Journal of Marine Animals and Their Ecology
Vol 6, No 2, 2013

Printed in Canada

Possible Sex Pheromones in the Blue Swimmer Crab *Portunus pelagicus*

Sugumar Vasudevan[1], Beema Mahin[1]

[1]Department of Oceanography and Coastal Area Studies, Alagappa University, Thondi Campus, Thondi – 623 409, Tamilnadu, India.

Abstract

The aim of the study is to identify the possible chemical attractants in the urine of the blue swimmer crab *Portunus pelagicus*. The behavioural response of the male crab to female urine was studied by a unique sponge assay and the urine samples of crabs were subjected to gas chromatography to identify potential chemical attractants. The sponge assay clearly depicted the attraction of the sexually receptive males to the sponges containing premoult and postmoult female urine. The fatty acid analysis revealed abundant levels of pentadecane, heptadecane and dichloroacetic acid in premoult and postmoult female urine. Octasiloxane, heneicosane and heptasiloxane were at higher concentrations in male urine. Many of the chemicals reported in the present study have been reported to be important constitutes for chemical communication system and potential sexual attractants in class Insecta. Gas chromatographic studies have ascertained the presence of chemical compounds in the urine of blue swimmer crabs which could play a putative role as pheromones in sexual attraction. [JMATE. 2013;6(2):12-19]

Keywords: Urine sponge assay, gas chromatography, fatty acid analysis

Introduction

The blue swimmer crab *Portunus pelagicus* (Linnaeus) is a widely distributed species which occurs in the Indian and the west Pacific oceans and plays an important role in the fishing industry at many locations (22). Furthermore, the blue swimmer crab has high aquaculture potential because it shows fast growth rates, high fecundity, and relatively short larval duration (18, 27). As the aquaculture interest for this species increases, a better understanding of the basic biological conditions required to optimize its production is needed (26).

Chemical signals between males and females play an important role in copulation of crustaceans. In many species, the male courting response is triggered by a pheromone released by the female (10, 30). Ryan was the first to postulate the presence of female sex pheromone in the blood of the spotted swimming crab *Portunus sanguinolentus* (28). In addition, in the common lobster *Homarus gammarus*, females have been found to orient towards the burrows of males when they received sex attractant signals produced by the males (8). Gleeson has described the presence of three pheromone-like compounds in the urine of the female blue crab *Callinectes sapidus* (12-15). Bouchard *et al*. reported the presence of female semiochemicals that evoked male precopulatory behaviour in the snow crab *Chinoecetes opilio* (4). The male, when attracted to the female, approaches it by rising its 2,3,4 pereiopods (1). Later the male guards the female under the abdomen until moulting. Whether this behaviour of the male was provoked by the urine of the precopulatory female suggesting the presence of sex pheromone as an attractant was confirmed using an actograph to measure their activity in a confined space (2, 6). The sensory receptors for the pheromone have been reported to be present in male antennules of the blue crab and in the green shore crab *Carcinus maenas* and confirmed by electrophysiological recordings from the male antennules of the green shore crab (1, 12, 16).

A number of chemicals have been reported to act as pheromones including tetrodotoxin, specific amino acids, purines, bile acids, gonadal steroids and F prostaglandins (9, 19, 21, 30, 31, 32). Gleeson postulated a molecular weight of 300-600 dalton (Da) for the molecules in the urine of pubertal females by Sephadex gel filtration calibrated with peptide markers (14). Furthermore, pheromones of the females also induce the conspecific females towards precopulatory behavior suggesting the sharing of chemical compounds not only in male and female urine but between females. To expand the knowledge on the sex attractants in blue crabs, this manuscript details chemical communication through sex pheromones between the female and male blue swimmer crab.

Received October 5, 2013; Accepted December 31, 2013
Correspondence: Dr. Sugumar Vasudevan
Department of Oceanography and Coastal Area Studies,
Alagappa University, Thondi Campus, Thondi – 623 409.
Email: mfsugumar@gmail.com
Phone :+91-9445139906

Materials and Methods

2.1 Collection of crabs

Male and female blue swimmer crabs were collected using trawls along the Thondi coast. The collected crabs were transported to the laboratory where they were maintained in tanks with filtered sea water at a salinity of 30-32 ppt and temperature ranging between 26-31ºC. The sexual maturity of the crabs was assessed and only mature male and female crabs were taken for the present study. The crabs were fed with the blue mussel *Mytilus edulis* twice a week.

