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Studies on contact sex pheromones of the caridean shrimp *Palaemonetes pugio*: I. Cuticular hydrocarbons associated with mate recognition

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Abstract

In the grass shrimp *Palaemonetes pugio*, evidence suggests that males respond to an insoluble substance (i.e., contact sex pheromone) in or on the exoskeleton of the postmolt parturial female. Cuticular hydrocarbons, glycoproteins, or other compounds present on the surface of the female might serve as recognition signals. Cuticular hydrocarbons are known to function as contact sex pheromones in many insect species. The purpose of this study was to test the hypothesis that the exoskeleton of postmolt parturial females contains cuticular compounds that might function as contact sex pheromones. Gas chromatography-mass spectrometry (GC-MS) analyses of chloroform–methanol extracts of the cuticle of postmolt parturial (sexually receptive) females (PPFs), postmolt nonparturial females (NPFs), postmolt males (PMMs), and intermolt females (IMFs) showed at least 55 compounds, 3 of which were unique to postmolt parturial females. Extracts of PPFs, NPFs, PMMs, and IMFs were characterized by 49, 50, 34, and 19 compounds, respectively, of which 22 could be identified. Twelve cuticular hydrocarbons and five fatty acids were found in the cuticular extractions of *P. pugio*. Multivariate analysis of GC-MS profiles demonstrated that the cuticular composition of postmolt parturial females differed significantly from that of nonparturial females, males, and intermolt females. A bioassay to test male response to cuticular extracts gave inconclusive results. 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*.

Key words: Crustacea, Malacostraca, cuticular hydrocarbons, mate recognition, sex pheromone, gas chromatography-mass spectrometry

Introduction

The term "mating system" refers to the behavioral tactics of males and females that enhances individual reproductive success (Emlen & Oring, 1977; Wickler & Seibt, 1981). In terms of fitness, a mate is a crucial resource. Mate acquisition is, thus, as important an adaptive process as is the avoidance of predators, the ability to find refuge, or the acquisition of food

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(Paterson, 1982). Many species have evolved a mate recognition system (MRS) to ensure mating success and thus to enhance reproductive fitness. The MRS of a species comprises the signals and preferences involved in communication, stimulation, and choice of sexual partners. Elements of a MRS include the coordination of reproductive timing and a finely tuned signal-receptor system (Lonsdale et al., 1996). Sexual signals are usually complicated and involve chemical, visual, behavioral and tactile stimuli.

In many decapod crustaceans, the timing of sexual activity is essential for successful matings. Females of many species can mate only during a limited period after a parturial (prespawning) molt. Females of caridean shrimp species are receptive to males only for a short time period, from a few minutes to a few days, following the parturial molt (Correa & Thiel, 2003; Bauer, 2004). In *Palaemonetes pugio*, females that have successfully copulated resist further attempts by males and become unattractive to them within 30 min of copulation. However, the attractiveness of unmated, postmolt parturial females was found to steadily decline over an 8-h period following the parturial molt (Bauer & Abdalla, 2001; Caskey & Bauer, 2005). Males of this species respond to attractive females only after touching them with the antennal flagella or the pereopods (Berg & Sandifer, 1984; Bauer & Abdalla, 2001; Caskey & Bauer, 2005). These findings suggest that a sexual signal used in the MRS of *P. pugio* may be an insoluble chemical substance (i.e., contact sex pheromone) present on the exoskeleton of newly molted parturial females. Females might be able to control the production of the sex pheromone based on whether or not she has successfully copulated.

However, other possible signals include tactile, visual, and behavioral stimuli. Caskey & Bauer (2005) showed that males exhibited mating behaviors towards postmolt parturial females but not toward postmolt nonparturial females (no ovarian development), newly-molted males, or intermolt females (hard-shelled females with no ovarian development). The lack of response to the soft cuticle of nonparturial females and postmolt males indicates that the soft cuticle (tactile stimuli) is not the sole cue for mate recognition in *P. pugio*.

Visual sexual communication involves cues such as color, shape, and size of morphological structures or resources. It has been hypothesized that in species that exhibit sexual dimorphism, like *P. pugio*, sexual recognition may be visual (Correa & Thiel, 2003). Males and females of *P. pugio* usually differ in size, with reproductive females usually larger than males. Other possible visual signals are not obvious to the human observer. In Caskey & Bauer (2005), physical contact was eliminated by a transparent barrier so that males could see but not touch the females. Males showed no difference in behavior when presented with a parturial female or a nonparturial female (Caskey & Bauer, 2005). While this negative evidence does not rule out visual stimuli, the reduced significance of visual cues in this species might be expected based on their turbid environment and generally nocturnal mating activity. Another possible explanation is that there may be a behavioral stimulus (e.g., courtship behavior) in the MRS of this species, and that both chemical and behavioral cues are required for successful mating. Berg and Sandifer (1984) noted no definitive female precopulatory behavior and that copulation occurred only upon antennal contact by males.

Bauer & Abdalla (2001) found that *P. pugio* employs a mating strategy in which male mating success depends primarily on their ability to find (and mate with) as many receptive females as possible. Males do not engage in precopulatory mate guarding such as grasping females prior to copulation, remaining in close proximity to the females, nor defending them from other males (Berg & Sandifer, 1984; Bauer & Abdalla, 2001). The only interactions between males and females involve momentary contacts with antennal flagella or thoracic appendages (Bauer & Abdalla, 2001). Upon locating a receptive female, males transfer sperm (within spermatophores) in a brief and simple copulation after which the pair immediately separates. With the exception of female cooperation in allowing males physical access to her, it is unlikely that there is a specific behavioral cue offered by the male in the MRS of *P. pugio*.

