Chemical Communication and Its Ecological Consequence in *Lysmata* Shrimp

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General Abstract

Male crustaceans must find and identify receptive females to mate successfully. Mate recognition depends mainly on sex pheromones, which are detected by antennae and antennules. Distance (soluble) pheromone mediates mating behaviour of some decapod crustaceans. Contact pheromone (an insoluble coating on the body surface) has been proposed but not confirmed to be used by male decapod crustaceans to detect females. Here we report for the first time the involvement of both distance and contact pheromones in the mating processes of Lysmata shrimp (a group of protandric simultaneous hermaphrodites), and we have partially identified the soluble and contact pheromones. Additionally, evolution of the sex pheromone system of Lysmata shrimp, and role of the sex pheromones in reproductive isolation of Lysmata shrimp were investigated.

Euhermaphrodite-phase (EP, simplified as female hereafter) shrimp can mate only as females during the small window of the postmoult period. Male shrimp (male-phase and inter-moult EP shrimp) recognized, tracked, and courted the receptive female shrimp based on both distance and contact sex pheromones, but responded aggressively to newly moulted male-phase (MP) shrimp. Male shrimp with their chemosensory appendages ablated appeared unable to identify EP shrimp and neither courted nor copulated with them. As reported for other decapod crustacean species, the outer flagella of the antennules house the receptors of the distance pheromone, whereas both antennae and antennules can detect the contact pheromone. Male shrimp could still mate with female successfully without the distance pheromone. These results suggest that contact pheromone, in addition to distance pheromone, is involved in mediating the mating behaviour of Lysmata shrimp. Moreover, contact pheromone may be more important than distance pheromone in the mating process.
Olfactory chemical cues used in mate recognition in decapod crustaceans have been well studied based on precopulatory behaviours; however, we still know very little about the chemical characteristics of these cues. In the present study, the water soluble sex pheromone of *Lysmata wurdemanni* was partially characterized. Female moulting water was collected and ultrafiltered using 1000 and 500 Dalton membranes, respectively. Afterwards, the supernatant of 500 Dalton was analyzed using HPLC with a Lichrosphere™ RP18 (C18) column. Behavioural bioassays showed that males did not respond to the supernatant of 1000 Dalton or to the 500 Dalton filtrate, but positively responded to the supernatant of 500 Dalton and to the 1000 Dalton filtrate, suggesting that the sex pheromone is likely to be a molecule between 500 and 1000 Dalton size. There was only one dominant peak (2.86 min) detectable in HPLC chromatogram of the supernatant of 500 Dalton, and behavioural bioassay confirmed that it is a bioactive component. Molecular size filtration can therefore be utilized in future studies to purify and eventually identify this pheromone.

A series of bioassays were ran to examine whether uridine-5'-di-phosphate (UDP), uridine-5'-tri-phosphate (UTP) or their mixtures elicit male mating behaviour in the peppermint shrimp (*Lysmata wurdemanni* and *L. boggessi*). The results show that the two shrimp species responded to UTP, but not to UDP. The mixtures of UTP and UDP did not maximize the male mating behaviour. Minimum effective concentration of UTP to the two shrimp species was between $10^{-6}$ and $10^{-7}$ M. The results suggest that UTP may be a major component of the distance pheromone in the two shrimp species. Male *L. boggessi* did not respond to the interspecific female moulting water, and number of male *L. wurdemanni* responded to interspecific female moulting water was markedly reduced, suggesting there might be other specific components in the sex pheromone to combine with UTP to make a species-specific blend.
In this study, for the first time the direct evidence for the presence of contact pheromones in a decapod crustacean, *Lysmata boggessi*, was demonstrated. Hexane-extracted chemicals from the exoskeleton of newly moulted and intermoult EP and MP shrimp were analyzed with gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). EP and MP shrimp shared a series of extractable cuticular compounds. The main difference is that there are 2 major peaks (at 13.81 min and 28.16 min) in the extracts of newly moulted EP shrimp; however, they are absent in those of intermoult EP, MP, and newly moulted MP shrimp. One of the identified compounds present in the newly moulted EP shrimp is (Z)-9-Octadecenamide. Behavioural assays indicate that male shrimp responded to cuticular extract of newly moulted EP shrimp, and (Z)-9-Octadecenamide is the major active component of the contact pheromones in *L. boggessi*.

The reproductive behaviours of the two protandric simultaneous hermaphroditic species (*L. amboinensis* and *L. boggessi*) that belong to two groups of *Lysmata* shrimp with different morphology, geographical distribution, and density were compared. *Lysmata amboinensis* occurs in tropical waters at low population densities, and *L. boggessi* is found in aggregation in sub-tropical and temperate areas. Results show that *L. amboinensis* was much less active during mating than *L. boggessi*. Male shrimp of *L. amboinensis* did not display obvious pre-copulation behaviour. They also took significantly longer to transfer spermatophores and lay eggs after mating than *L. boggessi* shrimp did. For *L. boggessi*, moulting time of female shrimp, copulation time and the interval between mouling and mating were significantly shorter when three male shrimp were present than when only one male shrimp was present. Our study suggests that the reproductive behavioural differences in the two shrimp species are the results of density-dependent effect.
To investigate the role of chemical cues in reproductive isolation in *Lysmata* shrimp, conspecific and interspecific crossing between two sympatric species of *Lysmata* shrimp (*L. wurdemanni* and *L. boggessi*) were conducted in the laboratory. Behavioural studies reveal that female *L. wurdemanni* accepted only conspecific male shrimp, whereas *L. boggessi* female could mate with an interspecific male if there was no conspecific male present. When males of both species were present, *L. boggessi* female always mated with the conspecific male. Male *L. boggessi* in general did not respond to the sex pheromone secreted by female *L. wurdemanni* and did not display any pre-copulatory behaviour to the newly moulted female *L. wurdemanni*. On the other hand, some male *L. wurdemanni* responded to female *L. boggessi*. Although mating was successful between *L. wurdemanni* males and *L. boggessi* females, the resulting embryos lived at most for 10 days and failed to hatch. The results indicate that the two species are both pre-zygotically and post-zygotically isolated. Behavioural observation suggests that chemical cues are mainly responsible for pre-zygotic isolation.
General Introduction

Animals behave in response to the information (e.g. signal or cue) they obtain. Thus, to better understand their behaviour we need to understand characteristics of the signals. Signal is “an act or structure that alters the behaviour of another organism, which evolved because of that effect, and which is effective because the receiver’s response has also evolved” (Maynard Smith and Harper, 2004). Organisms rely on signals, including visual, audio, mechanical, and chemical, to communicate with each other. One of the many chemical signals is pheromones. “Pheromones are the molecules used for communication between animals”, and are released by an individual and received by another individual of the same species to elicit a specific reaction (releaser pheromone) or a development process (primer pheromone) (Wyatt 2003). Functionally, pheromones are often divided into, for example, sex pheromones, aggregation pheromones, and alarm pheromones. Generally, the pheromonal signals are olfactory chemosensory cues. Distance pheromones travel in air or water from the signaler before they are detected. In addition, many chemical signals (contact pheromones) are detected by contact chemoreceptor. For example, pheromones on the cuticle of some insects are detected by antennae contact (Coyne & Oyama 1995; Inomata et al. 2000). Pheromones usually are small molecules having molecular weight of between 80 and 300 to diffuse in air and water easily (reviewed by Wyatt 2003). For aquatic pheromones, solubility is perhaps a key signal design feature. Aquatic animals use two main types of molecules as pheromones, small size molecules, such as the steroid-based in fish, and large polar molecules with high solubility, such as polypeptides in many marine invertebrates (reviewed by Wyatt 2003).
Chemical signals perhaps are more important for communication for aquatic animals than other signals because of low illumination and low sound transmission in water. Pheromonal communication has been found to be used widely for mate and species recognition by aquatic animals, such as rotifers (Gilbert 1963; Snell et al. 1995), worms (Hardege et al. 2004), crustaceans (Dunham 1978, 1988 for reviews), mollusks (Painter et al. 1999; Buresch et al. 2003), and fishes (Ahmed 2000 for a review). For example, rotifers use glycoproteins for mate recognition (Snell et al. 1995), worms’ sex pheromones are peptides (Hardege et al. 2004), and many fishes release steroids and prostaglandins as sex pheromones (e.g. Dulka et al. 1987). Generally, the communication signals are olfactory chemosensory cues. In addition, contact chemosensory cues have been reported to mediate sexual behaviour in some species of rotifers (e.g. Snell et al. 1995), squid (Buresch et al. 2003), and copepods (e.g. Ting et al. 2000).

