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HORMONAL AND PHEROMONAL EFFECTS OF 20-HYDROXYECDYSONE IN THE AMERICAN LOBSTER, HOMARUS AMERICANUS

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BY

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ABSTRACT

20-hydroxyecdysone (20E), the active principle of the molting hormone in the American lobster has significant effects on the animals' agonistic behavior and has been shown to influence the outcome of agonistic interactions. Animals injected with 20E are significantly more aggressive than saline-injected control animals, and premolt animals, which have high circulating levels of 20E in their hemolymph, are more successful than intermolt animals in agonistic interactions. 20E has been shown to act as an internal modulator of neuromuscular physiology: there is an increase in the amplitude of excitatory post-synaptic potentials in the claw opener muscle and a decrease of them in the abdomen when 20E is perfused across the neurons. In addition to its humoral action, 20E appears to be an important signaling molecule sensed by the animal's antennules, since the behavior of animals change when they are exposed to 20E. The purpose of this study was twofold: to reassess the internal hormonal effects of 20E on agonistic behavior in lobsters, and to provide biochemical evidence for the presence of 20E receptors on the antennules.

Fights were conducted between small lobsters injected with 20E and large lobsters injected with saline. The nephropores of each lobster were blocked to eliminate urine signals between the combatants. Using an ethogram, the frequency and intensity of aggressive, defensive and avoidance behaviors of animals in experimental fights were compared to those in control fights (large and small lobsters injected with saline). A significant difference was found in the aggressive content in the behavior of animals engaged in experimental fights and that of the animals engaged in control fights, such that the difference in aggressive content of defensive

behaviors between 20E injected animals and their opponents was less than its difference between saline-injected animals and their opponents. These results suggest the aggressiveness of the defensive behavior of smaller treated animals was closer to that of their larger opponents than the behavior of smaller control animals was to their opponents. A post-hoc analysis comparing the control animals in this study to control animals in a similar experiment in which lobsters were injected with 20E and allowed to urinate freely showed that blocking urine release changes the dynamics of an agonistic interaction between lobsters.

Since 20E was previously shown to affect the neuromuscular properties of the claw opener muscle, force experiments were performed to test the effect of ecdysteroids on the claw closer muscle. A customized force transducer was constructed to measure the force and duration generated by the closer muscle of male and female lobsters after injection with alpha-ecdysone or 20E. The differences in force and duration before and after injection of 20E or alpha-ecdysone was compared to their differences after injection of saline. Alpha-ecdysone significantly increased the force generated by female crusher and cutter claws, and 20E potentially increased the force in female crusher claws. The results suggest that circulating ecdysteroids influence the claw closer muscle of females and could be a factor influencing agonistic interactions.

Because previous behavioral experiments indicated that 20E could be perceived by lobsters and could alter their behavior, experiments were performed to determine whether a 20E receptor (EcR) existed on the antennules of lobsters. In order to visualize the presence of an EcR, various tissues from lobsters were dissected,

soluble and insoluble fractions extracted, and spot blots and Western blots performed. Spot blots indicate the presence of a 20E receptor in both the soluble (cytoplasmic/nuclear) and insoluble (membrane-associated) fractions of walking legs and eyestalks, but only in the membrane-associated fraction of the guard setae and aesthetasc sensilla. Western blots and Mass Spectrometry returned several different molecular weights for the EcR (75 kDa, 50 kDa, 40 kDa). The presence of an EcR in the membrane-associated fraction confirms that 20E can be perceived by the antennules of lobsters, while the various molecular weights suggest different isoforms may exist, which is consistent with various insect and crustacean species.

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Most importantly I need to thank my family for all of their moral (and sometimes financial) support over the course of my graduate school career. My parents have always been very supportive and I wouldn't have been able to complete my degree without their support. Finally, I need to thank my wife for her understanding of the time commitment needed to obtain a PhD. I will finally be able to answer her favorite question: 'When will you be done?'

PREFACE

This dissertation is prepared in manuscript format. Chapter one contains general background information and the rationale for each of the three major experiments conducted. Each experiment is separated into its own chapter and has been prepared for publication in the format of the relevant journal.

Chapter Two addresses the effects of 20-hydroxyecdysone (20E) on the agonistic interactions of lobsters, and has been prepared for *Biological Bulletin*. Appendix A containing all figures, tables and raw data not included in the paper prepared for publication is included at the end of the dissertation.

Chapter Three describes the location and molecular weights of a receptor for ecdysone (EcR) in lobsters, and has been prepared for *Chemical Senses*.

Chapter Four addresses the effects of 20E and alpha-ecdysone on the claw closer muscle in lobsters, and has been prepared for *Biological Bulletin*. Appendix B containing all figures, tables, and raw data not included in the paper prepared for publication is included at the end of the dissertation.

In summary, this dissertation addresses the pheromonal and hormonal actions of the molting hormone, 20E, on agonistic interactions in the American lobster, *Homarus americanus*. It is the first study to show biochemical evidence that a membrane bound EcR exists in lobsters, which could contribute to the immediate changes in agonistic behavior of lobsters exposed to 20E. It also describes the effects of alpha-ecdysone on the force produced by the claw closer muscle.

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CHAPTER 1:

Background

Background:

Agonistic encounters play an important role in the life of lobsters; they are involved in procuring shelters, defending and maintaining those shelters, mating, and foraging success (Atema and Cobb, 1980). Some of the factors affecting the outcomes of agonistic encounters include the physical characteristics of the animals, such as weight, carapace size, and chelae size. Large animals who weigh more and have greater carapace and chelae size (Scrivener, 1971) will often take on a dominant position over smaller animals (Cobb and Tamm, 1975). In staged encounters, larger lobsters will win significantly more encounters than smaller lobsters of the same sex (Scrivener, 1971). Size also plays a role in the formation of dominance hierarchies in lobsters; larger lobsters are dominant over smaller lobsters in social settings (Karnofsky, 1989). Similar hierarchies exist in crayfish, where larger animals are dominant over and have more access to food resources than smaller, subdominant males (Issa *et al*., 1999; Herberholz *et al*., 2007). Shelter competition between lobsters is influenced by size and prior residence; larger animals and animals who maintain prior residence are more aggressive and successful in obtaining/maintaining shelter (O'Neill and Cobb, 1979; Cromarty *et al*., 1999).

Effects of hormones on agonistic behavior:

Along with size, internal hormones and external chemical signals (pheromones) have been shown to influence the outcome of agonistic interactions between lobsters. The effects of hormones and pheromones are complex and can lead to a wide spectrum of effects on animals engaged in an agonistic interaction.

The hemolymph titers of the molting hormone, 20-hydroxyecdysone (20E), varies over the molt cycle of lobsters. Premolt animals ($D_1$and D_2 stage animals about to molt) with increased levels of ecdysone in their hemolymph and urine (Chang, 1985; Snyder and Chang, 1991a; Snyder and Chang, 1991b), are dominant over and more aggressive than their intermolt C-stage opponents in a confrontation (Tamm and Cobb, 1978). Evidence has been presented that injected 20E makes lobsters more aggressive, as in staged combats between large and small lobsters, smaller lobsters injected with the hormone are significantly more aggressive than small control animals injected with saline (Bolingbroke and Kass-Simon, 2001). The injection of 20E may mimic the increased 20E titers that occur in D-stage animals about to molt.

20-hydroxyecdysone isn't the only hormone shown to affect agonistic behavior; amines, such as serotonin and octopamine, have been shown to affect behavior (Kravitz, 1990; Kravitz, 2000). In lobsters, injection of serotonin causes sustained flexion of the limbs and abdomen: claws are opened and held forward, walking legs are flexed directly under the body, and tails are loosely flexed and tucked under the body (Livingstone *et al*., 1980). Octopamine has the opposite effect on the posture of lobsters; injection causes sustained extension of the limbs and abdomen, legs and tail are held rigidly straight just above the substrate, and the tail and claws are fully extended (Livingstone *et al*., 1980). Evidence has also been presented that injection of serotonin increases the aggression of previously subordinate lobsters (Huber *et al*., 1997). A dominance hierarchy was established between two lobsters, and subordinate animals were removed and injected with serotonin. The serotonin-injected subordinate animals were then re-introduced to the same dominant individual

from the first encounter. The fight duration and intensities of the serotonin-injected animals were three times as great as those of saline-injected control animals, which suggests that serotonin made the animals more aggressive (Huber *et al*., 1997).

Effects of hormones on neuromuscular physiology:

The effects of 20E and serotonin on behavior include their effects on neuromuscular physiology and synaptic transmission; 20E has been shown to affect the neuromuscular electrophysiology of the claw and abdomen. Over the molt cycle, animals in premolt stage D produce significantly larger excitatory junctional potentials (EJP's) and significantly fewer inhibitory junctional potentials (IJP's) in the claw opener muscle (Schwanke *et al.*, 1990). In the presence of 20E (which is present in greater quantities in the hemolymph of premolt animals), there is an increase in EJP amplitudes and miniature excitatory junctional potential (MEJP) frequency in the opener muscle (used in threat displays). There is also, a decrease in EJP amplitudes in the abdominal muscles used in the escape response, which corresponds to the effect of pre-molt and post-molt hemolymph on EJPs (Schwanke *et al*., 1990; Cromarty and Kass-Simon, 1998). These findings are consistent with the agonistic behavior of premolt animals.

In crayfish, 20E has also been shown to act as a hormone that alters the internal physiology of neurotransmitter release (Cooper and Ruffner, 1998; Cooper *et al*., 2003). Cooper and his colleagues have shown that 20E decreases the probability of neurotransmitter vesicle release in the walking legs, and that a mixture of 20E and serotonin increases neuron firing frequency in the slow-adapting muscle receptor

organ (MRO) of the abdomen. In early experiments (Fischer and Florey, 1983) both octopamine and serotonin were shown to increase nerve-evoked tension, amplitudes of EPSPs, amplitudes of synaptic currents and the effectiveness of excitation-contraction coupling through an increase in neurotransmitter release in the claw opener muscle of crayfish.

In concordance with their effects on postures and behavior mentioned above, in the neuro-muscular junctions of lobsters, octopamine and serotonin induce contractures and the appearance of Ca^{2+} action potentials. Serotonin facilitates transmitter release and dopamine relaxes muscle baseline tension, decreasing contraction (Kravitz, *et al*., 1980). Serotonin and octopamine were also found to act in the central ganglion and affect only slow (postural) flexor and extensor muscles, each with an opposite pattern of activation on the excitatory and inhibitory neurons that innervate the muscles. Octopamine acts on the excitatory extensor neuron and the inhibitory flexor neuron, and serotonin acts on the excitatory flexor neuron and inhibitory extensor neuron (Kravitz, *et al*., 1980). The results show that amines may act as neurohormones in the lobster nervous system, affecting behavior. High circulating levels of octopamine or serotonin could cause sustained extension or flexion of limbs, which could affect the mobility of a lobster. If a lobster is unable to flex or extend claws or walking legs, then they may be at a disadvantage in an agonistic interaction.

In the Pacific spiny lobster, serotonin significantly reduced the strength of graded synaptic transmission, and octopamine significantly increased the strength of graded synaptic transmission at all pyloric dilator synapses (Johnson and Harris-

Warwick, 1990). Serotonin was shown to reduce the pre- and post-synaptic input resistance, while octopamine did not change the input resistance. The results suggest that the different amines work through different mechanisms and affect the same neurons differently (Johnson and Harris-Warwick, 1990).

Ecdysteroids may also have differential effects on the neuromuscular properties of the claw closer muscle of crusher and cutter claws, particularly as the two claws differ in their muscle fiber types, motoneuron innervation and contractile properties (Govind, 1984). Fast twitch fibers quickly reach a maximal tension, which rapidly decays, while slow twitch fibers gradually increase in tension with a slow decay phase (Jahromi and Atwood, 1971; Costello and Govind, 1983). Crusher claw closer muscles contain only slow twitch fibers with long sarcomeres, which allows the crusher to maintain force for a long period of time (Lang *et al*., 1977; Govind and Lang, 1978; Kent and Govind, 1981; Govind, 1984). Cutter claws contain mostly fast twitch fibers with short sarcomeres and a small ventral band of slow twitch fibers, which leads to a quicker fatigue than crusher muscle (Lang *et al*., 1977; Govind and Lang, 1978; Kent and Govind, 1981; Govind, 1984). The closer muscle in both crusher and cutter claws are innervated by the fast closer excitor neuron (FCE) and the slow closer excitor neuron (SCE), however, cutter closer muscles have mostly FCE while crushers have a mixture of both (Wiersma, 1955; Costello *et al*., 1981; Govind, 1984). Generally, SCE synapses are more fatigue resistant and recover more quickly than FCE synapses, but the synapses at both the FCE and SCE in crusher claws are more resistant to fatigue than those in the cutter (Govind and Lang, 1974; Govind and Lang 1979).

During an agonistic interaction, lobsters perform many behaviors with their claws, including grabbing, hitting, pinching and claw locks (Mello *et al*., 1999; Bolingbroke *et al*. 2001; Coglianese *et al*., 2008; Reinhart *et al*., submitted; Sipala *et al*., unpublished). These claw behaviors are important, as the strength or duration of a squeeze/pinch may affect the outcome of the agonistic interaction. Also, a larger, thicker claw might deliver more force during a hit than a smaller claw, which could cause more harm. Based on the known neurophysiological effects of 20E, serotonin, and octopamine described above, the different neuromuscular properties of the crusher and cutter claw could lead to different responses to ecdysteroids. Any differential response by the claw closer muscle to ecdysteroids may affect the action of the claw during an agonistic encounter and therefore the outcome the interaction between animals. Such differences would be reflected in differences in patterns of behavior in pre- and intermolt animals and could help explain why premolt animals are more successful in agonistic encounters than intermolt animals.

Effects of pheromones on agonistic behavior:

Along with internal hormonal effects, external pheromonal signals have also been shown to affect the outcome of an agonistic interaction. Urine signaling appears to play a large role in determining the outcome of a fight: the ability of a lobster to 'smell' urine is very important in both establishing dominance hierarchies and in individual recognition in lobsters engaged in agonistic encounters. If urine release is blocked, then dominance hierarchies are not established (Karavanich and Atema 1998; Karavanich and Atema, 1991) and lobsters are not able to recognize each other in

subsequent encounters, which results in increased fighting before dominance is re-established during the secondary encounter (Kaplan, 1993; Karavanich and Atema, 1991). If the aesthetasc sensilla are removed or made anosmic, lobsters are not able to recognize previous opponents or established dominance relationships and spend more time fighting than lobsters who could smell normally (Johnson and Atema, 2005; Hoeppner, 1997). The same is true for crayfish, where ablation of aesthetasc sensilla results in fights of longer duration between previous combatants than unablated control pairs (Horner *et al.*, 2008). Further, there is evidence that both the timing of urine release and the contents of the urine affect agonistic encounters between lobsters. It has been shown that the winners of fights release significantly more urine than losers, and the removal or prevention of urine release in subsequent encounters between the same pair of animals abolishes a previously established dominance relationship (Breithaupt and Atema, 1993; Karavanich and Atema, 1998; Breithaupt *et al*., 1999; Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). These results highlight the importance of the urine as a means of assessing opponents in an agonistic encounter. If the urine signal or the ability to smell are removed, then the behavior of the lobster changes.

One component of urine that has been found to affect the outcome of agonistic encounters is the active principle of the molting hormone, 20E (Coglianese *et al*., 2008; Reinhart *et al*., submitted). Recent evidence presented by Coglianese *et al*. (2008), Reinhart *et al*. (submitted) and Cromarty *et al*. (unpublished) indicates that 20E acts as a pheromone that changes the behavior of other lobsters. Coglianese *et al*. (2008) found that the behavior of female animals exposed to a plume of 20E was

different than control animals exposed to a plume of artificial sea-water (ASW). In these experiments, the nephropores of each animal were blocked, thereby eliminating urine release into the tank, and 20E was puffed across the antennules of large lobsters while their small opponents were made anosmic. Large female lobsters who had a plume of 20E puffed across their antennules performed significantly more aggressive, defensive and avoidance behaviors than large control animals in staged confrontations. The small non-exposed animals became significantly more aggressive, presumably in response to the larger animal's overall arousal. The change in behavior of the exposed individual can be attributed to the "smelling" of the 20E in the odor plume, suggesting it acts as a pheromone. Reinhart *et al*. (submitted) performed the same experiment with male lobsters, and found that the behavior of males exposed to a plume of 20E was different than control animals exposed to a plume of artificial sea-water (ASW). Reinhart found that male lobsters exposed to 20E performed more defensive behaviors than ASW exposed control animals. Also, the opponents of the 20E exposed animals performed significantly more aggressive behaviors than the opponents of ASW exposed animals. These results also led to the conclusion that the change in behavior of the exposed individual could be attributed to the "smelling" of the 20E in the odor plume. One important distinction between the results of Coglianese and Reinhart is that males and females responded differently to 20E: Females responded to 20E exposure by becoming more aroused, increasing aggressive, defensive and avoidance behaviors, whereas males simply increased the frequency of defensive behaviors. In electrophysiological experiments, Cromarty *et al*. (unpublished) found that the olfactory receptor neurons (ORN bundles) of female lobsters exhibit a dose dependent

response to 20E, supporting that idea that 20E may be perceived by the antennules of lobsters during an agonistic encounter.

Chemoreception of ecdysteroids by an ecdysone receptor (EcR):

The perception of 20E by the antennules of lobsters suggests there is an ecdysone receptor (EcR) for 20E. Ecdysone and its active metabolite, 20-hydroxecdysone (20E), are steroid hormones that regulate molting in insects and crustaceans and coordinate alterations in the transcription of groups of genes required to control this process (Waddy *et al*., 1995). An EcR has been found in numerous insect and crustacean species, including *Drosophila* (Talbot *et al*., 1993), *Manduca sexta* (Fujiwara *et al*., 1995; Jindra *et al*., 1996), *Bombyx mori* (Kamimura et al., 1997) *Crangon crangon* (Verhaegen *et al*., 2011) and *Homarus americanus* (El Haj *et al*., 1994; Tarrant *et al*., 2011). Isoforms of the EcR have been found in many animals; *Drosophila* has three different isoforms, EcR-A (91 kDa), EcR-B1 (93 kDa) and EcR-B2 (73 kDa) (Talbot *et al*., 1993), and lobsters have two (Tarrant *et al*., 2011).

Molting is a slow process that takes place over several days or weeks, and activation of receptors for 20E most likely act via genomic mechanisms. Steroid hormones have been traditionally considered to work through genomic mechanisms, where steroids enter a cell, bind to a specific receptor in the cytosol or nucleus, and activate transcription that leads to changes in gene expression and results in the production of proteins that have a biological function (Losel *et al*., 2003). This mechanism is generally slow-acting. The pheromonal effects of 20E described above,

however, result in immediate changes in behavior, which cannot be explained by slow-acting genomic mechanisms.

A model for the fast-actions of steroid hormones involves a non-genomic mechanism, wherein steroids have an immediate effect on physiological function (Losel *et al*., 2003). The physiological effect of steroids that act through non-genomic mechanisms can be seen within seconds of exposure to the hormone, ruling out any models that involve changes in the transcription levels of genes. The activation of an outer membrane-bound receptor, or signaling via a second messenger pathway are likely causes of the immediate physiological changes observed to occur in response to the hormone (Losel *et al*., 2003).

In recent studies in numerous insects and crustaceans, ecdysteroids have been shown to have fast-acting effects, suggesting the presence of an additional non-classical steroid hormone signaling pathway (Spencer and Case, 1984; Cromarty and Kass-Simon, 1998; Cooper and Ruffner, 1998; Tomaschko, 1999; Thummel and Chory, 2002; Cooper *et al*., 2003; Schlattner *et al*., 2006). Rapid non-genomic effects have been found to act in exocrine glands, the central nervous system, motor neurons, neuromuscular junctions and sensory cells of numerous organisms (Schlattner *et al*., 2006). Compared to the prolonged and slow process of ecdysone induced molting, the non-genomic effects of ecdysone exposure are immediate; changes are sometimes observed within a matter of seconds or milliseconds (Tomaschko, 1999).

In the California spiny lobster, Spencer and Case (1984) found an increased action potential firing-frequency in the lateral antennule one second after exposure to both 20E and alpha ecdysone. In American lobsters, 20E has been shown to have

immediate effects on neuro-muscular properties. In the presence of 20E, there is an increase in EJP amplitudes and miniature excitatory junctional potential (MEJP) frequency in the opener muscle, as well as a decrease in EJP amplitudes in the abdomen (Cromarty and Kass-Simon, 1998). In *Drosophila*, Ruffner *et al*. (1999) found reduced transmitter release in the ventral abdominal muscles within one minute after exposure to 20E. In crayfish, there is a decreased amount of neurotransmitter release in the opener muscle of the first walking leg within 20 minutes after exposure to 20E (Cooper and Ruffner, 1998). Cooper *et al*. (2003) found increased action potential firing frequency in the muscle receptor organ of crayfish 10 seconds after exposure to 20E.

As described above, 20-hydroxyecdysone also appears to be a pheromone that immediately alters the agonistic behavior of lobsters (Coglianese *et al*., 2008; Reinhart *et al*., submitted) and is sensed by ORN in the antennules (Cromarty *et al*., unpublished). The external perception of 20E in lobsters and the immediate change in behavior of lobsters exposed to 20E suggest that a membrane bound receptor must be present on the aesthetasc sensilla. In spiny lobsters, *Panulirus argus*, the aesthetasc sensilla are the sensory cilia of the olfactory receptor cells whose nuclei are located within the antennules themselves (Ache and Derby, 1985; Grunert and Ache, 1988). Histological studies show that the aesthetasc sensilla are innervated by the dendritic extensions of multiple bipolar receptors, with the soma gathered in a cluster at the base of the sensillum inside the antennule itself (Ache and Derby, 1985; Grunert and Ache, 1988). The anatomy of the antennules in the Spiny lobster suggests that any receptor for 20E must be a membrane bound receptor, as no nucleus or cytoplasm exists in the

sensory hair itself. A similar morphology is presumed to exist in the American lobster, although the histology of the antennules in the American lobster has not been examined. The activation of membrane bound receptors is fast-acting, and could explain the immediate change in behavior observed by Coglianese *et al* (2008) and Reinhart *et al*. (submitted), and the immediate neural response to 20E observed by Cromarty *et al*. (unpublished).

The presence of a membrane bound ecdysone receptor has been isolated from the anterior silk gland of the silkworm, *Bombyx mori* (Elmogy *et al*., 2004). 20E aids in the initiation of apoptosis of the anterior silk gland, and Elmogy *et al*. (2004) found a putative membrane receptor located in the plasma membrane. This membrane receptor exhibited saturable binding to 20E and the authors suggest that the receptor is likely to be an integrated membrane protein. The presence of a membrane bound receptor for 20E in the silkworm supports the idea that a membrane bound receptor for 20E exists in other insect and crustacean tissues. Srivastava *et al*. (2005) discovered a G-protein coupled ecdysone receptor in *Drosophila*, so it is possible that an EcR in lobsters might act through a second messenger pathway.

Rationale of Dissertation:

This dissertation investigates both the internal and external effects of 20E on agonistic interactions in lobsters.

One of the questions raised by the Bolingbroke, Coglianese and Reinhart experiments (cited above) is what role do the internal hormonal effects and the external pheromonal effects of 20E play in the agonistic interactions of lobsters. In

Bolingbroke and Kass-Simon (2001) lobsters were injected with 20E and allowed to urinate, and both the hormonal and pheromonal effects of 20E were present. In Coglianese *et al*. (2008) and Reinhart *et al*. (submitted), lobsters had their nephropores blocked and were not injected, but 20E was puffed from one lobster onto another allowing for only the pheromonal signal of 20E. The purpose of the experiments in Chapter Two was to determine the effects of the 20E hormonal signal alone on the aggressive behaviors of female American lobsters, *Homarus americanus*. To do this, lobsters were injected with 20E and the nephropores were blocked, effectively eliminating urine released into the water.

The apparent pheromonal effects of 20E shown by Coglianese *et al*. (2008), Reinhart *et al*. (submitted) and corroborated by the response from ORN by Cromarty *et al*. (unpublished), lead to experiments in Chapter Three. The purpose of these experiments was to find biochemical evidence of a membrane bound EcR on the antennules of lobsters and to provide a molecular weight for the receptor. The presence of a membrane bound receptor would support the idea that lobsters are able to perceive 20E, and that the actions are too quick to be explained by genomic mechanisms.

The importance of claws in agonistic interactions and the effects of 20E on the neuromuscular physiology of lobsters and crayfish lead to the experiments in Chapter Four. The purpose of these experiments was to determine the effects of ecdysteroids, 20E and alpha-ecdysone on the claw closer muscle in lobsters. Since 20E affects neuromuscular physiology in the claw opener muscle and abdomen, it is possible that it affects the claw closer muscle. If 20E or alpha-ecdysone change neuromuscular

properties, such as increasing the force or duration of a squeeze, a lobster that has high circulating levels of ecdysteroids may have an advantage in an agonistic interaction.

Taken together, the experiments are aimed at further describing the hormonal and pheromonal effects of ecdysteroids on the physiology underlying agonistic behavior in *Homarus americanus*.

CHAPTER 2

Effects of injected 20-hydroxyecdysone on the agonistic behavior of American lobsters, *Homarus americanus*

by

Michael W. Sipala, Stuart I. Cromarty and G. Kass-Simon

is prepared for *Biological Bulletin*

Abstract:

In lobsters, 20-hydroxyecdysone (20E) has been shown to act as both an internal modulator of neuromuscular physiology and as an external pheromone that affects behavior. The purpose of this study was to reassess the internal hormonal effects of 20E on agonistic behavior in lobsters. Experimental fights were conducted between small lobsters injected with 20E and large lobsters injected with saline. Control fights consisted of small and large lobsters injected with saline. The nephropores of each lobster were blocked to eliminate urine signals between the combatants. Using an ethogram, the frequency and intensity of aggressive, defensive and avoidance behaviors of animals in experimental fights were compared to those in control fights. Significance was found between the differences in aggressive content of animals engaged in experimental fights to animals engaged in control fights. These results suggest the aggressiveness of the defensive behavior of smaller treated animals was more similar to that of their larger opponents than the aggressiveness of defensive behaviors of smaller control animals to their larger opponents. A post-hoc analysis comparing our control animals to control animals from a similar experiment in which lobsters were injected with 20E and allowed to urinate freely showed that blocking urine release changes the dynamics of an agonistic interaction between lobsters.

Introduction:

Agonistic encounters play an important role in the life of lobsters; they are involved in procuring shelters, defending and maintaining those shelters, mating, and foraging success (Atema and Cobb, 1980). Some of the factors affecting the outcomes of agonistic encounters include weight, carapace size, and chelae size; large animals weigh more and have greater carapace and chelae size (Scrivener, 1971). In staged encounters, larger lobsters will win significantly more encounters than smaller lobsters of the same sex (Scrivener, 1971). Size also plays a role in the formation of dominance hierarchies in lobsters, as larger lobsters are dominant over smaller lobsters in social settings (Karnofsky, 1989). This is also true of crayfish, where larger animals are dominant over and have more access to food resources than smaller, subdominant males (Issa *et al*., 1999; Herberholz *et al*., 2007).

Along with size, internal hormones and external chemical signals (pheromones) have been shown to influence the outcome of agonistic interactions between lobsters. The effects of hormones and pheromones are complex and can lead to a wide spectrum of effects on animals engaged in an agonistic interaction. Recent evidence indicates that 20-hydroxyecdysone (20E), a hormone that modulates molting in American lobsters, also acts as a pheromone (Coglianese *et al*., 2008; Reinhart *et al*., unpublished).

The hemolymph titers of the molting hormone, 20-hydroxyecdysone (20E), varies over the molt cycle. Premolt animals ($D_1$and D_2 stage animals about to molt) have increased levels of ecdysone in their hemolymph and urine (Chang, 1985; Snyder and Chang, 1991a; Snyder and Chang, 1991b), and are dominant over and more

aggressive than their intermolt C-stage opponents in a confrontation (Tamm and Cobb, 1978). Evidence has been presented that injected 20E makes lobsters more aggressive, as in staged combats between large and small lobsters, smaller lobsters injected with the hormone are significantly more aggressive than small control animals injected with saline (Bolingbroke and Kass-Simon, 2001). The injection of 20E may mimic the increased 20E titers that occur in D-stage animals about to molt (D1 and D2), which are correlated with increased aggression in D-stage animals (Tamm and Cobb, 1978).

20E has been shown to affect the neuromuscular electrophysiology of the claw and abdomen. In the claw opener muscle, the amount of opening depends on the patterned interaction between excitatory and inhibitory junctional potentials (Wilson and Davis, 1965; Kass-Simon and Govind, 1989). Over the molt cycle, animals in premolt stage D produce significantly larger excitatory junctional potentials (EJP's) and significantly fewer inhibitory junctional potentials (IJP's) in the claw opener muscle (Schwanke *et al.*, 1990). In the presence of 20E (which is present in greater quantities in the hemolymph of premolt animals), there is an increase in EJP amplitudes and miniature excitatory junctional potential (MEJP) frequency in the opener muscle (used in threat displays). Also, there is a decrease in EJP amplitudes in the abdomen (used in the escape response), which corresponds to the effect of pre-molt and post-molt hemolymph on EJPs (Schwanke *et al*., 1990; Cromarty and Kass-Simon, 1998). This correlates with the finding that lobsters are more aggressive just before molting.

In crayfish, 20E has been shown to act as a hormone that alters the internal physiology of neurotransmitter release (Cooper and Ruffner, 1998; Cooper *et al*., 2003). Cooper and his colleagues have shown that 20E decreases the probability of neurotransmitter vesicle release in the walking legs, and that a mixture of 20E and serotonin was effective in increasing neuron firing frequency in the slow-adapting muscle receptor organ (MRO) of the abdomen. In lobsters, injection of serotonin causes sustained flexion of the limbs and abdomen, where claws are opened and held forward, walking legs are flexed directly under the body, and tails are loosely flexed and tucked under the body (Livingstone *et al*., 1980). Furthermore, evidence has been presented that injection of serotonin increases the aggression of previously subordinate lobsters (Huber *et al*., 1997). Dominance hierarchies were established between two lobsters, subordinate animals were removed and injected with serotonin, and were then re-introduced to the same dominant individual from the first encounter. These serotonin injected animals had a fight duration and intensity level three times that of a saline injected control animal, which suggested that serotonin made them more aggressive (Huber *et al*., 1997). However, recent studies indicate that the removal of serotonin also increases the duration of fighting behavior in lobsters (Doernberg *et al*., 2001). This suggest that the concentration of serotonin, *per se*, is unlikely to be the determining factor in the level of aggression. This is consistent with the earlier biochemical studies indicating that serotonin does not vary significantly over the molt cycle (Fadool *et al*., 1989).

Internal hormonal effects are not the only factors that affect the outcome of a confrontation, as urine signaling appears to play a large role in determining the

outcome of a fight. There is evidence that both the timing of urine release and the contents of the urine affect agonistic encounters. It has been shown that the winners of fights release significantly more urine than the losers, and the removal or prevention of urine release in subsequent encounters between the same pair of animals abolishes a previously established dominance relationship (Breithaupt and Atema, 1993; Karavanich and Atema, 1998; Breithaupt *et al*., 1999; Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). One component of urine that has been found to affect the outcome of agonistic encounters is the active principle of the molting hormone, 20E (Coglianese *et al*., 2008; Reinhart *et al*., submitted).

Recent evidence presented by Coglianese *et al*. (2008), Reinhart *et al*. (submitted) and Cromarty *et al*. (unpublished) indicates that 20E may not only act as a hormone, but also as a pheromone that changes the behavior of other lobsters. Coglianese *et al*. (2008) found that the behavior of female animals exposed to a plume of 20E was different than control animals exposed to a plume of artificial sea-water (ASW). In these experiments, the nephropores of each animal were blocked, thereby eliminating urine release into the tank, and 20E was puffed across the antennules of large lobsters while their small opponents were made anosmic. Large female lobsters who had a plume of 20E puffed across their antennules performed significantly more aggressive, defensive and avoidance behaviors than large control animals in staged confrontations. The small non-exposed animals became significantly more aggressive, presumably in response to the larger animal's overall arousal. The change in behavior of the exposed individual can be attributed to the "smelling" of the 20E in the odor plume, suggesting it acts as a pheromone. Reinhart *et al*. (submitted) performed the

same experiment as Coglianese with male lobsters, and found that the behavior of males exposed to a plume of 20E was different than control animals exposed to a plume of artificial sea-water (ASW). Reinhart found that male lobsters exposed to 20E performed more defensive behaviors than ASW exposed control animals. Also, the opponents of the 20E exposed animals performed significantly more aggressive behaviors than the opponents of ASW exposed animals. These results also led to the conclusion that the change in behavior of the exposed individual could be attributed to the "smelling" of the 20E in the odor plume. In electrophysiological experiments, Cromarty *et al*. (unpublished) found that the olfactory receptor neurons (ORN bundles) exhibit a dose dependent response to 20E, supporting that idea that 20E may be perceived by the antennules of lobsters during an agonistic encounter.

One of the questions raised by the Bolingbroke, Coglianese and Reinhart experiments is what role do the internal hormonal effects and the external pheromonal effects of 20E play in the agonistic interactions of lobsters. In Bolingbroke and Kass-Simon (2001) lobsters were injected with 20E and allowed to urinate, and both the hormonal and pheromonal effects of 20E were present. In Coglianese *et al*. (2008) and Reinhart *et al*. (submitted), lobsters had their nephropores blocked and were not injected, but 20E was puffed from one lobster onto another allowing for only the pheromonal signal of 20E. The purpose of the present experiment was to determine the effects of the 20E hormonal signal alone on the aggressive behaviors of female American lobsters, *Homarus americanus*. To do this, lobsters were injected with 20E and the nephropores were blocked, effectively eliminating urine released into the water. Given that urine appears to affect the outcome of aggressive encounters and

that the injection of 20E appears to increase the aggressiveness of lobsters, blocking the pheromone signal leaves only the hormonal effect. The experimental set-up was identical to Bolingbroke and Kass-Simon (2001), with the exception that the nephropores were blocked on all animals.

Methods:

Animal Procurement and maintenance:

Female American lobsters, *Homarus americanus*, were obtained from local fisherman and the Rhode Island Department of Environmental Management from inshore waters off the coast of Narragansett Bay, RI. Animals were maintained in natural circulating unfiltered seawater tanks at the Narragansett Bay Campus, on a 12-hr light/dark cycle. Water temperature and salinity were ambient, ranging from 10-20°C and 28-33ppt, respectively. Animals were fed fish scraps, supplied by a local fish market, twice weekly. No animal was fed 48 hours prior to a fight. Animals were banded and kept in separate tanks with compartmentalized large gauge wire cages to prevent physical interactions between any lobsters prior to a fight. All animals were returned to Narragansett Bay after 2 weeks.

All lobsters used were intermolt C-stage animals in perfect condition, i.e., all eight walking legs, claws, antennae and antennules were intact, with no other signs of physical damage or shell disease. Animals for each fight were identified based on carapace length (at least a 10% difference) and claw length (at least a 5% difference for crusher and cutter claw). The small animals used for each fight ranged from 75-84.6mm carapace length and the large animals ranged from 83.4-97mm carapace length. Each individual pair within a fight differed by at least 10% in carapace length and 5% in claw length, allowing for consistency throughout the fights. Comparisons of the treated (T) and control (C) animals indicated that there were no significant differences in weight ($F_{1,18}$=0.05, P=0.8), carapace length ($F_{1,18}$ = 0.03, P=0.9), crusher ($F_{1,18}$=1.8, P=0.2) or cutter length ($F_{1,18}$=0.02, P=0.9). Comparisons of the opponents

of treated (OT) and opponents of control (OC) animals indicated that there were no significant differences in weight ($F_{1,18}$=0.002, P=0.97), carapace length ($F_{1,18}$= 0.08, P= 0.8), crusher ($F_{1,18}$=0.8, P=0.4) or cutter length ($F_{1,18}$=1.5, P=0.2). Comparisons of the T and OT animals indicated that there were significant differences in weight ($F_{1,18}$=12.5, P=0.002), carapace length ($F_{1,18}$=43.5, P= $3.4E^{-6}$), crusher ($F_{1,18}$=17.3, P=0.0006) and cutter length ($F_{1,18}$=8.6, P=0.009). Comparisons between C and OC animals indicated there were significant differences in weight ($F_{1,18}$=15.8, P=0.0009), carapace length ($F_{1,18}$=26.2, P=$7.2E^{-5}$), crusher ($F_{1,18}$=16, P=0.0008) and cutter length ($F_{1,18}$=11.6, P=0.003). All fights consisted of large versus small animals in order to bias the fight in favor of larger animals, who have been found to win significantly more encounters with smaller animals (Scrivener, 1971).

Experimental set-up:

A total of 20 fights (10 experimental and 10 control) were carried out between July-August 2005 and July-August 2006. Experimental fights consisted of a small 20-hydroxyecdysone-injected lobster pitted against a large saline-injected lobster; control fights consisted of saline-injected small lobsters versus saline-injected large animals. The large animal in all fights was identified by a rubber band placed on the endopodite of the crusher claw between the cheliped joint and the insertion of the dactyl. The band was placed in such a way that it did not interfere with the normal movement of the joint or the claw as a whole. In order to prevent urine release into the tank during the fights, the nephropores of each lobster were covered with aquarium tubing sealed at one end with sealing wax. Aquarium tubing was first cut to a size of approximately 2 cm, one end was blocked with sealing wax, allowed to cool and tested for leaks.

The nephropore blockers were glued over the nephropores with Super-Glue on the morning of the fight. Although the nephropores were blocked, the actual release of urine from the lobsters was not blocked during the fight, as the nephropore blockers collected the urine that was released by the lobsters during the fight.

The pre-fight injection protocol consisted of 4 injections of 20E or lobster saline 12 hours apart, and with 12 hours between the fourth injection and the fight. For example, a typical injection protocol was as follows: Day 1: 7PM; Day 2: 7AM and 7PM; Day 3: 7AM and fight at 7PM. Control animals were injected with saline with a composition in (mM/L) of: NaCl 472; KCl 10; $MgCL_2*6H_2O$ 7; $CaCl_2$ 16; glucose 11; Tris-maleate 10; pH 7.4 (Meiss and Govind, 1979). Experimental animals were injected with enough 20-hydroxyecdysone to result in a final hemolymph concentration inside the body of 600 ng/ml. Stock aliquots of 20E (1mg/mL saline) were frozen at –80°C. The volume of stock solutions injected was that which was estimated on the basis of the animals weight to result in a final hemolymph concentration of 600ng/ml. This weight/volume estimate was generated by Bolingbroke and Kass-Simon (2001), by measuring the hemolymph volume bled from lobsters of known weights and fitted to a linear curve, having the values: $y = 0.26x – 54.33$, where y is the hemolymph volume and x is the weight of the animal. The volume estimated from the equation was then used in a ratio to determine the amount of 20E stock solution needed to be injected in order to obtain a final concentration of 600ng/mL. The ratio used was: 0.0006mg/1mL = X mg/hemolymph volume of interest (y from the equation). For example, an animal with a total hemolymph volume of 50mL would receive an injection of 0.03 mL stock 20E solution, whereas

one with a hemolymph volume of 100mL would receive an injection of 0.06 mL stock solution. The amount of saline injected in the control animals was calculated the same way. A Dremel electric drill was used to drill a small hole through the outer portion of the carapace above the presumed level of the cardium through which a 20-gauge needle could be inserted into the remaining carapace layer. After injection, the hole was plugged with dental wax to prevent bleeding.

Fights took place in a 74cm diameter circular tank filled with approximately 2 feet of seawater. Prior to each fight, the tank was filled with new seawater and drained twice, in order to completely flush the tank. All fights were recorded with a SONY camcorder placed approximately 5 feet above the fighting arena. Taping was started prior to the introduction of lobsters into the fight tank, in order to ensure the first interactions of the fights were recorded; each fight lasted 30 minutes. Each fight was numbered in order on the camcorder tapes, and given a random alphabetical code by a colleague. The fights were then transferred from camcorder tapes to VHS tapes with the new alphabetical code, to preclude the possibility of recognizing the fight during subsequent analysis of the tapes.

Analysis:

Each fight was analyzed for aggressive, defensive and avoidance behaviors using the behavioral ethogram developed by Mello *et al*. (1999) and modified by Bolingbroke and Kass-Simon (2001) and Coglianese *et al*. (2008) (Table 1). The ethogram ranks each behavior on a numerical scale, called the Rank of Aggression scale, where the most aggressive behaviors receive the highest number, and the least aggressive behaviors (avoidance behaviors) receive the lowest numbers. For clarity, a

summary of the considerations used in the ethogram is repeated here: Aggressive behaviors are defined as any behavior in which the animal attempts to cause damage to their opponent or signal a threat of such a behavior. Defensive behaviors are defined as behaviors that attempt to ward off aggressive behaviors by an opponent. Avoidance behaviors are defined as any behaviors in which the animal attempts to get away from, or avoid its opponent. Along with behaviors directed towards opponents, behaviors designated as wall behaviors were also recorded. Wall behaviors are defined as any aggressive or defensive behavior that is directed towards the walls of the fighting tank, rather than towards the opponent. Definitions of all behaviors in the ethogram are listed in Table 2.

The fights were analyzed by two people, one of whom (MS) had initially staged the confrontations. The number of times each behavior was performed by each animal was noted (Frequency) and recorded into a computer program that kept a running total of the number of behaviors and also the rank of each behavior. After the frequency of behaviors were tabulated, two more parameters were calculated in order to assess the relative aggressiveness of each animal, the Rank Frequency and the Average Rank. The Rank Frequency (RF) for each animal was calculated by multiplying the Frequency of each behavior by its Rank of Aggression value, in order to reflect relative aggressive intensity. The RF value accounts for animals that may perform a low total number of aggressive behaviors, but perform many highly aggressive behaviors. The Average Rank (AR) is the mean of the ranks of all the behaviors for each animal. It is calculated for each individual by dividing the sum of all the Rank Frequencies for an animal by the sum of the total number of behaviors

performed by that animal (AR= Σ RF/ Σ F). For each pair of animals, the Frequency, Rank Frequency and Average Rank values of each animal were either added together (Treated + Opponent Treated) or subtracted from each other (Opponent Treated – Treated) for each category of behavior (aggressive, defensive and avoidance) both with and without wall behaviors. The summed values of the combatants in a fight represent the overall aggressive and defensive intensity of the fight, and the subtracted differences represent the relative aggressiveness or defensiveness of each individual combatant in the fight.

