. This suggests, as with the differences seen in the orienting responses, is that social status has influenced the depressor motor neurons in a fundamental way that does not require descending inputs from higher brain centers.

In summary, the social transitions that animals undergo during dominance formation are reflected in immediate differences in postural display in socially dominants and subordinates. As the dominance order matures the behavioral differences are translated into long-term differences in behavioral responses that are retained in the nervous system as illustrated in the reconfiguration of the neural circuit underlying behavior. Our results illustrate how social status enables the reconfiguration of the central nervous system to produce the desired behavioral response that would fit the
animals’ social standing. The mechanism of how social information are mediated that leads to the induction of circuit reconfiguration is not fully understood, suggesting a more fundamental level of analysis that remains to be investigated.
CHAPTER V
SEROTONERGIC MODULATION
OF THE LEG DEPRESSOR MOTOR NEURONS

Introduction

Neuromodulators have been implicated in affecting various aspects of nervous system function. Serotonin, octopamine, dopamine and proctolin among many other neuromodulators are implicated in affecting rhythmic motor pattern generation and movement (Marder & Thirumalai 2002, 2001; Landry, 2006; Rauscent et al., 2006), aggression (Ferrari et al., 2003; Olivier & Oorschot, 2005); and muscle properties (Erxleben et al., 1995). In particular, the neuromodulator, serotonin, has received great attention because of its apparent role in influencing a wide range of biological processes. It has been implicated in modulating cellular properties (Hultborn & Kiehn, 1992), neural network dynamics (Katz et al., 1994), initiating and modulating both motor output and behavior (Madriaga et al., 2004; Huber, 2005). In recent years it has become apparent that serotonin is also involved in regulating the social behavior of many species (Manuck et al., 2006; Miczek and Fish, 2006; Sneddon et al., 2000; Panksepp et al., 2003). In crayfish serotonin has been implicated in influencing a range of biological processes including the tailflip escape response (Yeh et al., 1996, 1997), body posture (Livingstone et al., 1980; Antonsen and Paul, 1997; Tierney and Mangiamele, 2001), and social interactions (Panksepp and Huber, 2002).

The effects of 5-HT on the neural circuit controlling leg movements have been extensively studied. Specifically, 5-HT is shown to modulate synaptic transmission
(Fischer and Florey, 1983; Delaney et al., 1991; Pearlstein et al., 1998; Wang and Zucker, 1998; Southard et al., 2000) and endogenous properties of the neurons that control leg locomotion (Rossi-Durand, 1993; Alvarado-Alvarez et al., 2000; Nagayama, 2002). Injection of 5-HT in freely behaving animals influences posture and walking (Tierney and Mangiamele, 2001), aggressiveness (Huber et al., 1997a, b), and social status in crayfish (Huber et al., 1997b, 2001). When 5-HT or octopamine is injected, they induce stereotypical dominant or subordinate postures, respectively (Livingstone et al., 1980; Kravitz, 1988). So our interest was to determine the functional role of serotonin in regulating the status-dependent response pattern of the depressor motor neurons previously described (chapter 4).

More recently, it was demonstrated not only that serotonin affects social behavior, but that the activities of the serotonergic neurons in the nervous system are also influenced by social dominance. This feedback interaction between behavior and cellular activity was demonstrated by Drummond and colleagues who recorded the response of the first abdominal serotonergic neurons (A1 5-HT neurons) to sensory stimulation in socially isolated, dominant, and subordinate animals (Drummond et al., 2001). They found that the pair of 5-HT neurons in the first abdominal ganglion (A1) displays similar social status-dependent response patterns to that of the depressor motor output described previously in chapter 4. Socially isolated and dominant animals show asymmetry in the response of the A1 5-HT neurons to sensory stimulation. Unilateral sensory stimulation led to an increase of the ipsilateral 5-HT neuron firing frequency while the contralateral neuron produced either no response or was modestly inhibited. In subordinate animals the 5-HT neurons displayed the same symmetrical
response pattern to that displayed by the depressor motor output. In half of the subordinates examined, the responses of the 5-HT neurons showed symmetrical excitation regardless of which side was stimulated while the remaining half showed symmetrical inhibition.

The striking similarities in the status-dependent response patterns of the 5-HT neurons and the depressor motor neurons suggests that release of 5-HT from the A1 5-HT neurons might either mediate or modulate the status-dependent depressor motor output. The axons of A1 5-HT neurons project rostrally to the 5th thoracic ganglion where axonal branches terminate in the ipsilateral hemiganglion (Beltz and Kravitz, 1983). Rossi-Durant and colleagues demonstrated that one axonal branch from the A1 5-HT neuron turns laterally and projects into the ipsilateral depressor nerve (Rossi-Durand, 1993). However, it is not yet determined whether this 5-HT axonal branch contacts the depressor motor neurons and, if so, what is the nature of their response. Collectively, these results suggest that the status-dependent response of the A1 5-HT neurons, hence, differential release of 5-HT due to the unilateral innervation of T5, might regulate the depressor motor output in a status-dependent manner.

In order to determine the effect of serotonin on the response pattern of the depressor motor output, we tested the effects of bath applied 5-HT. Second, while monitoring intracellularly the activity of the A1 5-HT neurons we manipulated their firing frequency to test their effects on the depressor motor neurons. We show that although 5-HT is important in affecting the activity of the depressor neurons, it is neither necessary nor sufficient to mediate the status-dependent responses of the motor output. Rather, 5-HT application has status-dependent effects on the tonic firing of the
depressor neurons. In addition, the A1 5-HT neurons seem to modulate the response of the depressor neurons by enhancing their sensitivity to synaptic input.

**Methods and Material**

*Animal maintenance*  Adult crayfish (*Procambarus clarkii*, 8-10cm) were purchased from a commercial supplier (Atchafalaya Biological Supply, Louisiana) then housed individually in 5L tanks each containing de-chlorinated water, a filter and air-stone for water oxygenation. The water was changed with fresh water once every two weeks. The animals were isolated from each other physically, visually, and chemically for a minimum period of one month and were fed shrimp pellets twice weekly.

*Pair formation*  Two previously isolated crayfish were paired for a period of at least two weeks and maximum one month. The animals were marked on the thorax with a permanent marker for identification one day prior pairing. On pairing day, the animals were removed from their isolated tanks and placed in the testing tank. The testing tank was divided into two chambers with an opaque divider preventing interaction between the two animals. Fifteen minutes of acclimation to the new environment were given then the divider was removed freeing them to interact. The agonistic interactions (attacks, approaches, escape tailflips, offensive tailflips, and retreats) between the two animals were recorded for the first 30 minutes of interactions and on every subsequent day. The animals were free to interact at all times during the pairing period, which lasted a minimum of 2 weeks and up to 1 month. Dominance between the paired animals was usually established within the first fifteen minutes of interactions on day one and was rarely reversed. Dominance was determined based on the total number of aggressive
and submissive behaviors an individual animal performed (See Issa et al, 1999 for detail).

**In vitro electrophysiological recordings** Prior to dissection an animal was chosen randomly then chilled in iced-water for ~20 minutes. The animal was then decapitated and the thorax and abdomen were pinned dorsal side-up. The nerve cord was isolated while keeping the 5th legs still attached to the nerve cord. The nerve cord was pinned ventral side up at the first abdominal ganglion (A1) for later intracellular recording from the ventrally located 5-HT neuron cell body, while the fifth thoracic ganglion was kept dorsal side-up for intracellular recording from the leg depressor motor neurons (Figure 5-1).

