Invertebrate Reproduction and Development, 53:2 (2009) 105–116
Balaban, Philadelphia/Rehovot

Studies on contact sex pheromones of the caridean shrimp *Palaemonetes pugio*: II. The role of glucosamine in mate recognition

JODI L. CASKEY[1*], GLEN M. WATSON[2] and RAYMOND T. BAUER[3]
Department of Biology, University of Louisiana at Lafayette, LA, USA
[1]Tel. +1 337.482.5231; Fax: +1 337.482.5834; email: jlc7280@louisiana.edu
[2]Tel. +1 337.482.6263; email: gmw5722@louisiana.edu
[3]Tel. +1 337.482.6435; email: rtbauer@louisiana.edu

Received 23 June 2009; Accepted 4 September 2009

Abstract

The role of surface glycoproteins in mate recognition of the shrimp *Palaemonetes pugio* was studied using mating bioassays and fluorescence microscopy. Our data indicate that a glycoprotein associated with female shrimp functions in mate recognition in *P. pugio*. This glycoprotein is likely to be a glucosamine or an *N*-acetylglucosamine-containing glycoprotein complemented by a lectin-like receptor on males. Significantly fewer copulations occurred in seawater augmented with glucosamine as compared to seawater augmented with glucose. Thus, it is likely that glucosamine competitively inhibits mate recognition by binding male receptors and significantly reducing recognition of parturial females. The antennal flagellum of decapod crustaceans has long been considered to be chemotactile with bimodal, touch/taste receptors. The fluorescent dye, Lucifer Yellow, is incorporated into antennal setae from the surrounding seawater. Furthermore, the fluorescent dye, DiI, stained neural tissues at the base of the antennal setae. Taken together, these results are consistent with a bimodal, sensory function. When fluo-treated male antennal setae were exposed to the carapace of postmolt, parturial females, an immediate and significant increase in fluorescence intensity was observed, suggesting that Ca^{2+} likely acts as a second messenger for the receptor system. Fluo-labeled antennal setae of both males and females showed larger increases in intracellular Ca^{2+} levels when exposed to glucosamine as compared to glucose. The effects produced by glucosamine were sexually dimorphic, with male setae displaying a significantly greater mean overall change in fluorescence intensity than female setae. The dimorphic response observed suggests that glucosamine or a related compound serves as a female signal (i.e., contact sex pheromone) in mate recognition.

Key words: Crustacea, Malacostraca, glycoprotein, glucosamine, mate recognition, pheromone

Introduction

Aquatic organisms detect, discriminate, and respond to a multitude of chemical signals in their environment (Atema, 1995; Zimmer & Butman, 2000; Rittschof & Cohen, 2004). Aquatic organisms have evolved the ability to employ a wide variety of compounds as

*Corresponding author.

chemical signals, including polypeptides, fatty acids, esters, steroids, and carbohydrates (Wyatt, 2003; Rittschof & Cohen, 2004). Often, chemical signals are derived from molecules already in use and are subsequently changed in the course of evolution to adopt a signal function (Wyatt, 2003). Thus, some pheromones evolved from compounds that originally had other functions or significance. Pheromones that have evolved from "leaking" hormones can illustrate the way that evolution can act on available chemical cues. In fish and lobsters, olfactory sex pheromones may have evolved from hormones or metabolites excreted in urine or "leaking" into the water across permeable membranes (see review by Derby & Sorensen, 2008). In the goldfish, *Carassius auratus*, ovulatory female goldfish release a mixture of compounds, two sex hormones and one metabolite, 12 h prior to spawning that function as a primer pheromone (Sorensen, 1992). All three components are olfactory stimulants that are detected by different, specific receptor sites of males indicating that there is also evolution of the receptors and response of the receiver (Wyatt, 2003; Derby & Sorensen, 2008). If detection of a particular compound leads to greater reproductive success, there will be selection for specialized receptors, more sensitive receptors, or receptors expressed in greater numbers.

An individual's ability to locate, to recognize, and to copulate with conspecifics serves to increase their reproductive fitness. In terms of fitness, a mate is a crucial resource. Thus, finding a mate is as important an adaptive process as is the avoidance of predators, the ability to find shelter, or the acquisition of food (Paterson, 1982). Many species have evolved a mate recognition system (MRS) to ensure mating success and thus to enhance reproductive fitness. Mate recognition, the behavioral response of an individual to an appropriate mating partner, involves several elements interacting to create a complex, co-adapted system that includes the coordination of reproductive timing and a finely tuned signal-receptor system (Paterson, 1982). The need for synchronous reproductive readiness is especially important for many decapods, including caridean shrimps, because sexual receptivity of females is linked to the reproductive (parturial) molt (Bauer, 2004). Female carideans are receptive only for a short time period, from a few minutes to a few days, following the parturial molt (Correa & Thiel, 2003; Bauer, 2004).

Ekerholm & Hallberg (2005) proposed that mating in crustaceans is highly dependent upon chemical cues (i.e., sex pheromones) that may guarantee that a male and a female are in contact during the limited reproductive time period. The key role of sex pheromones in mate recognition of many decapod crustaceans has been well documented (see reviews by Dunham, 1978, 1988). In decapods such as lobsters (Atema, 1986; Bushmann & Atema, 2000), crayfishes (Ameyaw-Akumfi & Hazlett, 1975; Stebbing et al., 2003), and brachyuran crabs (Ryan, 1966; Gleeson, 1980; Asai et al., 2000; Kamio et al., 2000, 2002; Bublitz et al., 2008), the urine of females contains a water-soluble pheromone that acts over distances to attract mating partners. However, as Burkenroad (1947) first proposed, females of some caridean shrimp species may possess a "nondiffusible coating of the integument" that serves as a mate recognition cue (i.e., contact sex pheromone). Behavioral evidence suggests the existence of such contact sex pheromones in caridean shrimps (Kamiguchi, 1972; Bauer, 1979; Diaz & Thiel, 2004; Caskey & Bauer, 2005).

In the caridean shrimp *Palaemonetes pugio*, several studies have shown that males respond to sexually-attractive females only after touching them with the antennal flagella or the pereopods (Berg & Sandifer, 1984; Bauer & Abdalla, 2001; Caskey & Bauer, 2005). Caskey & Bauer (2005) examined visual, tactile and behavioral cues as sexual signals and concluded that a chemical signal, in the form of a contact sex pheromone, plays a role in the MRS of *P. pugio*. This conclusion leads to the hypothesis that males are responding to an insoluble substance (i.e., contact pheromone) present on the newly-molted parturial female. An unmated, parturial female's ability to successfully attract and copulate with males gradually decreases with time (Caskey & Bauer, 2005). After approximately 8 h, the unmated female will either reabsorb or abort her eggs, resulting in decreased reproductive fitness. Thus, by using a contact sex pheromone to advertise her sexually receptive condition, the female increases her chance of successfully reproducing. Furthermore, we propose that males receive and interpret this signal by employing receptors primarily located on their antennal sensilla. We propose that this putative signal-receptor system comprises an essential component of the mate recognition system of *P. pugio*.

A survey of the literature indicates that two groups of compounds may function as mate recognition signals in crustaceans: cuticular hydrocarbons or surface-bound glycoproteins. In some insect species, epicuticular hydrocarbons serve as both inter- and intraspecific chemical messengers (Howard & Blomquist, 1982, 2005). Cuticular hydrocarbons (CHCs) have been shown to function as contact sex pheromones in many insects, including Coleoptera (Fukaya, 2003; Ginzel & Hanks, 2003; Ginzel et al., 2003a, 2003b), Diptera (Howard & Blomquist, 1982, 2005; Stoffolano et al., 1997), Lepidoptera (Gries et al., 2002; Francke et al., 2004; Leal et al., 2006) and Hymenoptera (Ayasse et al., 2001). Caskey et al. (2009) examined the presence and

identity of cuticular compounds that might serve as contact sex pheromones in *P. pugio*.