Chemical communication has been well documented in many decapod crustaceans (Dunham 1978, 1988 for reviews), such as crabs (Ryan 1966; Gleeson 1980; Seifert 1982; Asai et al. 2000; Hardege et al. 2002; Kamio et al. 2002), lobsters (Atema 1984, Karavanich and Atema 1998), crayfishes (Ameyaw-Akumfi & Hazlett 1975; Tierney 1984; Breithaupt and Eger 2002; Stebbing et al 2003; Skog et al. 2009), and snapping shrimp (Obermeier and Schmitz 2004). Chemical signals have been demonstrated to play roles in social dominance (i.e. dominance signals) (Karavanich and Atema 1998; Breithaupt and Eger 2002; Obermeier and Schmitz 2004; Skog et al. 2009) and mate recognition (i.e. sex pheromones) (Ryan 1966; Dunham 1978, 1988; Gleeson 1980; Seifert 1982; Asai et al. 2000; Hardege et al. 2002; Kamio et al. 2002) in decapod crustaceans. Many decapod crustaceans use urine-borne chemical signals to elicit specific responses. For example, dominance signals in snapping shrimp (Obermeier and Schmitz
2004), crayfishes (Breithaupt and Eger 2002) and lobsters (Karavanich and Atema 1998; Skog et al. 2009) are released through urine. To attract mating partners, female crabs (Ryan 1966; Gleeson 1980; Seifert 1982; Asai et al. 2000; Hardege et al. 2002; Kamio et al. 2002), lobsters (Atema 1984), crayfishes (Ameyaw-Akumfi & Hazlett 1975; Tierney 1984; Stebbing et al 2003) emit distance (soluble) pheromones in the urine. However, as Burkenroad (1947) proposed, some shrimp may, in addition to distance pheromone, possess an insoluble substance coating a receptive female’s body (contact pheromone), which enables the male to recognize the receptive female for copulation (Kamiguchi 1972; Bauer 1979; Sarojini et al. 1984). So far there is no report confirming the existence of both distance and contact sex pheromones involved in a decapod crustacean species. Two caridean shrimp species, *Palaemon paucidens* (Kamiguchi 1972) and *Heptacarpus paludicola* (Bauer 1979), might depend on both distance and contact pheromones for sex recognition in mating. Existence of contact pheromone in *P. paucidens* has not been tested (Kamiguchi 1972). *H. paludicola* might release distance pheromone, but the pheromone seem not to be involved in the mating process, as copulation occurs upon physical contact (Bauer 1979). Behavioural evidence suggests the existence of such contact pheromone in the caridean shrimp *Palaemonetes pugio* (Caskey and Bauer 2005).

To date, efforts to characterize distance pheromones of crustaceans have been made in lobsters (e.g. Atema and Gagosian 1973; Gagosian and Atema 1973), crabs (e.g. Gleeson et al. 1984; Asai et al. 2000; Hardege et al. 2002; Kamio et al. 2002) and the amphipod *Microdeutopus gryllotalpa* (Borowsky et al. 1987). Sex pheromones in the hair crab *Erimacrus isenbeckii* are identified as ceramides; however, result of behavioural assay does not support it (Asai et al. 2000). The pheromones in the crab *Carcinus maenas* are molecules of 500 -1000 Dalton (Hardege et al. 2002), and similarly the molecular weight of waterborne pheromone in the crab *Temessus cheiragonus* is less than 1000
Dalton (Kamio et al. 2002). It is only known that the bioactive waterborne substance of *M. gryllotalpa* is polar (Borowsky et al. 1987). Recently, uridine-5’-di-phosphate (UDP) is identified as the main components of the pheromones in the crab *Carcinus maenas* (Bublitz 2007) and the snow crab (*Chionoeetes opilio*). Yellowline arrow crab (*Stenorhynchus sticornis*) also displays mating behavioural response to UDP (Bublitz 2007). Further test demonstrates that uridine-5’-tri-phosphate (UTP) is also a component of the pheromones in the crab, *C. maenas* (Fletcher 2007). Because both UTP and UDP are linked to the pathway of chitin biosynthesis (Stevenson 1972) and may be released during the moulting process, it is reasonable to believe that decapod crustaceans would use either or mixture of them as sex pheromone. In this study, soluble sex pheromone of *L. wurdemanni* was characterised.

Contact sex pheromones are well known and have been identified as cuticular hydrocarbons in a variety of insect species including *Drosophila* and a variety of beetles (e.g., Linn and Roelofs 1995; Etges and Jackson 2001; Zhang et al. 2003; Sugeno et al. 2006); but they have not been well investigated in crustaceans. Currently, a contact pheromone found to be a glycoprotein has been identified in the harpacticoid copepod, *Tigriopus japonicus* (Ting et al. 2000). However, glycoproteins are not the contact pheromones in *Lysmata* shrimp (Zhang et al. in press). The characteristics of the contact pheromones of decapod crustaceans remain unknown. In this study, contact pheromone of *L. boggessi* was investigated.

Speciation in animals is often characterized by the presence of pre-zygotic (ethological barriers to inter-specific mating) and post-zygotic isolation (infertility and/or non-viability of inter-specific hybrids). Pre-zygotic factors include mating recognition and morphological constraints (e.g. Collins and Tuskes 1979; Gardner 1997; Coyne and Orr 2004). Genetic incompatibility is referred to as the post-zygotic element
It has been realized that chemical and visual, and other cues are involved in behavioural isolation, of which chemical cues are often predominant in many taxa, such as insects (e.g. Collins and Tuskes 1979), reptiles (e.g. snake, Shine et al. 2002), and amphibians (e.g. salamander, Rollmann et al. 2000). Pheromones have been demonstrated to be associated with speciation (e.g. Linn and Roelofs 1995 for a review; Shine et al. 2002) and pheromonal difference among sympatric species may provide the basis for species recognition and avoidance of interspecific mating in salamanders (Rollmann et al. 2000), lizards (e.g. Cooper and Vitt 1984, 1987), snakes (e.g. Shine et al. 2002), insects (e.g. Collins and Tuskes 1979) and decapod crustaceans (Dunham 1978, 1988 for reviews). This study focused on the role of chemical cues in behavioural isolation in *Lysmata* species.

Shrimps in the genus *Lysmata* have attracted much attention because they have an unusual reproductive system, protandric simultaneous hermaphroditism (review, Bauer 2000). Several studies have been conducted on their reproductive biology (e.g. Bauer and Holt 1998; Fiedler 1998; Lin and Zhang 2001; Baeza and Bauer 2004; Zhang and Lin 2004a, b, 2005a, b, 2006). Gonads of the shrimp are ovotestes that have testis and ovarian portions (Bauer and Holt 1998; Fiedler 1998). Individuals mature first as males (male phase-MP), i.e. testis portion mature first. As shrimp grow, the ovarian portion may also develop, so that the gonad is able to produce both eggs and sperm simultaneously, a condition called simultaneous hermaphrodite that has both male and female functions (Bauer and Holt 1998; Fiedler 1998). This phase has been termed the female-phase by Bauer and Holt (1998). To describe the phase more accurately, it has been changed to euhermaphrodite-phase (true hermaphrodite) by Lin and Zhang (2001). The intermoulting euhermaphrodite-phase shrimp (EP) that functions as a male is able to mate with the
newly moulted EP shrimp that plays the female role. Same as other caridean shrimp, maturation of oocytes in the ovary, moulting, mating, spawning, embryonic development, and hatching are interrelated processes in EP shrimp of *Lysmata* species. For example, EP shrimp of *L. wurdemanni* take about 11 days to complete a moult cycle at temperature of 26-27 °C (personal observation). When ovary matures, EP shrimp moults and mates with MP shrimp or intermoult EP shrimp immediately after moulting, then spawns eggs about 3 hours after mating, the embryos attaching on the abdomen of the EP shrimp develop about 10 days prior to hatching, and the EP shrimp moults again 12-24 hr after hatching (personal observation). Normally larvae moult at least 12 times to become postlarvae in about 25 days (Zhang et al. 1998). Male function matures in about 6-10 days later at about 1.3 cm total length (TL) (Zhang and Lin 2005b) and female function matures in about another 14 days at about 2.4 cm TL in *L. wurdemanni* (Lin and Zhang 2001; Zhang and Lin 2005b).

Several hrs before EP shrimp moult, most male-role shrimp display pre-copulatory behaviour (Zhang and Lin 2004a, b). Two pre-copulatory behaviours have been observed: flirt (swim around and approach the receptive female-role shrimp) and chase (actively follow the receptive female-role shrimp within 1 min of the female moulting) (Zhang and Lin 2004a, b). Behavioural evidence indicates that these male-role shrimp obviously track and locate the receptive females by distance pheromone (Giri 2002). However, some male-role shrimp do not have obvious pre-copulatory behaviour. After the female moults, both female- and male-role shrimp swim around, mating occurs immediately when the male-role shrimp contacts the female-role shrimp with its antenna/antennules (Zhang and Lin 2004a, b). This suggests the presence of contact pheromone to mediate the copulation process.
The *Lysmata* shrimp may be an ideal model to examine whether evolution of mating system is associated with social environment because there is a distinct dichotomy in sociobiology of *Lysmata* specie and two mating systems (pure searching and mate guarding) may have been evolved in *Lysmata* shrimp. Low density/pair-living species (e.g., *L. amboinensis*, *L. debelius* and *L. grabhami*) live in tropical waters and are specialized fish cleaners; while group-living species (e.g., *L. boggessi*, *L. seticaudata*, and *L. wurdemanni*) live mostly in sub-tropical and temperate areas and are unspecialized, facultative fish cleaners (Wirtz 1997; Fiedler 1998; Bauer 2000). Male mating tactics in *L. wurdemanni* and *L. boggessi* can be classified as “pure searching”, since they are continuously “on the prowl” for a receptive female (Bauer and Holt 1998; Zhang and Lin 2004a, b). When one is encountered, copulation occurs almost immediately after a brief interaction (Bauer and Holt 1998; Bauer 2002; Zhang and Lin 2004a, b). Several pre-copulatory behaviours of *L. amboinensis* have been described based on observations of one pair (Fiedler 2000). It seems that male-role *L. amboinensis* do not actively search for female-role shrimp as *L. wurdemanni* and *L. boggessi* do. Moreover, if more than 3 EP shrimp are held in a tank, fighting may occur between male-role shrimp, especially when an EP shrimp would moult (personal observation), i.e. mate guarding may have evolved in *L. amboinensis*. This behavioural difference might be the results of adaptation to their respective social environment (density), i.e. low male competition at low density causes inactive pre-copulatory behaviour. This study investigated the difference in reproductive behaviour between *L. amboinensis* (low density species) and *L. boggessi* (high density species) to compare the chemical communication in the two groups of shrimp.

