CHEMICAL CUES ARE USED BY MALE HORSESHOE CRABS, *Limulus polyphemus*,
TO LOCATE AND SELECT MATES

By

KATHARINE M. SAUNDERS

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2011

To Mom, Dad, Ben, and Dave

ACKNOWLEDGMENTS

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Abstract of Thesis Presented to the Graduate School
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Katharine M. Saunders
May 2011

Chair: H. Jane Brockmann
Major: Zoology

Chemical cues have an important role during mating for both male and female

horseshoe crabs, *Limulus polyphemus.* The use of multisensory cues to locate mates

can increase an organism's success by acting as a back-up plan when one system fails,

by providing additional information to the receiver, and by increasing their ability to

detect mates using senses that have different ranges in a variable aquatic environment.

During the breeding season, females migrate into shore during high tides to spawn.

Males attach to females as they approach the beach or are attracted to pairs already

spawning. Vision is well established as an important cue in attracting unattached males.

Although chemoreception is well known in other marine arthropods, and horseshoe

crabs have the anatomy available, there are few studies on chemical cues in this

species. Experiments are presented here that provide evidence for chemical cue use.

Male and female horseshoe crabs exhibit alternative reproductive tactics. Much is

known about the use of cues by females and alpha males to locate and choose mates,

but few studies have shown what cues satellite males use. Female horseshoe crabs

can exhibit both polyandry and monandry. The experiments in this study, found a

difference in satellite male attraction between monandrous and polyandrous nesting

pairs, showing that satellite males are using chemical cues from already nesting pairs to most efficiently allocate their reproductive effort. Also, by removing the possible effect of other satellite males on the chemical cues, the source of the chemical cue was determined to be the nesting pair. Certain females benefit in their reproductive success with the additional fertilizations of satellite males, and they may be soliciting fertilizations from unattached males.

CHAPTER 1
MALE HORSESHOE CRABS USE MULTIPLE SENSORY CUES TO LOCATE MATES

Introductory Remarks

Animals locate mates using a complex array of cues that are detected by a variety of sensory modalities including acoustic, visual, chemical, and tactile. In some cases cues are emitted and received simultaneously in multisensory channels (called multimodal signals), but in other cases signals (called unimodal composite signals) are emitted and received sequentially in different channels (Smith, 1977; Partan and Marler, 2005). Both types of multisensory cues are used by invertebrates when locating mates (Atema, 1995; Dunham and Oh, 1996; Partan and Marler, 1999; Candolin, 2003). For example, in the butterfly *Bicyclus anynana*, females simultaneously use visual and chemical cues from males to choose mates, suggesting that these different sensory modalities have equal importance in mate choice (Constanzo and Monteiro, 2007). On the other hand in a species of wolf spider, visual and chemical cues are used in different ways during the mating process and provide different information (Rypstra et al., 2009).

The ability to use multisensory channels to detect signals can be beneficial to an individual's survival in several ways. First, having the option of more than one sensory system to perceive the environment can function as a back-up plan. Environments can often be noisy and a second channel for sensing cues can increase the receiver's ability to detect a signal (Wiley, 1994) and process the information (Johnstone, 1996). Second, multisensory signals can transmit more information than a signal sent through a single sensory channel. Signaling in multiple modalities may enhance the overall complexity of the signal and increase the chance of a response from the receiver. This is true for a wolf spider signal, in which seismic signals, in addition to visual cues (tufts of bristles on

the male's legs), increase the female's responsiveness when compared to males that use only one signaling channel (Uetz et al., 2009). Third, because of modality-specific mechanisms for mate attraction and detection (Bradbury and Vehrencamp, 1998), multisensory signaling can be adaptive when individuals experience variable or changing conditions or environments. For example, in an aquatic environment, chemical gradients may be used over long ranges (migrating salmon) or short ranges (lobster mating), and the useful range for vision varies from hundreds of meters in clear water in the day, to less than a meter in murky water or at night. Having the ability to perceive a signal in a different modality depending on the state of the environment can improve detection. For example, a stomatopod fighting under varying light conditions will use visual cues at high light intensity and switch to chemical cues when less light is available (Cheroske et al., 2009). Thus, by using either multimodal or composite multisensory signals, individuals can improve their ability to detect and reliably respond to important cues in the environment.

In some species females or males produce specific sexual signals, such as sex pheromones, songs or visual displays, which increase the ability of one sex to find the other to the benefit of both (Bradbury and Vehrencamp, 1998). In other species, however, members of one sex, usually males, use cues from potential mates that were inadvertently transmitted, such as when a male responds to the shape, or size of a female or vibrations from female movements (Maynard Smith and Harper, 2003). In such cases the male receiver may have evolved enhanced abilities to detect cues from the female, but the female sender has not evolved specific signals to attract mates (Wyatt, 2004). Over the course of evolution, such inadvertent and unavoidable stimuli

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may be modified to enhance mate attraction or mate choice to the female's benefit in which case they would then be considered communication signals (Greenfield, 2002). In practice, however, it is difficult to tell whether a cue is a signal or simply a source of information to which males are responding. Here we review what is known about the cues and possible signals used by male horseshoe crabs, *Limulus polyphemus*, to locate mates, provide new information on their use of chemical cues, and discuss their use of multisensory cues to detect and respond to mates under different conditions.

Male horseshoe crabs have two mating tactics: some find females offshore and come to the nesting beach holding onto the female's opisthosoma (attached males in amplexus), whereas other males remain unpaired and approach the nesting beach alone during the high tide when pairs are spawning (Brockmann and Penn, 1992). These unattached males crowd around the nesting pairs as "satellites" and may form large mating groups (Brockmann, 1996). Horseshoe crabs are unique among arthropods in that females dig into the substrate and release their eggs into the environment where fertilization takes place outside the female's body (Giese and Kanatani, 1987). After spawning, the pair leaves the beach and the eggs develop in the sand. When the attached male is the only male present, he is the only one to fertilize the eggs laid by the female, but when satellites are present (or when satellites have recently been present during the egg laying), they share much of the paternity (Brockmann et al., 1994). Satellite males compete for position around the female and when they are over the female's incurrent canal and under the front margin of the attached male's carapace, they have the highest paternity (Brockmann et al., 2000). Attached and unattached males do not differ in size but they do differ in condition and

age, with attached males being on average younger than unattached males (Brockmann and Penn, 1992; Penn and Brockmann, 1995). Furthermore, when males are unable to attach because their first pair of appendages have been experimentally covered, those in good condition remain offshore whereas those in poorer condition come to the nesting beach and join spawning pairs as satellites (Brockmann, 2002). This means that the two male mating patterns are condition-dependent tactics and not just a result of a male's ability to locate a female. What cues do males that employ either tactic use to locate females and do females do anything to attract males? First we examine the use of visual cues and then we discuss the use of other cues, including chemical cues.

Evidence for Visual Cue Use by Male Horseshoe Crabs in Locating Mates

The lateral eyes of horseshoe crabs are a classic preparation in neuroscience and several important properties of visual systems, such as lateral inhibition, were first discovered in this species (Barlow and Powers, 2003). Vision is widely used by chelicerates and aquatic arthropods in orienting to landmarks (Herrnkind, 1972), in escaping from predators (Locket, 2001), in searching for food (Su et al., 2007), in agonistic encounters (Bruski and Dunham, 1987), and in locating and identifying mates (Christy, 2007). For example, the males of many crustaceans wave their chelipeds in species-typical displays that attract mates (Hazlett, 1972). Male wolf spiders even respond to video presentations of females so it is clear that no other cues are needed to elicit approach (Uetz and Roberts, 2002). But until recently, little was known about the ways in which vision contributed to normal behavior. We now know, thanks primarily to the work of Robert Barlow and his colleagues, that vision plays an important role in horseshoe crab mating behavior.

