Chapter 20
Approaches to a Molecular Identification of Sex Pheromones in Blue Crabs

Michiya Kamio and Charles D. Derby

Abstract Molecular identification of sex pheromones in marine crustaceans has proven to be very difficult, and so far no unequivocal identification for any decapod crustacean has been published. Some of these difficulties are common to other animals – pheromones are often blends of molecules at low concentrations. Some difficulties are more specific to marine crustaceans – pheromones are often small and polar molecules that are difficult to separate from salts in their source (often urine) or carrier medium (sea water). These difficulties led us to take on new approaches as we searched for sex pheromones in the blue crab *Callinectes sapidus*. Premolt pubertal female blue crabs that are ready to mate release a pheromone in their urine. This pheromone is detected by male crabs using specific chemical sensors – aesthetasc sensilla on the antennules. Male blue crabs respond to the pheromone with courtship stationary paddling, a distinctive behavior that is useful in bioassays for pheromone identification. We used bioassay-guided fractionation to demonstrate that the pheromone of female blue crabs is of low molecular mass (<1,000 Da) and possibly a mixture. We used liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance, biomarker targeting, and metabolomics approaches to isolate molecules specific to premolt pubertal females and that are thus candidate pheromones. Our working hypothesis is that female blue crabs release a species-specific sex pheromone in their urine that is composed of two functional classes of molecules, both of which are small and polar. One class distinguishes females from males and thus is a sex-specific signal, and a second class distinguishes blue crabs from other species and thus constitutes a species-specific signal.

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20.1 Introduction

Blue crabs are known to all who frequent shallow waters along the east coast of North America. Their abundance and tasty meat have made them a major commercial fishery for hundreds of years. Lifestyles of people, even cultures, have been defined by them, as chronicled in William Warner's book, "Beautiful Swimmers: Watermen, Crabs and the Chesapeake Bay." In fact, the blue crab's scientific name, *Callinectes sapidus*, given in 1896 by Dr. Mary Jane Rathbun, at the time one of the foremost authorities on crustacean systematics, means "good-tasting beautiful swimmer." That they are, but they are much more, as we hope we convey in this chapter.

The life history of blue crabs is well known because of their abundance and economic value. A recent scholarly book, "The Blue Crab, *C. sapidus*," edited by Victor Kennedy and Eugene Cronin, makes the interested reader up to date on what scientists know about blue crabs. Our research focus, and that of this chapter, is on sexual behavior of blue crabs, including pair bonding and mating, and the role of chemical communication in these behaviors. Because the pheromones of reproductive females are only released during a few days of their entire life, as described below, and in very small amounts, we use the advantages of the commercial fishery to obtain these chemicals in amounts sufficient to perform the requisite chemical analyses and behavioral bioassays.

Identifying pheromones of crustaceans, especially sex pheromones, is a challenging prospect, as is obvious to anyone who reads this book. In fact, there are very few successes in molecular identification of pheromones of crustaceans. Examples include larval settlement factors of barnacles, which are large α_2-macroglobulin glycoproteins called "settlement inducing protein complex" (Dreanno et al. 2006a, b, 2007). The pheromone used by male copepods, *Tigriopus japonicus*, to recognize females has been partially characterized – and interestingly, it too has similarity to α_2-macroglobulin (Ting and Snell 2003). Despite many attempts to identify sex pheromones of decapod crustaceans such as crabs, crayfish, clawed lobsters, and spiny lobsters (for example, see Gleeson 1991; Asai et al. 2000, 2001; Hardege et al. 2002; Kamio et al. 2002, 2003; Atema and Steinbach 2007), there are no published papers that present a convincing demonstration of the molecular identity of their pheromones (but see the chapter in this book by Hardege and Terschak on the shore crab *Carcinus maenas*).

Our own individual experiences that have led us to attack this problem are quite different. One of us – Michiya Kamio – has been well trained in chemistry, in addition to behavioral biology, and has applied these skills to the search for crustacean sex pheromones starting with graduate school. The other – Charles Derby – has always been interested in the chemistry of crustacean pheromones, but lacking the training, willing collaborators, and especially in the earlier years, adequate technology, focused on other aspects of crustacean chemical senses. Several years ago, we decided that it was a good time to bring our skills together and start a

collaboration focused on blue crab sex pheromones. Julia Kubanek was our first collaborator, and we have since brought in others who have specialized analytical chemistry skills to help in our work. We also were fortunate to have the advice, assistance, and inspiration of Richard Gleeson, who did groundbreaking work on chemical communication in blue crab reproductive behavior (Gleeson 1980, 1982, 1991; Gleeson et al. 1984, 1987). In this chapter, we outline the experimental strategies and techniques that we are using to try to identify the sex pheromones of blue crabs. We also consider chemical signals in the context of blue crab behavioral ecology, chemical ecology, and interspecies ecological interactions in natural habitats. We are working towards a more general understanding of the mating and reproductive behavior of blue crabs.