Gas chromatography-mass spectrometry (GC-MS) analyses of chloroform-methanol extracts of the cuticle of postmolt parturial (sexually receptive) females, postmolt nonparturial females, postmolt males, and intermolt females showed 55 compounds, 3 of which were unique to postmolt parturial females (Caskey et al., 2009). Twelve cuticular hydrocarbons and five fatty acids were found in the cuticular extractions of *P. pugio* and multivariate analysis revealed that the cuticular composition of postmolt parturial females differed significantly from that of nonparturial females, males, and intermolt females (Caskey et al., 2009). Although GC-MS enabled identification and partial quantification of some cuticular compounds, it is still undetermined which, if any, compounds function in mate recognition in *P. pugio*.

Carbohydrate residues of glycoproteins bound to body surfaces or substrates are chemical signals for several non-crustacean groups: rotifers (Snell et al., 1995); mollusks (Krug & Manzi, 1999); cnidarians (Morse & Morse, 1991); and polychaetes (Kirchman et al., 1982; Maki & Mitchell, 1985). There is increasing evidence that two forms of carbohydrates are used by crustaceans as chemical signal molecules: carbohydrate residues of glycoproteins and modified amino sugars hydrolyzed from proteoglycans (Rittschof & Cohen, 2004). Copepods are planktonic crustaceans that use dissolved and surface-bound chemical cues for mate recognition (Lonsdale et al., 1998). The functional importance of carbohydrate residues of surface-bound glycoproteins of the harpacticoid copepods (*Tigriopus japonicus* and *Coullana* spp.), particularly *N*-acetyl-glucosamine, has been demonstrated by lectin and monosaccharide-binding assays (Lonsdale et al., 1996; Frey et al., 1998; Kelly & Snell, 1998). Lectins are non-immunological proteins that bind to specific carbohydrate residues non-covalently (Sharon & Lis, 1989). Probing an intact animal with lectins of different, specific carbohydrate affinities reveals the location of surface glycoproteins and the composition of some of its monosaccharides (Kelly & Snell, 1998). In harpacticoid copepods, specific, surface glycoproteins correlate with structures important to sex and developmental stage as well as function in recognition of species, sex, and stage of potential mates (Snell & Carmona, 1994; Kelly & Snell, 1998; Ting et al., 2000).

This study tests the hypothesis that glucosamine is an important component of the mate recognition system of *P. pugio*. We also examine the possibility that receptors for glucosamine are located on antennal setae.

Materials and Methods

Competition bioassay by glycan

The term glycan, used interchangeably with carbohydrate and sugar, refers to any sugar or assembly of sugars, in free form or attached to another molecule. The addition of soluble glycans or structural mimics into a biological system can interrupt the interactions between the receptor and the ligand (Varki & Lowe, 2009). Based on the hypothesis that mate recognition in *Palaemonetes pugio* is based on the recognition of a glycan (glucosamine) by a specific glycan-binding protein, the introduction of a sufficient concentration of a soluble glucosamine may inhibit mate recognition. Mating experiments in which males were exposed to glucosamine (experimental) or glucose (control) were used to test this hypothesis. Shrimps used in all experiments and observations in this study were collected and maintained as described in Caskey et al. (this issue). Preliminary observations indicated that a 50 mM solution of glucosamine did not noticeably affect the shrimps' behavior or appearance. In replicates (n = 20), a postmolt, parturial female (sexually receptive), <4 h postmolt, was placed with two males in a 15 L testing chamber filled with 10 L of 50 mM solution of glucosamine (experimental) or glucose (control) for 30 min. Prior to testing, the males and the female were separated by a partition and acclimated to the testing chamber for 5 min. Because there is no precopulatory behavior in these shrimp, copulation was considered the positive response. The occurrence of copulations was determined from time-lapse video surveillance. Twenty replicates of each treatment were performed using 40 different pairs of shrimp. Previously, it was found that in seawater alone, 28 of 40 males copulated with postmolt, parturial females (Caskey & Bauer, 2005). This result was used as a standard against which shrimp exposed to glycans in seawater were compared. The null hypothesis of no difference in the frequency of copulations among the treatment groups was tested using the Cochran-Mantel-Haenszel (CMH) Row Mean Scores test (Proc FREQ, SAS, 2004).

Retrograde DiI labeling

Long-chain dialkylcarbocyanines, in particular DiI (1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate), are widely used as anterograde and retrograde neuronal tracers in living (Honig & Hume, 1986, 1989) and fixed (Godement et al., 1987; Baker & Reeset, 1993) tissues. DiI is a fluorescent lipophilic dye (excitation 549 nm, emission 565 nm) that uniformly labels neurons via lateral diffusion in the plasma

membrane. Bundy & Paffenhöfer (1993) showed that, in crustaceans, neurons and their extensions into sensilla could be identified using DiI labeling when the exoskeleton is transparent. DiI was used to determine whether neural components were contained within antennal setae of *P. pugio*.

Antennal flagella were fixed in a 3 ml solution 1% glutaraldehyde, and 3% paraformaldehyde in Millonig buffer augmented with 3% NaCl, for 1 h and then rinsed in PBS (phosphate buffer solution) for 15 min. DiI crystals mixed into an inert, water-resistant gel/oil improves the penetration of the dye into bundled neurons, labeling axons both on and below the surface. DiI crystals, mixed with viscous immersion oil, were applied to the flagellum, kept moist with PBS, sealed in a Petri dish and covered in aluminum foil for 1 week. The crystals were removed using a mild detergent and rinsed in deionized water to remove all detergent. Antennae were viewed using rhodamine fluorescence microscopy (LOMO Lumam model RP011-T, LOMO America, Prospect Heights, IL, USA). The principal objective used was a 100X oil-immersion fluorite (n.a. = 1.30). Images were obtained using a STL-11000 SBIG cooled CCD camera (SBIG, Santa Barbara, CA) controlled by Maxim-DL software (Diffraction Limited, Ontario, Canada). The fluorescence light source was a 100-W mercury lamp.

Lucifer Yellow uptake

To determine if the antennal setae of *P. pugio* were able to uptake polar molecules such as glycoproteins from the environment, we used Lucifer Yellow CH (LY; Sigma), a water-soluble fluorescent dye. The polar nature of LY makes it unable to passively diffuse across the plasma membrane and requires active transport to enter a cell. Excised antennal flagella were prepared by placing them in a solution of one mg of LY in one ml of 5 ppt natural seawater (NSW) water for 5 min. Antennal flagella were then rinsed in NSW for 1 min, wet mounted, and were imaged as was described above.

Time-course experiments for intracellular Ca^{2+}

To test whether the mate recognition system of *P. pugio* includes setae receptors that employ Ca^{2+} as a second messenger, a series of three time-course experiments were performed on fluo-labeled antennal setae. Fluo is a calcium indicator that is only weakly fluorescent in the absence of calcium and significantly increases in fluorescence in the presence of calcium.

(1) Natural stimuli — To determine the effects of natural stimuli on intracellular Ca^{2+}, fluo-labeled male antennae were imaged before and after they were touched to carapaces of intact, postmolt, parturial females (experimental) or postmolt, nonparturial females (control) (n = 5 each). Antennal sensilla were touched by the exoskeleton (dorsal carapace) of anesthetized postmolt, parturial and postmolt, nonparturial females (<4 h postmolt). Female shrimp were anesthetized by placing them in a refrigerator until they ceased swimming and were kept in chilled water set on top of ice between time points.