In a number of elegant experiments, Barlow, Powers and their collaborators demonstrated that male horseshoe crabs are attracted to unpaired females by visual cues (Barlow and Powers, 2003). The lateral compound eyes of horseshoe crabs are modulated by physiological and structural light adaptation processes (Pieprzyk et al., 2003) and by inputs from a circadian clock such that at night their sensitivity to light increases up to one million times (Dalal and Battelle, 2010), which allows the animals to see as well at night as during the day (Powers and Barlow, 1985; Herzog et al., 1996). Even on a new moon night, males appear to respond visually to females nearly as well as during the day (Krutky et al., 2000)! In a field experiment on Cape Cod, Massachusetts, Barlow et al. (1982) observed male responses to cement models that were placed in shallow water 4-m below the high tide line. They presented nine models of equal area simultaneously: three different shapes (hemisphere, cube and female *Limulus*) painted three different shades (black, white and gray). Males approached the models when they were within about a meter, attached to the models and sometimes even released sperm in the presence of the models. They were significantly more attracted to the horseshoe crab shape than the hemisphere and both were much more attractive than the cube. While males readily attached to models shaped like horseshoe crabs, they only approached and circled the hemisphere without contacting it, suggesting that secondary visual or tactile cues were used as the male approached closer (Powers and Barlow, 1981). Contrast with the background also mattered as males were more likely to approach the black or gray models than the white ones (Barlow et al., 1982). Size also influenced their response, with the larger objects tested being more attractive than the smaller ones (7, 15, 22 and 30 cm models were tested)

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(Herzog et al., 1996). Males blinded by black acrylic paint did not respond to the models and sometimes buried in the sand. Females and juveniles turned away and avoided the objects (Powers et al., 1991; Ridings et al., 2002).

Using an overhead video camera that extended over a shallow inshore area, they determined that males oriented toward dark objects from about the same distance away (1 m) in the day as at night (Powers et al., 1991). By using a camera mounted on the carapace of a horseshoe crab and simultaneously recording from the optic nerve, they found that the eye responds vigorously to crab-sized objects moving across the visual field (Barlow et al., 2001). Males also responded well to the flickering light from overhead waves that reflected off the carapace of a potential mate. This likely helps males detect a female irrespective of the contrast of her carapace with the background (Passaglia et al., 1995). Thus, the eye transmits to the brain neural images of objects having the size, contrast, reflective properties and motion of potential mates (Passaglia et al., 1997). Based on their experiments with visual cues, Barlow and Powers (2003) concluded, "The great attraction of the males to the cement castings proved that chemical cues (pheromones) were not involved."

Schwab and Brockmann (2007) showed that unattached males also use visual cues when approaching pairs nesting along the shoreline in a Florida Gulf coast population (Seahorse Key) of horseshoe crabs. They presented cement model horseshoe crab pairs (made from molds of normal-sized male and female horseshoe crabs from the Florida population) in amplexus near other nesting crabs (unattached males were present but no crabs had been nesting at the spots where the models were placed) (Fig. 1-1). They compared the response of unattached males to two model pairs

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presented simultaneously 1 m apart, which differed in size (prosoma width of female models: 17.5 cm and 23 cm; males: 13 cm). Unattached males were significantly more attracted to the larger of the two model pairs, which differed in size by only 5.5 cm. Since models were used, no cues other than visual ones were available to males in this experiment before contact was made. This means that visual cues were used by males to locate spawning pairs onshore just as they were used when pairing in deeper water offshore.

Clearly, then, both attached and satellite males use visual cues to locate mates. However, since many males have eyes that are in poor condition (Brockmann and Penn, 1992; Penn and Brockmann, 1995; Wasserman and Cheng, 1996; Duffy et al., 2006); since horseshoe crabs often nest under visually limited conditions (e.g. high turbidity, low contrast between animals and substrate); and since some females are completely buried when joined by satellite males (Schwab and Brockmann, 2007), non-visual cue use also seems likely. In the next section we discuss what is known about tactile and other non-visual cues and then we focus on chemical cue use.

Evidence for Tactile Cue Use by Male Horseshoe Crabs in Locating Mates

Horseshoe crabs are covered with mechanoreceptors. They are found on the chelae of the walking legs (Wyse, 1971), at the base of the tail (Eldredge, 1970), on the lateral spines of the opisthosoma (Eagles, 1973) and they cover the entire dorsal surface of the prosoma (Thompson and Page, 1975) with particularly dense concentrations (they can be seen on the surface as small black dots) around the median and lateral eyes (Kaplan et al., 1976). Certainly, when you hold a horseshoe crab by its prosoma, you know it detects your grasp, since its chelae are directed toward your fingers (Brockmann, personal observation). Some of the mechanoreceptors

on the chelae respond to force applied to the unsclerotized cuticle of the grasping surfaces of the tarsal pads (Wyse, 1971). Not only do chelae respond to tactile stimulation but they also respond to chemical cues, water flow, osmotic changes and thermal stimulation. It is not clear whether these are additional response properties of the chemo- and mechano-receptors present on the chelae or whether separate thermo- and osmo-receptors are also involved. Mechanoreceptors may be involved in mating based on the observation that a male works his way around a female or a nesting pair before attaching or settling over the incurrent canal (Barlow and Powers, 2003). Also, when an attached male is touched by a satellite, he responds vigorously by pulling himself forward on the female, rocking from side to side and leaning toward the intruder, or wagging his telson from side to side (Brockmann, 1990; Brockmann, 2003). Stroking the side of the male's carapace will provoke the same response (M.D. Smith, personal observation). Males will attach to a variety of objects other than females, including other males, dead females, a cinder block, driftwood, a black frisbee left on the beach, shoes, beer cans (personal observations) or even a diamondback terrapin (R. Weber, personal observation). These observations suggest that males use tactile cues and that visual cues may not be available at all times when attaching to females and interacting with attached males or satellites.

Near-field acoustic and substrate vibrational cues are widely used by other marine arthropods (Salmon and Horch, 1972) and particularly by chelicerates (Hill, 2009) when locating prey (Brownell, 2001) or attracting mates (Proctor, 1992; Elias et al., 2010). However, there is no information on whether horseshoe crabs respond to such cues or not. Certainly horseshoe crabs respond to water currents (Ehlinger and Tankersley,

2003; Botton et al., 2010) and their mechanoreceptors are very sensitive to water currents (Wyse, 1971). Individuals face into currents and respond to wave surge when approaching the breeding beach (Rudloe and Herrnkind, 1976, 1980). Sensory processing of hydrodynamic cues could lead to behaviors that attract horseshoe crabs to mating beaches as has been found in some crustaceans (Mellon, 2007). On a smaller spatial scale, it is possible that males are also responding to the respiratory currents of females and pairs. Of course, such currents also carry chemical cues to which the males may be responding as well (Quinn et al., 1998).

Evidence for Chemical Cue Use by Male Horseshoe Crabs in Locating Mates: Previous Studies

Unlike many other arthropods, horseshoe crabs lack antennae or other specialized appendages for detecting environmental chemicals. Nonetheless they are well endowed with chemoreceptors, which can be found on the flabellum, located at the base of the fifth pair of legs (Waterman and Travis, 1953), on the bases of the legs (gnathobases) around the mouth (Barber, 1956), on the chilaria (Hayes and Barber, 1982) and on the claws of the walking legs (Wyse, 1971; Hayes, 1985). These structures respond to stimulation from various chemicals such as amino acids associated with food. There are also chemoreceptors on the gills (Page, 1973) that respond to oxygen in the water (Crabtree and Page, 1974; Thompson and Page, 1975). Wyse (1971) demonstrated contact chemoreception experimentally and he suggested that horseshoe crabs could also sense distant chemical cues (Quinn et al., 1998). Further, the brains of horseshoe crabs have particularly large mushroom bodies (corpora pedunculata) that make up 80% of the total brain volume. These structures receive their inputs from chemoreceptors on the legs and gills (Loesel and Heuer, 2010) and are known to serve

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as centers for sensory integration and learning in other arthropods. The physical evidence is overwhelming that horseshoe crabs have a rich chemosensory life.

There are already a number of contexts in which horseshoe crabs have been documented to use chemical cues. For example, larvae and juveniles are known to respond to chemical cues from suitable habitats and they orient away from visual targets when accompanied by conspecific odor (Medina and Tankersley, 2010). In the Cape Cod population, horseshoe crabs can locate clams (a preferred food) even when the clams are completely buried (Smith, 1953). In the Delaware Bay, Botton et al. (1988) suggest that females use chemical cues, including hydrogen sulfide receptors, when locating their nesting beaches. In the Florida Gulf coast population and in Delaware Bay, we have observed males circling over an area where females had recently been nesting (Cohen and Brockmann, 1983; Hassler and Brockmann, 2001) suggesting that they were responding to chemical cues left by the departing pairs. In an ablation experiment, Patten (1894) showed that males could no longer find females once their olfactory organ (the region around the ventral eye) had been removed. Hanström (1926) replicated Patten's study using better controls and concluded that it was likely that males located females using chemoreceptors in this antero-ventral region. Given their extensive system of chemoreceptors, the behavior described above and the results of ablation experiments, it seems likely that male horseshoe crabs respond to chemical cues from females in locating mates.