In the present study, the mechanism of mate recognition in *Lysmata* shrimp (using *L. wurdemanni* as the model species), including chemical communication cues, whether both distance and contact pheromones are involved in mating, was investigated (Chapter...
1, published in *Anim. Behav.*, 2006, 71:1191–1196); afterwards, both the soluble
(Chapters 2, published in *Mar. Biol.*, in press, and 3; *L. boggessi* and *L. wurdemanni* were
the model species) and contact pheromones (Chapter 4, *L. boggessi* was the model
species) were characterized, respectively, and ecological consequence of chemical cues in
the *Lysmata* shrimp (*L. amboinensis*, *L. boggessi*, and *L. wurdemanni* were the model
species) was examined in Chapters 5 (published in *J. Mar. Biol. Assoc. UK* 2007, 87:

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Chapter 1

Mate Recognition in a Simultaneous Hermaphroditic Shrimp, *Lysmata wurdemanni*  
(Caridea: Hippolytidae)
Abstract

Male crustaceans must find and identify receptive females to mate successfully. Sex recognition depends mainly on sex pheromones, which are detected by antennae and antennules. Distance (soluble) pheromone mediates mating behaviour in some decapod crustaceans. Contact pheromone (an insoluble coating on the body surface) has been proposed but not confirmed to be used by male decapod crustaceans to detect females. Here we report for the first time the involvement of both distance and contact pheromones in the mating processes of a decapod crustacean, *Lysmata wurdemanni*, a protandric simultaneous hermaphrodite. Euhermaphrodite-phase (EP) shrimp can mate only as females during the small window of postmoult period, during which they secrete both distance and contact sex pheromones. Male shrimp tracked, recognized and courted the receptive EP shrimp based on both distance and contact sex pheromones, but responded aggressively to newly moulted male shrimp. Male shrimp with their chemosensory appendages ablated appeared unable to identify EP shrimp and neither courted nor copulated with them. As reported for other decapod crustacean species, the outer flagella of the antennules house the receptors of the distance pheromone, whereas both antennae and antennules can detect the contact pheromone. Shrimp could still mate successfully without the distance pheromone. These results suggest that contact pheromone, in addition to distance pheromone, is involved in mediating the mating behaviour of *L. wurdemanni*. Moreover, contact pheromone may be more important than distance pheromone in the mating process. Selection of the sex pheromone system of *L. wurdemanni* may be driven by social environment.
**Introduction**

Mating systems, defined as behavioural strategies used by individuals to obtain mates, greatly affect the genetic structure and the traits that are favoured by selection in a species (e.g. Emlen & Oring 1977). Sexual roles and the evolution of pheromones are both keys to understanding mating systems. Pheromonal communication is used widely for mate and species recognition by animals such as rotifers (Gilbert 1963; Snell et al. 1995), crustaceans (reviewed in Dunham 1978, 1988), insects (Coyne & Oyama 1995; Inomata et al. 2000), molluscs (Painter et al. 1999; Buresch et al. 2003), fish (reviewed in Ahmed 2000) and amphibians (reviewed in Kikuyama et al. 2002). Generally, the communication signals are olfactory chemosensory cues. In addition, contact chemosensory cues have been reported to mediate sexual behaviours in some species of rotifers (e.g. Snell et al. 1995), squid (Buresch et al. 2003), copepods (e.g. Ting et al. 2000) and insects (e.g. Zhang et al. 2003). It has been well documented that, in many decapod crustaceans (reviewed in Dunham 1978, 1988), such as crabs (Ryan 1966; Gleeson 1980; Seifert 1982; Hardege et al. 2002), lobsters (reviewed in Atema 1984) and crayfish (Ameyaw-Akumfi & Hazlett 1975; Tierney et al. 1984), the females secrete distance (soluble) pheromone in their urine to attract mating partners. However, as Burkenroad (1947) proposed, females of some shrimp species may also secrete an insoluble substance that coats the female’s body (contact pheromone) when they are receptive, allowing males to recognize them (Kamiguchi 1972; Bauer 1979; Sarojini et al. 1984). So far there has been no evidence of the involvement of both distance and contact pheromones in the mating process of a decapod crustacean. Two caridean shrimp species, *Palaemon paucidens* (Kamiguchi 1972) and *Heptacarpus paludicola* (Bauer 1979), might depend on both distance and contact pheromones for sex recognition in mating. The existence of contact pheromone in *P. paucidens* has not been tested (Kamiguchi 1972).
*Heptacarpus paludicola* might release distance pheromone, but the pheromone does not seem to be involved in the mating process, because copulation occurs upon physical contact (Bauer 1979). The caridean *Lysmata* shrimp has an unusual reproductive system, protandric simultaneous hermaphrodite (Bauer 2000). A shrimp first matures as a male, but with growth may change into euhermaphrodite. A euhermaphrodite-phase (EP) shrimp with both male and female functions can mate as a female (during postmoult) as well as a male (during intermoult) (Bauer 2000; Lin & Zhang 2001). In *L. wurdemanni*, most male-role shrimp have a active precopulatory behaviour (Zhang & Lin 2004a): flirt (swim around and approach the receptive female-role shrimp) and chase (actively follow the receptive female-role shrimp within 1 min before the female moults). Behavioural evidence indicates that these male-role shrimp obviously track and locate the receptive females by distance pheromone (T. Giri, unpublished data). However, some male-role shrimp do not have obvious precopulatory behaviour. After the female moults, both female- and male-role shrimp swim around, and mating occurs immediately when the male-role shrimp contacts the female-role shrimp with its antenna/antennules (Zhang & Lin 2004a). This contact behaviour suggests that the presence of contact pheromone in this species is to mediate the copulation process. A related species, *L. rathbunae*, has a similar behaviour (personal observation). In the present study, we investigated the mechanism of mate recognition in *L. wurdemanni*, including chemical communication cues, whether both distance and contact pheromones are involved in mating and the sites for pheromone reception.

**Methods**

This study was carried out from April to October 2004 at Florida Institute of Technology’s Vero Beach Marine Laboratory. *Lysmata wurdemanni* were reared in the
laboratory from broodstock shrimp originally collected from Port Aransas, Texas, U.S.A. The shrimp, between 2.6 and 4.4 cm in total length (TL), were housed in 20-litre tanks with flow-through seawater of 35% salinity at 26–28°C and were fed frozen adult *Artemia* once daily. When EP shrimp were about to moult (EP shrimp moult about 15 h after hatching under 26–28°C), they were moved to a 10-litre white bucket for the series of observations. Only male-phase (MP) shrimp were used to serve the male role in this study. No individual shrimp was used more than once. To simplify, we use ‘male’ and ‘female’ hereafter to represent the male role and female role, respectively. The copulation behaviour was observed and recorded with a Sony camcorder using fluorescent illumination.

**Mating Behaviour**

Mating behaviour was identified according to the following criteria (Zhang & Lin 2004a). The suitor approaches and follows (to catch up and flirt with) the premoult female shrimp about 1 h before the female shrimp moults, and is most active (male follows female more closely) within 1 min of the moulting. The male approaches the female and contacts her repeatedly with his head. This behaviour lasts from several seconds to minutes, and is then repeated. Thereafter, the male shrimp follows the female. Some male shrimp stay beside the females and mate with them immediately after the females completes moulting. Males follow newly moulted females consistently after antenna/antennule contact, and grasp the females and bring their ventral surfaces into contact. Normally, there is no interaction between the male and female shrimp if they encounter each other more than 2 h before the female moults (Zhang & Lin 2004a).

Female shrimp spawn about 3 h after copulation. The shrimp do not self-fertilize or store sperm. Spermatophores are generally deposited on the bases between the fourth
and fifth pereiopods (Zhang & Lin 2004a). The eggs are fertilized when they are extruded and are attached on the abdomen.

Female and male shrimp were kept in a 10-litre bucket for spawning. Subsamples of the eggs (at least 30 from each shrimp) were sampled with forceps and examined under a compound microscope about 6 h after the spawning (when the first division is completed) to assess mating success. If more than 90% of the eggs (embryos) were developing, the mating was considered successful (Zhang & Lin 2004a).

**Distance Pheromone**

We first compared the behaviour of male shrimp towards newly moulted female and male shrimp to examine whether both sexual phases secrete the sex pheromones. To ensure successful observations, each 10-litre bucket contained two male shrimp of different sizes so that they could be easily distinguished, and one female shrimp that was about to moult (some male shrimp are inactive during pre- and postmoult periods of parturial EP shrimp: Zhang & Lin 2004a). There is no (intermoult) male–male interaction in this species during mating when multiple male and female shrimp are present (Bauer 2002). Behaviour of the shrimp in the 15 replicate buckets was observed and recorded using the method described above.