(2) Artificial stimuli, glycans in NSW — The effects of artificial stimuli on intracellular Ca^{2+} levels of fluo-labeled male (n = 10) and female (n = 5) antennal flagella, prepared in NSW, were tested by imaging fluo-labeled antennae before and after exposure to 50 mM glucosamine (experimental) or glucose (control).

(3) Artificial stimuli, glycans in ASW — To determine the source of elevated intracellular Ca^{2+}, fluo-labeled male antennae were prepared in Ca^{2+}-free artificial seawater (ASW) prior to exposure to glucosamine (n = 5). Since the data did not meet the criteria of normality for analysis of variance (Sokal & Rohlf, 1981), the nonparametric Kruskal–Wallis (K–W) test was used to test the hypothesis of no difference between groups for each time period. In addition, the overall mean change in fluorescence among groups was tested using a K–W test ($P \leq 0.05$). Multiple comparisons were made so the significance level of 0.05 was adjusted by dividing by the number of comparisons.

Fluo-labeling of antennal setae and microscopy

Natural seawater (NSW) or Ca^{2+}-free artificial seawater (Ca^{2+}-free ASW) served as media during labeling, washing, and imaging viewing of the antennal flagella. Preliminary studies, using the exclusion dye Trypan Blue, indicated that excised antennal setae were viable for >4 h. For each replicate, antennal flagella were excised and placed in 200 μl of SW treated with 2 μl fluo-reagent solution for 30 min. Antennae were rinsed for 10 min, placed on a slide, secured with petroleum jelly across each end of antennae, and 200 μl of fresh medium was added. In order to establish baseline Ca^{2+} fluorescence levels, a control image was obtained prior to treatment. An image was produced immediately upon introduction of treatment (0 min) and subsequently every two min for 20 min. Specimens remained in the dark between exposures and a single image was produced at each time interval. Antennal flagella were viewed at a magnification of 10× (n.a. = 0.40) using an Olympus inverted microscope. The fluorescence light source used was a 100-W halogen lamp set at 12 V. Images were taken with a cooled CCD (Starlight Xpress HX516) high resolution camera, 60 s exposure time, and analyzed using Maxim software.

Mean fluorescence intensity (grey value) was recorded from the same point location for each time period. These values were then subtracted from the control to give an overall change in intracellular Ca^{2+} at each time period. Mean Ca^{2+} fluorescence from replicate experiments were calculated for each time point, which provided an index of the fluorescence intensity over time and allowing for day to day variation between replicates. Data are plotted as mean fluorescence intensity measured in grey values. Because the images were digitalized to 16-bit precision there were 65,536 possible grey values between black, 0, and white at 65,536. Using Maxim DL software, difference images were produced for time points 0 min through 10 min by subtracting the control image from the experimental image. Difference images were then uniformly contrasted.

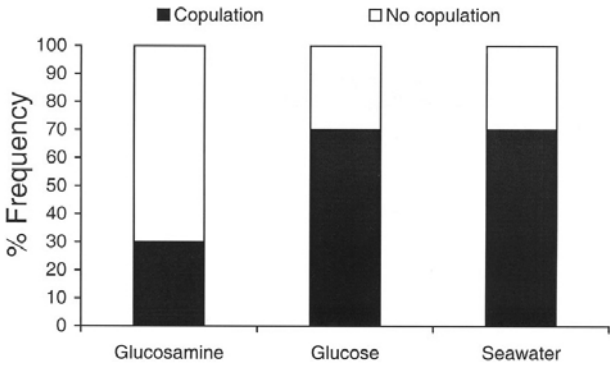

Fig. 1. Frequency of copulations based on exposure to either glucosamine or glucose (n = 20 for glucose and n = 20 for glucosamine trials). These frequencies are compared to data from Caskey & Bauer (2005) which measured male response to postmolt, parturial females in seawater alone (n = 40).

Reagents

Fluo reagent, for use in labeling of antennal setae, was made by dissolving 25 mg of pluronic acid in 75 μl of dimethylsulfoxide (DMSO) and adding 25 μl of this solution to 50 μg of Fluo-3, AM (Invitrogen). Ca^{2+}-free artificial seawater (ASW) was prepared in deionized water as follows: NaCl (448 mM), KCl (9 mM), $MgCl_2$-$6H_2O$ (23 mM), $MgSO_4$-$7H_2O$ (25 mM), $NaHCO_3$ (2 mM), and EGTA (8 mM) (Sigma). For Ca^{2+} imaging, 1 M solutions of glucosamine and glucose were diluted to 50 mM concentrations by adding 4 μl to 200 μl SW containing antennal flagellum. Seawater of 5 ppt, the salinity of the habitat from which shrimps were collected, was used in observations and experiments.

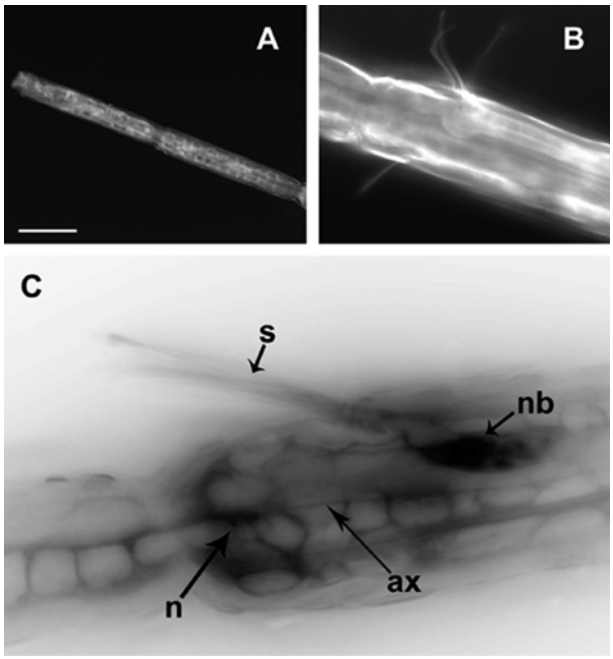

Fig. 2. Retrograde DiI labeling of antennal setae of *Palaemonetes pugio*. A: Distal articles of DiI treated antennal flagellum. B: Antennal flagellum showing DiI labeled setae. C: An inverted 3-D RGB image of an article of the antennal flagella of *P. pugio* with a cluster of 4–6 setae (s). A single neuron (n) as well as a large neuron bundle (nb) are found at the base of a single seta and a cluster of setae, respectively. Long axons (ax) run longitudinally down the antennae spanning several articles. Scale bar = 200 µm in A, 100 µm in B, and 40 µm in C.

Results

Competition bioassay by glycan

The hypothesis that the frequency of copulations is independent of the treatment was rejected (CMH Row Mean Scores, DF = 2, P = 0.0072). The frequency of copulations with parturial females (<4 h postmolt) by males in the absence of introduced glycans is 0.70 (Caskey & Bauer, 2005). In the presence of glucose, the frequency of copulations was the same as in the absence of any introduced glycans, 0.70 (Fig. 1). However, when males were exposed to glucosamine, the frequency of copulations with postmolt, parturial females significantly decreased to 0.30 (Fig. 1).

Retrograde DiI labeling

Retrograde DiI labeling of putative neural processes within simple and denticulate setae of the antennal flagellum of *P. pugio* occurred after treatment and incubation with DiI. The bright fluorescence indicates the presence of neural innervation along the setal shaft to the distal tip (Fig. 2A-B). The antennal flagellum of a male using rhodamine fluorescence microscopy shows

stained nerve fibers spanning several antennal articles (Fig. 2A-B). These long axons run longitudinally along each side of the antennal flagellum presumably linking neurons among within adjacent articles (Fig. 2A, C). A large neuron bundle is found at the base of the cluster of 4 to 6 setae which is approximately 40 microns in length. The long axons can be seen running lengthwise down the antennae.