2.2 Pheromonal Assay

2.2.1 Collection of Urine

Crabs were immobilized on a wooden board and the third maxillipeds were lifted up to expose the antennal gland opening. The gill cavity openings were simultaneously blocked and the operculum was lifted up. This resulted in massive flow of urine from the opening which was collected using pipettes and stored in tubes. Urine collected from premoult female (n=6), postmoult female (n=5) and male (n=5) crabs were separately pooled and stored at –20ºC until analysis (2).

2.2.2 Sponge assay

The sponge assay was carried out to study the response of male crabs to female urine as per the methodology of Stebbing *et al.* (32). Individual mature males were placed in 3 opaque tanks containing 2 litres of seawater. Males were left to acclimatize for 2 hours before the start of the experiment. The sponge assay was carried out with sponges coupled to a lead weight (6 g) to prevent them from floating. A tube was attached to the sponge to inject the samples to minimize disturbance. The sponges were separately and sequentially injected with urine of: (a) male crab, (b) premoult and (c) postmoult female crabs. The behavioural response of the male crab was classified as Quiescent (male remaining motionless), Motile (male moving around the tank) or Handling (male making contact with, seizing, and/or mounting the sponge).

The sponge and tube was placed inside the tank during the 2 hour acclimatization period, to ensure any behavioural responses during the following experiments were not due to moving the sponges. For each experiment, first 20 ml of seawater (blank) was injected into the sponge via the tube. Then, after 20 minutes of observation, 20 ml of the sample was injected followed by 20 minutes observation. The time taken for the behavioural responses of the male during the experiments was recorded. The results of the study were subjected to paired t-test which looks at the difference between paired values in two samples, taking into account the variation of values within each sample. It provides a t-value which is related to the size of the difference between the means of the two samples compared. The larger the t value is, the larger the difference. It also provides a p-value which helps to decide whether or not to accept the null hypothesis.

2.3 Gas Chromatographic Assay of Urine Samples

2.3.1 Extraction and Estimation of Lipids

Estimation of total lipids was done according to the methodology of Barnes and Blackstock (3). Extraction of lipids for the quantitative determination of lipids by sulphovanillin method was done following the procedure of Folch *et al*. (11). 1 ml of the urine sample was taken in a test tube containing 5 ml of chloroform: methanol (2:1). To this mixture 0.5 ml of 0.9% sodium chloride solution was added and shaken well. This mixture was then transferred to a separating funnel and allowed to stand for 12-14 hours. The lower layer was collected from the separating funnel in a beaker and volume was made up to 5 ml with chloroform and evaporated to dryness at room temperature. The dry content was used for further analysis as detailed below.

Methylation of lipids was carried out as per the methodology of Kroppenstedt (20). The dry content was remixed with methanol and centrifuged at 4000 rpm. 100 µl of the supernatant was mixed with 1 ml of saponification reagent, vortexed and boiled for 30 minutes. Then 2 ml of methylation reagent was added and boiled in a water bath at 90ºC for 15 minutes and cooled to room temperature. The mixture was then treated with 1.25 ml of extraction solvent and left undisturbed for the separation of the phases. The lower aqueous phase was discarded and to the upper organic phase, 3 ml of base wash was added and transferred to a GC vial.

2.3.2. Instrumentation and Analytical Conditions

A GLC (Clarus 500, Perkin Elmer - Mass, USA) equipped with a Mass detector Turbo mass gold (Perkin Elmer - Mass, USA) was used to separate and quantify the fatty acids present in the urine samples. The column used for separation was an Elite-5MS (5% Diphenyl/ 95% Dimethyl ply siloxane), 30 X 0.25 mm X 0.25 mm df column with a automatic sample injector. During the 2 ml sample loading which took 2 minutes, the injector temperature was 250°C and the oven temperature was 110°C. The oven temperature was programmed to increase at a rate of 10ºC/minute up to 200°C and then the temperature was increased finally to 280°C at a rate of 5°C/minute for 9 minutes. The total GC running time was standardized to 36 minutes and the analytes detected were characterized by their retention time (RT) by comparison to the standard fatty acid chromatogram.