Upon examining and discounting visual, tactile (i.e., the soft nature of the postmolt cuticle), and behavioral cues as essential sexual signals, the current evidence suggests that chemical signals in the form of a contact sex pheromone are the primary cues in the MRS of *P. pugio*. Ekerholm & Hallberg (2005) proposed that in decapod crustaceans mating is highly dependent upon chemical cues and that these chemical cues may guarantee that a male and a female are in contact during the limited reproductive time period. Female sex pheromones have been suggested for decapods such as lobsters (Atema, 1986; Bushmann & Atema, 2000), crayfishes (Ameyaw-Akumfi & Hazlett, 1975; Stebbing et al., 2003), brachyuran crabs (Ryan, 1966; Kittredge et al., 1971; Gleeson, 1980; Asai et al., 2000; Kamio et al., 2000, 2002; Bublitz et al., 2008), and shrimps (Kamiguchi, 1972; Díaz & Thiel, 2004; Caskey & Bauer, 2005). In most studies, olfactory (water soluble) pheromones have been suggested by the behavior of males and females. Remarkably, few attempts have been made to identify aquatic pheromones. Most information

on the existence of sex pheromones, primarily olfactory pheromones, in decapods is based on behavioral evidence (Atema & Steinbach, 2007). Knowledge about contact pheromones in caridean shrimps is limited to behavioral observations indicating their existence. Burkenroad (1947) first suggested, based on anecdotal mating observations in *P. vulgaris* (Say), that the "recognition-mark" might be a "nondiffusible coating" on the female exoskeleton. More extensive studies on the mating behavior of caridean shrimps by Bauer (1979), Bauer & Abdalla (2001), Díaz & Thiel (2004), and Caskey & Bauer (2005) agree with Burkenroad's hypothesis. However, the chemical nature of the compound(s) involved is not known.

This study sought to determine the presence and identity of cuticular compounds that might serve as contact sex pheromones in *P. pugio*. The vast entomological literature was used for background information and methodologies. An epicuticular lipid layer comprising long-chain fatty acids and hydrocarbons covers the outer surface of the cuticle in insects (Simmons et al., 2003). In some insect species, epicuticular hydrocarbons serve as 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). Analyses of many insect species indicate that, in general, hydrocarbon profiles tend to be species specific (Howard & Blomquist, 2005). If this is true in other arthropods such as crustaceans, the cuticular hydrocarbon profile of *P. pugio* might serve as a species-specific sex pheromone.

In this study, we tested the hypothesis that chemical extracts of the cuticle of postmolt parturial females contains compounds that are sufficiently distinct from those of other recently molted individuals that they could serve as a female contact sex pheromone. Cuticular compounds were extracted and identified with gas chromatography-mass spectrometry and analyzed using analysis of variance and principal component analyses. A bioassay to detect male response to cuticular compounds extracted from sexually receptive and attractive males was performed.

Materials and Methods

Culture conditions

Individuals of *Palaemonetes pugio* (Caridea: Palaemonidae) were collected in a salt marsh habitat at Cypremort Point, Vermilion Bay, Louisiana, USA (29° 43′ N, 91°51′ W) during the spring to early autumn reproductive season. Shrimps were maintained under a 13 h light: 11 h dark cycle on a recirculating water table at 5 ppt salinity and a water temperature of 21–24°C. Shrimps in varying reproductive condition (treatments, n = 20 individuals each) were used for extractions: intermolt (hard cuticle) females without embryos; postmolt parturial females (soft cuticle and full ovarian development); postmolt nonparturial females (soft cuticle and no ovarian development), and postmolt (soft cuticle) males. All postmolt individuals were <4 h postmolt and intermolt females were at least 4–5 days postmolt. Time of the molt was determined from 24 h time-lapse video surveillance illuminated by overhead fluorescent light during "day" hours and infrared lamps (880 nm wavelength) during "night" hours. Videos were reviewed to determine time of the molt; if the individual was <4 h postmolt, the individual was removed for immediate chemical extraction.

Extraction of cuticle

Mature females of this species range in size from 6–8 mm and males from 4–6 mm carapace length (CL). Each shrimp was immersed in an 11 ml screw-top glass vial in 6 ml of deionized water for 30 s to remove salt and other material from the exoskeleton. Individuals were then immersed in 6 ml of methanol for 30 s to make the surface miscible with the extraction solvent. The final immersion of the individual was in chloroform–methanol (2:1) for 5 min to extract compounds from the surface of the shrimp. Then, the shrimp were removed, the extracts gravity filtered using Fisherbrand® Filter Paper (Qualitative P8, Coarse, Fast Flow Rate, 12.5 cm dia.) into a clean 11 ml vial with a Teflon-lined lid, and stored at −4°C until methylation and analysis. Ethereal diazomethane was freshly prepared for methylation of the cuticular extractions (Cohen, 1984). Each extract was methylated by placing 0.5 ml of the extract in a target vial and drying with N_2 to less than 100 μl. Then 0.5 ml of ethereal diazomethane was added to the vial. After 5 min, the solutions were completely dried using N_2 and dissolved in 1 ml of ethyl acetate.

Gas chromatography-mass spectrometry

The cuticular extracts were analyzed using gas chromatography-mass spectrometry (GC-MS) with a Hewlett-Packard (HP) 6890 Series GC system and HP 5973 Mass Selective Detector. The GC was equipped with a capillary column (Zebron, MS-5, 30 m length, 250 μm diameter, 0.1 μm coating of 5% phenyl and 95% dimethylpolysiloxane). Two μl were injected

(250 °C, pulsed splitless) into the column and the oven temperature was ramped from 120 °C (2 min) to 320 °C at 20 °C min^{-1} and kept at the final temperature for 2 min. Helium was used as the carrier gas with constant flow conditions (50 cm min^{-1}). The subsequent mass spectra (70 keV ionization) were compared to controls and among the treatment groups.