Extracellular pin electrodes were placed on the second sensory nerves at A1 for sensory stimulation and one recording electrode was placed on the depressor nerve (Figure 5-1). All signals were amplified using A-M Systems amplifiers and then the signals were digitized by Spike2 for storage and analysis. All depressor motor nerves were spike sorted using the Spike 2 software. In order to further refine the sorted depressor units all sorted spikes were further analyzed using the principle component analysis software provided with Spike2 software.

One intracellular electrode was used to record from the A1 5-HT neuron. The electrode was filled with either 10% Dextran Rhodamine that was diluted in 2M potassium acetate or 5% Lucifer Yellow and had a resistance ranging from 35-45 MΩ. The signals were amplified using an Axon Instrument axoclamp 2A then digitized using Spike2 interface (Cambridge Electronics Design) and software for analysis and permanent storage.
Figure 5-1: experimental setup.

Isolated nerve cord preparation showing the dissected coxopodite segment of the 5th leg and the relevant motor nerves that exit from the 5th thoracic ganglion. An extra-cellular recording electrode was placed on the depressor nerve. Stimulating electrodes were placed on both the left and right sensory second nerves (N2) at the 1st abdominal ganglion. The firing activity of the A1 5-HT cell, ipsi-lateral to the depressor nerve being recorded, was monitored intracellularly from the cell soma during N2 sensory stimulation (inset).
Previously it was demonstrated that extended dissections depletes 5-HT expressing cells of 5-HT (Musolf et al., 2004). Depletion of 5-HT can be reversed by bathing the preparation with 5-HT, thus, allowing serotonergic neurons to readily take up 5-HT. In the present study, dissections took on average 2-3 hours, which may have caused the depletion of 5-HT from the CNS. To circumvent this potential problem we bathed the preparations with 20uM of 5-HT allowing the depleted 5-HT neurons to take up 5-HT (Musolf and Edwards, 2000). This was followed by a wash period with normal saline for 1hr.

**Drug application**

Exogenous application of 50 µM of 5-HT (Sigma Aldrich; serotonin creatinine sulfate complex) was perfused at a rate of 1mL per minute for 45 minutes that was followed by a wash period that lasted 2 hours. Methiothepin Mesylate salt is a 5-HT$_{2β}$ receptor antagonist, and it was applied at a concentration of 10µM and a rate of 1ml/min for a duration of 20min then washed away with normal saline for 2 hours.

**Immunohistochemistry**

**Backfills** The depressor nerve was backfilled with 0.25% Texas Red (TR) dye. The depressor nerve was placed in a vaseline well containing TR dye. Then the nerve was cut, thus allowing the dye to backfill. The nerve was allowed to fill for 24 hours, after which it was put in fixative for immunocytochemical processing.

**5-HT Staining protocol** The preparations were fixed for 24 hours with paraformaldehyde. They were rinsed in saline for approximately 10 minutes, dehydrated in an alcohol series (10min intervals) to 100% then re-hydrated down to buffer then were rinsed in PBTX buffer 5 times for 50 minutes each. The preparations were then incubated in 60 µl
anti-mouse 1° 5-HT anti-body, in a mixture of 150 µl normal goat serum, 2790 µl of 0.25% PBTX, and 0.1% sodium azide for 2-3 nights. The preparations were washed with PBTX buffer 6 times at 50 min intervals then incubated in 60ul of 2° anti-body Alexa Fluor 488 goat anti-mouse and 2940 µl of PBTX for 1-2 nights. Then the preparations were washed with PBTX 6 times with 50 minutes in between the washes. De-hydrated and mounted the preparations in Methyl Salicylate.

5-HT₁ Receptor staining protocol The preparations were fixed for 24 hours with paraformaldehyde. Then they were rinsed in saline for approximately 10 minutes. Dehydrated to 100% alcohol (10min intervals) then re-hydrated down to buffer. The preparations were then rinsed in PBTX buffer 5 times for 50 minutes each. The preparations were incubated in 20 µl 1° 5-HT₁ anti-body (Am-3), 930 µl of 0.25% PBTX, 0.1% sodium azide and 50 µl normal goat serum for 2-3 nights. Then we washed the preparations with PBTX 6 times with 50 minutes in between the washes. Then they were incubated in 20 µl of 2° anti-body Alexa Fluor 488 goat anti-Rabbit and 980 µl of PBTX for Am-3 for 1-2 nights. Then we washed the preparations with PBTX 6 times with 50 minutes in between the washes. De-hydrate and mount the preparations in Methyl Salicylate.

Results

Effect of exogenous application of 5-HT on depressor nerve activity

Application of 5-HT had two effects on the activity of the depressor nerve. 5-HT influenced (i) the tonic firing of the depressor nerve and (ii) its response to sensory nerve stimulation. First, 5-HT significantly decreased the overall tonic firing activity of
the depressor in dominants and increased it but insignificantly in social isolates and subordinates (Figure 5-2). The average firing frequencies for all three conditions (control, 5-HT, and wash) were calculated by averaging ten 5 second recording samples of tonic firing. Control samples were taken 5 minutes prior to the onset of 5-HT application (control), 5-HT recording samples were taken 40 minutes into 5-HT application (5-HT), and the wash samples were taken 1 hour after 5-HT application was stopped (wash). In social isolates 5-HT application increased the tonic firing of the depressor nerve in 8 out of 11 preparations and the remaining 3 preparations showed a modest decrease (Figure 5-2A). In subordinate animals, 5-HT application led to an increase in the firing frequency of the depressor nerve in 5 out of 7 preparations. The firing frequency was unchanged in one preparation, and one preparation showed a modest decrease (Figure 5-2C). The average increase of the tonic firing frequency in both social isolates and subordinates was not statistically significant (One-way ANOVA. Isolates $P=0.1632$; subordinates $P=0.3046$). In dominants, 5-HT significantly decreased the tonic firing of the depressor nerve in 6 out of 7 preparations, and one preparation showed an increase (Figure 5-2B; One-way ANOVA; Friedman test post test, $P=0.0272$). The tonic firing of the depressor nerve returned back to control values within one hour of wash for all preparations except for one socially isolated and one subordinate animals where firing continues to increase (t-test Wilcoxon signed rank test comparison of control and wash; isolates $P=1.0547$; subordinates $P=0.2188$). In dominant animals even after one hour of wash the tonic firing of the depressor nerve continued to be significantly different than control (t-test Wilcoxon signed rank test, $P=0.0313$).
Figure 5-2: Effect of 5-HT application on the tonic firing activity of the depressor nerve in socially experienced animals.

Graphs at right panel show individual response of the depressor nerve for each animal to bath application of 5-HT in isolates (A) dominants (B) and subordinates (C). Graphs at left panel show the averaged responses for each social state. (A) in 10 out of 11 isolated crayfish the tonic activity of the depressor nerve increased when 5-HT was applied, one animal showed no response. (B) in 6 out of 7 dominant crayfish the tonic activity of the depressor nerve decreased when 5-HT was applied and the response of the depressor for one animal showed an increase. (C) in 5 out of 7 subordinate crayfish the tonic activity of the depressor nerve increased when 5-HT was applied, one animal showed a decrease, and one animal showed no response. The averaged data indicates that there was a statistically significant decrease in the tonic activity of the depressor nerve when 5-HT is applied in dominants, and this decrease was washed away. Although there was an increase in the tonic activity of the depressor nerve in isolates and subordinates; this increase was not statistically significant One-way ANOVA with repeated measures. Friedman test, $P<0.05$. 