20.2 Features of Mating Behavior in Blue Crabs and the Role of Chemical Communication

20.2.1 Molting and Mating

Molting and mating are closely linked in blue crabs, as it is in many (but not all) decapod crustaceans (Jivoff et al. 2007). Blue crabs, like other arthropods, grow by molting. Male blue crabs grow continuously throughout their lives and become sexually active after reaching a mature size. Female blue crabs, on the other hand, grow until a final molt, called the pubertal or terminal molt. Following this molt, females are sexually mature and reproductive and are often called "pubertal females." They have an enlarged abdomen for incubating the fertilized eggs.

Female blue crabs mate only at this time of their life (Fig. 20.1). Timing is critical. They must mate just after the terminal molt, before their new hard shell forms. Before mating, females must find a suitable mate. They do this at a severely vulnerable stage of their lives. Both males and females take on a significant risk and energetic investment, along with the promise of reproductive gain, in mating. Molting itself is a risky endeavor. Animals in the stage immediately before molting, i.e., premolt, are not very active, mobile, or strong, and molting animals lose all mobility. The state immediately after molting, i.e., postmolt, is also risky. At this time, they are soft because their new shell has not yet hardened. It is these immediate postmolt crabs, both females and males, that are the gastronomic delicacy known as "soft shell crabs." For females, the risks of being soft-shelled are further compounded by the aggressive behavior of male crabs during mating. How do female crabs survive the risks of mating in this vulnerable state?

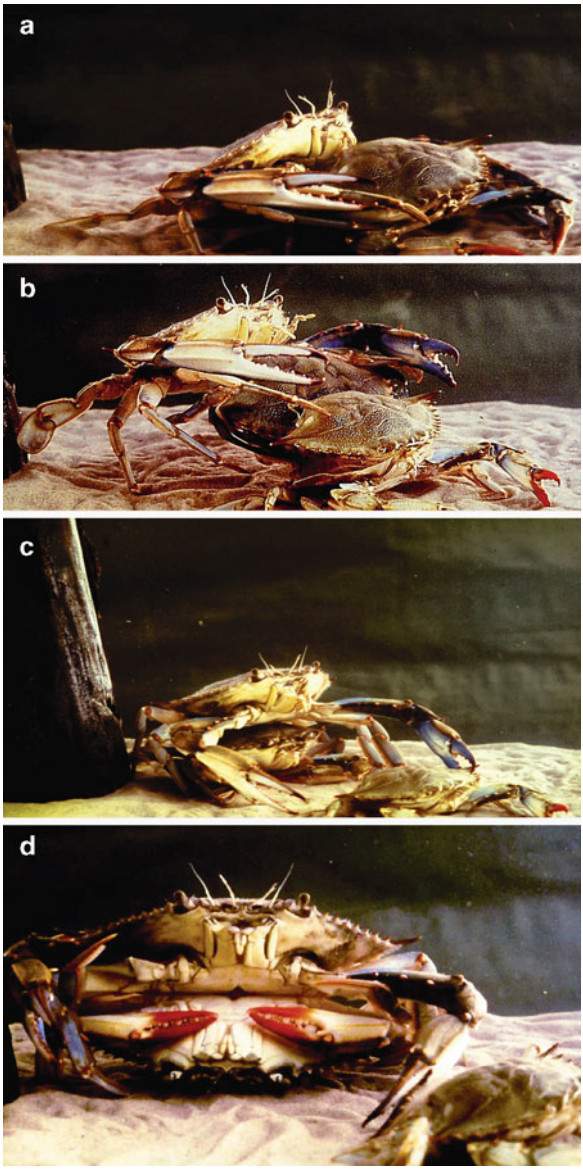

Fig. 20.1 Series of photographs of a male–female pair, beginning with cradle carry and culminating in copulation. (**a**) Male crab (*left*), with his blue-tipped claws, cradle carries the smaller, red-tipped female (*right*) as she is beginning to molt. (**b**) Female continues to back out of her shell, under the protection of the male. (**c**) Female completes molting, with her old shell, or exuvium, littering the substrate in front of her and the male returning to his cradle carrying position. (**d**) Male and female copulate. This previously unpublished series of images is courtesy of Dr. Richard Gleeson and used with his permission

20.2.2 *Bidirectional Chemical Communication Between Males and Females During Courtship Behavior in Natural Environment*

Most animals mate in a narrow time window of their lives and therefore must signal to each other their readiness to reproduce. Collectively, they do so through a diversity of sensory channels within environmental and evolutionary constraints (Bradbury and Vehrencamp 1998). This is also true for many crabs (e.g. Hardege and Terschak, Chap. 19), including blue crabs, as described here. The female blue crab communicates that she is approaching her terminal molt, and the male communicates his interest in taking her as a mate. They do this through multiple sensory channels. One is visual signaling, including male preferences for the red color on female claws (Teytaud 1971; Baldwin and Johnsen 2009). Another means of communicating sexual readiness is chemical signaling, including reciprocal release and detection of sexual pheromones. As the female approaches her terminal molt, she produces a pheromone that changes the behavior of males. Several scientists, most notably Richard Gleeson, have helped us understand this chemical communication in blue crabs (Gleeson 1980, 1982, 1991; Gleeson et al. 1984, 1987; Bushmann 1999; Jivoff et al. 2007).