Single factor Analyses of Variance (ANOVA) were used to determine significance between control and experimental animals. In our analysis, small experimental animals (20E injected) (Treated, T) were compared to small control animals (saline injected) (Control, C), and large experimental animals (opponents of 20E injected animals (OT)) were compared to large control animals (opponents of saline injected small animals (OC)). ANOVA's were performed on all three parameters measured, Frequency, Rank Frequency and Average Rank for all behaviors recorded (aggressive, defensive, avoidance) both with and without wall behaviors. ANOVA's were also performed on each pair within a fight; large experimental plus small experimental vs. large control plus small control (OT + T vs. OC + C), large experimental minus small experimental vs. large control minus small control (OT – T vs. OC- C). Values were considered significant at $p \leq 0.05$ and potentially significant (strong trend) at $0.05 \leq p \leq 0.08$.

Post-Hoc Analysis:

During the course of analyzing the data of the present study, it became apparent the results were different from those of Bolingbroke and Kass-Simon (2001). Since no significant differences were found among the aggressive or avoidance behaviors and only one significant difference was found in defensive behaviors, the question arose as to whether blocking the nephropores changes the dynamics of agonistic interactions in lobsters. To address this question, a post-hoc analysis was performed to compare the control animals in the present study (blocked) with the control animals of Bolingbroke and Kass-Simon (2001) (unblocked). The total Frequency and Average Rank of aggressive, defensive and avoidance behaviors (including wall behaviors) were compared using two-sample, two-tailed T-tests with equal variance. The following comparisons were made: Sipala Control (C_S) vs. Bolingbroke Control (C_B), Sipala Opponent Control (OC_S) vs. Bolingbroke Opponent Control (OC_B), Sipala Opponent Control plus Control ($OC_S + C_S$) vs. Bolingbroke Opponent Control plus Control ($OC_B + C_B$), and Sipala Opponent Control minus Control ($OC_S - C_S$) vs. Bolingbroke Opponent Control minus Control ($OC_B - C_B$). Values were considered significant if the p-value was ≤ 0.05.

Results:

All Behaviors (Aggressive, Defensive and Avoidance):

No significant differences were found for all behaviors between treated animals (T) and their counterpart controls (C) in any of the parameters measured with or without wall behaviors (Frequency, Rank Frequency and Average Rank). Nor were there any significant differences found between the opponents of treated animals (OT) and their counterpart controls (OC) for any parameters measured with or without wall behaviors. Similarly, there were no significant differences found between OT + T vs. OC + C, or between OT – T vs. OC – C for any parameters measured with or without wall behavior.

Aggressive Behaviors:

No significant differences were found for any aggressive behaviors between treated animals (T) and their counterpart controls (C) in any of the parameters measured with or without wall behaviors (Frequency, Rank Frequency and Average Rank). Nor were there any significant differences found between the opponents of treated animals (OT) and their counterpart controls (OC) for any parameters measured with or without wall behaviors. Similarly, there were no significant differences found between OT + T vs. OC + C, or between OT – T vs. OC – C for any parameters measured with or without wall behavior.

Defensive Behaviors:

No significant differences were found for any defensive behaviors between treated animals (T) and their counterpart controls (C) in any of the parameters measured with or without wall behaviors (Frequency, Rank Frequency and Average

Rank). Nor were there any significant differences found between the opponents of treated animals (OT) and their counterpart controls (OC) for any parameters measured with or without wall behaviors. Similarly, there were no significant differences found between OT + T vs. OC + C for any parameters with or without wall behaviors.

Significance was found in the differences in Average Rank between the OT - T vs. OC – C with wall behaviors (41.8 and 60.7, respectively; $F_{1,18}$=4.9, P=0.04) (Figure 1) indicating that the disparity between the two combatants in control fights might have been greater than in treated fights. Since the Average Rank is larger for the control fights, these results indicate that the aggressiveness of the defensive behavior of smaller treated animals was more similar to that of their larger opponents than the aggressiveness of the defensive behaviors of smaller control animals and their larger opponents. No other significant differences were found between OT – T and OC - C animals for any parameters measured with or without wall behaviors.

Avoidance Behaviors:

No significant differences were found for any avoidance behaviors between treated animals (T) and their counterpart controls (C) in any of the parameters measured (Frequency, Rank Frequency and Average Rank). Nor were there any significant differences found between the opponents of treated animals (OT) and their counterpart controls (OC) for any parameters measured.

Post-Hoc Results:

The post-hoc analysis results suggests that the removal of the urine/chemical signal from an agonistic interaction changes the behaviors of the combatants in the following ways.

Aggressive Behaviors:

Significant differences in the Frequency of aggressive behaviors were found between the control experiments in which the nephropores were blocked (Sipala) and those in which they were not blocked (Bolingbroke). C_S animals performed significantly more aggressive behaviors than C_B animals (183.4 and 93.2, respectively, p=0.004), while OC_B animals performed significantly more aggressive behaviors than OC_S animals (359.9 and 242.2, respectively, p=0.009) (Figure 2). When the aggressive behaviors of the control animal were subtracted from the behaviors of the opponent control animal in a single fight, the difference between OC_B-C_B was larger than the difference between OC_S-C_S (266.7 and 59, respectively, p=0.001) (Figure 2).

Defensive Behaviors:

Significant differences in the Frequency of defensive behaviors were found between the experiments such that C_B animals performed significantly more defensive behaviors than C_S animals (110.8 and 51.3, respectively, p=0.0000004) (Figure 3). When the defensive behaviors of the two animals in a single fight were added together, OC_B+C_B performed significantly more defensive behaviors than OC_S+C_S (152.2 and 99.1, respectively, p=0.001) (Figure 3). When the defensive behaviors of the control animal were subtracted from the behaviors of the opponent control animal in a single fight, OC_B-C_B was larger than OC_S-C_S (-69.4 and -3.5, respectively, p=0.0002) (Figure 3).

Avoidance Behaviors:

Significant differences in the Frequency of avoidance behaviors were found between the experiments such that C_B animals performed significantly more avoidance behaviors than C_S animals (81.5 and 59.6, respectively, p=0.03) (Figure 4). When the avoidance behaviors of the two animals in a single fight were added together, OC_B+C_B performed significantly more avoidance behaviors than OC_S+C_S (135.2 and 107.5, respectively, p=0.007) (Figure 4).

Discussion:

The purpose of this study was to determine whether increased blood titers of 20-hydroxyecdysone affected the aggressive behavior of female American lobsters, *Homarus americanus*. The only significant difference found in the present study was the difference in Average Rank of Defensive Behaviors between the OT - T and OC – C with wall behaviors (41.8 and 60.7, respectively; $F_{1,18}$=4.9, P=0.04), indicating that the difference in the aggressiveness of defensive behaviors of treated animals was more similar to that of their opponents than the aggressiveness of the defensive behaviors of control animals and their opponents. Therefore, the disparity between the two combatants is greater in the control fights than in the fights with treated animals**.** This could be due to the hormonal effect of 20E making treated animals more aggressive than saline-injected control animals. The increased aggression of the treated animals could cause the OT animals to increase their level of aggression in defensive response, which could lead to a decreased difference in the total amount of defensive behaviors between the T and OT animals. It was expected that the injection of the hormone alone (with no urine release) would increase the aggressiveness of lobsters due to its effects on physiological processes: increase in EJP amplitudes and miniature excitatory junctional potential (MEJP) frequency in the opener muscle, as well as a decrease in EJP amplitudes in the abdomen (Cromarty and Kass-Simon, 1998). However, our experiments failed to find any significant differences in aggressive behaviors between hormone-injected treated animals and saline-injected control animals (or their opponents) for any category of behaviors tested. The lack of significance in other parameters raised the possibility that an experimental artifact had

been created by removing the urine signal from the interaction, since in comparable studies in which an olfactory signal was present, a number of significant differences were found (Bolingbroke and Kass-Simon, 2001; Coglianese *et al*., 2008; Reinhart *et al*., unpublished).

This led to a post-hoc analysis to compare the behaviors of the control animals of the present study with the control animals in the Bolingbroke and Kass-Simon study, since the only treatment differences between these two sets of animals was that the control animals in the Bolingbroke and Kass-Simon study were capable of receiving a urine signal, while those in the present study were not. The differences in behaviors found between the present experiment and Bolingbroke and Kass-Simon (2001) indicates that removing the urine signal changes the dynamics of agonistic interactions in lobsters. Since the only obvious consistent difference between the control animals was the lack of a urine signal in the present study, the difference in behavior may be due to the removal of the urine signal.

In an agonistic interaction between a large and a small lobster, large lobsters win significantly more encounters than smaller lobsters (Scrivener, 1971). Concomitantly, larger animals will often take on a dominant position over smaller animals (Cobb and Tamm, 1975), evicting them from shelters and initiating more confrontations than smaller animals (Karnofsky, 1989). Furthermore, once dominance is established between two lobsters in a staged confrontation, dominant 'winners' continue to perform aggressive behaviors toward the subordinate 'losers,' who perform submissive or avoidance behaviors (Karavanich and Atema, 1998). The present study and Bolingbroke and Kass-Simon (2001) do not directly assess

dominance or 'winners' and 'losers' in a fight, but addresses the aggressiveness of the behaviors of the lobsters engaged in the interaction. Since the fights consist of a large animal versus a small animal, the fight is biased in favor of the larger animal, and the behaviors of each animal in the interaction should follow those described in the previous experiments: larger animals should be dominant over smaller animals and perform more aggressive behaviors, while smaller animals should be submissive and perform more submissive/avoidance behaviors.

The control animals in Bolingbroke and Kass-Simon (2001) (C_B and OC_B) appear to follow similar behavioral patterns to the normal dominant/subordinate interaction, while C_S and OC_S animals do not. The differences found between OC_B-C_B and OC_S-C_S suggests that there is a smaller difference in the total number of aggressive behaviors between the lobsters in a single fight when the urine signal is removed. In OC_B animals, the total number of aggressive behaviors is greater than that of the C_B animals, leading to a larger difference. The difference in the aggressive behaviors between OC_S and C_S was much smaller than that of Bolingbroke's, which means that the number of aggressive behaviors of the smaller animal was closer to that of the larger animal. Once the urine signal is removed, small animals that would ordinarily become less aggressive in an interaction with a larger opponent did not, which accounts for the smaller difference in aggressive behaviors between $OC_S - C_S$ than $OC_B - C_B$. The removal of urine changes the behavior of a small animal engaged agonistic interaction with a larger animal is that C_S animals perform significantly more aggressive behaviors than C_B animals, and C_B animals perform significantly more defensive and avoidance behaviors.

OC_B animals performed significantly more aggressive behaviors than OC_S animals, which contradicts the finding that non-urine signaled control animals are more aggressive than urine-signaled controls. Since small C_S did not become more defensive or subordinate, the large OC_S did not become more aggressive in response to a more defensive subordinate smaller animal. This may be why OC_B perform more aggressive behaviors than OC_S. Another reason OC_B may be more aggressive is explained by the fact that $OC_B + C_B$ perform significantly more defensive and avoidance behaviors than $OC_S + C_S$. Since C_B immediately perform more defensive and avoidant behaviors in response to a larger dominant animal, the additive value of those behaviors in the individual fight is very high due to the high frequency of behaviors by the smaller animal. This could further explain why OC_B are more aggressive than OC_S, as the larger OC_B would increase its aggressiveness in response to a smaller subordinate animal, while OC_S would not increase their aggressiveness because their smaller opponent does not exhibit the same frequency of defensive and avoidance behaviors as a urine-signaled animal.

These results together suggest that blocked animals engaged in an agonistic interaction are not able to assess each other through urine signals, resulting in a change of the dynamics of the encounter. The ability of each lobster to asses each other via urine signals is an important determinant in the outcome of agonistic interactions (Breithaupt and Atema, 1993; Karavanich and Atema, 1998; Breithaupt *et al*., 1999; Breithaupt and Atema, 2000; Breithaupt and Eger, 2002), and the lack of urine signals in the present study affects the outcome of the encounter. Bolingbroke's OC is able to receive a urine signal from its smaller combatant, alerting the larger animal that the

smaller lobster is weaker, and therefore increases its aggressiveness. Sipala's OC does not receive a urine signal, so it is not able to assess the strength of its smaller combatant, and its aggressiveness does not increase as much as Bolingbroke's OC. Bolingbroke's OC aggressiveness has been increased by the perceived weakness of its smaller combatant, and since Sipala's OC can't assess its opponent, it is less aggressive than Bolingbroke's OC. Conversely, Sipala's C did not receive a urine signal from a blocked larger opponent, and therefore increased its aggressiveness due to the lack of a 'strong' signal from a larger animal.

The ability of a lobster to 'smell' urine is very important in both establishing dominance hierarchies and individual recognition in lobsters engaged in agonistic encounters. If urine release is blocked, then dominance hierarchies are not established (Karavanich and Atema 1998; Karavanich and Atema, 1991) and lobsters are not able to recognize each other in subsequent encounters, leading to increased fighting before dominance is re-established (Kaplan, 1993; Karavanich and Atema, 1991). Furthermore, if the aesthetasc sensilla are removed or made anosmic, lobsters are not able to recognize previous opponents or established dominance relationships and spend more time fighting than lobsters who could smell normally (Johnson and Atema, 2005; Hoeppner, 1997). The same is true for crayfish, as the ablation of aesthetasc sensilla results in fights of longer duration between previous combatants than unablated control pairs (Horner *et al.*, 2008). These results highlight the importance of lobsters being able to smell urine and gain some kind of assessment of the animal opposite them in an agonistic encounter. If the urine signal or the ability to smell are removed, then the behavior of the lobster changes. The lobsters in the

present study were not able to smell the urine of their opponent lobsters, which may have prevented them from assessing their opponent, thereby affecting their behavior.

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Table 1. Rank of Aggression Ethogram

Aggressive Behaviors	Defensive Behaviors	Avoidance Behaviors
152 Agressive Tail Flip	*82 Hitting Wall*	28 Avoidance Stretch
150 Agressive Subsequent Swim	*80 Small Ready to Wall*	26 Back/Front Truce
148 Pulling	*78 Antenna Whip to Wall*	24 Appendage Release
146 Grabbing	*76 Advance to Wall*	22 Mutual Avoidance
144 Pushing	*74 Claw Touch to Wall*	20 Antenna Retraction
142 Claw Lock	*72 Antenna Touch to Wall*	18 Withdrawal
140 Meral Spread	*70 Antenna Point to Wall*	16 Freeing
138 Large Ready	*68 Backing into Wall*	14 Walk Away
136 Lunge	*66 Avoidance Stretch to Wall*	12 Turn Away
134 Hitting/Pinching	*64 Climbing Wall*	10 Retreat
132 Agg Antenna Whip	62 Hitting	8 Backing Away
130 Lean On	60 Shielding	6 Superman Swim
128 Backing Into	58 Claw Extension	4 Avoidance Tail Flip
126 Walk Over/On	56 Forward/Backward Walk	2 Avoidance Subsequent Swim
124 Aggressive Claw Touch	54 Holding	
122 Aggressive Tail Touch	52 Defensive Antenna Whip	
120 Aggressive Antenna Touch	50 Defensive Antenna Touch	
118 En Passant	48 Defensive Antenna Point	
116 Walk By	46 Defensive Antenna Wave	
114 Advance	44 Defensive Position	
112 Small Ready Position	42 Defensive Turn Into	
110 Turn Into	40 Defensive Claw Touch	
108 Aggressive Antenna Wave	38 Defensive Tail Touch	
106 Aggressive Antenna Point	36 Defensive Walk By	
104 Meral Spread To Wall	34 Backing Into	
102 Hitting Wall	32 Squeeze By	
100 Climbing Wall	30 Walk Under	
98 Large Ready to Wall		
96 Antenna Whip to Wall		
94 Advance to Wall		
92 Claw Touch to Wall		
90 Antenna Touch to Wall		
88 Antenna Point to Wall		
86 Backing into Wall		
84 Facing		

Table 2. Description of Ethogram behaviors (numbers indicate the rank of each behavior).

Behavior	Description
Advance (114)	Forward movement towards the opponent
Antenna point (Aggressive 106, Defensive 48)	Movement of antennae toward opponent without physical contact with the opponent
Antenna retraction (20)	Moving antenna back away from opponent along axis of body
Antenna touch (Aggressive 120, Defensive 50)	A single touch of the opponent with an antenna
Antenna wave (Aggressive 108, Defensive 46)	Lateral sweep of the antennae without physical contact with opponent
Antenna whipping (Aggressive 132, Defensive 52)	Hitting opponent with antennae
Appendage release (24)	Animal releases opponent's appendage
Avoidance stretch (28)	An extension of the entire body with chelipeds and tail extended, such that the animal has made its profile as thing and long as possible
Back/front truce (26)	Opponents aligned with the tail of one in close proximity or touching the opponent
Backing away (8)	Walking backward with contact while facing an opponent
Backing into (Aggressive 128, Defensive 34)	Walking backward toward or into an opponent with abdomen flexed
Claw extension (58)	Use of one claw to shield and the other to fend off an opponent
Claw lock (142)	Mutual grasping and holding of opponent's claws
Claw touch (Aggressive 124, Defensive 40)	Use of claw to touch opponent's claw
Defensive position (42)	Claws closed together in front of body (protecting)
En passant (118)	Walking past opponent while brushing against it
Facing (standoff) (84)	Lobsters face each other without contact for 10-60 s
Forward/backward walk (56)	Indecisive movement (approach/retreat or retreat/approach)
Freeing (16)	Withdrawal of an appendage or body part from being held
Grab (146)	Animal seizes or attempts to seize the opponent's appendage or body part
Hitting (Aggressive 134, Defensive 62)	Swinging claws and touching various parts of opponent's body
Holding (54)	Standing one's ground in the face of an aggressive act by opponent
Lean on (130)	One or both claws pressing on claw or carapace of the opponent
Lunge (136)	Rapid striking out at opponent with claw

Meral spread (140)	Claws open and raised slightly to either side of rostrum; not always completely perpendicular to carapace (true meral spreads as defined in the literature were rarely seen and usually only when the animals were placed in the fighting tank)
Mutual avoidance (22)	Both animals are somewhat in an avoidance stretch or defensive position against side of the tank, separated by a distance of approximately 1/4 of the tank's circumference
Pinching (Aggressive 134, Defensive 62)	Tips of dactyl and propodite are forcefully applied to opponent
Pulling (148)	An attempt to pull off an opponent's appendage with backward movement (if the movement consisted of a tail flip and pulling, it was simply referred to as an aggressive tail flip)
Pushing (144)	Forced movement of the opponent with fixed claws; forceful displacement of the opponent
Ready position (Large Ready 138, Small Ready 112)	Claws in front of head preparing to confront; the large ready differs from meral spread in that the claws are not raised as high or spread as far apart; the small ready is a lesser large ready
Retreat (10)	Walking backward without contact while facing an opponent
Shielding (60)	Claws slightly separated in front of body to fend off opponent
Squeeze by (32)	Animal moves between opponent and the wall in avoidance stretch
Subsequent swims (Aggressive 150, Avoidance 2)	Successive abdominal flexions following the initial aggressive or avoidance tail flip
Superman swim (6)	Tail flip away from opponent with full extension of the body
Tail flip	Contraction of the abdomen to propel animal backwards
(1) Aggressive (152)	Used to pull opponent's appendage (usually a claw)
(2) Avoidance (4)	Used to escape from opponent
Tail touch (Aggressive 122, Defensive 38)	Use of claw to touch opponent's tail
Turning away (12)	Animal turns away from opponent
Turning into (110)	Animal turns to face opponent
Walk away (14)	Animal simply walks away from opponent (leaves interaction)
Walk by (Aggressive 116, Defensive 36)	One animal passes closely by its opponent without contact
Walk over (126)	One animal walks on top of its opponent
Walk under (30)	One animal walks under its opponent
Withdrawal (18)	Forward movement away from an opponent without physical contact

Figure 1. Average rank values of defensive behaviors with wall behaviors for OT-T vs. OC-C. Values are means ± SEM, N = 10. Asterisk indicates significant difference (P = 0.04).

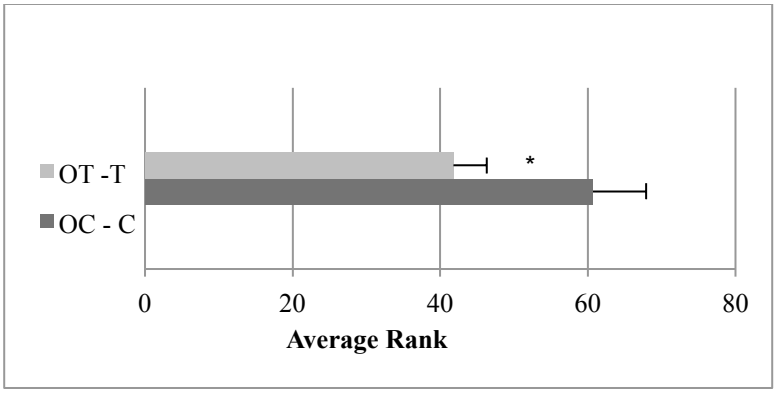
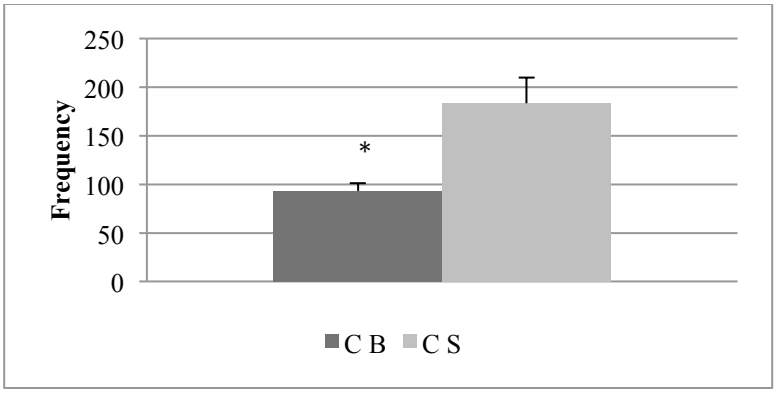

OT -T
OC - C
*
0
20
40
60
80
Average Rank

Figure 2a. Comparison of frequency of Aggressive behaviors between C_B and C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.004).

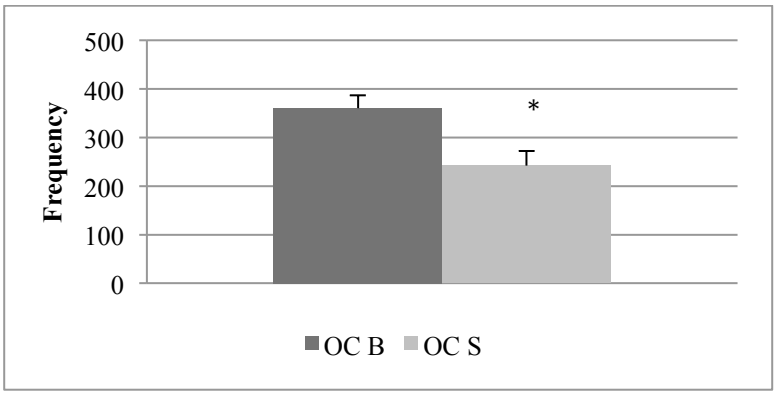
Figure 2b. Comparison of frequency of Aggressive behaviors between OC_B and OC_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.009).

Figure 2c. Comparison of frequency of Aggressive behaviors between $OC_B - C_B$ and $OC_S - C_S$. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.001)

2a.


250
200
150
100
50
0
Frequency
*
C B
C S

2b.


500
400
300
200
100
0
Frequency
*
OC B
OC S

2c.

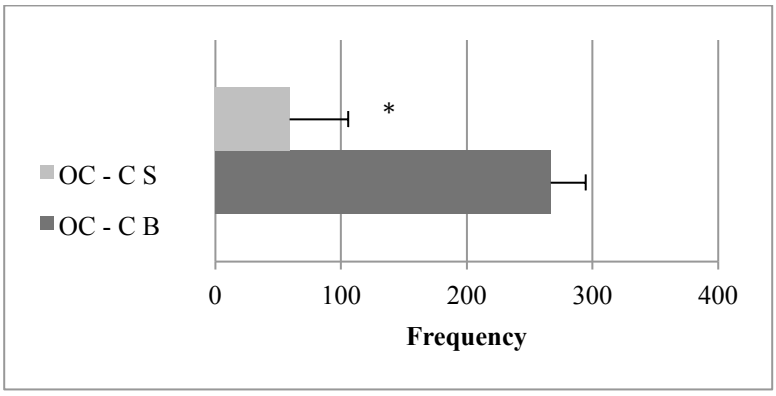
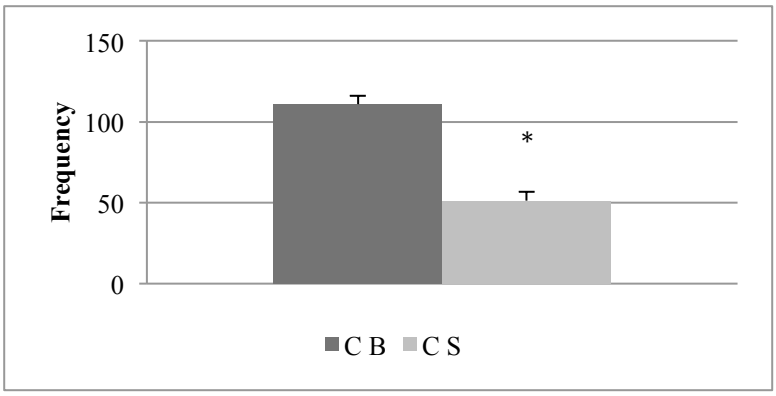

OC - C S
OC - C B
*
0
100
200
300
400
Frequency

Figure 3a. Comparison of Frequency of Defensive behaviors between C_B and C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.0000004).

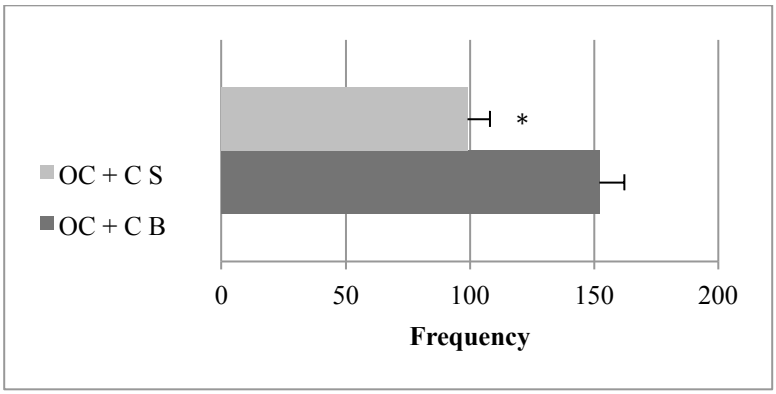
Figure 3b. Comparison of Frequency of Defensive behaviors between $OC_B + C_B$ and $OC_S + C_S$. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.0001).

Figure 3c. Comparison of Frequency of Defensive behaviors between $OC_B - C_B$ and $OC_S - C_S$. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.0002).

3a.


150
100
50
0
Frequency
*
C B
C S

3b.


OC + C S
OC + C B
*
0
50
100
150
200
Frequency

3c.

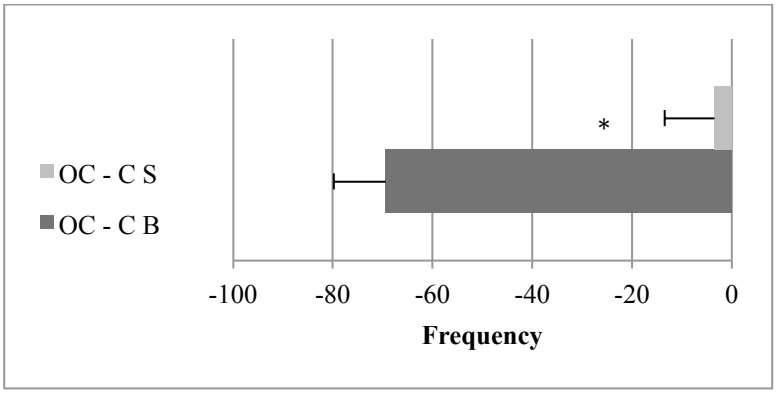
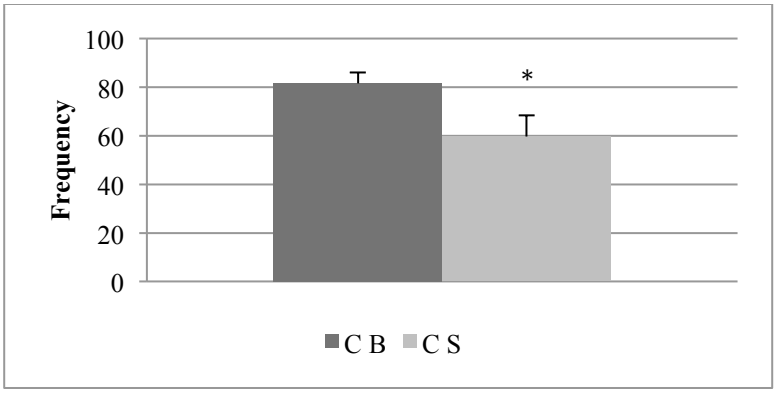

OC - C S
OC - C B
*
-100
-80
-60
-40
-20
0
Frequency

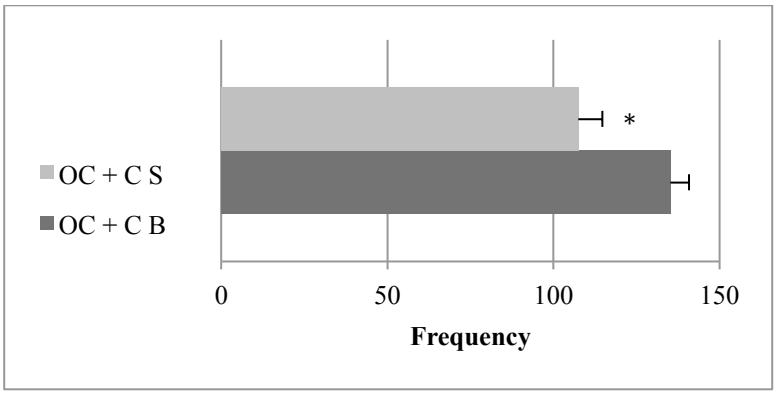
Figure 4a. Comparison of Frequency of Avoidance behaviors between C_B and C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.03).

Figure 4b. Comparison of Frequency of Avoidance behaviors between OC_B + C_B and OC_S + C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.007).

4a.


100
80
60
40
20
0
Frequency
*
C B
C S

4b.


OC + C S
OC + C B
*
0
50
100
150
Frequency

CHAPTER 3

Biochemical evidence for the molecular weight of a membrane-associated and cytoplasmic EcR in the tissues of the American lobster, *Homarus americanus*

by

Michael W. Sipala, Lenore M. Martin, Stuart I. Cromarty and G. Kass-Simon

is prepared for *Chemical Senses*

Abstract:

20-hydroxyecdysone (20E), the active principle of the molting hormone in the American lobster, *Homarus americanus*, has significant effects on the animals' agonistic behavior. In addition to its humoral action, 20E appears to be an important signaling molecule sensed by the animal's antennules, since the behavior of animals change when they are exposed to 20E. In this study, we provide biochemical evidence for the presence of 20E receptors on the antennules of lobsters, along with their molecular weights. Various tissues from lobsters were dissected, soluble and insoluble fractions extracted, and spot blots and Western blots performed. Spot blots indicate the presence of a 20E receptor in both the soluble (cytoplasmic/nuclear) and insoluble (membrane-associated) fractions of walking legs and eyestalks, but only in the membrane-associated fraction of the guard setae and aesthetasc sensilla. Western blots and Mass Spectrometry returned several different molecular weights for the EcR (75 kDa, 50 kDa, 40 kDa). The presence of an EcR in the membrane-associated fraction confirms that 20E may be perceived by the antennules of lobsters, while the various molecular weights suggest different isoforms may exist, which is consistent with various insect and crustacean species.

Introduction:

Ecdysone and its active metabolite, 20-hydroxecdysone (20E), are steroid hormones that regulate molting in insects and crustaceans and coordinate alterations in the transcription of groups of genes required to control this process (Waddy *et al*., 1995). Molting is a slow process that takes place over several days or weeks, however, in recent studies in numerous insects and crustaceans, Ecdysteroids have been shown to have fast-acting effects, suggesting the presence of an additional non-classical steroid hormone signaling pathway (Spencer and Case, 1984; Cromarty and Kass-Simon, 1998; Cooper and Ruffner, 1998; Tomaschko, 1999; Thummel and Chory, 2002; Cooper *et al*., 2003; Schlattner *et al*., 2006).

Steroid hormones have been traditionally considered to work through genomic mechanisms, where steroids enter a cell, bind to a specific receptor in the cytosol or nucleus, and activate transcription that leads to changes in gene expression and results in the production of proteins that have a biological function (Losel *et al*., 2003). This mechanism is generally slow-acting, as it sometimes takes several hours or days to alter patterns of gene transcription after the hormone enters the cell. A second model for the actions of steroid hormones involves a non-genomic mechanism, wherein steroids have an immediate effect on physiological function that cannot be explained by the slower classical mechanisms (Losel *et al*., 2003). The physiological effect of steroids that act through non-genomic mechanisms can be seen within seconds of exposure to the hormone, ruling out any models that involve changes in the transcription levels of genes. The activation of an outer membrane-bound receptor, or signaling via a second messenger pathway are likely causes of the immediate

physiological changes observed to occur in response to the hormone (Losel *et al*., 2003).

Rapid non-genomic effects have been found to act in exocrine glands, the central nervous system, motor neurons, neuromuscular junctions and sensory cells of numerous organisms (Schlattner *et al*., 2006). Compared to the prolonged and slow process of ecdysone induced molting, the non-genomic effects of ecdysone exposure are immediate; changes are sometimes observed within a matter of seconds (Tomaschko, 1999). In the California spiny lobster, Spencer and Case (1984) found an increased action potential firing frequency in the lateral antennule 1 second after exposure to both 20E and alpha ecdysone. In American lobsters, 20E has been shown to have immediate effects on neuro-muscular properties. In the presence of 20E, there is an increase in EJP amplitudes and miniature excitatory junctional potential (MEJP) frequency in the opener muscle, as well as a decrease in EJP amplitudes in the abdomen (Cromarty and Kass-Simon, 1998). In *Drosophila*, Ruffner *et al*. (1999) found reduced transmitter release in the ventral abdominal muscles within 1 minute after exposure to 20E. In crayfish, Cooper and Ruffner (1998) found a decreased amount of neurotransmitter release in the opener muscle of the first walking leg within 20 minutes after exposure to 20E. Cooper *et al*. (2003) found increased action potential firing frequency in the muscle receptor organ of crayfish 10 seconds after exposure to 20E.

Recent evidence presented by Coglianese *et al*. (2008) and Reinhart *et al*. (submitted) indicates that 20E acts as a pheromone that leads to an immediate change in the behavior of lobsters. Coglianese *et al*. (2008) found that the behavior of female

animals exposed to a plume of 20E was different than control animals exposed to a plume of artificial sea-water (ASW). Large female lobsters who had a plume of 20E puffed across their antennules performed significantly more aggressive, defensive and avoidance behaviors than large control animals in staged confrontations. The change in behavior of the exposed individual was attributed to the perception of the 20E in the odor plume, suggesting it acts as a pheromone. Reinhart *et al*. (submitted) performed a similar experiment as Coglianese with male lobsters, and found that the behavior of males exposed to a plume of 20E was also different than control animals exposed to a plume of artificial sea-water (ASW). Reinhart found that male lobsters exposed to 20E performed more defensive behaviors than ASW exposed control animals. These results led to the conclusion that the change in behavior of the exposed individual could be attributed to the perception of the 20E in the odor plume.

In earlier electrophysiological experiments, Cromarty *et al*. (unpublished) found that the olfactory receptor neurons (ORN bundles) in the antennules of American lobsters exhibit a dose-dependent response to 20E, which supports the idea that 20E may be perceived by the antennules of lobsters and this perception is responsible for the alteration of behavior during an agonistic encounter. Because the physiological and behavioral changes occur within milliseconds or seconds of ecdysone exposure, these result together suggest the presence of a non-genomic receptor on the lateral antennules.

Here, we present evidence for a membrane-bound and nuclear ecdysone receptor (EcR) with comparable molecular weights in various tissues of the American lobster, *Homarus americanus*. The presence of a receptor for 20E in the membrane

fraction suggests the possibility that a membrane bound receptor is responsible for the immediate physiological response exhibited by lobsters exposed to 20E.

Methods:

Animal procurement and maintenance:

Female American lobsters, *Homarus americanus*, were obtained from local fisherman and the Rhode Island Department of Environmental Management from inshore waters off the coast of Narragansett Bay, RI. Animals were maintained in circulating unfiltered natural seawater tanks at the Narragansett Bay Campus, on a 12-hr light/dark cycle. Water temperature and salinity were ambient, ranging from 10-20°C and 28-33ppt, respectively. Animals were fed fish scraps twice weekly, banded and kept in separate tanks with compartmentalized large gauge wire cages. All animals were returned to Narragansett Bay after 2 weeks. All lobsters used were intermolt C-stage animals in perfect condition, all eight walking legs, claws, antennae and antennules were intact with no other signs of physical damage or shell disease. Samples of each tissue types were dissected from live animals, and each sample consisted of the same tissue type from at least 3 different animals

Tissue Dissection:

We tested a variety of tissues for the presence of a receptor for 20E including walking legs (WL), eyestalk (EY), intact lateral antennule (IA), guard setae (GS), aesthetasc sensilla (SE), cuticle from antenna (CUA) and cuticle from carapace (CUC). Once each tissue was removed from a lobster, it was immediately placed into a scintillation vial with lobster saline and protease inhibitors (10 mM Tris base, 5 mM Maleic acid, 472 mM NaCl, 10 mM KCl, 16 mM $CaCl_2$, 7 mM MgCl2, D-glucose, Complete mini EDTA-free protease inhibitor cocktail tablets, pH=7.4) in ice before further maceration was performed. WL muscle tissue was dissected out of the first two

pair of walking legs; EY was cut out of the head of the animal, leaving only the y-organ. Cuticle from the antenna (CUA) and the carapace (CUC) was shaved off, under saline, with razor blades. When cuticle was shaved, care was taken to only scrape the outermost layer of the cuticle in order to avoid cutting into the softer inner layers. The lateral antennule (IA) was cut off of the lobster, and chopped up into small pieces. The GS and SE were separated from each other under a dissecting microscope as follows: the GS were plucked off of the antennule with a fine pair of forceps leaving the SE on the antennule. The SE were then carefully shaved off with a Feather Razor blade (#11) taking great care to prevent contamination or cutting into the softer layers of the antennules. For Western Blots and Mass Spectrometry, GS and SE were combined together (GS+SE) in order to increase the overall protein concentration. We collected *Drosophila* (D) larvae and pupae as a control for the presence of 20E, and placed them into the same saline with protease inhibitors as the lobster tissue samples.

Homogenization and Membrane Protein Fractionation:

The tissue was placed into a homogenization tube and macerated sequentially with two different buffers: homogenization buffer (20 mM Tris-HCl, 2 mM EDTA) and detergent Buffer (0.5X homogenization Buffer, 1% (v/v) Triton X-114). The transfer of the smaller GS and SE were performed under a dissecting microscope to ensure adequate sample was collected for maceration. The tissue was first ground up with homogenization buffer in a 1:1 ratio using a Dounce Homogenizer and the liquid portion was transferred to a micro-centrifuge tube. Detergent buffer was then added to the homogenization tube along with homogenization buffer (1:1 ratio), and further

macerated. The residual liquid from this procedure was placed in a separate micro-centrifuge tube. All samples were then sonicated with a Branson Model 184 V sonicator (Branson Instruments, Danbury, CT) with a 3 mm tip operating at 50/60 Hz for 3 separate 5 second bursts in order to ensure that tissue was fully disrupted before the final extraction process. After all samples were sonicated, the insoluble pellet was removed by centrifugation for 10 min at 5,000 RPM. The resultant supernatants were transferred to new tubes with 500 ul aqueous sucrose (0.5X Homogenization Buffer, 6% (w/v) Sucrose, 0.06%(v/v) Triton X-114). The homogenate was vortexed and placed on a heating block at 37°C for 10 minutes, until the cloud point was reached. If the cloud point was not reached after 10 minutes, more detergent buffer was added drop-wise until the solution appeared cloudy. After the cloud point was reached, the samples were spun for 3 minutes at 2,000 RPM to separate the soluble (aqueous) from the insoluble (pellet) fractions. The aqueous supernatant was transferred to a new micro-centrifuge tube to separate it from the remaining pellet.

Protein Characterization by Western Blotting:

The homogenized samples were then either spotted directly onto nitrocellulose paper or loaded into a 10% SDS-PAGE gel. Samples run on SDS-PAGE gels were then transferred to nitrocellulose. A total of three spot blots were made during each experiment: one spot blot for an amido black total protein stain and two blots for staining with two different anti-mouse secondary antibodies (Biotin-linked goat anti-mouse and LICOR Odyssey Goat anti-mouse IR DYE 680LT). We used two different types of standards as controls for the two different types of blots: BSA was used to quantify the total protein blot and a standard mouse IGG was used for the two

antibody blots. The total protein stain spot blot was stained with 0.25 % Amido Black in order to determine if protein was indeed extracted for each sample. This blot was later compared to the blots labeled with EcR-specific antibodies in order to eliminate background artifacts. If a receptor was positively identified on an antibody spot blot, but inadequate protein was extracted from that tissue sample on the amido black spot, then that result could be thrown out as background staining.