The effects of 5-HT on the tonic firing activity of the depressor nerve consequently affected the depressor’s response to sensory stimulation. In social isolates the depressor nerve showed asymmetrical response pattern to unilateral stimulus prior to 5-HT application (Figure 5-3 A, control). In 8 out of 11 social isolates 5-HT application increased the tonic firing frequency of the depressor nerve (Figure 5-3 A, 5-HT, and Figure 5-2 A). This increase was sufficiently high leading the depressor neurons in most preparations to reach their maximal firing rate. Consequently, the depressor nerve was prevented from responding further to sensory stimulation (Figure 5-3 A). One hour after 5-HT was washed the depressor nerve reverted back to the same asymmetrical response to sensory stimulation as that during control (Figure 5-3 A, wash). In one preparation out of 8 the tonic firing continued to increase during wash. Interestingly, the depressor nerve displayed an asymmetrical response as that of control despite the high firing frequency. In this instance, the increase of tonic firing and response of the depressor nerve to sensory stimulation was due to recruitment of new depressor units that were previously not active. This indicates that individual motor units may reach their maximal firing frequency in the presence of 5-HT as observed in most preparations, but the firing rate of the depressor nerve may increase even further with the recruitment of more motor units. In dominant animals the significant decrease in the firing frequency of depressor tonic activity appeared to have led to a basement effect that prevented the depressor nerve from responding to sensory stimulation (Figure 5-3 B). During control tests that preceded 5-HT application, the depressor nerve displayed an asymmetrical response pattern to unilateral stimulation in all dominants tested. When 5-HT was applied and reached its maximal concentration 40 minutes into its application
**Figure 5-3:** Effect of 5-HT application on the response of the depressor nerve to ipsilateral and contralateral sensory stimulation in socially isolated (A), dominant (B) and subordinate (C) animals.

Control traces are illustrated in black, stimuli delivered in the presence of 5-HT are illustrated in red, and traces of stimuli recorded after 1 hour of wash are illustrated in blue. Bottom graphs for each social phenotype show the instantaneous firing frequency of the depressor nerve for all three experimental conditions for ipsilateral and contralateral stimulations.
the depressor nerve tonic activity significantly decreased, and it ceased responding to sensory stimulations (Figure 5-3 B, 5-HT, n=6 out of 7). One hour after 5-HT was washed the tonic firing remained significantly slower than control values (Figure 5-2 B), but its response to sensory stimulation reverted to asymmetrical pattern as that of control (Figure 5-3 B, wash). In subordinate animals 5-HT application increased the tonic firing activity of the depressor nerve. Although this increase was not statistically significant it was sufficient to block the response of the depressor nerve to sensory stimulation (Figure 5-3 C). Before 5-HT application the depressor nerve displayed symmetrical response pattern to unilateral sensory stimulation (Figure 5-3 C, control). When 5-HT was applied and as it reached its maximal concentration in the bath (40 minutes into application) the depressor nerve stopped responding to sensory stimulations (Figure 5-3 C, 5-HT, n=7). The effect of 5-HT on the depressor’s tonic firing and response to sensory stimulation was washed away within one hour in 6 out of 7 animals tested (Figure 5-3 C, wash). In the remaining preparation the tonic firing continued to increase even two hours into the wash, and the depressor continued to be unresponsive to sensory stimulations.

Among a total of 13 known depressor motor neurons approximately 1-7 units fired tonically and responded to sensory stimulation in each preparation tested. So we were interested to determine whether 5-HT had different effects on each of the depressor units as compared to an overall nerve activity. To this end, we spike sorted the depressor nerve for each preparation (see Methods for details on spike sorting). We found that the response of individual depressor motor neurons to 5-HT was varied (Appendix 2). Small to mid range size depressor units, which responded vigorously to
sensory stimulation, were more affected by 5-HT application compared to large depressor units. When 5-HT was applied, the firing rate of mid-size units either increased in social isolates and subordinates (Appendix 2 A, C) or significantly decreased in dominants (Appendix 2 B) as illustrated previously in figure 5-3. This response seemed to prevent these units from responding to sensory stimulation. When 5-HT was washed their responses returned to that of control. The change in the tonic firing frequency of large depressor units varied from little change (Appendix 2 A) to large change in firing rate (Appendix 2 B). The large units did not respond significantly to sensory stimulation in the absence or presence of 5-HT (Appendix 2 A and B).

**Effect of bath application of Methiothepin on depressor nerve activity**

To further investigate the role of 5-HT on the depressor nerve activity and its response to sensory N2 stimulation we bath applied methiothepin, which was previously demonstrated to be a highly specific 5-HT$_{2B}$ receptor antagonist in crayfish (Spitzer et al., 2008). Prior to methiothepin application we tested the response of the depressor nerve to unilateral sensory stimulation. We found that social isolates and dominants displayed the typical asymmetrical response patterns demonstrated previously (Figure 5-4 A, control) while subordinates displayed a symmetrical response pattern. When methiothepin was applied it induced two effects on depressor nerve activity that was common to all three social phenotypes. First, it decreased the overall tonic firing of the depressor nerve (Figure 5-4 A, methiothepin, and Figure 5-4 B). Second, it blocked the depressor nerve response to sensory N2 stimulation (Figure 5-4 A, methiothepin, and Figure 5-4 C). The decrease of the depressor nerve tonic activity and its response to
Figure 5-4: Effect of methiothepin (5-HT₂B antagonist) on the response of the depressor nerve to N2 sensory stimulation.

(A) An example of the response of the depressor nerve to N2 sensory stimulation in a dominant animal. Control, ipsi-lateral stimulation enhanced the firing activity of the depressor nerve while contra-lateral stimulation had little effect. Methiothepin had two effects on the activity of the depressor nerve. First, methiothepin significantly decreased the tonic firing activity of the depressor nerve (B). This effect could not be washed away after one hour of wash but did wash away after 2 hours. Secondly, methiothepin blocked the peak response of the depressor nerve to N2 sensory stimulation, and its effect was washable within one hour of its application (C).
sensory stimulation after methiothepin application was significant and consistent across all three social groups. For that reason the results of all animals were combined (Figure 5-3 B; ANOVA, post test Friedman test; \(^{P<0.001}\); two tailed, 95% confidence. Isolates=6; dominants=6; subordinates=5; ±SEM). The average change in the tonic firing of the depressor nerve was calculated by averaging five seconds periods recordings of tonic activity across animals. The effect of methiothepin on tonic firing was measured when methiothepin reached maximal concentration 20 minutes into its application; while the wash values were calculated two hours after methiothepin was washed. One hour of wash was not sufficient to wash away the effects methiothepin induced. However, after two hours of wash both tonic firing and depressor response to sensory N2 stimulation returned to control measurements.

**Anatomical contact between the serotonergic and the depressor motor neurons**

We conducted an immunohistochemical staining for serotonin of the CNS and backfilled the depressor nerve with Texas Red heavy weight dye (10,000 MW) to determine possible anatomical contact between the depressor neurons and 5-HT immunoreactive neurons (Figure 5-5). Our focus was on the A1 5-HT neurons that are known to have their axons unilaterally extend and innervate the fifth thoracic (T5) hemигангlion (Beltz and Kravitz, 1983; Rossi-Durand, 1993). We focused on one major branch of the A1 5-HT axon in T5 that curves distally and innervates the depressor nerve. Confocal imaging of the T5 hemигангlion show possible contact between the ipsilateral A1 5-HT axonal branch and a number of the depressor motor neurons (Figure 5-5 B). The image illustrated in this figure is a confocal image consisting of a stack of 7
Figure 5-5: Putative cellular contact between the A1 5-HT cell axon and the depressor motor neurons and expression of the 5-HT$_{1crust}$ receptor by the depressor motor neurons.