What is the effect of the female pheromone on males? Male blue crabs respond to the female pheromone with a series of behaviors. First, they search for the source of the pheromone by walking upstream while sensing features of the chemical plume containing the pheromone (Gleeson 1991; Kamio et al. 2008; Dickman et al. 2009). If the searching male finds a female and she is accessible to him, he immediately grabs her and initiates "cradle carry" behavior (Fig. 20.1c). In this stereotypical behavior, the larger male holds the female under his abdomen and both animals are positioned with their dorsal side upwards. However, if the male detects a female but cannot reach her because she is inaccessible to him, then he performs a highly stereotyped behavior called "courtship stationary paddling" (Fig. 20.2). The term "courtship display" has a broader meaning, since it includes standing high on legs, spreading chelae, holding paddles up over the carapace, and performing courtship stationary paddling. Inaccessibility in the natural environment occurs when the female is hiding, which females do around the time of molting. Inaccessibility can be simulated in the laboratory by caging the female. When a male performs courtship stationary paddling, he elevates his body by standing high on his legs, opens his chelae, and paddles his last pair of legs, which in this species have paddles at the end and thus are called swimming legs.

What is the function of courtship display and courtship stationary paddling? It is probably a multimodal signal to females. It probably contains a visual signal, since sexually receptive females respond to a model of a male crab in the stationary posture of courtship display (Teytaud 1971) and the red color on the female claws can induce courtship by males (Baldwin and Johnsen 2009). The dynamic component of the display, paddling, probably also provides a characteristic and conspicuous visual stimulus, though this has not been experimentally tested. The water current generated

Fig. 20.2 Courtship stationary paddling of a male blue crab (*right*) towards a female (*left*) that is inaccessible to the male due to a barrier between them. This behavioral display has both postural and dynamic components. The male stands erect on extended legs and laterally spreads his chelae. He also rhythmically waves his last pair of legs, which have paddles and also are used for swimming. This behavior includes chemical and visual components. See text for more explanation. Photograph by Peter Essick, used with permission

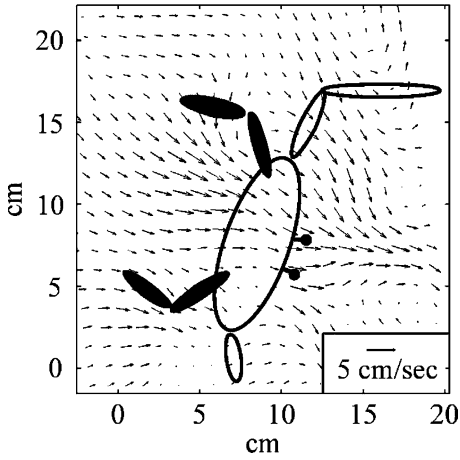

Fig. 20.3 Water currents generated by the courtship stationary paddling of male blue crab. View from above, the male performing courtship stationary paddling. Particle imaging velocimetry was used to visualize water currents generated by this behavior. *Arrows* indicate the direction of the currents, with the length of the *arrows* being proportional to the velocity. Results show that the water current was directed away from the male at a mean velocity of 3.1 cm/s. From Kamio et al. (2008), reproduced with permission of The Journal of Experimental Biology

by paddling may act as a mechanical signal to females. We know for certain that courtship stationary paddling creates a water current directed away from the male and toward the female (Kamio et al. 2008; Fig. 20.3). We also know that males release a pheromone to which pubertal females respond. These females move upstream over a

few meters in a flow scented with water from reproductive males (Gleeson 1991), and they will perform other behaviors that facilitate pair formation (Rittschof 2005). We conclude that courtship stationary paddling is a form of chemical signaling that induces females to move towards males, leading to pair formation, cradle carry, and eventually mating.

Our notion is that courtship stationary paddling is an adaptation to the environmental conditions in which mating occurs (Kamio et al. 2008; Fig. 20.4). Females approaching their terminal molt move to areas such as salt marshes, which have plentiful submerged vegetation and other refuges for females to hide from predators. We hypothesize that courtship stationary paddling is a context-dependent behavior that males produce only when females are inaccessible and that functions

Fig. 20.4 This drawing portrays how we envision blue crabs communicate their sexual status to each other, in their natural environment, leading to pair bonding and mating. A pair of blue crabs residing in a salt marsh is shown, with the female on the *left* and the male on the *right*. When premolt females such as this one approach their terminal molt at which time they become sexually mature and mate, they hide in grass beds to avoid predators such as sea turtles, sharks, and egrets, and release a pheromone that attracts males. If a male can easily reach the female, he picks her up and initiates cradle carrying behavior as shown in Fig. 20.1c. But if he cannot easily reach the female, he performs a courtship stationary paddling, as shown in this illustration and in Fig. 20.2. This behavior generates a water current that delivers his pheromone to the hidden female, as depicted in Fig. 20.3. Once the female detects and locates the source of the pheromone, she will move out of hiding so that the male can initiate cradle carrying behavior. Drawing by Jorge A. Varela Ramos

to deliver his sex pheromone to the female, in an effort to entice her to emerge from her refuge and engage in cradle carry.