Results

3.1 Sponge Assay

Pheromonal assay using sponges injected with urine samples revealed the movement of males towards the sponges witnessing the presence of a chemical attractant in the samples (Figure 1). Figures 2-4 show the average time that the male spent exhibiting varying behaviour before and after the injection of the samples into the sponge. There was a significant decrease in the time spent quiescent after the addition of premoult female urine (219±82 sec; t = - 5.698, p= 0.001) and postmoult female urine (386±50 sec; t = - 4.320, p=0.001). There was a significant (474±98 sec; t = 0.857, p=0.05) increase in the time spent motile after addition of premoult female urine. The handling time increased significantly (346±65 sec; t = 5:893, p=0.001) after the addition of the premoult female urine. There were no significant differences in the time spent quiescent (485±35 sec; t = -0.254, p=0.9115), motile (172±15 sec; t = 0.071, p=0.9700), or handling (20±05 sec; t= -0.241, p=0.9512) for before and after introduction of the male urine.

3.2 GC-Assay of the Urine Samples

In the urine samples, the presence of as much as 30 compounds in premount females, 126 compounds in postmoult females and 28 compounds in the males was

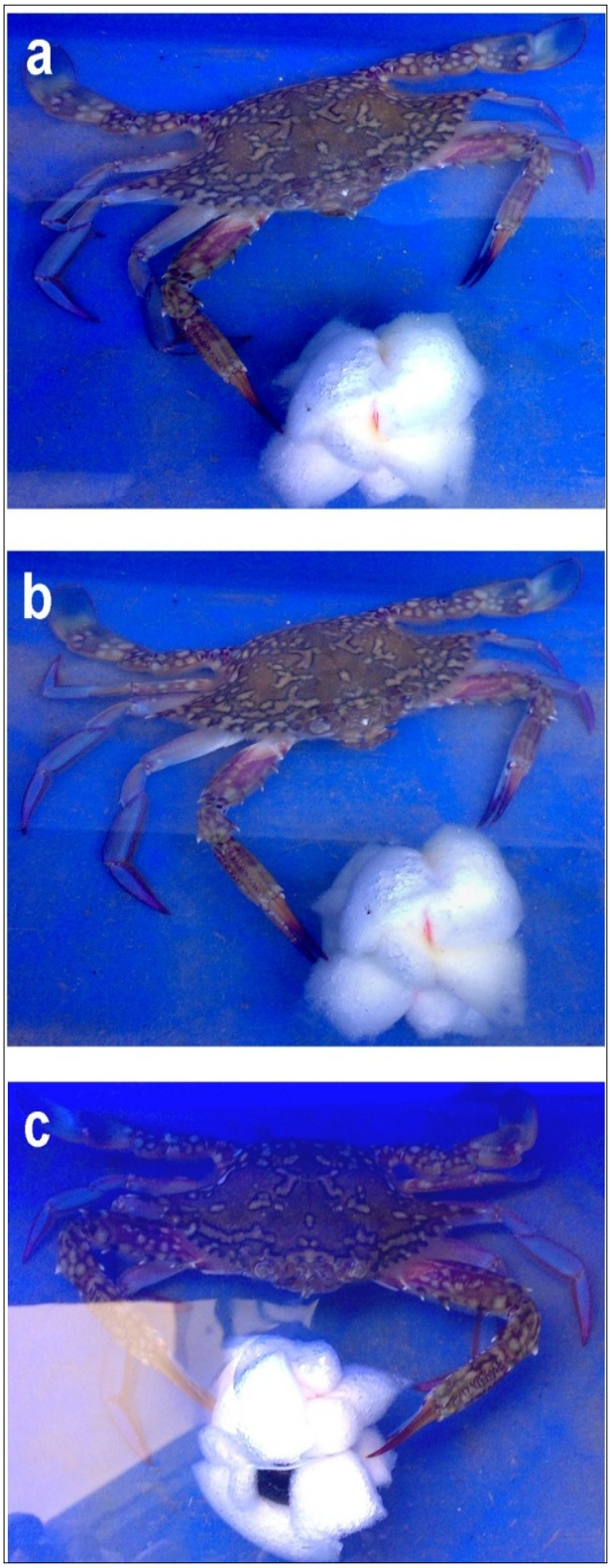

Figure 1: Sequential images of male P. pelagicus behaviour to a sponge containing premoult female urine (a) approaching; (b) touching; (c) handling. Reproduced with permission of author.