Lucifer Yellow uptake

After a 5 min exposure to LY, fluorescence (LY uptake) was localized to areas of the antennal flagellum in which setae (indicated by arrows) were located (Fig. 3A). Setae, which occur in clusters of 4–6 (cs) as well as singly (ss), fluoresce due to active uptake of LY particles from the environment (Fig. 3B). This simple experiment demonstrates the ability of putative pheromones, i.e., glycoproteins, to access receptors on the antennal setae of males.

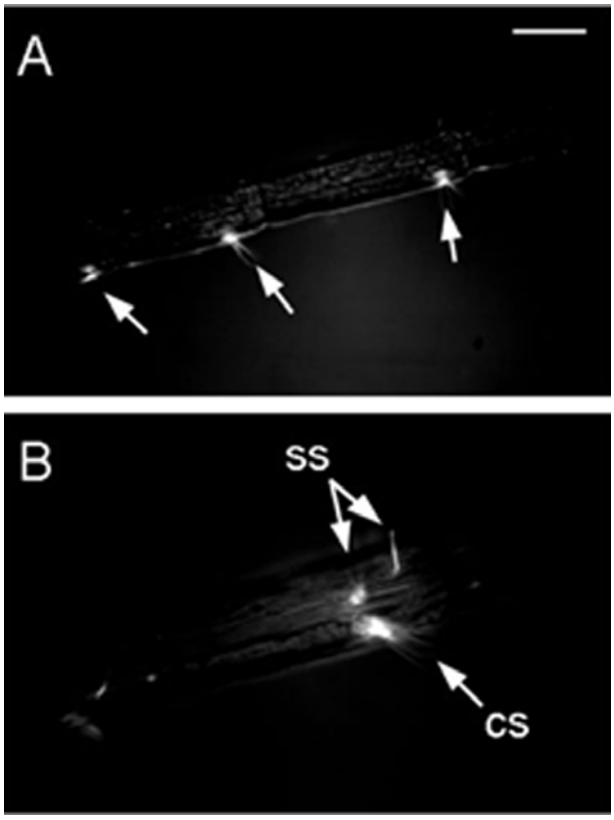

Fig. 3. Lucifer Yellow (LY) uptake. A: Several articles of male antennal flagellum exposed to LY for 5 min. Arrows indicate location of setae. B: Single article of male antennal flagellum exposed to LY for 5 min. LY uptake was localized to setae, with fluorescence found at clusters of 4–6 setae (cs) and single setae (ss). Scale bar = 100 µm in A and 50 µm in B.

Time-course experiments for intracellular Ca^{2+}

To determine if intracellular Ca^{2+} increases in response to natural stimuli, fluo-treated male antennal setae were exposed to the exoskeleton of postmolt, parturial females and postmolt, non-parturial females. The overall mean change in fluorescence of male antennal setae exposed to postmolt, parturial females or postmolt, nonparturial females differed significantly (K–W test, P <0.0001) (Fig. 4). When exposed to postmolt, parturial females, a large initial increase was seen which differed significantly at 0 min and 2 min from that of postmolt, nonparturial females (Table 1). Fluorescence intensity steadily increased, peaked at 10 min, and then decreased for the remaining 10 min period (Fig. 4). When exposed to the exoskeleton of postmolt, non-parturial females, the maximum fluorescence levels were reached at 6 min, followed by a gradual decrease for the remaining 14 min resulting in a net decrease in fluorescence levels (Fig. 4).

Because an increase in intracellular Ca^{2+} was produced by exposure to natural stimuli, the effect of artificial stimuli, glucosamine and glucose, on fluo-treated male antennal setae was tested. The mean overall change in intracellular Ca^{2+} levels of male antennal setae in NSW, exposed to either glucosamine or glucose, differed significantly (K–W test, P <0.0001). A larger immediate increase was seen (0 min) in response to glucosamine as compared to glucose but did not differ significantly (Fig. 5A, Table 1). This increase was sustained in the continued presence of glucosamine but not glucose (Fig. 5A). Although the mean fluorescence at each time period was greater in the presence of glucosamine than glucose, no statistical significance was found (Table 1). A comparable initial increase was seen in antennal setae prepared in Ca^{2+}-free ASW; however, the sustained increase seen in setae prepared in NSW was not also observed in ASW (Fig. 5C). The overall mean change in fluorescence between male setae prepared in NSW and male setae prepared in ASW differed significantly (K-W test, P = 0.001).

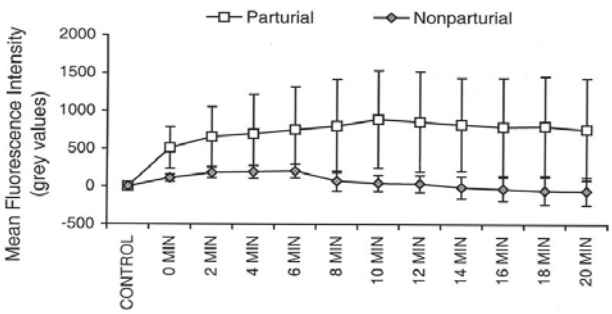

Fig. 4. Mean fluorescence intensity (± SD) of fluo-treated male antennal setae exposed to the carapace of postmolt, parturial females (n = 5) and postmolt, nonparturial females (n = 5).

Table 1. Kruskal–Wallis tests of the null hypothesis of no difference in mean fluorescence intensity of male antennal setae exposed to natural stimuli (postmolt, parturial or postmolt, nonparturial females) and male or female antennal setae exposed to artificial stimuli (glucosamine and glucose). Male antennae prepared in Ca^{2+}-free ASW exposed to glucosamine were compared with setae prepared in NSW. MGA = male glucosamine; MGL = male glucose; FGA = female glucosamine; FGL = female glucose; ASW = Ca^{2+}-free artificial seawater; NSW = natural seawater; PPF = postmolt, parturial females; NPF = postmolt, nonparturial females. For multiple comparisons, the significance level (0.05) was adjusted by dividing by the number of comparisons; *$P \leq 0.017$; $^{\dagger}P \leq 0.025$

	0 min	2 min	4 min	6 min	8 min	10 min	12 min	14 min	16 min	18 min	20 min
PPF vs NPF	0.009	0.016	0.117	0.117	0.076	0.117	0.117	0.076	0.076	0.076	0.076
MGA vs MGL	0.112*	0.174*	0.059*	0.096*	0.112*	0.131*	0.151*	0.151*	0.174*	0.174*	0.174*
FGA vs FGL	0.047†	0.076†	0.076†	0.047†	0.076†	0.175†	0.347†	0.465†	0.602†	0.917†	0.917†
ASW vs NSW	0.540*	0.624*	0.806*	0.903*	0.806*	0.713*	0.713*	0.806*	0.806*	0.806*	0.806*
MGA vs FGA	0.903*	0.903*	0.624*	0.624*	0.713*	0.391*	0.540*	0.540*	0.391*	0.462*	0.540*

As in males, the overall mean change in intracellular Ca^{2+} levels of female antennal setae differed significantly when exposed to either glucosamine or glucose (K–W test, $P < 0.0001$) (Fig. 5B). Female setae showed gradual increases in fluorescence in response to glucosamine but showed no increase in the presence of glucose (Fig. 5B). Fluo-treated female antennal setae exposed to glucosamine exhibited a smaller, but significant, overall change in fluorescence than male antennal setae when exposed to glucosamine (K–W test, $P < 0.0001$). In both males and females, the general trend of increasing fluorescence in the presence of glucosamine is comparable to the response to postmolt, parturial females (Fig. 4, 5A,B) and glucose is comparable to postmolt, nonparturial females (Fig. 4, 5A,B).