Chemical signals and pheromones are known from other chelicerates (Gaffin and Brownell, 1992) and are widely used by marine arthropods to locate and choose mates. These include lobsters (Atema and Engstrom, 1971; Atema, 1995; Bushmann and

Atema, 2000), blue crabs (Gleeson 1980) and other decapod crustaceans (Atema and

Steinbach, 2007). In these cases, there is a specific identified chemical component of

the signal that is necessary for mating to occur. Amphipods also use their

chemosensory system during reproductive behavior to locate females, but males use a

chemical cue present in the exoskeleton of a newly molted female (Borowsky and

Borowsky, 1987). In contrast to other modalities such as visual or acoustic cues,

chemical cues move through the aquatic environment by molecular diffusion and can be

aided by different types of flow (Atema, 1995; Zimmer and Butman, 2000; Hay, 2009),

which means that they can be detected over a wide range of distances and therefore

are a particularly important sensory mode for organisms in locating mates, often in

combination with other sensory modalities. However, in spite of the widespread use of

chemical cues and pheromones by marine arthropods and the well-known presence of

chemoreceptors in horseshoe crabs, few experimental studies have been conducted to

evaluate chemical cue use by horseshoe crabs.

Hassler and Brockmann (2001) conducted two experiments specifically designed

to test the use of chemical cues by unattached males when locating spawning pairs

along the shoreline. In both experiments cement horseshoe crab models were placed

on the shoreline where they were approached by unattached males (Fig. 1-1). These

models were prepared by filling the shells of dead female horseshoe crabs (that had

been cleaned out and sun dried to reduce odors) with concrete (the concrete filling was

necessary to keep the models in place during the experiment). In their first experiment,

Hassler and Brockmann (2001) concurrently placed a model over each of three nearby

sites, one where a group had been recently nesting (the group was removed and

replaced by the model), one where a lone pair had been recently nesting (the pair was removed and replaced by the model), and one nearby site where no pair had been nesting (a model was placed on the sand). Over the next 10 min, they counted the number of unattached males that approached each of the three models. Since they used cement models, there were no vibrational or auditory cues available and the visual cues were randomized among treatments. Hassler and Brockmann (2001) found that unattached males were significantly more likely to approach the model that had been placed over the site of a nesting group than the site where a pair had been located and both were significantly more likely to attract males than a site where no crabs had been nesting. The numbers of unattached males attracted to the models continued to increase over the first 6 min and then declined slightly by 10 min. The experiment was run in two variants; in one all satellites were allowed to remain with the models after they had approached and in the other all satellites that arrived at the models were removed. Both showed similar, significant effects. In a second experiment using a paired design, Hassler and Brockmann (2001) placed a sponge filled with water from a pair with many satellites under one model and a control sponge filled with seawater under a second model. Unattached males were significantly more likely to approach the model with the 'many satellites' sponge than the model with the seawater-filled sponge. Taken together, these experiments provide clear evidence that unattached males use chemical cues when approaching nesting pairs.

Although these experiments were carefully conducted in two different populations (Florida Gulf coast and Delaware Bay) using several sets of cement models (to prevent pseudoreplication) and although these models were randomly assigned to the different

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treatment groups, in retrospect when we consider the male's visual sensitivity (Schwab and Brockmann, 2007), there were possible confounds in the Hassler and Brockmann (2001) study. Since the models were made from dead horseshoe crabs, there might have been slight differences between the models in color, height or width to which the unattached males may have been responding. Further, male models were not used in this experiment so the unattached males were not responding to a pair but to an unattached female and unattached females are rare near shore in both Florida and Delaware Bay.

These problems were remedied by Schwab and Brockmann (2007) who evaluated the importance of one chemical cue, the odor of eggs. Since horseshoe crab eggs are known to produce chemical cues (Shoger and Bishop, 1967; Ferrari and Targett, 2003), eggs seemed a likely source of cues for satellite males. Using the same procedure described above (Fig. 1-1), they placed two large cement model pairs along the shoreline. Under each model they placed a screen bag; the experimental bag contained freshly spawned eggs and the control bag was empty. Males were equally attracted to the experimental and control models but once they had joined a model, they remained significantly longer with the experimental (with eggs) than with the control (no eggs). This result was not surprising as they also reported that satellite males often (38% of the time) joined pairs before any eggs had been laid so the presence of eggs was not a prerequisite for satellite attraction. Nonetheless, egg odor appears to be a likely cue used by satellite males to remain with a spawning pair.

The new study presented here further evaluates the use of chemical cues by unattached males from the same Florida population as the Hassler and Brockmann

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(2001) and Schwab and Brockmann (2007) studies. In this experiment cement models of pairs were made from a mold, which controlled for extraneous visual cues associated with the pair.

Experimental Study on Chemical Cue Use by Florida Horseshoe Crabs

Materials and Methods

This experiment was conducted to evaluate the hypothesis that satellite male horseshoe crabs respond to chemical cues when locating spawning pairs along the shoreline. It was conducted at the University of Florida Marine Laboratory at Seahorse Key from 20 September–18 October 2008 around a new or full moon when the highest high tides in a month occur and when the most horseshoe crabs are present (at this time of year the highest high tides are at night) (Cohen and Brockmann, 1983; Barlow et al., 1986). Seahorse Key is an island that is part of the Cedar Keys National Wildlife Refuge on the Florida Gulf Coast. About an hour before the predicted maximum high tide, we walked along the beach until we found an area without nesting groups but where unattached males were observed close to the shoreline. We established two contiguous 1.7 × 1 m arenas on the beach and marked the corners of the arena with survey flags. We placed equal-sized, black, cement horseshoe crab models in the center of each arena (Fig. 1-1) 1 m apart. They were placed perpendicular to the shoreline, with the female's anterior end facing toward the land and with the male behind the female, so that the water washed over the posterior half of each and the front of each carapace was above water. The two model pairs were intended to look like two mating pairs along the shoreline to any approaching satellite males.

After the models were in place, we collected water in sponges from two sources. The experimental treatment used water from female horseshoe crabs already nesting

with satellites along the beach. The water was collected by gently removing sand from around a well-buried female with at least one satellite male (see methods from Hassler and Brockmann, 2001). We slowly lifted one side of the female's carapace, squeezed a sponge and held it underneath the female and allowed it to absorb the surrounding water for 3 sec. The control treatment used an identical sponge filled with plain seawater collected near the arenas. We used cellulose, household sponges, cut in half to make two 7 ×5.5 ×1.5 cm squares (no "antimicrobial" sponges were used to prevent alteration to the cues). The experimental treatment sponge was placed under one model pair in the arena and the control treatment sponge under the other. We used new sponges with each trial and we randomly assigned the treatments to the two arenas. We immediately started the 10 min trial and recorded the number of unpaired males that crossed into each arena, the amount of time that each male spent in each arena, and which arena was entered first.

Results

Significantly more satellite males entered the arena with the experimental treatment sponge than the arena with the control treatment sponge (Wilcoxon signed-rank test, W= 79, P= 0.02, n= 16; Fig. 1-2). The experimental treatment was significantly more likely to be the first arena to have a satellite male enter an arena than the control treatment (Chi-square test, χ^2=6.25, P= 0.01, n= 16; Fig. 1-3). The time spent by unattached males in the experimental treatment arena (average = 120.06 sec) was not significantly different from the time spent in the control arena (average = 109.94 sec; Wilcoxon signed-rank test, W= 30, P=0.20, n= 16).

Discussion

This study demonstrates that unattached male horseshoe crabs use chemical cues when locating pairs along the shoreline. More males entered the experimental arena, and this was the first arena to attract a satellite male in more trials when compared with the control arena. By using identical cement models so that visual cues were the same, we demonstrate that chemical cues attract satellite males. When the unattached males were moving along the shore searching for mates, they were under water, i.e. below the level where the waves break, and presumably could not see the models that were placed at the shoreline and out of the water. For this reason we suggest that males may not be using visual cues at all as they approach, but cue in on chemical cues from the spawning pairs.

Chemical Cues in Horseshoe Crabs

The new experimental study described here makes it clear that unattached males are using chemical cues to locate spawning pairs. The new study confirms the findings from the Hassler and Brockmann (2001) study and improves the methods by holding tactile and visual cues the same and by presenting realistic cement models of a male-female pair nesting along the shoreline. An experiment on a New Hampshire population eliminated visual cues altogether and showed that unattached males are attracted to spawning pairs with chemical cues alone when nesting in shallow water near shore (Saunders et al., 2010). These studies combined with those in the literature clearly demonstrate that unattached male horseshoe crabs are using chemical cues along with visual cues when locating mates. What is less clear is the source of those chemical cues.