After pair formation, the male crab will hold and protect the female for a period of time necessary for her to molt, then mate, and then for her shell to harden to the point that she can defend herself and protect their reproductive investment. This period of cradle carrying may last several days. It is possible that contact pheromones mediate interactions of male and female crabs after they find each other, including copulation, as is the case in other decapod crustaceans (Kamio et al. 2002; Díaz and Thiel 2004; Caskey and Bauer 2005), but this has not been studied. The process of molting and mating is intricate, gentle, and seemingly choreographed. A series of photographs of this process is shown in Fig. 20.1. The female backs out of her shell with the male's assistance, still guarded by the male. The male flips her over, and they copulate, a process that can last for many hours. The male then turns her over again, and resumes cradle carrying for 4–5 days until her shell hardens and the male releases her (Jivoff et al. 2007).

20.2.3 Site of Release of Pheromones

The female's pheromone that induces courtship stationary paddling by males is in her urine (Gleeson 1980). Since crabs and other crustaceans can control the release of their urine, they can control the release of pheromone. Female blue crabs may squirt the pheromone towards males at select times to maximize the use of this limited resource, although this has not been experimentally demonstrated. Bushmann's work (1999) suggests that the female's pheromone may be released not only in urine, but also from other, unidentified sources. The site of release of the male pheromone is not known, but we hypothesize that it is also in his urine, as it acts from a distance (Gleeson 1991).

20.2.4 Receptor Organs for Detecting Pheromones

What are the mechanisms responsible for sensing pheromones? The male detects the female's pheromone using only one of his many types of sensors – the aesthetasc sensilla. These are located on the distal end of their first antennae, or antennules. Blue crab aesthetascs are densely packaged on the antennule, and each aesthetasc contains approximately 150 olfactory receptor neurons (Gleeson et al. 1996; Cate et al. 1999). Aesthetascs are a type of sensillum found in many crustaceans, even the more ancient and primitive forms (Hallberg and Skog, Chap. 6; Schmidt and Mellon, Chap. 7). They are the only sensilla known to be exclusively chemosensory. They are considered olfactory because of homologies with olfactory sensors of terrestrial arthropods and analogies with olfactory systems of the vertebrates (Schmidt and Ache 1996). Aesthetascs are innervated by

olfactory neurons sensitive to a range of odors, including food-related chemicals. The emerging view is that they are the only antennular sensors containing receptors for pheromones and other intraspecific cues mediating sex, social interactions, aggregations, and alarm (Gleeson 1982; Shabani et al. 2009; Schmidt and Mellon, Chap. 7). Males lacking aesthetascs cannot detect the female's sex pheromone and will not mate with her (Gleeson 1982, 1991). The location of the female's sensors of the male pheromone is not known.

20.3 Experimental Approaches to Identifying Sex Pheromones of Female Blue Crabs

20.3.1 Possible Experimental Approaches

The molecules serving as cues, signals, or pheromones can be identified by a variety of experimental approaches. One approach that has been highly successful, leading to the identification of many bioactive cues, is bioassay-guided fractionation (Hay et al. 1998; Koehn and Carter 2005). Since the pheromone of female blue crabs is not a protein and is present in freeze-dried urine (Gleeson et al. 1984; Gleeson 1991), we focused on identifying relatively small, polar molecules present in the urine. This approach uses isolation of molecules from the natural source of the signals, which are termed natural products, together with bioassays. It begins by separating a natural product with bioactivity into fractions based on physicochemical properties such as polarity, molecular size, or molecular charge (Sarker et al. 2006). An example is using gel filtration to separate compounds based on molecular size, then testing the resultant fractions for bioactivity using behavioral assays. High throughput methods such as electrophysiological screening can speed up the process of identifying molecules that are detected by an animal. Examples of this approach include mouse (Lin et al. 2005), cockroach (Nojima et al. 2005), and sea louse (Ingvarsdóttir et al. 2002a; Fields et al. 2007). But ultimately behavioral assays are required to know the behavioral relevance of the molecules. Bioassay-guided fractionation requires a comparison of the bioactivity of the fractions against the original material and the recombined fractions, the latter to determine if the activity was lost during fractionation either by degradation or loss of bioactive molecules or the effect of trace solvents that may be added to the fractions. A negative control allows identification of fractions containing the bioactivity.

The active components can be extracted from tissues, organs, or secretions. These can then be separated and purified based on differences in polarity, size, and ionic charge using solvent–solvent partitioning, liquid chromatography (LC), or high performance liquid chromatography (HPLC) with a variety of separation modes such as size exclusion, ion exchange, normal phase, and reversed phase. Once separated to sufficient purity, the bioactive molecules can be identified through a combination of spectroscopic methods such as mass spectrometry

(MS), nuclear magnetic resonance (NMR) spectroscopy, infrared spectroscopy (IR), ultraviolet spectroscopy (UV), circular dichroism (CD) spectroscopy, X-ray crystallography, or other approaches including organic synthesis of the candidate compounds, as has been already demonstrated for other pheromones and natural products (Haynes and Millars 1998; Koehn and Carter 2005; Sorensen and Hoye 2007). Databases such as SciFinder Scholar (http://www.cas.org/SCIFINDER/SCHOLAR/index.html) and Marinlit (http://www.chem.canterbury.ac.nz/marinlit/marinlit.shtml) are useful in identifying the molecular structures, by comparing spectroscopic data, molecular formulae, and proposed structures of the purified bioactive molecules against those in the databases for identified molecules.