Difference images of male antennal setae show the immediate and sustained increase in intracellular Ca^{2+} in the presence of glucosamine (Fig. 6A–F). The greatest increase of intracellular Ca^{2+} (white) was localized to the cluster of setae and in the soma of the neurons of these setae (Fig. 6A). A single seta increases in fluorescence at 4 min (Fig. 6C) and increases over the next 6 min (Fig. 6D–F). Overall, the Ca^{2+} fluorescence increased over time in the presence of glucosamine but not glucose. When exposed to glucose, the fluorescence intensity seen at 0 min (Fig. 7A) starts to gradually decrease over time (Fig. 7B–F). As in response to glucosamine, the most intense fluorescence (higher Ca^{2+} levels) is restricted to the setal cluster and their corresponding neurons; however, the increase of intracellular Ca^{2+} is greater in response to glucosamine than to glucose.

Discussion

The purpose of this paper was to examine the biological role of glucosamine and the functional role of antennal setae in the mate recognition system of *Palaemonetes pugio*. In recent years, there has been a growing recognition that glycans (i.e., carbohydrates, sugars) are used as signals for the initiation of a wide variety of biological processes (Etzler & Esko, 2009). Surface glycoproteins function in mate recognition in the rotifer *Brachionus plicatilis* (Snell et al., 1995) and in the harpacticoid copepods *Tigriopus japonicus* (Kelly et al., 1998; Ting et al., 2000) and *Coullana* spp. (Lonsdale et al., 1996; Frey et al., 1998). The results of this study suggest that surface glycoproteins might serve as mate recognition cues for the caridean shrimp *P. pugio*. Our data indicate that the compound associated with mate recognition in *P. pugio* is likely to be glucosamine or a glucosamine-containing glycoprotein.

The addition of soluble glycans or lectins into a system can cause interference with the interactions between endogenous receptors and ligands through competitive inhibition (Varki & Lowe, 2009). In this study, the introduction of glucosamine into the surrounding seawater appeared to reduce mate recognition through competitive inhibition of natural ligands. In animals, glycan-receptor interactions tend to be of high specificity and low affinity and the ability of these low affinity sites to mediate biologically relevant interactions appears to require multiple binding sites (multivalency) (Varki et al., 2009). Multivalency or low affinity binding could explain why mate recognition was

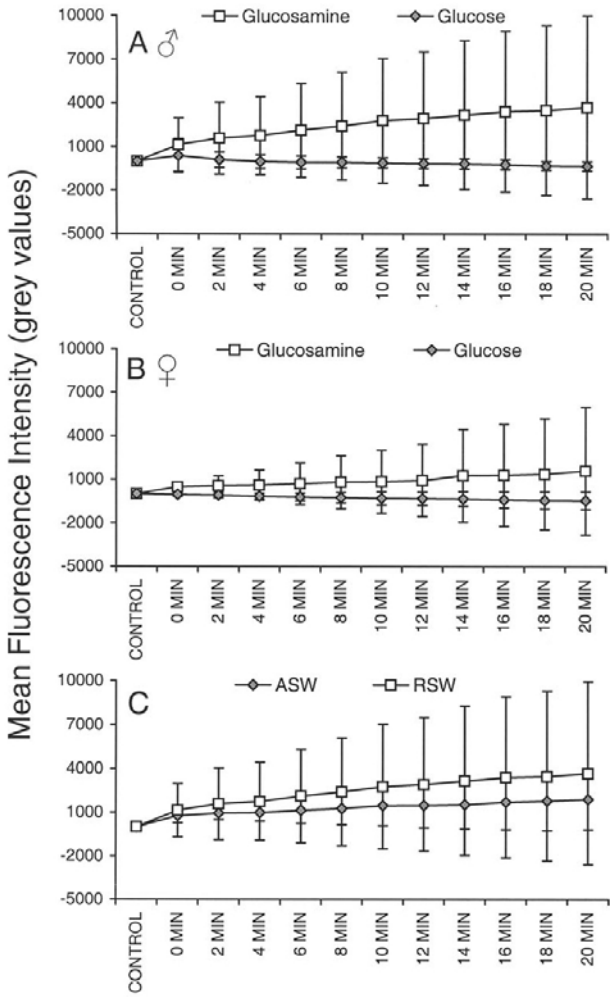

Fig. 5. Mean fluorescence intensity (± SD) of fluo-treated antennal sensilla of *Palaemonetes pugio* over a 20 min time period. A: Male setae exposed to 50 mM solutions of glucosamine and glucose, n = 10. B: Female setae exposed to 50 mM solutions of glucosamine and glucose, n = 5. C: Male antennal setae treated with Fluo 3-AM in calcium free seawater (ASW), n = 5, and natural seawater (NSW), n = 10.

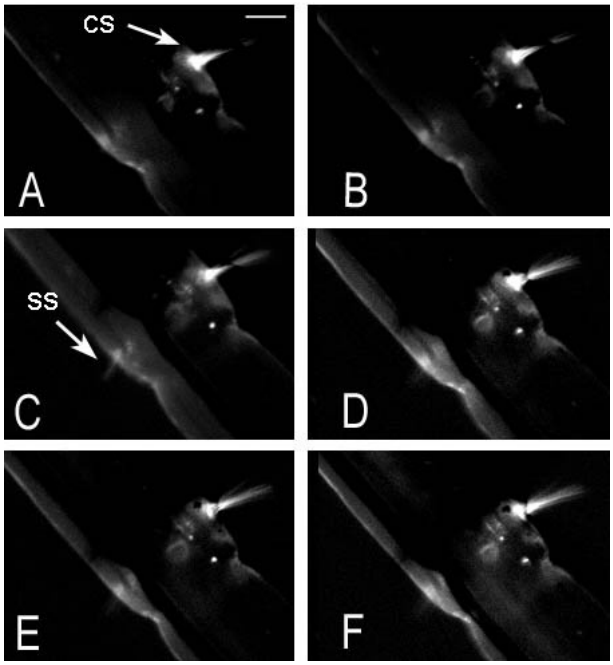

Fig. 6. Difference images of fluo-treated male antennal setae exposed to 50 mM glucosamine. Difference images were produced for time points 0 min through 10 min by digitally subtracting the control image from the experimental image. Thus, the images show changes in fluorescence caused by the experimental treatment. Fluorescence intensity decreases from white (highest fluorescence) to black (no fluorescence). A: 0 min; B: 2 min; C: 4 min; D: 6 min; E: 8 min; F: 10 min. cs = cluster of 4–6 setae; ss = single seta. Scale bar = 50 μm.

not inhibited in 100% of the glucosamine replicates. Either of these scenarios would make it difficult for soluble glucosamine to fully occupy all of the receptors all of the time and, thereby, to fully obscure the receptor site for female-derived ligands. Perhaps there were unbound receptors able to recognize sexually receptive females despite the presence and relatively high concentration of glucosamine. Much remains unknown about this complex signaling system. It is possible that the introduced glucosamine may not function as the sole mate recognition signal, but in combination with some other, unknown compound.