The staining protocol for the Biotin linked Goat Anti-Mouse secondary antibody was as follows. The blots were blocked overnight in the fridge with 5% (w/v) Bovine Serum Albumin (BSA) in Tris Buffered Saline (TBS) (20mM Tris-HCl, 150 mM NaCl, pH=7.5), and washed in TBS twice, for 5 min each the next morning. Each blot was then submerged in a solution of the primary antibody, DDA2.7 (EcR common supernatant, Hybridoma Bank U. Iowa), for 1 hour, and then washed 3 times with TBS (15 min, 5 min and 5 min). The blot were treated for 45 min with Biotin Linked Goat Anti-Mouse secondary antibody dissolved in 1% BSA/TBS, and washed with TBS 3 times (15min, 5min, 5min). The blot was then stained with a 1:1,000 Streptavidin-linked Alkaline Phosphatase (Zymed Laboratories) dissolved in 1% BSA/TBS for 30 minutes and then washed with TBST (TBS with Tween-20) 3 times (15 min, 5 min, 5 min) followed by a final wash with TBS for 5 min. Each nitrocellulose membrane was washed off with distilled water, placed into a clean petri dish, and a small amount of Fast Red dye (Fast Red Substrate pack, Zymed Laboratories) was poured into the dish on top of the membrane. The dye was allowed to soak into the blot for about 1-2 minutes, until the standards clearly showed up and other positive results became clear. The blots were washed with distilled water again

to prevent development of excess background color, and were then scanned on the computer. The EcR-positive results for the Fast Red appeared as red spots.

The staining protocol for the LICOR Odyssey Goat anti-mouse IR DYE 680LT was as follows. The blots were blocked with Casein blocking buffer for one hour at room temperature and washed in TBS three times for five minutes each. Each blot was then submerged in a solution of the primary antibody, DDA2.7 (EcR common supernatant, Hybridoma Bank University of Iowa), for 24 hours at 4°C and then washed three times with TBS (15 min, 5 min and 5 min). The blots were then stained with LICOR Odyssey Goat anti-mouse IR DYE 680LT for one hour at room temperature and washed with TBST (TBS with Tween-20) three times (15 min, 5 min, 5 min) followed by a final wash with TBS for five min. Membranes were then visualized with the LICOR Odyssey scanner at a 700nm wavelength to visualize the presence of a receptor.

In order to determine the molecular weights of the lobster receptors for 20E expressed in different tissue fractions, SDS-PAGE and Western Blots were employed. SDS gels (10 lanes each) were prepared with 5% Acrylamide stacking and 10% Acrylamide resolving gels using the BIO-RAD Mini Trans Assembly Kit. Samples were first mixed with an equal volume of 2x sample loading buffer (10% (w/v) SDS, 4X Stacking buffer, 80% (w/v) Glycerol, DTT, 1% (w/v) bromophenol blue), vortexed and centrifuged at 11,000 rpm for 10 seconds, heated at 100°C for 5 minutes, vortexed and centrifuged again at 11,000 rpm for 10 sec. Approximately 18 uL of protein sample and 10 uL of standard sample were loaded into each lane of the gel. Standards included: Precision Plus Protein Unstained Standards, Biotinylated SDS-PAGE

Standards, Kaleidoscope Standards and LICOR Odyssey fluorescent molecular weight markers. Gels were run at a constant voltage of 125 Volts for 2 hours in 1X Tank Buffer (0.025M Tris Base, 0.192M Glycine, 0.1% SDS). Gels were cut into two identical halves, one was stained with GelCode Blue reagent, the other used to transfer to a nitrocellulose membrane. The GelCode half was rinsed in double distilled water for 15 minutes to remove SDS before GelCode stain was added, and the gel remained in darkness on the shaker table at room temperature overnight. The gel was then washed with distilled water, photographed and scanned into the computer. The Western Blot half was first washed in transfer buffer (80% tank buffer, 20% methanol HPLC grade) for 10 minutes and then assembled in the Mini Trans-Blot assembly kit. Transfer blots were run at a constant current of 150 Volts for 30 min in transfer buffer. The staining procedure for the Western Blots is the same as for the LICOR antibody stained dot blots, described above.

In order to confirm bands found on Western Blots and to specify accurate molecular weights of the receptor, we ran Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry. Samples were mixed with a 7% ACN/TFA (Acetonitrile, Trifluoroacetic acid) solution, added to a sinapinic acid matrix and loaded onto a gold chip. The gold chip was placed into a Ciphergen Mass Spectrometer, and hit with a laser at an intensity of 150-200.

Results:

Spot blot results indicate the presence of an EcR in both the soluble and insoluble fractions of walking legs and eyestalks, but only in the membrane associated fraction of the guard setae and aesthetasc sensilla (Table 1; Figure 1). No evidence of a receptor was found in the outer layer of the cuticle for the antenna or carapace. Spot blots on D control were positive for both the soluble and insoluble fractions (Table 1; Figure 1).

Western Blots confirmed the presence of the receptor in WL, EY and D control and yielded molecular weights for the receptor. The membrane bound WL fraction yielded two bands at approximately 75 kDa and 40 kDa whereas the cytoplasmic/nuclear fraction yielded three bands at approximately 75 kDa, 50 kDa and 40 kDa (Table 1; Figure 2). The cytoplasmic/nuclear fraction of EY yielded two bands at approximately 50 kDa and 40 kDa. (Table 1; Figure 3) The D control yielded bands for both the membrane and cytoplasmic/nuclear fractions (75, 50, 40 kDa and 60, 50, 40 kDa, respectively) (Table 1; Figure 4). We were unable to obtain a Western Blot for GS or SE, as we were unable to obtain a sufficient concentration of protein to show up on the Western Blot. Our maximum concentration of 3.8 ug/uL from 90 noses was considerably less than the 8 ug/uL we had previously found to be necessary for visualization on Western Blots.

All bands found on Western Blots were consistent with peaks found by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The membrane and cytoplasmic/nuclear fractions of WL, EY and GS+SE consistently returned peaks of 75/76 kDa and 39/38/37 kDa on the Mass Spec (Table 1; Figure 5; Figure 6; Figure 7).

The D control returned peaks of 83 kDa, 48 kDa, 39 kDa for the membrane fraction and 65 kDa, 46 kDa, 41 kDa for the cytoplasmic/nuclear fraction (Table 1; Figure 8).

Discussion:

In this study, we have presented the first biochemical evidence for a membrane bound EcR in the guard setae, aesthetasc sensilla, walking legs and eyestalks of lobsters. The presence of a membrane bound receptor could explain the immediate actions of 20E that cannot be explained by genomic mechanisms. Spot blots indicated the presence of the EcR in both the membrane and nuclear/cytoplasm of WL and EY. Western Blot and Mass Spec analysis of WL and EY consistently returned molecular weights of 75 kDa and 40 kDa for both the cytoplasmic/nuclear and membrane fractions. Spot blots indicated the presence of a receptor in only the membrane bound fraction of GS and SE, and never the nuclear/cytoplasmic fraction. Mass Spec analysis of GS+SE consistently returned molecular weights of 75 kDa and 38 kDa for the membrane fraction. Although we were not able to obtain a Western Blot for GS or SE, spot blot data coupled with MALDI results indicate that there is an EcR whose molecular weights are consistent with those found in WL and EY.

In spiny lobsters, *Panulirus argus*, the aesthetasc sensilla are the sensory cilia of the olfactory receptor cells whose nuclei are located within the antennules themselves (Ache and Derby, 1985; Grunert and Ache, 1988). Histological studies shows that the aesthetasc sensilla are innervated by the dendritic extensions of multiple bipolar receptors, with the soma gathered in a cluster at the base of the sensillum inside the antennule itself (Ache and Derby, 1985; Grunert and Ache, 1988). Although we have not studied the histology of the antennules in the American lobster, we have made the assumption that a similar morphological arrangement exists, and our spot blot data appears to confirm this. Since we shaved the sensilla off of the

antennule at the base, we would have separated the cell bodies from the dendritic extensions that extend into the sensillum itself. Therefore, our GS and SE samples did not contain any cell bodies (nucleus or cytoplasm), and positive staining for the EcR must represent a membrane receptor, not a cytoplasmic one.

We found several molecular weights for the EcR, which suggest that different isoforms exist in lobsters. Isoforms of the EcR have been found in several insect and crustacean species, including *Drosophila* (Talbot *et al*., 1993), *Manduca sexta* (Fujiwara *et al*., 1995; Jindra *et al*., 1996), *Bombyx mori* (Kamimura et al., 1997) *Crangon crangon* (Verhaegen *et al*., 2011) and *Homarus americanus* (Tarrant *et al*., 2011). In *Drosophila*, three different isoforms exist for the EcR: EcR-A (91 kDa), EcR-B1 (93 kDa) and EcR-B2 (73 kDa) (Talbot *et al*., 1993). Ann Tarrant *et al*. (2011) sequenced the EcR in American lobsters, and found two splice variants with different amino acid sequences, predicted at 60.1 kDa, but expressed as 63.3 kDa when expressed in a plasmid.

The molecular weights of the EcR reported here for WL and EY are not consistent with those reported by El Haj *et al*. (1994) (97-116kDa) or Tarrant *et al*. (2011), (60.1kDa sequence data and 63.3 kDa expression plasmids). We do not know the reason why we didn't obtain similar molecular weights for the EcR in our samples, however, there are some possibilities for the differences. The differences found between our work and Tarrant's work might be attributed to the fact that Tarrant *et al*. (2011) derived their results by synthesizing and expressing cDNA in a plasmid, whereas we obtained our results directly from dissected tissue. A possible reason for this could be due to alternative splicing of the EcR, which has been widely reported in

crustaceans (Chung *et al*., 1998; Wu *et al*., 2004; Kim *et al*., 2005; Asazuma *et al*., 2007; Kato *et al.*, 2007). Also, Tarrant *et al*. (2011) states that EcR variants they found were identified fortuitously during cloning and suggest that additional variants of the EcR may be found from different tissue types. Tarrant used tissue from the abdomen, claw, hepatopancreas and ovary, while we used tissue from the WL, EY, GS and SE. The different tissues used could express different splice variants of the EcR, which could explain the differences found between our data. Finally, the pictures of the gels published in Tarrant *et al*. (2011) do not coincide with the text that reports the molecular weight of 60.1/63.3kDa. The gels pictured appear to show bands about halfway between 52 kDa and 38 kDa, which would correspond with our data that has bands at approximately 40 kDa on Western Blots and mass spec.

We are not sure why both our data and Tarrant's data are different from El Haj *et al*. (1994). El Haj reported only the presence of one receptor at approximately 97-116 kDa, with no possible isoforms in WL and EY tissue. It may be that the dissection and extraction method that El Haj *et al*. (1994) employed did not separate different isoforms, or there may be a possible dimerization of the two isoforms. A second possibility could be due to the fact that El Haj used juvenile premolt animals, and we used adult intermolt animals. In crustaceans, it has been shown that there is differential expression of the EcR over the molt cycle (Durica *et al*., 1999; Asazuma *et al*., 2007; Kato *et al*., 2007; Hirano *et al*., 2008).

The molecular weights we obtained for *Drosophila* (75/60, 50, 40 kDa) are not consistent with the three isoforms reported by Talbot *et al*. (1993) (93, 91, 73 kDa), however, a similar argument to the differences we found in lobster molecular weights

can be made. Talbot *et al*. (1993) employed a plasmid method and transfected S2 cells with the expression construct of each isoform, while we extracted the receptor from live pupae and larvae. It is possible that different tissues used or alternative splicing in those tissues could account for the differences we observed.

The mechanism of non-genomic actions of Ecdysone in insects and crustaceans has not been fully described, although several studies suggest possible modes of action. Srivastava *et al*. (2005) discovered a G-protein coupled dopamine/ecdysteroid receptor in *Drosophila,* the *Drosophila melanogaster* dopamine/ecdysteroid receptor, which confirms to the second messenger system hypothesis for the non-genomic action of steroids. The *Drosophila* dopamine/ecdysteroid receptor is able to bind dopamine, 20E and alpha ecdysone. Its genetic sequence is homologous to the vertebrate β-adrenergic receptor, and activates two different second messenger pathways depending upon whether dopamine or ecdysteroids are bound to the receptor. Once dopamine binds, cAMP levels increase inside the cell and the phosphoinositide-3-kinase pathway is activated. When 20E or alpha ecdysone binds, the mitogen-activated protein kinase pathway is activated (Srivastava *et al.*, 2005). With respect to the non-genomic functions of the ecdysone receptor, the presence of a putative membrane bound ecdysone receptor has been isolated from the plasma membrane of the anterior silk gland of the silkworm, *Bombyx mori* (Elmogy *et al*., 2004). The receptor (57 kDa) exhibited rapid saturable binding to PonasteroneA, and these quick association/dissociation kinetics are characteristic of membrane bound receptors, not nuclear/cytoplasmic receptors (Elmogy *et al*., 2004). The presence of a membrane bound receptor for 20E in the silkworm correlates with

our finding of a membrane bound receptor for 20E in lobsters. These membrane bound receptors could act through a second messenger pathway, as observed in *Drosophila*, or through another pathway initiated in the membrane.

In summary, we have shown the presence of a receptor for 20E in both the membrane and nucleus/cytoplasm for WL, and EY, but only the membrane of GS and SE. These results were obtained consistently multiple times through spot blots and Western Blots, and the molecular weights were confirmed with Mass Spec. Our present findings, together with our previous electrophysiological and behavioral studies indicate that in lobsters, a fast-acting membrane-bound ecdysone receptor exists and that either external or internal exposure to 20E contributes to changes in the animal's behavior.

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Table 1. Summary of Spot Blots, Western Blots and Mass Spectrometry.

Abbreviations: WL=Walking legs, EY= Eyestalk, GS= Guard Setae, SE= Aesthetasc sensilla, D= Drosophila, (+) = Positive for EcR, (-) = Negative for EcR, P= Pellet/membrane fraction, S= Soluble/nuclear/cytoplasmic fraction. Molecular weights are in kDa. Results were replicated 5 times for spot blots, 3 times for Western Blots, and 4 times for Mass Spectrometry.

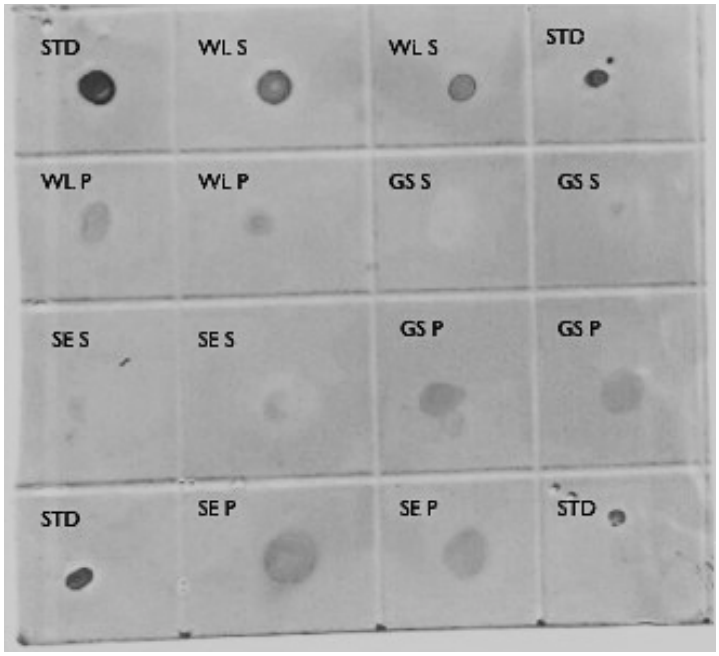
Tissue	Dot Blot	Western Blot	Mass Spec
WL P	+	75, 40	75, 39
WL S	+	83, 75, 50, 40	83, 75, 47, 39
EY P	+	N/A	75, 39
EY S	+	50, 40	75, 65, 37
GS + SE P	+	N/A	76, 38
GS + SE S	-	N/A	75, 38
D P	+	75, 50, 40	83, 48, 39
D S	+	60, 50, 40	65, 46, 41

Figure 1a. Representative Spot blot of Walking legs (WL), Guard Setae (GS) and Aesthetasc Sensilla (SE) with Fast Red Stain. P = Pellet/membrane fraction, S = Soluble/cytoplasmic/nuclear fraction, STD = Biotin Standard.

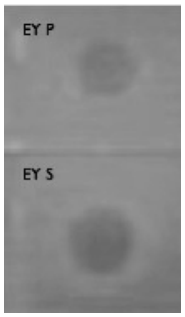
Figure 1b. Spot blot of Eyestalk (EY) with Fast Red Stain. P = Pellet/membrane fraction, S = Soluble/cytoplasmic/nuclear fraction.

Figure 1c. Spot blot of Drosophila (D) control with Fast Red stain. P = pellet/membrane fraction, S = soluble/cytoplasmic/nuclear fraction, STD = Biotin standard

1a.


STD
WL S
WL S
STD
WL P
WL P
GS S
GS S
SE S
SE S
GS P
GS P
STD
SE P
SE P
STD

1b.


EY P
EY S

1c.

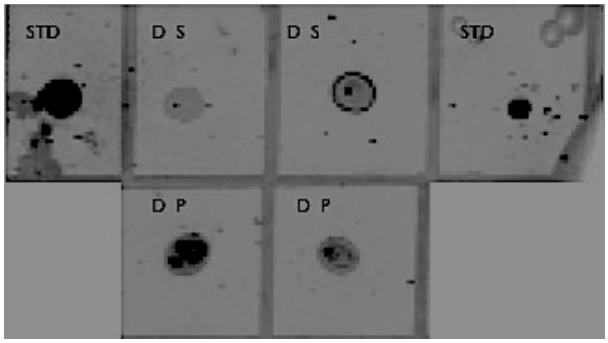
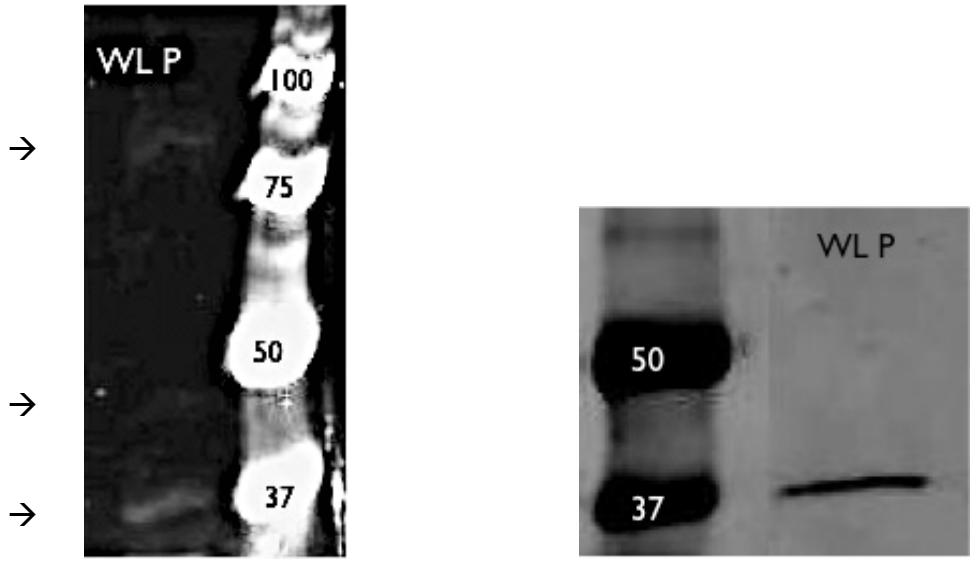

STD
D S
D S
STD
D P
D P

Figure 2a. Representative Western Blots for Walking leg Pellet (Membrane) fraction. LICOR Odyssey fluorescent molecular weight marker bands are labeled with appropriate molecular weight in kDa.

Figure 2b. Representative Western Blot for Walking leg soluble (nuclear/cytoplasmic) fraction. LICOR Odyssey fluorescent molecular weight marker bands are labeled with appropriate molecular weights in kDa.

2a.



2b.

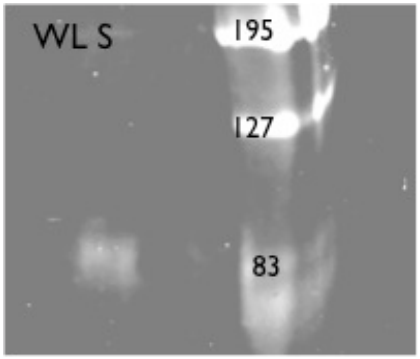


Figure 3. Representative Western Blot for Eyestalk soluble (cytoplasmic/nuclear) fraction. LICOR Odyssey fluorescent molecular weight marker bands are labeled with appropriate molecular weights in kDa.

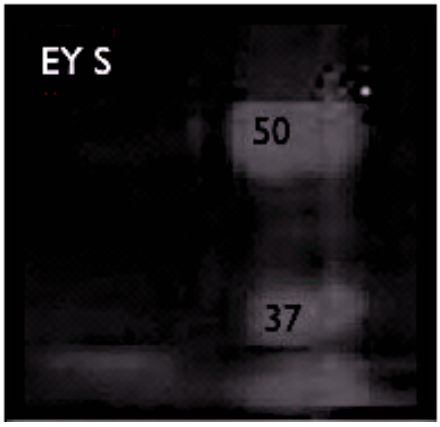
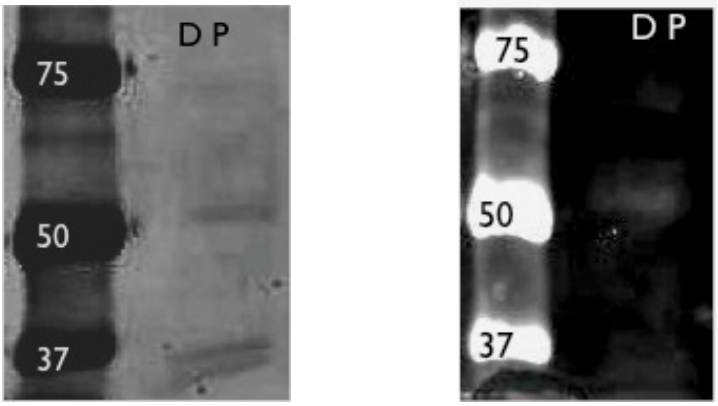

EY S
50
37

Figure 4a. Representative Western blots for Drosophila pellet (membrane) fraction. LICOR Odyssey fluorescent molecular weight marker bands are labeled with appropriate molecular weights in kDa.

Figure 4b. Representative Western blot for Drosophila soluble (cytoplasmic/nuclear) fraction. LICOR Odyssey fluorescent molecular weight marker bands are labeled with appropriate molecular weights in kDa.

4a.



4b.

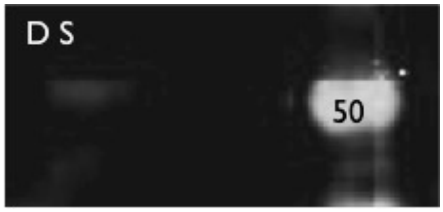
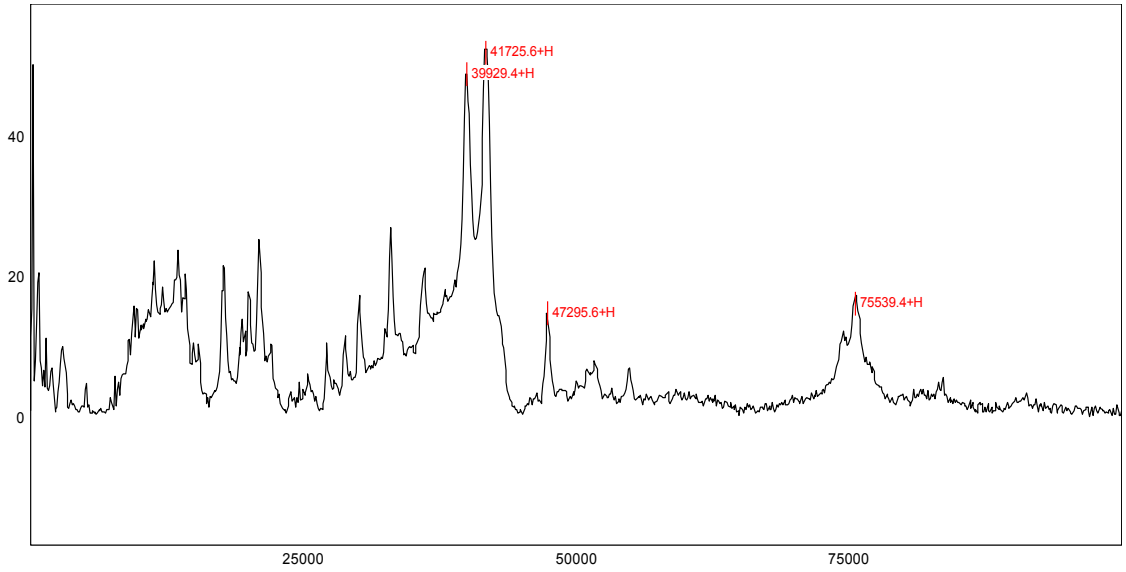


Figure 5a. Mass Spectrometry results for Walking leg pellet (membrane) fraction.

Figure 5b. Mass Spectrometry results for Walking leg soluble (cytoplasmic/nuclear) fraction.

5a.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39929.4+H
47295.6+H
75539.4+H
40
20
0
25000
50000
75000

5b.

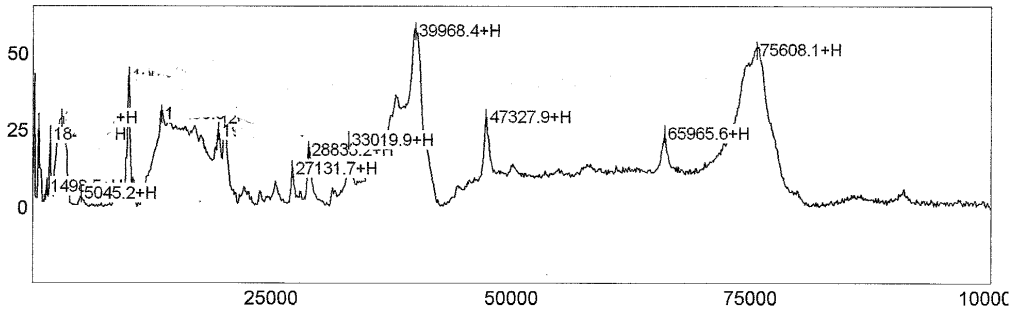
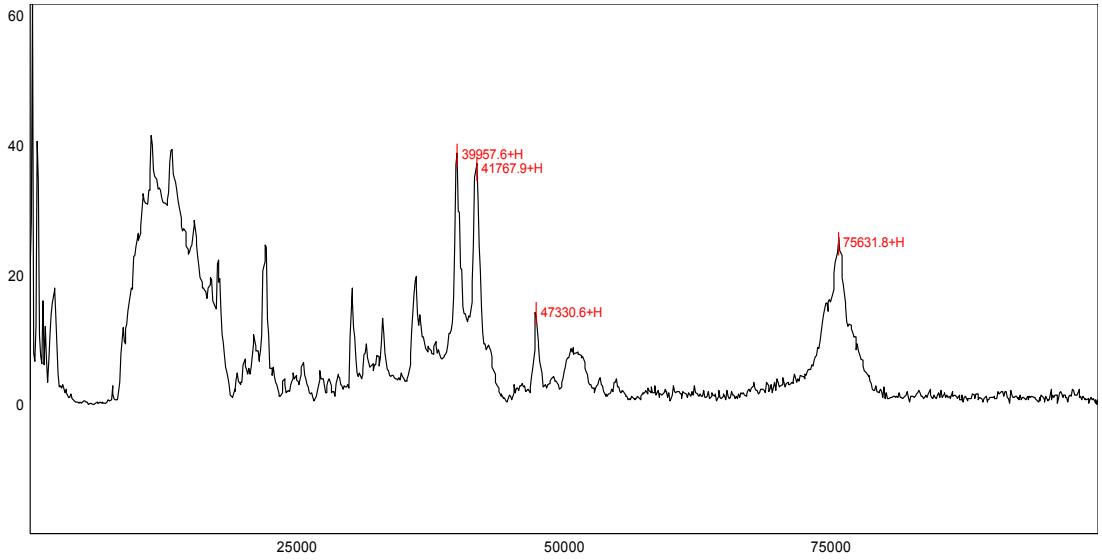

39968.4+H
75608.1+H
47327.9+H
65965.6+H
33019.9+H
27131.7+H
50
25
0
25000
50000
75000
10000

Figure 6a. Mass Spectrometry results for Eyestalk pellet (membrane) fractions.

Figure 6b. Mass Spectrometry results for Eyestalk soluble (cytoplasmic/nuclear) fractions.

6a.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20
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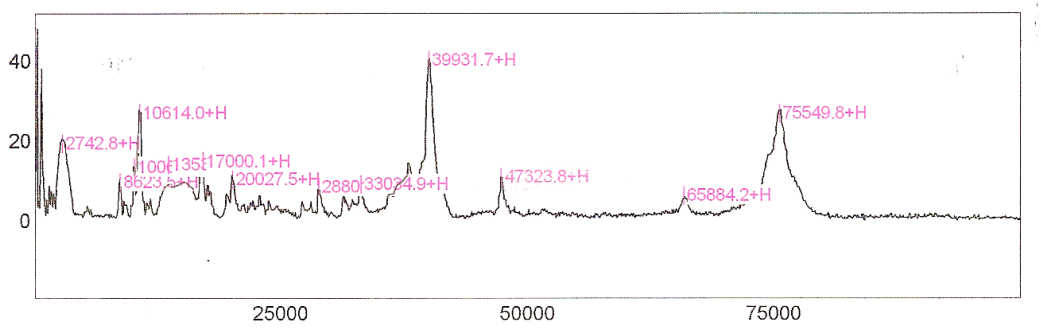
41767.9+H
75631.8+H
47330.6+H
25000
50000
75000

6b.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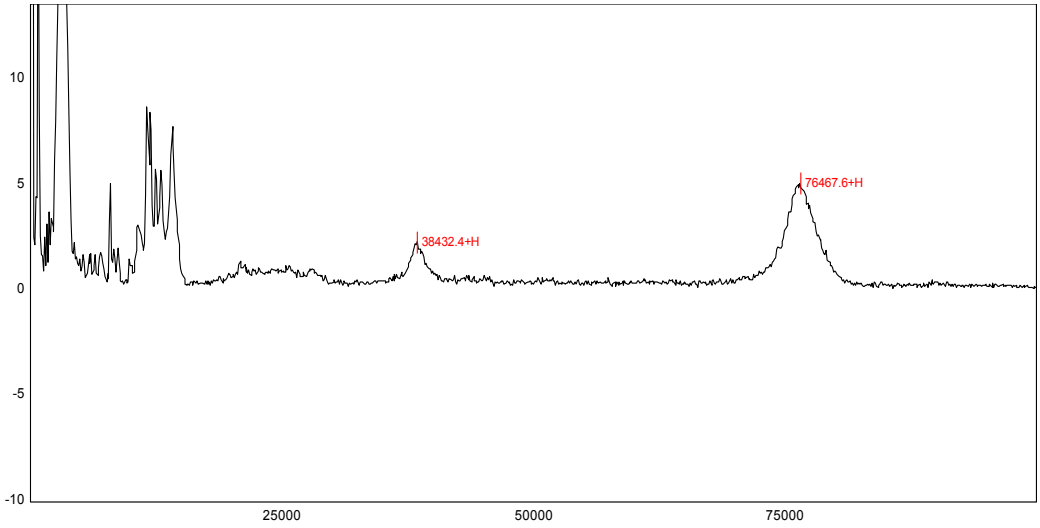
20
0
39931.7+H
10614.0+H
12742.8+H
17000.1+H
20027.5+H
47323.8+H
75549.8+H
65884.2+H
25000
50000
75000

Figure 7a. Mass Spectrometry results for Guard Setae and Aesthetasc sensilla pellet (membrane) combined.

Figure 7b. Mass Spectrometry results for Guard Setae and Aesthetasc sensilla soluble (cytoplasmic/nuclear) combined.

7a.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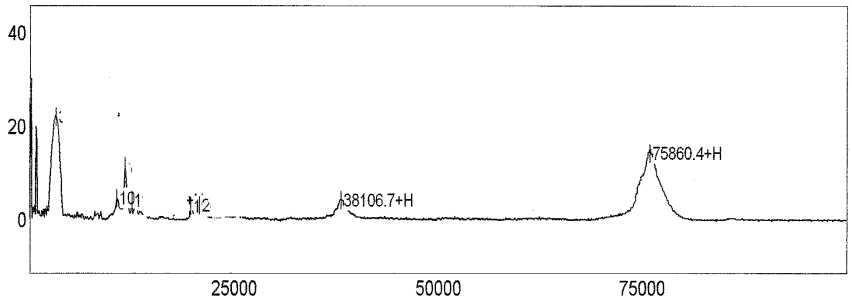
-10
25000
50000
75000
38432.4+H
76467.6+H

7b.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20
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25000
50000
75000
101
112
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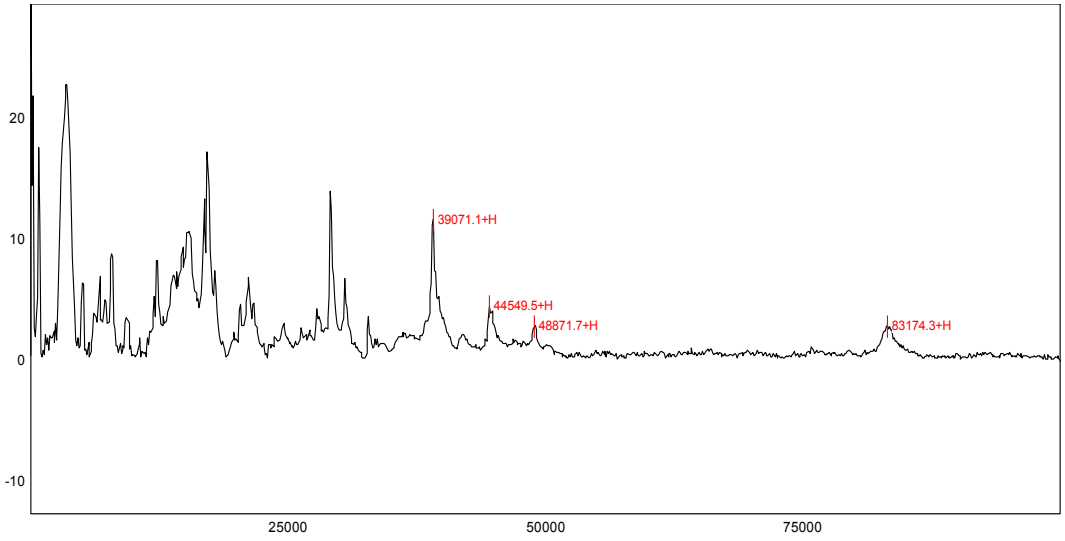
Figure 8a. Mass Spectrometry results for Drosophila pellet (membrane) fraction.

Figure 8b. Mass Spectrometry results for Drosophila soluble (cytoplasmic/nuclear) fraction.

8a.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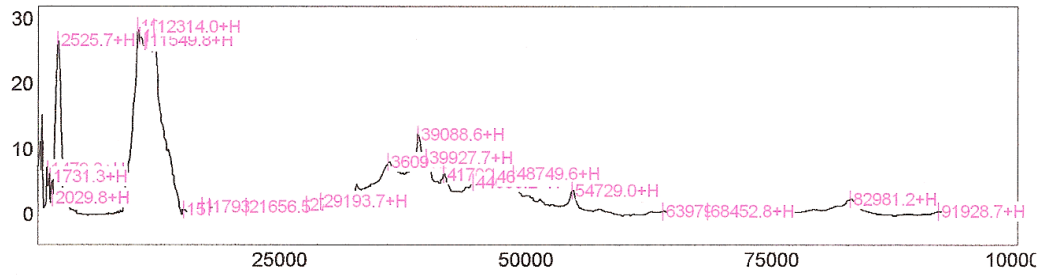
8b.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39927.7+H
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54729.0+H
1731.3+H
2029.8+H
29193.7+H
68452.8+H
82981.2+H
91928.7+H

CHAPTER 4

Effect of injected ecdysteroids on force generation in the claw closer muscle of the American lobster, *Homarus americanus*

by

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is prepared for *Biological Bulletin*

Abstract:

20-hydroxyecdysone (20E), the active principle of the molting hormone, has been shown to affect the outcome of agonistic interactions in lobsters. Lobsters injected with 20E are more aggressive than non-treated animals, and premolt lobsters, which have high circulating levels of 20E, have a higher success rate in agonistic encounters. 20E has been shown to increase the amplitude of excitatory post-synaptic potentials in the claw opener muscle and decrease them in the abdomen. To test whether ecdysteroids also affect the closer muscle, a customized force transducer was constructed to measure the force and duration generated by the closer muscle of male and female lobsters after injection with alpha-ecdysone or 20E. The difference in force and duration before and after injection of 20E or alpha-ecdysone was compared to their differences after injection of saline. Alpha-ecdysone significantly increased the force generated by female crusher and cutter claws, and 20E also potentially increased the force in female crusher claws. The results suggest that circulating ecdysteroids influence the claw closer muscle and could be a factor influencing agonistic interactions.

Introduction:

Agonistic encounters play an important role in the life of lobsters; they are involved in procuring shelters, defending and maintaining those shelters, mating, and foraging success (Atema and Cobb, 1980). Some of the factors affecting the outcomes of agonistic encounters include physical characteristics of the animals, such as weight, carapace size, and chelae size; larger animals weigh more and have greater carapace and chelae size (Scrivener, 1971). In staged encounters, larger lobsters will win significantly more encounters than smaller lobsters of the same sex (Scrivener, 1971). Size also plays a role in the formation of dominance hierarchies in lobsters, as larger lobsters are dominant over smaller lobsters in social settings (Karnofsky, 1989). This is also true in crayfish, where larger animals are dominant over and have more access to food resources than do smaller, subdominant males (Issa *et al*., 1999; Herberholz *et al*., 2007).

During an agonistic interaction, lobsters perform many behaviors with their claws, including grabbing, hitting, pinching and claw locks (Mello *et al*., 1999; Bolingbroke *et al*. 2001; Coglianese *et al*., 2008; Reinhart *et al*., submitted; Sipala *et al*., unpublished). These claw behaviors are important, as the strength or duration of a squeeze/pinch may affect the outcome of the agonistic interaction. On average, male lobsters have larger crusher and cutter claws than female lobsters of the same carapace size (Elner and Campbell, 1981). This is also true in crayfish, where for a given body length, males have larger chelae that generate a greater force than do the chelae of females of the same size (Wilson *et al*., 2009).

Along with body size, hormones have been shown to influence the outcome of agonistic interactions in crustaceans. In lobsters, the hemolymph titers of the molting hormone, 20-hydroxyecdysone (20E), varies over the molt cycle. Premolt animals ($D_1$and D_2 stage animals about to molt) have increased levels of ecdysones in their hemolymph and urine (Chang, 1985; Snyder and Chang, 1991a; Snyder and Chang, 1991b), and are dominant over and more aggressive than their intermolt C-stage opponents in a confrontation (Tamm and Cobb, 1978). Evidence has been presented that injected 20E makes lobsters more aggressive; in staged combats between large and small lobsters, smaller lobsters injected with the hormone are significantly more aggressive than small control animals injected with saline (Bolingbroke and Kass-Simon, 2001). The injection of 20E may mimic the increased 20E titers that occur in D-stage animals about to molt (D1 and D2), which are correlated with increased aggression in D-stage animals (Tamm and Cobb, 1978).

One reason circulating 20E may affect the outcome of an agonistic interaction has to do with its effect on neuromuscular physiology. 20-hydroxyecdysone has been shown to affect the neuromuscular electrophysiology of the claw and abdomen in lobsters (Cromarty and Kass-Simon, 1998), as well as alter neurotransmitter release in crayfish (Cooper and Ruffner, 1998; Cooper *et al*., 2003). 20E decreases the probability of vesicular neurotransmitter release in the walking legs (Cooper and Ruffner, 1998), and a mixture of 20E and serotonin increased neuron firing frequency in the slow-adapting muscle receptor organ (MRO) of the abdomen (Cooper *et al*., 2003). In the claw opener muscle of lobsters, animals in premolt stage D produce significantly larger excitatory junctional potentials (EJP's) and significantly fewer

inhibitory junctional potentials (IJP's) than intermolt animals (Schwanke *et al.*, 1990). In the presence of 20E (which is present in greater quantities in the hemolymph of premolt animals), there is an increase in EJP amplitudes and frequency of miniature excitatory junctional potentials (MEJP) in the opener muscle (used in threat displays) (Cromarty and Kass-Simon, 1998). There is also a decrease in EJP amplitudes in the abdomen (used in the escape response) (Cromarty and Kass-Simon, 1998), which corresponds to the effect of pre-molt and post-molt hemolymph on EJPs (Schwanke *et al*., 1990).

Ecdysteroids may also have differential effects on the neuromuscular properties of claw closer muscle of crusher and cutter claws, as the two claws differ in their muscle fiber types, motoneuron innervation and contractile properties (Govind, 1984). Fast twitch fibers quickly reach a maximal tension, which rapidly decays, while slow twitch fibers gradually increase in tension with a slow decay phase (Jahromi and Atwood, 1971; Costello and Govind, 1983). Crusher claw closer muscles contain only slow twitch fibers with long sarcomeres, which allows the crusher to maintain force for a long period of time (Lang *et al*., 1977; Govind and Lang, 1978; Kent and Govind, 1981; Govind, 1984). Cutter claws contain mostly fast twitch fibers with short sarcomeres and a small ventral band of slow twitch fibers, which leads to a quicker fatigue than crusher muscle (Lang *et al*., 1977; Govind and Lang, 1978; Kent and Govind, 1981; Govind, 1984). The closer muscle in both crusher and cutter claws are innervated by the fast closer excitor neuron (FCE) and the slow closer excitor neuron (SCE), however, cutter closer muscles have mostly FCE while crushers have a mixture of both (Wiersma, 1955; Costello *et al*., 1981; Govind,

1984). Generally, SCE synapses are more fatigue resistant and recover more quickly than FCE synapses, but the synapses at both the FCE and SCE in crusher claws are more resistant to fatigue than those in the cutter (Govind and Lang, 1974; Govind and Lang 1979).