S. No.	Compounds	Retention time (RT) (minutes)	Premoult female urine		Postmoult female urine		Male urine	
			Peak Height (milli absorbance units)	Area %	Peak Height (milli absorbance units)	Area %	Peak Height (milli absorbance units)	Area %
1.	Hexa & Penta decanoic acid	14.01	503716	16.64	1688027	10.70	863808	23.11
2.	Dimethyl Benzaldehyde	5.48	274186	14.21	-	-	323259	13.99
3.	Octa & Heptadecanoic acid	16.78	247994	9.30	669586	4.96	347113	10.69
4.	Heptacosane & Eicosane	11.40	104757	3.31	-	-	99373	2.33
5.	Phenol and Toluene	9.10	101592	5.69	305110	2.15	-	-
6.	Hexadecane	8.87	88822	3.03	404650	2.52	-	-
7.	Methyl tetredecanoate & Tridecanoic acid	11.55	86689	2.78	-	-	165008	3.99
8.	Eicosane & Dodecane	6.70	46116	2.40	-	-	-	-
9.	Nonadecene	12.34	-	-	286691	1.79	-	-
10.	Benzoic acid	9.32	43599	2.28	-	-	-	-
11.	Butylated hydroxy toluene	9.19	-	-	752187	5.03	443192	14.23

Table 1. Chemical compounds occurring in abundance in premoult female, postmoult female and male urine as per GC chromatogram in blue swimmer crabs.

identified by GC chromatogram (Figures. 5a-c). Table 1 depicts the compounds found in common in the urine of premoult female, postmoult female and male respectively with their retention time (RT). Hexadecanoic acid (RT - 14.01 minutes) is abundant in all the three samples, followed by dimethyl benzaldehyde (RT – 5.48 minutes), octadecanoic acid and heptadecanoic acid (RT - 16.76 minutes) and other compounds such as eicosane, dodecane, hexadecane, benzoic acid, methyl tetradeconate, phenol and toluene.

Pentadecane, heptadecane and dichloroacetic acid are compounds found in both premoult and postmoult female urine in higher amounts. Predominant levels of of hexasiloxane, undecanoic acid, dodecanoic acid, and tetracosane were also observed in premoult urine whereas pentacosane, hentriacontane, and decanol were present in considerable amounts in postmoult urine. Male urine is characterized by higher concentrations of octasiloxane, heneicosane and heptasiloxane.

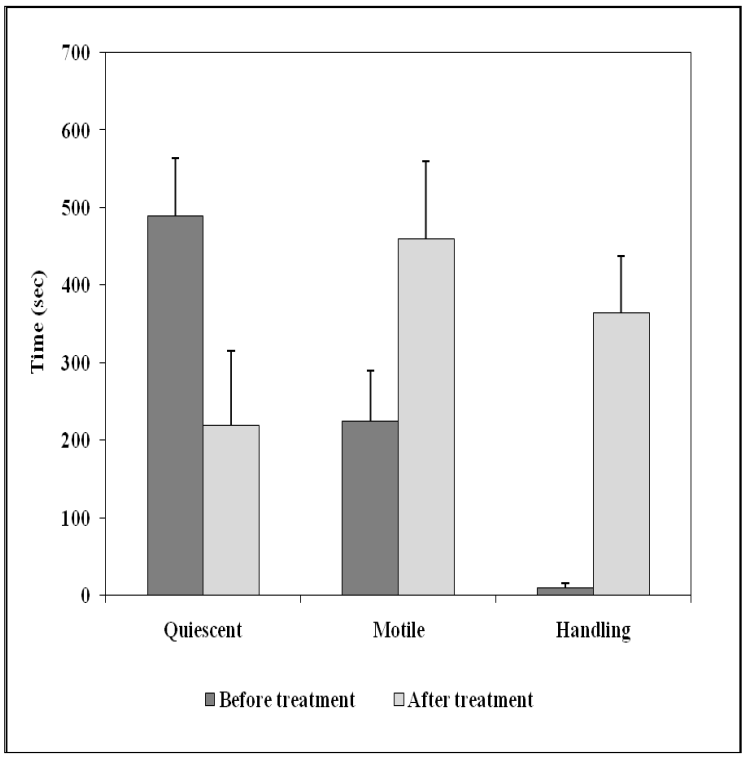

Figure 2: Behavioural variations of male blue swimmer crabs before and after premoult female urine sponge assay.