One potential limitation in the use of bioassay-guided fractionation is revealed when a pheromone or other natural product under study is a mixture and the full expression of that mixture's bioactivity requires the simultaneous presence of several or all of its compounds. For example, if more than one fraction has components of the mixture but each fraction contributes a small portion of the mixture's activity, then identification of the bioactive molecules can be difficult. Another example is when there are synergistic interactions among bioactive molecules in different fractions, such that once fractionated, no single fraction has activity.

Although bioassay-guided fractionation has proven successful for identifying many bioactive molecules, other experimental approaches can be useful as well. One of these is biomarker targeting. A biomarker is any molecule that is associated with a specific physiological state or condition, being either unique to or in significantly higher or lower concentration in individuals in that state or condition. Consequently, biomarker targeting is the search for the identity of those distinctive molecules. For example, biomarker targeting in the study of a disease involves identifying molecules in urine, blood, or some other body fluid or odor that indicate that disease's specific occurrence (Soga et al. 2006). Another example of biomarker target is the search for pheromones. Ovulated female masu salmon release L-kynurenine in their urine as a sex pheromone (Yambe et al. 2006). Although L-kynurenine was isolated by bioassay-guided fractionation, direct analysis of urine from mature and immature males and females detected L-kynurenine only in ovulated female urine. Since L-kynurenine has a characteristic ultraviolet absorption, comparative analysis using HPLC equipped with a photodiode array and/or MS identified this compound as a biomarker of a female that is releasing the pheromone. A limitation of biomarker targeting is that it has the potential to lead to false positives because the molecules that are in relatively high or low abundance in the target sample are not necessarily contributing the most, or at all, to that sample's bioactivity. And of course, whatever molecules are identified by this technique must be tested in behavioral assays for their biological relevance.

As described below, we have used biomarker targeting to search for blue crab sex pheromones by seeking molecules that distinguish urine of pubertal females from urines of other females, premolt males, juveniles, and any other urine that does not have pheromonal bioactivity.

20.3.2 Application Towards Identifying Sex Pheromones from Female Blue Crabs

20.3.2.1 Bioassay-Guided Fractionation

Richard Gleeson provided a preliminary characterization of the molecules in urine of pubertal females that induce courtship stationary paddling in males. He reported that the pheromone is 300–600 Da and stable at high temperature, 95°C (Gleeson 1991). This was based on gel filtration using a Sephadex column calibrated with peptide markers. Gleeson also used the other separation methods – ultrafiltration using YC-2 filter andYM-05 filter (Amicon) – to retain the pheromone in a fraction with mass of 500–1,000 Da, then using reverse phase chromatography for chemical separation. Fractions were bioassayed using a behavioral test based on a male crab performing cradle carry behavior towards a target animal in the same aquarium. In nature, the target animal is a premolt pubertal female. Adding to the bioassay, a target animal that does not release the pheromone but does provide a physical presence is important as it increases the responsiveness of the male test animal to the pheromone without increasing the response to sea water, a negative control stimulus. Gleeson used as the target animal another male that had been rendered unresponsive to the female pheromone through antennular ablation. Gleeson et al. (1984) further showed that the active component is not crustecdysone, a molecule previously reported to be a female sex pheromone in other crab species (Kittredge et al. 1971), though this result has been questioned (Hayden et al. 2007).

Our bioassay-guided fractionation began as did Gleeson et al.'s (1984) by separating premolt pubertal female urine into three fractions with molecular size of $<$500, 500–1,000, or $>$1,000 Da, using ultrafiltration with YM-1 and YC-05 membranes. Our study differed from that of Gleeson in two ways. First, we used a nonmating postpubertal molt female as the target animal. Second, we used courtship stationary paddling as our behavioral indicator of responsiveness to pheromone, because it is distinct, easily quantifiable, and most importantly, is the only behavior produced by males specifically in response to pubertal female urine. Other behaviors such as grasping and lateral spread of chelae were evoked by pubertal female urine, but not significantly more than by urine from nonreproductive females or urine from males. We found that the $<$500 and 500–1,000 Da fractions of female urine were the only stimuli to evoke significantly more courtship stationary paddling than the negative control, sea water (Fig. 20.5). This result is partially consistent with that of Gleeson (1991), but differs in revealing activity in a fraction $<$500 Da. Thus, the sex pheromone of blue crabs includes small, polar molecules. This is also the case with some other decapod crustaceans (see Hardege and Terschak, Chap. 19). However, not all crustacean sex pheromones are small and polar (hydrophilic): those of sea lice appear to be small nonpolar (lipophilic) molecules (Ingvarsdóttir et al. 2002b), and those of copepods are large proteins (Ting and Snell 2003).