To test the response of male shrimp to newly moulted males, the same trial was carried out using male instead of female shrimp that were about to moult. Before the study, all of the male shrimp were housed individually in 20-litre buckets to determine their moult cycle.
Contact Pheromone

We performed the following experiment to test whether male shrimp could detect the newly moulted female shrimp with the contact pheromone. Distance pheromone was removed by rinsing. To eliminate possible stress or trauma on female shrimp while rinsing off the distance pheromone, a parturial female shrimp was maintained in a 4-litre flow-through system that held 1 litre of water. The female shrimp was held in the static water with aeration when she moulted. Immediately after moulting, 500 ml of the moulting water was collected for another bioassay (see below), and the tank was returned to continuous flow through for at least 30 min. Afterwards, the water in the tank was drained and refilled five times to remove the distance pheromone as complete as possible. The water from the final rinse was collected to test whether the pheromone was still present (see below). The rinsed newly moulted female shrimp was kept in the final rinse water for 1 min (because mating would occur within 1 min after the female was placed into the bucket) before being introduced into the 10-litre bucket with the two males to test whether the distance pheromone was released after the female was introduced into the 10-litre testing bucket. The water collected from the final rinse was confirmed to contain no effective distance pheromone with the following procedure. Two male shrimp of different sizes were placed in a rectangular tank (20 × 40 × 24 cm) containing 6 litres of seawater and presented with regular seawater, followed by the final rinse water. In another tank, two other male shrimp of different sizes were exposed to regular seawater, followed by the moulted water in which the parturial female shrimp had moulted (and therefore should contain the distance pheromone). The water was slowly added (2-3 drops/second) at 4-5 cm away from the tested male shrimp through a pipette. Responses displayed by the shrimp were recorded with a Sony camcorder. Fifteen replicates were conducted.
After rinsing, the newly moulted female was introduced into the 10-litre bucket containing two males. We compared behaviour of the shrimp in the 15 replicate buckets with those of the same number of controls where the newly moulted male shrimp replaced the newly moulted female shrimp. If the newly moulted female did not release sufficient distance pheromone within 1 min (because copulation occurred within 1 min) and the male shrimp could still identify and mate with the female (mating occurred 2–10 s after the female was introduced into the observation bucket, see Results), this meant that males recognized the females based only on the contact pheromone that the female secreted.

We investigated interspecific mating between *L. wurdemanni* and *L. rathbunae* to further verify the presence of the contact pheromone and whether the pheromone was species specific. *Lysmata wurdemanni* shrimp were offspring from the broodstock shrimp originally collected from Port Aransas, Texas, U.S.A., and *L. rathbunae* shrimp were offspring from adults collected from Hernando Beach, Florida. The two species were of similar sizes, between 2.6 and 4.4 cm in total length (TL). We tested 10 pairs of each male–female combination, *L. wurdemanni* male × *L. rathbunae* female and *L. wurdemanni* female × *L. rathbunae* male, and 10 pairs of intraspecific combinations for each species served as the control. Observations were made using the same methods for intraspecific mating (Zhang and Lin 2004a, b).

**Antenna/Antennules Ablation**

We conducted an ablation experiment to confirm the role of the antenna and antennule of the male shrimp in detecting distance and contact pheromones. This experiment was conducted in 10-litre buckets each containing two males with antennae (ref. to the second pair of antennae) and/or antennules ablated, and one intact female that
was about to moult. The excision experiment consisted of five treatments: (1) only antennae were ablated; (2) inner flagella of antennules and whole antennae were ablated; (3) outer flagella of antennules and whole antennae were ablated; (4) whole antennules were removed; (5) both antennae and antennules were completely ablated. Intact male shrimp served as controls. Antennae/antennules were excised at the base using sharp-tipped forceps. Ablation was conducted one day before the mating observation. No individual shrimp was used more than once in the ablation experiment. Each treatment and control had 15 replicates. We compared precopulatory behaviour 1 h before the female shrimp moulted, copulation attempts and mating success between the treatments. These behaviours were used to interpret the responses to distance and contact pheromones.

Results

**Distance Pheromone**

Intraspecific mating behaviour of male shrimp towards the female shrimp was the same as reported before (Zhang & Lin 2004a). The behaviour of male to premoult female and male shrimp was significantly different ($\chi^2$ test: $G = 61.52, P < 0.001$). Only three of the 30 male shrimp did not respond to the premoult female shrimp, whereas none of the males responded to the premoult males (Table 1). Male shrimp did not display the approach and follow behaviour towards the newly moulted male shrimp. The response of males to newly moulted female and male shrimp was also significantly different ($\chi^2$ test: $G = 11.84, P < 0.001$). After a newly moulted male shrimp was detected by antenna/antennule contact, 15 of the 30 males launched a sudden attack against the newly moulted male that then flipped to escape from the attack, whereas there was no obvious
attack on the other 15 males (Table 1). In contrast, males followed and mated with the newly moulted females (Table 1).

**Contact Pheromone**

Male shrimp did not respond to the final rinse water, indicating that there was no effective distance pheromone in the water. This result was significantly different from that for male shrimp towards the moulted water ($2 \times 2$ G test: $G = 42.90, P < 0.001$) in which the male followed the movement of the pipette that delivered the moulted water.

After the rinsed newly moulted female shrimp was introduced, both male shrimp strolled slowly and mating between one of the males and the newly moulted female took place soon afterwards (mean ± SD 4.0 ± 2.1 s; range 2–10 s; n =15). Copulation occurred immediately after the male shrimp contacted the female with his antenna/antennule. All of the 15 matings resulted in the production of fertilized eggs. The precopulation behaviour of male shrimp was significantly affected by the distance pheromone (Chi-square test: $\chi^2 = 270.00, P < 0.001$). Male shrimp did not display the approach and follow behaviour towards the newly moulted, rinsed females as they did towards premoult females when the distance pheromone was present. Only eight of the 30 male-role shrimp recognized the newly moulted male shrimp, and displayed attack behaviour towards the male shrimp.

Matings in all the intraspecific pairings of both species were successful. In *L. wurdemanni* male × *L. rathbunae* female pairs, 14 of the 20 males displayed the same flirt, chase and postmoult behaviour as did 19 of the 20 males in the intraspecific mating of both species. The number of male *L. wurdemanni* that displayed precopulation behaviour towards female *L. rathbunae* was significantly different from the intraspecific (*L. wurdemanni*) mating ($2 \times 2$ G test: $G = 4.80, P < 0.05$). Eight of the 10 *L.
wurdemanni male and *L. rathbunae* female pairs mated successfully. The difference in mating success between interspecific pairs (*L. wurdemanni* male × *L. rathbunae* female) and intraspecific pairs (*L. wurdemanni*) was not significant (2 × 2 G test, $G = 2.40, P > 0.05$). The eggs were fertilized and developed to early embryo stages in all of the matings. However, in *L. wurdemanni* female × *L. rathbunae* male pairs, approach and follow behaviour was not obvious. Most male *L. rathbunae* did not respond actively to postmoult *L. wurdemanni* females. Only four of the 20 males showed a response towards the newly moulted females, compared to 19 of the 20 males in the intraspecific pairs. The difference in the number of male *L. rathbunae* that displayed precopulation behaviour towards interspecific *L. wurdemanni* female and intraspecific *L. rathbunae* females was significant (2 × 2 G test: $G = 25.50, P < 0.001$).

**Antenna/Antennules Ablation**

When only antennae were ablated, precopulation behaviour was not significantly affected (Chi-square test: $\chi^2 = 1.48, P > 0.05$). When antennae and the outer flagella of antennules were ablated, precopulatory behaviour of males was significantly influenced (Chi-square test: $\chi^2 = 270.00, P < 0.0001$). Ablation of antennae and the inner rami of antennules did not significantly affect the number of shrimp displaying the precopulatory behaviour (Chi-square test: $\chi^2 = 3.33, P > 0.05$). When entire antennules were removed, precopulatory behaviour of males was significantly affected (Chi-square test: $\chi^2 = 270.00, P < 0.0001$). Precopulation behaviour was also significantly affected when both antennae and antennules were ablated (Chi-square test: $\chi^2 = 270.00, P < 0.0001$). Mating was not influenced except in the treatment in which both antennae and antennules of the male shrimp were ablated (Table 2).
Discussion

The results show that both distance and contact pheromones are involved in the mating process of *Lysmata wurdemanni*. Male shrimp courted pre- and postmoult female shrimp, did not respond to premoult male shrimp, and displayed aggressive behaviour towards newly moulted males. The differences in responses of male shrimp towards female and male shrimp indicate the presence of both distance and contact pheromones that are secreted only by females. Although the females with the distance pheromone rinsed away did not elicit approaching and following behaviour in the males, the males did copulate with the females, suggesting the existence of contact pheromone. Male shrimp with chemosensory appendages ablated appeared unable to identify female shrimp (they neither courted nor copulated with them), further suggesting that chemical communication mediated mating in *L. wurdemanni*. Male *L. wurdemanni* could detect and recognize pre- and postmoult female *L. rathbunae*, but male *L. rathbunae* could not detect and recognize pre- and postmoult female *L. wurdemanni*, suggesting that the two shrimp secrete specific chemical signals and further confirming that contact pheromone is one of the mediators of the mating process in shrimp.