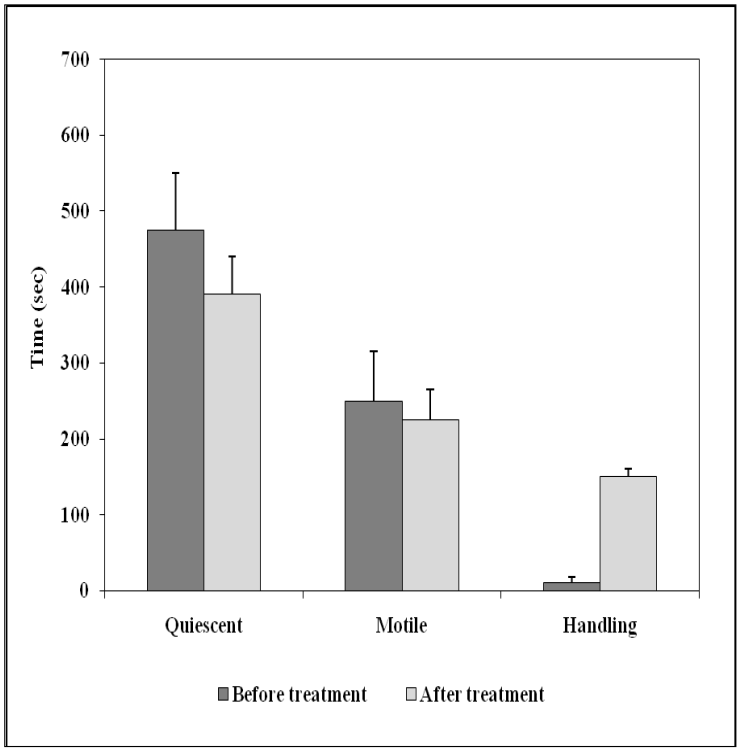

Figure 3: Behavioural variations of male blue swimmer crabs before and after postmoult female urine sponge assay.

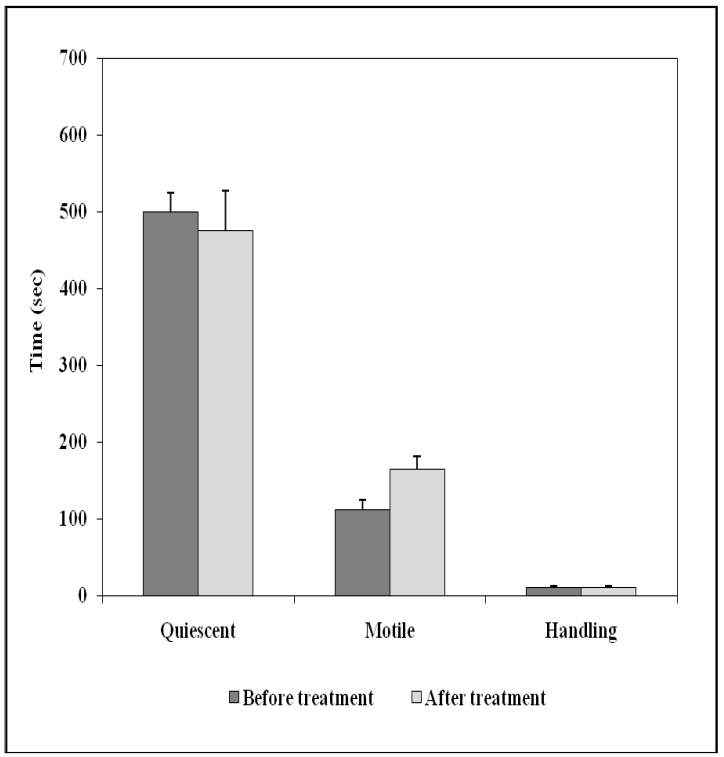

Figure 4: Behavioural variations of male blue swimmer crabs before and after male urine sponge assay.

Discussion

The present study using a sponge assay clearly depicts that pheromones are released by female blue swimmer crabs that stimulates mating behaviour in males. This is evident from the increase in the amount of time that the animal spent handling the sponges after the addition of premoult and postmoult female urine. The lack of significant differences when mature males were tested with male urine suggests that chemical compounds in the male urine do not elicit any mating behaviour in males. Similar results were witnessed by sponge assay in signal crayfish *Pacifastacus leniusculus,* proving the presence of pheromone-like compounds in the water conditioned with mature females (32).

