

Male horseshoe crabs *Limulus polyphemus* use multiple sensory cues to locate mates

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Abstract 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. In this contribution we review the sensory cues that male horseshoe crabs *Limulus polyphemus* are known to use when locating mates and then provide new data that shed light on this subject. 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 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. We show that the attraction, and retention, of attached and satellite males to actively spawning females and mating pairs involves multimodal cues [Current Zoology 56 (5): 485–498, 2010].

Key words Multimodal communication, Horseshoe crabs, Sensory system, Mating cues

1 Introduction

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 in multisensory channels simultaneously (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

Received July 04, 2010; accepted Aug. 28, 2010

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(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 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, we provide new information on their use of chemical cues, and we 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), 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 in high density Delaware Bay (Brockmann, 1996; Smith et al., 2010) and Florida (Brockmann and Penn, 1992; Penn and Brockmann, 1995) populations but not in the low density population in Long Island Sound (Mattei et al., 2010). 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.

2 Evidence for Visual Cue Use by Male Horseshoe Crabs when 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). But until recently, little was known about the ways in which vision contributed to normal behavior. Vision is widely used by chelicerates and aquatic arthropods in orienting to landmarks (Herrnkind, 1972), in escaping from predators (Lockett, 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). 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 will allow 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) (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 horse-

shoe 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). They compared the response of unattached males to two model pairs 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 during 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.

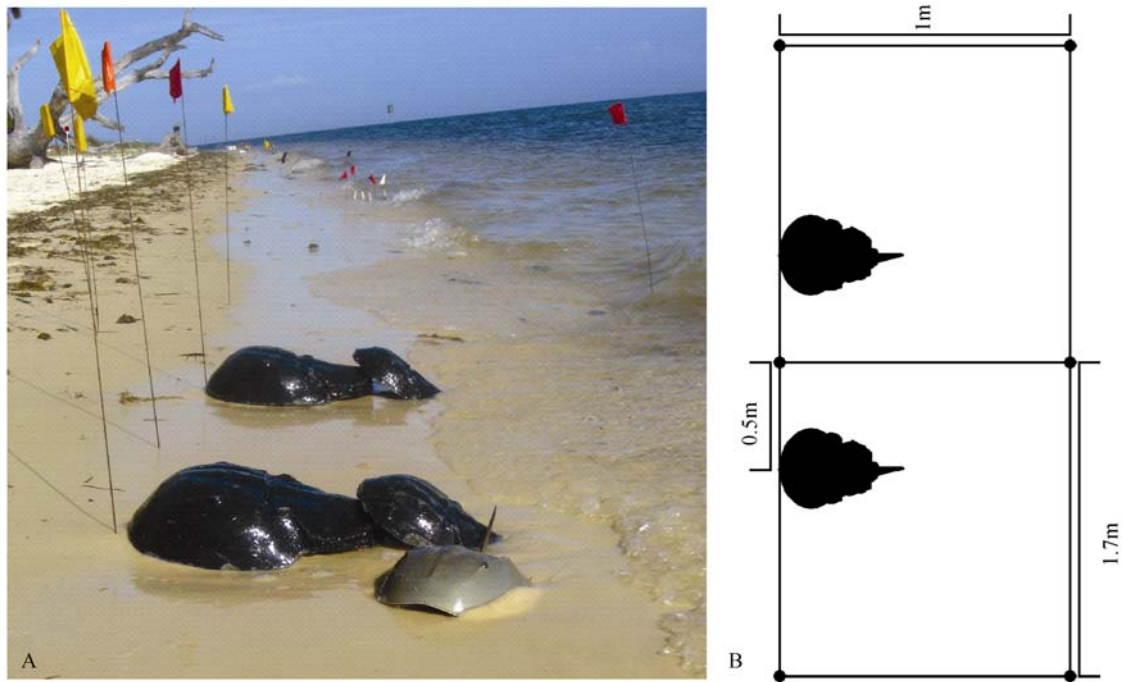


Fig. 1 Experimental setup for Florida study

A. Photograph of the experimental setup in Florida 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**.

3 Evidence for Tactile Cues used 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 incumbent 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 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).

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

A number of behavioral observations also support the use of chemical cues by horseshoe crabs. 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 soft-shelled 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.

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, 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). 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 treatment groups, in retrospect when we consider the male's visual sensitivity (Schwab and Brockmann, 2007), there were possible sources of error 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), 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%) 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.

One of the new studies presented here (section 5) further evaluates the use of chemical cues by unattached males from the same Florida population as the Hassler and Brockmann (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. In the second study using animals from a New Hampshire population (section 6), the problems associated with the use of models were avoided altogether by placing mating pairs or control individuals under an enclosure, so odor could diffuse out but visual cues were not available.