Diffusible distance pheromone that is used as an attractant have been reported in many decapod crustacean species (e.g. reviewed in Dunham 1978, 1988). Distance pheromone triggers the precopulatory behaviour in *L. wurdemanni*, because the male approached and followed the pre-molt female and the female molt water. However, the male shrimp depend on contact pheromone to identify the receptive females. Even if the males did not display any precopulatory behaviour towards the premoult females, they could eventually detect the newly moulted female shrimp via contact pheromone and thus mated successfully (Zhang & Lin 2004a, b; present study). Male shrimp did not respond
to the premoult males and attacked the newly moulted males, suggesting that both distance and contact pheromones are secreted only by freshly molted female shrimp.

The results confirm the existence of contact pheromone in decapod crustaceans for the first time. The soft exoskeleton of the newly moulted female shrimp has been proposed as an essential condition for mating (Kamiguchi 1972). Behavioural differences in responses of male shrimp to newly moulted female and male shrimp indicate that the female was identified by the contact pheromone coated on the exoskeleton, not just by the soft exoskeleton. This difference may not be enough to conclude that contact pheromone is present. However, the males’ response to rinsed, newly moulted females and the interspecific mating results clearly show that contact pheromone is involved in the mating process, because male shrimp did not display precopulation behaviour to either the rinsed female shrimp or to the final rinse water, in which the soluble sexually attractive substance had been rinsed away. Although no distance pheromone was present, male shrimp could still recognize the newly moulted female based on contact pheromone on the exoskeleton of the female shrimp. Thus, contact pheromone allows the male to recognize the newly moulted female, not just newly moulted shrimp.

Interspecific mating results show that male *L. rathbunae* did not recognize newly moulted female *L. wurdemanni*; however, male *L. wurdemanni* could recognize female *L. rathbunae*. The behavioural difference suggests that recognition between male *L. rathbunae* and female *L. wurdemanni* or between male *L. wurdemanni* and female *L. wurdemanni* is determined by contact pheromone, not the soft exoskeleton. This is an interesting finding because pheromones are often considered to be highly species specific.
Why there is a difference in contact pheromone specificity between these two species is unknown and evolution of chemical signals in the genus *Lysmata* is worth investigation.

Antennules have been reported to act as distant chemo sensors for detecting soluble pheromones, with the site of reception located in the outer flagella of the antennules of male crabs (Gleeson 1980), crayfish (Tierney et al. 1984) and shrimp (present study). Results of the present study indicate that both antennae and antennules can detect the contact pheromones. When we ablated, respectively, antennae and the outer flagella of antennules, antennae and the inner flagella of antennules, and antennules, receptive female shrimp could be identified and mating was still successful. As long as the outer flagella of antennules were present, male shrimp would display precopulation behaviour. The aesthetascs (chemoreceptive hairs) in the outer antennular flagella are believed to be responsible for detecting the distance pheromones (e.g. Tierney et al. 1986; Cate & Derby 2001). Our preliminary observation showed that the outer antennular flagella of *L. wurdemanni* bear aesthetascs. The sensor (setae) on antennae and antennules responsible for detecting contact pheromone is unknown. In addition to chemical cues, visual cues are also used for sexual communication in decapod crustaceans (e.g. Dr’az & Thiel 2004). However, the antennae/antennules ablation experiment in the present study indicates that visual cues are not involved in the mate recognition of *L. wurdemanni*. When both antennae and antennules were ablated, only one of the 30 male shrimp mated successfully. *Lysmata wurdemanni* moult, mate and spawn mainly at night, when visual cues are ineffective.

Unlike olfactory pheromones, contact pheromone is not common in aquatic animals. So far contact pheromone has been found only in rotifers (Gilbert 1963; Snell
et al. 1995), squid (Buresch et al. 2003) and copepods (Ting et al. 2000). The presence of both distance and contact pheromones in crustacean decapods has not been reported before. Why shrimp have both kinds of pheromones to mediate mating behaviour is unknown. This may be the result of environmental adaptation, because ecology influences the evolution of pheromone communication (Theissen 1977) and mating systems (Emlen & Oring 1977). Social environment may be a key factor in affecting the evolution of pheromone communication systems. Individuals in a high-density population may need contact pheromone as a mate recognition signal. All of the species known to have contact pheromone (Gilbert 1963; Snell et al. 1995; Ting et al. 2000; Buresch et al. 2003) live in high densities. *Lysmata wurdemanni* and *L. rathbunae* are also highly aggregated. Correlation between male mating tactics and sex pheromones has not been thoroughly analysed. Male mating tactics of decapod crustaceans are classified basically into pure searching, mate guarding and monogamy (Wickler & Seibt 1981; Bauer 2004). Sex recognition in female guarding and monogamous species seems to depend mostly on distance pheromones. However, pure-searching species seem to depend mainly on contact pheromone. The pure searching species are generally nonterritorial, mobile and live in high densities so that males have many opportunities to encounter females (Wickler & Seibt 1981; Bauer 2004). Contact pheromone in high-density populations benefits both males and females. In a high-density population, synchronous moulting is common (e.g. Anderson et al. 1985). Male shrimp might be confused if only distance pheromone is present. Contact pheromone would allow males to recognize individual newly moulted females and ensure mating success. Although *L. wurdemanni* and *L. rathbunae* are highly aggregated, some other species in the genus (e.g. *L. grabhami*, *L. amboinensis* and *L. debelius*) live in pairs or low densities. Whether these low density
species also have both distance and contact pheromones is not known. It would be fruitful to examine more species in the genus, as well as other species with different mating systems, to understand the evolution of the pheromones and their roles in speciation, since sex pheromones have been referred to as one of the reproductive isolating mechanisms (e.g. Shine et al. 2002) and one of the most important signals to identify species (Paterson 1978).

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Table 1 Behavioural response of male shrimp toward female shrimp and other male shrimp

<table>
<thead>
<tr>
<th></th>
<th>Pre-moult behaviour (Approach, Follow)</th>
<th>Post-moult behaviour</th>
<th>Copulation</th>
<th>Aggression</th>
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<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>
| Female         | 27                                    | 3                    | 30         | 0          | 0          | 30
| Male           | 0                                     | 30                   | 0          | 0          | 15         | 15

Pre- and post-moult behaviour indicate response of male shrimp toward the female/male shrimp before and after female/male shrimp moulted. For pre-moult behaviour, positive response (+) indicates that the male shrimp displayed pre-copulatory behaviour, including approach and follow; negative response (-) represents no response to pre-moult female/male shrimp. For post-moult behaviour, positive response (+) indicates that the male shrimp responded (+) by mating with (copulation) or attacking (aggression) the newly moulted female/male shrimp. Shrimp without any response is shown as negative (-).
Table 2 Effect of ablation of male shrimp on their responses to female shrimp.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response to the receptive female shrimp</th>
<th>Mating success</th>
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<tbody>
<tr>
<td></td>
<td>Distance pheromone</td>
<td>Contact pheromone</td>
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<tr>
<td></td>
<td>(Pre-copulatory behaviour)</td>
<td>(Copulation attempt)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>AT ablated</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>AT and OF of ATL ablated</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>AT and IF of ATL ablated</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>ATL ablated</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>AT and ATL ablated</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>27</td>
<td>3</td>
</tr>
</tbody>
</table>

Positive response (+) indicates that the male-phase shrimp either displayed pre-copulatory behaviour toward the female shrimp (such as approach and follow, evidence for the existence of distance pheromone) or attempted copulation upon physically contacting the female shrimp with antenna/antennules. Negative (-) represents no response.

AT = antennae, ATL = antennules, IF = inner flagella, OF = outer flagella.
Chapter 2

Characterization of soluble sex pheromone in a simultaneous hermaphroditic shrimp, \textit{Lysmata wurdemanni}
Abstract

Olfactory chemical cues have been described to play important roles in the control of mate recognition in many decapod crustaceans. However, we still know very little about the chemical characteristics of the cues that coordinate pre-copulative behaviour. In this study we partially characterized a water-borne sex pheromone of a marine shrimp, *Lysmata wurdemanni*. Female moulting water was collected and ultra-filtered using 1000 and 500 Dalton membranes, respectively, and analyzed using HPLC with a Lichrosphere™ RP18 (C18) column. The sex pheromone is likely to be a molecule between 500 and 1000 Daltons in size because behavioural bioassays showed that males responded to the supernatant of 500 Dalton and to the 1000 Dalton filtrate, but did not respond to the supernatant of 1000 Dalton or to the 500 Dalton filtrate. There was only one dominant peak (2.86 min) detectable in HPLC chromatograms of the supernatant of the 500 Dalton filtration. This peak showed a UV absorbance maximum at 274 nm, similar to the recently identified shore crab sex pheromone Uridine-di-phosphate (UDP). Behavioural bioassays confirmed that this peak is a bioactive component of a potential pheromone bouquet, but is different from UDP, which showed no bioactivity in *Lysmata wurdemanni*. Our results lay the foundation for future studies to purify and eventually identify this sex pheromone.
**Introduction**

In many decapod crustaceans (Dunham 1978, 1988 for reviews), such as crabs (Ryan 1966, Gleeson 1980, Seifert 1982, Hardege et al., 2002, Kamio et al. 2002), lobsters (Atema 1984 for a review), and crayfish (Ameyaw-Akumfi and Hazlett 1975, Tierney et al. 1984, Stebbing et al. 2003), females emit distance function waterborne sex pheromones with the urine to attract mating partners. However, the purification and characterization of such distance pheromones of decapod crustaceans is only progressing slowly. Efforts have been made in lobsters (Atema and Gagosian 1973, Gagosian and Atema 1973), crabs (Gleeson et al. 1984, Asai et al. 2000, Hardege et al. 2002, Kamio et al. 2002) and amphipod *Microdeutopus gryllotalpa* (Borowsky et al. 1987). To date, we only know that ceramides are potentially the distance sex pheromones in the hair crab *Erimacrus isenbeckii*, although no behavioural data exist to confirm this hypothesis (Asai et al. 2000). Preliminary purifications using the shore crab *Carcinus maenas*, (Hardege et al. 2002) and the helmet crab *Temessus cheiragonus*, (Kamio et al. 2002) showed that these cues are small, water soluble molecules under 1000 Daltons in size, mainly between 500 -1000 Daltons. It is only recently that uridine-di-phosphate (UDP) was identified to be the sex pheromone of the shore crab, *C. maenas* and was shown to induce mating in a number of decapod crustaceans (Bublitz et al 2008, Fletcher and Hardege 2009).