The purpose of this study was to determine whether alpha-ecdysone and its active principle, 20E, alter the squeezing properties of the crusher and cutter closer muscle in male and female lobsters.

Methods:

Animal procurement and maintenance:

Male and female American lobsters, *Homarus americanus*, were obtained from local fisherman and the Rhode Island Department of Environmental Management from inshore waters off the coast of Narragansett Bay, RI. Animals were maintained in natural circulating unfiltered seawater tanks at the Narragansett Bay Campus, on a 12-hr light/dark cycle. Water temperature and salinity were ambient, ranging from 10-20°C and 28-33ppt, respectively. Animals were fed fish scraps, supplied by a local fish market, twice weekly. All lobsters used were intermolt C-stage animals in perfect condition, i.e., all eight walking legs, claws, antennae and antennules were intact, with no other signs of physical damage or shell disease. The tanks were compartmentalized, so animals could not physically interact with each other prior to use. After animals were weighed and measured, one claw was chosen to be the test claw and left unbanned for the entirety of the experiment. This allowed the claw to have free movement prior to the testing period in order to prevent atrophy of the claw. The other claw was banded for the entirety of the experiment, which ensured that the unbanned claw was the only claw that could grab the force transducer used to measure force.

Preparation of test substances and injection protocol:

In order to prevent bias during data acquisition and analysis, one of us number coded stock aliquots of 20E, alpha-ecdysone and saline. Each number was then assigned to a given sex and claw type, ensuring that male and female cutters and crushers were allotted equal numbers of 20E, alpha-ecdysone and saline. The

following experiments were performed: Male cutter and crusher with 20E, alpha-ecdysone, and saline and female cutter and crusher with 20E, alpha-ecdysone, and saline.

20E and alpha-ecdysone aliquots were made at a concentration of 1mg/ml, and frozen at –80°C. Saline aliquots had a composition in (mM/L) of: NaCl 472; KCl 10; $MgCL_2*6H_2O$ 7; $CaCl_2$ 16; glucose 11; Tris-maleate 10; pH 7.4 (Meiss and Govind, 1979) and were frozen at –80°C. Experimental animals were injected with enough 20E or alpha-ecdysone to result in a final hemolymph concentration inside the body of 600 ng/ml. The volume of stock solutions injected was that which was estimated on the basis of the animals weight to result in a final hemolymph concentration of 600ng/ml. This weight/volume estimate was generated by Bolingbroke and Kass-Simon (2001), by measuring the hemolymph volume bled from lobsters of known weights and fitted to a linear curve, having the values: $y = 0.26x – 54.33$, where y is the hemolymph volume and x is the weight of the animal. The volume estimated from the equation was then used in a ratio to determine the amount of 20E stock solution needed to be injected in order to obtain a final concentration of 600ng/mL. The ratio used was: 0.0006mg/1mL = X mg/hemolymph volume of interest (y from the equation). The amount of saline injected in the control animals was calculated the same way. A Dremel electric drill was used to drill a small hole through the outer portion of the carapace above the presumed level of the cardium through which a 20-gauge needle could be inserted into the remaining carapace layer. After injection, the hole was plugged with dental wax to prevent bleeding.

Force Measurements:

In order to measure the force generated by the claw, a customized force transducer with strain gauges leading to a customized Wheatstone bridge was constructed similar to that described by Wilson *et al*. (2009). The transducer consisted of six metal mending braces (Stanley zinc plated 3 in. X 5/8 in.) stacked on top of each other, with the top and bottom braces offset from the other four to create a squeezing area for the claw. The mending braces were held together with a screw and bolt to prevent movement or breaking when the lobster squeezed. Strain gauges (Omega Precision Linear, 1000 Ohm resistance) were glued with epoxy onto the underside of each offset mending brace and wires were soldered onto the leads of the strain gauge, and plugged into the Wheatstone bridge. In order to waterproof the transducer, shrink tubing was placed over every exposed wire connection point, and epoxy was layered over the strain gauges and shrink tubing to create a tight seal. The Wheatstone bridge was connected to a Power Lab (ADInstruments), which recorded the deflections in each strain gauge. The transducer was calibrated by hanging known weights from each squeezing surface to determine the change in voltage for each weight. These results were graphed and a best-fit line and equation were generated. Since there were two strain gauges attached to each offset mending brace, the total force for one squeeze was obtained by adding the force from each strain gauge. A sample Power Lab recording is provided (Figure 1).

Experimental protocol:

The squeeze injection protocol consisted of an initial pre-squeeze followed by 4 injections of 20E, alpha-ecdysone or saline 12 hours apart. The post-squeeze was

taken 12 hours after the fourth injection. All pre- and post-squeezes were taken after 7PM.

A total of three pre-squeezes and three post-squeezes were recorded from each subject. During the acquisition of squeezes, lobsters were placed into 10-gallon opaque Tupperware bins filled with approximately nine gallons of water and allowed to acclimate for one hour. All experiments were performed under red light to mimic nighttime conditions. The squeezing protocol was as follows: the transducer was slowly lowered into the water and the lobster was allowed to grab and release the transducer. Once the lobster released the transducer, the transducer was slowly removed from the bin and re-introduced for the second squeeze one minute after the first squeeze ended. The third squeeze was recorded one minute after the second squeeze ended. In some instances, three squeezes could not be elicited from a lobster, or some squeezes were not forceful enough to be picked up by the force transducer. The transducer was only presented three times to each lobster for a maximum of one minute each, with one minute in between each presentation. Only measurable squeezes were used for analysis, any instances where no measurable squeezes were elicited were thrown out. Because several trials had to be discarded, the following sample sizes for each experiment were analyzed: N=9 (FM 20E cutter, FM alpha cutter, FM saline cutter, M 20E cutter), N=8 (FM 20E crusher, FM alpha crusher, FM saline crusher, M saline cutter, M saline crusher), N=7 (M 20E crusher, M alpha cutter) and N=6 (M alpha crusher).

Squeezes were analyzed for force and duration using the Power Lab analysis tools. Force was determined by measuring the maximum amplitude of each squeeze,

and duration was determined by measuring the total length of each squeeze. The difference between the maximum of the three pre-squeeze values and the maximum of the three post-squeeze values were calculated and compared for each treatment in each claw category (i.e. male cutters, male crushers, female cutters, female crushers). The average difference (Post-Pre) for each test were compared with Student's t-tests. Values were considered significantly different at $P \leq 0.05$ and potentially significant (trend) at $0.07 \leq P < 0.05$.

Results:

In female crusher claws, the average difference between post and pre-squeezes for animals injected with alpha-ecdysone (71.4 N) was greater than the average difference between post and pre-squeezes for those treated with saline (-55.5 N) (Student's t-test, P=0.009) (Figure 2). In female cutter claws, the average difference between post and pre-squeezes for animals treated with alpha-ecdysone (18.1 N) was greater than the average difference between post and pre-squeezes for those treated with saline (-17.7 N) (Student's t-test, P=0.024) (Figure 3). Potential significance was found in the force of female crusher claws treated with 20E compared to saline (109.2 N vs. -55.5 N, Student's t-test, p=0.07). No other significant differences were found in crusher or cutter claws for any other parameter measured in males or females.

An analysis was made of all the pre-squeezes for all animals (control, before hormone injection) to compare the force and duration generated by crusher and cutter claws for males and females. The average maximum force generated by crusher claws (93.9 Newtons) was greater than that by cutter claws (46.7 Newtons) (Student's t-test, p=0.02), while no differences were found between maximum duration of the squeeze by crusher and cutter claws. Also, there were no sex differences in the average force or duration from cutter or crusher claws when all pre-squeezes were analyzed. The average force generated by male (43.1 N) and female (49.9 N) cutter claws were not significantly different (Student's t-test, P= 0.3), nor were the average force generated by male (93.1 N) and female (93.7 N) crusher claws (Student's t-test, P= 0.5).

Discussion:

Our results suggest alpha ecdysone causes a significant increase in the maximum force produced in female cutter and crusher claws. This increase in force caused by alpha-ecdysone may contribute to the fact that lobsters are more successful in agonistic interactions immediately prior to the molt. Levels of alpha-ecdysone and 20E spike immediately prior to molt (Chang, 1985; Snyder and Chang, 1991a; Snyder and Chang, 1991b) so increased levels of alpha-ecdysone may increase the force generated by claws, which could help the lobster gain an advantage in a fight. If a lobster is able to squeeze with a stronger force than its opponent, then the opponent may be able to sense that strength and withdraw from the confrontation. Intermolt lobsters, who have lower levels of circulating alpha-ecdysone, may produce a lower closing force than premolt lobsters, which could be a reason they lose more encounters with premolt lobsters. Since claws are an important factor in the outcome of an agonistic interaction, anything that changes the mechanisms of the claw could affect the agonistic interaction itself.

No differences were found in male cutter or crusher claws for force or duration with 20E or alpha-ecdysone. The fact that no differences were found in male lobsters with either alpha-ecdysone or 20E suggests that ecdysteroids have differential effects on the closer muscle in males and females. However, Cromarty *et al*. (1998) showed that 20E has a significant effect on the claw opener and abdominal flexor muscles in male lobsters. Given that 20E affects the opener and abdominal flexor muscles in males, we expected the closer muscle to respond to 20E in males and females. There were no changes in the closing force among male claws, but there was a potentially

significant response in females (see below). It is possible that differential sexual responses occur in the claw closer muscle, as differential responses to ecdysteroid exposure have been shown in previous studies (Coglianese *et al*., 2008; Reinhart *et al*., submitted). Coglianese *et al*. (2008) puffed 20E across the antennules of female lobsters engaged in an agonistic interaction, and Reinhart *et al*. (submitted) performed the same experiment with male lobsters. Females responded to 20E exposure by becoming more aroused, increasing aggressive, defensive and avoidance behaviors, whereas males simply increased the frequency of defensive behaviors. It is possible that a similar sex-dependent differential response to ecdysteroids may exist in the claw closer muscle.

One reason our results with 20E did not prove to be significant could be due to the high variance in our data. In female crushers injected with 20E, the average difference between the post and pre-squeezes was 109.2 N, which was not significant at the 5% level when compared to saline (-55.5N), although a trend or potential significance at the 7% level was demonstrated (Student's t-test, P=0.07). However, the mean difference in 20E was actually greater than the difference between alpha-ecdysone (96.9N) and saline (-55.5N), which was significant. The standard error in crusher claws treated with 20E was ±96.9 N, while the standard error in crusher claws treated with alpha was only ±39.8N. One cause of the large variance was the fact that there were instances where lobsters did not squeeze the transducer at all or squeezed with such low force that it did not register on the transducer. Further, the fact that differences in the crusher claw of females were potentially significant but that

differences in the cutter claw were not could be attributed to the different muscle fiber types and motoneuron innervation in crusher and cutter claws, describe earlier.

A second reason that the effects of 20E were not significant may be due to metabolic considerations. Since we injected 20E into the abdomen, but tested the effects at the periphery, it is possible the injected 20E became degraded or metabolized before it reached its target area. In contrast, because alpha-ecdysone is converted into 20E in the peripheral tissues by 20-hydroxylase (Mykles, 2010), this conversion within the target tissue would result in an exposure to 20E in the muscle. It is possible that the alpha-ecdysone we injected made its way into the peripheral tissues and was converted into 20E, and once converted, it may be the 20E that is having the effect on the closer muscle, and not alpha-ecdysone. In this regard, we have reported as unpublished observations in an earlier study that we were unable to show that alpha-ecdysone caused a change in the neuromuscular properties of the claw or abdomen (Schwanke *et al*., 1990). It is also possible that the injected 20E was excreted so that its concentration would have been too low to have an effect on the claw closer muscle. Cromarty *et al*. (1998) perfused 20E directly over the neuro-muscular preparation, so there was no metabolism or excretion of 20E before it could have an effect, unlike our injections.

Although our data had a high variance, analysis of all pre-squeeze data revealed similar patterns and trends for force observed in other crustaceans. In crustaceans, in general, larger animals have larger claws that generate greater force than smaller claws on smaller animals (Brown *et al*., 1979; Elner and Campbell, 1981; Lee, 1993; Gabbanini *et al*., 1995; Vye *et al*., 1997; Wilson *et al*., 2009). In lobsters,

Vye *et al.* (1997) found a general increase in contraction force with an increase in claw dimensions, although results in that study were also highly variable. Another characteristic of crustaceans is that crusher claws, or major chelae, generate a greater overall force than cutter, or minor, chelae (Brown *et al*., 1979; Elner and Campbell, 1981; Govind and Blundon, 1985; Vye *et al*., 1997). Our data indicates that crusher claws generate more force than cutter claws, on average; crusher claws have many slow twitch fibers with long sarcomeres, while cutter claws have fast twitch fibers with short sarcomeres (Lang et al., 1977; Govind and Lang, 1978; Kent and Govind, 1981; Govind, 1984). Finally, Elner and Campbell (1981) did not find any significant sexual differences in the force generated by the claws, a finding that is corroborated by our data.

In summary, alpha-ecdysone causes a significant increase in the force of crusher and cutter claws of female American lobsters, and a potentially significant increase in the force of female crusher claws treated with 20E, suggesting that circulating ecdysteroids could be a factor that leads to the success of premolt animals in agonistic interactions.

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Figure 1. Sample Power Lab recording.

Each window represent the force exerted on one of the two strain gauges. The force from each window was added together to obtain the overall force of the squeeze.

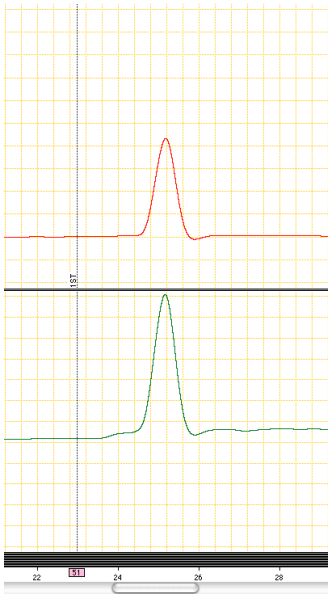

1ST
22
51
24
26
28

Figure 2. Average difference in force in female crusher claws.

Values are means $\pm$ SEM, N = 8 for each treatment. Asterisk indicates significant difference from saline (P = 0.009).

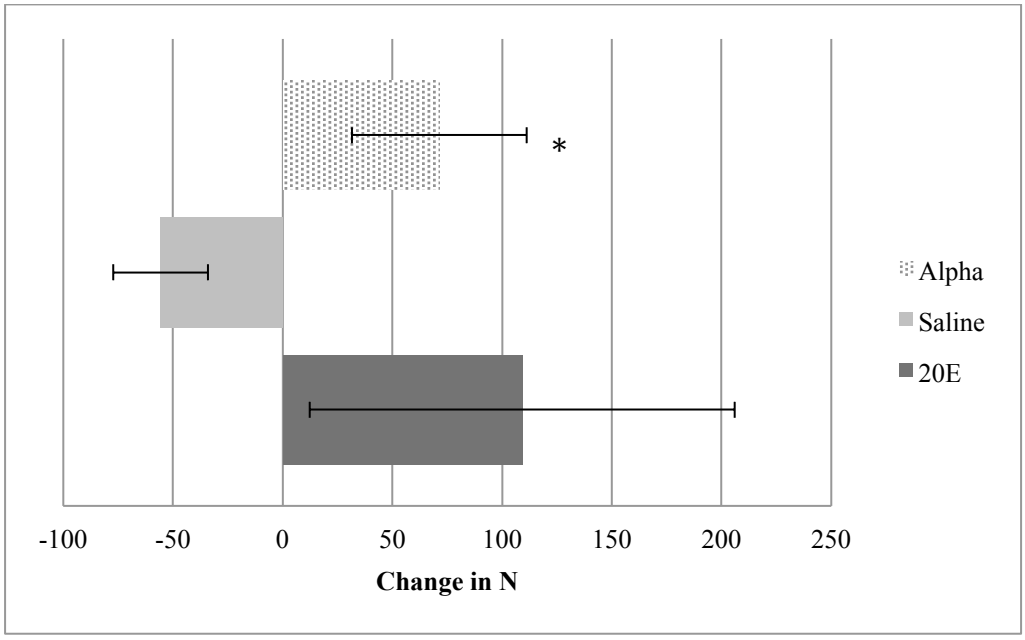
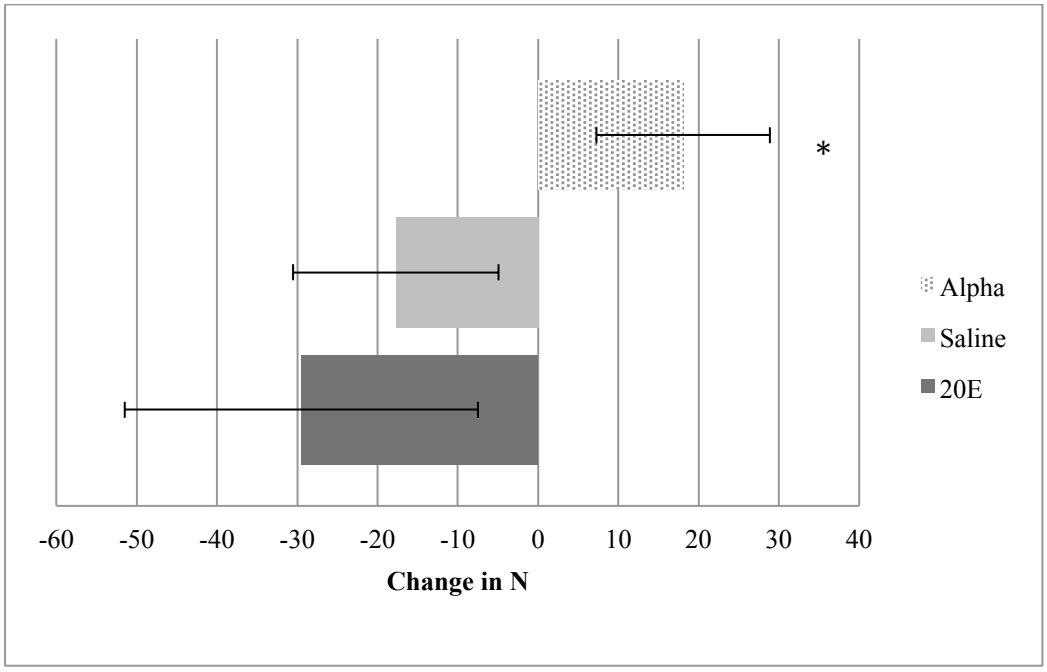

*
Alpha
Saline
20E
-100
-50
0
50
100
150
200
250
Change in N

Figure 3. Average difference in force in female cutter claws.

Values are means $\pm$ SEM, N = 9 per treatment. Asterisk indicates significant difference from saline (P = 0.02).


*
Alpha
Saline
20E
-60
-50
-40
-30
-20
-10
0
10
20
30
40
Change in N

APPENDIX A

Ancillary material for Chapter 2:

Effects of injected 20-hydroxyecdysone on the agonistic behavior of American lobsters, *Homarus americanus*

Summary Tables

Table 1. Summary of Aggressive behaviors.

Values are mean ± SEM. Abbreviations: F=Frequency,

RF = Rank Frequency, AR = Average Rank.

Aggressive Behaviors

		C	T	P-value
F	**Wall**	183.4±26.4	215.2±28.0	0.42
	No Wall	160.7±22.4	188.8±26.1	0.42
RF	**Wall**	21834.6±3167.2	25633.4±3394.9	0.42
	No Wall	19739±2822.25	23195±3259.0	0.43
AR	**Wall**	119.1±1.1	118.8±1.1	0.88
	No Wall	122.5±0.96	122.4±0.58	0.97
		OC	**OT**	**P-value**
F	**Wall**	242.4±30.6	225.6±31.3	0.71
	No Wall	198.4±27.5	201.7±24.8	0.93
RF	**Wall**	28759±3747.0	27373.4±3709.4	0.8
	No Wall	24684.6±3525.5	25155±3107.2	0.92
AR	**Wall**	118.3±1.5	121.7±0.8	0.06
	No Wall	123.9±0.6	124.7±0.4	0.32
		OC+C	**OT+T**	**P-value**
F	**Wall**	425.8±32.9	440.8±19.9	0.7
	No Wall	359.1±33.2	390.5±19.0	0.42
RF	**Wall**	50593.6±4225.9	53006.8±2449.7	0.63
	No Wall	44423.6±4313.5	48349.1±2427.6	0.44
AR	**Wall**	118.4±1.2	120.2±0.9	0.26
	No Wall	123.3±0.70	123.7±0.41	0.6
		OC-C	**OT-T**	**P-value**
F	**Wall**	59±46.7	10.4±55.9	0.51
	No Wall	37.7±37.7	12.9±47.2	0.69
RF	**Wall**	6897.4±5504.2	1740±6676.1	0.56
	No Wall	4945.6±4709.8	1960±5887.3	0.7
AR	**Wall**	117.8±3.1	118.8±3.9	0.85
	No Wall	123.1±2.2	120.7±3.3	0.55

Table 2. Summary of Defensive Behaviors.

Values are mean ± SEM. Abbreviations: F=Frequency,

RF = Rank Frequency, AR = Average Rank.

Defensive Behaviors

		C	T	P-value
F	**Wall**	51.3±5.5	58.7±9.5	0.51
	No Wall	42.8±4.2	53.2±8.1	0.27
RF	**Wall**	2765±348.8	2841.6±499.6	0.9
	No Wall	1968.8±185.2	2427±357.0	0.27
AR	**Wall**	50.2±1.6	48.2±1.2	0.33
	No Wall	46.5±1.1	45.9±0.54	0.68

		OC	OT	P-value
F	**Wall**	47.8±7.7	52.8±10.1	0.7
	No Wall	45±7.9	48.6±9.7	0.78
RF	**Wall**	2303.6±353.2	2521.8±459.1	0.71
	No Wall	2097.8±352.4	2218.4±415.5	0.83
AR	**Wall**	48.4±1.2	48.0±1.3	0.83
	No Wall	46.9±0.6	46.3±1.1	0.59

		OC+C	OT+T	P-value
F	**Wall**	99.1±9.0	111.5±15.8	0.51
	No Wall	87.8±9.0	101.8±14.2	0.42
RF	**Wall**	4884.4±422.5	5363.4±775.7	0.59
	No Wall	4067.6±402.0	4646.2±616.1	0.44
AR	**Wall**	49.5±1.1	48.2±1.1	0.41
	No Wall	46.53±0.7	46.0±0.7	0.62

		OC-C	OT-T	P-value
F	**Wall**	-3.5±9.9	-5.9±11.6	0.88
	No Wall	2.2±8.8	-4.6±10.7	0.63
RF	**Wall**	-277.2±504.4	-319.8±564.9	0.96
	No Wall	128±394.2	-209.4±469.8	0.59
AR	**Wall**	60.7±7.2	41.8±4.5	0.04
	No Wall	26.0±7.5	42.2±2.8	0.06

Table 3. Summary of Avoidance behaviors

Values are mean ± SEM. Abbreviations: F=Frequency,

RF = Rank Frequency, AR = Average Rank.

Avoidance Behaviors

		C	T	P-value
F	**Wall**	59.6±8.7	54.4±4.7	0.61
RF	**Wall**	780.2±81.0	676±43.7	0.27
AR	**Wall**	13.8±0.7	12.8±0.6	0.27

		OC	OT	P-value
F	**Wall**	47.9±2.1	53.9±5.4	0.32
RF	**Wall**	660.2±30.2	738.6±72.3	0.33
AR	**Wall**	13.8±0.4	13.8±0.4	0.96
		OC+C	**OT+T**	**P-value**
F	**Wall**	107.5±7.2	108.3±7.3	0.94
RF	**Wall**	1441.2±82.1	1414±84.7	0.82
AR	**Wall**	13.5±0.4	13.2±0.4	0.56

		OC-C	OT-T	P-value
F	**Wall**	-11.7±10.4	-0.5±7.1	0.39
RF	**Wall**	-120±90.8	62.6±84.3	0.16
AR	**Wall**	14.8±6.4	5.9±4.3	0.26

Table 4. Summary of All behaviors

Values are mean ± SEM. Abbreviations: F=Frequency,

RF = Rank Frequency, AR = Average Rank.

All Behaviors

		C	T	P-value
F	**Wall**	294.3± 25.8	328.3± 29.9	0.4
	No Wall	263±22.7	296.4±29.7	0.38
RF	**Wall**	25195.6± 3083.9	10959.1±3465.6	0.41
	No Wall	22480.6±2811.2	26298.8±3441.1	0.4
AR	**Wall**	84.6±4.4	86.4±4.1	0.76
	No Wall	84.3±4.8	86.0±4.4	0.79

		OC	OT	P-value
F	**Wall**	338.1±30.9	332.3±34.3	0.9
	No Wall	291.2±29.7	304.2±27.6	0.75
RF	**Wall**	31722.8±3764.5	30633.8±3664.5	0.84
	No Wall	25975.8±3860.4	28112±3025.2	0.67
AR	**Wall**	91.4±3.3	91.4±3.1	0.99
	No Wall	86.8±5.0	91.7±3.2	0.42

		OC+C	OT+T	P-value
F	**Wall**	632.4±43.1	660.6±36.9	0.63
	No Wall	554.2±43.4	600.6±33.4	0.41
RF	**Wall**	56911.2±4577.5	59784.8±3162.5	0.61
	No Wall	49914.8±4695.0	54410.8±2938.1	0.43
AR	**Wall**	89.5±1.5	89.6±1.8	0.97
	No Wall	89.3±1.8	90.7±1.4	0.52

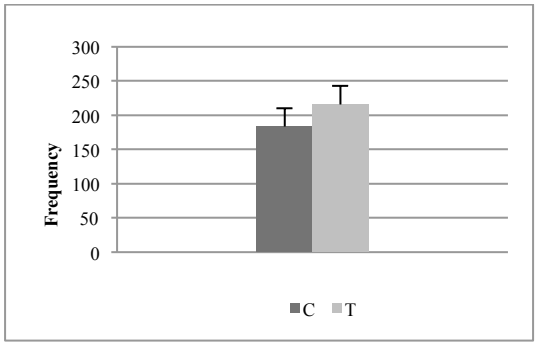
		OC-C	OT-T	P-value
F	**Wall**	43.8±37.3	13.1±51.1	0.63
	No Wall	28.2±30.1	7.8±46.7	0.72
RF	**Wall**	6527.2±5138.4	2354±6223.9	0.61
	No Wall	4953.6±4471.8	1833.2±5774.4	0.67
AR	**Wall**	137.7±14.0	108.9±54.4	0.61
	No Wall	162.3±32.2	52.9±149.1	0.48

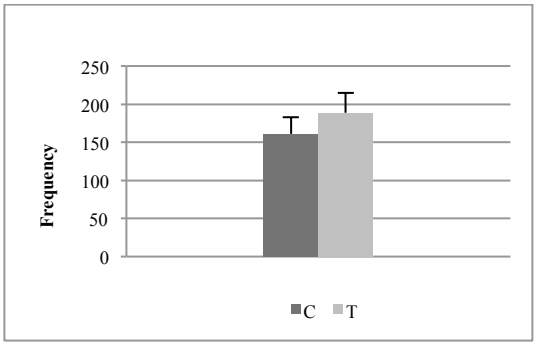
Figures

Figure 1. Aggressive Behaviors of C vs. T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average Rank, with and without wall behaviors. Values are mean ± SEM, N = 10.

With Wall Without Wall

1a.





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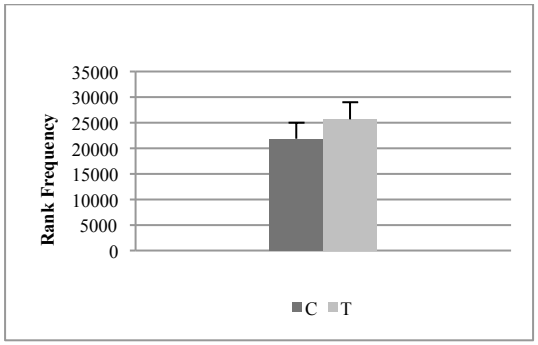

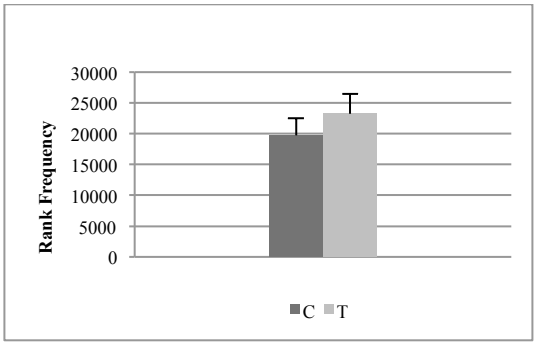

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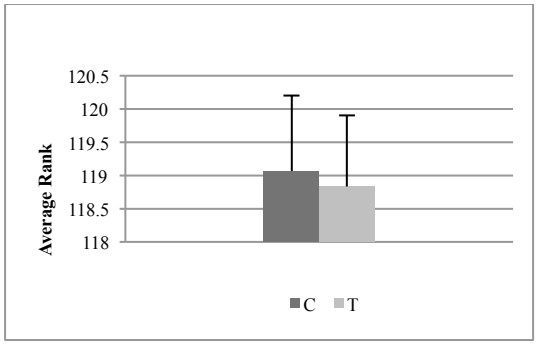


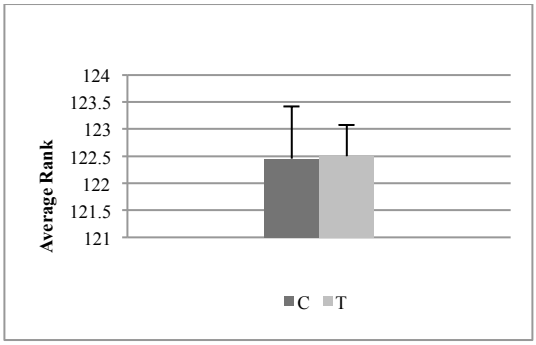
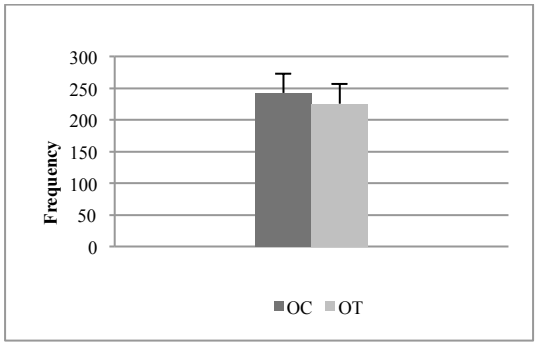
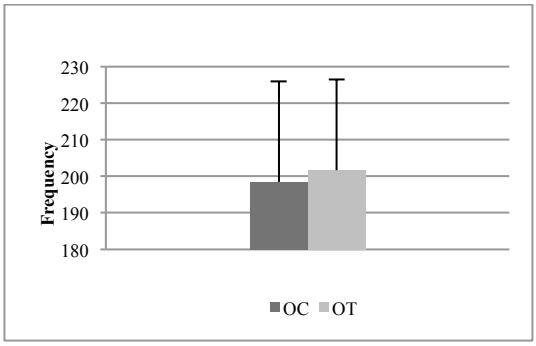


Figure 2. Aggressive Behaviors of OC vs. OT for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean $\pm$ SEM, N = 10.

With Wall Without Wall

2a.





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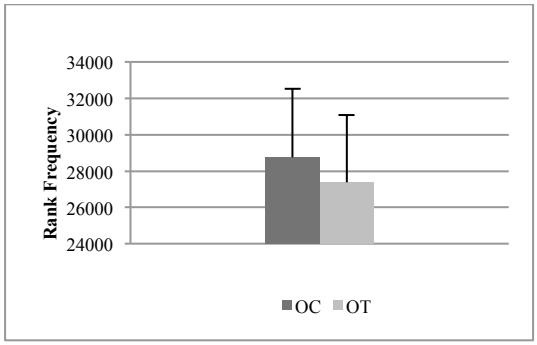

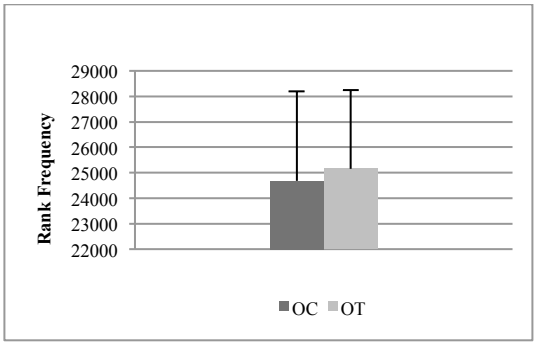

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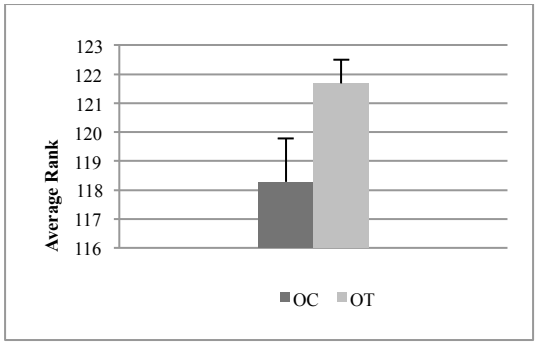


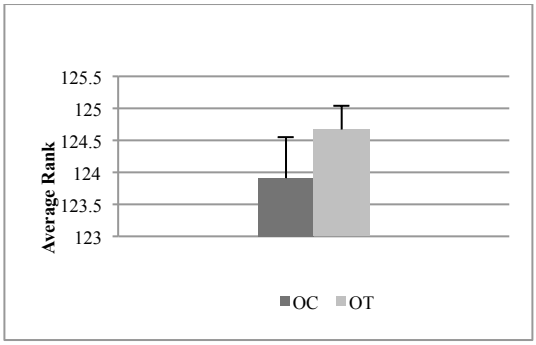
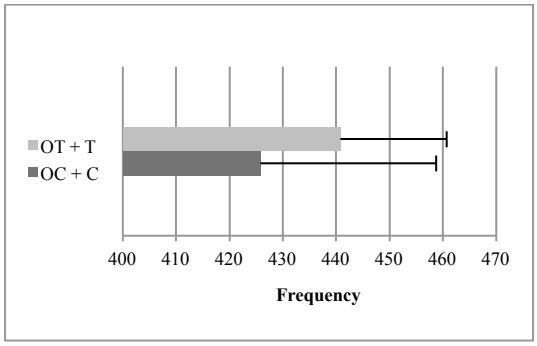
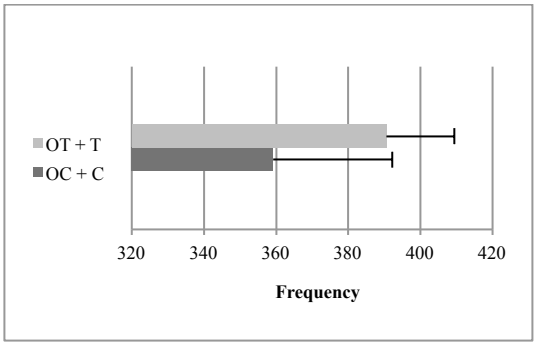


Figure 3. Aggressive Behaviors of OC + C vs. OT + T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean $\pm$ SEM, N = 10.

With Wall Without Wall

3a.





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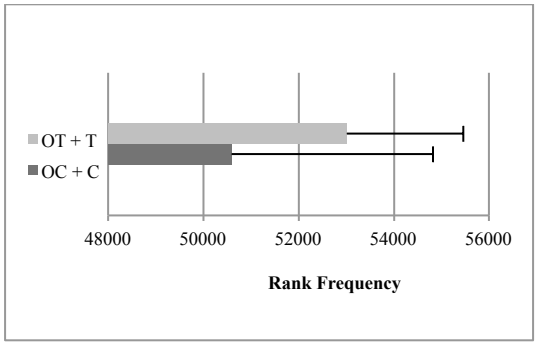

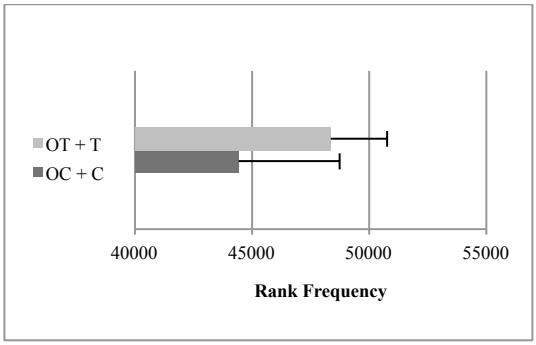

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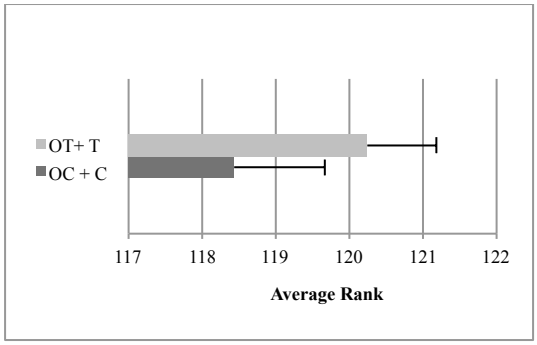


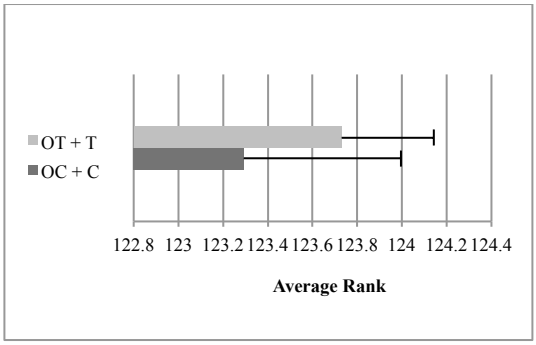
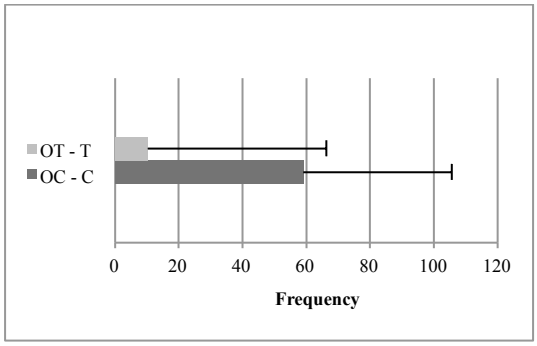
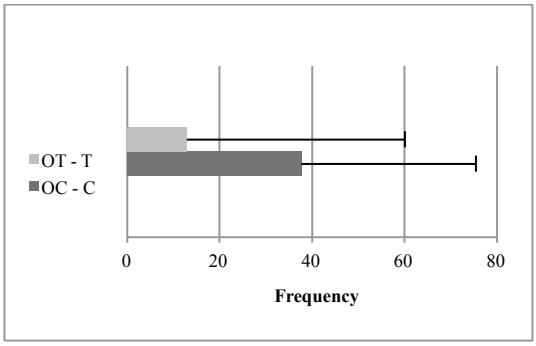


Figure 4. Aggressive Behaviors of OC - C vs. OT - T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean $\pm$ SEM, N = 10.

With Wall Without Wall

4a.





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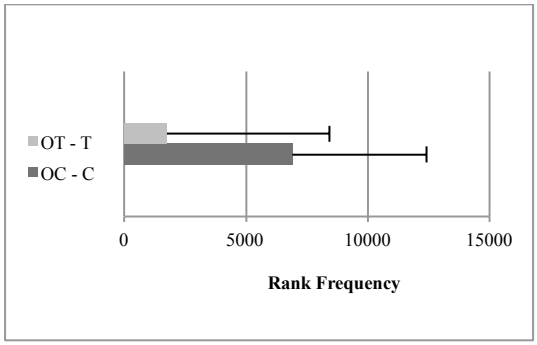

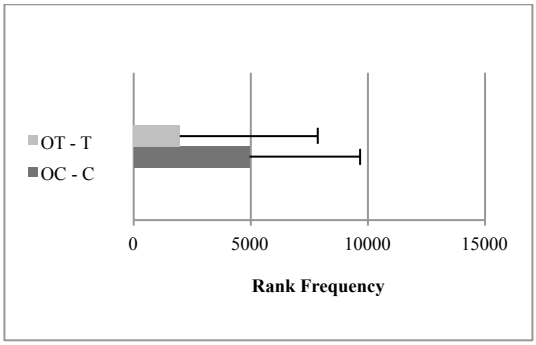

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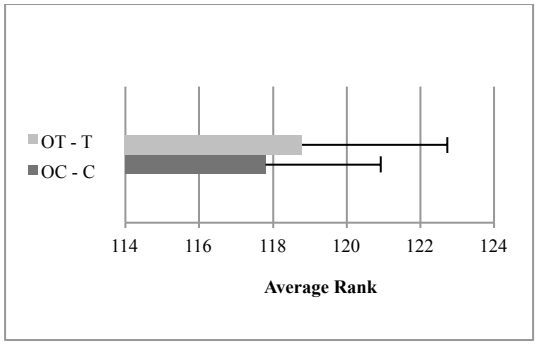


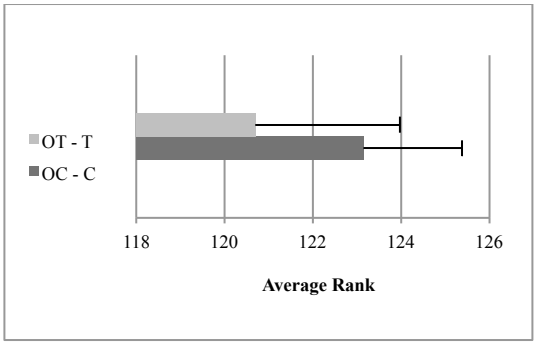
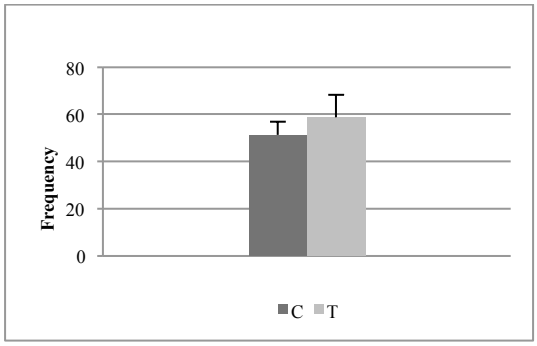
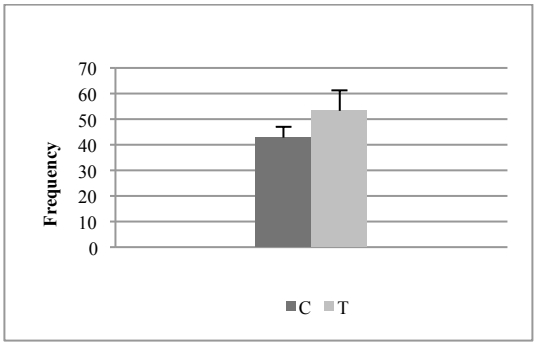


Figure 5. Defensive Behaviors of C vs. T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean $\pm$ SEM, N = 10.