5 Experimental Study on Chemical Cue Use by Florida Horseshoe Crabs

This experiment (conducted by Saunders and Brockmann) was conducted to evaluate the hypothesis that satellite male horseshoe crabs respond to chemical cues when locating spawning pairs along the shoreline.

5.1 Materials and Methods

This experiment 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. 1b) 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 $7 \times 5.5 \times 1.5$ cm squares (no "antimicrobial" sponges were used). The experi-

mental 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.

5.2 Results

Significantly more satellite males entered the arena with the experimental treatment sponge than the arena with the control treatment sponge (Wilcoxon signed-ranks test, $W_s=79$, $P=0.02$, $n=16$; Fig. 2a). 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, $\chi^2=6.25$, $P=0.01$, $n=16$; Fig. 2b). The time spent by unattached males in the experimental treatment arena (median = 100 sec) was not significantly different from the time spent in the control arena (median = 34 sec; Wilcoxon signed-ranks test, $W_s=30$, $P=0.20$, $n=16$).

5.3 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

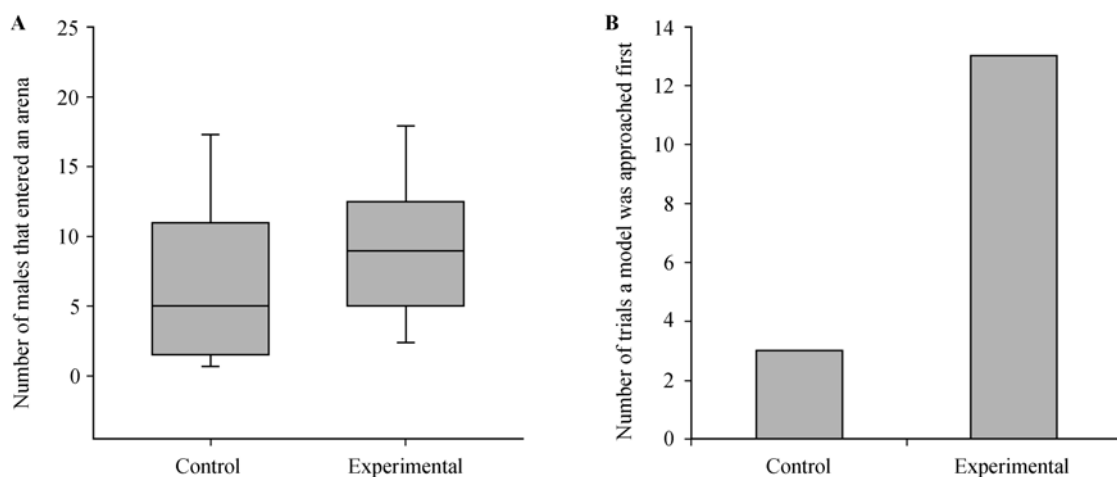


Fig. 2 Summary of Florida study on the attraction of males to chemical cues from spawning pairs

A. 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-ranks test, $W_s=79$, $P=0.02$, $n=16$). **B.** The number of trials in which a male first entered the experimental and control model arenas (Chi-square test, $\chi^2=6.25$, $P=0.01$, $n=16$).

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.

6 Experimental Study on Chemical Cue Use in New Hampshire Horseshoe Crabs

This study (conducted by Watson and Jury), tested the hypothesis that male horseshoe crabs were responding to chemical cues when locating spawning pairs near shore using a different methodology that further reduces the potential influence of visual cues.

6.1 Materials and Methods

In May and June 1984–1987, experiments on horseshoe crabs were conducted during daylight hours on a mud flat near the Jackson Estuarine Lab in Great Bay Estuary, New Hampshire. Horseshoe crabs regularly nest in this area and there is often a strongly male-biased sex ratio (Watson et al., 2009). Square enclosures (1 m sq. and 0.5 m high) were built with angle iron and opaque acrylic plastic (3.2 mm thick) and numerous (approximately 5/cm²) small (1.5 mm) holes were drilled around the lower half of each enclosure. Thus, animals could not see in or out of the enclosure but water, and potential chemical cues, could flow through. Moreover, the walls were double layered and baffled, so the holes in the outer wall did not match the holes in the inner wall, further ensuring that visual cues were absent for approaching males.