Qualitative analysis

To qualitatively compare treatments and determine differences in surface compounds present among treatments, an elimination method was used in which substances occurring in the controls were eliminated as possible target compounds. Similarly, substances occurring in only one replicate of a single treatment group were considered biologically irrelevant and not considered in further analysis. Compounds were identified using retention times (Rt.) and the mass spectral database of the National Institute of Standards and Technology (NIST 98). The quality of a compound was defined as the percentage of the mass fragments of that compound shared with a chromatogram of a pure sample of that compound. If the mean quality of a compound was over 85%, then identification was considered reliable.

Quantitative analysis

There was no indication of which cuticular compounds might function in mate recognition, so a four-step quantitative analysis was applied. We normalized each compound by dividing the total ionizable compounds (TIC) of each compound by the TIC of all compounds in that extract. This allowed direct comparison across samples. Relative values were normalized for parametric statistical procedures by dividing each compound by cholesterol (retention time of 12.41 min), which occurred in all 80 replicates. The ratios now represented a compositional data set in which the ratios summed to one. In response to this unit-sum constraint, log contrasts were taken (Aitchison, 1986). Additionally, log transformation helped to meet the assumptions of analysis of variance (ANOVA). Log (x+1) (as in Simmons et al., 2003) transformation was used because many of the compounds were not found in all treatment groups or replicates, yielding values of zero.

Next, one-way ANOVAs (Proc GLM, SAS, 2004) were run for each compound to determine which of them differed significantly among treatment groups. Compounds that were significant at the $p \leq 0.05$ level were used in a multivariate analysis of variance (MANOVA) model (Proc GLM, MANOVA, SAS, 2004). The MANOVA model tested whether or not the number of compounds found and the quantity of those compounds differed significantly among all treatment groups. An *a priori* contrast determined if the cuticular extracts of postmolt parturial females differed from other extracts, i.e., intermolt females, postmolt males, and postmolt nonparturial females. Lastly, the transformed data were subjected to principal component analysis, PCA (Proc PRINCOMP, SAS, 2004) to describe trends in the data and to describe these trends based on shared correlations. Because the proportion of variation explained by a principal component may not be related to its biological activity (Blows & Allan, 1998), all ten prin-cipal components (PC 1-10) are considered in relation to the role of cuticular compounds in mate recognition.

Bioassay

An attempt was made to conduct a bioassay using small sponge-tipped rods treated with cuticular extracts. For each replicate (n = 10) of this experiment, each side of a small (8×13 mm) latex sponge was treated with 30 μl of cuticular extract of a particular treatment and allowed to dry. The sponge was then attached to a thin, clear plastic tube and suspended in the testing arena (30 cm L, 9 cm W, 15 cm H) containing two males acclimated for five min. Male copulatory response to the sponge was scored as pheromone activity. Positive responses were considered to be direct forward movements into the sponge with the anterior portion of the body, grasping of the sponge with the chelipeds, or climbing on the sponge. Comparisons of male responses to cuticular extracts of postmolt parturial females, postmolt nonparturial females, intermolt females, and postmolt males were made. A negative control of chloroform-methanol and a positive food control (homogenized shrimp tails) were used. The null hypothesis of no difference in male responses to sponges among treatments was tested using the CMH Row Mean Scores test (Proc FREQ, SAS, 2004).

Results

Qualitative analysis

The GC-MS analyses identified 55 compounds among the four treatment groups (Table 1). The single most abundant compound in all replicates was cholesterol (Rt. 12.41 min) (Fig. 1). Many of the most abundant compounds identified were not considered for analysis because they were either contaminants, e.g., bis (2-ethylhexyl) phthalate (Rt. 9.85 min), or were found in our control chloroform-methanol profile, e.g., benzenesulfonic acid, 4 methyl (Rt. 4.65 min), butylated

Table 1. Fifty-five peaks (retention time = Rt., shown in min) found in the chloroform–methanol extracts of postmolt parturial females (PPF), postmolt nonparturial females (NPF), postmolt males (PMM), and intermolt females (IMF) (compounds that could be identified are listed in Table 2). Number of replicates (of 20) in which this compound could be identified are listed. *Indicates compounds that differed significantly among treatment groups ($P \leq 0.05$, ANOVA) and were used in the MANOVA model

Rt. (min)	PPF	NPF	PMM	IMF	Rt. (min)	PPF	NPF	PMM	IMF
3.92	4	8	5	1	**8.66***	13	12	2	1
4.18*	1	4	0	0	**8.70**	1	3	0	0
5.42	1	2	0	0	**8.76***	11	16	9	1
5.50	0	1	2	0	**8.88***	10	5	1	3
5.55	0	1	2	0	**9.18**	3	7	6	1
5.59	0	2	2	0	**9.22**	4	4	2	0
5.75	0	1	4	0	**9.47***	10	11	0	0
5.84	0	3	1	0	**9.53***	4	8	0	0
5.94*	10	11	8	7	**9.56***	2	6	0	0
6.05	2	1	0	0	**9.60**	3	7	6	1
6.08	2	2	0	0	**9.62***	4	7	0	0
6.39	2	0	0	0	**10.00***	8	10	6	1
6.52	2	2	0	0	**10.23***	4	7	0	0
6.68	2	1	0	0	**10.40***	3	9	5	1
6.72	2	0	0	0	**10.63**	9	6	3	7
6.80	4	5	0	1	**10.80***	3	7	6	1
7.08	1	4	0	0	**10.95***	1	9	1	2
7.11	2	1	0	0	**11.21**	3	7	6	1
7.24	3	1	0	0	**11.27**	2	0	0	0
7.49	1	1	1	0	**11.37**	4	4	1	0
7.51*	10	8	1	2	**11.65**	3	7	6	1
7.55	3	1	1	0	**11.85**	2	0	1	0
7.80	1	4	0	0	**11.88***	3	0	1	0
7.86*	9	9	6	4	**11.97**	2	3	0	0
7.91	2	2	0	0	**12.41***	20	20	20	20
8.31	3	6	6	1	**12.57**	2	2	1	0
8.36	3	2	2	0	**12.62***	4	10	1	0
8.63*	7	3	0	0					