Unlike crabs, lobsters, and crayfish, distance sex pheromones are not commonly described to exist in shrimps and have only been found in the rock shrimp *Rhynchocinetes typus* and several species in the genus *Lysmata*. Male rock shrimps release such distance sex pheromones that attract females (Díaz and Thiel 2004), and euhermaphrodites of several species of *Lysmata* emit distance sex pheromones to attract males (Giri 2002, Zhang and Lin 2006, Zhang et al. 2007). Burkenroad (1947) proposed
that some shrimps might possess water insoluble or slightly water soluble substances that coat a receptive female’s body. The contact pheromones allow a male to recognize a receptive female (Kamiguchi 1972, Bauer 1979, Caskey and Bauer 2005, Zhang and Lin 2006). As such, *Lysmata* shrimps are the only decapod crustaceans proposed to use both distance and contact chemical cues for the coordination of the mating process (Zhang and Lin 2006).

Shrimps in the genus *Lysmata* have attracted much attention because they have an unusual reproductive system, protandric simultaneous hermaphroditism (review, Bauer 2000). Several studies have been conducted on their reproductive biology (e.g. Bauer and Holt 1998; Fiedler 1998; Lin and Zhang 2001; Baeza and Bauer 2004; Zhang and Lin 2004, 2005a, b, c, 2006). Gonads of the shrimp are ovotestes that have testis and ovarian portions (Bauer and Holt 1998; Fiedler 1998). Individuals mature first as males (male phase), i.e. testis portion mature first. As shrimp grow, the ovarian portion may also develop (i.e. sex change), so that the gonad is able to produce both eggs and sperm simultaneously, a condition called simultaneous hermaphrodite that has both male and female functions (Bauer and Holt 1998; Fiedler 1998). Sex change, for example in *Lysmata wurdemanni*, is mainly socially controlled (Lin and Zhang 2001, Baeza and Bauer 2004, Zhang and Lin 2007), and some male phase shrimp may not change sex to become euhermaphrodite (Baldwin and Bauer 2003, Zhang and Lin 2007). This condition has been termed the female-phase by Bauer and Holt (1998). To describe the condition more accurately, we changed it to euhermaphrodite-phase (true hermaphrodite) (Lin and Zhang 2001). The intermoult euhermaphrodite-phase shrimp that functions as a male is able to mate with the newly moulted euhermaphrodite-phase shrimp that plays the female role. Pre-copulatory mating behaviour of male-role *L. wurdemanni* has been well studied (Bauer and Holt 1998; Bauer 2002; Zhang and Lin 2004, 2006; Zhang et al. 2009). Male
mating behaviour in *L. wurdemanni* can be classified as searching behaviour since they are in continuous motion; this being interpreted a searching for a receptive female (Bauer and Holt 1998; Bauer 2002; Zhang and Lin 2004, 2006; Zhang et al. 2009). When a receptive female is encountered, copulation occurs almost immediately after a brief interaction (Bauer and Holt 1998; Bauer 2002; Zhang and Lin 2004, 2006; Zhang et al. 2009). During the mating process of *Lysmata* shrimp species, the waterborne distance pheromones elicit searching behaviour, and the contact pheromones are responsible for recognizing a receptive female (Zhang and Lin 2006). Nevertheless the chemical nature of these cues in *Lysmata boggessi* and *L. wurdemanni* (Zhang and Lin 2006) and other *Lysmata* shrimp species (Zhang et al. 2007, 2009) remain unknown.

Molecular evolution is usually conservative in animals (Wyatt 2003). For example, elephant and moths use highly similar compounds as their pheromones (Kelly 1996), and the main compositions of cuticular hydrocarbons (CHC’s) are the same in the pheromones of ants (Liu et al. 2003) and fruit flies (Ishii et al. 2001). Examples of aquatic cues include ketones that function as distance cues in polychaete worms as well as alarm signals in ants (Zeeck et al. 1988). Hence, the molecular weights and structures of the pheromone compound(s) of the shrimps and crabs could be similar. However, the recently identified sex pheromone of the shore crab *Carcinus maenas*, uridine-di-phosphate (UDP), did not elicit a full pre-copulatory response in lobsters, mud crabs and swimming crabs tested (Bublitz et al 2008). This suggests a substantial level of species-specificity of the waterborne soluble sex pheromones in decapod crustaceans. As such it remains to be examined whether nucleotides also function as sex pheromones in *Lysmata* shrimps. In this study, we attempted to identify and partially characterize the distance sex pheromone in the species *Lysmata wurdemanni* and tested the hypothesis that the shore crab pheromone UDP is also used in the shrimp.
Materials and methods

Collection and maintenance of shrimp

*Lysmata wurdemanni* shrimp were collected from Florida Keys, Florida, U.S. The shrimp, between 2.6 and 4.4 cm in total length (TL), were housed in 20-L tanks with flow-through seawater of 35‰ at 26 - 28 °C. They were fed frozen adult *Artemia* once daily.

Estimation of molecular weight and HPLC analysis

About 6 hrs before euhermaphrodite-phase shrimp moult (about 15 hrs after hatching under 26 - 28 °C, when the ovary is fully developed), they were moved to a 1-L beaker containing 500 ml filtered (0.45 μm) seawater. Each beaker contained one shrimp. The seawater in which euhermaphrodite-phase shrimp moulted (moulting water) was filtered with 0.2 μm filters, and then stored at -20 °C for further analysis. Water samples from 20 euhermaphrodite-phase shrimp were combined for concentrating the bioactive compounds. The sample molecular weight was estimated according to behavioural assay results (see below) of ultra-filtered water samples. The volume of moulting water was reduced using a freeze dryer, and the sample taken up in 400 ml of ultra-pure water. Afterwards, this moulting water sample was ultra-filtered using an Amicon® stirred cell (1000 and 500 Dalton) ultra-filtration kit to remove large molecules and to desalt it and at the same time to further concentrate the bioactive compounds. The sample molecular weight was determined with a refractometer (Atago).
Desalted samples (supernatants) were subsequently analyzed in triplicate using HPLC (Agilent model 1100). A Lichrosphere™ RP18 (C18) column (250 × 4.6 mm) (Phenomenex: Macclesfield, Cheshire, UK), at 30°C, was used with ultra-pure water (isocratic, 1.0 ml min⁻¹) as mobile phase. An Agilent Diode Array Detector (DAD, model G1513B) was used, and we collected all of the peaks visible in the chromatogram with UV absorbance spectra in scan mode ranging from 190 nm to 400 nm (optimal wavelength data presented in Figure was at 210 nm). One hundred microlitres of the sample was injected, and the analysis had a running time of 20 min. Fractions between the retention times of 1.0 min and 8.0 min were collected every min for biological assays.

Behavioural bioassay

The remaining water samples (i.e. the supernatants) after HPLC analysis and the filtrates (about 200 ml each) were all used for the behavioural bioassay. All samples were diluted with seawater to a total volume of 500 ml to enable comparison of bioassay results. The bioassay procedure followed the method described by Zhang and Lin (2006). Only male-phase shrimp were used to serve the male role in the bioassay. No individual shrimp was used more than once. To simplify the terminology, ‘male and female’ are used to represent male-role and female-role shrimp, respectively, throughout the paper hereafter. Behavioural bioassays were conducted in rectangular tanks (20 × 40 × 24 cm) containing 6-L of seawater. One male was acclimated in the tank with aeration and fed with Artemia sp. nauplii for 24 h before the bioassay. Regular seawater was presented first for 3-5 min, followed by the supernatants or filtrates, or sample fractions, or moult water in which the parturial female shrimp had moulted. In another tank, another male shrimp was exposed to regular seawater (as control). Water was added (2-3 drops/s) near
(4 – 5 cm away) the tested male through a plastic tube of 3.0 mm inside diameter. If the male approached the tube, then the tube was moved slowly to see whether the male would follow the movement. Twenty replicates for each treatment were conducted and responses displayed by the shrimp were recorded with a Sony camcorder (DCR-200) and analyzed. A positive behavioural response of male to the tested water, (i.e. seawater, sample water, female moulting water and sample fractions), was defined as approach and follow: male would approach the tube and stay seconds to tens of seconds, and males may follow the movement of the tube, and may swim fast around the periphery of the tank as well. A $2 \times 2$ G-test with Bonferroni correction was applied to test the homogeneity of the male’s responses (number of males that displayed positive responses) with significance at $P<0.05$ compared to regular seawater and water samples, to ultra-filtered water samples (supernatants or filtrates), to female moulting water, and to sample fractions (Sokal and Rohlf, 1995).