Mate recognition involves not only an intraspecific signal, i.e. sex pheromones, but also specialized receptors to receive and to interpret the signal. Chemoreception is the ability to detect and respond to specific chemicals in the environment. Crustacean chemoreceptors fall into one of two categories: unimodal, olfactory receptors or bimodal, touch/taste receptors (Hallberg & Hansson, 1999). The antennal flagellum of decapod crustaceans is hypothesized to function in contact chemoreception by having bimodal, touch/taste receptors (Bauer, 2004). *P. pugio* have two types of antennal setae, simple and denticulate, that are hypothesized to be bimodal, functioning as both mechano- and chemoreceptors (Bauer & Caskey, 2006). Both simple and denticulate setae have terminal pores which are typically, but not solely, indicative of a chemosensory function (Schmidt & Gnatzy, 1984; Cate & Derby, 2002; Schmidt & Derby, 2005; Bauer & Caskey, 2006). The uptake of LY into setae from the surrounding seawater indicates that water-soluble molecules including glycoproteins have access to the cells (and their cell-surface receptor proteins) lying underneath the cuticle. Presumably, the LY diffuses through the terminal pores. This study using DiI indicates the presence of neurons at setae. Presumably, such neurons have a sensory function. It remains to be seen if

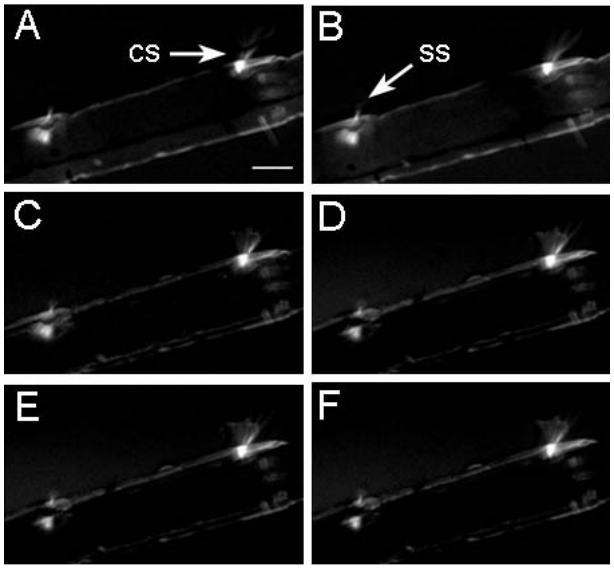

Fig. 7. Difference images of fluo-treated male antennal setae exposed to 50 mM glucose. Difference images were produced for time points 0 min through 10 min by digitally subtracting the control image from the experimental image. Thus, the images show changes in fluorescence caused by the experimental treatment. Fluorescence intensity decreases from white (highest fluorescence) to black (no fluorescence). A: 0 min; B: 2 min; C: 4 min; D: 6 min; E: 8 min; F: 10 min. cs = cluster of 4–6 setae; ss = single seta. Scale bar = 50 μm.

they function as both chemo- and mechanoreceptors. Further study of the innervation of these setae with transmission electron microscopy would be useful in determining if their neuron ultrastructure is that typical of crustacean bimodal receptors (Derby, 1989).

Intracellular Ca^{2+} levels were found to increase in setae exposed to glucosamine and to contact with postmolt, parturial (sexually receptive) females, but not to glucose or to contact with postmolt, nonparturial females. When comparing the mean overall change in fluorescence (intracellular Ca^{2+}) we found significant differences between the treatment groups; however, when comparing individual time points, we often did not. Given the low N of our treatments and this high variability of the data, the general trends of the data should be emphasized more than the statistical significance of the data at each time period. The high variability of response to glucosamine by male antennae may reflect individual variability in male readiness to respond to females in nature. Even in untreated seawater, 30% of males tested in mating experiments did not respond to contact with receptive females (Caskey & Bauer, 2005). This lack of response might be due to variation in male physiological state, e.g., molt stage.

Intracellular levels of Ca^{2+} increased in setae on antennae excised from male shrimp when the setae were touched with intact carapaces from postmolt, parturial females. These data suggest that Ca^{2+} functions as a second messenger in the mate recognition system of *P. pugio*. Although levels of intracellular Ca^{2+} also increased following contact with carapaces of nonparturial females, the initial increase (0 min and 2 min) was significantly smaller than that following contact with postmolt, parturial females. Thus, it appears that males can discriminate between reproductive (postmolt, parturial) and nonreproductive (nonparturial) females on the basis of contact chemoreception mediated through their antennae. Interestingly, glycans added to the seawater containing the excised antennae from males also induced elevations in intracellular levels of Ca^{2+} in setae. Furthermore, glucosamine induced a larger increase in levels of intracellular Ca^{2+} than did glucose. These data (along with those from our bioassay) suggest that the receptors on the plasma membrane of the sensory neurons in setae bind to glucosamine or compounds such as glycoproteins that are conjugated to N-acetylglucosamine. Upon exposure to glucosamine, we find an immediate elevation in intracellular levels of Ca^{2+} that is sustained for minutes in excised antennae held in NSW but not also in ASW. Thus, the initial increase in cytoplasmic Ca^{2+} may involve a release of Ca^{2+} ions from internal storage vesicle. Furthermore, the maintenance of sustained elevated levels of Ca^{2+} likely requires replenishment from external sources (seawater).

The effect of glucosamine on intracellular Ca^{2+} was sexually dimorphic, with a smaller overall effect, both immediate and sustained, on female antennal setae. Sexual dimorphism in morphological structures is usually indicative of sexual differences in function. These morphological variations in males and females are known to control functions or behaviors that are expressed in only one sex (Weissburg, 2001). Many such behaviors mediate intraspecific communication. For animals that use sex-specific chemical signals in mating, observations indicate that the ability of males to utilize these chemicals depends on the existence of structures that are often lacking in females. In planktonic copepods, the chemosensory system is typically enhanced by the doubling of the aesthetascs, by the enlargement of existing aesthetascs, or by the transformation of seta-like elements into aesthetasc-like elements (Boxshall & Huys, 1998). These differences may confer a greater sensitivity to chemosensory signals, such as pheromones produced by receptive females.

As previously stated, both males and females have two types of antennal setae, but males have significantly more antennal setae (Bauer & Caskey, 2006). This, coupled with the differential change in intracellular Ca^{2+}

levels of males and females upon exposure to glucosamine, indicates that males have an enhanced ability to respond to glucosamine compared to that of females. Perhaps male shrimp rely more heavily on chemoreception than females during courtship. *Palaemonetes pugio* employs a pure-searching mating strategy in which male mating success depends primarily on their ability to find and mate with as many females as possible (Bauer & Abdalla, 2001; Correa & Thiel, 2003). To search efficiently, these males roam through the population and continually contact conspecifics until they find a receptive mate (Correa & Thiel, 2003). For males of *P. pugio*, having more chemoreceptive setae as well as a stronger response to a mate recognition cue is an adaptive advantage.

Conclusions

According to our current model, the mate recognition signal of *P. pugio* appears to be a surface glycoprotein. Data suggest the presence of a glucosamine or a glucosamine-containing glycoprotein present on the exoskeleton of postmolt, parturial females that functions in mate recognition. Following molting, parturial female shrimp produce an unknown glycoprotein that is capable of diffusion. Males possess chemoreceptors for this putative glycoprotein on the plasma membrane of sensory neurons. Antennal setae were found to be capable of the uptake of water-soluble molecules from the environment and to contain neural tissue. Increases in intracellular Ca^{2+} in response to natural stimuli (i.e., carapaces of postmolt, parturial females) as well as by glucosamine suggests that Ca^{2+} functions as a second messenger in this pathway. Terminal pores in the setae allow the glycoproteins to bind the receptors, but because the receptors likely have low affinity binding, the sensory capabilities of the setae are restricted to close proximity to the source of the glycoproteins. In other words, chemoreception occurs only when the setae are virtually in contact with the female carapace. Hence, the system is correctly identified as "contact chemoreception."