The experimental enclosure was placed over a haphazardly chosen mating pair at high tide while they were nesting in <0.5 m water so animals could be observed from the beach. In general, experiments were initiated as early in the incoming portion of the tidal cycle as possible so that fewer animals were on the beaches, there was a paucity of mating pairs and nests, and many unattached males would be entering the area during the subsequent hours. All other mating pairs were removed from the area surrounding the test enclosure, for a distance of 5 m on either side of the mating pair (at this beach the density of animals is fairly low, so often this was not even necessary). Control enclosures were placed no closer than 5 m from the enclosure containing the mating pair, and all animals within 5 m of these enclosures were also removed. Using this general procedure we conducted three experiments: 1) a control, empty enclosure was placed approximately 5 m away

from the experimental enclosure containing a mating pair; 2) a control, empty enclosure was placed approximately 5 m away from the experimental enclosure and both the control and the experimental enclosure had two cement casts of female *Limulus* (similar in size to the female under the experimental enclosure) placed outside and next to the enclosures so approaching males would have the opportunity to receive a chemical, a visual and a tactile cue and; 3) an empty enclosure was placed 5 m away from an experimental enclosure (containing a mating pair) and two additional enclosures containing either an unattached male or an unpaired female (removed from her attached male as she approached the beach) were randomly placed approximately 5m from each of the other enclosures. Thus a total of four enclosures per replicate were tested at a given time for this last experiment. For each experiment the number of unattached males that approached within 0.5 m of each enclosure every 5 min (experiments 1 & 2) or every 10 min (experiment 3) were counted for a 30 min observation period. Males were not removed after they were counted and numbers are presented as the total number of satellite males counted over the observation period.

6.2 Results

In experiment 1, the enclosure containing a mating pair attracted significantly more satellite males than the enclosure with no pair present (Fig. 3a; Wilcoxon matched pairs, $n=19$, $P<0.005$). Similarly, in experiment 2, when the enclosures had cement models present nearby, more satellite males were attracted to the enclosure with a visually shielded pair than the empty control (Fig. 3b; Wilcoxon matched pairs, $n=9$, $P<0.05$). Furthermore, the addition of the cement models showed a tendency either for males remaining longer around the enclosure, more attraction of males to the model/enclosure combination, or both (Fig. 4; Mann Whitney $P=0.057$ at the 30 min interval). The manner in which the models influenced males was not examined further by, for example, directly comparing the attraction of males to enclosures containing mating pairs that had cement models vs. no models. Finally, in the third experiment, in which four choices were presented, more unattached males approached the enclosure with the pair than any of the other choices (Fig. 3c; Kruskal Wallis, $n=9$, $df=3$, $P<0.05$).

6.3 Discussion

Unattached males regularly visited all the enclosures in the New Hampshire experiments, but the enclosures with a visually shielded spawning pair attracted significantly more males than the controls. This supports the

hypothesis that satellite males are using chemical cues to locate mating pairs on the beach. The enclosures were providing visual cues to the horseshoe crabs, but they did not resemble horseshoe crabs. They were much larger than horseshoe crabs and they extended from the substrate to the surface, thus it is unlikely that they were mistaken for a conspecific. It is also possible that the satellite males detected sounds produced by the mating pair, but we are not aware of any studies indicating that horseshoe crabs are capable of sensing acoustic stimuli. In a comparison between enclosures containing mating pairs with (Exp. 2) and without (Exp. 1) surrounding cement models, satellite males showed a tendency to remain at the enclosure longer, or more unattached males arrived later in the observation period (Fig. 4). However, experiments 1 and 2 were not run concurrently and the number of crabs available to be attracted may have differed between trial days and there was not a direct comparison of enclosure choices with and without cement models with a mating pair present. Anecdotally we also observed that unattached males clasped the cement models next to the experimental enclosure containing a spawning pair (and expected chemical cues), but not when they were next to the control enclosure. This supports our general hypothesis that horseshoe crab satellite males use multiple sensory modalities to locate spawning pairs.

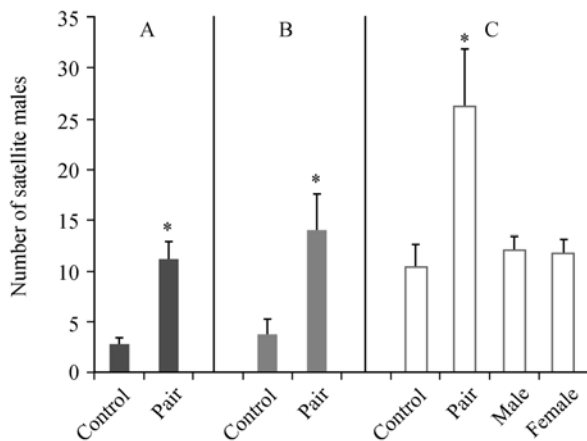


Fig. 3 Summary of New Hampshire satellite male attraction studies

A. Experiment 1: the number (mean \pm SEM) of unattached males attracted to mating pairs in enclosures was significantly greater when compared with empty enclosures (Wilcoxon matched pairs, $n=19$, $P<0.005$). B. Experiment 2: the number of unattached males attracted to mating pairs in enclosures was also significantly greater when compared with empty enclosures with the addition of two cement models of *Limulus* outside each enclosure (Wilcoxon matched pairs, $n=9$, $P<0.05$). C. Experiment 3: the number of unattached males attracted to mating pairs in enclosures was significantly greater than attraction to enclosures containing an unattached male, an unattached female or an empty enclosure (Kruskal Wallis, $n=9$, $P<0.05$).