(A) Immunocytochemical staining of 5-HT (green) in the 5th thoracic hemiganglion of the CNS and the depressor motor neurons backfilled with Texas Red dye. Arrow: the A1 5-HT cell axon that project into the depressor nerve. (B) Enlarged view (x60 objective) of the boxed area shown in (A) of possible contact between the A1 5-HT cell and the depressor motor neurons. Arrowheads indicate putative contact points. (C) Extracellular electrophysiological recording of the depressor nerve firing activity when the A1 5-HT cell was activated by positive current injection. Spike sorted analysis shows that one unit in the depressor nerve correlates one-to-one with the A1 5-HT cell intracellular recording (overlay of 10 spikes). (D-G) expression of the 5-HT$_{1crust}$ expression (green) by the depressor motor neurons. (D) Texas Red backfilled staining of three depressor cell bodies. (E) Immunocytochemical staining of 5-HT$_{1crust}$ (green) by a depressor MN (arrow) same cell body indicated in part D. (F) overlay of part D and part E showing that only one cell expresses the 5-HT$_{1crust}$ receptor (arrow). (G) Expression of the 5-HT$_{1crust}$ on the axons of the depressor motor neurons approximately in the same axonal region of possible synaptic contact by the A1 5-HT cell shown in part B.
slices each ∼2 µm in thickness. These images suggest that the A1 5-HT neuron may directly contact some of the depressor motor neurons proximal to the ganglion. However, further examination using electron microscopy is necessary to conclusively verify the occurrence of this anatomical connection.

Further, we have electrophysiological evidence suggesting that the A1 5-HT axon innervates the depressor nerve (Figure 5-5 C). Spike sorted analysis of the depressor motor neuron units indicated that one of the small units in the depressor nerve correlates and is time locked with the intracellularly recorded A1 5-HT neuron spike with a spike conduction delay of approximately 24ms. The ability to record the A1 5-HT spike in the depressor nerve was found to be conditional on the position of the recording site along the depressor nerve root. Distal recording of depressor activity immediately proximal to the depressor muscle failed to record the activity of 5-HT neuron. More medially located recordings of depressor activity on different preparations were more reliable in recording the 5-HT neuron firing as illustrated in Figure 5-1C. This suggests that the A1 5-HT axon innervation of the depressor nerve does not extend to the motor nerve endings, indicating that the A1 5-HT neuron does not directly modulate the depressor muscle. Instead it might modulate the activity of the depressor motor neurons. This data corresponds with the immunohistochemical data that shows the 5-HT axonal branch converging onto the depressor motor neurons only proximally with little 5-HT immuno-reactivity distally.

We conducted immunohistochemical mapping of the 5-HT_{1α} crust receptor in T5 (Figure 5-5 D-G) to determine whether the depressor motor neurons express this 5-HT receptor. Confocal imaging of T5 indicated Immuno-labeling of the 5-HT_{1α} crust in the
same putative contact region between the A1 5-HT axon and the depressor motor neurons (n=5). Receptor labeling was primarily localized to the surface of the depressor motor neurons with little labeling within the axons. Conversely, the cell bodies of the depressor motor neurons showed receptor labeling throughout the cytoplasm. It is worthy to mention that not all depressor motor neurons express this particular receptor (in each nerve cord 4 or 5 cell bodies labeled for the 5-HT$_{1\alpha}$ crust receptor out of ~10 successfully backfilled cells bodies). The widespread labeling of the receptor in the cell bodies and localized expression on the membrane of the axons suggests that expression of the 5-HT$_{1\alpha}$ crust receptor occurs in the cell bodies and then is transported and inserted in axonal regions.

In this study we focused exclusively on one axonal branch of A1 5-HT neuron that innervates the depressor nerve. However, serotonergic labeling in the 5$^{th}$ thoracic ganglion is extensive as illustrated in Figure 5-5 A and other previous studies (Beltz and Kravitz, 1983, Real and Czternasty, 1990). We were unable to identify specific sights and possible contact points between the 5-HT and the depressor neurons. Nonetheless, our results do not exclude the possibility of a paracrine release of 5-HT in the 5$^{th}$ ganglionic neuropil that could act on the depressor motor neurons.

*Modulation of the depressor motor neurons by the A1 5-HT cells*

The unilateral projections of the A1 5-HT neurons into the 5$^{th}$ thoracic ganglion and the putative anatomical connectivity between them and the depressor neurons suggest that the 5-HT neurons are suited to modulate the activity of the depressor neurons. To test this hypothesis, we measured the response of the depressor nerve to
stimulation of N2 sensory afferents in the first abdominal ganglion with and without activation of the ipsilateral 5-HT neuron (Figure 5-6). As a control, we measured the response of the depressor nerve to ipsilateral N2 sensory stimulation while the 5-HT neuron was kept silent by injecting negative DC current in the cell soma. We repeated the test as the 5-HT neuron was driven at a rate of ~10 Hz by injecting positive current. We found that the response of the depressor nerve to N2 sensory stimulation was enhanced when the 5-HT neuron was active as compared to when it was silent (Figure 5-6 A, n=5). Conversely, 5-HT neuron stimulation did not change the response of the depressor nerve to stimulation of the contralateral N2 sensory stimulation (Figure 5-6 B, n=5). This result suggests that the A1 5-HT neurons enhance the firing activity of the depressor motor neurons, but the modulatory effect is limited to the ipsilateral side.

The A1 5-HT neurons fire tonically at a rate of 2-4 Hz (Beltz & Kravitz, 1983, Drummond et al., 2001). This suggests that the 5-HT neurons persistently release 5-HT so to modulate their postsynaptic targets tonically. We had previously demonstrated status-dependent tonic firing of the depressor nerve in in vitro preparations (Chapter 4). So our hypothesis was that the A1 5-HT neurons tonic activity may also depend on social experience. So we compared the tonic firing of the A1 5-HT neurons in all three social phenotypes. However, we found that the tonic firing of the A1 5-HT neurons did not differ among isolates, dominants, and subordinates (Figure 5-7).

**Discussion**

Here we studied the effects of exogenous application of 5-HT and stimulation of the A1 5-HT neurons on the firing activity of the depressor nerve in socially isolated,
Figure 5-6: Effect of 5-HT cell stimulation on the response pattern of the depressor nerve to N2 sensory stimulation.

(A) Stimulation of the left sensory nerve while keeping the left 5-HT cell silent activates the left depressor nerve (black) and the right depressor nerve shows little response (blue). (A1) Driving the left 5-HT cell by current injection enhances the response of the left depressor nerve to stimulation of the left sensory nerve but the response of the right depressor is unaffected (Red). (A2) Comparison of the response frequency of the left/right depressor nerves to left sensory stimulation in the absence and presence of 5-HT cell stimulation.

(B) Stimulation of the right sensory nerve while keeping the left 5-HT cell silent activates the right depressor nerve (blue) and the left depressor nerve shows little response (black). (B1) Driving the left 5-HT cell by current injection during sensory stimulation of the right sensory nerve does not enhance the response of the right depressor nerve nor the response of the left depressor is affected (Red). (B2) Comparison of the response frequency of the left/right depressor nerves to right sensory stimulation in the absence and presence of 5-HT cell stimulation. R = right; L = left
**Figure 5-7:** Basal tonic firing frequency of the A1 5-HT cells among animals of different social experience.