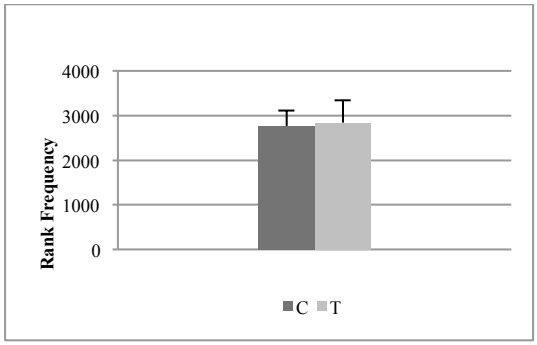
With Wall Without Wall

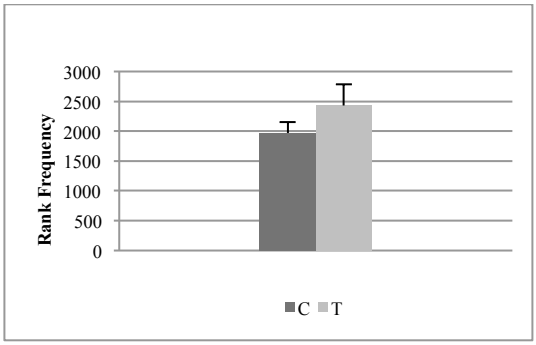
5a.



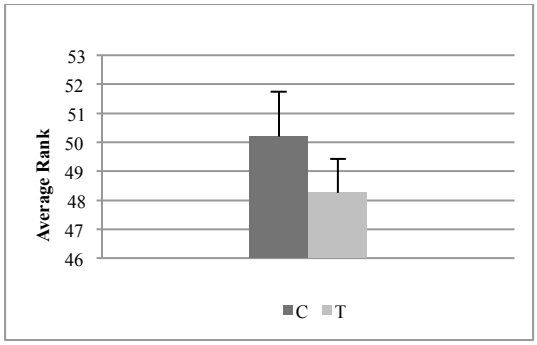


5b.





5c.



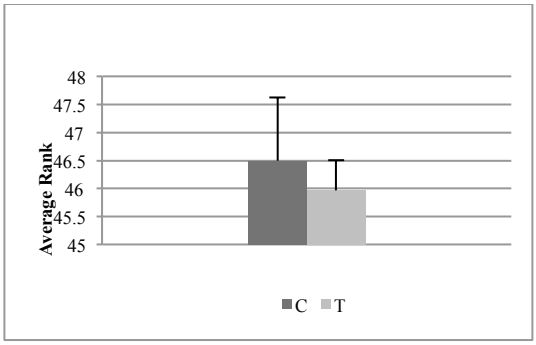
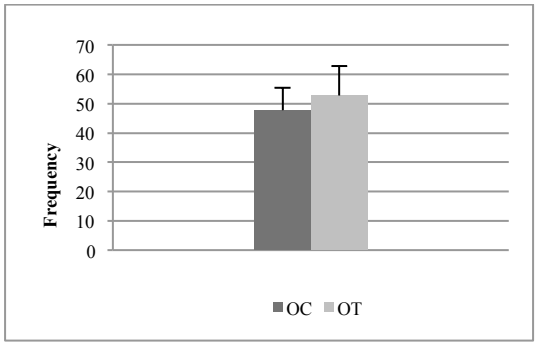
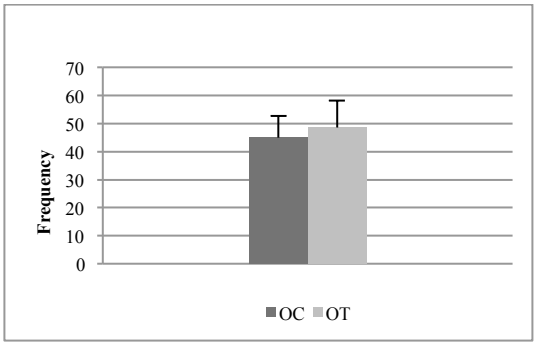


Figure 6. Defensive Behaviors of OC vs. OT for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean $\pm$ SEM, N = 10.

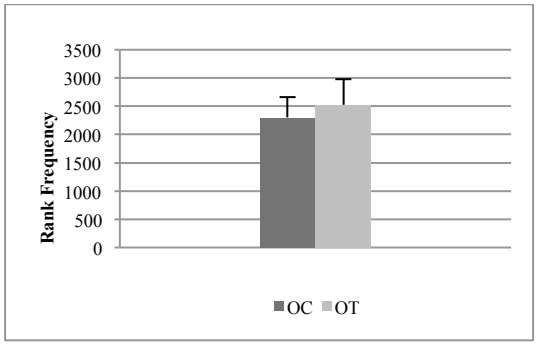
With Wall Without Wall

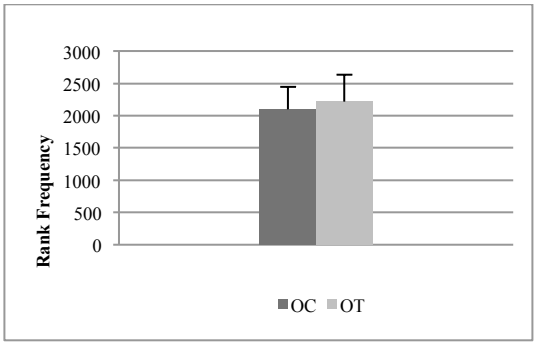
6a.



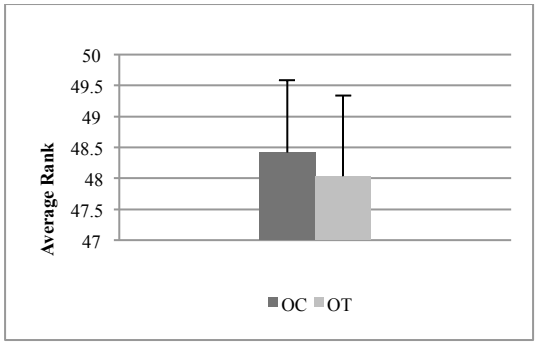


6b.





6c.



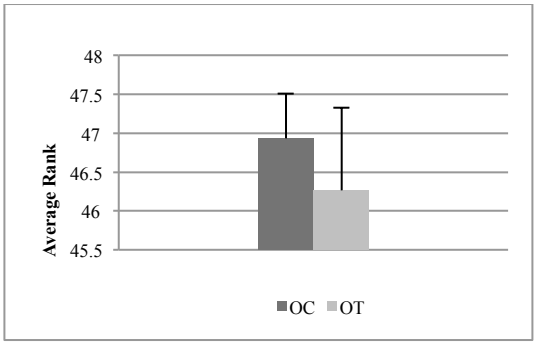
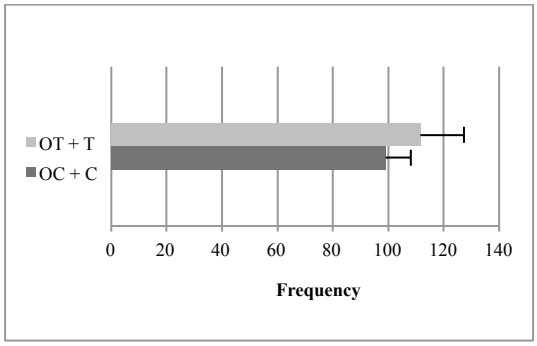
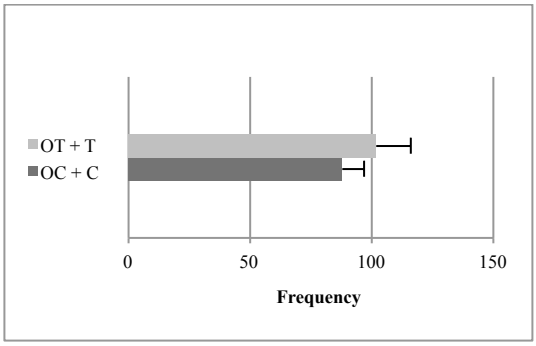


Figure 7. Defensive Behaviors of OC + C vs. OT + T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean ± SEM, N = 10.

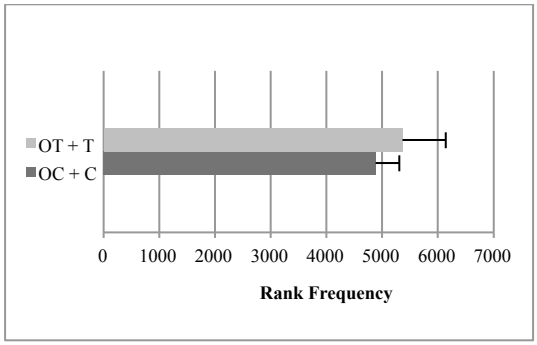
With Wall Without Wall

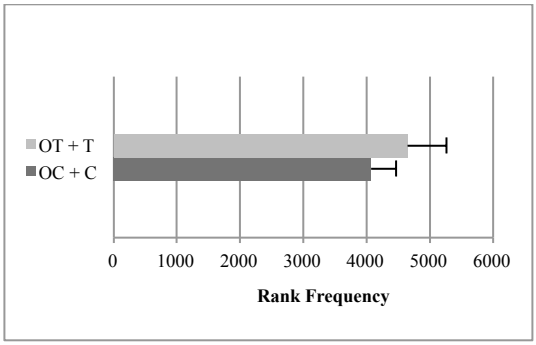
7a.



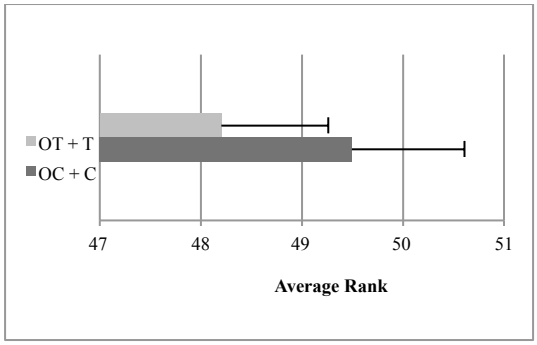


7b.





7c.



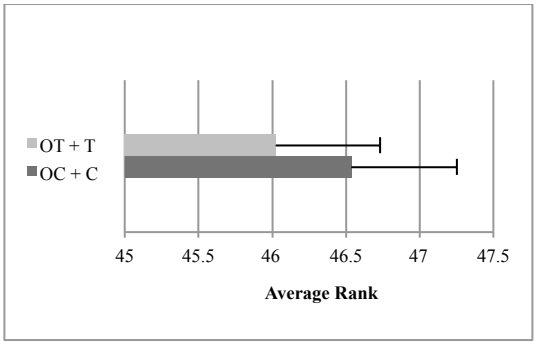
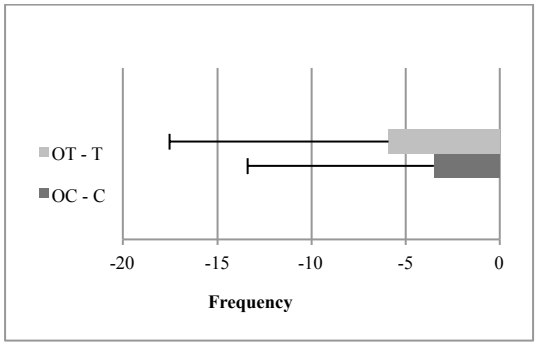
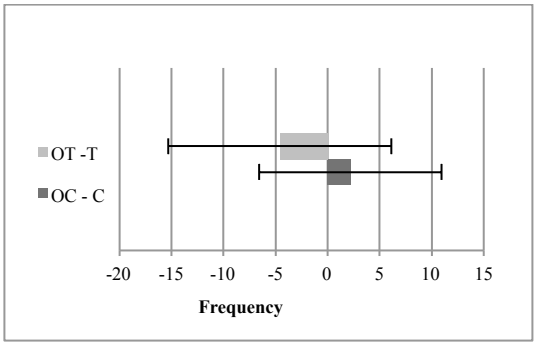


Figure 8. Defensive Behaviors of OC - C vs. OT - T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.04).

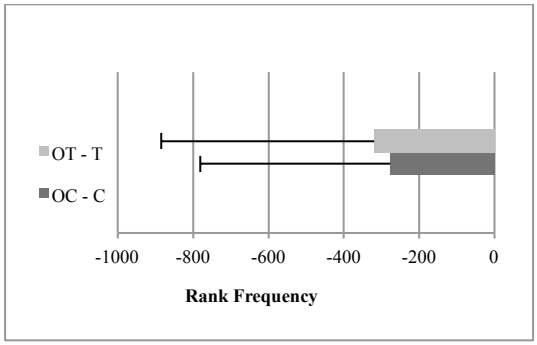
With Wall Without Wall

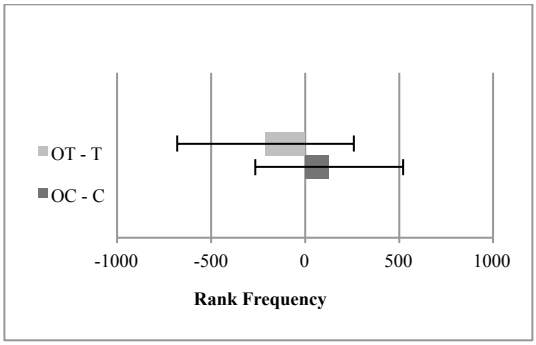
8a.



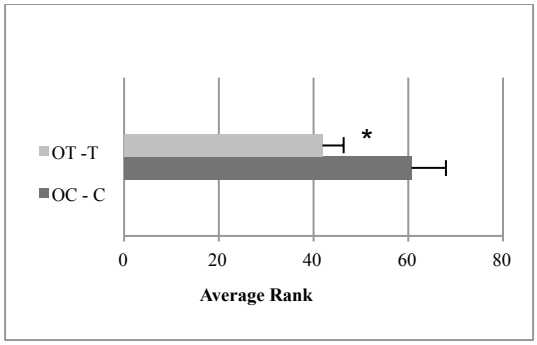


8b.





8c.



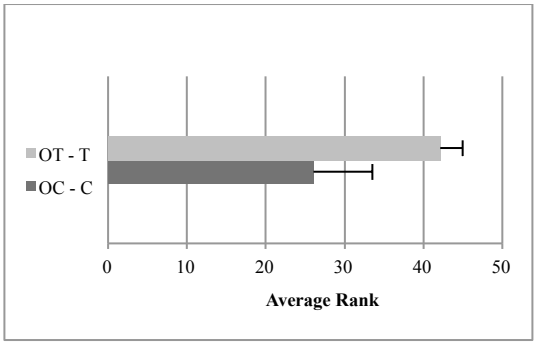
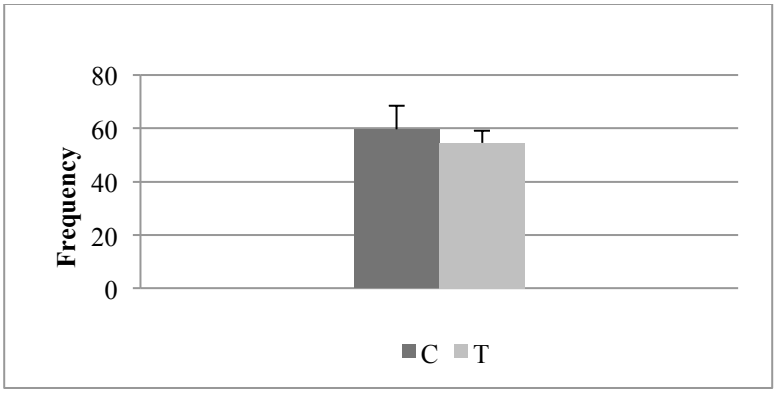
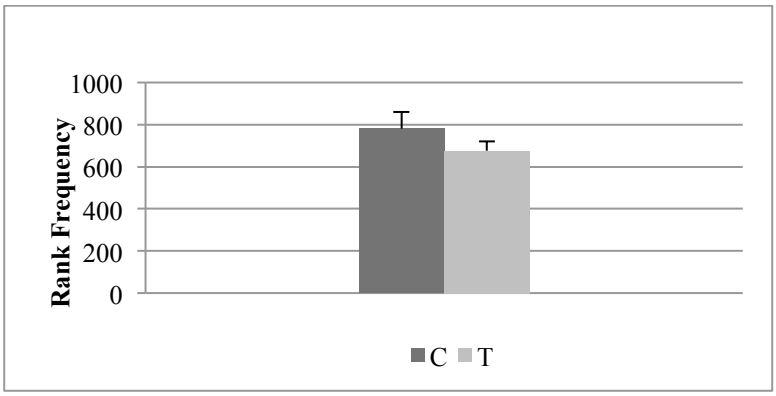


Figure 9. Avoidance Behaviors of C vs. T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank. Values are mean ± SEM, N = 10.

9a.


80
60
40
20
0
Frequency
C
T

9b.


1000
800
600
400
200
0
Rank Frequency
C
T

9c.

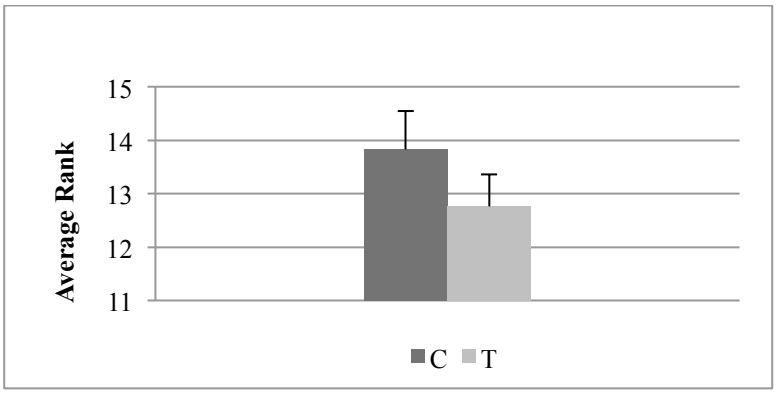
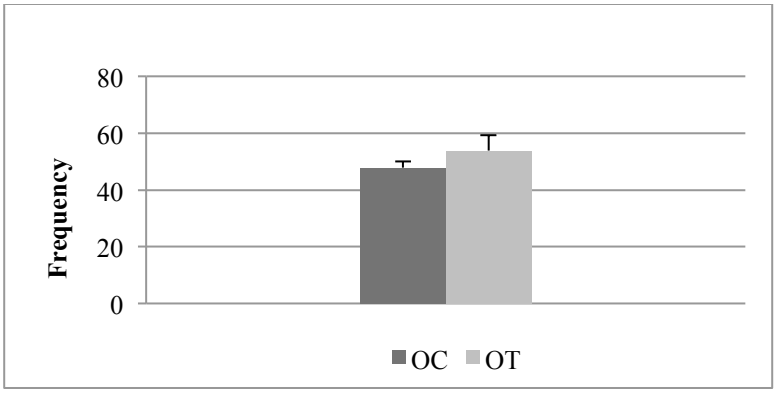
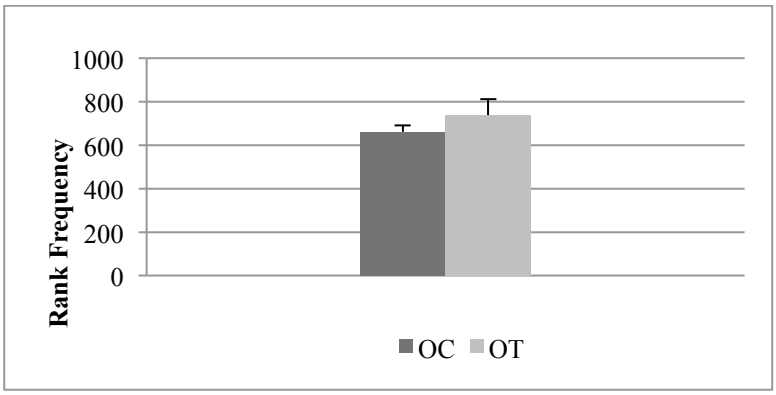

15
14
13
12
11
Average Rank
C
T

Figure 10. Avoidance Behaviors of OC vs. OT for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank. Values are mean ± SEM, N = 10.

10a.


80
60
40
20
0
Frequency
OC
OT

10b.


1000
800
600
400
200
0
Rank Frequency
OC
OT

10c.

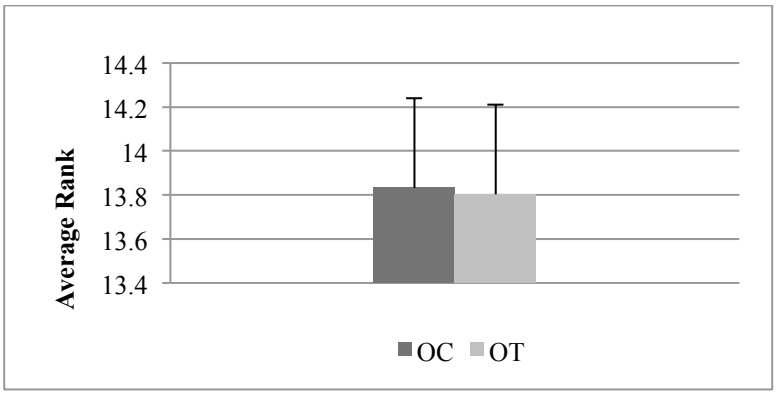
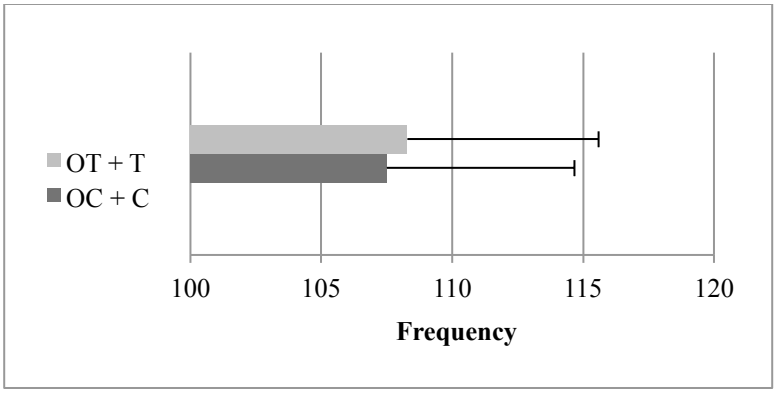
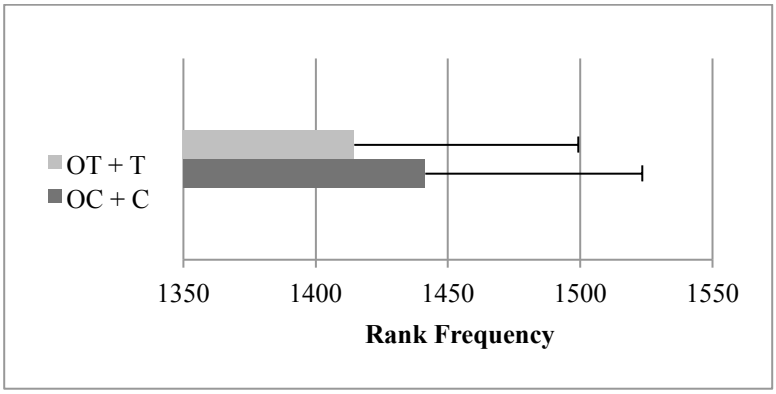

14.4
14.2
14
13.8
13.6
13.4
Average Rank
OC
OT

Figure 11. Avoidance Behaviors of OC + C vs. OT + T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank. Values are mean ± SEM, N = 10.

11a.


OT + T
OC + C
100
105
110
115
120
Frequency

11b.


OT + T
OC + C
1350
1400
1450
1500
1550
Rank Frequency

11c.

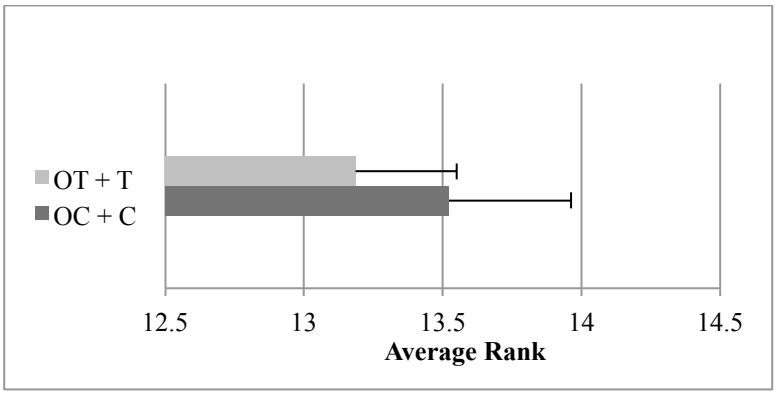
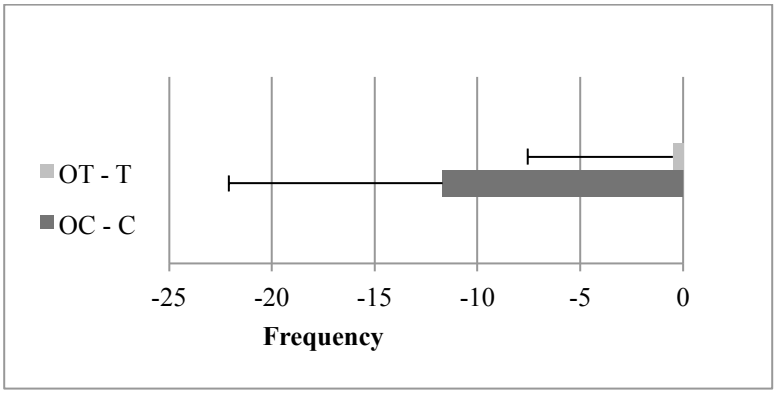
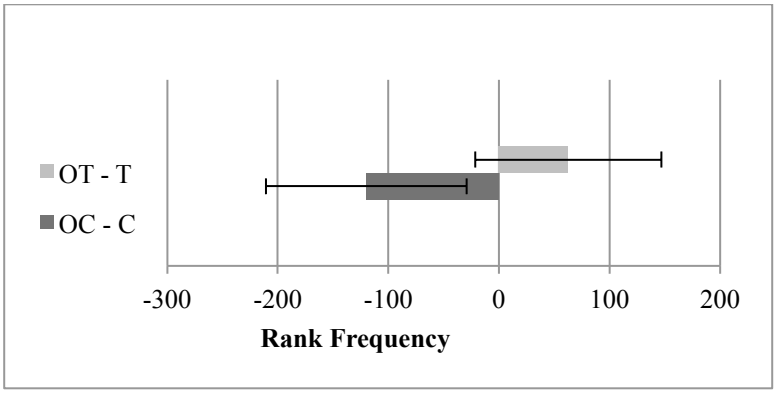

OT + T
OC + C
12.5
13
13.5
14
14.5
Average Rank

Figure 12. Avoidance Behaviors of OC - C vs. OT - T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank. Values are mean ± SEM, N = 10.

12a.


OT - T
OC - C
-25
-20
-15
-10
-5
0
Frequency

12b.


OT - T
OC - C
-300
-200
-100
0
100
200
Rank Frequency

12c.

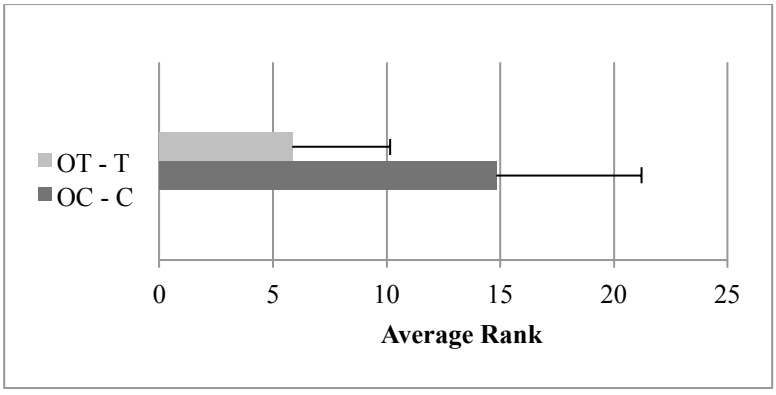
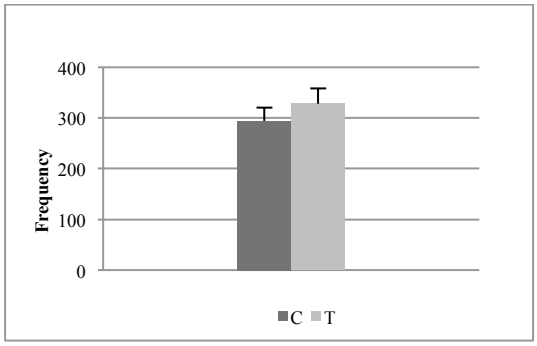
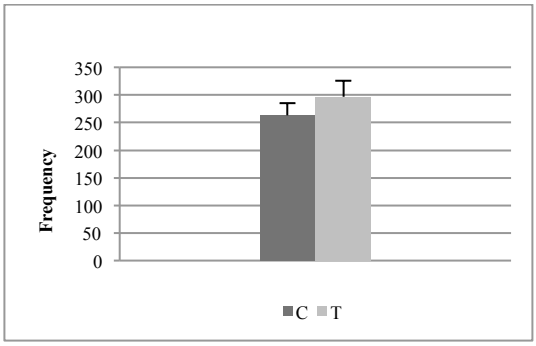

OT - T
OC - C
0
5
10
15
20
25
Average Rank

Figure 13. All behaviors of C vs. T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean ± SEM, N = 10.

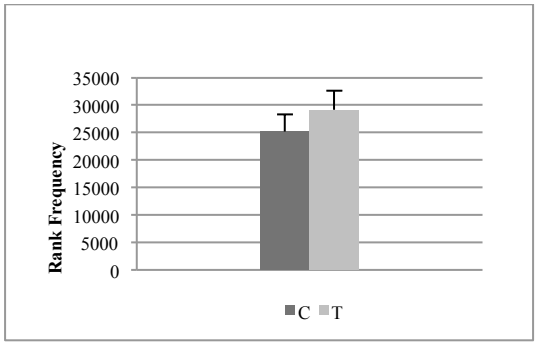
With Wall Without Wall

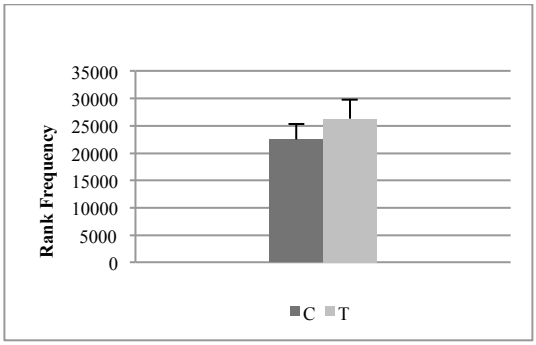
13a.



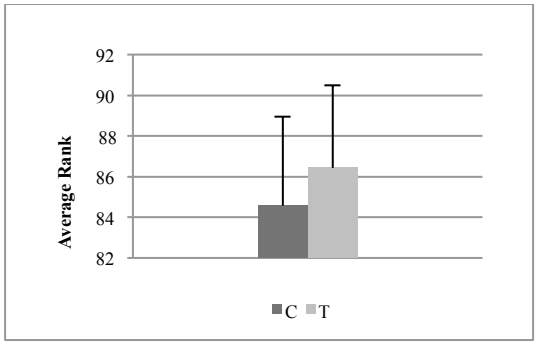


13b.





13c.



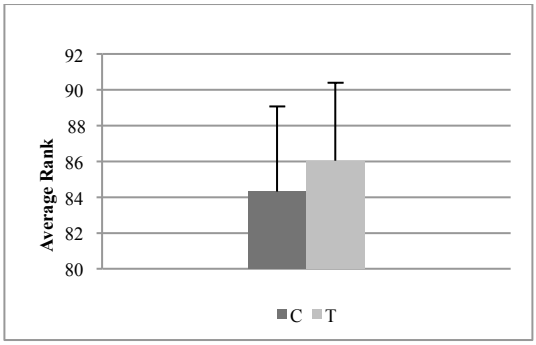
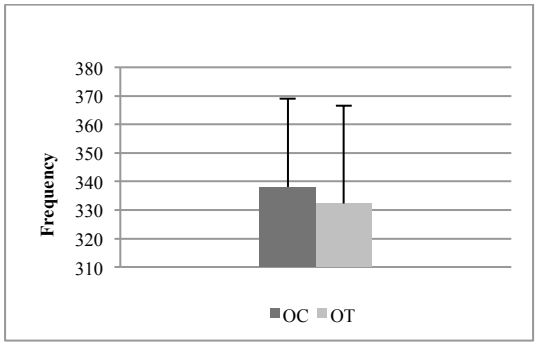
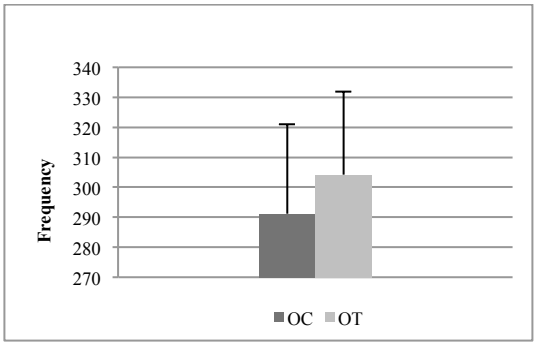


Figure 14. All behaviors of OC vs. OT for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean ± SEM, N = 10.

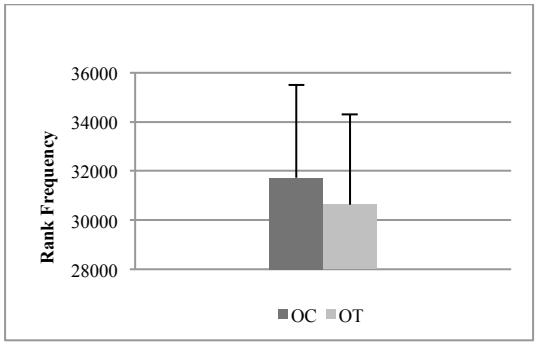
With Wall Without Wall

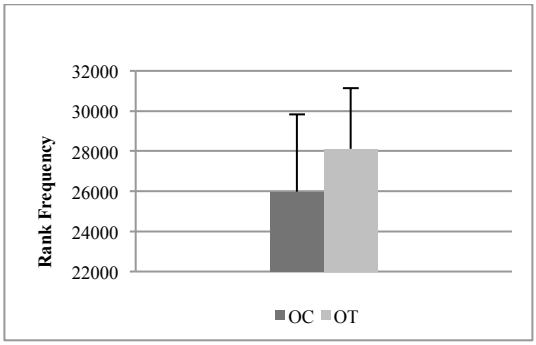
14a.



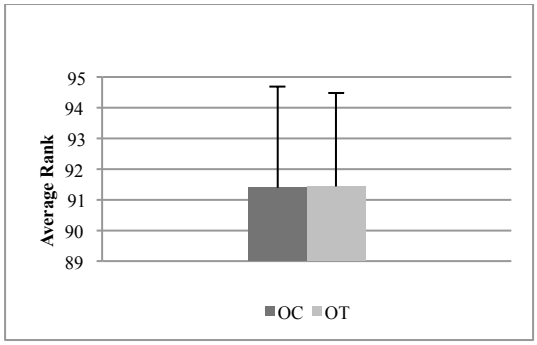


14b.





14c.



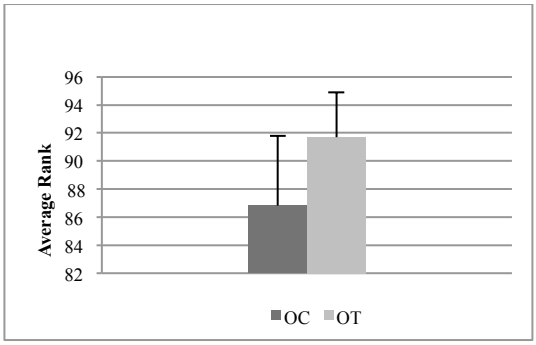
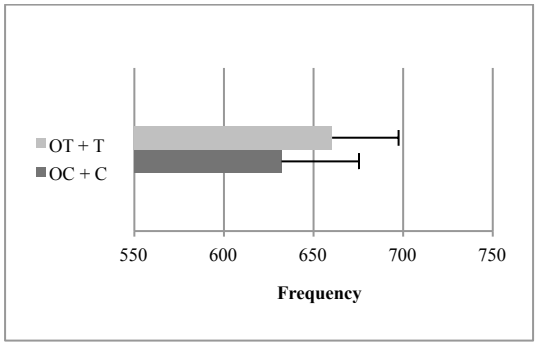
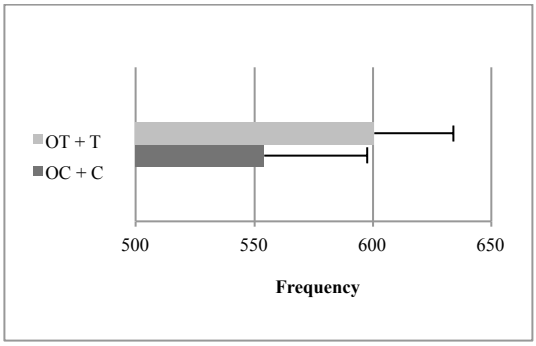


Figure 15. All behaviors of OC + C vs. OT + T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean ± SEM, N = 10.

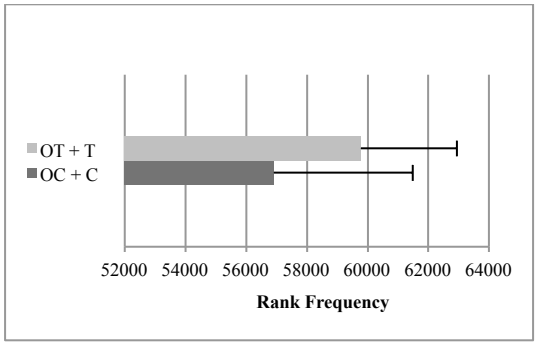
With Wall Without Wall

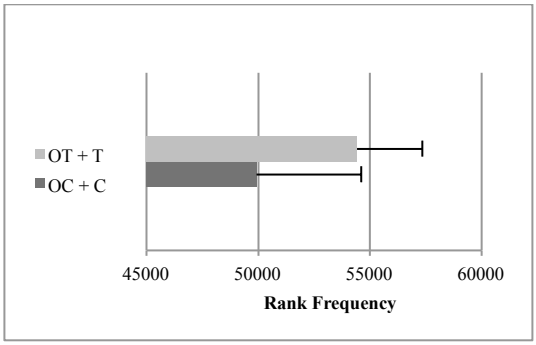
15a.



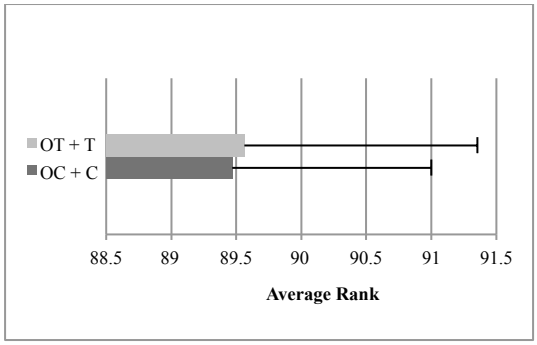


15b.





15c.



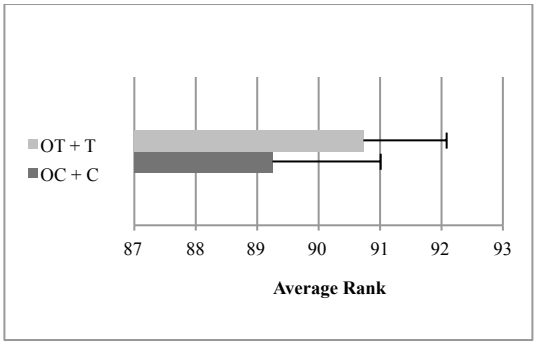
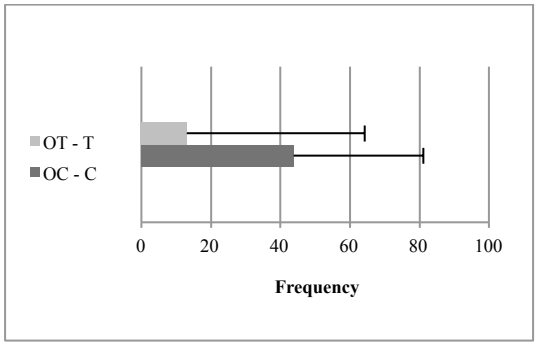
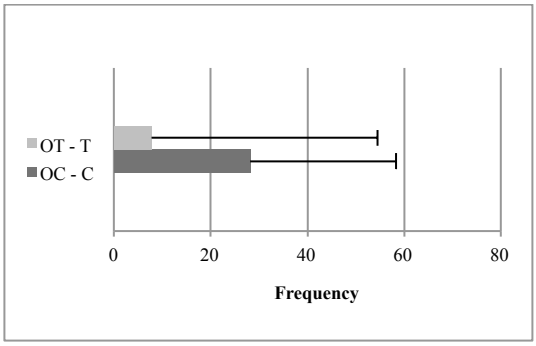


Figure 16. All behaviors of OC - C vs. OT - T for (**a**) Frequency, (**b**) Rank Frequency and (**c**) Average rank, with and without wall behaviors. Values are mean $\pm$ SEM, N = 10.