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The chemical cues that attract males could be from a number of different sources. The local environment is one possibility if horseshoe crabs are attracted to high quality areas for egg development, such as patches of sand with high oxygen content. While this "environmental source" hypothesis might be important for horseshoe crabs in finding suitable beaches, there are several reasons why this is probably not a significant factor for locating potential mates and mating (Hassler and Brockmann, 2001). (a) Whenever unattached males were present, some pairs nested with satellites and some without satellites even at high densities (6%–88% of pairs were nesting in groups in Delaware [Brockmann, 1996]), which implies that pairs differed in their attractiveness to unattached males. (b) Groups were not clumped but rather were interspersed with singly mating pairs over the entire active section of the beach. (c) The experiment presented here eliminates the possibility of environmental chemical cues by removing the cue from its original environment. The experimental setup was placed in an area where no horseshoe crabs were spawning in the immediate vicinity, showing that other pairs were not attracted to these areas from any environmental cue. The fact that males were nonetheless attracted to the models means that the most likely source for the chemical cues is the nesting pair or group rather than the substrate or immediate surroundings.

For a number of reasons, the nesting pair as the source of the chemical cues is a more likely explanation than is attractants originating from satellite males. First, the "pair source" hypothesis accounts for the first satellite male to arrive at a pair (Hassler and Brockmann, 2001) whereas the "satellite attraction" hypothesis does not. Second, a New Hampshire study demonstrated that unattached males were attracted to spawning

pairs without satellite males being present by surrounding a pair with a wall to eliminate

visual cues (Saunders et al., 2010). Third, paternity analyses demonstrate that satellite

male fertilization success is diminished by the presence of additional males (when more

than two satellites are present, (Brockmann et al., 2000) so it is unlikely that satellite

males would be producing a chemical cue that would attract competitors that reduce

their success. However, it is possible that satellites are attracted by the presence of

large quantities of sperm or other unavoidable by-product associated with large groups.

Fourth, evidence for individual differences in chemical cues from pairs comes from

individually marked animals. Pairs that attracted satellites on one high tide were

significantly more likely to attract unattached males on subsequent high tides when

compared with pairs that did not initially attract satellites (Hassler and Brockmann,

2001). Finally, pairs that were allowed to retain one satellite were no more likely to

attract satellites than pairs where all satellites had been removed (Brockmann, 1996;

Hassler and Brockmann, 2001). Taken together, these results strongly suggest that

unattached males are attracted by chemical cues emanating from the nesting pair.

Multimodal Cue Use by Male Horseshoe Crabs

The types of cues that male horseshoe crabs use to find mates depend on the

sequence of behaviors expressed at different distances from a potential mate. First,

when an unattached male encounters an unattached female offshore as she is

migrating to the nesting beach, it is likely he uses visual cues, turning toward objects of

the appropriate size, shape and contrast (Barlow et al., 1982; Herzog et al., 1996).

While chemical, vibrational or tactile may not be necessary for him to find a female, the

studies so far do not exclude the possibility that males use such cues in addition to

vision. Second, when unattached males are on or near the shoreline, both chemical and

visual cues attract males to spawning pairs, as shown by the experiments presented here and in the literature (Hassler and Brockmann, 2001; Schwab and Brockmann, 2007; Saunders, et al. 2010). Third, after a male has made contact with a female or pair, he may be using cues from any of the sensory modalities including visual, tactile (including contact and currents), and chemosensory. It is generally agreed that tactile cues are important as the male orients around the female to attach or to take up a satellite position (Brockmann, 1990; Barlow and Powers, 2003; Brockmann, 2003) but no specific tests have been conducted to evaluate the cues used at this stage in the sequence. Finally, whether a male will stay attached to a female or remain a satellite of a pair are determined by the presence of chemical cues. Schwab and Brockmann (2007) demonstrated that the presence of freshly laid eggs under a model increased the time that a satellite remained with the model pair. Undoubtedly, tactile cues also affect attachment and satellite persistence since males quickly detach from many (although certainly not all) inappropriate objects. Saunders et al. (2010) suggests that, in the presence of odors, males will remain attached to a model of a horseshoe crab longer than when appropriate odors are not present, reinforcing the hypothesis that multiple sensory cues contribute to the full suite of mating behaviors expressed by horseshoe crabs. Why do males use an array of sensory modalities at different stages in the mate-locating process?

First, the use of several different sensory modalities to find potential mates may result from the need to have a backup when one system fails. For example, the lateral eyes that are used in locating mates (Barlow and Powers, 2003) may become fouled with encrusting organisms or deteriorated (due to the action of chitinoclastic bacteria

and other agents). When this occurs, the eyes have reduced visual acuity (Wasserman and Cheng, 1996). Males with such visual impairments are unable to respond to females offshore and do not orient to or attach to females (Barlow et al., 1982). Unattached males are much more likely to have deteriorated, fouled or damaged eyes than attached males and thus it may be difficult for these males to detect mates visually (Duffy et al., 2006). By using chemical cues, these males can still locate females on the shoreline.

In addition, the simultaneous use of several different sensory cues may also be advantageous if it can provide more overall information to the receiver. There is evidence that different females may be perceived differently by unattached males. When satellites were removed from nesting pairs, pairs that had more satellites before the removal were more likely to regain satellites after the removal (Brockmann, 1996). This consistency in the attractiveness of pairs remained from one tide to the next (Hassler and Brockmann, 2001) and was associated with differences between the pairs and the ability of males to fertilize the female's eggs (Johnson and Brockmann, unpubl.). In some cases females will leave the beach as soon as a satellite joins the pair (Johnson and Brockmann, 2010). This suggests that selection would favor unattached males that could distinguish between pairs that are likely to leave versus continue to nest when approached. Further, while a male horseshoe crab has enough visual acuity to recognize another horseshoe crab, it is probably difficult for him to determine if it is male or female (while females are typically larger, some small females are the same size as large males). Thus, chemical cues might help males distinguish females from

males, as well as mating pairs from single animals, and unattached males may be using multimodal cues to gain additional information when searching for mates.

Finally, in changing, variable, and unpredictable environments, the use of multiple sensory modalities can improve the animal's ability to detect the relevant information such as the presence of a nesting female. While the contrast between the sandy bottom in Cape Cod and some mid-Atlantic and Florida spawning beaches is very high and thus makes it fairly easy for a male horseshoe crab to visualize a large, brown, female either in the day or at night, this is not always the case either in other areas, or at all times. In New Hampshire, areas of the Chesapeake Bay, and many other estuaries and embayments, the water is typically quite turbid and the sediment dark. In addition, a male can use different sensory cues when offshore or near shore since some cues are better detected over longer or shorter ranges or can be enhanced based on different hydrodynamic conditions. Also, because visual and chemical cues are transmitted differently, one channel could provide more complete information depending on the environment the horseshoe crab is currently experiencing. Spawning pairs may even be above the waterline or the females may be completely buried depending on the beach characteristics and selection will favor males that can find females under all these different conditions.

The use of multimodal cues by unattached male horseshoe crabs is advantageous because they may lose one of the sensory systems, because of the increased information the different channels can provide, and because of the wide range of environments they experience. The strong evidence presented here for an important

role of chemical cues in addition to visual cues when locating mates suggests there

might be still other modes of sensing that have yet to be studied.

Figure 1-1. A. Photograph of the experimental setup with the cement models in place
and a male horseshoe crab that has entered one of the arenas. B. Schematic
showing dots where survey flags were placed to mark out the arena, lines as
distances between the flags, and the large black shapes representing the
horseshoe crab cement model pairs. The diagram is oriented in the same way
as the photograph in A.

Figure 1-2. The number of male horseshoe crabs entering the arena with the model pair covering a sponge filled with water collected from a pair with satellites compared to the number that entered the arena with a model covering a sponge with seawater (Wilcoxon signed-rank test, W= 79, *P*= 0.02, *n*= 16).

Figure 1-3. The number of trials in which a male first entered the experimental and control model arenas (Chi-square test, χ^2=6.25, P= 0.01, n= 16).