Status-dependent differences were not significant in the mean firing frequency of the A1 5-HT cells. Filled dots represent averaged frequency measurements of individual animals. Bars represent the averaged data of all the filled dots. Statistical test: two-tailed one-way ANOVA.
dominant and subordinate animals. Our objective was to determine the functional role of
the A1 5-HT neurons in contributing to the status-dependent response patterns of the
stimulus evoked depressor motor output (chapter 4). It was previously demonstrated
that the A1 5-HT neurons show the same social status response dependency as that of
the depressor motor output (Drummond et al., 2001). Our hypothesis was that the
status-dependent release of 5-HT from the 5-HT neurons mediates the status-
dependent evoked responses of the depressor nerves. To test this hypothesis we
supplemented the differential endogenous release of 5-HT by bath applying it. We
expected that the bilateral 5-HT exposure would convert the asymmetrical response of
the depressor nerve to a unilateral sensory stimulus typically observed in isolates and
dominants to the symmetrical response observed in subordinates. We found that 5-HT
application affects the depressor nerve’s tonic firing differently in socially experienced
animals; however, these tonic firing responses seem to have confounded the effects to
determine conclusively how 5-HT affects the response of the depressor nerve to
sensory stimulation.

First, we found that 5-HT application has different effects on the tonic firing of the
depressor in social isolates and subordinates compared to dominant animals. When
applied, 5-HT increased the depressor nerve tonic firing frequency in social isolates and
subordinates but significantly decreased it in dominants (Figure 5-2). The 5-HT induced
increase in tonic firing of the depressor nerves would translate into an increase in body
posture in a behaving animal. This is consistent with the finding that injection of 5-HT in
a freely behaving crayfish increases its body posture. However, this study was
conducted on animals housed communally and of unknown social status (Livingstone et
al., 1980). The effects of 5-HT injections on socially experienced animals are yet to be determined. If the in vitro results presented here are of any guide then it would be expected that injections of 5-HT into dominant animals would lead a decrease of body posture and an increase of body posture in subordinates. This hypothesis is counterintuitive to the current thinking that 5-HT always promotes dominant like behavior. However, these effects are likely to depend on the basal concentration of 5-HT in the blood, which might differ between socially dominant and subordinate animals. Tierney and Mangiamele studied the behavioral response of communal crayfish to various concentrations of exogenously administered 5-HT (Tierney and Mangiamele, 2001). They showed that the effects of 5-HT at low concentrations had little effect on body posture. Intermediate concentrations elicited the raised body posture as previously described (Livingston et al., 1980; Antonsen & Paul, 1997). But high concentrations of 5-HT induced the opposite effect by lowering the body posture.

The effects of 5-HT application on the depressor nerve's tonic firing had influenced the response of the depressor nerve to N2 sensory stimulation. Regardless of the social status the depressor nerve failed to respond to sensory stimulation in the presence of 5-HT (Figure 5-3). In the majority of social isolates and subordinates, 5-HT sufficiently increased the tonic firing of the depressor nerve causing it to reach near maximal firing rate. This effect prevented the depressor nerve from responding further to sensory stimulation. The maximal rate of firing was a reflection of the motor units that were firing at that time. However, recruitment of additional depressor neurons that were previously silent may increase the rate of firing even further. In dominant animals the significant decrease in depressor firing when 5-HT was applied also led to a similar
effect in preventing the depressor nerve from responding to sensory stimulation. These effects are likely due to direct action of 5-HT by modulating the sensitivity of the depressor neurons and indirect action by acting on intermediate targets that further affected the response of the depressor neurons activity.

**A1 5-HT neurons modulation of the depressor nerve**

The pair of serotonergic neurons in the first abdominal ganglion was previously shown to display the same social status response dependency as that of the depressor motor output (Drummond et al., 2001). Additionally, the A1 5-HT neurons were identified to be part of the neural circuit that might connect with the depressor motor neurons and enhance their firing activity to unilateral sensory stimulation (Figure 5-5 and Figure 5-6).

In lobster, the A1 serotonergic neurons are neurosecretory and neuromodulatory and are known to modulate the neurons controlling abdominal posture (Ma et al., 1992). The homologous serotonergic neurons in crayfish show a similar function (Drummond and Edwards, 1998). Increasing the firing rate of these cells in lobster promotes central and peripheral release of serotonin (Beltz and Kravitz, 1987). The similarities in anatomical innervation and known functions of the serotonergic neurons in lobster and crayfish suggest that the serotonergic neurons in lobster are likely to show similar status-dependent responses and modulatory role in enhancing the depressor motor output as seen in crayfish.

The known effects of these serotonergic neurons correspond with our findings of the behavioral and neural responses to sensory stimulation. These neurons are shown to promote abdominal flexion responses in both lobsters and crayfish (Ma et al, 1992;
Drummond and Edwards, 1998). Here we show that the A1 5-HT neurons also enhance the firing activity of leg depressor motor neurons to the same unexpected sensory touch (figure 5-6). The unilateral thoracic projections of each of the serotonergic neurons (Beltz and Kravitz, 1983) may enable the asymmetric firing patterns seen in isolates and dominants to assist in the unilateral enhancement of the firing activity of the depressor motor neurons during turning responses. Conversely, bilateral excitation of the 5-HT neurons that is seen in half of the subordinates may correspond with the bilateral enhancement of the depressor nerve to unilateral sensory stimulation (Drummond et al., 2001). Bilateral enhancement of the depressor nerves would enable the bilateral depression of the 5th legs as seen in half of the subordinates (Chapter 4; Figure 4-2 C). On the other hand, bilateral inhibition of the 5-HT neurons seen in the other half of subordinates suggests that the firing activity of the depressor motor neurons will not be enhanced and thus would lead to a lowering of body posture as seen in the other half of subordinates (Chapter 4; Figure 4-2 D). As part of the neural circuit underlying the orienting response, the activities of the 5-HT neurons are coordinated to enhance the sensitivity of the depressor motor neurons to sensory input. The effect of 5-HT neuron stimulation on the response of the depressor nerve to sensory stimulation was more reliable when the preparations were first bathed in 5-HT solution then 5-HT was washed away. If 5-HT was not bath applied prior to stimulation of the 5-HT neuron the results were variable. This interesting caveat is likely due to the 5-HT neurons taking up the supplied 5-HT prior to selectively stimulating them (Musolf and Edwards, 2000). This is likely due to the depletion of 5-HT during the course of dissection and sensory nerve
stimulation, although long-term lingering effects of the applied 5-HT that might have sensitized the response of the depressor nerve cannot be excluded.

**Status-dependent expression of serotonergic receptors**

Serotonin is implicated in behavioral differences in dominant and subordinate lobsters and crayfish (Antonsen & Paul, 1997; Kravitz et al., 1983; Kravitz, 1988; Livingstone et al., 1980). It is hypothesized that changes in the effect of serotonin occur through changes in the population of serotonin receptors (Yeh et al, 1996, Yeh et al., 1997). The opposing excitatory and inhibitory effects of 5-HT on the lateral giant neuron in dominant and subordinate animals is mediated by a change in the balance of the various 5-HT receptors. When the 5-HT agonist $\alpha$-methyl-5-HT was applied it induced the facilitatory effect of 5-HT in dominant animals and had a similar effect in subordinates. On other hand, when (m-chlorophenyl)-piperazine (m-CPP), another 5-HT agonist, was applied, it induced the inhibitory effects of 5-HT in subordinates and had similar effects in dominants (Yeh et al., 1997). These results suggest that both types of animals express both types of receptors but to varying degrees. Later it was demonstrated that the expression level of the 5-HT$_{1\alpha \text{crust}}$ receptor in the entire crayfish CNS is influenced by the social experience (Spitzer et al., 2003). These results demonstrate that social experience influences not only the supply of 5-HT but also receptor expression, but it is not yet known where in the nervous system the pattern of 5-HT receptor expression is being regulated. The neural circuits that are differentially influenced by 5-HT are likely to regulate their expression of the 5-HT receptors in accordance to social status. My immunohistochemical results show that the depressor
motor neurons express the 5-HT\textsubscript{1A} crust receptor (Figure 4-5), and that there was variability in the number of cells that express the receptor. However, given that the results were obtained from communal animals it is not possible to conclude that social status regulates the number of depressor neurons that express the 5-HT\textsubscript{1A} crust receptor or the level of its expression.