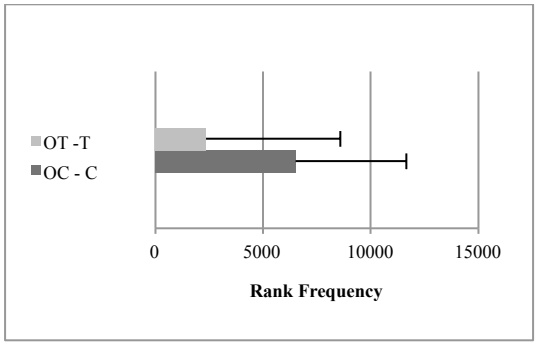
With Wall Without Wall

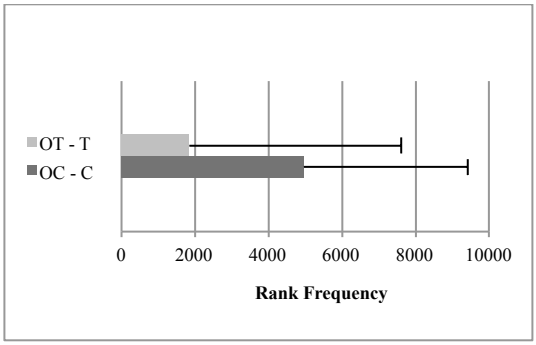
16a.



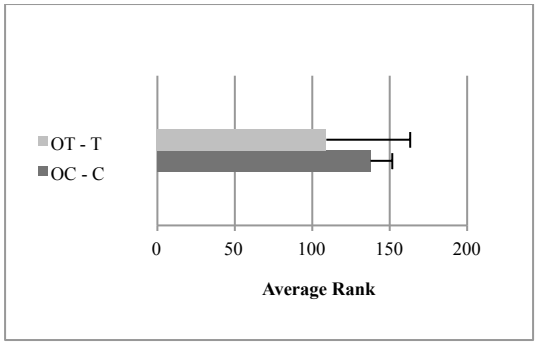


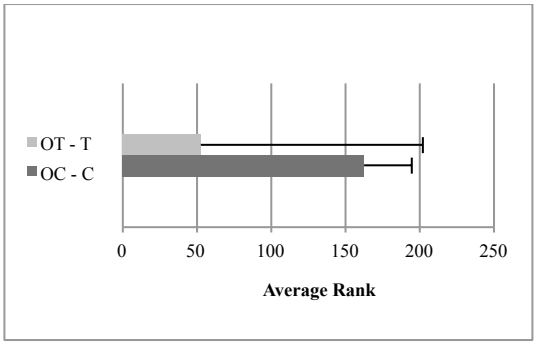
16b.





16c.





Raw ANOVA Results for Aggressive Behaviors

Treated vs. Control with Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	1834	183.4	6957.6
Treated	10	2152	215.2	7825.956

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5056.2	1	5056.2	0.68403	0.419032	4.413863
Within Groups	133052	18	7391.778			
Total	138108.2	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	218346	21834.6	1E+08
Treated	10	256334	25633.4	1.15E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	72154407	1	72154407	0.669438	0.423947	4.413863
Within Groups	1.94E+09	18	1.08E+08			
Total	2.01E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	1190.659	119.0659	12.80557
Treated	10	1188.328	118.8328	11.35925

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.271552	1	0.271552	0.022475	0.882498	4.413863
Within Groups	217.4834	18	12.08241			
Total	217.7549	19				

Treated vs. Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	1607	160.7	5029.567
Treated	10	1888	188.8	6793.289

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3948.05	1	3948.05	0.667867	0.424482	4.413863
Within Groups	106405.7	18	5911.428			
Total	110353.8	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	197390	19739	79650944
Treated	10	231950	23195	1.06E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	59719680	1	59719680	0.642615	0.433221	4.413863
Within Groups	1.67E+09	18	92932327			
Total	1.73E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	1224.533	122.4533	9.289223
Treated	10	1224.987	122.4987	3.326318

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.010327	1	0.010327	0.001637	0.96817	4.413863
Within Groups	113.5399	18	6.30777			
Total	113.5502	19				

Opponent Treated vs. Opponent Control with Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	2424	242.4	9348.489
OT	10	2256	225.6	9773.6

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1411.2	1	1411.2	0.147599	0.705341	4.413863
Within Groups	172098.8	18	9561.044			
Total	173510	19				

Anova: Single Factor Rank. Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	287590	28759	1.4E+08
OT	10	273734	27373.4	1.38E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9599436.8	1	9599437	0.06906	0.795694	4.413863
Within Groups	2502010186	18	1.39E+08			
Total	2511609623	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	1182.806	118.2806	22.80397
OT	10	1216.8	121.68	6.786022

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	57.78159	1	57.78159	3.905482	0.063659	4.413863
Within Groups	266.309914	18	14.795			
Total	324.091504	19				

Opponent Treated vs. Opponent Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	1984	198.4	7588.711
OT	10	2017	201.7	6134.9

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	54.45	1	54.45	0.007935	0.930002	4.413863
Within Groups	123512.5	18	6861.806			
Total	123567	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	246846	24684.6	1.24E+08
OT	10	251550	25155	96546170

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1106381	1	1106381	0.01002	0.921372	4.413863
Within Groups	1.99E+09	18	1.1E+08			
Total	1.99E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	1239.048	123.9048	4.099544
OT	10	1246.664	124.6664	1.390572

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.900365	1	2.900365	1.056577	0.317613	4.413863
Within Groups	49.41104	18	2.745058			
Total	52.31141	19				

Opponent Treated + Treated vs. Opponent Control + Control with Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	4258	425.8	10845.51
OT + T	10	4408	440.8	3960.844

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1125	1	1125	0.151962	0.701242	4.413863
Within Groups	133257.2	18	7403.178			
Total	134382.2	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	505936	50593.6	1.79E+08
OT + T	10	530068	53006.8	60009835

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	29117671	1	29117671	0.244076	0.627252	4.413863
Within Groups	2.15E+09	18	1.19E+08			
Total	2.18E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	1184.321	118.4321	15.17183
OT + T	10	1202.438	120.2438	8.771298

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	16.41067	1	16.41067	1.370804	0.256941	4.413863
Within Groups	215.4881	18	11.97156			
Total	231.8988	19				

Opponent Treated + Treated vs. Opponent Control + Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	3591	359.1	11002.54
OT + T	10	3905	390.5	3596.5

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4929.8	1	4929.8	0.675359	0.421942	4.413863
Within Groups	131391.4	18	7299.522			
Total	136321.2	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	444236	44423.6	1.86E+08
OT + T	10	483491	48349.1	58932064

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	77047751	1	77047751	0.628981	0.438059	4.413863
Within Groups	2.2E+09	18	1.22E+08			
Total	2.28E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	1232.928	123.2928	4.933435
OT + T	10	1237.312	123.7312	1.688359

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.96106	1	0.96106	0.290272	0.596647	4.413863
Within Groups	59.59615	18	3.310897			
Total	60.55721	19				

Opponent Treated – Treated vs. Opponent Control – Control with Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	590	59	21766.67
OT - T	10	104	10.4	31238.27

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11809.8	1	11809.8	0.445611	0.512895	4.413863
Within Groups	477044.4	18	26502.47			
Total	488854.2	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	68974	6897.4	3.03E+08
OT - T	10	17400	1740	4.46E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.33E+08	1	1.33E+08	0.355282	0.55856	4.413863
Within Groups	6.74E+09	18	3.74E+08			
Total	6.87E+09	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	1178.062	117.8062	97.42113
OT - T	10	1187.947	118.7947	154.7494

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.885482	1	4.885482	0.038747	0.846155	4.413863
Within Groups	2269.535	18	126.0853			
Total	2274.421	19				

Opponent Treated – Treated vs. Opponent Control – Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	377	37.7	14234.01
OT - T	10	129	12.9	22259.88

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3075.2	1	3075.2	0.168532	0.686265	4.413863
Within Groups	328445	18	18246.94			
Total	331520.2	19				

Anova: Single Factor Rank freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	49456	4945.6	2.22E+08
OT - T	10	19600	1960	3.47E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	44569037	1	44569037	0.156816	0.69676	4.413863
Within Groups	5.12E+09	18	2.84E+08			
Total	5.16E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	1231.331	123.1331	50.29025
OT - T	10	1206.857	120.6857	107.8314

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	29.94969	1	29.94969	0.378818	0.54594	4.413863
Within Groups	1423.095	18	79.06081			
Total	1453.044	19				

Raw ANOVA Results for Defensive Behaviors

Treated vs. Control with Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	513	51.3	304.4556
Treated	10	587	58.7	908.2333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	273.8	1	273.8	0.451559	0.51013	4.413863
Within Groups	10914.2	18	606.3444			
Total	11188	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	27650	2765	1216754
Treated	10	28416	2841.6	2496249

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	29337.8	1	29337.8	0.015803	0.901356	4.413863
Within Groups	33417024	18	1856501			
Total	33446362	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	501.8963	50.18963	24.18563
Treated	10	482.4797	48.24797	13.62584

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.85016	1	18.85016	0.997061	0.331258	4.413863
Within Groups	340.3032	18	18.90573			
Total	359.1533	19				

Treated vs. Control NO Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	428	42.8	174.1778
Treated	10	532	53.2	655.7333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	540.8	1	540.8	1.303272	0.268577	4.413863
Within Groups	7469.2	18	414.9556			
Total	8010	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	19698	1969.8	343140
Treated	10	24278	2427.8	1274736

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1048820	1	1048820	1.29654	0.269775	4.413863
Within Groups	14560879	18	808937.7			
Total	15609699	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	464.9686	46.49686	12.86561
Treated	10	459.6522	45.96522	2.967726

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.41317	1	1.41317	0.178506	0.677665	4.413863
Within Groups	142.5	18	7.916669			
Total	143.9132	19				

Opponent Treated vs. Opponent Control with Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	478	47.8	592.8444
OT	10	528	52.8	1022.844

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	125	1	125	0.154733	0.698673	4.413863
Within Groups	14541.2	18	807.8444			
Total	14666.2	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	23036	2303.6	1247200
OT	10	25218	2521.8	2107853

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	238056.2	1	238056.2	0.141909	0.710794	4.413863
Within Groups	30195474	18	1677526			
Total	30433530	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	484.1482	48.41482	13.74324
OT	10	480.3356	48.03356	17.12279

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.7267952	1	0.726795	0.047094	0.830641	4.413863
Within Groups	277.79431	18	15.43302			
Total	278.52111	19				

Opponent Treated vs. Opponent Control NO Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	450	45	616.6667
OT	10	486	48.6	933.3778

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	64.8	1	64.8	0.083611	0.775767	4.413863
Within Groups	13950.4	18	775.0222			
Total	14015.2	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	20978	2097.8	1242052
OT	10	22184	2218.4	1726314

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	72721.8	1	72721.8	0.048998	0.827308	4.413863
Within Groups	26715290	18	1484183			
Total	26788012	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	469.3429	46.93429	3.299859
OT	10	462.6269	46.26269	11.40981

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.255242	1	2.255242	0.306634	0.586567	4.413863
Within Groups	132.387	18	7.354833			
Total	134.6422	19				

Opponent Treated + Treated vs. Opponent Control + Control with Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	991	99.1	817.2111
OT + T	10	1115	111.5	2510.278

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	768.8	1	768.8	0.46209	0.505298	4.413863
Within Groups	29947.4	18	1663.744			
Total	30716.2	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	48844	4884.4	1784769
OT + T	10	53634	5363.4	6017297

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1147205	1	1147205	0.294077	0.59427	4.413863
Within Groups	70218591	18	3901033			
Total	71365796	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	494.9751	49.49751	12.35639
OT + T	10	482.0354	48.20354	11.25684

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.371787	1	8.371787	0.709076	0.4108	4.413863
Within Groups	212.5191	18	11.80662			
Total	220.8909	19				

Opponent Treated + Treated vs. Opponent Control + Control NO Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	878	87.8	812.4
OT + T	10	1018	101.8	2028.178

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	980	1	980	0.69	0.417047	4.413863
Within Groups	25565.2	18	1420.289			
Total	26545.2	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	40676	4067.6	1616163
OT + T	10	46462	4646.2	3795323

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1673890	1	1673890	0.618643	0.441785	4.413863
Within Groups	48703378	18	2705743			
Total	50377268	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	465.3583	46.53583	5.153623
OT + T	10	460.2278	46.02278	5.021844

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.316095	1	1.316095	0.25868	0.617206	4.413863
Within Groups	91.5792	18	5.087733			
Total	92.89529	19				

Opponent Treated – Treated vs. Opponent Control – Control with Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	-35	-3.5	977.3889
OT - T	10	-59	-5.9	1351.878

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	28.8	1	28.8	0.024729	0.876795	4.413863
Within Groups	20963.4	18	1164.633			
Total	20992.2	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	-2772	-277.2	2543793
OT - T	10	-3198	-319.8	3190906

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9073.8	1	9073.8	0.003165	0.955759	4.413863
Within Groups	51612293	18	2867350			
Total	51621367	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	606.8085	60.68085	521.5665
OT - T	10	418.095	41.8095	205.7023

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1780.639	1	1780.639	4.896783	0.040067	4.413863
Within Groups	6545.419	18	363.6344			
Total	8326.057	19				

Opponent Treated – Treated vs. Opponent Control – Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	22	2.2	769.2889
OT - T	10	-46	-4.6	1150.044

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	231.2	1	231.2	0.240917	0.629475	4.413863
Within Groups	17274	18	959.6667			
Total	17505.2	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	1280	128	1554220
OT - T	10	-2094	-209.4	2206775

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	569193.8	1	569193.8	0.302683	0.588968	4.413863
Within Groups	33848960	18	1880498			
Total	34418154	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	260.4619	26.04619	557.6866
OT - T	10	421.5217	42.15217	78.91879

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1297.012	1	1297.012	4.074777	0.05868	4.413863
Within Groups	5729.448	18	318.3027			
Total	7026.461	19				

Raw ANOVA Results for Avoidance Behaviors

Treated vs. Control

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	596	59.6	752.933333
Treated	10	544	54.4	223.6

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	135.2	1	135.2	0.27689787	0.605165	4.413863
Within Groups	8788.8	18	488.2666667			
Total	8924	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	7802	780.2	65615.5111
Treated	10	6760	676	19069.3333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	54288.2	1	54288.2	1.28212316	0.272364	4.413863
Within Groups	762163.6	18	42342.42222			
Total	816451.8	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	138.312738	13.83127375	5.21350351
Treated	10	127.668734	12.7668734	3.51846065

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.664740502	1	5.664740502	1.29747223	0.269608	4.413863
Within Groups	78.5876774	18	4.365982078			
Total	84.2524179	19				

Opponent Treated vs. Opponent Control

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	479	47.9	46.1
OT	10	539	53.9	292.1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	180	1	180	1.064459	0.315865	4.413863
Within Groups	3043.8	18	169.1			
Total	3223.8	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	6602	660.2	9141.733
OT	10	7386	738.6	52328.93

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	30732.8	1	30732.8	0.999918	0.330584	4.413863
Within Groups	553236	18	30735.33			
Total	583968.8	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	138.3155	13.83155	1.658761
OT	10	138.0209	13.80209	1.67861

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00434	1	0.00434	0.002601	0.959887	4.413863
Within Groups	30.03634	18	1.668686			
Total	30.04068	19				

Opponent Treated + Treated vs. Opponent Control + Control

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	1075	107.5	515.3889
OT + T	10	1083	108.3	531.5667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.2	1	3.2	0.006113	0.938543	4.413863
Within Groups	9422.6	18	523.4778			
Total	9425.8	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	14414	1441.4	67371.6
OT + T	10	14146	1414.6	71804.49

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3591.2	1	3591.2	0.051607	0.822851	4.413863
Within Groups	1252585	18	69588.04			
Total	1256176	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	135.2344	13.52344	1.928329
OT + T	10	131.8638	13.18638	1.332888

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.568024	1	0.568024	0.348351	0.562385	4.413863
Within Groups	29.35095	18	1.630609			
Total	29.91898	19				

Opponent Treated – Treated vs. Opponent Control – Control

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	-117	-11.7	1082.678
OT - T	10	-5	-0.5	499.8333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	627.2	1	627.2	0.792664	0.385041	4.413863
Within Groups	14242.6	18	791.2556			
Total	14869.8	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	-1200	-120	82383.11
OT - T	10	626	62.6	70992.04

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	166713.8	1	166713.8	2.173935	0.157642	4.413863
Within Groups	1380376	18	76687.58			
Total	1547090	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	148.3986	14.83986	406.768
OT - T	10	58.55238	5.855238	185.191

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	403.617	1	403.617	1.363665	0.258139	4.413863
Within Groups	5327.631	18	295.9795			
Total	5731.248	19				

Raw ANOVA Results for All Behaviors

Treated vs. Control With Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	2943	294.3	6680.233
Treated	10	3283	328.3	8926.9

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5780	1	5780	0.740687	0.40076	4.413863
Within Groups	140464.2	18	7803.567			
Total	146244.2	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	251956	25195.6	95104594
Treated	10	291510	29151	1.2E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	78225946	1	78225946	0.726989	0.405064	4.413863
Within Groups	1.94E+09	18	1.08E+08			
Total	2.02E+09	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	845.7928	84.57928	192.8573
Treated	10	864.2942	86.42942	164.9526

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.11509	1	17.11509	0.095666	0.760646	4.413863
Within Groups	3220.289	18	178.9049			
Total	3237.404	19				

Treated vs. Control NO Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	2630	263	5132.222
Treated	10	2964	296.4	8838.489

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5577.8	1	5577.8	0.798499	0.383335	4.413863
Within Groups	125736.4	18	6985.356			
Total	131314.2	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	224806	22480.6	79026825
Treated	10	262988	26298.8	1.18E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	72893256	1	72893256	0.738384	0.401478	4.413863
Within Groups	1.78E+09	18	98719975			
Total	1.85E+09	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	10	843.1233	84.31233	226.0213
Treated	10	860.4558	86.04558	191.4534

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15.02066	1	15.02066	0.07196	0.791555	4.413863
Within Groups	3757.272	18	208.7374			
Total	3772.293	19				

Opponent Treated vs. Opponent Control with Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	3381	338.1	9572.544
OT	10	3323	332.3	11778.46

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	168.2	1	168.2	0.015756	0.901502	4.413863
Within Groups	192159	18	10675.5			
Total	192327.2	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	317228	31722.8	1.42E+08
OT	10	306338	30633.8	1.34E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5929605	1	5929605	0.042969	0.838111	4.413863
Within Groups	2.48E+09	18	1.38E+08			
Total	2.49E+09	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	913.8978	91.38978	108.3895
OT	10	914.2705	91.42705	93.27825

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.006944	1	0.006944	6.89E-05	0.99347	4.413863
Within Groups	1815.01	18	100.8339			
Total	1815.017	19				

Opponent Treated vs. Opponent Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	2912	291.2	8832.844
OT	10	3042	304.2	7643.956

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	845	1	845	0.102568	0.752457	4.413863
Within Groups	148291.2	18	8238.4			
Total	149136.2	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	259758	25975.8	1.49E+08
OT	10	281120	28112	91516793

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	22816752	1	22816752	0.189708	0.668337	4.413863
Within Groups	2.16E+09	18	1.2E+08			
Total	2.19E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC	10	868.2302	86.82302	247.2662
OT	10	917.0062	91.70062	103.2282

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	118.9549	1	118.9549	0.678784	0.420789	4.413863
Within Groups	3154.449	18	175.2472			
Total	3273.404	19				

Opponent Treated + Treated vs. Opponent Control + Control with Wall

Anova: Single Factor **Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	6324	632.4	18598.93
OT + T	10	6606	660.6	13605.16

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3976.2	1	3976.2	0.246938	0.625254	4.413863
Within Groups	289836.8	18	16102.04			
Total	293813	19				

Anova: Single Factor **Rank Freq.**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	569112	56911.2	2.1E+08
OT + T	10	597848	59784.8	1E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	41287885	1	41287885	0.266763	0.611799	4.413863
Within Groups	2.79E+09	18	1.55E+08			
Total	2.83E+09	19				

Anova: Single Factor **Avg. Rank**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	894.7054	89.47054	23.41045
OT + T	10	895.6116	89.56116	32.16944

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.041059	1	0.041059	0.001477	0.969762	4.413863
Within Groups	500.219	18	27.78995			
Total	500.2601	19				

Opponent Treated + Treated vs. Opponent Control + Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	5542	554.2	18853.07
OT + T	10	6006	600.6	11150.04

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10764.8	1	10764.8	0.717579	0.408062	4.413863
Within Groups	270028	18	15001.56			
Total	280792.8	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	499148	49914.8	2.2E+08
OT + T	10	544108	54410.8	86324436

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.01E+08	1	1.01E+08	0.658958	0.427533	4.413863
Within Groups	2.76E+09	18	1.53E+08			
Total	2.86E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC + C	10	892.57	89.257	30.78775
OT + T	10	907.2821	90.72821	18.44761

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.8223	1	10.8223	0.439615	0.51571	4.413863
Within Groups	443.1182	18	24.61768			
Total	453.9405	19				

Opponent Treated – Treated vs. Opponent Control – Control with Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	438	43.8	13906.62
OT - T	10	131	13.1	26131.66

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4712.45	1	4712.45	0.235397	0.633403	4.413863
Within Groups	360344.5	18	20019.14			
Total	365057	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	65272	6527.2	2.64E+08
OT - T	10	23548	2354.8	3.89E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	87044609	1	87044609	0.26674	0.611814	4.413863
Within Groups	5.87E+09	18	3.26E+08			
Total	5.96E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	1376.872	137.6872	1948.372
OT - T	10	1089.487	108.9487	29563.6

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4129.505	1	4129.505	0.262091	0.614911	4.413863
Within Groups	283607.7	18	15755.99			
Total	287737.3	19				

Opponent Treated - Treated vs. Opponent Control - Control NO Wall

Anova: Single Factor Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	282	28.2	9077.067
OT - T	10	78	7.8	21814.84

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2080.8	1	2080.8	0.134715	0.717872	4.413863
Within Groups	278027.2	18	15445.96			
Total	280108	19				

Anova: Single Factor Rank Freq.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	49536	4953.6	2E+08
OT - T	10	18332	1833.2	3.33E+08

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	48684481	1	48684481	0.182541	0.674266	4.413863
Within Groups	4.8E+09	18	2.67E+08			
Total	4.85E+09	19				

Anova: Single Factor Avg. Rank

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
OC - C	10	1622.696	162.2696	10396.03
OT - T	10	528.7513	52.87513	222267.5

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	59835.75	1	59835.75	0.514354	0.482464	4.413863
Within Groups	2093972	18	116331.8			
Total	2153808	19				

Post-Hoc Summary Tables

Table 5. Summary of Aggressive behaviors for Bolingbroke vs. Sipala. Values are means ± SEM, N = 10. Significant P-values are in bold. Abbreviations: F=Frequency, RF = Rank Frequency, AR = Average Rank.

Aggressive Behaviors

Control	Bolingbroke	Sipala	P-value
F	93.2	183.4 ± 26.4	**0.004**
RF	10989.8	21834.6 ± 3167.2	**0.004**
AR	119.1	119.1	0.9

OC	Bolingbroke	Sipala	P-value
F	359.9 ± 26.7	242.2 ± 30.6	**0.009**
RF	43702.8 ± 3317.1	28759 ± 3747.0	**0.007**
AR	121.5 ± 2.3	118.3 ± 1.5	0.25

OC+C	Bolingbroke	Sipala	P-value
F	453.1 ± 28.1	425.8 ± 32.9	0.5
RF	54692.6 ± 3385.7	50593.6 ± 4225.9	0.5
AR	120.9 ± 2.5	118.4 ± 1.2	0.4

OC-C	Bolingbroke	Sipala	P-value
F	266.7 ± 27.9	59 ± 46.7	**0.001**
RF	32713 ± 3506.1	6897.4 ± 5504.2	**0.0009**
AR	122.8 ± 3.2	117.8 ± 3.1	0.3

Table 6. Summary of Defensive behaviors for Bolingbroke vs. Sipala

Values are means ± SEM, N = 10. Significant P-values are in bold.

Abbreviations: F=Frequency, RF = Rank Frequency, AR = Average Rank.

Defensive Behaviors

Control	Bolingbroke	Sipala	P-value
F	110.8 ± 5.3	51.3 ± 5.5	**0.0000004**
RF	6035.6 ± 379.2	2765 ± 348.8	**0.000006**
AR	54.4 ± 2.1	50.2 ± 1.6	0.1

OC	Bolingbroke	Sipala	P-value
F	41.4 ± 8.8	47.8 ± 7.7	0.6
RF	2246.2 ± 517.4	2303.6 ± 353.2	0.9
AR	54.9 ± 4.9	48.4 ± 1.2	0.2

OC+C	Bolingbroke	Sipala	P-value
F	152.2 ± 10.1	99.1 ± 9.0	**0.0001**
RF	8278.8 ± 649.6	4884.4 ± 422.5	**0.0004**
AR	54.5 ± 2.7	49.5 ± 1.1	**0.09**

OC-C	Bolingbroke	Sipala	P-value
F	-69.4 ± 10.4	-3.5 ± 9.9	**0.0002**
RF	-3789 ± 634.3	-277.2 ± 504.4	**0.0004**
AR	57.9 ± 3.3	60.7 ± 7.2	0.7

Table 7. Summary of Avoidance behaviors for Bolingbroke vs. Sipala. Values are means ± SEM, N = 10. Significant P-values are in bold. Abbreviations: F=Frequency, RF = Rank Frequency, AR = Average Rank.

Avoidance Behaviors

Control	Bolingbroke	Sipala	P-value
F	81.5 ± 4.7	59.6 ± 8.7	**0.03**
RF	1044.8 ± 56.2	780.2 ± 81.0	**0.01**
AR	12.9 ± 0.4	13.8 ± 0.7	0.3

OC	Bolingbroke	Sipala	P-value
F	53.7 ± 2.3	47.9 ± 2.1	0.08
RF	736.8 ± 47.5	660.2 ± 30.2	0.2
AR	13.6 ± 0.4	13.8 ± 0.4	0.7

OC+C	Bolingbroke	Sipala	P-value
F	135.2 ± 5.6	107.5 ± 7.2	**0.007**
RF	1781.6 ± 84.6	1441.4 ± 82.1	**0.009**
AR	13.2 ± 0.3	13.5 ± 0.4	0.6

OC-C	Bolingbroke	Sipala	P-value
F	-27.8 ± 4.9	-11.7 ± 10.4	0.2
RF	-308 ± 60.6	-120 ± 90.8	0.1
AR	11.3 ± 1.6	14.8 ± 6.4	0.6

Table 8. Summary of All behaviors for Bolingbroke vs. Sipala.

Values are means ± SEM, N = 10. Significant P-values are in bold.

Abbreviations: F=Frequency, RF = Rank Frequency, AR = Average Rank.

All Behaviors

Control	Bolingbroke	Sipala	P-value
F	285.5 ± 13.9	294.3 ± 25.8	0.8
RF	18070.2 ± 1149.5	25195.6 ± 3083.9	**0.04**
AR	63 ± 1.8	84.6	**0.0003**

OC	Bolingbroke	Sipala	P-value
F	456 ± 26.2	338.1 ± 97.8	**0.009**
RF	46685.8 ± 3201.7	31722.8 ± 3764.5	**0.007**
AR	101.9 ± 2.0	91.4 ± 3.3	**0.01**

OC+C	Bolingbroke	Sipala	P-value
F	741.5 ± 33.7	632.4 ± 43.1	0.06
RF	64752.4 ± 3581.1	56911.2 ± 4577.5	0.2
AR	86.9 ± 1.4	89.5 ± 1.5	0.2

OC-C	Bolingbroke	Sipala	P-value
F	170.5 ± 25.1	43.8 ± 37.3	**0.01**
RF	28615.6 ± 3211.6	6527.2 ± 5138.4	**0.002**
AR	182.9 ± 16.8	137.7 ± 13.9	**0.05**

Post-Hoc Figures

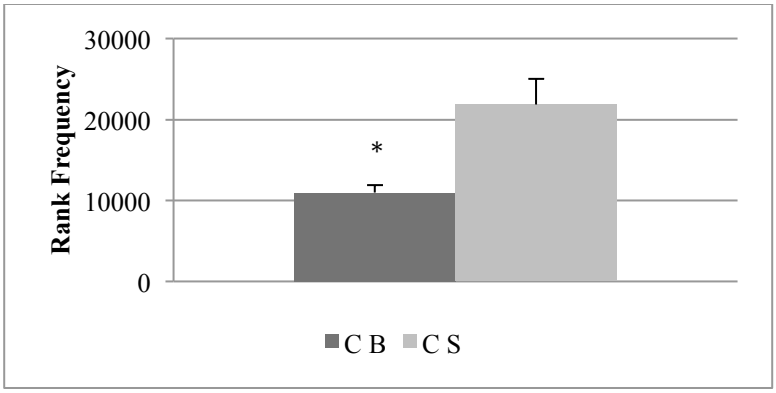
Graphs of significant comparisons

Figure 17a. Comparison of Rank frequency of Aggressive behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.004).

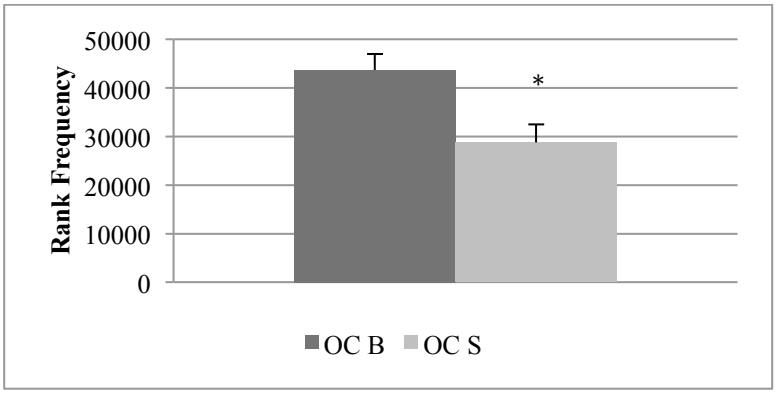
Figure 17b. Comparison of Rank frequency of Aggressive behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.007).

Figure 17c. Comparison of Rank frequency of Aggressive behaviors for $OC_B - C_B$ vs. $OC_S - C_S$. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.0009).

17a.


30000
20000
10000
0
Rank Frequency
*
C B
C S

17b.


50000
40000
30000
20000
10000
0
Rank Frequency
*
OC B
OC S

17c.

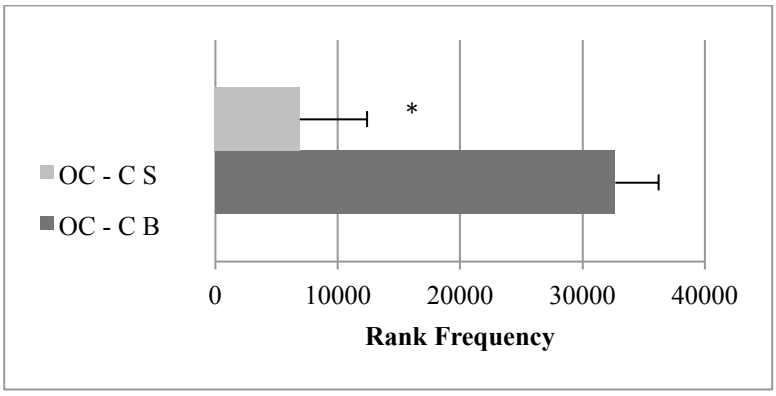
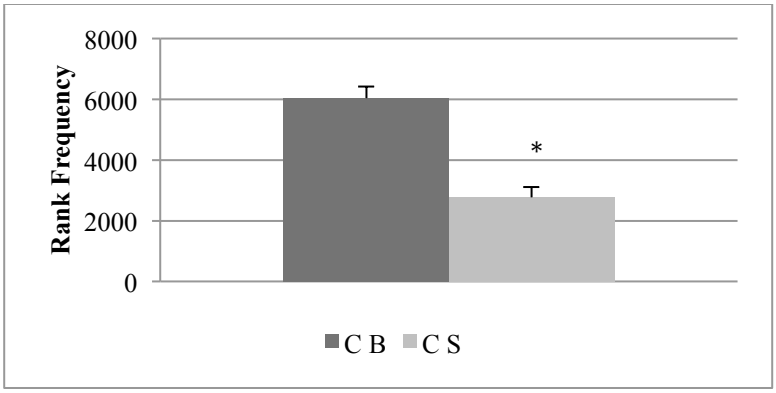

OC - C S
OC - C B
*
0
10000
20000
30000
40000
Rank Frequency

Figure 18a. Comparison of Rank frequency of Defensive behaviors for C_B vs. C_S. Values are mean $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.000006).

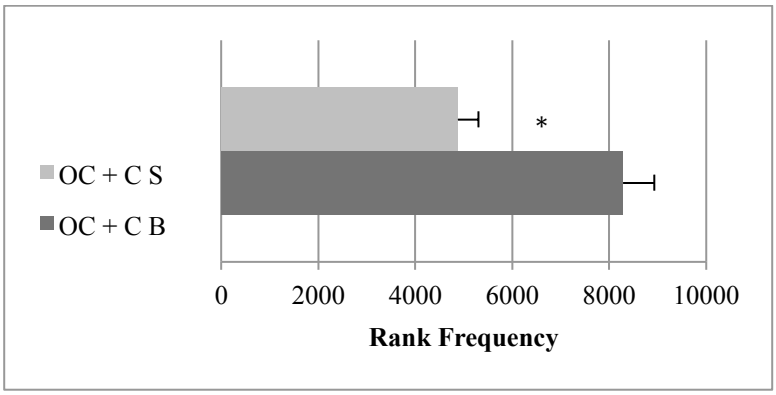
Figure 18b. Comparison of Rank frequency of Defensive behaviors for $OC_B + C_B$ vs. OC_S vs. C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.0004).

Figure 18c. Comparison of Rank frequency of Defensive behaviors for $OC_B - C_B$ vs. $OC_S - C_S$. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.0004).

18a.


8000
6000
4000
2000
0
Rank Frequency
*
C B
C S

18b.


OC + C S
OC + C B
*
0
2000
4000
6000
8000
10000
Rank Frequency

18c.

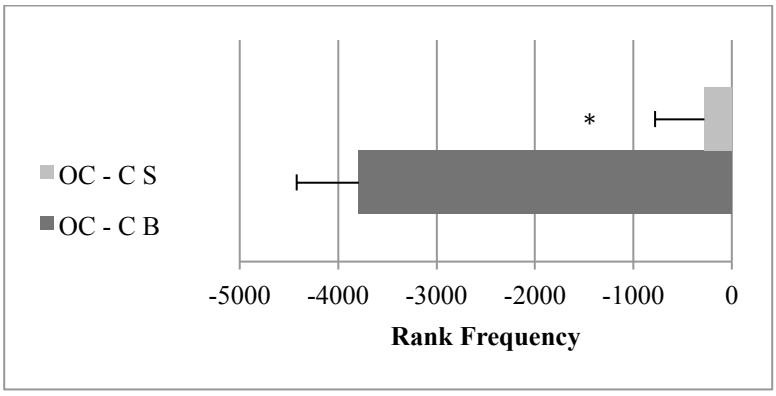
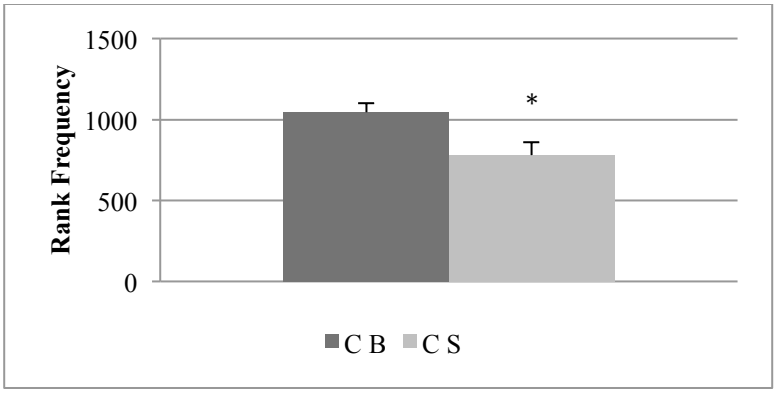

OC - C S
OC - C B
*
-5000
-4000
-3000
-2000
-1000
0
Rank Frequency

Figure 19a. Comparison of Rank frequency of Avoidance behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.01).

Figure 19b. Comparison of Rank frequency of Avoidance behaviors for $OC_B + C_B$ vs. $OC_S + C_S$. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.009).

19a.


1500
1000
500
0
Rank Frequency
*
C B
C S

19b.

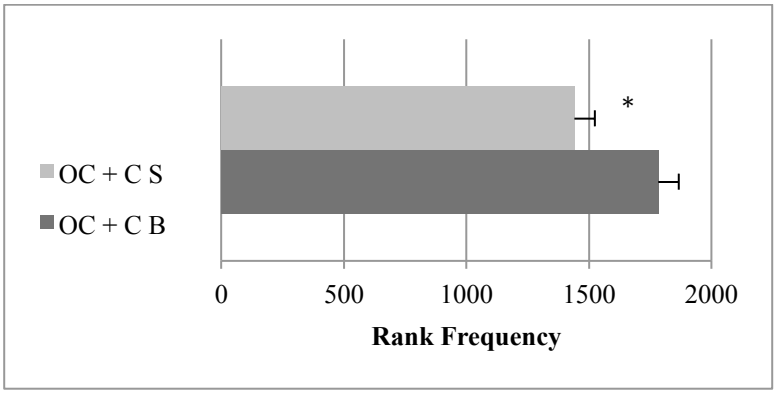
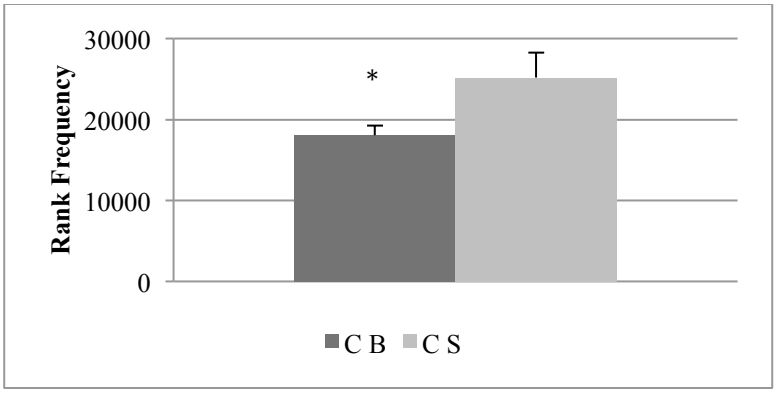

OC + C S
OC + C B
*
0
500
1000
1500
2000
Rank Frequency

Figure 20a. Comparison of Rank Frequency of All Behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.04).

Figure 20b. Comparison of Average rank of All Behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.0003).

20a.


30000
20000
10000
0
Rank Frequency
*
C B
C S

20b.

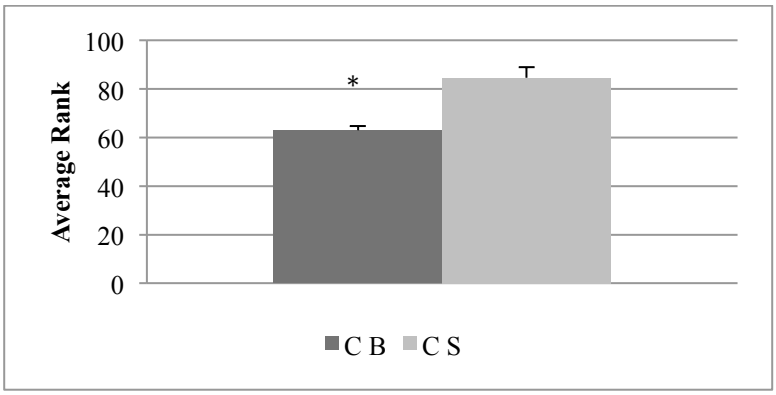
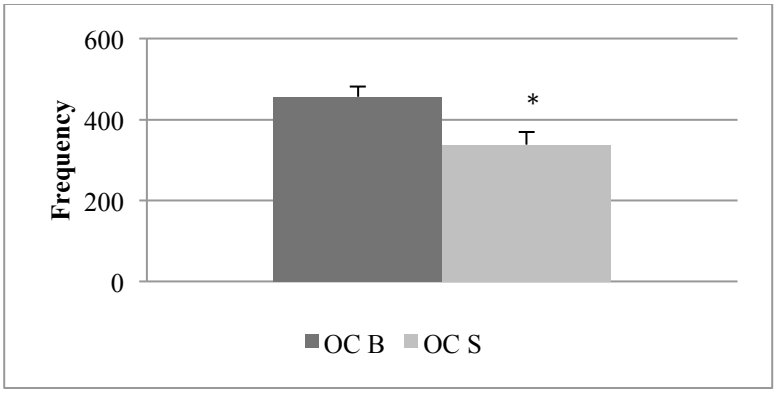

100
80
60
40
20
0
Average Rank
*
C B
C S

Figure 21a. Comparison of Frequency of All Behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.009).