CHAPTER 2
SATELLITE MALE HORSESHOE CRABS USE CHEMICAL CUES TO LOCATE
FEMALES WITH ALTERNATIVE REPRODUCTIVE TACTICS
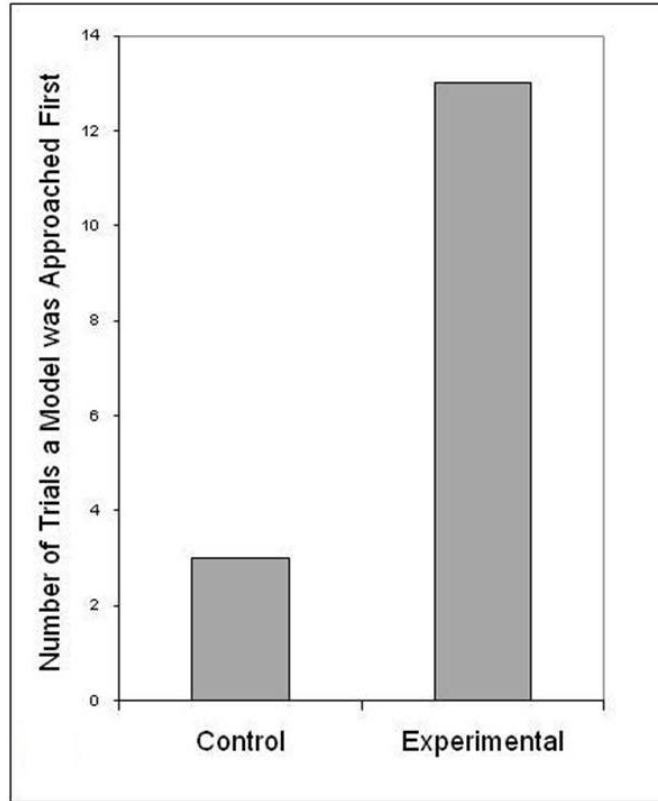
Alternative reproductive tactics may provide a means for individuals to further increase their fitness over what they would expect to achieve if all individuals in the population used the same reproductive tactic. Alternative reproductive tactics have been widely studied across a range of taxonomic groups (horned beetles, Eberhard, 1982; isopods, Shuster, 1987; swordtail fish, Ryan and Causey, 1989; lizards, Sinervo and Lively, 1996; wading bird, Lank et al., 1995), but most examples are in the male sex (Henson and Warner, 1997). These tactics can take many forms such as a territorial, courting or alpha male and the alternative strategy as a sneaker, satellite or female mimic (Brockmann, 2008). Cues are used by both sexes to locate and evaluate mates. Mating cues have been shown to be used by females and alpha males, but few studies have asked what mating cues satellite or sneaker males employ. Satellite males are often attracted to breeding pairs or to the territories of breeding males (Goncalves et al., 2003; Fukuyama, 1991). However, the proximate mechanisms used by the satellite males to choose a pair or female have not been studied. One study found that sand goby sneaker males chose nests to parasitize based on female courting behavior, suggesting a visual cue was important, but the study did not identify specifically what cue the sneaker was using (Svensson and Kvarnemo, 2007).

Male horseshoe crabs follow one of two condition-dependent alternative reproductive tactics (Brockmann and Penn, 1992; Brockmann, 2002). Attached males find females offshore and arrive at the beach holding onto a female ready to spawn, whereas unattached or "satellite" males approach the beach alone and join a pair that is already spawning in the sand (Brockmann and Penn, 1992). Attached and unattached

males differ in condition and age with attached males being on average younger than unattached males (Brockmann and Penn, 1992; Penn and Brockmann, 1995). There can be high variation in the number of satellites around a spawning female that is buried in the sand with her attached male (Brockmann, 1996). Fertilization takes place outside of the female's body in the sand. When no satellites are present, the attached male fertilizes all the eggs but when satellites are present they compete with the attached male and other satellite males for optimal positions around the female. The position that yields the highest paternity is over the female's incurrent canal and under the front margin of the attached male's carapace (Brockmann et al., 1994). When in this position the satellite often outcompetes the attached male (Brockmann et al., 2000).

Female alternative reproductive tactics also occur in horseshoe crabs. Some females (polyandrous) attract males and other females (monandrous) do not attract or possibly avoid satellite males (Johnson and Brockmann, 2010). These differences are consistent: females with a nesting group are more likely to regain satellites after they have been removed (Brockmann, 1996) when compared with females without satellites; and females with higher numbers of satellites at one high tide are more likely to have higher numbers of satellites when nesting on future high tides (Hassler and Brockmann, 2001). Monandrous females are on average slightly smaller than polyandrous females and they lay fewer eggs but the difference is not great (Hassler and Brockmann, 2001). Johnson and Brockmann (2010) found that normally polyandrous females have lower reproductive success when their eggs are fertilized only by their attached male's sperm as compared with their success when their eggs are fertilized by a satellite male's sperm; and normally monandrous females have lower reproductive success when

forced to nest with satellites or when their eggs are fertilized by satellite sperm than when fertilized only by their attached male's sperm. So monandrous females are less attractive to satellite males for a number of reasons but how do unattached males that are approaching the beach distinguish monandrous from polyandrous females?

Visual and chemical cues are used by male horseshoe crabs in locating mates. Hassler and Brockmann (2001) found that satellite males were attracted to areas where females with many satellites have previously nested. Schwab and Brockmann (2007) found that visual cues play an important part in satellite males locating nesting pairs on the shore. In chapter 1 I confirmed that satellite males are attracted to chemical cues from females with many satellites by controlling for visual cues. These studies suggest that polyandrous and monandrous pairs differ in the cues they produce, and satellite males can detect these differences. It is not known who produces the chemical cue: the female, both the female and attached male (nesting pair), or the satellite males. It seems unlikely that satellites would be attracting other satellites because they compete for fertilizations at a nesting female when other satellite males are present (Brockmann et al., 2000), but inadvertent or unavoidable cues such as the presence of sperm could possibly provide cues. Cues from satellites also do not explain how the initial satellite joins a nesting pair. In this study, I looked for differences in chemical cues between monandrous and polyandrous nesting groups. Are chemical cues the mechanism by which satellite males decide which nesting group to join? To determine the source of the chemical cue, I removed the satellite male as a factor and compared polyandrous and monandrous females (with their attached males since females will not spawn without an attached male).

Materials and Methods

My study was conducted at the University of Florida Marine Laboratory at Seahorse Key, an island that is part of the Cedar Keys National Wildlife Refuge on the Gulf Coast of Florida. The experiments took place on the southwest- facing beach from 11 September - 8 October 2009 and 31 March – 16 May 2010 during both the night and day high tides. These dates were chosen because they occurred at the times of the new and full moons when the greatest numbers of horseshoe crabs were present on the high tides (Cohen and Brockmann, 1983; Barlow et al., 1986). During these high tides I observed lone males moving along the shoreline without a female, attached males holding onto a female approaching the beach to begin nesting, and satellite males that had joined a pair once they began nesting. Female horseshoe crabs were observed in two states: as a monandrous female nesting with only an attached male, or as a polyandrous female nesting with an attached and at least one satellite male, i.e. a male that is physically in contact with the female, her attached male or another satellite. A female's status was determined by observing whether satellites joined the nesting female or if she nested with just an attached male.

Satellite Male Preference Experiment

One hour before the predicted maximum high tide, I walked along the beach looking for an area on the beach where mating pairs were absent, but where satellite males could be observed swimming near the shore. I identified two 1.7 x 1 m areas next to one another as the test arena, marking the corners with survey flags. Two cement models of female horseshoe crabs of equal size were placed in the test arena, each in its own arena 1 m apart; a cement model male was placed on the posterior part of the opisthosoma of each female so that they looked like a normal nesting pair (Fig. 1-1). I

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located the pairs at the water's edge so that the anterior ends of the models remained above water.

I then collected water using household, non- "antimicrobial" sponges, cut in half to be 7 x 5.5 x 1.5 cm in size. The sponges were used to collect water from two sources. The polyandrous treatment used water from female horseshoe crabs already nesting with satellites along the beach and the monandrous treatment used water from females nesting only with their attached males. I gently removed sand from the sides of each female that was buried and nesting, carefully lifting one side of the female's carapace just enough so that I could reach underneath and use the sponge to absorb water from underneath her for 3 seconds. After the water was collected I allowed the female to settle back into her nesting hole and she generally continued nesting without interruption. The monandrous pair was observed for 2 minutes after the water was collected to make sure that no satellites joined and to determine that this female was indeed monandrous. The polyandrous treatment sponge was placed under one model pair in the experimental arena and the monandrous treatment sponge was placed under the other model pair and the 10-min trial began. For each trial we collected water from new females with new sponges, found a new location for the testing arena, and alternated which side of the arena was designated for each treatment. We recorded the number of unpaired males that crossed into each arena, the amount of time that each male spent in each arena, and which arena was entered first.