**Multitasking of the serotonergic neurons**

The dual-functionality of the serotonergic neurons in behavioral control (i.e. posture, locomotion, orientation and bending) has been described in a number of other systems (Jing and Gillette, 2003; Kristan and Nusbaum, 1982). In *Pleurobranchaea californica*, the As1-3 serotonergic neurons are components of two neural circuits: escape swimming and avoidance turning. The As1-3 5-HT neurons show laterality to a noxious sensory stimulus and enhance the unilateral response of the A4 interneurons that mediate the avoidance turning behavior (Jing and Gillette, 2003). A stronger aversive stimulus, however, activates the swim escape circuit incorporating the As1-3 neurons within it. Our results indicate that in crayfish the reconfiguration of the neural circuit containing the serotonergic neurons depends on the social experience of the animals rather than on stimulus strength. Analogous to the serotonergic neurons in the leech that act synaptically and neurohormonally to control the swimming behavior (Kristan and Nusbaum, 1982), the A1 5-HT neurons in crayfish are likely to play a dual role in postural regulation and orientation to sensory stimulation. As with the leech dual mode of action (synaptic and paracrine), 5-HT can be synaptically targeted to influence depressor motor output in response to a transient sensory stimulus. Secondly, through
paracrine release the A1 5-HT neurons can act globally in the neuropil of the thoracic ganglion to chronically regulate the tonic firing of the depressor motor output. Here we have identified one possible synaptic contact point between the A1 5-HT neurons and the depressor motor neurons (Figure 5-5). Yet the extensive serotonergic labeling in the 5th thoracic ganglion suggests that it is also possible for the 5-HT neurons to release 5-HT humorally in the neuropil.

**Conclusion**

The neuromodulator serotonin is implicated in influencing the social interactions and aggressive behavior in crayfish. In this study we sought to determine the role of the A1 5-HT neurons in regulating the firing activity of the depressor motor neurons. We found that exogenous application of 5-HT differentially modulated the tonic activity of the leg depressor neurons. However, we were unable to determine conclusively the effect of 5-HT application on the response of the depressor neurons to sensory stimulation because the effects on depressors’ tonic activity confounded the effects to N2 sensory stimulation. In an effort to minimize the drawbacks of bath application of 5-HT that might cause indirect and secondary effects on depressor activity we tested how direct activation of the A1 5-HT neurons would affect depressor activity. We found that stimulation of the ipsilateral 5-HT neurons modulate the response of the depressor nerve to sensory stimulation. However, the 5-HT neurons are neither sufficient nor necessary in mediating the depressor motor output to sensory stimulation. Rather the A1 5-HT neurons play a modulatory role in enhancing the sensitivity of the depressor neurons to sensory stimulation. We identified possible contact point on the axonal
branches of the depressor where the A1 5-HT neurons might exert their modulatory actions. But given the extensive serotonergic branching in the 5th thoracic ganglion other possible serotonergic neuron innervations are likely to exist whose effects remain to be determined.
CHAPTER VI
GENERAL DISCUSSION

Utilizing behavioral, electrophysiological and histochemical techniques, this work advances our knowledge of the extent to which social interactions affect behavior patterns, social dynamics and nervous system function. In the short-term, formation of social dominance has immediate effects on the activation patterns of the tailflip escape circuits in newly formed dominant and subordinate animals. Secondly, formation of a dominance order illustrated in the form of ritualized pseudo-copulatory behavior that signifies the acceptance of a social rank benefits pair members in promoting social harmony by reducing aggression. Thirdly, social dominance reconfigures neural circuits controlling leg movements to produce the proper behavioral response that fits the animals’ social standing.

Social dominance and ritualized behavior

By definition social animals are highly interactive with conspecifics, and with social interactions conflicts often arise. Competition for scarce resources such as food, shelter and mates are usually important reasons that lead to tension among group members (Wilson, 1975). Almost every social species has evolved to deal with this problem by forming social hierarchies of various structural types (Wilson, 1975). Numerous examples of social hierarchies have been described, from the highly structured and rigid eusocial ant colonies (Wilson; 1971) and honeybees (Brian; 1983) to the dynamic and complex societies of chimpanzees (de Waal; 2000) and bonobos (de Waal; 1995). Regardless of the complexity of the social structure, social hierarchies
evolved as organizing mechanisms to minimize social conflicts. Thus, valuable resources are divided among members in accordance to their social rank as the case in crayfish (Herberholz et al., 2007), sex as in hyenas (East and Hofer, 2001) or relatedness and affiliation to high-ranking members as in primates (de Waal, 1995).

The various behavioral elements involved during social interactions have been scrutinized in great detail to determine the mechanisms of how group members come to decide their rankings (Doolan and Macdonald, 1997a,b; Huber and Kravitz, 1995; Herberholz et al., 2001; Issa et al., 1999; Schenkel, 1947; Van Hooff, 1973). Despite some distinguishing differences, aggression has emerged across species as an effective and invariable instrument by which social hierarchies are initially organized (Bovbjerg, 1953; Fernald, 1977; Schenkel, 1947; Wilson; 1971). Aggression plays an essential role in the initial formation of dominance hierarchies of unfamiliar crayfish (Bovbjerg 1953 Issa et al., 1999), cichlid fish (Peeke et al., 1971; Fernald, 1977), Wolves (Scott, 1967), spotted hyenas (Owens and Owens, 1996), monkeys and chimpanzees (Tokuda and Jenson, 1968; Teas et al., 2006; Noë et al., 1980; de Waal and Hoekstra, 1980). However, despite its usefulness as an organizing agent of social hierarchies, aggression is inherently destabilizing and provides the greatest long-term threat for group harmony as illustrated in studies on Japanese macaques (Tokuda and Jenson, 1968).

In order to maintain a stable social structure without having to continuously resort to violence, many social animals have evolved “ritualized” behavior that signifies their dominance relationships. By so doing group members would assess the strength of an opponent and avoid the costs associated with fighting (i.e. time and energy expenditure,
injuries and death). Schenkel was one of the earliest to study ritualized behavior in (1947) while observing the behavior of wolf packs (Schenkel, 1947, 1967). He described the various torso, head and neck postures that are displayed by both dominants and subordinates. He concluded that the objective of these displays is to signify the dominance relationships between animals without having to escalate tension. However, the most essential aspect about these behavioral displays is not the individual behavioral components, rather the entire behavioral repertoire displayed by both animals that progresses according to a specified sequence. Ritualized behavior was later described in many social animals with varying degrees of complexities (Huxley, 1966). A classic example of ritualized behavior was discovered by Tinbergen who observed that a series of visual cues alternate between male and female stickleback in a specified sequence, resulting in a reaction chain that concludes in spawning (Tinbergen, 1948). In primates ritualized behavior has received great attention in part to the unusual behavioral displays involved in signifying dominance. Bonobos engage in a ritualized “penis fencing” behavior in order to defuse tension between combatants (de Waal, 2001). And male-male mounting of chimpanzees and monkeys in which the dominant male mounts the subordinate male is another example where social animals use a ritualized behavior in order to avoid an increase in aggressive activity (Reichard and Boesch, 2003; Edwards and Todd, 1991).