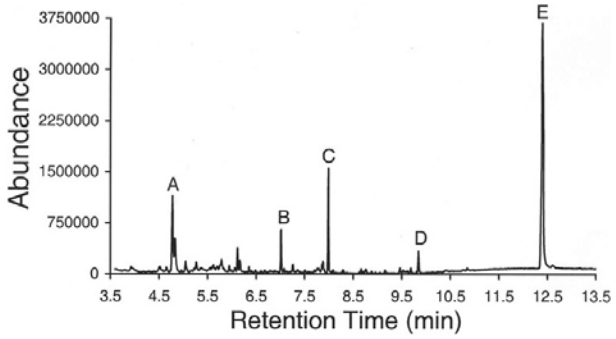

Fig. 1. GC-MS chromatograph of a representative cuticular extract of *Palaemonetes pugio*. Six most frequently occurring and abundant peaks, labeled in the figure, were not considered for analysis because they were either a contaminant or were found in the control: (A) benzenesulfonic acid, 4 methyl (Rt. 4.65 min) and butylated hydroxytoluene (Rt. 4.77 min); (B) pentadecanoic acid, ME (Rt. 7.01 min); (C) heptadecanoic acid, ME (Rt. 7.99 min); (D) bis (2-ethylhexyl) phthalate (Rt. 9.85 min); (E) cholesterol (Rt. 12.41 min). ME = methyl ester.

hydroxytoluene (Rt. 4.77 min), pentadecanoic acid, methyl ester (Rt. 7.01 min), and heptadecanoic acid, methyl ester (Rt. 7.99 min) (Fig. 1). As a result, mean peak areas were calculated for all 55 compounds found in the treatment groups to create representative cuticular profiles of postmolt parturial females (PPFs), postmolt nonparturial females (NPFs), postmolt males (PMMs), and intermolt females (IMFs) (Fig. 2A–D). NPFs, PPFs, PMMs, and IMFs had 50, 49, 34, and 19 compounds, respectively (Table 1), of which 22 could be identified (Table 2). Cuticular extracts of PPFs showed 3 unique compounds with retention times (Rt.) of 6.39, 6.72 and 11.27 min (Table 1). These compounds occurred in only 2 replicates, PPF 4 and PPF 5. The low relative abundance of the compounds and the low frequency of occurrence did not allow for their identification. There were 18 compounds common to all treatment groups (Table 1). Cholesterol (Rt. 12.41 min) was the only compound found in all 80 replicates. Tricosane, (8.76 min), and methyl tetradecanoate, (5.94 min), were the

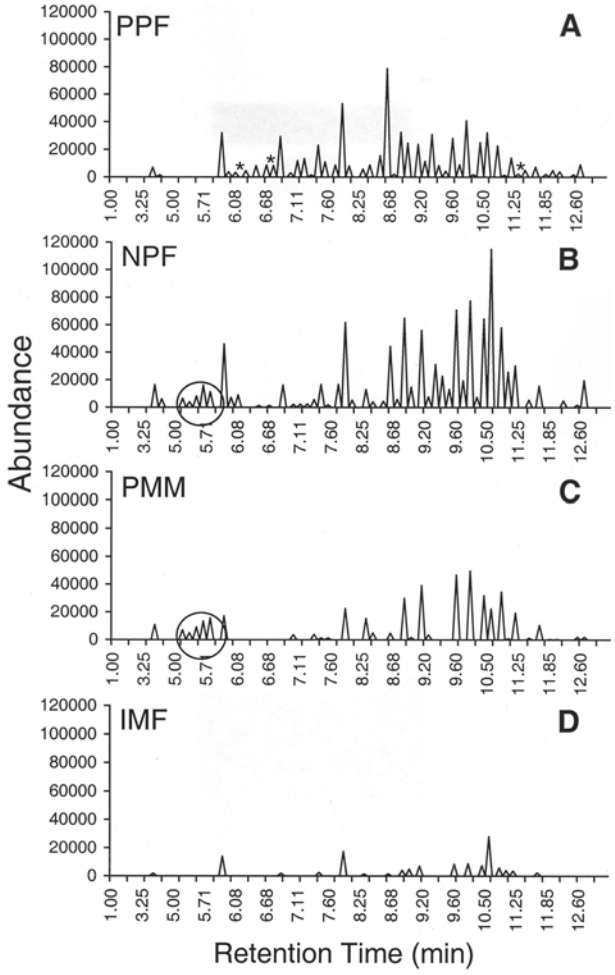

Fig. 2. Representative chromatographs from A: postmolt parturial females (PPF); B: postmolt nonparturial females (NPF); C: postmolt males (PMM); and D: intermolt females (IMF). Mean peak areas (abundance) were taken for all compounds within each treatment and graphed against retention time (min) to produce a composite chromatograph representing the cuticular profile for each treatment. *Indicates unique peaks. Circled peaks (Rt. 5.50, 5.55, 5.59, 5.75, and 5.84 min) were found only in NPF and PMM cuticular extracts.

next two most common compounds that were present in 47 and 45% of all samples, respectively. A total of 12 hydrocarbons were found in the extracts of *P. pugio*: cyclotetradecane, cyclohexadecane, docosane, tricosane, tetracosane, cycloeicosane, pentacosane, hexacosane, heptacosane, octacosane, tetracohexaene, and eicosane (Table 2). Two amino acids, L-phenylalanine and L-tyrosine (Rt. 4.18 and 6.05 min, respectively), were also identified (Table 2).