**Results**

The molecular weight of the pheromone(s) is likely to be between 500 and 1000 Daltons, because males only responded to the 1000 Dalton filtrate and the supernatant of 500 Dalton filtration, but did not respond to the 500 Dalton filtrate or the supernatant of 1000 Dalton (Fig. 1). All males tested displayed the typical approach behaviour (about 5-10 seconds after the tested samples were introduced) and the typical follow response when exposed to the fraction of 1000 Dalton filtrate/supernatant of 500 Dalton. No significant difference ($2 \times 2$ G-test, $G_{adj} = 2.432$, $P > 0.05$) was found between the number (14/15) of males that displayed a positive response to the original female moulting water (Fig. 1) and this fraction. In the control (regular seawater), only one male displayed the approach behaviour, and none showed the follow response. The fast swimming behaviour was observed in those males exposed to the 500-1000 Dalton water sample and also when exposed to the female moulting water sample. At the beginning of the bioassays, males
were generally calm and mainly stationary or moved slowly within a small range. About 2 min after exposure to the pheromone containing 500-1000 Dalton sample or the female moulting water was introduced to the tank all males would actively swim around the periphery of the tank.

Seven fractions were collected at retention time of 1.0 to 8.0 min. The male shrimp only displayed obvious response to the fraction collected between 2.0 and 3.0 min. Of the 20 male-phase shrimp tested, 13 displayed both the approach and the follow behaviour compared to 3 in the control ($2\times2$ G-test, $G_{adj} = 10.614$, $P < 0.01$).

HPLC analysis of the bioactive sample indicates that female *Lysmata wurdemanni* may only release a limited number of compounds. The HPLC chromatogram of the supernatant of 500 Dalton only showed one major peak at 2.86 min (Fig. 2A) that is characterised by a UV absorbance maximum at 274 nm (Fig. 2B). This suggests that the pheromone might be a single molecule or is a mixture of compounds co-eluting under the given HPLC analysis conditions. There were no large peaks other than the salt front in the 500 Dalton filtrate or in the supernatant of the 1000 Dalton filtration (Fig. 2A). The retention time, molecular weight (and UV spectrum) of the bioactive fraction is very similar to that described by Hardege et al. (2002) for the female sex pheromone in *Carcinus maenas* using the same analytical conditions. We therefore also co-injected synthetic shore crab pheromone (UDP, see Bublitz et al. 2008) and found that the peak at 2.86 min now increased but also showed a large tailing shoulder. Although the HPLC analysis made it unlikely that UDP is the sex pheromone in female *Lysmata wurdemanni* we also tested synthetic UDP in the behavioural bioassay for confirmation. When exposed to $10^{-4}$M solution of UDP, none of the 30 male shrimp displayed the typical approach behaviour whilst 27 of the 30 males responded to female moulting water.
Discussion

Although efforts to chemically characterise pheromones in decapod crustaceans have been made for more than 20 years, their chemical structures remain largely unknown. The only identified sex pheromones are ceramides that are thought to be sex pheromones of the hair crab *Erimacrus isenbeckii* (Asai et al. 2000). However, bioassay results do not conclusively support this hypothesis and further tests are required to confirm their biological functions (Asai et al. 2000). The waterborne sex pheromones in crabs have been described to be polar molecules of <1000 Daltons (Hardege et al. 2002, Kamio et al. 2002). Waterborne sex pheromones eliciting male searching behaviour in shrimps are not as commonly described as in crabs, lobsters or crayfish. The coordination of the reproduction in most shrimps studied (mostly in carideans) has been hypothesized to rely on contact chemical cues to complete copulation (Bauer 1979, Caskey and Bauer 2005). Shrimp species in *Lysmata* are the only group known to depend on both soluble and contact pheromones in controlling their reproductive process (Zhang and Lin 2006, Zhang et al. 2007). Besides the existing behavioural evidence (Zhang and Lin 2006, Zhang et al. 2007), we report here the partial characterization of the soluble sex pheromone for the first time and further confirm the existence of such a waterborne pheromone in the shrimp *Lysmata wurdemanni*. Our bioassay results indicate that the molecular weight of the pheromone molecule is between 500 and 1000 Daltons. We also found little evidence for the existence of significant quantities of odour compounds <500 or >1000 Dalton in female moulting water. This is in accordance with the findings in decapod crabs (Hardege et al. 2002, Kamio et al. 2002). Under similar analytical conditions, Hardege et al. (2002) found the female sex pheromone in *C. maenas* to have a retention time very close to the 2.86 min (Fig. 2) that we found for the pheromone in *Lysmata wurdemanni*. Although our chemical data show similarities (i.e. UV
specification, peak retention, molecular weight filtration etc.) between the *C. maenas* pheromone and the shrimp cue, the peaks are slightly different and the results of our bioassays showed no bioactivity of UDP. This suggests that the cues of *C. maenas* and *Lysmata* shrimp are different albeit potentially similar. In many insects the pheromone evolution has been found to be very conservative (Ishii et al. 2001, Liu et al. 2003). Future studies using the purification protocols successfully applied to the shore crab pheromone are required to identify the shrimp cues. UDP, the sex pheromone of *C. maenas* has been found to have bioactivity on other decapod crustaceans, i.e. in *Majidae*, but not in lobsters or in mud crabs (Bublitz et al 2008). Further purification and structural identification of the signal molecules are required to understand the pheromone evolution in decapod crustaceans.

The existence of female sex pheromones has been predicted in almost all those crustacean species where female ecdysis and mating are linked, i.e. mating occurs shortly after parturial female moult (Christoffersen 1978). Early reports indicated that 20-hydroxyecdysone, the crustacean moulting hormone, is the sex pheromone of several crab species (Kittredge et al. 1971). Many previous studies, such as Gleeson et al. (1984), have since provided convincing evidence that, for a number of crab species, such as *Callinectes sapidus*, this is not the case. The present study confirms that in *Lysmata wurdemanni*, 20-hydroxyecdysone is not a bioactive component in the female moulting water sample and indeed was not detectable in the sample at all, this being similar to what was found in shore crab *Carcinus maenas* (Hardege et al. 2002). Additionally, male shrimp did not show active response to all other fractions collected, including the fraction at 5.0 to 6.0 min, except for the fraction collected at 2.5 to 3.0 min.

The behavioural assay methods developed in a previous study (Zhang and Lin, 2006) and modified in the current study are reliable for *Lysmata* shrimps that do not
exhibit active or vigorous swimming. Compared with the previous study, we allowed the tested males to acclimate to the environment longer (one day vs. several hrs). As such the males were calm prior to testing, which resulted in distinct behavioural difference of the males between non-female-conditioned seawater and moulting water sample treatments. For example, fast swimming was seldom found in the males of the control (regular seawater) in the present study.

Further studies should focus on the identification of the sex pheromones in *Lysmata wurdemanni* and other species to fully understand their characteristics. This would be helpful to understand pheromone evolution, reproductive isolation and speciation (Theissen 1977) in *Lysmata*, an important group with an unusual reproductive system, protandric simultaneous hermaphroditism (review in Bauer 2002).

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Fig. 1. *Lysmata wurdemanni*. Behavioral responses of male shrimps (out of 20) to the supernatants of 500 and 1000 Dalton (500 S, 1000 S) filtrations of moulting water, and 500 and 1000 Dalton filtrate (500 F, 1000 F), female moult water (FMW), and seawater (SW). No significant difference ($2 \times 2$ G-test, $P > 0.05$, $G_{adj} = 2.432$) among SW, 1000 S, and 500 F, and among FMW, 1000 F, and 500 S; numbers of males displayed approach or follow in FMW, 1000 F, and 500 S were significantly higher ($2 \times 2$ G-test, $P < 0.001$, $G_{adj} = 24.611$) than SW, 1000 S, and 500 F.
Fig. 2. *Lysmata wurdemani*. HPLC chromatogram of the supernatant of 500 Dalton of female moult seawater of *Lysmata wurdemanni*. A Lichrosphere™ RP18 (C18) column (250 × 4.6 mm; Phenomenex) was used with ultra-pure water (isocratic, 1.0 ml min⁻¹) as mobile phase (UV detection at 190 nm - 400nm).
Fig. 3. *Lysmata wurdemai*. UV spectrum of the largest peak at 2.86 min to show the maximum absorbance at 274 nm.
Chapter 3

UTP is able to elicit courtship behaviour of two marine shrimps, *Lysmata wurdemanni* and *L. boggessi*
Abstract