Perhaps the most unusual feature of the ritualized male-male mounting behavior is that it co-opted one behavior that evolved for sexual reproduction (mounting/mating) and utilized it in a completely different social context that signifies social dominance. Such ritual has been described in many vertebrate species such as chimpanzees,
monkeys, dogs, cats and birds (de Waal, 1980; de Waal, 1995; Wagner, 1996). Thus, male-male mounting as a social dominance ritual was thought of as an attribute unique only to vertebrates. Hence, our finding that ritualized pseudo-copulation between male crayfish during social interactions that serves a similar function to that of vertebrates is surprising (Issa and Edwards, 2006). As with vertebrates, male-male mounting in crayfish signifies the acceptance of a social rank, and its repeated occurrence reasserts the social status co.

A similar ritual behavior is well documented in human populations especially among incarcerated men, and as with other animal species its presence appears to serve the functional role of facilitating hierarchical stability (Dummond, 2003; Robertson, 2003). Sexual domination of new prison inmates expedites the stability of the social order. However, inmates that refuse sexual targeting are further victimized and experience higher murder rates compared to subordinate inmates that accept their submissive roles for the duration of confinement (Robertson, 2003). The behavioral similarity of intra-sexual mounting across taxa and its effects on social dynamics attests to its importance in shaping and stabilizing social groups.

Why sex?

The sexual content of male-male mounting is a rather puzzling aspect of this ritual when considering its objective of signifying social dominance. Why does it require a sexual act to signify dominance between two males? Intra-sexual mounting is a viable option where combatants can physically interact to assess their strengths without the possibility of inflicting injuries on one another. When considering the alternative of
increased aggression to maintain the social hierarchy, it is not hard to envision the evolution of intra-sexual mounting as an alternative strategy. This would be very important especially between genetically related combatants as is the case with the razorbills (Wagner, 1996; Reichard and Boesch, 2003). In razorbills, as with many other bird species, mature offspring often remain with their family members to help their parents with raising their siblings until a proper nest site becomes available to start their own families. However, it is possible for the eldest offspring to challenge the father for the alpha position and take over the nest site. When such conflicts arise, the alpha males would mount their offspring to reassert their social dominance (Wagner, 1996). If aggression is the only mean by which such a conflict can be resolved then that would lead to two negative consequences for the entire family. First, increased aggression would likely lead the father to kill its offspring, which is evolutionarily not favored. Second, increased aggression would lead the father to evict its offspring from the nest site, which would lead to the loss of an extra member that would help with raising younger siblings and defend the nest sight against rival families.

**Effect of social dominance on activation of neural circuits**

The social interactions described in the previous chapters illustrate the richness of crayfish behavior patterns during dominance formation. Underlying these behaviors are neural networks that are constantly adapting to changing sensory social cues as animals interact to establish their social ranks. In chapter 2, I showed that the pattern of coordination shifts when an animal disengages the fighting and switches from aggressive to submissive behavior. For instance in figure 2-4 or figure 3-2, as the
animals began to interact, aggressive behaviors were more frequent than defensive ones. Once the dominance relationship was formed, the switch in behavioral patterns was abrupt. The frequency of the defensive behavior suddenly increased and aggressive behavior was decreased. This behavioral switch is more prominent and sudden among the emerging subordinates.

Most remarkable was the change observed in the activation pattern of the medial giant escape tailflips. Prior to dominance formation, MG and non-giant escapes by the emerging subordinates were infrequent and were performed when the subordinate was physically attacked by the emerging dominant. This observation is consistent with the current data that tailflips mediated by the MG circuit are reflexive and require phasic and tactile stimuli for activation. However, after dominance formation, many of the MG escape tailflips observed were elicited without physical contact. Many of the MG tailflips were induced by simple approach or threatening displays by the dominant. This suggests that the activation threshold of the MG circuit suddenly changed from being high to low as the dominance decision is formed. Similar status-dependent changes of the MG excitability were more recently found when socially experienced animals were presented with a looming threatening stimulus (Herberholz et al., in preparation). When tested shortly after forming a dominance relationship dominant animals were significantly less likely to elicit MG tailflips to a looming shadow stimulus compared to subordinates.

The rapid switch in the excitability of neural circuits has been documented previously in crayfish. The threshold for the lateral giant escape circuit to a threatening stimulus was found to vary according to food size (Bellman and Krasne, 1983). If the
animal is threatened during feeding on a readily portable meal with a weak stimulus, it would tailflip quickly away while still holding the food, suggesting that the LG circuit excitability is increased during feeding. Conversely, even a strong stimulus will not induce a LG tailflip when the food is heavy for transport suggesting a threshold increase of the LG circuit. The switch in behavior from feeding to escaping is attributed to the descending tonic inhibition" that regulate the excitability of the LG neurons (Vu et al., 1993; Vu and Krasne, 1993). The tonic inhibition is activated when a behavior that is counterproductive to escape is activated (Beall et al., 1990). This form of network management, the balance of which is dictated by current environmental and social conditions, offers the animal control of behavioral switching and allows an immediate behavioral adaptation to changing social conditions.

The neural networks that mediate other aggressive behavior (i.e. attacks, approaches, offensive tailflips) are also likely to experience similar adaptations. As illustrated in Figure 2-4, once dominance is established, the patterns of attacks, retreats and offensive tailflips also changes according to social rank. Thus it is reasonable to hypothesize that these behavioral changes are probably accompanied by changes in the networks that mediate them.

The complexities of these behaviors pose the problem of being able to study the adaptations in the neural networks that mediate them. For instance, an attack consists of forward walking while the body posture is raised, and a retreat consists of backward walking while body posture is lowered. The neural elements of these behaviors consist of the walking and posture command networks that are tightly coordinated. Although we know many of the circuits that mediate walking and posture, our knowledge of how they
are coordinated to produce an attack or a retreat, or how an animal switches between the two behaviors, is very limited.

**The role of serotonin in social behavior and network function**

Inhibitory mechanisms might regulate the immediate switch in the excitability of networks and their corresponding behaviors, but it does not account for the long-term behavioral and physiological changes observed that suggest a more fundamental change of the nervous system.

Long-term effects of cellular function can be induced by a variety of mechanisms: morphological changes in dendritic connectivity, gene expression, second messenger cascades and/or changes in the firing pattern of neural circuits induced by extrinsic or intrinsic release of neuromodulators.

Numerous steroid hormones (Bolingbroke and Kass-Simon, 2001) and peptide stress hormones (Chang et al., 1999b; Kravitz et al., 2001) were shown to affect the social behavior of crayfish. But in recent years, most attention has been focused on the role serotonin and octopamine play in regulating the social behavior in crustaceans (Kravitz, 2000). Serotonin injection into crayfish and other decapods crustaceans induces a dominant like posture; while octopamine injections induce subordinate like postures (Antonsen and Paul, 1997; Livingstone et al., 1980). While the overall behavior patterns of subordinate animals do not change after serotonin injections (Tierney and Mangiamele, 2001), serotonin injection does change the subordinate animals' decision to retreat from a fight as indicated by an increase in the duration of agonistic interactions (Huber et al., 1997, 2001; Huber and Delago, 1998). Targets of serotonin actions include the abdominal postural circuits (Djokaj et al., 2001; Harris-Warrick and
Kravitz, 1984, 1985), escape circuits (Glanzman and Krasne, 1983), locomotion (Gill and Skorupski, 1996; Pearlstein et al., 1998; Rossi-Durand, 1993), and swimmeret beating (Barthe et al., 1993). Interestingly, some of these systems are active during agonistic interactions.