Three saturated fatty acids (SFAs) were found in all treatment groups at Rt. 7.51, hexadecanoic acid (16:0); Rt. 7.86, octadecanoic acid (18:0); and Rt. 8.88, eicosanoic acid (20:0) (Table 2). Two polyunsaturated

Table 2. Compound identifications. Compounds were identified using NIST 98 compound identification software. Identifications were considered valid if the quality was over 85%. Quality given is an average of all qualities over 80%. ME = methyl ester

Rt. (min)	Compound	Quality
4.18	L-Phenylalanine, ME	80
5.75	Benzene sulfonamide	91
5.94	Methyl tetradecanoate	91
6.05	L-Tyrosine, ME	85
6.80	Cyclotetradecane	96
7.51	Hexadecanoic acid, ME	92
7.80	Cyclohexadecane	98
7.86	Octadecanoic acid, ME	99
8.31	Docosane	92
8.63	Eicosatetraenoic acid, ME	83
8.66	Eicosapentanoic acid, ME	88
8.76	Tricosane	95
8.88	Eicosanoic acid, ME	94
9.18	Tetracosane	98
9.22	Cycloeicosane	88
9.60	Pentacosane	97
10.00	Hexacosane	97
10.40	Heptacosane	97
10.80	Octacosane	91
10.95	Tetracohexaene	95
11.21	Eicosane	93
12.41	Cholesterol	99

fatty acids (PUFAs) were found in the cuticular extractions of *P. pugio*: eicosatetraenoic acid (20:4ω3) and eicosapentaenoic acid (20:5ω6), Rt. 8.63 and 8.66 min, respectively (Table 2). Hexadecanoic (palmitic) acid occurred most frequently in PPFs (10 of 20 replicates) and NPFs (8 of 20 replicates) (Table 1). Octadecanoic (stearic) acid occurred at equal frequency in PPFs and NPFs (Table 1). Eicosatetraenoic acid was found only in PPFs and NPFs, in 7 and 3 replicates, respectively (Table 1). Eicosapentaenoic acid was found in all four treatment groups; however, it occurred with greater frequency in PPFs, 13 replicates, and NPFs, 12 replicates, than in PMMs and IMFs where it occurred in only 2 and 1 replicates respectively (Table 1).

PPFs lacked several compounds found in NPF and PMM cuticular extracts (Rt. 5.50, 5.55, 5.59, 5.75, and 5.84 min) (Table 1, Fig. 2A–C). In general, the cuticular hydrocarbons of PPFs occurred in lower concentrations than in NPFs and PMMs (Fig. 2A–C). With the exception of cyclotetradecane (Rt. 6.80) all cuticular hydrocarbons were found in higher concentrations in NPFs than in PPFs (Fig. 2A–B). Seven of the 12 cuticular hydrocarbons identified occurred in lower concentrations in the cuticular extracts of PPFs than in PMMs (docosane, tetracosane, pentacosane, hexacosane, hepta-

cosane, octacosane, and eicosane) (Fig. 2A, C). The fewest number and lowest concentrations of cuticular compounds were found in cuticles of IMFs (Fig. 2D).

Quantitative analysis

Based on individual ANOVAs, 20 compounds were significant ($P \leq 0.05$) and used for the multivariate analysis of variance (Table 1). The MANOVA model tested and rejected the overall hypothesis that the cuticular extracts of *P. pugio* did not differ among the treatment groups (Wilks $\lambda = 0.0644$, $F_{60,171} = 4.29$, $P < 0.0001$). An *a priori* contrast showed that cuticular compounds of PPFs differed significantly from those of IMFs, NPFs, and PMMs (Wilks $\lambda = 0.3389$, $F_{20,57} = 5.56$, $P < 0.0001$). Overall, PPFs and NPFs were more similar to each other than to PMMs or IMFs (Table 1, Fig. 2A–D).

The percentage of the variance explained by each of the ten principal components was 19.1, 15.4, 13.7, 9.9, 4.8, 4.1, 3.5, 3.3, 2.7, and 2.5, respectively. The biological interpretation of the principal components (PCs) is best approached by an examination of whether they can discriminate between naturally occurring biological groupings (Blows & Allan, 1998). A plot of the largest principal components, based on eigenvectors and proportion of variation explained, provides a rapid means of visualizing similarities or differences among the variables. The variables that contributed to each PC were ranked by the highest correlation to each principal component. Eigenvectors over 0.250 were considered to have contributed significantly to that principal component.