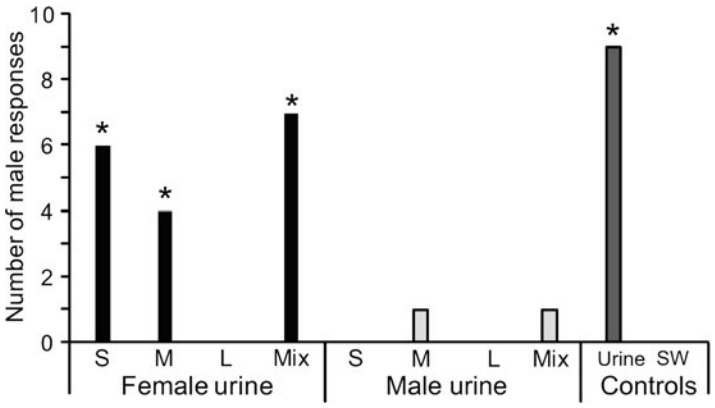

Fig. 20.5 Using bioassay-guided fractionation to identify the molecular size of female sex pheromone. The dependent measure is the number of male blue crabs out of 72 tested that performed courtship stationary paddling. Stimuli were male or female urine fractionated into the indicated molecular sizes (*S* small <500 Da, *M* medium 500–1,000 Da, *L* large >1,000 Da, a mixture of *S*+*M*+*L* Mix) and positive control (unprocessed pubertal female urine = Urine) and a negative control (*SW* sea water). Friedman ANOVA shows an overall difference in the responsiveness to these stimuli ($P < 0.0001$, $n = 10$). An asterisk marks stimuli that elicit significantly more males to respond compared to the sea water control (Wilcoxon post hoc tests, $P < 0.05$)

The advantage of this bioassay-guided fractionation method is that it enables discrimination of components with sex pheromone activity from those that do not. Serial repetition of separation guided by bioassay should lead to purification of the sex pheromone molecules. The disadvantage of the application of this experimental approach to blue crabs is that the bioassay is time-consuming. Courtship stationary paddling is strictly specific to courtship, and males produce it only when females are inaccessible (Kamio et al. 2008); consequently, under the conditions of our laboratory assay, only 10% of males showed courtship stationary paddling to the target animal in the presence of premolt pubertal female urine, the positive control. This low occurrence of the courtship stationary paddling is problematic for bioassay-guided fractionation because it slows the process. We are currently modifying our bioassay in an effort to increase the responsiveness of males. For example, we know that visual signals from females (Baldwin and Johnsen 2009) and inaccessibility of females (Kamio et al. 2008) contribute to the male's courtship stationary paddling. Adding these features to our bioassay will, we hope, make it more useful.

20.3.2.2 Biomarker Targeting

Another approach to isolating sex pheromones of blue crabs takes advantage of the economic importance of these animals (Kamio 2009). The significant fishery for hard-shell and soft-shell crabs gives us access to large numbers of crabs of either sex and any molt stage, including pubertal females. Comparison of the chemical

composition of urine from these animals allows identification of molecules that are unique to or concentrated in each type, and therefore candidate sex pheromones. In our search for female sex pheromones, we compared the chemical composition of urine from premolt pubertal females against the urine of intermolt females, premolt males, and other stages. The following section describes our use of analytical techniques in biomarker targeting of the female sex pheromone.

Liquid Chromatography-Mass Spectrometry (LC-MS)-Based Biomarker Targeting

LC-MS separates compounds in a mixture using HPLC, identifies the molecular weight of compounds using MS, and determines the relative concentration of each. Applying principal component analysis (PCA) to these data allows us to determine the relative similarity in the overall compositions of mixtures, based on all molecules that are separable, identifiable, and quantifiable. From this, we can identify which components are most different in those mixtures.

In collaboration with Tomoyoshi Soga and Yuji Kakazu of Keio University, Japan, we used LC-MS PCA to compare urine from four premolt males, urine from five premolt females, and three samples of deionized water as a control. The results are shown in Fig. 20.6. The LC-MS detected hundreds of compounds based on molecular weight and retention time in the male and/or female urines and determined their relative concentrations. PCA shows that two principal components (PC1 and PC2) explained 70% of the variance in the data (PC1, 57%; PC2, 13%). Furthermore, the four male urines form a group (represented by a dashed oval in the two-dimensional PC space of Fig. 20.6), the five female urines form a different group (represented by another dashed oval), and both of these are distinct from the group of water controls (Fig. 20.6). This indicates that the overall compositions of the male urines are more similar to each other than to the female urines, and vice versa, and both are very different from water. In other words, despite variations between animals in the chemical compositions of their urine, there is a "male type" urine and a "female type" urine. Further analysis using tandem MS experiments can identify which of the compounds is specific to male urine or female urine, as well as those that are common to both male and female urines.

NMR-Based Biomarker Targeting

An advantage of NMR-based biomarker targeting is that it does not require any separation methods and thus we can use more complex mixtures. But this advantage brings with it a challenge: it can be difficult to identify the compounds in the complex NMR spectrum. A limitation is that NMR, especially 1D-NMR, alone usually yields partial structures even for a pure compound. 2D-NMR gives more information and resolution for structure analysis, but its lower sensitivity is a disadvantage. Complete molecular identification often requires a combination of approaches.