Acknowledgments

This work was funded in part by the Graduate Student Organization of the University of Louisiana at Lafayette, the National Oceanographic and Atmospheric Administration's Louisiana Sea Grant Program (R/SA-03 to RTB), and graduate fellowships from the American Microscopical Society and the Crustacean Society to JLC. We wish to thank Drs. Scott France and Hans Laufer for their valuable comments and suggestions. This is contribution No. 134 of the Laboratory for Crustacean Research.

References

Ameyaw-Akumfi, C. & Hazlett, B.A. 1975. Sex recognition in the crayfish *Procambarus clarkii*. *Science*, 190, 1225–1226.

Asai, N., Fusetani, N., Matsunaga, S. & Sasaki, J. 2000. Sex pheromones of the hair crab *Erimacrus isenbeckii*. Part 1: Isolation and structures of novel ceramides. *Tetrahedron*, 56, 9895–9899.

Atema, J. 1986. Review of sexual selection and chemical communication in the lobster, *Homarus americanus*. *Canadian Journal of Fisheries and Aquatic Science*, 43, 2283–2290.

Atema, J. 1995. Chemical signals in the marine environment: dispersal, detection, and temporal signal analysis. *Proceedings of the National Academy of Science U.S.A*, 92, 62–66.

Ayasse, M., Paxton, R.J. & Tengo, J. 2001. Mating behavior and chemical communication in the order Hymenoptera. *Annual Review of Entomology*, 46, 31–78.

Baker, J.E. & Reeset, B.E. 1993. Using confocal laser scanning microscopy to investigate the organization and development of neuronal projections labeled with DiI. *Methods in Cell Biology*, 38, 325–344.

Bauer, R.T. 1979. Sex attraction and recognition in the caridean shrimp *Heptacarpus* Holmes (Decapoda: Hippolytidae). *Marine Behaviour and Physiology*, 6, 157–174.

Bauer, R.T. 2004. *Remarkable Shrimps: Adaptations and Natural History of the Carideans*. University of Oklahoma Press, Norman.

Bauer, R.T. & Abdalla, J.H. 2001. Male mating tactics in the shrimp *Palaemonetes pugio*: Precopulatory mate guarding vs. pure searching. *Ethology*, 107, 185–199.

Bauer, R.T. & Caskey, J.L. 2006. Flagellar setae of the second antennae in decapod shrimps: sexual dimorphism and possible role in detection of contact sex pheromones. *Invertebrate Reproduction and Development*, 49, 51–60.

Berg, A.B. & Sandifer, P.A. 1984. Mating behavior of the grass shrimp *Palaemonetes pugio*. *Journal of Crustacean Biology*, 4, 417–424.

Boxshall, G.A. & Huys, R. 1998. The ontogeny and phylogeny of copepod antennules. *Philosophical Transactions of the Royal Society of London Series B*, 353, 765–786.

Bublitz, R., Sainte-Marie, B., Newcomb-Hodgetts, C., Fletcher, N., Smith, M. & Hardege, J. 2008. Interspecific activity of the sex pheromone of the European shore crab (*Carcinus maenas*). *Behaviour*, 145, 1465–1478.

Bundy, M.H. & Paffenhöfer, G.A. 1993. Innervation of copepod antennules investigated using laser scanning confocal microscopy. *Marine Ecology Progress Series*, 102, 1–14.

Burkenroad, M.D. 1947. Reproductive activities of decapod Crustacea. *American Naturalist*, 81, 392–398.

Bushmann, P.J. & Atema, J. 2000. Chemically mediated mate location and evaluation in the lobster, *Homarus americanus*. *Journal of Chemical Ecology*, 26, 883–899.

Caskey, J.L. & Bauer, R.T. 2005. Behavioral tests for a possible contact sex pheromone in *Palaemonetes pugio*. *Journal of Crustacean Biology*, 25, 571–576.

Caskey, J.L., Hasenstein, K.H. & Bauer, R.T. 2009. Studies on contact sex pheromones of the caridean shrimp *Palaemonetes pugio*: I. Cuticular hydrocarbons associated with mate recognition. *Invertebrate Reproduction and Development*, 53(2), 93–103.

Cate, H.S. & Derby, C.D. 2002. Ultrastructure and physiology of the hooded sensillum, a bi-modal chemomechanosensillum of lobsters. *Journal of Comparative Neurology*, 442, 293–307.

Correa, C. & Thiel, M. 2003. Mating systems in caridean shrimp (Decapoda: Caridea) and their evolutionary consequences for sexual dimorphism and reproductive biology. *Revista Chilena Historia Natural*, 76, 187–203.

Derby, C.D. 1989. Physiology of sensory neurons in morphologically identified cuticular sensilla of crustaceans. In: *Functional Morphology of Feeding and Grooming*, B.E. Felgenhauer, L. Watling & A.B. Thistle (eds), A.A. Balkema Press, Rottterdam, pp. 27–73.

Derby, C.D. & Sorensen, P.W. 2008. Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans. *Journal of Chemical Ecology*, 34, 898–914.

Díaz, E.R. & Thiel, M. 2004. Chemical and visual communication during mate searching in the rock shrimp. *Biological Bulletin*, 206, 134–143.

Dunham, P.J. 1978. Sex pheromones in Crustacea. *Biological Reviews of the Cambridge Philosophical Society*, 53, 555–583.

Dunham, P.J. 1988. Pheromones and behavior in Crustacea. In: *Endocrinology of Selected Invertebrate Types*, H. Laufer & G.H. Downer (eds), Alan R. Liss, Inc., New York, pp. 375–392.

Ekerholm, M. & Hallberg, E. 2005. Primer and short-range releaser pheromone properties of premolt female urine from the shore crab *Carcinus maenas*. *Journal of Chemical Ecology*, 31, 1845–1864.

Etzler, M.E. & Esko, J.D. 2009. Free glycans as signaling molecules. In: *Essentials of Glycobiology*, A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, & M.E. Etzler (eds), Cold Springs Harbor Laboratory Press, Cold Springs Harbor, New York, pp. 523–530.

Francke, W., Karaluis, V., Plass, E., Lehmann, L., Dos Santos, A., Bûda, V., Borg-Karlson, A.-K. & Mozûraitis, R. 2004. New type of Sesiidae sex pheromone identified from the hornet moth *Sesia apiformis*. *Journal of Chemical Ecology*, 30, 805–817.

Frey, M.A., Lonsdale, D.J. & Snell, T.W. 1998. The influence of contact chemical signals on mate recognition in a harpacticoid copepod. *Philosophical Transactions of the Royal Society of London Series B*, 353, 745–751.

Fukaya, M. 2003. Recent advances in sex pheromone studies in the white-spotted longicorn beetle, *Anoplophora malasiaca*. *Japan Agricultural Research Quarterly*, 37, 83–88.

Ginzel, M.D. & Hanks, L.M. 2003. Contact pheromones as mate recognition cues of four species of long-horned beetles (Coleoptera: Cerambycidae). *Journal of Insect Behaviour*, 16, 181–187.

Ginzel, M.D., Millar, J.G. & Hanks, L.M. 2003a. (Z)-9-Pentacosene-contact sex pheromone of the locust borer, *Megacyllene robiniae*. *Chemoecology*, 13, 135–141.

Ginzel, M.D., Blomquist, G.J., Millar, J.G. & Hanks, L.M. 2003b. Role of contact pheromones in mate recognition in *Xylotrechus colonus*. *Journal of Chemical Ecology*, 29, 533–545.

Gleeson, R.A. 1980. Pheromone communication in the reproductive behavior of the blue crab *Callinectes sapidus*. *Marine Behaviour and Physiology*, 7, 119–134.

Godement, P., Vanselow, J., Thanos, S. & Bonhoeffer, F. 1987. A study in developing visual systems with a new method of staining neurons and their processes in fixed tissue. *Development*, 101, 697–713.