Chemical Cue Differences without Satellites

In this experiment I removed the satellite male as a source of attraction to a nesting pair. I did this by collecting chemical cues from polyandrous pairs before they

were joined by any satellites. I conducted two experiments that differed in the amount of time between collecting the chemical cue and conducting the experiment.

Experiment 1

From 1 to 2 hours before the predicted maximum high tide, I walked along the beach looking for unattached males that were moving along the shoreline. When I encountered one, I followed him to a nesting pair that did not already have a satellite male present. As the lone male approached the nesting pair and just before he joined the pair, I took a water sample from the nesting pair in the same way as described above and placed the sponge with the sample in a plastic bag. I then allowed the lone male to join the pair as a satellite, and if he did join, I stored the sponge in a cooler with ice packs. I marked the now polyandrous group with a flag and observed them for ten minutes to make sure both the pair and the satellite continued nesting. As soon as the water was collected from this polyandrous group, I found a nearby monandrous pair and collected a water sample in the same way. I marked this monandrous pair with a flag and also observed them for ten minutes (while the water sample was stored in the cooler) to establish whether they remained monandrous (Johnson and Brockmann, 2010). By collecting the sample before the satellite male joined the pair, I could tell whether satellites were cueing in on the odors of other satellite males when they approached a polyandrous pair or whether they were attracted to pairs before they became polyandrous.

When the 10-minute observation period was nearly over, I set up the test arenas and models as described for the Satellite Male Preference Experiment. Since the effect of the satellite male preference experiment was much weaker than the initial presence of chemical cues experiment (see chapter 1), in this experiment I put more space (1 m)

between the two treatment arenas and made them slightly smaller (1 x 1 m) to make it clearer to which arena the unattached males were attracted (Fig. 2-1). I placed one sponge (that had been stored for 10 min in the cooler) under each of the two models (one with water from a polyandrous pair before satellites had arrived and the other with water from a monandrous pair). During the 10-min trial, I recorded which arena was entered first, the number of unpaired males that crossed into each arena, the number that joined (made physical contact with) the models, the amount of time that each male spent in each arena and the amount of time that each male spent in physical contact with each model.

Experiment 2

Experiment 1 did not show a strong difference in the attraction of satellite males to cues from polyandrous and monandrous pairs, both without satellites (see results below). While this result might mean that unattached males were using cues from other satellites rather than from the pair, it might also indicate that the cue was degrading during the 10-min period in which the sample was held prior to conducting the trial. This seems likely given that Hassler and Brockmann (2001) found some evidence for chemical cues diminishing in attractiveness to satellites over the 10 min trials. To eliminate this possibility, I conducted the same experiment again with a few small changes that limited the amount of time between collection of the cue and execution of the trial. I set up the experimental arena as in experiment 1 (Fig. 2-1) in advance and then I followed an unattached male along the shore as he approached nesting pairs without satellites. I collected water from the pair and then let the male join the pair. Once the cue from the now polyandrous pair was collected, I also collected water with a sponge from a nearby monandrous pair so the cues were collected at the same time. I

observed the polyandrous pair for 2 minutes to confirm that the female was amenable to the satellite male remaining with the pair and then ran the 10-minute trial using the two collected water samples, as described in experiment 1. While I ran the experiment, a field assistant continued to watch the monandrous pair for 2 minutes after the collection of the cue to make sure she remained monandrous and she continued to watch both pairs for an additional 10 min to make sure that they remained monandrous or polyandrous. Having a field assistant continue to observe the pairs from which water was collected while I ran the trial with the recently collected water decreased the time over which the chemical cues were degrading. I recorded the same response variables as in Experiment 2.

Statistical Analysis

The experiments described in this study were conducted using a paired design, i.e. I compared the response of approaching unattached males to the presence of chemical cues under two simultaneously presented models. The data for the first arena entered were analyzed using a Chi-Square test. To analyze the time spent in the arenas or with models, I measured the amount of time that each male spent with the models or in the arena and took the mean of those numbers. For those means and for counts of the number of crabs in the arenas, I used a Wilcoxon Signed Rank Test (SigmaStat 3.5). This is a paired design statistical test that does not include trials in which the polyandrous and monandrous treatments had the same values for the variable. Because of this, the sample size for the chi-square data might differ from the Wilcoxon Signed Rank test data.

<div align="center">

Results

</div>

Satellite Male Preference Experiment

Significantly more males entered the arena with the polyandrous (median=6) mating group cue than the arena with the monandrous (median=3.5) pair cue (Wilcoxon Signed Rank Test, W=118, p=0.001, n=16; Fig. 2-2). The arena with the polyandrous mating cues was also the first approached in more trials (11) than the monandrous model arena (7), but this was not a significant difference (Chi-Square Test, χ^2=0.5, p=0.24, n=18; Fig. 2-3). There was no discernable difference (Wilcoxon Signed Rank Test, W=7.5, p=0.44, n=18) in the mean of the times spent by all males in the polyandrous cue arena (61.33 sec) versus the monandrous cue arena (62.11 sec).

Chemical Cue Differences without Satellites

Experiment 1

The number of males entering the arena with the cue from the polyandrous pair (median=2), before they had satellites, was greater in more trials (72%) than the number of males entering the monandrous cue arena (median=1), but this was not significant (Wilcoxon Signed Rank Test, W=59, p=0.102, n=18; Fig. 2-4). The arena with the polyandrous pair cues was more often the arena entered first during a trial (68% of trials), but this was also not significant (Chi-Square Test, χ^2=1.9, p=0.17, n=19; Fig. 2-5). There were more males joining the model with the polyandrous cue (median=1) in more trials (61%) than the model with the monandrous cue (median=1), though not significantly more (Wilcoxon Signed Rank Test, W=15, p=0.305, n=13). The mean time that a male spent in an arena was greater for the polyandrous pair cue (42.85 sec) than for the monandrous cue (38.99 sec), but this was not significant (Wilcoxon Signed-Rank Test, W=74, p=0.069, n=19). The mean time that a male joined a model was greater,

though not significantly, for the monandrous pair cue model (43.85 sec) than for the model with the polyandrous cue (26.87 sec) (Wilcoxon Signed Rank Test, W=9, p=0.41, n=16).

Experiment 2

The number of males entering the arena with the cue from the polyandrous pair (median=4) was significantly greater than the number of males entering the monandrous (median=3) cue arena (Wilcoxon Signed Rank Test, W=69, p=0.05, n=17; Fig. 2-6). The arena with water from the polyandrous pair (before they had satellites) was entered first more often (63%) during a trial, but this was not significant (Chi-Square Test, χ^2=0.84, p=0.36, n=19; Fig. 2-7). More males joined the model with water from the polyandrous pair (median=3) in more trials (71%) than the model with water from the monandrous pair (median=2), and this was a stronger effect than in experiment 1, but it was still not significant (Wilcoxon Signed Rank Test, W=60, p=0.08, n=17). The mean time that a male spent in an arena was greater for the polyandrous pair (51.69 sec) than for the monandrous pair (50.37 sec) but this was not significant (Wilcoxon Signed Rank Test, W=18, p=0.36, n=19), and the mean time a male was in contact with the polyandrous pair model was greater (73.53 sec) than the monandrous model (56.5 sec) but this was also not significant (Wilcoxon Signed Rank Test, W=16, p=0.38, n=19).

Discussion

Satellite male horseshoe crabs were significantly more likely to enter the arena and approach the model with a sponge with water collected from polyandrous females with satellites than the model with a sponge containing water from a monandrous female. This supports the hypothesis that satellite male horseshoe crabs are

distinguishing between polyandrous and monandrous nesting groups by using a chemical cue. The arena with the model with water from the polyandrous pair was also the first arena entered by unattached males in more trials than the arena with the monandrous cues, although this was not significant. This experiment did not distinguish the source of the attraction, which may have been a cue from the female, the nesting pair or other satellite males. To distinguish the source, I compared the response of unattached males to water taken from a polyandrous female before any satellite males were present with water taken from a monandrous pair. The first experiment showed no differences suggesting either that males were attracted to satellites or that the cues had deteriorated over the observation experiment. The second experiment showed a significant difference suggesting that the cues had deteriorated or the 10 min observation period. The results of this experiment mean that males can distinguish monandrous from polyandrous pairs as they approach a spawning pair even when no other satellites are present. This means that the attractant must be given off either by the female, her attached male or an interaction of the two.