The hydrocarbons (docosane, tricosane, tetracosane, pentacosane, hexacosane, heptacosane, octacosane, and eicosane) were identified as those compounds that contributed significantly to PC1. The variable PC1 (Fig. 3A) separates treatment groups based on the presence and frequency of hydrocarbons. Hydrocarbons were found in all treatment groups, with IMFs having a single replicate showing hydrocarbons. PPFs, NPFs, and PMMs have the highest number of replicates with hydrocarbons and thus show the most separation along PC1 (Fig. 3A–B). Compounds found only in PPFs and/or NPFs (Rt. 6.39, 6.52, 6.68, 6.72, 6.80, 7.11, 7.24, 7.55, and 11.27) were those that contributed strongly to PC2. As a result, PPFs and NPFs separated more along PC2 than did PMMs and IMFs (Fig. 3A, C). These compounds occurred infrequently within PPFs and NPFs so the separation along PC2 was not dramatic. Compounds found primarily in NPFs and PMMs (Rt. 5.42, 5.50, 5.55, 5.59, 5.75, 6.05, and 6.08) were those that contributed strongly to PC3. Therefore, the variable PC3 separated NPFs and PMMs from the other two

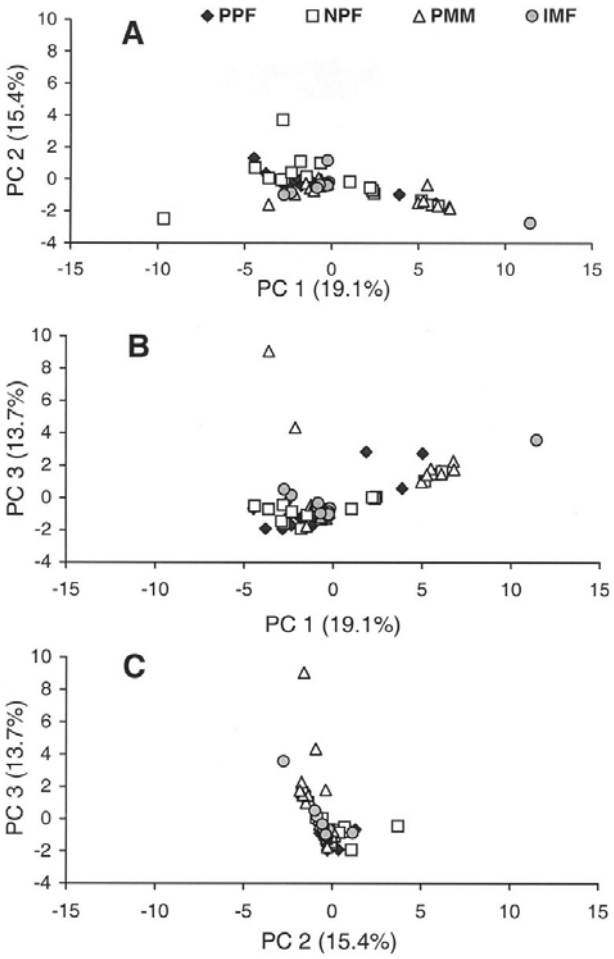

Fig. 3. Principal components analysis (PCA) has revealed three independent trends underlying the 54 compounds found in the cuticular extracts of *Palaemonetes pugio*. Principal components (PC) 1, 2, and 3 represent cuticular hydrocarbons, postmolt females (parturial and nonparturial), and postmolt males and nonparturial females, respectively. A: Principal components (PC) 1 (19.1%) and 2 (15.4%) explaining 34.5% of the variation in the transformed peak areas. B: PC 1 (19.1%) and 3 (13.7%) explaining 33.8% of the variation in the transformed peak areas. C: PC 2 (15.4%) and 3 (13.7) explaining 29.1% of the variation in the transformed peak areas. PPF, postmolt parturial females; NPF, postmolt nonparturial females; PMM, postmolt parturial males; IMF, intermolt males. n = 20 for all treatments.

treatment groups (Fig. 3B–C) There was no overlap among the compounds that contributed to the three major principal components, suggesting that there are three independent levels of organization: hydrocarbons (PC1), postmolt females (parturial and nonparturial) (PC2), and postmolt non-reproductive individuals (nonparturial females and males) (PC3). A fourth level of organization was revealed upon examining PCs 4, 5, and 6 together. The fatty acids contributed strongly to these principal components, as well as the unidentified compounds of Rt. 9.47, 9.53, 9.56, and 9.62 min.

Table 3. Number of replicates with observed contacts (0, 1, 2) by males in response to sponges treated with the cuticular extracts of postmolt, parturial females (PPFs), postmolt, non-parturial females (NPFs), intermolt females (IMFs), postmolt males (PMMs), positive food control (PC), and a negative control (NC). n = 10 for each treatment

	Number of contacts		
Treatment	0	1	2
PC	7	2	1
NC	8	1	1
PPF	7	1	2
NPF	8	2	0
IMF	9	0	1
PMM	7	1	2

Bioassay

Overall, males showed a lack of interest in sponges treated with cuticular extracts as well as positive control treated with food extract (Table 3). No more than two contacts with the test sponge per replicate were observed in any treatment. Sponges treated with the cuticular extracts of PPFs and IMFs evoked at least one positive response in 3 of 10 replicates, as did the positive food control (Table 3). Sponges treated with cuticular extracts of NPFs and chloroform-methanol (negative control) elicited contact in 2 of 10 replicates. Males responded to sponges treated with cuticular extracts of IMFs with 2 contacts in only a single replicate (Table 3). There was no difference in the number of contacts with the test sponge among treatments (CMH Row Mean Scores Test, $P = 0.845$).