The chemical nature of the compounds present in the urine of premoult female, postmoult female and male which may act as sex attractants were identified in blue swimmer crabs. Specifically, hexadecanoic acid methyl ester, the compound found in higher concentration in all the samples in the present study, together with hexadecane and octadecanoic acid, has been reported to be an efficient sex attractant in the

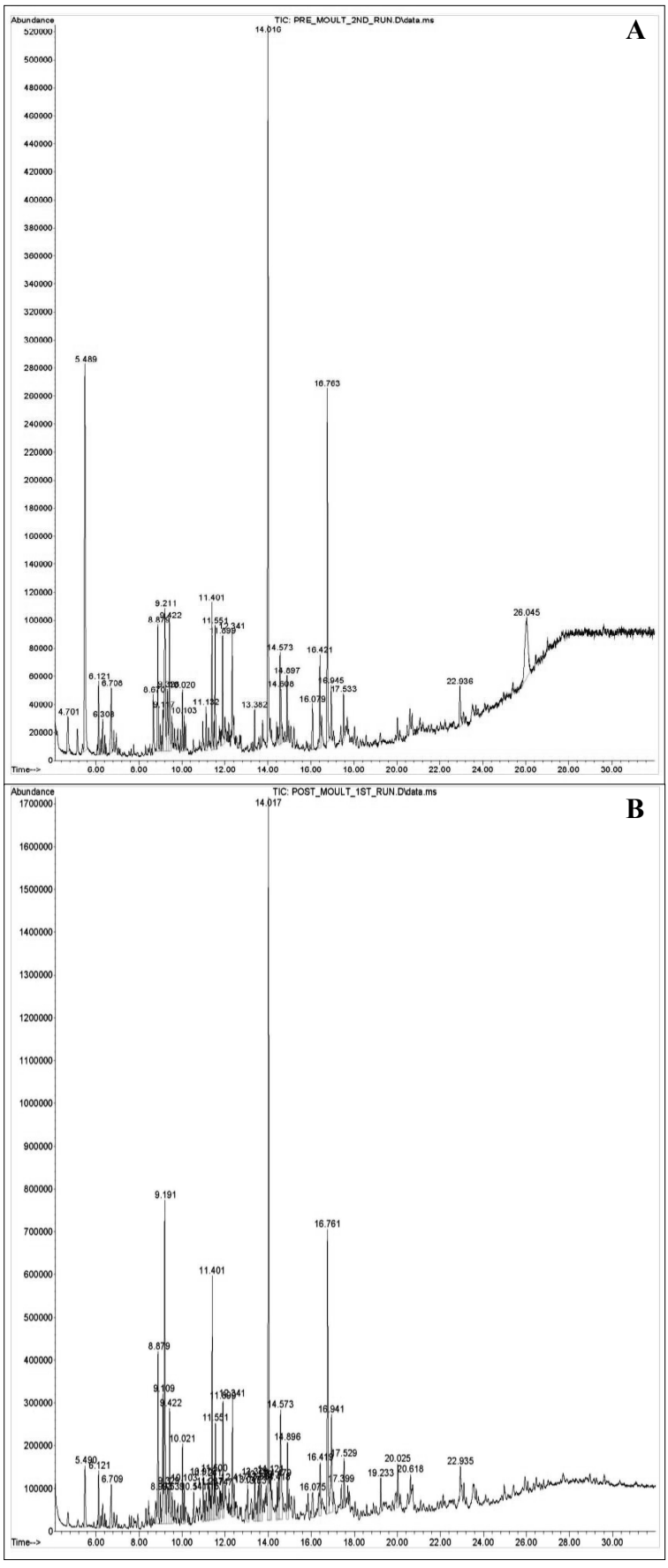

Figure 5: GC Chromatogram showing the compounds present in the (a) premoult urine; (b) postmoult urine; (c) male urine.

marine insect the water strider *Trochopus plumbeus* (5). The present study is the first report on the identification of hexadecanoic acid methyl ester in the urine of crabs**.** The compounds found in the urine samples might act as specific sex attractants for attracting the opposite sex. For example, males might get attracted towards the females due to the presence of unique compounds in female urine such as: benzoquinone, undecanoic acid, dodecanoic acid, nonadecane, decanol, hentricosane, pentacosane. Similarly, females might get attracted towards the males due to the occurrence of specific compounds in male urine such as heneicosane and pyrazin. Interestingly, pentadecane present in the female urine has been reported to be an alarm pheromone in the family Formicinae of Insecta (24).