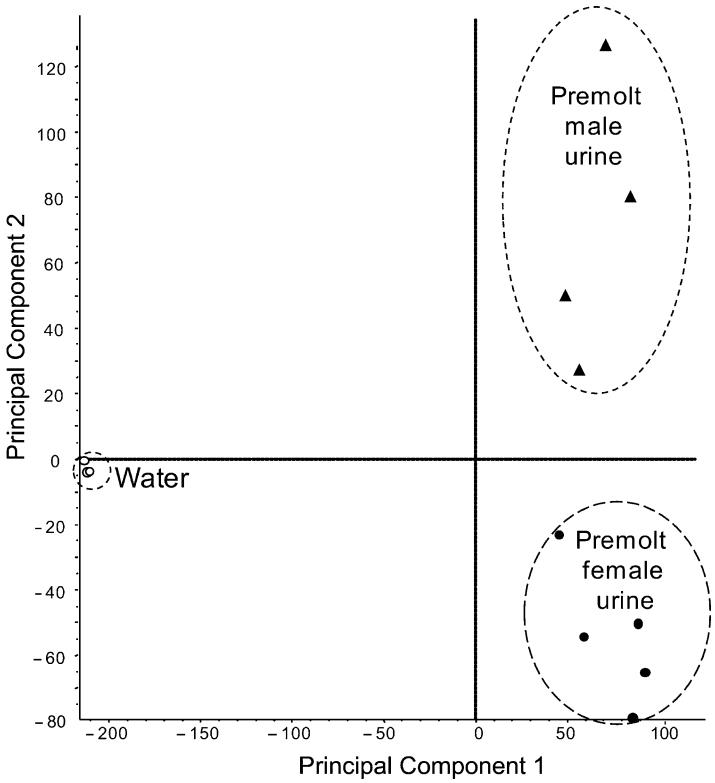

Fig. 20.6 Results of principal components analysis on HPLC-Mass Spectrometry data for premolt male and premolt pubertal female urines. See text for explanation

We used 1D ^{1}H NMR, which analyzes all protons in a mixture. As a first step towards identifying female sex pheromone molecules, we started by detecting features in the spectra, i.e., molecules specific to premolt female urine. ^{1}H NMR spectra of 0.2 mL equivalent of urine from male and female juveniles and premolt, postmolt, and intermolt mature crabs were obtained on 800 MHz NMR equipped with a cold probe. To obtain higher sensitivity in our experiment, we substituted hydrogen atoms with deuterium by using deuterium oxide as a solvent and removing water by freeze-drying. To avoid chemical shift changes of the compounds in urine due to differences in pH, we standardized the pH of the samples by adding phosphate buffer. After spectrum binning, the data for each molting stage were mathematically analyzed by PCA, in collaboration with Koichi Matsumura of Monell Chemical Senses Center. Urines from premolt stage males and females were clearly different in PCA (Fig. 20.7): the first two principal components explained 78% of the variance in the data (PC1, 43%; PC2, 35%), indicating that the NMR spectrum contained enough information to discriminate between the urine of male and female premolt crabs. We are currently pursuing this difference with

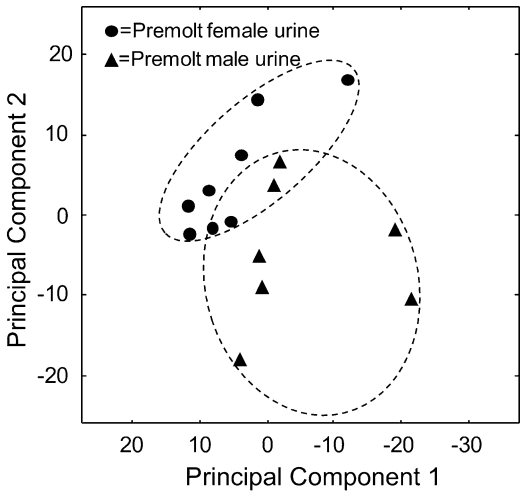

Fig. 20.7 Results of principal components analysis of NMR data for premolt male and premolt pubertal female urines. The results are from ^{1}H NMR data, which observes all protons in the mixture except exchangeable protons. See text for explanation

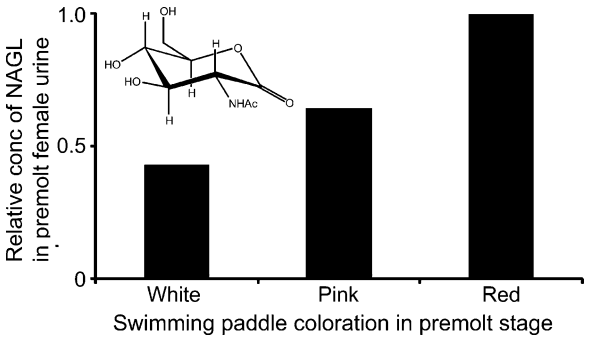

Fig. 20.8 Increase of concentration of N-acetylglucosamino-1,5-lactone, or NAGL (molecular structure shown in *inset*), in urine of premolt females as they approach molting. Coloration of swimming legs of blue crabs indicates their molting stage. *White* indicates early, *pink* means middle, and *red* means late premolt stage (Kennedy and Cronin 2007). Quantification by ^{1}H NMR showed that the concentration of NAGL increased as crabs progressed in molt stage from *white to pink to red*. Urine sample for each stage is pooled from many females

the aim of identifying the compounds specific to premolt females. We are also performing 2D-NMR on the samples.