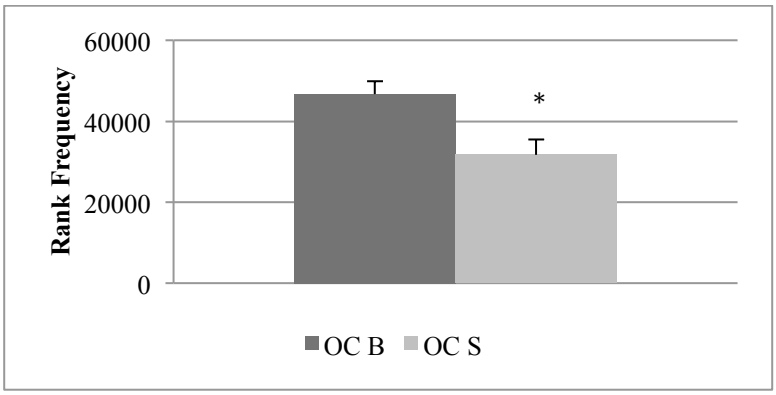
Figure 21b. Comparison of Rank frequency of All Behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.007).

Figure 21c. Comparison of Average rank of All Behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.01).

21a.


600
400
200
0
Frequency
*
OC B
OC S

21b.


60000
40000
20000
0
Rank Frequency
*
OC B
OC S

21c.

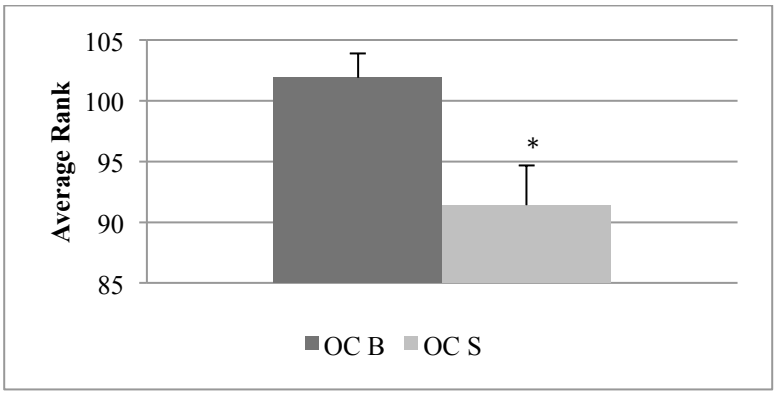
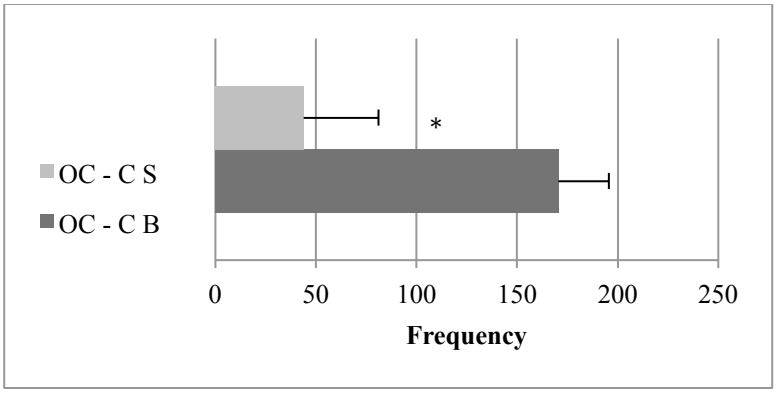

105
100
95
90
85
Average Rank
*
OC B
OC S

Figure 22a. Comparison of Frequency of All Behaviors for OC_B - C_B vs. OC_S - C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.01).

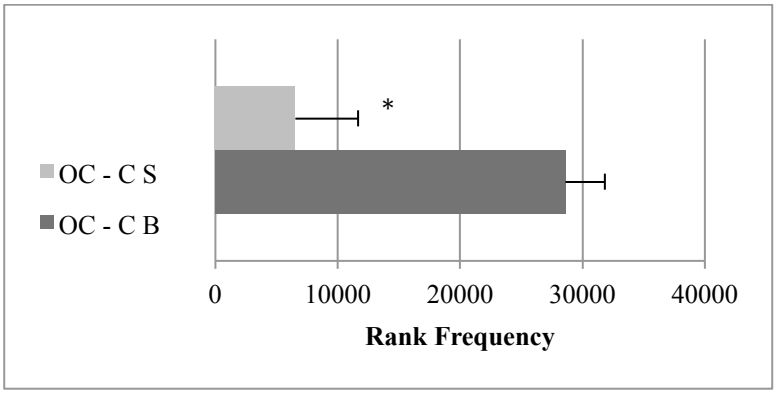
Figure 22b. Comparison of Rank Frequency of All Behaviors for OC_B - C_B vs. OC_S - C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.002).

Figure 22c. Comparison of Average rank of All Behaviors for OC_B - C_B vs. OC_S - C_S. Values are means $\pm$ SEM, N = 10. Asterisk indicates significant difference (P = 0.05).

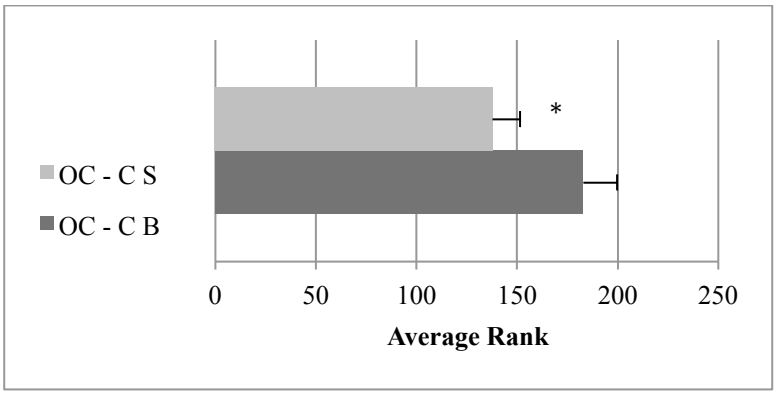
22a.


OC - C S
OC - C B
*
0
50
100
150
200
250
Frequency

22b.


OC - C S
OC - C B
*
0
10000
20000
30000
40000
Rank Frequency

22c.


OC - C S
OC - C B
*
0
50
100
150
200
250
Average Rank

Post-Hoc Figures

Graphs of non-significant comparisons

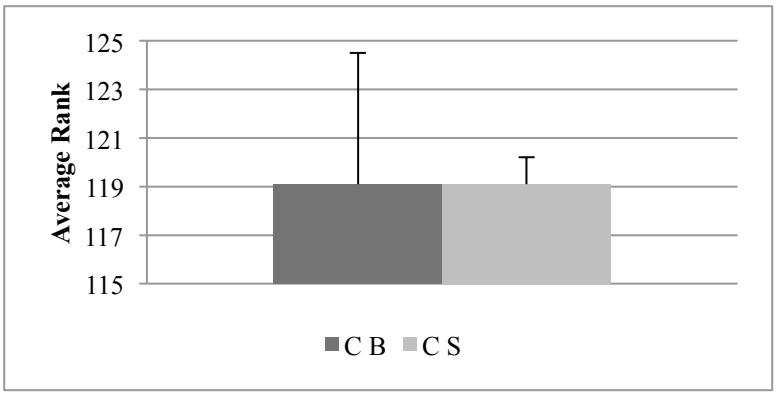
Figure 23a. Comparison of Average rank of Aggressive behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N = 10.

Figure 23b. Comparison of Average rank of Aggressive behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10.

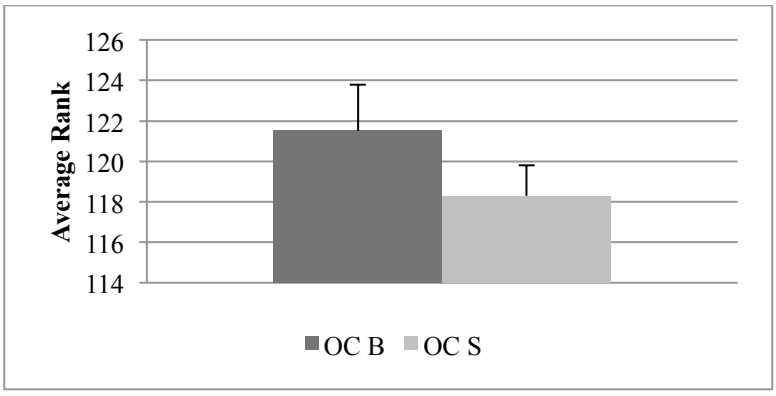
Figure 23c. Comparison of Average rank of Aggressive behaviors for $OC_B + C_B$ vs. $OC_S + C_S$. Values are means $\pm$ SEM, N = 10.

Figure 23d. Comparison of Average rank of Aggressive behaviors for $OC_B - C_B$ vs. $OC_S - C_S$. Values are means $\pm$ SEM, N = 10.

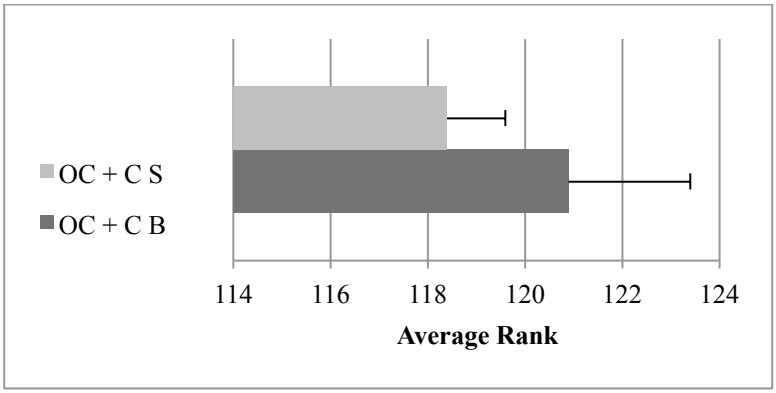
23a.



23b.



23c.



23d.

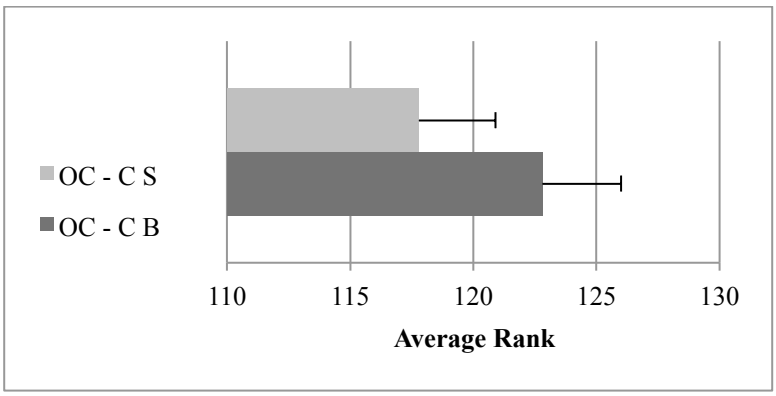
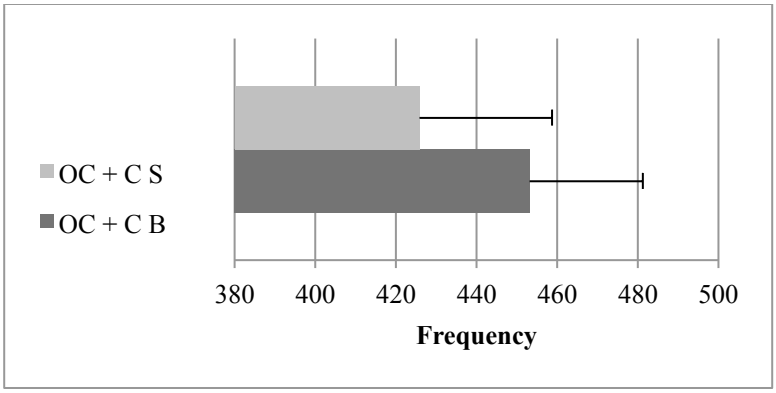


Figure 24. Comparison of (**a**) Frequency and (**b**) Rank frequency of Aggressive behaviors for $OC_B + C_B$ vs. $OC_S + C_S$. Values are means $\pm$ SEM, N = 10.

24a.


OC + C S
OC + C B
380
400
420
440
460
480
500
Frequency

24b.

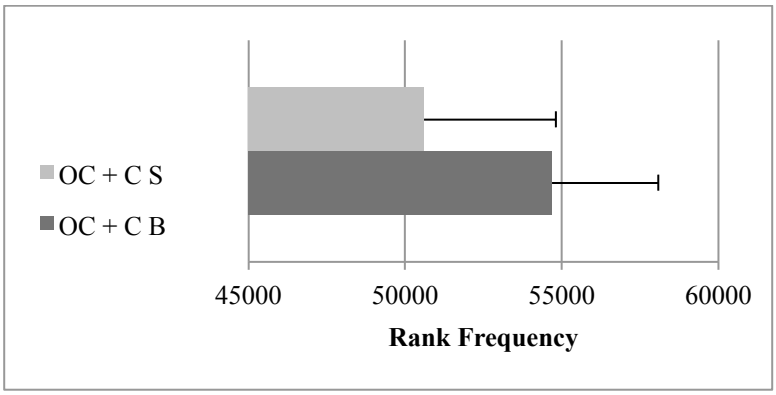

OC + C S
OC + C B
45000
50000
55000
60000
Rank Frequency

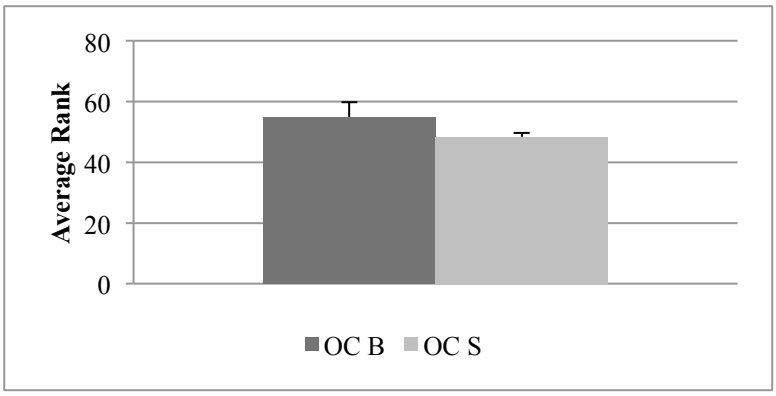
Figure 25a. Comparison of Average rank of Defensive behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N = 10.

Figure 25b. Comparison of Average rank of Defensive behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10.

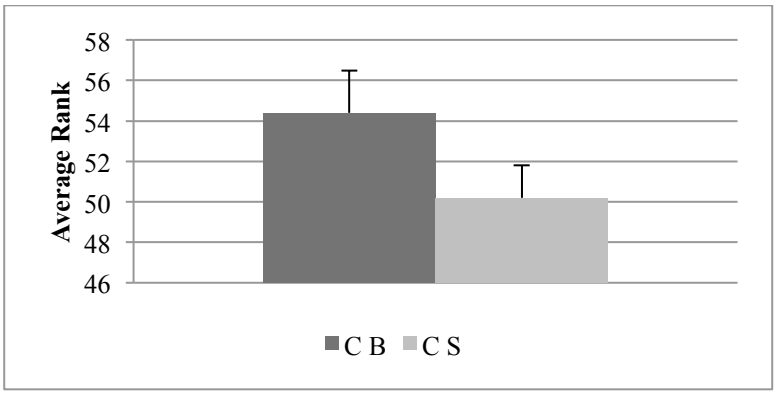
Figure 25c. Comparison of Average rank of Defensive behaviors for $OC_B - C_B$ vs. $OC_S - C_S$. Values are means $\pm$ SEM, N = 10.

Figure 25d. Comparison of Average rank of Defensive behaviors for $OC_B + C_B$ vs. $OC_S + C_S$. Values are means $\pm$ SEM, N = 10.

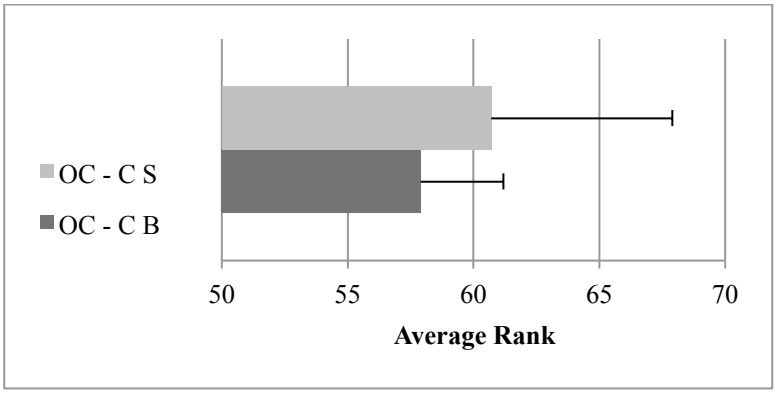
25a.



25b.



25c.



25d.

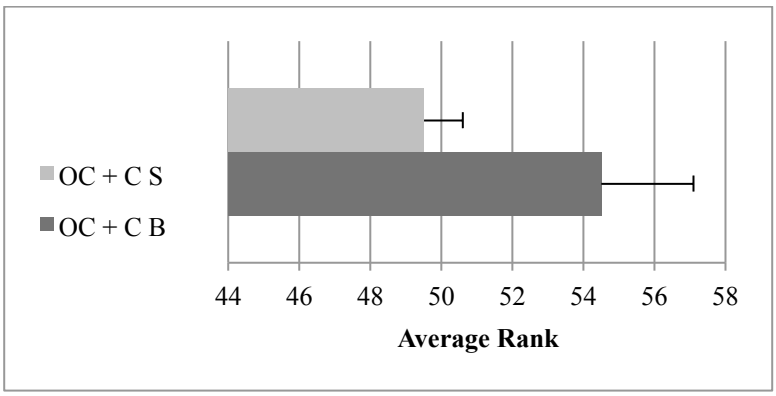
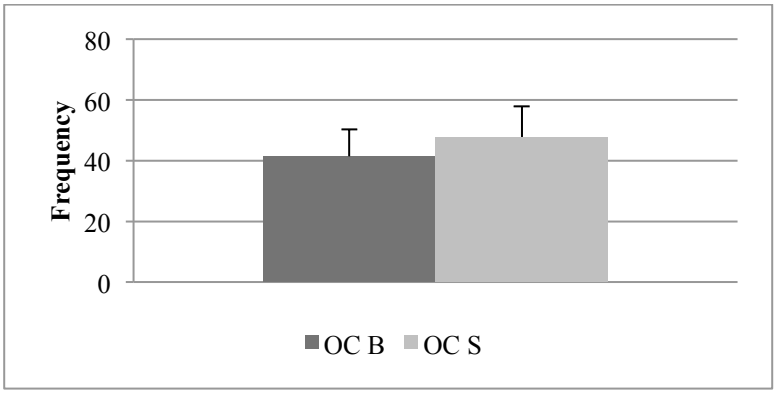


Figure 26. Comparison of (**a**) Frequency and (**b**) Rank frequency of Defensive behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM.

26a.


80
60
40
20
0
Frequency
OC B
OC S

26b.

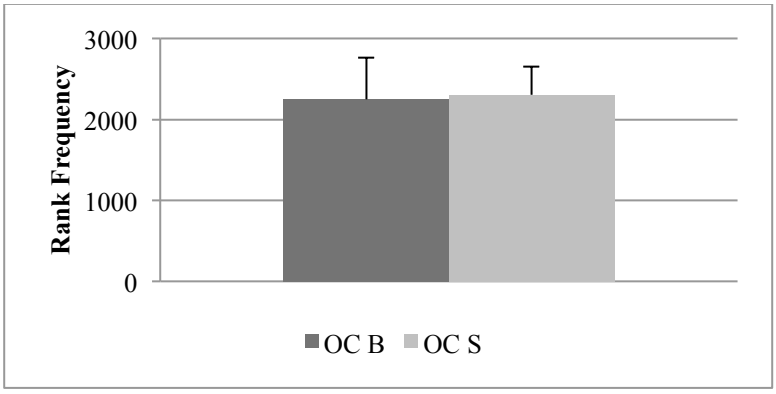

3000
2000
1000
0
Rank Frequency
OC B
OC S

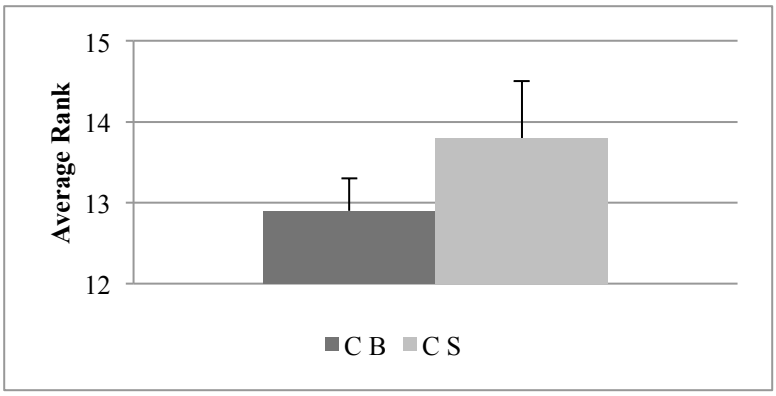
Figure 27a. Comparison of Average rank of Avoidance behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N = 10.

Figure 27b. Comparison of Average rank of Avoidance behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10.

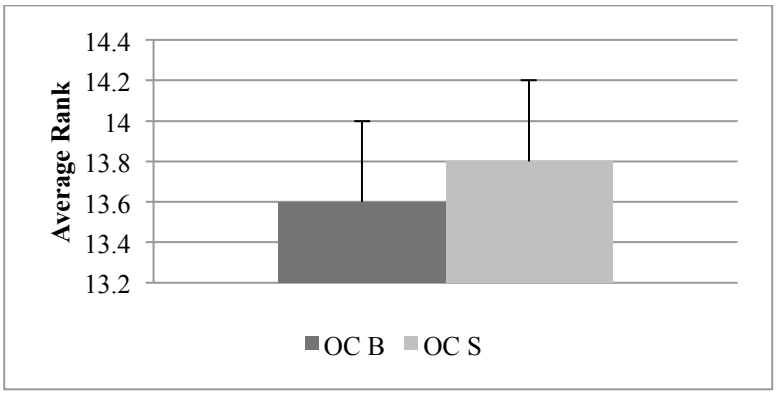
Figure 27c. Comparison of Average rank of Avoidance behaviors for $OC_B + C_B$ vs. $OC_S + C_S$. Values are means $\pm$ SEM, N = 10.

Figure 27d. Comparison of Average rank of Avoidance behaviors for $OC_B - C_B$ vs. $OC_S - C_S$. Values are means $\pm$ SEM, N = 10.

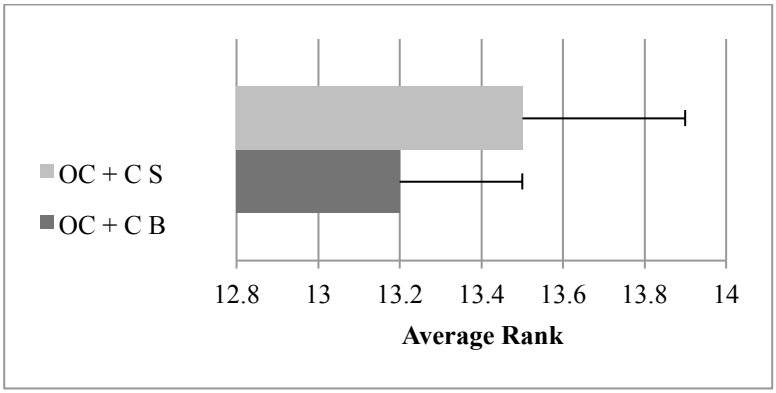
27a.



27b.



27c.



27d.

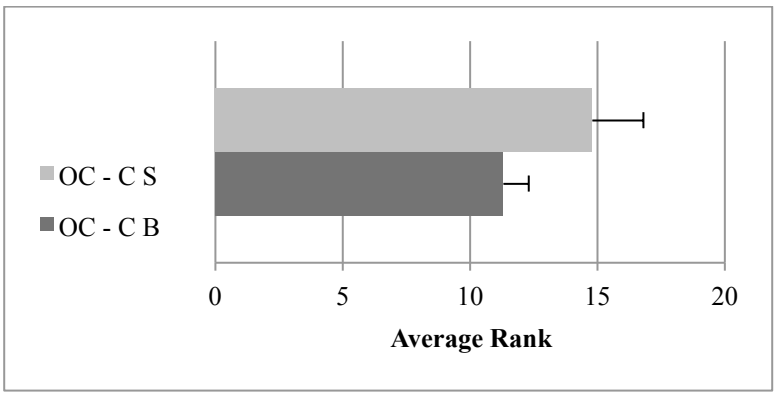
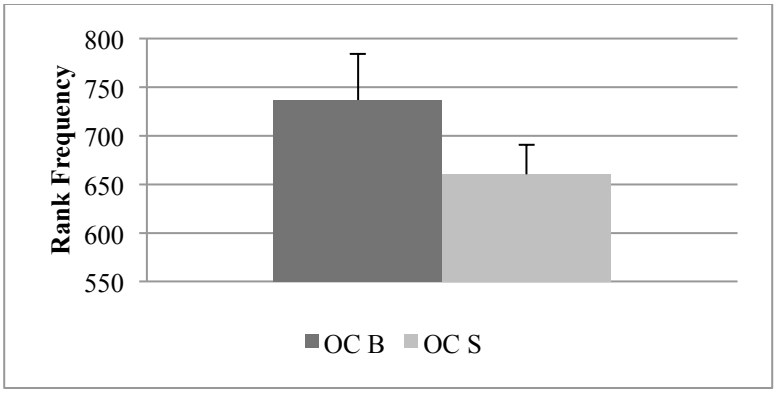


Figure 28a. Comparison of Rank frequency of Avoidance behaviors for OC_B vs. OC_S. Values are means $\pm$ SEM, N = 10.

Figure 28b. Comparison of Rank Frequency of Avoidance behaviors for $OC_B - C_B$ vs. $OC_S - C_S$. Values are means $\pm$ SEM, N = 10.

28a.


800
750
700
650
600
550
Rank Frequency
OC B
OC S

28b.

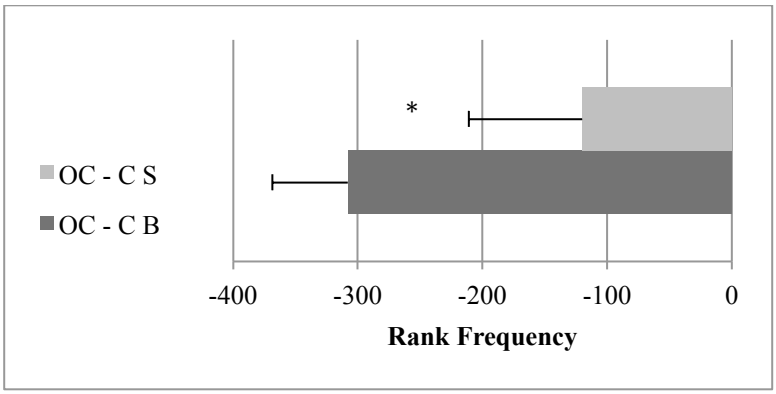

*
OC - C S
OC - C B
-400
-300
-200
-100
0
Rank Frequency

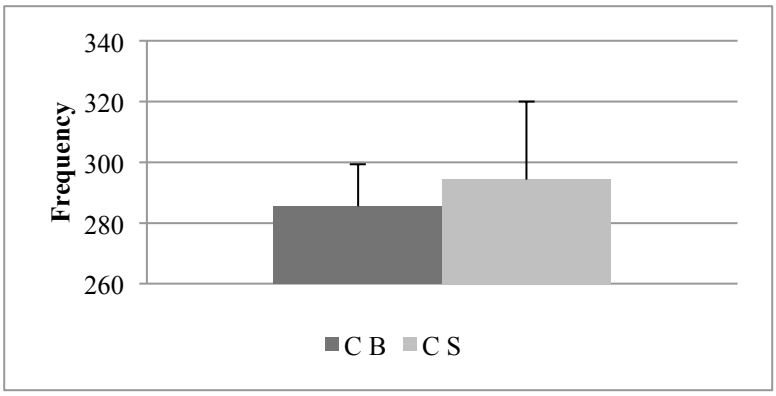
Figure 29a. Comparison of Frequency of All behaviors for C_B vs. C_S. Values are means $\pm$ SEM, N =10.

Figure 29b. Comparison of Frequency of All behaviors for $OC_B + C_B$ vs. $OC_S + C_s$. Values are means $\pm$ SEM, N = 10.

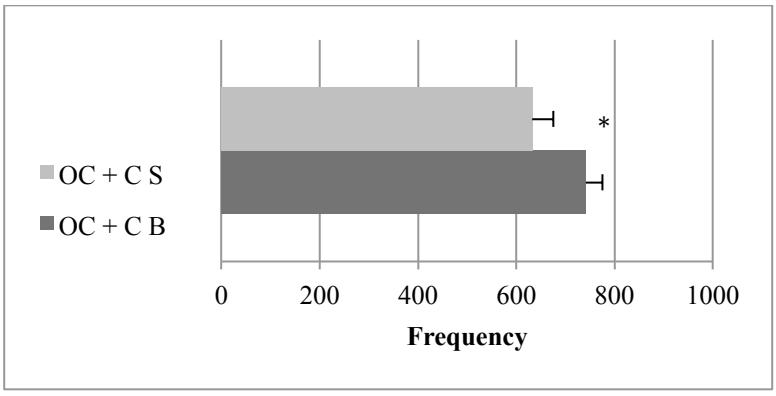
Figure 29c. Comparison of Rank frequency of All behaviors for $OC_B + C_B$ vs. $OC_S + C_S$. Values are means $\pm$ SEM.

Figure 29d. Comparison of Average rank of All behaviors for $OC_B + C_B$ vs. $OC_S + C_S$. Values are means $\pm$ SEM.

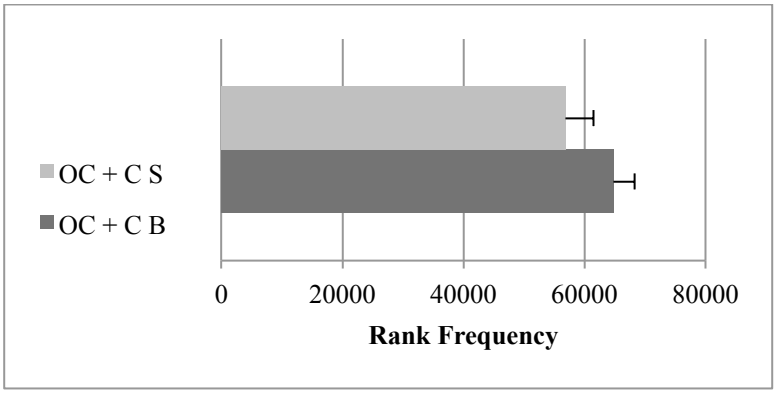
29a.



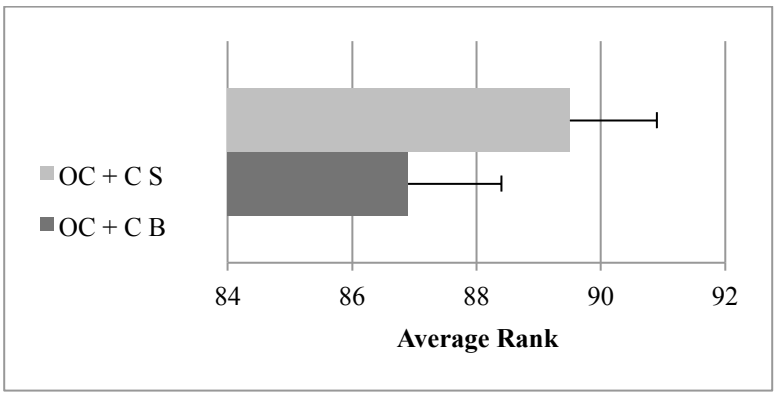
29b.



29c.



29d.



APPENDIX B

Ancillary material for Chapter 4:

Effect of injected ecdysteroids on force generation in the claw closer muscle of the American lobster, *Homarus americanus*

Force transducer:

The force transducer (Figure 1) has two squeezing surfaces (top and bottom) with two different strain gauges, and each strain gauge was calibrated by hanging known weights. The calibration graphs with subsequent best-fit equations for each strain gauge are given below (Figure 3 and Figure 4). Two different force transducers were constructed green and red, based on the color of hook-up wire used. Normally, on a given experimental night, I would use each transducer for half of the experiments in order to ensure that no one transducer was over-used. The same transducer was used for pre- and post-squeezes on the same lobster.

Since there are two squeezing surfaces and two strain gauges, the total force exerted by the claw was obtained by adding the force of each strain gauge. This accounts for the force that is exerted on each squeezing surface, and not just the top or bottom squeezing surface. In order to accurately asses the force, it is necessary to include the force exerted on both squeezing surfaces.

Analysis of integral of squeeze:

Along with force and duration described in the manuscript, we also analyzed the integral of the squeeze (area under the curve). The integral was calculated using the Power Lab integral function, and the maximum pre-squeeze integral was subtracted from the maximum post-squeeze integral. No significant differences were found for the integral for any parameters measured, however, a potentially significant trend was found in female cutter claws treated with 20E (Student's t-test, $p = 0.06$) (Table 1). These results suggest that 20E potentially lowers the force duration, which

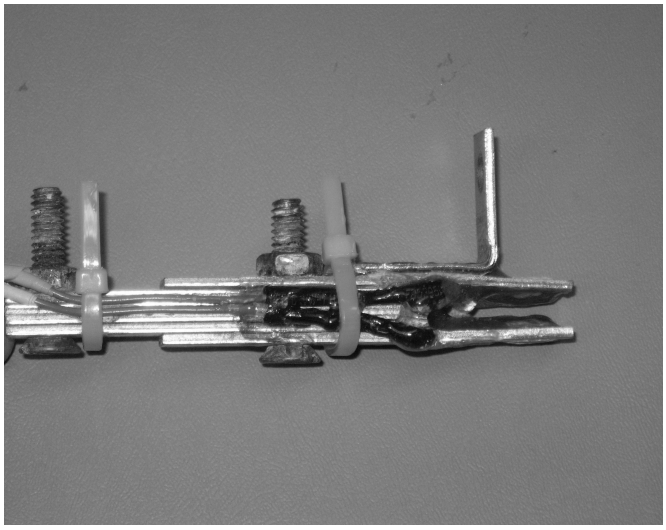
is in contrast the fact that the force itself is potentially greater in the presence of 20E, and that the duration was not significantly affected.

Figures

Figure 1a. Force transducer.

Figure 1b. Lobster squeezing transducer

1a.



1b.

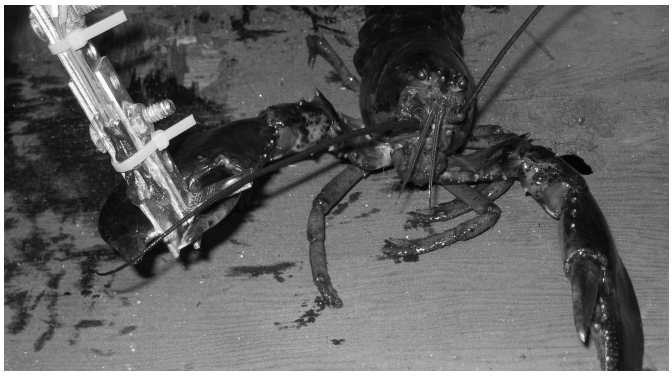


Figure 2a. Experimental set-up.

Force transducer is inside the Tupperware bin, connected to the Wheatstone bridges on the table.

Figure 2b. Wheatstone bridges and Power Lab.

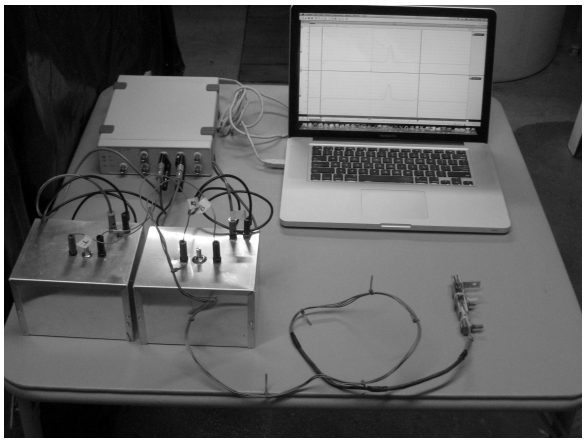
Transducer is on the table, leading into the Wheatstone bridges, which are connected to the Power Lab.

Figure 2c. Diagram of entire measuring circuit.

Each strain gauge glued on the underside of the transducer squeezing surface connect to their own Wheatstone bridge. The first Wheatstone bridge connects to Channel 1 on the Power Lab, the second Wheatstone bridge connects to Channel 2. The Power Lab connects to the computer.

2a.

2b.



2c.

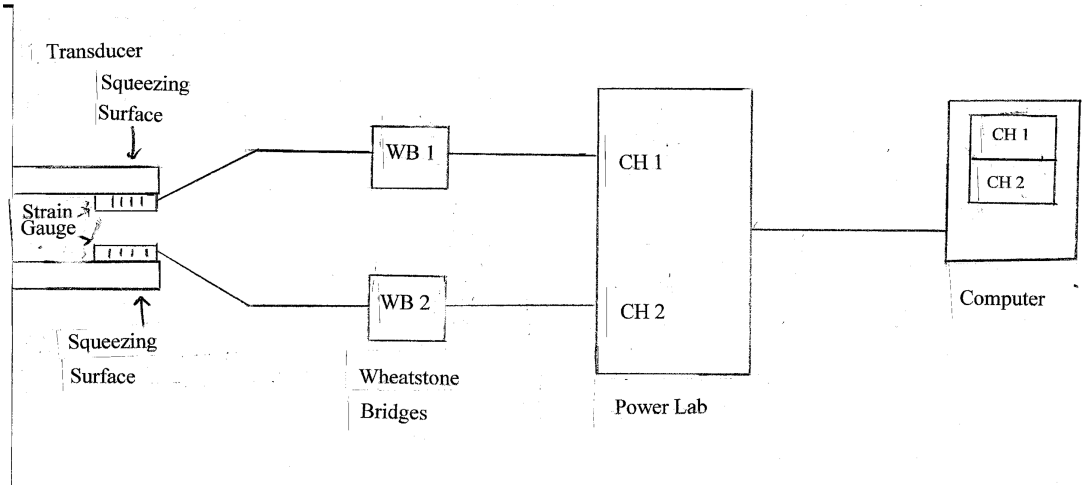

Transducer
Squeezing
Surface
Strain
Gauge
Squeezing
Surface
WB 1
WB 2
Wheatstone
Bridges
CH 1
CH 2
Power Lab
CH 1
CH 2
Computer

Figure 3. Wheatstone bridge circuit.

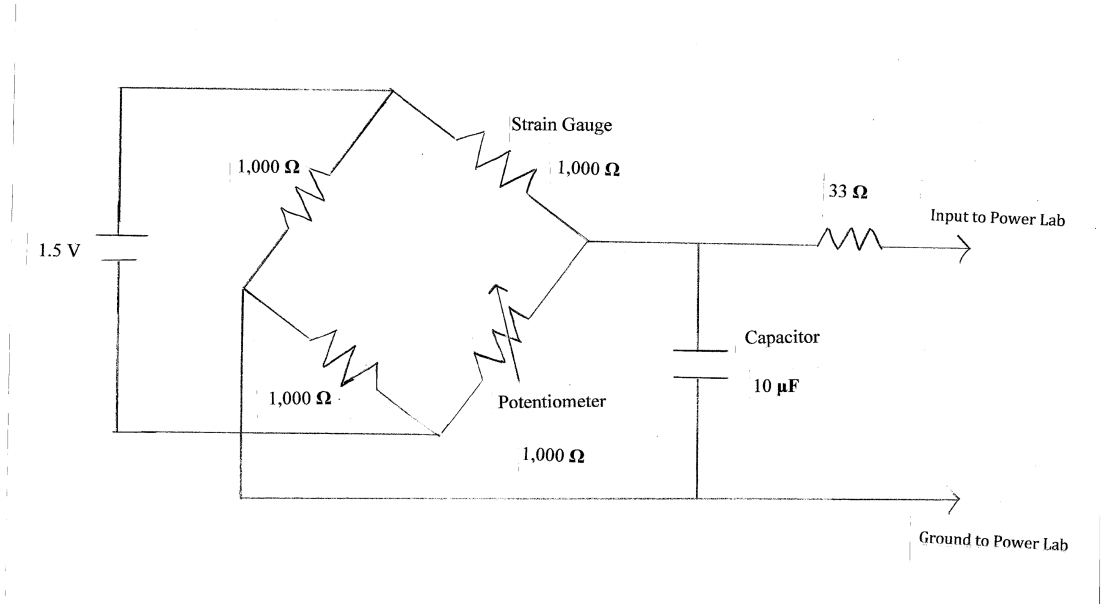
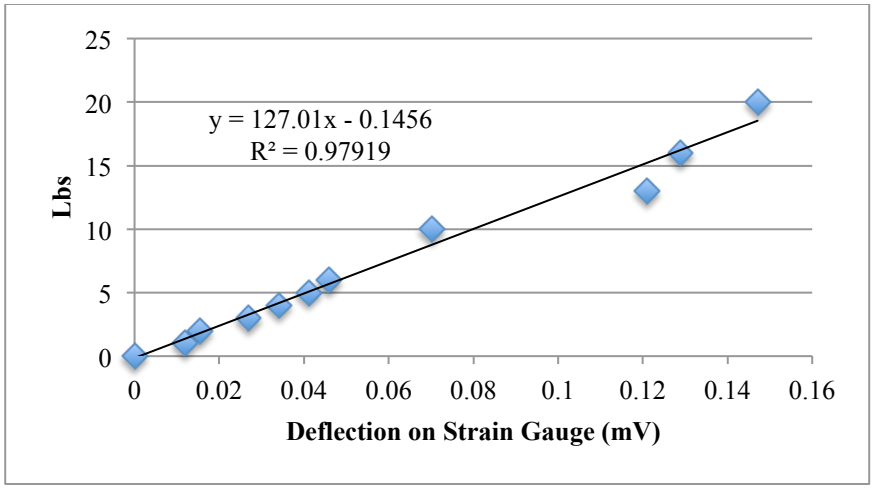

Strain Gauge
1,000 Ω
1,000 Ω
33 Ω
Input to Power Lab
1.5 V
Capacitor
10 µF
1,000 Ω
Potentiometer
1,000 Ω
Ground to Power Lab

Figure 4. Calibration graph for (**a**) the top strain gauge and (**b**) the bottom strain gauge of the green transducer.