Pheromonal assays carried out in the blood spotted swimming crab, green shore crab and blue crab have also revealed the presence of sex attractants in the premoult and postmoult urine of females

(2, 12, 28). Besides the presence of nitrogenous compounds like ammonia, urea, amino acids in the urine of crustaceans, steroids and prostaglandins have also been identified as sex pheromones in the urine of crabs (7).

Many authors have proposed that the crustacean moulting hormone 20-hydroxyecdysone, stimulating ovarian development in crustaceans, could act as a sex pheromone signal (26, 29). However, the present study confirmed that in blue swimmer crabs, 20-hydroxyecdysone was not a bioactive component in the female urine nor was it detectable in any samples. This remains analogous to the reports on the absence of 20-hydroxyecdysone in the urine of blue crab and green shore crab (15, 17). Reho and Dough have obtained US patent right for the invention of chemical composite attractants in male urine for the premoult female (25). Their invention demonstrates the ability of an artificial chemical composition to attract pre-molting female blue crabs and the ability of such composition to elicit, in these females, a sexual response normally issued in response to a male blue crab. GC results of the present study clearly depict the presence of some of the compounds in the urine of male and female blue swimmer crabs such as eicosane, tetracosane, heptadecane, nonadecene, pentacosane, hexacosane, heneicosane and tricosane, suggesting these compounds to be efficient partners in the chemical attraction of blue swimmer crabs.

Conclusion

Urine sponge assay and GC studies have confirmed the presence of chemical attractants in the urine of premoult and postmoult female crabs. This preliminary approach identifying the chemical nature of the compounds in the urine samples could be extended to the comparison and identification of molecules unique to any species of crab.

Acknowledgments
This work was supported by grants from Alagappa University Research Fund (AURF) (A13/AURF/2009 dt.30.09.2009) through Alagappa University, Tamil Nadu and from University Grants Commission, Government of India, New Delhi. (UGC- F.No.39-566/2010 SR).

References

1. Bamber SD, Naylor E. Chemical communication and behavioural interaction between sexually mature male and female shore crabs (*Carcinus maenas*). *Journal of Marine Biological Association of United Kingdom* 76: 691-699. 1996.
2. Bamber SD, Naylor E. Sites of release of putative sex pheromone and sexual behaviour in female *Carcinus maenas* (Crustacea: Decapoda). *Estuarine, Coastal Shelf Science* 44: 195-202. 1997.
3. Barnes H, Blockstock J. Estimation of lipids in marine animals and tissues: Detailed investigation of the sulpho phosphovanillin method for total lipids. *Journal of Experimental Marine Biology and Ecology* 12: 103-118. 1973.
4. Bouchard S, Sainte-Marie B, McNeil JN. Indirect evidence indicates female semiochemicals release male precopulatory behaviour in the snow crab, *Chinoecetes opilio* (*Brachyura: Majidae*). *Chemoecology* 7: 39-44. 1996.
5. Cheng L, Roussis V. Sex attractant in the marine insect *Trochopus plumbeus*: a preliminary report. *Marine Ecology Progress Series* 170: 283-286. 1998.
6. Christofferson JP. Evidence for the controlled release of a crustacean sex pheromone. *Journal of Chemical Ecology* 4: 633-639. 1978.
7. Claybrook DL. The biology of crustaceans. In: *Internal Anatomy and Physiological Regulation, edited by* Mantel A: *Academic Press*, London, pp. 163-213. 1983.
8. Cowan D, Atema J. Moult staggering and serial monogamy in American lobsters, *Homarus americanus*. *Animal Behaviour* 39: 1199-1206. 1990.
9. Doving KB, Selset R, Thommesen G. Olfactory sensitivity to bile acids in salmonid fishes. *Acta Physiologica Scandinavica* 108: 123-131. 1980.
10. Dunham PJ. Pheromones and behaviour in Crustacea. In: *Endocrinology of Selected Invertebrate Types*, edited by Laufer F, Downer M: Alan R. Liss. Inc., New York, pp. 375–392. 1988.