It is unknown whether the chemical cue that attracts satellites is a pheromone or a by-product of spawning behavior. It seems unlikely that the attached male would be attracting satellite males because the presence of satellites greatly diminishes the attached male's share of the fertilizations (Brockmann et al., 2000). It is possible however, that the satellite males are, for example, detecting a lack of sperm from the attached male of the polyandrous female or some other inadvertent cue, perhaps in combination with cues from the female. Alternatively, the polyandrous female may be attracting satellite males with a specific chemical cue (pheromone), which could benefit

the female by increasing her reproductive success by ensuring that her eggs are fertilized by a male other than her attached male (Johnson and Brockmann, 2010).

Alternative reproductive tactics evolve because they provide an individual with a way to increase their mating and reproductive success. Non-satellite males and females use specific cues to locate and choose their mates (Bernal et al., 2009), so there must be a proximate mechanism for satellite males to locate mates as well. Satellite or sneaker males may use the same cues to locate mates as the primary males. Alternatively, satellite males may be using an alpha male's characteristics to find a mating opportunity as in the green frog, *Rana clamitans*, where satellite males place themselves near males with attractive territories (Wells, 1977), or satellite males place themselves near the most attractively calling males (Perrill et al., 1982; Byrne and Roberts, 2004). In the case of horseshoe crabs, attached males are known to use visual cues to locate females (chapter 1) whereas unattached males are known to use chemical cues to locate pairs (chapter 2). If unattached males are attracted by inadvertent cues from the attached male, then this is similar to satellite or male sneaker frogs that are attracted to the calling males that are most likely to increase their fitness. However, if the cue that attracts satellite male horseshoe crabs is a pheromone from the female then this is one of the few cases in which females are known to actively attract satellite males, as has been shown in some species of birds (Birkhead and Moller, 1992). Female choice for nests with satellite males is known in bluegill sunfish as this will increase the amount of parental care the offspring receive (Neff, 2008). Variation in female choice of mates could also explain the existence of male alternative reproductive strategies (Alonzo and Warner, 2000).

Polyandry is now known to be quite common in mating systems (Jennions and Petrie, 2000) and is certainly common in species with male alternative reproductive tactics. Two possible advantages have been proposed. First, a female may mate with multiple males to ensure high genetic quality of her offspring (Jennions and Petrie, 2000), if the female's current attached male is not of high quality and the female is directly soliciting satellite males to join the group. The female has the potential to increase the fitness of her offspring by now having satellites compete with the attached male for a share of the paternity (Brockmann et al., 1994). A second advantage to multiple mating is that the polyandrous female's current attached male may not be of general lower genetic quality, but may be genetically incompatible with this particular female (Zeh and Zeh, 1996), and having satellite males present increases the chances that there will be compatible sperm present. In this case females and satellite males both benefit from polyandry. In order for a satellite to take advantage of this reproductive opportunity, selection favors satellite males that can detect the cues that will maximize his success and allow him to allocate his effort most efficiently. In the case of horseshoe crabs, I've shown that the satellite male ability to detect a difference in chemical cues between monandrous and polyandrous pairs can benefit the females since females are using alternative tactics to increase their reproductive success (Johnson and Brockmann, 2010).

Table 2-1. These are the results values from each of the four experiments (both chapter 1 and 2). S= seawater treatment, M= monandrous treatment, P= polyandrous treatment, *p*= the p-value from the statistical test performed.

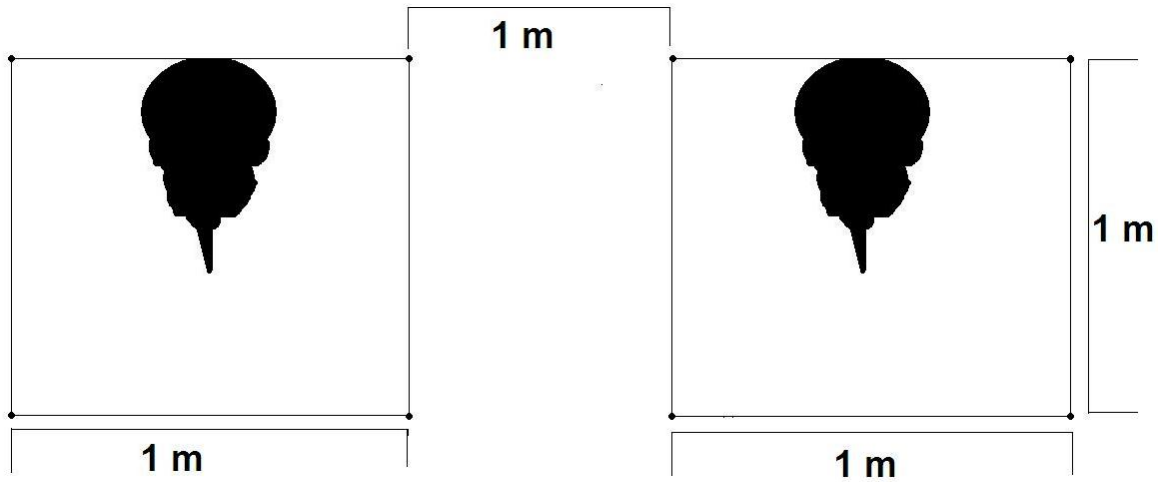| Experiment	Value	# Trials entered first	Median # entered	Avg. time in arena (sec)	Median # joined	Avg. time joined (sec)
Chapter 1	S	3	5	109.94	NA	NA
	P	13	9	120.06	NA	NA
	p	0.02	0.01	0.20	NA	NA
Satellite Male	M	7	3.5	62.11	NA	NA
Preference	P	11	6	61.33	NA	NA
Experiment	*p*	0.24	0.001	0.44	NA	NA
Chem. Cue	M	6	1	38.99	1	43.85
Differences	P	13	2	42.85	1.05	1
without	*p*	0.17	0.10	0.07	0.31	0.41
Satellites 1						
Chem. Cue	M	7	3	50.37	2	56.5
Differences	P	12	4	51.69	3	73.53
without	*p*	0.36	0.05	0.36	0.08	0.38
Satellites 2						

Figure 2-1. The experimental arenas used in the cue source experiments 1 and 2. Equal-sized 1 x 1 m arenas separated by 1 m were set up at the shoreline with the cement model pairs facing land.
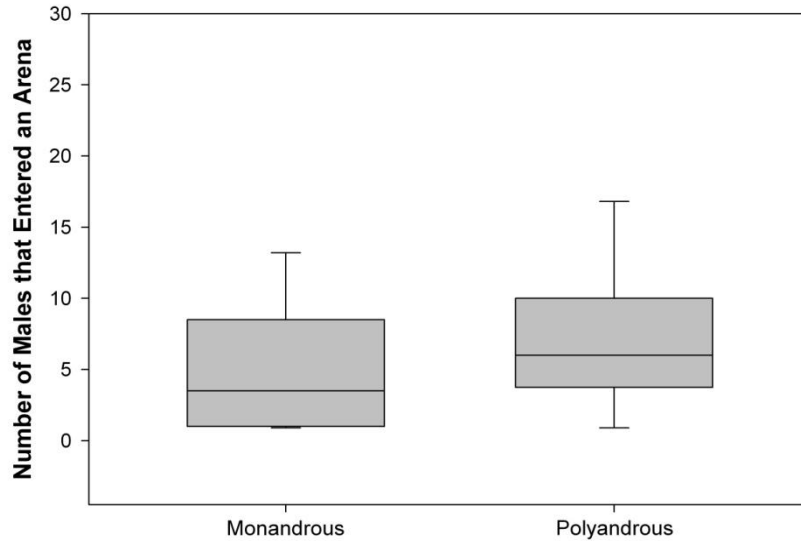
Figure 2-2. The number of male horseshoe crabs entering the arena with the model pair covering a sponge filled with water collected from a polyandrous female compared to the number that entered the arena with a model covering a sponge with water from a monandrous female in the female tactics chemical cues experiment (Wilcoxon Signed Rank test, W= 118, P= 0.001, n= 16).This was significant.
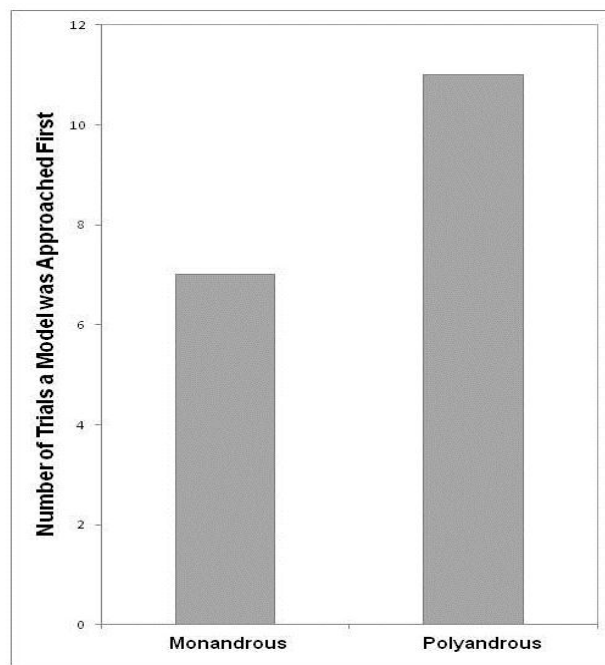