Discussion

Burkenroad (1947), studying *Palaemonetes vulgaris*, first proposed that a "nondiffusible coating of the integument of the female" (i.e., contact sex pheromone) was involved in sex attraction of a caridean shrimp. However, to date, no contact sex pheromones have been identified and no studies have examined the role of cuticular hydrocarbons in mate recognition in crustaceans. We identified 12 cuticular hydrocarbons (cyclotetradecane, cyclohexadecane, docosane, tricosane, tetracosane, cycloeicosane, pentacosane, hexacosane, heptacosane, octacosane, tetracohexaene, and eicosane) that occurred in 3 or more treatment groups. All of the above hydrocarbons have been identified as a pheromone or a component of a pheromone blend in various insect species, with most functioning as contact sex pheromones and a few others serving as alarm pheromones (Howard & Blomquist, 2005). In this study, the cuticular extracts of postmolt parturial females (PPFs) differed qualitatively and quantitatively from postmolt nonparturial females (NPFs), postmolt males (PMMs), and intermolt females (IMFs).

Sex pheromones of insects are often a blend of cuticular hydrocarbons that vary in concentration between males and females or between reproductive females and non-reproductive females. Among the Diptera, several species of fruit flies of the genus *Drosophila*, tsetse flies of the genus *Glossina*, and the house fly *Musca domestica* have sexually dimorphic hydrocarbon profiles (Singer, 1998). The main hydrocarbon component of the sex pheromone of the housefly is (Z)-9-tricosene, which is found only on the cuticle of females (Carlson et al., 1971). Ginzel et al. (2003a) identified (Z)-9-pentacosene as the contact sex pheromone of the locust borer, *Megacyllene robiniae* (Coleoptera). Ginzel et al. (2003b) identified the female sex pheromone of the rustic borer *Xylotrechus colonus* (Coleoptera) as a blend of pentacosane, 9-methylpentacosane and 3-methylpentacosane, which was either absent or present in very small quantities on males.

The cuticular extracts of *P. pugio* showed considerable variation in the number and concentration of the compounds identified within each treatment. The crustacean cuticle undergoes dramatic changes in the hours prior to and following ecdysis (Travis, 1955; Skinner, 1962). This could account for some, if not most, of the variation seen among the treatment groups. The hydrocarbons found in our extracts are not unique to postmolt parturial females; however, this does not eliminate them as pheromone candidates. Many insect species use a blend of cuticular hydrocarbons as their sex pheromone (Howard & Blomquist, 2005). It is possible that the MRS of *P. pugio* could employ a mixture of cuticular hydrocarbons in mate recognition. Often, chemical signals are derived from molecules already in use and evolve through a gradual process of small changes in components, such as the loss or gain of a single component or a change in the relative proportion of components over time (Roelofs & Brown, 1982; Wyatt, 2003). The cuticular extracts of *P. pugio* show several compounds absent from PPFs but present in NPFs and PMMs (Rt. 5.50, 5.59, 5.59, 5.75, and 5.84 min). There is also a general reduction in the concentration of cuticular hydrocarbons and an increase in fatty acid concentration in PPFs. This observation suggests that the loss or change in a single compound or several compounds in PPFs may function in mate recognition of *P. pugio*. Cuticular compounds were particularly scarce in IMFs, which might be the basis of their rejection as potential mating partners by males.

Three saturated fatty acids, SFAs, and two polyunsaturated fatty acids, PUFAs, were found in the cuticular extracts of *P. pugio*. Fatty acids and their

derivatives are involved in events associated with reproduction in invertebrates such as oocyte maturation in *Ascidia* and *Asterias*, gonad maturation in penaeid shrimps, egg production in a fresh water snail, and vitellogenesis in some decapods (Gonzolez-Baro & Pollero, 1988; Spaziani et al., 1993; Spaziani & Hinsch, 1997; Styrishave & Anderson, 2000). Parturial females had fully developed vitellogenic oocytes at the time of extraction. Four of the five fatty acids found occurred in higher concentrations in PPFs than in all other treatment groups, the exception being octadecanoic acid. One possible explanation is that vitellogenic oocytes of PPFs require a higher concentration of these fatty acids. In order for these fatty acids to function in mate recognition, males must be able to detect their presence. One hypothesis is that these fatty acids may be able to pass through the soft, uncalcified cuticle of PPFs. Males, upon contacting the female an antennal flagellum, might detect these compounds, eliciting copulatory behavior.

A sponge bioassay was attempted with cuticular extracts of each treatment. Males did not respond to sponges treated with the cuticular extracts of postmolt parturial females with greater frequency than to other treatments. It should be noted that a positive food control (homogenized shrimp tails) did not elicit a high frequency of contacts from males, which indicates a problem with the bioassay. In many cases, specific behavioral acts are often hard to discriminate from other activities, making identification of active material extremely difficult. Precopulatory behavior such as grasping (of the female in nature, of the extract-treated sponge in the bioassay) was not observed in *P. pugio*. Further refinement of the bioassay or development of another bioassay technique is needed to test the biological significance of the hydrocarbons and fatty acids found on the cuticle of *P. pugio*.

From an evolutionary standpoint, pheromones provide an adaptive advantage for females to attract males of their species. Females of this species are only attractive to males during a short time following the parturial molt and producing a sex attractant during this period most likely assures successful mating. Also, given her "soft" and vulnerable condition, it may be an advantage to attract males with water-insoluble compounds that require physical contact. *Palaemonetes pugio* occurs in aggregations, and physical contact (via antennal flagella) among individuals is frequent. A contact pheromone may reduce the number of males attracted and prevent physical harassment and injury (Bauer & Abdalla, 2001).