Best-fit line equation is included in graph.

4a.


y = 127.01x - 0.1456
R² = 0.97919
Lbs
Deflection on Strain Gauge (mV)
0
5
10
15
20
25
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16

4b.

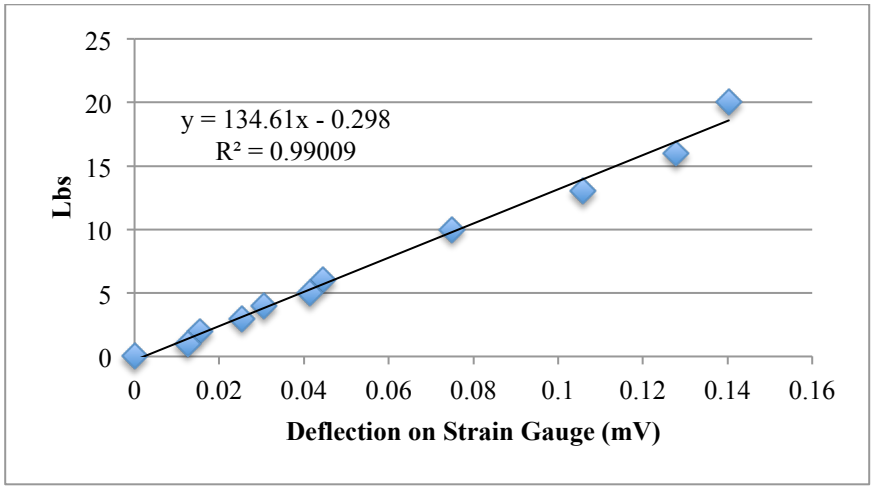
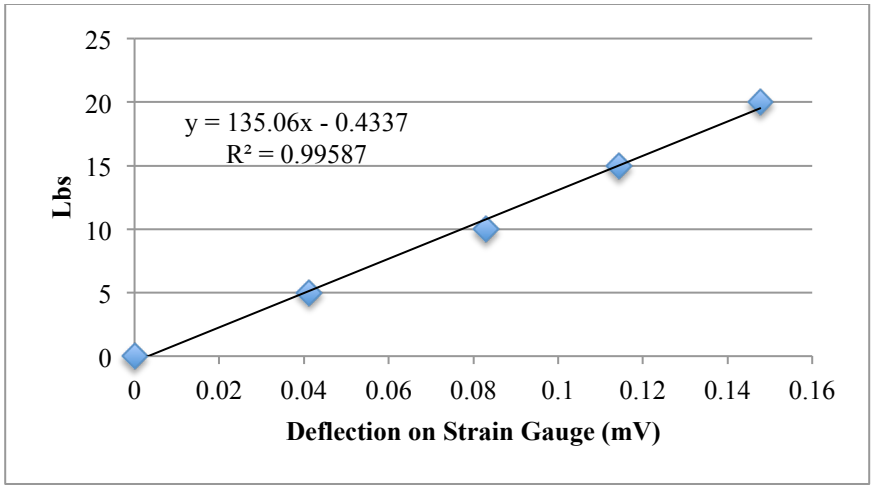

y = 134.61x - 0.298
R² = 0.99009
Lbs
Deflection on Strain Gauge (mV)
0
5
10
15
20
25
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16

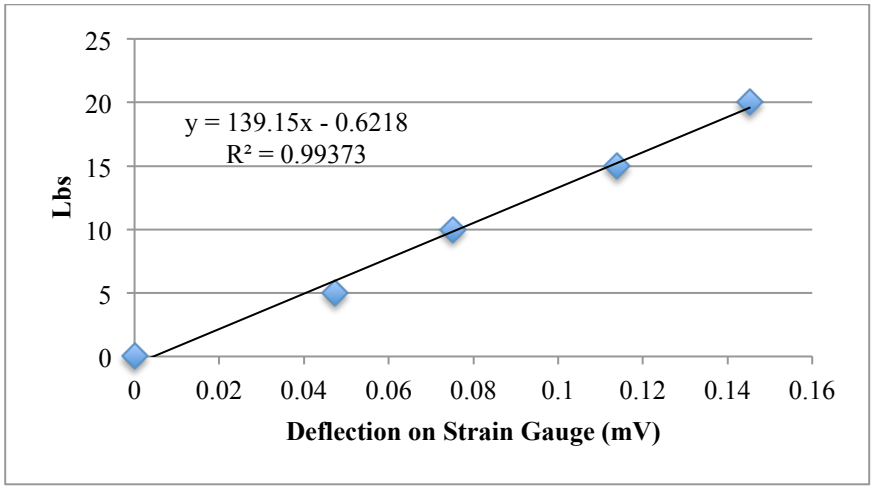
Figure 5. Calibration graph for (**a**) the top strain gauge and (**b**) the bottom strain gauge of the red transducer.

Best-fit line equation is included in graph.

5a.


y = 135.06x - 0.4337
R² = 0.99587
Lbs
25
20
15
10
5
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
Deflection on Strain Gauge (mV)

5b.


y = 139.15x - 0.6218
R² = 0.99373
Lbs
25
20
15
10
5
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
Deflection on Strain Gauge (mV)

Summary and Raw Data Tables

Table 1. Summary of the average difference between post-squeezes and pre-squeezes for force, duration and integral.

Values are means $\pm$ SEM. Force measured in pounds, duration measured in seconds and integral measured in lbs/sec. Significant P-values are in bold.

FEMALE	20E	Saline	P
Force			
Crusher	24.5 ± 23.1 (N=8)	-12.5 ± 5.2 (N=8)	0.07
Cutter	-6.63 ± 4.9 (N=9)	-3.98 ± 2.9 (N=9)	0.3
Duration			
Crusher	0.44 ± 0.8 (N=8)	1.71 ± 1.2 (N=8)	0.2
Cutter	-1.38 ± 0.4 (N=9)	-1.13 ± 0.9 (N=9)	0.2
Integral			
Crusher	38.0 ± 32.8 (N=8)	-4.35 ± 12.7 (N=8)	0.1
Cutter	-1.38 ± 8.1 (N=9)	-12.35 ± 8.1 (N=9)	0.06

FEMALE	Alpha	Saline	P
Force			
Crusher	16.1 ± 9.5 (N=8)	-12.5 ± 5.2 (N=8)	**0.009**
Cutter	4.07 ± 2.4 (N=9)	-3.98 ± 2.9 (N=9)	**0.02**
Duration			
Crusher	1.16 ± 0.8 (N=8)	1.71 ± 1.2 (N=8)	0.4
Cutter	-0.32 ± 0.7 (N=9)	-1.13 ± 0.9 (N=9)	0.2
Integral			
Crusher	17.6 ± 8.3 (N=8)	-4.35 ± 12.7 (N=8)	0.08
Cutter	-1.38 ± 8.1 (N=9)	-12.35 ± 8.1 (N=9)	0.1

Male

MALE	20E	Saline	P
Force			
Crusher	-4.29 ± 16.6 (N=7)	-2.58 ± 3.3 (N=8)	0.5
Cutter	2.7 ± 3.4 (N=9)	0.93 ± 3.7 (N=8)	0.4
Duration			
Crusher	1.03 ± 0.8 (N=7)	0.39 ± 0.6 (N=8)	0.3
Cutter	-0.18 ± 0.7 (N=9)	0.17 ± 0.9 (N=8)	0.4
Integral			
Crusher	-3.16 ± 10.6 (N=7)	-1.31 ± 2.9 (N=8)	0.4
Cutter	2.54 ± 3.6 (N=9)	1.91 ± 11.5 (N=8)	0.5

Male

MALE	Alpha	Saline	P
Force			
Crusher	1.32 ± 4.7 (N=6)	-2.58 ± 3.3 (N=8)	0.2
Cutter	8.49 ± 10.2 (N=7)	0.93 ± 3.7 (N=8)	0.2
Duration			
Crusher	-0.89 ± 3.1 (N=6)	0.39 ± 0.6 (N=8)	0.3
Cutter	-0.58 ± 0.7 (N=7)	0.17 ± 0.9 (N=8)	0.3
Integral			
Crusher	-2.91 ± 12.7 (N=6)	-1.31 ± 2.9 (N=8)	0.4
Cutter	9.79 ± 17.1 (N=7)	1.91 ± 11.5 (N=8)	0.4

Table 2. Raw data for female cutter claws treated with 20E.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

20 HYROXYECDYSONE FEMALE CUTTER

		P	R	E			PO	S	T	
Lobster		**1**	**2**	**3**	**X**		**1**	**2**	**3**	**X**
1	Force	9.05024	2.07353	3.91072	**5.01149667**		2.2790338	0.8750314	2.0694998	**1.74118833**
	Duration	3.401	1.152	2.278	**2.277**		3.381	1.821	2.0694998	**2.42383327**
	Integral	18.44151	0.9958	4.796	**8.07777**		3.694	0.23008	1.465172	**1.79641733**
2	Force	32.85622	33.08084	10.043807	**25.3269557**		3.962466	1.0962965	0.31555	**1.7914375**
	Duration	4.112	2.387	2.213	**2.904**		1.486	1.587	1.218	**1.43033333**
	Integral	50.5115	35.2392	9.9903	**31.9136667**		1.3872	0.7714	0.2684	**0.809**
3	Force	6.9576789	8.205262	6.4686547	**7.21053187**		15.428826	5.2750466	13.006813	**11.2368952**
	Duration	5.965	5.796	6.767	**6.176**		2.87	7.285	5.503	**5.21933333**
	Integral	29.17737	37.7142	30.0137	**32.3017567**		20.258	22.5989	47.0978	**29.9849**
4	Force	52.385949	12.71815	23.031812	**29.378637**		20.21814	16.74519	1.6211091	**12.8614797**
	Duration	1.299	1.277	4.126	**2.234**		3.433	3.293	3.032	**3.25266667**
	Integral	29.9571	6.72167	29.32268	**22.0004833**		33.05283	22.01699	3.06797	**19.3792633**
5	Force	5.420121	4.867652	6.570493	**5.619422**		8.0737382	2.384693	1.1509879	**3.86980637**
	Duration	2.614	5.001	2.739	**3.45133333**		1.1	4.119	1.969	**2.396**
	Integral	3.42263	15.0197	10.8539	**9.76541**		3.24536	5.4706	0.58294	**3.09963333**
6	Force	8.230884	3.614534	15.847904	**9.23110733**		2.465016	8.242045	8.7287098	**6.47859027**
	Duration	4.319	1.2	1.318	**2.279**		1.195	1.105	2.646	**1.64866667**
	Integral	13.98617	1.57795	9.61035	**8.39149**		0.75038	3.5754	6.6108	**3.64552667**
7	Force	4.1154112	2.0223546	4.8297033	**3.65582303**		13.417941	3.7454413	7.414291	**8.19255777**
	Duration	4.072	1.983	1.947	**2.66733333**		6.269	1.354	5.335	**4.31933333**
	Integral	10.58749	1.88163	4.36691	**5.61201**		52.5196	5.135171	25.07875	**27.5778403**
8	Force	4.0441994	1.7858803	3.0963363	**2.975472**		8.000834	3.0917299	8.498502	**6.5303553**
	Duration	3.332	1.675	3.96	**2.989**		3.321	3.308	2.327	**2.98533333**
	Integral	6.14769	1.08969	6.76848	**4.66862**		15.14099	3.64632	9.78153	**9.52294667**
9	Force	6.0067644	4.0312329	13.552694	**7.86356377**		1.324143	4.282848	7.2760595	**4.29435017**
	Duration	4.66	5.308	1.247	**3.73833333**		2.421	4.961	4.158	**3.84666667**
	Integral	13.9001	9.35407	6.33334	**9.86250333**		1.12048	18.90263	19.45512	**13.15941**

Table 3. Raw data for male cutter claws treated with 20E.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

20 HYDROXYECDYSONE MALE CUTTER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	16.41445	7.229097	7.4803	**10.3746157**		1.7394154	10.8913	5.046495	**5.89240347**
	Duration	1.566	1.544	1.253	**1.45433333**		1.995	1.295	1.846	**1.712**
	Integral	10.25008	4.788405	3.843889	**6.29412467**		1.443891	5.60901	3.687419	**3.58010667**
2	Force	5.84916	2.9654195	10.130628	**6.31506917**		1.856462	19.569801	2.311837	**7.9127**
	Duration	2.218	1.565	5.87	**3.21766667**		1.376	4.41	2.329	**2.705**
	Integral	4.84816	1.54241	40.98065	**15.7904067**		0.316377	39.44593	3.30927	**14.3571923**
3	Force	1.6927894	5.6614912	8.0138481	**5.12270957**		2.7466138	5.445809	5.1909274	**4.46111673**
	Duration	4.061	4.903	4.331	**4.43166667**		5.659	9.568	7.421	**7.54933333**
	Integral	2.7334	19.10785	31.8089	**17.8833833**		9.68815	29.4948	35.6451	**24.9426833**
4	Force	10.488074	8.4567366	2.0694171	**7.00474257**		34.503811	10.686512	6.6399226	**17.2767485**
	Duration	4.553	2.925	2.336	**3.27133333**		3.026	5.295	1.027	**3.116**
	Integral	17.9146	13.5642	3.13557	**11.5381233**		44.7325	25.35491	2.250921	**24.112777**
5	Force	10.82793	3.2836501	5.399905	**6.50382837**		2.379071	1.419534	0.5097716	**1.43612553**
	Duration	5.076	1.412	4.121	**3.53633333**		1.922	1.721	2.022	**1.88833333**
	Integral	24.862094	1.08082	13.64801	**13.1969747**		1.566144	1.08082	13.64801	**5.431658**
6	Force	2.0377019	5.5502019	1.349123	**2.97900893**		16.247852	3.9316046	7.8473679	**9.34227483**
	Duration	1.679	5.062	3.863	**3.53466667**		3.506	1.647	4.395	**3.18266667**
	Integral	1.47618	11.86034	0.80522	**4.71391333**		21.0363	3.17933	18.61127	**14.2756333**
7	Force	4.9390454	2.1902784	0.8979451	**2.6757563**		4.6085004	1.8678749	0.8932864	**2.4565539**
	Duration	0.896	0.924	1.578	**1.13266667**		1.042	0.731	1.421	**1.06466667**
	Integral	1.52573	0.61455	0.2789	**0.80639333**		1.77687	0.65035	0.0492	**0.82547333**
8	Force	1.478924	0.9718518	3.1894581	**1.88007797**		2.5103406	3.2769202		**2.8936304**
	Duration	3.722	1.157	2.048	**2.309**		1.272	0.935		**1.1035**
	Integral	3.19293	0.129525	3.73638	**2.352945**		0.83771	0.314944		**0.576327**
9	Force	2.7384553	8.8701596	4.470295	**5.35963663**		2.653336	2.9784501	5.941867	**3.85788437**
	Duration	2.445	2.936	1.513	**2.298**		2.215	2.278	3.298	**2.597**
	Integral	7.5692	16.2052	0.21222	**7.99554**		1.53382	3.29471	19.233896	**8.02080867**

Table 4. Raw data for female cutter claws treated with alpha-ecdysone.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

ALPHA FEMALE CUTTER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	2.0963914	4.0552874	7.2105458	**4.45407487**		3.80311	0.1122303	2.0866042	**2.00064817**
	Duration	3.038	1.158	8.552	**4.24933333**		4.926	1.89	1.437	**2.751**
	Integral	3.9492	2.08098	42.6461	**16.2254267**		10.56003	0.1123	0.49305	**3.72179333**
2	Force	2.1162904	4.756125	2.0992211	**2.9905455**		14.5526	12.453263	3.8979304	**10.3012645**
	Duration	2.303	1.811	2.892	**2.33533333**		2.467	2.092	1.083	**1.88066667**
	Integral	3.26062	4.08819	2.76836	**3.37239**		16.25135	12.2709	0.850569	**9.79093967**
3	Force	2.8569911	1.25455	0.4215512	**1.51103077**		8.3567185	2.26986	1.8205912	**4.14905657**
	Duration	10.772	0.931	6.87	**6.191**		13.803	1.922	2.522	**6.08233333**
	Integral	43.3988	0.1831015	3.405206	**15.6623692**		58.3822	1.530522	1.422145	**20.4449557**
4	Force	11.620538	11.136651	6.195657	**9.65094867**		6.088306	4.230468	3.912969	**4.74391433**
	Duration	5.819	7.592	7.841	**7.084**		6.402	4.248	3.942	**4.864**
	Integral	46.4957	67.6752	43.1189	**52.4299333**		21.3827	5.92187	4.79013	**10.6982333**
5	Force	2.281925	6.010635	5.018789	**4.43711633**		24.345887	22.661675		**23.503781**
	Duration	1.075	1.935	1.528	**1.51266667**		1.636	4.215		**2.9255**
	Integral	0.34983	6.56697	2.17349	**3.03009667**		17.61763	38.0856		**27.851615**
6	Force	6.1590022	7.7475831	5.8126385	**6.5730746**		5.1424132	1.0078345	6.5197083	**4.22331867**
	Duration	4.116	4.616	3.223	**3.985**		1.57	1.536	2.308	**1.80466667**
	Integral	17.11929	7.4282	11.2811	**11.9428633**		3.14623	0.45828	7.1545	**3.58633667**
7	Force	2.0173803	1.8494342	1.085495	**1.65076983**		3.2159073	5.4165489	8.7867441	**5.8064001**
	Duration	1.728	1.338	2.463	**1.843**		2.504	1.291	2.561	**2.11866667**
	Integral	0.65345	1.32577	1.04323	**1.00748333**		3.8673	2.9555	13.43059	**6.75113**
8	Force	5.7878406	3.00682	7.43125	**5.40863687**		10.337351	8.741443	10.821661	**9.96681833**
	Duration	4.468	5.489	4.25	**4.73566667**		3.596	2.969	3.454	**3.33966667**
	Integral	14.42448	10.71646	23.99829	**16.3797433**		20.10671	14.83494	26.05856	**20.3334033**
9	Force	2.2156507	2.1692096	3.298174	**2.56101143**		6.290429	4.221033	4.0357357	**4.8490659**
	Duration	1.626	1.214	2.051	**1.63033333**		1.096	1.945	3.411	**2.15066667**
	Integral	0.53263	1.034485	2.174711	**1.24727533**		2.298818	1.63761	5.23895	**3.05845933**

Table 5. Raw data for male cutter claws treated with alpha-ecdysone.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

ALPHA MALE CUTTER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	5.3885447	2.5736841	3.1265481	**3.69625897**		44.23486	73.14291	21.332299	**46.2366897**
	Duration	3.437	2.744	3.932	**3.371**		5.939	2.274	5.35	**4.521**
	Integral	7.9951	2.7537	8.02254	**6.25711333**		112.7609	94.5897	37.7585	**81.7030333**
2	Force	4.7777346	2.3377137	0.679178	**2.59820877**		16.724276	9.72766	11.961157	**12.8043643**
	Duration	2.539	8.285	3.764	**4.86266667**		7.335	1.797	9.481	**6.20433333**
	Integral	6.38205	28.3209	1.94206	**12.2150033**		19.75322	9.16116	53.1423	**27.3522267**
3	Force	8.908907	17.282613	6.268309	**10.819943**		8.5410046	2.799784	3.204825	**4.84853787**
	Duration	1.85	2.424	3.071	**2.44833333**		1.258	2.669	1.27	**1.73233333**
	Integral	8.71012	26.09908	10.41885	**15.0760167**		3.8687	3.31483	0.040554	**2.408028**
4	Force	26.55169	2.2945772	1.1073346	**9.98453393**		0	0	0	**0**
	Duration	5.703	2.609	5.502	**4.60466667**		0	0	0	**0**
	Integral	73.3926	1.769496	7.916534	**27.6928767**		0	0	0	**0**
5	Force	7.042225	5.9932256	9.337486	**7.45764553**		2.628566	2.4819466	4.748393	**3.28630187**
	Duration	4.446	4.157	5.496	**4.69966667**		0.919	1.826	2.162	**1.63566667**
	Integral	21.05614	6.18374	31.88079	**19.70689**		0.68249	1.502299	3.9206	**2.03512967**
6	Force	5.5178157	4.8218516	2.869386	**4.40301777**		2.989142	3.8065596	3.7888819	**3.5281945**
	Duration	1.004	1.254	2.16	**1.47266667**		1.33	1.163	1.446	**1.313**
	Integral	1.847595	2.95138	1.460013	**2.08632933**		0.82702	1.188874	1.78564	**1.267178**
7	Force	1.8408189	1.2796292	1.7035623	**1.60800347**		2.4278923	1.5889607	1.1402959	**1.71904963**
	Duration	1.464	4.23	1.518	**2.404**		3.178	3.194	1.472	**2.61466667**
	Integral	1.8116	3.2658	0.7605	**1.94596667**		2.69223	2.03968	0.32764	**1.68651667**
8	Force	1.1102927	1.5567071		**1.3334999**		0	0	0	**0**
	Duration	2.253	2.356		**2.3045**		0	0	0	**0**
	Integral	0.669199	1.84997		**1.2595845**		0	0	0	**0**
9	Force	8.768083	10.019182	7.36514	**8.71746833**		4.201407	3.751551	4.030737	**3.994565**
	Duration	4.56	2.812	2.289	**3.22033333**		2.362	2.746	2.811	**2.63966667**
	Integral	14.0227	2.17844	5.93632	**7.37915333**		0.977096	4.90102	3.19959	**3.025902**

Table 6. Raw data for female cutter claws treated with saline.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

SALINE FEMALE CUTTER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	2.111865	2.94335	31.27713	12.1107817		2.30936	7.852285	4.025227	4.72895733
	Duration	1.848	0.95	3.12	1.97266667		2.537	2.148	1.293	1.99266667
	Integral	1.2985848	0.8163451	30.77143	10.96212		2.12463	8.2891	3.049067	4.487599
2	Force	10.831761	11.653966		11.2428635		3.44932	2.752365	1.719089	2.640258
	Duration	9.076	1.285		5.1805		1.759	1.33	2.2	1.763
	Integral	70.38408	7.0029		38.69349		2.22308	1.64456	0.738045	1.53522833
3	Force	17.280702	2.2256314	2.4050365	7.30378997		8.5056206	11.68752	2.800558	7.6645662
	Duration	2.842	4.513	1.792	3.049		1.077	1.301	1.325	1.23433333
	Integral	19.940612	8.50173	1.31174	9.91802733		3.9371	6.7754	1.599767	4.104089
4	Force	10.545247	10.62744	3.425951	8.199546		9.275045	6.584382	5.594311	7.151246
	Duration	2.429	2.66	2.247	2.44533333		1.427	1.061	0.933	1.14033333
	Integral	16.3437	17.4981	4.6471	12.8296333		4.28229	3.1282	0.9619	2.79079667
5	Force	0.7331126	1.006371		0.8697418		3.046287	1.421703	4.255225	2.90773833
	Duration	2.375	0.775		1.575		2.107	1.382	4.426	2.63833333
	Integral	0.30099	0.20506		0.253025		5.44961	0.58155	10.9947	5.67528667
6	Force	4.421081	2.0293956	4.275547	3.5753412		2.1392068	2.6725992	1.9078071	2.23987103
	Duration	4.061	1.319	2.747	2.709		2.965	2.236	1.605	2.26866667
	Integral	10.45342	0.62553	8.44608	6.50834333		3.00796	3.4107	0.49173	2.30346333
7	Force	7.1587137	6.1074344	4.9906132	6.0855871		5.0986852	4.31492	3.6835203	4.3657085
	Duration	1.998	3.929	2.029	2.652		3.169	4.574	3.789	3.844
	Integral	5.19425	8.62718	3.9981	5.93984333		5.64093	8.7749	6.16749	6.86110667
8	Force	14.035893	2.2430107	5.621238	7.30004723		5.9848118	9.6344805	8.702278	8.1071901
	Duration	4.14	0.794	3.093	2.67566667		1.82	3.696	2.079	2.53166667
	Integral	27.5367	0.427642	10.3005	12.7549473		4.6391	13.1606	3.1535	6.9844
9	Force	1.1021143	5.4014866	2.2761171	2.92657267		4.141713	13.07921	6.175599	7.79884067
	Duration	1.892	2.698	1.908	2.166		3.093	3.246	2.902	3.08033333
	Integral	0.46538	5.421684	2.0121	2.63305467		7.614	21.8438	8.2032	12.5536667

Table 7. Raw data for male cutter claws treated with saline.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

SALINE MALE CUTTER

		P	R	E			PO	S	T	
Lobster		**1**	**2**	**3**	**X**		**1**	**2**	**3**	X
1	Force	13.36587	4.2493358	3.5089495	**7.0413851**		4.9801255	1.0521745	4.1972836	**3.4098612**
	Duration	1.173	0.971	1.742	**1.29533333**		4.26	2.438	1.359	**2.68566667**
	Integral	6.8005	2.00693	3.05309	**3.95350667**		11.6801	1.4318	1.98415	**5.03201667**
2	Force	4.35008	3.3889333	17.816258	**8.51842377**		39.64905	7.157992	4.5555591	**17.120867**
	Duration	2.87	2.077	2.393	**2.44666667**		3.015	0.9444	5.717	**3.22546667**
	Integral	3.3282	2.43031	19.35799	**8.37216667**		70.2113	2.26635	12.7582	**28.41195**
3	Force	3.7639541	1.9576856		**2.86081985**		4.51378	1.3598968	3.1446835	**3.0061201**
	Duration	5.386	0.681		**3.0335**		3.186	2.139	2.351	**2.55866667**
	Integral	10.14905	0.492109		**5.3205795**		1.28563	0.422475	3.7321619	**1.8134223**
4	Force	7.988394	2.531566	9.053437	**6.52446567**		7.52571	4.6667706	5.254489	**5.81565653**
	Duration	2.126	0.882	1.598	**1.53533333**		5.357	3.713	5.186	**4.752**
	Integral	7.80827	0.4688	5.02851	**4.43519333**		28.8908	5.4662	22.1393	**18.8321**
5	Force	1.1841947	2.1489882	8.8229899	**4.0520576**		3.865064	5.3212614	1.8971428	**3.6944894**
	Duration	5.631	5.075	1.007	**3.90433333**		2.309	5.582	4.575	**4.15533333**
	Integral	9.73449	7.500276	4.279591	**7.17145233**		2.93491	15.85581	7.64573	**8.81215**
6	Force	5.7286417	4.5802871	16.53859	**8.94917293**		4.487174	5.9745329	2.7310704	**4.39759243**
	Duration	3.532	7.468	7.84	**6.28**		3.571	3.599	2.505	**3.225**
	Integral	11.3512	20.0919	71.8581	**34.4337333**		6.3199	7.1068	3.7219	**5.7162**
7	Force	7.8482329	4.657503	12.932196	**8.47931063**		3.8167295	12.0686207		**7.9426751**
	Duration	3.101	2.59	3.409	**3.03333333**		4.581	4.295		**4.438**
	Integral	9.3788	2.25287	20.3163	**10.6493233**		5.64134	15.4856		**10.56347**
8	Force	0.4707664	1.1689532	2.9530533	**1.5309243**		12.69436	7.495322	7.0121009	**9.06726097**
	Duration	1.045	1.75	3.283	**2.026**		1.275	0.929	1.401	**1.20166667**
	Integral	0.0582	0.271759	2.78977	**1.03990967**		11.17166	3.0094	1.55801	**5.24635667**

Table 8. Raw data for female crusher claws treated with 20E.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

20 HYROXYECDYSONE FEMALE CRUSHER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	5.4033198	2.4031463		3.90323305		1.3632788	13.386459	3.843526	6.1977546
	Duration	2.194	4.355		3.2745		4.971	5.567	2.392	4.31
	Integral	7.3742	6.11705		6.745625		5.93536	34.6834	4.88955	15.1694367
2	Force	1.580466	2.235838		1.908152		1.239145			1.239145
	Duration	1.695	0.716		1.2055		2.831			2.831
	Integral	0.946826	0.01075		0.478788		1.7253			1.7253
3	Force	2.853289	2.8127007	0.2913144	1.98576803		3.9341404			3.9341404
	Duration	1.611	5.662	2.027	3.1		1.471			1.471
	Integral	2.1737616	11.96262	0.85022	4.99553387		2.27104			2.27104
4	Force	29.833238	4.028276	99.98436	44.6152913		162.5798	3.912		83.2459
	Duration	6.252	7.315	2.033	5.2		6.579	3.074		4.8265
	Integral	63.65756	12.856098	54.15795	43.5572027		317.2589	4.331841		160.795371
5	Force	93.749105	3.1097436		48.4294243		5.353455	0.425355	3.781853	3.18688767
	Duration	2.459	3.159		2.809		1.297	1.141	5.818	2.752
	Integral	69.7225	5.67402		37.69826		1.42509	0.30578	9.85741	3.86276
6	Force	4.5777294	35.079968	6.540451	15.3993828		153.00642	36.421484	10.321377	66.5830937
	Duration	5.817	4.62	5.44	5.29233333		2.123	5.071	8.928	5.374
	Integral	10.9327	50.3442	18.1236	26.4668333		93.2639	48.2922	43.6534	61.7365
7	Force	6.126222	5.9012522	6.260089	6.0958544		6.9716	5.4095012	6.4588522	6.27998447
	Duration	7.487	6.795	2.076	5.45266667		6.582	5.065	9.09	6.91233333
	Integral	18.48322	13.62549	5.7084	12.6057033		20.1312	20.1986	44.7157	28.3485
8	Force	1.8452	3.293036	3.6758522	2.9380294		99.084295	8.5278892	10.335663	39.3159491
	Duration	4.197	3.354	7.698	5.083		1.142	6.385	2.159	3.22866667
	Integral	3.9637	6.8411	24.7176	11.8408		47.4834	27.5818	10.7131	28.5927667

Table 9. Raw data for male crusher claws treated with 20E.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

20 HYDROXYECDYSONE MALE CRUSHER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	1.8915267			1.8915267		4.637597	4.14425		4.3909235
	Duration	1.272			1.272		1.23	2.88		2.055
	Integral	0.508525			0.508525		2.3539	6.0436		4.19875
2	Force	0			0		0			0
	Duration				#DIV/0!					#DIV/0!
	Integral				#DIV/0!					#DIV/0!
3	Force	60.915129	5.668918	2.8912091	23.158419		37.67633	1.987263		19.8317965
	Duration	1.35	5.512	2.763	3.2083333		1.005	1.86		1.4325
	Integral	29.67	11.6899	3.5555	14.9718		14.72688	1.71511		8.220995
4	Force	7.5241017	4.375521	3.9878314	5.295818		2.5550196	9.724785	2.243246	4.84101687
	Duration	1.582	4.492	1.956	2.6766667		5.256	1.338	4.862	3.81866667
	Integral	2.991719	2.97433	2.074813	2.6802873		4.852285	4.74486	6.34614	5.31442833
5	Force	6.6809453	1.6916042		4.1862748		64.485541	8.3563113	1.1275936	24.656482
	Duration	1.204	1.911		1.5575		1.689	3.112	3.372	2.72433333
	Integral	5.7667	0.45955		3.113125		33.1535	17.02085	2.95023	17.7081933
6	Force	2.938888	48.224655	0.8534218	17.338988		41.980371	62.864058	0.6845968	35.1763419
	Duration	1.662	2.64	1.261	1.8543333		6.013	3.848	6.663	5.508
	Integral	0.61913	50.9878	0.69469	17.433873		48.9853	48.824	7.27101	35.02677
7	Force	47.821914	125.01961	0.768772	57.870099		37.66508	27.837087	23.24368	29.581949
	Duration	1.536	1.785	3.283	2.2013333		5.448	4.784	2.75	4.32733333
	Integral	28.948	89.2042	2.15373	40.101977		30.0467	21.0263	12.5592	21.2107333
8	Force	8.392836	1.8648873	10.566673	6.9414654		13.737613	1.25455	4.5257441	6.50596903
	Duration	1.539	5.23	3.652	3.4736667		5.867	6.036	4.656	5.51966667
	Integral	3.81975	7.939	17.1288	9.6291833		34.8457	5.4819	11.9647	17.4307667

Table 10. Raw data for female crusher claws treated with alpha-ecdysone.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

ALPHA FEMALE CRUSHER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	4.1232327	1.0254322		2.57433245		3.872523	5.36108		4.6168015
	Duration	2.048	1.176		1.612		2.131	5.987		4.059
	Integral	1.85748	0.33819		1.097835		2.27722	12.63111		7.454165
2	Force	6.4620822	17.2995041		11.8807932		2.25616	16.709701	6.254141	8.40666733
	Duration	2.679	3.114		2.8965		1.164	4.309	6.614	4.029
	Integral	11.02505	13.96978		12.497415		1.21361	37.0489	22.195	20.1525033
3	Force	19.016007	6.1979668	4.3844539	9.86614257		46.188313	18.45949	6.327329	23.6583773
	Duration	1.154	1.786	0.86	1.26666667		1.091	1.53	3.692	2.10433333
	Integral	8.59105	2.648805	1.2259	4.15525167		20.2162	10.37247	7.79861	12.79576
4	Force	2.9102606	2.9080909	6.432052	4.08346783		83.61964	24.60536		54.1125
	Duration	6.313	4.873	1.415	4.20033333		3.787	4.062		3.9245
	Integral	8.94725	1.112558	4.49087	4.850226		39.9458	16.3701		28.15795
5	Force	1.316461	4.8956512	6.4056144	4.20590887		12.19668	4.3620316	4.635549	7.06475353
	Duration	4.728	5.434	6.267	5.47633333		6.492	5.026	8.4	6.63933333
	Integral	1.48422	23.8111	19.0951	14.7968067		23.6025	19.1162	13.49617	18.73829
6	Force	3.319252	4.866476	3.7425206	3.97608287		24.539007	12.538366	2.68451187	13.2539616
	Duration	5.848	2.454	1.805	3.369		6.415	6.672	4.065	5.71733333
	Integral	6.0439	12.4208	5.1057	7.8568		79.7763	59.6664	3.1894	47.5440333
7	Force	4.769335	4.418475	5.261946	4.81658533		0.7062064	2.607511	4.9673238	2.76034707
	Duration	1.19	3.63	4.559	3.12633333		3.691	1.002	5.699	3.464
	Integral	1.84848	6.79377	10.4784	6.37355		1.3614	0.85804	8.8927	3.70404667
8	Force	2.726149	1.064553		1.895351		0.627891	0.4297553	0.9860591	0.68123513
	Duration	1.294	3.464		2.379		1.188	0.871	1.563	1.20733333
	Integral	0.85465	1.723996		1.289323		0.11961	0.18894	0.11886	0.14247

Table 11. Raw data for male crusher claws treated with alpha-ecdysone.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

ALPHA MALE CRUSHER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	2.2358375	1.7798716	1.1530958	**1.72293497**		5.3513928	6.9874244	2.4794605	**4.9394259**
	Duration	1.313	1.627	1.029	**1.323**		1.07	3.461	2.227	**2.25266667**
	Integral	1.3344	1.0122	0.31031	**0.88563667**		1.51939	13.47646	1.59549	**5.53044667**
2	Force	1.978007	5.3559765	3.731568	**3.68851717**		0			**0**
	Duration	1.014	3.769	2.302	**2.36166667**		0			**0**
	Integral	0.72696	8.50782	4.61861	**4.61779667**		0			**0**
3	Force	3.040371			**3.040371**		7.830557	2.509115		**5.169836**
	Duration	2.689			**2.689**		2.008	9.926		**5.967**
	Integral	4.3849			**4.3849**		6.02548	4.90439		**5.464935**
4	Force	24.814074	4.5575803	6.1058322	**11.8258288**		0.6180265	7.1564311	2.359978	**3.3781452**
	Duration	3.628	10.395	17.256	**10.4263333**		1.609	0.978	0.877	**1.15466667**
	Integral	25.5342	33.04821	66.10815	**41.56352**		0.38432	2.03026	0.29088	**0.90182**
5	Force	1.677643	3.6426414	4.8214846	**3.38058967**		1.3159302	2.112445	3.0968336	**2.1750696**
	Duration	2.635	0.976	0.94	**1.517**		9.4	2.352	3.784	**5.17866667**
	Integral	0.49282	1.21102	1.25012	**0.98465333**		8.94487	2.202144	8.48247	**6.54316133**
6	Force	0.7264654			**0.7264654**		1.1076897			**1.1076897**
	Duration	1.302			**1.302**		1.255			**1.255**
	Integral	0.378997			**0.378997**		0.9041			**0.9041**
7	Force	17.010786	5.355486	3.458778	**8.60835**		2.860073	34.36355		**18.6118115**
	Duration	1.129	2.338	3.983	**2.48333333**		4.091	4.002		**4.0465**
	Integral	7.30819	5.7553	9.7865	**7.61666333**		5.62519	34.4073		**20.016245**
8	Force	0.1578628			**0.1578628**		0			**0**
	Duration	1.317			**1.317**		0			**0**
	Integral	0.034172			**0.034172**		0			**0**

Table 12. Raw data for female crusher claws treated with saline.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

SALINE FEMALE CRUSHER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	26.060983			26.060983		5.92675	0.9060428	4.569534	3.8007756
	Duration	2.957			2.957		3.054	1.026	0.903	1.661
	Integral	24.75407			24.75407		4.7766	0.64676	0.23799	1.88711667
2	Force	7.4153053	24.343198		15.8792517		1.262941	4.736664	2.176143	2.72524933
	Duration	1.024	2.378		1.701		0.985	1.151	5.929	2.68833333
	Integral	4.0098	13.0905		8.55015		0.40306	1.6575	7.95615	3.33890333
3	Force	3.323264	12.7906156		8.0569398		1.739228	3.466564	1.442025	2.215939
	Duration	1.045	2.798		1.9215		1.378	0.926	1.003	1.10233333
	Integral	1.9688	4.7637		3.36625		0.50699	0.41017	0.51376	0.47697333
4	Force	8.062779	1.7201769	10.1942841	6.65908		6.681188	6.1058617	3.359399	5.38214957
	Duration	4.067	0.868	1.112	2.01566667		13.219	9.784	6.128	9.71033333
	Integral	15.93626	0.44415	3.11818	6.49953		74.8782	5.12215	12.50558	30.83531
5	Force	0.6820355	1.1829755		0.9325055		3.420018	3.744989	3.442364	3.53579033
	Duration	0.957	4.804		2.8805		6.008	1.607	3.55	3.72166667
	Integral	0.058398	2.6267		1.342549		9.8666	2.07859	4.93809	5.62776
6	Force	79.63378	6.599033	6.6294912	30.9541014		36.97463	1.68341	2.665876	13.7746387
	Duration	3.98	1.046	3.506	2.844		4.305	3.334	1.89	3.17633333
	Integral	103.2392	2.54193	12.3038	39.3616433		31.197	0.42316	0.13962	10.5865933
7	Force	6.3357	0.1781218	1.4491843	2.65433537		2.5060079	1.662414	0.313288	1.4939033
	Duration	2.765	3.202	4.29	3.419		3.701	1.881	4.363	3.315
	Integral	0.66179	0.7981	3.28838	1.58275667		4.4728	1.71783	1.71535	2.63532667
8	Force	3.646677			3.646677		0.263209	0.1578628		0.2105359
	Duration	0.939			0.939		1.614	1.573		1.5935
	Integral	0.81635			0.81635		0.01205	0.02493		0.01849

Table 13. Raw data for male crusher claws treated with saline.

Force is measured in pounds, duration is measured in seconds, and integral is measured in lbs/sec.

SALINE MALE CRUSHER

Lobster		P 1	R 2	E 3	X		PO 1	S 2	T 3	X
1	Force	2.2334592	58.02276	3.9182721	21.3914971		71.112028	46.83659	16.83359	44.9274027
	Duration	1.034	1.217	2.959	1.73666667		1.318	1.195	1.233	1.24866667
	Integral	0.301077	28.0017	6.7103	11.6710257		30.7977	24.0047	12.9182	22.5735333
2	Force	0.4522936	0.410425		0.4313593		2.431465			2.431465
	Duration	2.112	1.493		1.8025		1.271			1.271
	Integral	0.03309	0.04403		0.03856		0.88349			0.88349
3	Force	14.897254	1.1245		8.010877		2.2347022	2.859061	1.5410298	2.21159767
	Duration	2.235	5.771		4.003		8.498	8.273	4.384	7.05166667
	Integral	11.49818	1.65276		6.57547		8.07924	10.25196	5.1335	7.82156667
4	Force	2.5238573			2.5238573		0.3050782			0.3050782
	Duration	4.314			4.314		4.556			4.556
	Integral	5.43236			5.43236		1.1224			1.1224
5	Force	19.845675	7.5801822	6.163228	11.1963617		4.082706	2.1943429	5.340257	3.8724353
	Duration	1.561	4.145	5.538	3.748		2.734	1.308	8.005	4.01566667
	Integral	10.23529	7.189886	17.74474	11.7233053		2.56204	1.175054	22.74448	8.82719133
6	Force	4.6960212	2.7718197	0.7282288	2.73202323		1.7717966	0.291024		1.0314103
	Duration	8.323	5.556	1.971	5.28333333		9.44	1.515		5.4775
	Integral	27.5587	11.4225	0.47679	13.1526633		10.51361	0.27602		5.394815
7	Force	9.713184	16.96775	7.8603346	11.5137562		7.267189	1.0481328	2.1129914	3.4761044
	Duration	2.28	3.48	0.894	2.218		3.272	3.355	1.48	2.70233333
	Integral	7.98393	15.12339	2.9554	8.68757333		8.27722	1.06272	0.81105	3.38366333
8	Force	1.9544741	8.1246365	1.9082404	3.99578367		13.825174	4.5505439	6.463665	8.2797943
	Duration	3.355	1.257	0.897	1.83633333		2.507	0.785	1.185	1.49233333
	Integral	2.34964	3.2121	0.42646	1.99606667		13.5661	1.28947	2.30576	5.72044333

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