What role do the serotonergic neurons play in regulating these circuits? The serotonergic neurons are thought to act as “gain-setting” neurons on the various targets they innervate (Kravitz, 1988, 2000; Ma et al., 1992). Thus, their role is to enhance the excitation or inhibition of the targets on which they act. The mechanism by which the 5-HT cells modulate their targets might depend on the expression of different serotonergic receptors (Spitzer et al., 2005) and their second messenger cascades (Krasne et al., submitted) or even on the pattern of serotonin release (Teshiba et al., 2001). In the first case, it is shown that the expression level of the 5-HT subcrust receptor in the crayfish CNS may depend on the social experience of animals (Spitzer et al., 2005). But how status-dependent changes in the levels and/or patterns of receptor expression affects circuit activity is limited only to the activation of the LG tailflip circuit (Yeh et al., 1997). It is possible that the status-dependent responses of the depressor motor neurons are achieved by the balance of receptor expression that promotes neural excitation or inhibition. Immunohistochemical staining showed that the depressor motor neurons express the 5-HT subcrust receptor (Figure 4-5), and that application of the 5-HT sub antagonist decreases the tonic firing and response of the depressors to sensory stimulation (Figure 4-7). Changes in the level and the type of serotonergic receptors might enhance or depress the response of the depressor motor neurons to synaptic inputs. Whether social status affects the level and expression pattern of the 5-HT receptors and their
second messenger elements and how the response of the depressor might be affected remained unknown.

Complementing differences in the level and pattern of receptor expression are differences in serotonin release. Teshiba and colleagues have shown that the response of the lateral giant neuron (LG) to sensory nerve stimulation is influenced by exogenous application of 5-HT, and that the response pattern of the LG depends not only on the concentration of 5-HT applied but also on the rate and duration of its application (Teshiba et al., 2001). Drummond and colleagues have shown that the response of the first abdominal and fifth thoracic serotonergic neurons to sensory stimulation is influenced by social status. These response differences suggest that status-dependent differences in endogenous release of serotonin, although serotonin release has not been measured in those experiments (Drummond et al., 2001). Thus, it is reasonable to hypothesize that the status-dependent differences observed in the depressor motor output are due to a combination of differences in (1) serotonergic release, (2) pattern of serotonergic receptor expression by the depressor neurons and (3) the unilateral innervation of the 5-HT cells onto their target cells. These processes working congruently lead to the three status-dependent response patterns observed in the depressor motor neurons. Although this hypothesis may account for the status-dependent response patterns observed in the motor output, it does not explain how the response of the 5-HT cells is influenced by social status. The results of Drummond and colleagues suggest that the influence of social status also occurs presynaptically to the 5-HT cells, perhaps, in the sensory synaptic pathway that precedes the 5-HT cells. We know little about the direct synaptic connection between the sensory afferents and the
A1 5-HT cells. Cobalt backfills of the second sensory nerves (Leise et al., 1987) show that the dendritic arborization for the majority of the sensory neurons innervate approximately the same ganglionic region of the A1 5-HT cells dendrites, and some sensory neurons cross the midline and innervate the contralateral hemiganglion. However, it is still not known whether there is direct synaptic contact between the sensory neurons and the 5-HT cells. Nevertheless, it is possible that the 5-HT cells might receive direct bilateral sensory input. This sensory to 5-HT cell pathway along with other polysynaptic commissural interneuronal pathways discussed previously are likely targets for social status-dependent plasticity.

**General conclusion**

This dissertation work and many studies that have been cited herein have demonstrated the powerful effects social status has on the social behavior and the nervous system. The similarities in nervous system function and evolution of many animal species, even across the vertebrate/invertebrate divide, suggests that the proposed neural mechanisms underlying the social status-dependent effects are likely to be applicable to many social animals. However, this work leaves us with numerous important questions unanswered. On the behavioral level, the social cues required in the induction of the neural differences observed remain unknown. We know very little of the social trigger required of an animal to decide its social fate and become a subordinate. Is it a summation of many sensory cues culminating in the crossing of a behavioral threshold that leads to a behavioral decision? If so, how does that translate to changes in brain activity? What are the properties of the descending inputs that
regulate the activity of the local thoracic circuits regulating the legs, and how are they affected by social status?

Serotonin was the primary neuromodulator that was explored here; however, other neuromodulators and hormones are likely to be equally important in regulating social behavior. Octopamine was previously shown to influence the social behavior, cell function and circuit activation in crayfish, while hormones were demonstrated to affect synaptic activity and behavior of both crayfish and lobsters. How these neuromodulators and hormones work congruently in the living animal to influence network function and produce the rich behavioral repertoires with varying internal and external parameters and varying time scale is an open question.
REFERENCES


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Appendix 1

in vivo recordings:
Electrode implantation technique

Animals were anesthetized by placing them in ice-water for a period of ~20 minutes. The thoracic region including all legs and claws were then rapped with cold and wet paper towels then bound with rubber bands to immobilize the animals. The animals were then placed on a wax platform and held in place to prevent any movement. Fresh cold water was periodically squirted onto the paper towels to keep the animals wet. A small incision was made in the ventral part of the epodeme of the coxopodite segment. The incision was proximal to the thoraxco-coxo joint where the depressor nerve innervates the coxopodite segment ventrally (Figure Appendix 1A). I used a sharp 1mm thick stainless steel to make the incision. The incision was large enough to expose the depressor muscle and depressor nerve. A pair of insulated hook electrodes was placed around the depressor nerve once the nerve was identified. In order to prevent electrode movement and minimize muscle potentials crosstalk, an insulation of tissue adhesive (3M VetbondTM, part #1469SB) was placed around the hook electrode and depressor nerve (Figure Appendix 1C). The adhesive is typically used to bond tissue during veterinary procedures, especially oral surgeries. The adhesive usually hardened within approximately 15-20 minutes. The exoskeleton surrounding the incision was dried thoroughly then a silicone elastomers cure (World Precision Instruments, Kwik-CastTM & Kwik-SilTM) was placed onto the insulated part of the wire and the exoskeleton. This cemented the outer electrode to the exoskeleton and prevented it from any movement (Figure Appendix 1C).
Appendix 2: Two examples of the effect of 5-HT application on the tonic firing and response of the depressor motor neurons to sensory stimulation in a socially isolated (A), dominant (B) animals, and subordinate (C).

(A, B, C) Extracellular recording of a depressor nerve and its response to unilateral sensory stimulation during control (top), 5-HT application (middle) and wash (bottom) in a socially isolated (A), dominant (B) and subordinate (C). Two depressor units were tonically firing that are labelled green (small unit) and black (large unit).

(A1, B1, C1) The instantaneous firing frequency for each of the two depressor units during the three conditions for each social phenotype.
APPENDIX 2

A

Control

Ipsi-lateral stim.

Contra-lateral stim.

5-HT

Wash

Ipsi-lateral stim.

Contra-lateral stim.

B

Control

Ipsi-lateral stim.

Contra-lateral stim.

5-HT

Wash

Ipsi-lateral stim.

Contra-lateral stim.
APPENDIX 2 continue

C

Ipsi-lateral stim. Contra-lateral stim.

Control

5-HT

Wash

C1

Instantaneous Freq. (Hz)

Ipsi-lateral stim. Contra-lateral stim.

3ms

1 sec

30 Hz

154