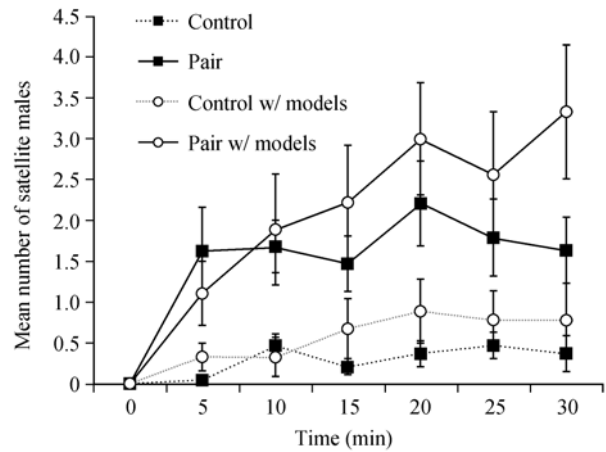


Fig. 4 Time course of satellite male attraction with and without cement models

The mean \pm SEM of satellite males attracted in experiment 1 without models (dark squares, $n=19$) and experiment 2 with models (light circles, $n=9$) in the New Hampshire studies are shown for each five minute observation interval. In both experiments the enclosures containing a mating pair attracted significantly more satellite males than the controls. Furthermore there was a tendency for the enclosures with cement models to attract (or retain) more satellite males by the end of the 30 minute trial (Mann Whitney $P=0.057$).

7 General Discussion of Chemical Cues in Horseshoe Crabs

The new experimental studies described here make it clear that in populations of *Limulus polyphemus* from Florida to New Hampshire, unattached males are using chemical cues to locate spawning pairs. The new Florida study confirms the findings from the Hassler and Brockmann (2001) study by holding tactile and visual cues the same and by presenting realistic cement models of a male-female pair nesting along the shoreline. One of the New Hampshire experiments 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. 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.

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) When nesting is complete and the pair has left the beach, unattached males circle around the depression that was left in the sand by the recently departed pair (Cohen and Brockmann, 1983). While unattached males may be using tactile or visual cues to locate these small depressions in the sand, chemical cues from the departed pair seem a more likely explanation for the satellites' behavior. (b) 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. (c) Groups were not clumped but rather were interspersed with singly mating pairs over the entire active section of the beach. (d) The new Florida experiment 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, the New Hampshire study demonstrates that unattached males were attracted by spawning pairs without satellite males being present. 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 sub-

sequent 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.

Although, we can narrow down the cue source as being from the nesting pair, we still cannot say whether it is the female, her attached male or both that is producing the cue. Therefore, it is also unknown whether this chemical cue is actually a pheromone or a by-product of spawning behavior that unattached males are using to locate mates. Like other animals with an explosive breeding system (Brockmann, 1990) where there is intense competition among males to actively locate females, there appears to be little selection on females to expend energy in attracting mates (Brockmann, 2003).

The nature of the chemical compound is also unknown. Horseshoe crabs secrete an anti-fouling and cytolytic substance from dermal glands located over the entire surface of the carapace (Harrington et al., 2008), which could be a possible source of attractant. A chemical signal from horseshoe crabs is known to attract the flatworm *Bdelloura candida*, a parasite of horseshoe crabs (Chevalier and Steinbach, 1969). From the Florida chemical cues experiment, we know that the chemical attractant is something that can be isolated from its source and can still attract unattached male horseshoe crabs, and the NH experiment demonstrates that the compound is not produced by lone females or males. Hassler and Brockmann (2001) showed that unattached males were still attracted to the cue even after the nesting pairs were removed but the number of males being attracted declined over time. This might point to degradation of the compound over time, although the wave action might have also caused the compound to dissipate.

8 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). 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, the cues that affect whether a male will stay attached to a female or remain a satellite of a pair are determined by the presence of chemical cues. In the New Hampshire study presented here, males attached to female models placed around enclosures that contained a mating pair, but not models placed around a control enclosure. In addition, 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. The New Hampshire study 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 back up 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 chitino-clastic 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.

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. Thus, 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. 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.

Acknowledgments We thank the Jackson Estuarine Lab in Great Bay Estuary, New Hampshire and the University of Florida Marine Laboratory at Seahorse Key and its Director, Harvey Lillywhite, and the Lower Suwannee National Wildlife Refuge and its Manager, John Kasbohm for their support of this project. Kimberley Barbeitos de Sousa, Lindsay Keegan, Jill Turnbull and Jim Groome provided field assistance. This research was supported by the National Science Foundation IOB-0641750 to Jane Brockmann.

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