This NMR-based biomarker targeting has proven useful for identifying molting biomarkers. Comparison of urine of premolt and intermolt males and females identified a molt-specific compound. We observed a clear difference in the spectra of the 500–1,000 Da fraction of urines from premolt vs. intermolt animals. This signal was isolated using HPLC and identified as N-acetylglucosamino-1,5-lactone (NAGL), whose structure is shown in Fig. 20.8. Its concentration increases as female crabs approach molting (Fig. 20.8). NAGL has never before been reported

as a natural product. It is known to be an inhibitor of N-acetylglucosaminidase of limpets, mice, bovines, and *E. coli* (Findlay et al. 1958; Yem and Wu 1976; Legler et al. 1991). It may be part of the metabolic pathway of chitin (Merzendorfer and Zimoch 2003). We bioassayed NAGL using sexually receptive males and found that they detect NAGL, but do not perform courtship stationary paddling to it. Thus, NAGL by itself is not sufficient as the female sex pheromone. It might be a component of a pheromone blend, contributing in an additive or synergistic way with other components of the blend. It is also possible that NAGL is an indicator of molting animals. These possibilities should be experimentally tested. It is also worth noting that P-31 NMR was used to identify the phospholipid 2-aminoethyl phosphonate as being specifically enriched in the gills of male blue crabs (Kleps et al. 2007), but it was not reported to be in the urine and nor is it reported to have any sex pheromone activity.

Our current working hypothesis is that the female sex pheromone is a multicomponent mixture that is constituted by a combination of compounds specific to pubertal females as well as others that are distinctive of blue crabs, i.e., having species specificity but lacking sex specificity. Evidence of species specificity comes from the work by Gleeson (reported in Bublitz et al. 2008) that showed that male blue crabs do not express courtship behavior to the sex pheromone of shore crabs, *C. maenas*. Sex pheromones of many organisms are blends, including insects and mammals (Wyatt 2003). Blends can provide species specificity that is not possible in single molecules. One experimental observation supporting the blend hypothesis for blue crabs is that males responded to two molecular size fractions (see Fig. 20.5), suggesting that more than one molecule is involved. A test of this idea requires formulating an artificial premolt female urine containing female-specific and blue crab species-specific compounds, and bioassaying it against sexually active males.

20.4 Summary and Conclusions

Molecular identification of sex pheromones in marine crustaceans has proven to be very difficult, to the point that no unequivocal identification for any decapod crustacean has been published (but see Hardege and Terschak, Chap. 19). Several factors have made successful identification difficult. Some difficulties are common to searches in other animals: pheromones are likely blends of molecules whose components are at very low concentration. In addition, pheromones of marine crustaceans are often small and polar molecules, making them difficult to separate from salts and other small ions in their background, i.e., sea water or urine. These difficulties have led us to take on new approaches to pheromone identification and to select the blue crab *C. sapidus* as our experimental model.

The blue crab is a good model for pheromone research. Pubertal females approaching the stage where they molt into a mature female release a pheromone in their urine. This pheromone is detected by male crabs using as sensors the

aesthetascs on their antennules. Males respond with a very distinctive behavior, called courtship stationary paddling, as well as other behaviors. The distinctiveness of courtship stationary paddling makes it useful as a specific behavioral indicator of the presence of the pheromone; however, this advantage is counterbalanced by the fact that the behavior is not reliably released by the pheromone, and thus, there are no false positives, but many false negatives. Fortunately, several studies of the behavioral ecology of blue crabs have helped to identify why courtship stationary paddling is used by blue crabs in mating. This should lead to modification of bioassays that can decrease the rate of false negatives. The commercial fishery for blue crabs has given us access to animals of all stages of maturity and molting, and thus to sources of the pheromones. We have used a combination of experimental approaches, including commonly used (bioassay-guided fractionation) and new (biomarker targeting), to show that the female pheromone is less than 1,000 Da and possibly a mixture. From the urine, we isolated some molecules that are specific to premolt pubertal females and thus are candidate pheromones. These approaches have yielded promising results, and we hope that more work will uncover the identity of the pheromones.

Generalizations from our results and from other studies of decapod crustaceans must be done with caution, since results for all species are preliminary or fragmentary. Our working hypothesis is that a "species-specific sex pheromone" (though see Wyatt, Chap. 2) is composed of two functional classes of molecules, both of which are small and polar. One class of molecules distinguishes female vs. male, and thus is a sex-specific signal that could be, but is not necessarily, unique to blue crabs. The second set of molecules distinguishes blue crabs from other species, thus constituting a species-specific signal. Thus, we expect the species-specific sex pheromone to be a blend of molecules which contains a variety of messages and information.

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