Gries, G., Schaefer, P.W., Gries, R., Fan, Y.–B., Higashiura, Y. & Tanaka, B. 2002. 2-Methyl-(Z)-7-Octadecene: sex pheromone of allopatric *Lymantria lucescens* and *L. serva*. *Journal of Chemical Ecology*, 28, 469–478.

Hallberg, E. & Hansson, B.S. 1999. Arthropod sensilla: morphology and phylogenetic considerations. *Microscopy and Research Technique*, 47, 428–439.

Honig, M.G. and Hume, R.I. 1986. Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. *Journal of Cell Biology*, 103, 171–187.

Honig, M.G. & Hume, R.I. 1989. DiI and DiO: versatile fluorescent dyes for neuronal labeling and pathway tracing. *Trends in Neuroscience*, 12, 333–341.

Howard, R.W. & Blomquist, G.J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annual Review of Entomology*, 27, 149–172.

Howard, R.W. & Blomquist, G.J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology*, 50, 371–393.

Kamiguchi, Y. 1972. Mating behavior in the freshwater prawn, *Palaemon paucidens*. A study of the sex pheromone and its effects on males. *Journal of the Faculty of Science Hokkaido, Series IV, Zoology*, 18, 347–355.

Kamio, M., Matsunaga, S. & Fusetani, N. 2000. Studies on sex pheromones of the helmet crab, *Telmessus cheiragonus*. 1. An assay based on precopulatory mate guarding. *Zoological Science*, 17, 731–733.

Kamio, M., Matsunaga, S. & Fusetani, N. 2002. Copulation pheromone in the crab *Telmessus chieragonus* (Brachyura: Decapoda). *Marine Ecology Progress Series*, 234, 183–190.

Kelly, L.S. & Snell, T.W. 1998. Role of surface glycoproteins in mate-guarding of the marine harpacticoid *Tigriopus japonicus*. *Marine Biology*, 130, 605–612.

Kelly, L.S., Snell, T.W. & Lonsdale, D.J. 1998. Chemical communication during mating of the harpacticoid *Tigriopus japonicus*. *Philosophical Transactions of the Royal Society of London Series B*, 353, 737–744.

Kirchman, D., Graham, S., Reish, D. & Mitchell, R. 1982. Bacteria induce settlement and metamorphosis of *Janua* (*Dexiospira*) *brasiliensis* Grube (Polychaeta: Spirorbidae). *Journal of Experimental Marine Biology and Ecology*, 56, 153–163.

Krug, P.J. & Manzi, A.E. 1999. Waterborne and surface-associated carbohydrates as settlement cues for larvae of the specialist marine herbivore *Alderia modesta*. *Bio-*

logical Bulletin, 197, 94–103.

Leal, W.S., Parra-Pedrazzoli, A.L., Cossé, A.A., Murata, Y., Bento, J.M.S. & Vilela, E.F. 2006. Identification, synthesis, and field evaluation of the sex pheromone from the citrus leaf miner, *Phyllocnistis citrella*. *Journal of Chemical Ecology*, 32, 155–167.

Lonsdale, D.J., Snell, T.W. & Frey, M.A. 1996. Lectin binding to surface glycoproteins on *Coullana* spp. (Copepoda: Harpacticoida) can inhibit mate guarding. *Marine Freshwater Behaviour and Physiology*, 27, 153–162.

Lonsdale, D.J., Frey, M.A. & Snell, T.W. 1998. The role of chemical cues in copepod reproduction. *Journal of Marine Research*, 15, 1–12.

Maki, J.S. & Mitchell, R. 1985. Involvement of lectins in the settlement and metamorphosis of marine invertebrate larvae. *Bulletin of Marine Science*, 37, 675–683.

Morse, D.E. & Morse, A.N.C. 1991. Enzymatic characterization of the morphogen recognized by *Agaricia humilis* (Scleractinian coral) larvae. *Biological Bulletin*, 181, 104–122.

Paterson, H.E.H. 1982. Perspective on speciation by reinforcement. *South African Journal of Science*, 78, 53–57.

Rittschof, D. & Cohen, J.H. 2004. Crustacean peptide and peptide-like pheromones and kairomones. *Peptides*, 25, 1503–1516.

Ryan, E.P. 1966. Pheromone—evidence in a decapod crustacean. *Science*, 151, 340–341.

SAS Institute Inc. Base SAS 9.1.3 Procedure Guide. 2004. SAS Institute, Inc., Cary, N.C.

Schmidt, M. & Derby, C.D. 2005. Non-olfactory chemoreceptors in asymmetric setae activate antennular grooming behavior in the Caribbean spiny lobster *Panulirus argus*. *Journal of Experimental Biology*, 208, 233–248.

Schmidt, M. & Gnatzy, W. 1994. Are the funnel-canal organs the 'campaniform sensilla' of the shore crab, *Carcinus maenas* (Decapoda, Crustacea)? II. Ultrastructure. *Cell and Tissue Research*, 237, 81–93.

Sharon, N. & Lis, H. 1989. Lectins as cell recognition molecules. *Science*, 246, 227–234.

Snell, T.W. & Carmona, M.J. 1994. Surface glycoproteins in copepods: potential signals for mate recognition. *Hydrobiologia*, 292/293, 255–263.

Snell, T.W., Rico-Martinez, R., Kelly, L.S. & Battle, T.E. 1995. Identification of a sex pheromone from a rotifer. *Marine Biology*, 123, 347–353.

Sokal, R.R. & Rohlf, F.J. 1981. *Biometry* (2nd ed.). W.H. Freeman and Company, New York.

Sorensen, P.W. 1992. Hormonally derived sex pheromones in goldfish: a model for understanding the evolution of sex pheromone systems in fish. *Biological Bulletin*, 183, 173–177.

Stebbing, P.D., Bentley, M.G., and Watson, G.J. 2003. Mating behavior and evidence for a female released courtship pheromone in the signal crayfish *Pacifastacus leniusculus*. *Journal of Chemical Ecology*, 29, 465–475.

Stoffolano, J.G., Schauber, E., Yin, C.–M., Tillman, J.A. & Blomquist, G.J. 1997. Cuticular hydrocarbons and their role in copulatory behavior in *Phormia regina* (Meigen). *Journal of Insect Physiology*, 43, 1065–1076.

Ting, J.H., Kelly, L.S. & Snell, T.W. 2000. Identification of sex, age, and species specific proteins on the surface of the harpacticoid copepod *Tigriopus japonicus*. *Marine Biology*, 137, 31–37.

Varki, A. & Lowe, J.B. 2009. Biological roles of glycans. In: *Essentials of Glycobiology*, A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart & M.E. Etzler (eds), Cold Springs Harbor Laboratory Press, Cold Springs Harbor, New York, pp. 75–88.

Varki, A., Etzler, M.E., Cummings, R.D. & Esko, J.D. 2009. Discovery and classification of glycan-binding proteins. In: *Essentials of Glycobiology*, A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart & M.E. Etzler (eds), Cold Springs Harbor Laboratory Press, Cold Springs Harbor, New York, pp. 375–386.

Weissburg, M.J. 2001. Sex, sensitivity, and second messengers: different effect of cyclic nucleotide mediated inhibition in the chemosensory system of fiddler crabs. *Journal of Comparative Physiology, A*, 187, 489–498.

Wyatt, T.D. 2003. *Pheromones and Animal Behavior: Communication by Smell and Taste*. Cambridge University Press, Cambridge.

Zimmer, R.K. & Butman, R.A. 2000. Chemical signaling processes in the marine environment. *Biological Bulletin*, 198, 168–187.