This study did not demonstrate conclusively that cuticular compounds serve as contact sex pheromones in *P. pugio*, but it did show that the cuticle of *P. pugio* contains a mixture of hydrocarbons and fatty acids that might serve this purpose. If these cuticular compounds (hydrocarbons or fatty acids) function in the mate recognition system of *P. pugio*, the sex pheromone may be a particular blend or concentration of cuticular compounds rather than a single compound. This is suggested by the lack of frequently-occurring unique compounds in postmolt parturial females. The other alternative is that, although the profile of extractable hydrocarbons of receptive females differs from that of other individuals, these compounds simply do not serve as contact pheromones in shrimps as they do in many insects. Another possibility for the mate recognition cue of *P. pugio* is a surface glycoprotein. Strong evidence that cuticular glycoproteins serve as contact pheromones in other crustaceans (harpacticoid copepods) has been given by Frey et al. (1998) and Kelly & Snell (1998), similar to studies in rotifers (Snell et al., 1995). Marlowe et al. (1994) and Shafer et al. (1994) studied the cuticular proteins of decapod crustaceans and showed that the glycoprotein composition of the cuticle changes throughout the molt. This study investigated hydrophobic compounds in *P. pugio*; Caskey et al. (2009) examines the role of glucosamine in mate recognition in an attempt to elucidate the chemical nature of the contact sex pheromone of *P. pugio*.

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References

Aitchinson, J. 1986. *The Statistical Analysis of Compositional Data*. Chapman & Hall, London.

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. & Steinbach, M.A. 2007. Chemical communication and social behavior of the lobster *Homarus americanus* and other decapod Crustacea. In: *Evolutionary Ecology of Social and Sexual Systems*, J.E. Duffy & M. Thiel (eds.), Oxford University Press, New York, pp. 115–144.

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

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.

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

Blows, M.W. & Allan, R.A. 1998. Levels of mate recognition within and between two *Drosophila* species and their hybrids. *American Naturalist*, 152, 826–837.

Bublitz, R., Sainte-Marie, B., Newcombe-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.

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.

Carlson, D.A., Mayer, M.S., Silhacek, D.L., James, J.D., Beroza, M. & Bierl, B.A. 1971. Sex attractant pheromone of the housefly: isolation, identification, and synthesis. *Science*, 174, 76–78.

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., Watson, G.M. & Bauer, R.T. 2009. Studies on contact sex pheromones of the caridean shrimp *Palaemonetes pugio*: II. The role of glucosamine in mate recognition. *Invertebrate Reproduction and Development*, 53(2), 105–116.

Cohen, J. 1984. Convenient apparatus for the generation of small amounts of diazomethane. *Journal of Chromatography*, 303, 193–196.

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.

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

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.

Emlen, S.T. & Oring, L.W. 1977. Ecology, sexual selection, and the evolution of mating systems. *Science*, 197, 215–233.

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.

Gonzalez-Baro, M.R. & Pollero, R.J. 1988. Lipid characterization and distribution among tissues of the freshwater crustacean *Macrobrachium borelli* during annual cycle. *Comparative Biochemistry and Physiology. B*, 91, 711–715.

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.

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

Kittredge, J.S., Terry, M. & Takahashi, F. 1971. Sex pheromone activity of the molting hormone, crustecdysone, on male crabs. *Fisheries Bulletin*, 69, 337–343.

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.

Marlowe, R.L., Dillaman, R.M. & Roer, R.D. 1994. Lectin binding by crustacean cuticle: the cuticle of *Callinectes sapidus* throughout the molt cycle, and the intermolt cuticle of *Procambarus clarkii* and *Ocypode quadrata*. *Journal of Crustacean Biology*, 14, 231–246.

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

Roelofs, W.L. & Brown, R.L. 1982. Pheromones and evolutionary relationships of Torticidae. *Annual Review of Ecology and Systematics*, 13, 395–422.

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.

Shafer, T.H., Roer, R.D., Miller, C.G. & Dillaman, R.M. 1994. Postecdysial changes in the protein and glycoprotein composition of the cuticle of the blue crab *Callinectes sapidus*. *Journal of Crustacean Biology*, 14, 210–219.

Simmons, L.W., Alcock, J. & Reeder, A. 2003. The role of cuticular hydrocarbons in male attraction and repulsion by female Dawson's burrowing bee, *Amegilla dawsoni*. *Animal Behaviour*, 66, 677–685.

Singer, T.L. 1998. Roles of hydrocarbons in the recognition systems of insects. *American Zoologist*, 38, 394–405.

Skinner, D.M. 1962. The structure and metabolism of a crustacean integumentary tissue during a molt cycle. *Biological Bulletin*, 123, 635–647.

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.

Spaziani, E.P. & Hinsch, G.W. 1997. Variation in selected unsaturated fatty acids during vitellogenesis in the Florida freshwater crayfish *Procambarus paeninsulanus*. *Invertebrate Reproduction and Development*, 32, 21–25.

Spaziani, E.P., Hinsch, G.W. & Edwards, S.C. 1993. Changes in prostaglandin E_2 and $F_{2\acute{a}}$ during vitellogenesis in the Florida crayfish *Procambarus paeninsulanus*. *Journal of Comparative Physiology, B*, 163, 541–545.

Stebbing, P.D., Bentley, M.G. & 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.

Styrishave, B. & Andersen, O. 2000. Seasonal variations in hepatopancreas fatty acid profiles of two colour forms of shore crabs, *Carcinus maenas*. *Marine Biology*, 137, 415–422.

Travis, D.F. 1955. The molting cycle of the spiny lobster, *Panulirus argus* Latrielle. II. Pre-ecdysial histological and histochemical changes in the hepatopancreas and integumental tissues. *Biological Bulletin*, 108, 88–112.

Wickler, W. & Seibt, U. 1981. Monogamy in Crustacea and Man. Z. Tierpsychol, 57, 215–234.

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