11. Folch J, Lee M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry* 226: 477-509. 1957.
12. Gleeson RA. Pheromone communication in the reproductive behaviour of the blue crab, *Callinectes sapidus*. *Marine Behaviour and Physiology* 7: 119–134. 1980.
13. Gleeson RA. Morphological and behavioral identification of the sensory structures mediating pheromone reception in the blue crab, *Callinectes sapidus*. *Biological Bulletin* 163: 162-171. 1982.
14. Gleeson RA. Intrinsic factors mediating pheromone communication in the blue crab, *Callinectes sapidus*. In: *Crustacean Sexual Biology*, edited by Bauer M, Martin E: Columbia University Press, New York, pp. 17-32. 1991.
15. Gleeson RA, Adams MA, Smith AB. Characterization of a sex pheromone in the blue crab, *Callinectes sapidus*: Crustecdysone studies. *Journal of Chemical Ecology* 10: 913-921. 1984.
16. Gomez G, Atema J. Temporal resolution in olfaction: stimulus integration time of lobster chemoreceptor cells. *Journal of Experimental Zoology* 199: 1771-1779. 1996.
17. Hardege JD, Jennings A, Hayden D. Novel behavioral assay and partial purification of a female-derived sex pheromone in *Carcinus maenas*. *Marine Ecology Progress Series* 244: 179-189. 2002.
18. Josileen J, Menon NG. Growth of the blue swimmer crab, *Portunus pelagicus* in captivity. *Crustaceana* 78: 1-18. 2005.
19. Kawabata K. Induction of sexual behaviour in male fish, *Rhodeus ocellatus ocellatus* by amino acids. *Amino Acids* 5(3): 323-327. 1993.
20. Kroppenstedt RM. Fatty acid and menaquinone analysis of actinomycetes and related organisms. In: *Chemical Methods in Bacterial Systematics*, edited by Goodfellow M, Minnikin J: Academic Press, New York, pp. 173-199. 1985.
21. Matsumura K. Production of tetrodotoxin in puffer fish embryos. *Environmental Toxicology and Pharmacology* 6(4): 217-219. 1998.
22. Meagher TD. Ecology of the crab *Portunus pelagicus* (Crustacea Portunidae) in Southwestern Australia. *Dissertation, University of Western Australia, Australia*. 1971.
23. Pfeiffer W, Walz U, Wolf R, Wernado M. Effects of steroid hormones and other substances on alarm substance cells and mucous cells in the epidermis of the European minnow, *Phoxinus phoxinus* (L.), and other Ostariophysi (Pisces). *Journal of Fisheries Biology* 27: 553-570. 1985.
24. Regnier FE, Wilson EO. The alarm-defence system of the ant *Acanthomyops claviger*. *Journal of Insect Physiology* 14(7): 955-970. 1968.
25. Reho JH, Dough WG. Chemical composite attractant of premoulting female blue crabs, United States Patent Application Publication US2004/0258658 A1. 2004.
26. Romano N, Zeng C. The effects of salinity on the survival, growth and haemolymph osmolality of early juvenile blue swimmer crab *Portunus pelagicus*. *Aquaculture* 260: 151-162. 2006.
27. Romano N, Zeng C. Blue swimmer crabs-emerging species in Asia. *Global Aquaculture Advocate* 11(3): 34-36. 2008.
28. Ryan EP. Pheromone: Evidence in a decapod crustacean. *Science* 151: 340-341. 1966.
29. Seifert P. Studies on the sex pheromone of the shore crab, *Carcinus maenas*, with special regard to ecdysone excretion. *Ophelia* 21: 147-158. 1982.
30. Sorensen PW, Christenses TA, Stacey NE. Discrimination of pheromonal cues in fish: emerging parallels with insect. *Current Opinion in Neurobiology* 13: 689-695. 1998.
31. Sorensen PW, Hara TJ, Stacey NE, Dulka JG. Extreme olfactory specificity of male goldfish to the preovulatory steroidal pheromone 17q20P-dihydroxy-4-pregnen-3-one. *Journal of Comparative Physiology* 166A: 373-383. 1990.
32. Stebbing PD., Bentley MG, Watson GJ. Mating behaviour and evidence for a female released courtship pheromone signal in the crayfish *Pacifastacus leniusculus*. *Journal of Chemical Ecology* 29: 465-475. 2003.