Figure 2-3. The number of trials in which a male first entered the polyandrous and monandrous model arenas in the female tactics chemical cues experiment (Chi-square test, χ^2= 0.5, P= 0.24, n= 18). This was not significant.
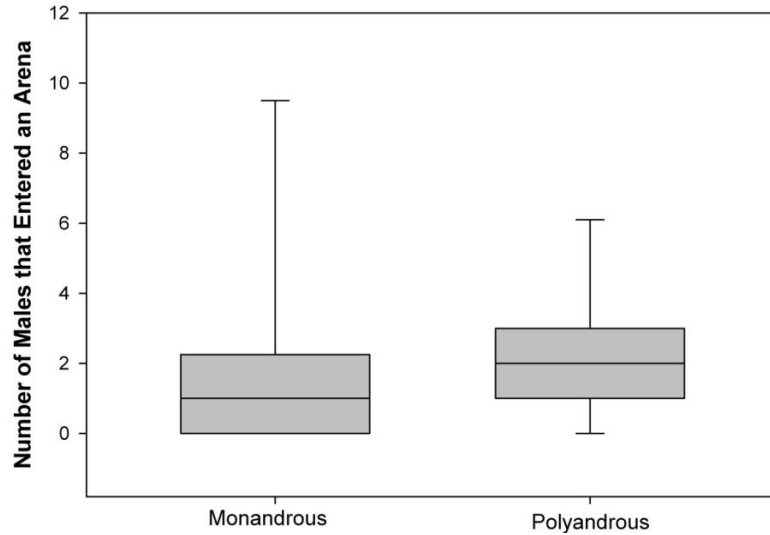
Figure 2-4. The number of male horseshoe crabs entering the arena with the model pair covering a sponge filled with water collected from a polyandrous pair compared to the number that entered the arena with a model covering a sponge with water from a monandrous pair in female cue source experiment 1 (Wilcoxon Signed Rank test, W= 59, *P*= 0.102, *n*= 18).This was not significant.

Figure 2-5. The number of trials in which a male first entered the polyandrous and monandrous model arenas in cue source experiment 1 (Chi-square test, χ^2= 1.9, *P*= 0.17, *n*= 19). This was not significant.

Figure 2-6. The number of male horseshoe crabs entering the arena with the model pair covering a sponge filled with water collected from a polyandrous pair compared to the number that entered the arena with a model covering a sponge with water from a monandrous pair in female cue source experiment 2 (Wilcoxon Signed Rank test, $W= 69$, $P= 0.05$, $n= 17$).This was not significant.
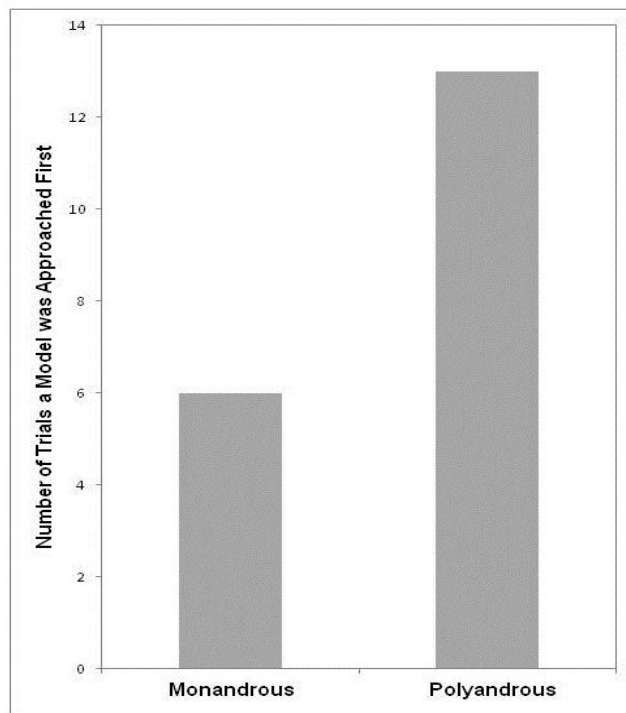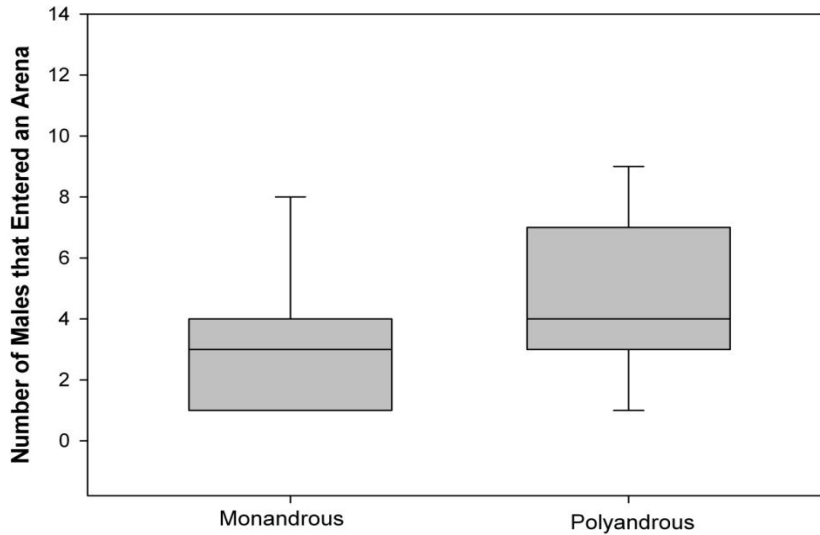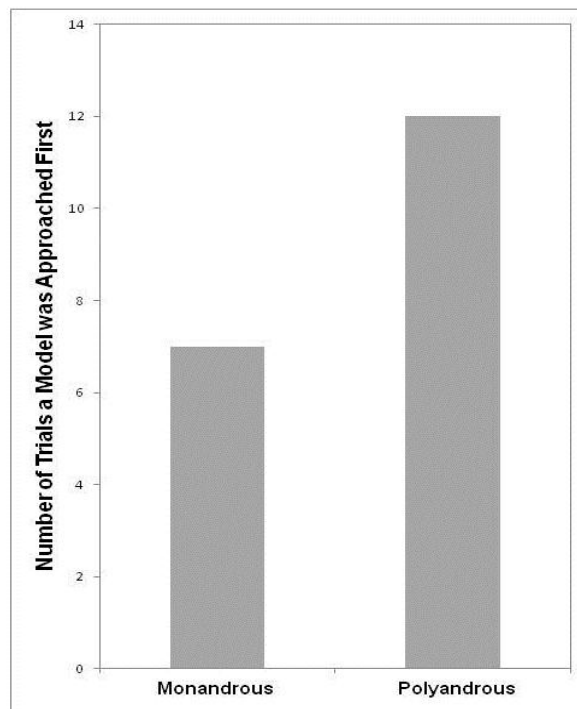

Figure 2-7. The number of trials in which a male first entered the polyandrous and monandrous model arenas in cue source experiment 2 (Chi-square test, $\chi^2= 0.84$, $P= 0.36$, $n= 19$). This was not significant.

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BIOGRAPHICAL SKETCH

Katharine Saunders grew up in Texas and first became interested in science through Texas A&M University Galveston Sea Camp in high school. Here she learned a lot about the behavior and biology of marine mammals and the kind of techniques used in this type of research. This led her to enter the Evolution, Ecology, and Behavior Biology Program at The University of Texas at Austin. Here, she immediately became involved in behavioral research studying sleep behavior in honeybees. In the summers of 2006 and 2007, she was accepted into a National Science Foundation Research Experience for Undergraduates at Northern Arizona University. It was here that she stumbled upon research in sexual selection and mating systems and strategies under Dr. Stephen Shuster and this led her to find a focus in research and apply for graduate school. She entered a Master of Science program at the University of Florida in 2008 working under Dr. Jane Brockmann, studying chemical cues and alternative strategies in the horseshoe crab. In her time at the University of Florida, she has taught four semesters of Introductory Biology Laboratory and presented her horseshoe crab research at conferences both in Florida and in France.