Sex pheromones are known to control pre-mating pair formation in a number of crustaceans but to date their chemical nature remains largely unknown. Here we report a series of behavioural bioassays to examine whether the recently identified sex pheromone of the shore crab, *Carcinus maenas*, uridine-5'-di-phosphate (UDP), and the related nucleotide uridine-5'-tri-phosphate (UTP) or their mixtures elicit male mating behaviour in two species of peppermint shrimps (*Lysmata wurdemanni* and *L. boggessi*). Our results show that the two shrimp species responded to UTP with male mating behaviour, but not to UDP or mixtures of UDP and UTP. The minimum effective concentration of UTP to the two shrimp species was between $10^{-6}$ and $10^{-7}$M, a level comparable to the shore crabs. The results suggest that UTP may be a major component of the sex pheromone bouquet in the two shrimp species. Male *L. boggessi* did not respond to the moulting water of female *L. wurdemanni*, and the number of male *L. wurdemanni* responded to moulting water of female *L. boggessi* was low. This suggests the existence of other species-specific components in the sex pheromone bouquet that in combination with UTP ensure species specificity.
Introduction

It has been well documented that in many decapod crustaceans (Dunham 1978, 1988 for reviews), such as crabs (e.g. Ryan 1966; Gleeson 1980; Seifert 1982; Hardege et al. 2002; Kamio et al. 2002), lobsters (Atema 1984 for a review), crayfishes (e.g. Ameyaw-Akumfi & Hazlett 1975; Tierney 1984; Stebbing et al. 2003), rock shrimp *Rhynchocinetes typus* (Díaz & Thiel 2004) and several shrimp species in the genus *Lysmata* (Giri 2002; Zhang & Lin 2006; Zhang et al. 2007), the females emit distance (soluble) pheromones to attract mating partners. In *Lysmata* species, distance pheromones elicit a pre-copulatory behaviour, i.e. searching behaviour (Zhang & Lin 2006). To date, efforts to chemically characterize such distance pheromones of crustaceans have been made in lobsters (e.g. Atema & Gagosian 1973; Gagosian & Atema 1973), crabs (e.g. Gleeson et al. 1984; Asai et al. 2000; Hardege et al. 2002; Kamio et al. 2002) and amphipod *Microdeutopus gryllotalpa* (Borowsky et al. 1987), but little progress has been made. Sex pheromones in the hair crab *Erimacrus isenbeckii* were identified as ceramides; however behavioural assays did not support this (Asai et al. 2000), nor the initial hypothesis (Kittredge 1971) that the moulting hormone 20-hydroxyecdysone would function as sex pheromone. The sex pheromones in helmet crabs *Temessus cheiragonus* (Kamio et al. 2002) and in the shore crab *Carcinus maenas* are molecules of less than 1000 Daltons (Hardege et al. 2002, Hayden et al. 2007), and described as being highly polar in *M. gryllotalpa* (Borowsky et al. 1987). Recently, a nucleotide, uridine-5’-di-phosphate (UDP) was identified as the main component of the sex pheromone bouquet in the crab *Carcinus maenas* (Bublitz 2007). Other crustaceans including the snow crab (*Chionoecetes opilio*) and yellowline arrow crab (*Stenorhynchus sticornis*) also display mating behavioural response to UDP (Bublitz et al. 2008). UDP and the related
triphosphate uridine-5’-tri-phosphate (UTP) are linked to the pathway of chitin biosynthesis (Stevenson 1972) in decapod crustaceans and are released during moulting, one and/or both of them or their mixture may serve as sex pheromone in a number of species where reproduction is linked with the female moulting. We recently investigated the waterborne odour cues in two Lysmata shrimp (L. wurdemanni and L. boggessi) and found peaks in chromatograms of urine samples that have similar retention times and UV spectra as to these nucleotides in the bioactive fractions (Zhang et al. unpublished data). In this study we therefore ran a series of bioassays to test whether the two Lysmata shrimp species (L. wurdemanni and L. boggessi) exhibit pre-copulatory behavioural response to UTP and/or UDP.

**Materials and Methods**

*Animal maintenance.* Lysmata wurdemanni and L. boggessi were collected at Sebastian inlet and Hernando Beach, Florida, USA, respectively. They (2.6 – 4.0 cm total length) were individually maintained in 20-L tanks with flow-through seawater of 35‰ salinity and 26-28 °C temperature and fed frozen adult Artemia sp. once daily.

*Chemical stimuli.* UTP and UDP were purchased from Sigma Chemical Co. UTP, UDP and their mixtures in three ratios (80%UTP+20%UDP, 50%UTP+50%UDP, 20%UTP+80%UDP) at 10⁻⁴ M were prepared. Female conditioned seawater (500 ml) in which euhermaphrodite-phase (EP) shrimp of Lysmata wurdemanni and L. boggessi moulted was also tested. Regular seawater served as control.
**Determination of minimum active stimulus concentration.** Because male shrimp did not display behavioural responses to UDP, we focused on determining the minimum concentration of UTP required to elicit a behavioural response in male shrimp. This was done by determining the concentration-response for 30 individuals. Shrimp were presented with log-step concentrations (descending from $10^{-4}$ M till no behavioural response was observed) of each individual chemical compound. Regular seawater served as control. For this test, no individuals were used more than once.

**Behavioural bioassay.** Behavioural responses of the shrimp to soluble sex pheromone, as well as the method for determining and quantifying those behaviours have been described in a previous study (Zhang & Lin, 2006), but were slightly modified in this study. Two male-phase (MP) with different size and one intermoult EP shrimp were used to serve the male role in this assay. Different size and status of shrimp made them be identified easily. No individual shrimp was used more than once. To simplify the procedure, males were used to represent the male-role throughout the paper hereafter. Bioassays were conducted in rectangular tanks ($20 \times 40 \times 24$ cm) containing 6-L of seawater. One day before the observations, males to be tested were housed in the tank with aeration and fed with *Artemia* sp. nauplii to let them acclimate to the environment. Control seawater was presented first, followed by the individual compounds, or mixtures, or female conditioned seawater. The experiments were undertaken blind with the experimenter not informed as to the stimuli being tested. Water was added (2-3 drops/second) near (4 – 5 cm away) the tested male through a tube of 3.0 mm inside diameter. If the male approached the tube, then the tube was moved slowly around the male to see whether the male would follow the movement. Thirty males for each stimulus and control seawater were tested and responses displayed by the shrimp were recorded with a Sony camcorder and analyzed.
Positive responses of male shrimp were defined as approach and follow: male would approach the tube and stay seconds to over 10 seconds, and some males may follow the movement of the tube. A 2×2 Fisher’s test was applied to test the homogeneity of male’s response (number of male displayed positive response) to regular seawater, stimuli, and female conditioned water (Sokal & Rohlf 1995).

**Results**

Most male shrimp of both species displayed the approach response when exposed to UTP, the three mixtures of UTP and UDP, and the conspecific female moult water whereas no response to UDP or control seawater occurred (Fig. 1). There was a significant degree of heterospecificity as although male *Lysmata boggessi* did not show approaching response to the moult water of female *L. wurdemanni*, 13 of the 30 male *L. wurdemanni* did respond to the moult water of female *L. boggessi* (Fig. 1).

The number of males responding to UTP solutions decreased with the decreasing concentration of this nucleotide within the UTP/UDP mixtures presented (Fig. 2). The minimum effective concentration of UTP to induce the mating behaviour in males of the two shrimp species was between $10^{-6}$ and $10^{-7}$ M (Fig. 2).

**Discussion**

The results presented in this study suggest that UTP may be a major component of the soluble sex pheromone in the two shrimp species, *Lysmata wurdemanni* and *L. boggessi*. UTP attracts male *Lysmata wurdemanni* and *L. boggessi* and leads a courtship behaviour, approach and follow, which is exactly the same as the behaviour that female moulting water elicits. Recently, a closely related nucleotide, UDP, the
dephosphorylation product of UTP in energy consuming reactions, has been identified as the main component of the sex pheromone in the crab, *Carcinus maenas* (Bublitz 2007). Interestingly UDP is not entirely species specific and also evokes sexual behaviour in other crab species, such as the snow crab (*Chionoecetes opilio*) and yellowline arrow crab (*Stenorhynchus sticornis*) (Bublitz et al. 2008). However, *L. wurdemanni* and *L. boggessi* did not show positive responses to UDP even at a relatively high concentration of $10^{-4}$ M. This suggests that decapod crustaceans may have developed similar strategies in production of their sex pheromones. Both UTP and UDP are linked to the pathway of chitin ($\beta$-1,4 linked glucose) biosynthesis during moulting process (Stevenson 1972). During the last step of the chitin biosynthesis nucleotides function as phosphate donors for the enzymatic reactions of UDP-acetylglucosamine into chitin, which produces UDP (Merzendorfer & Zimoch 2003). Decapod crustaceans, especially those species where reproduction is linked to ecdysis using either UTP or UDP, a mixture of both, or related compounds as their sex pheromones. For example, although UDP is the main component of the sex pheromone in *C. maenas* (Bublitz 2007), UTP also elicits some degree of sexual behaviour in shore crabs albeit at a significantly higher threshold concentration (Fletcher 2008). The effective concentration of pheromones that aquatic animal release is described to vary from $10^{-6}$ to $10^{-7}$ M (Wyatt 2003). Result from the test with different UTP concentrations suggests that biologically potent pheromones that *Lysmata* shrimp release are minimum at $10^{-6}$ - $10^{-7}$ M, which is similar to other aquatic animals.

Male *L. boggessi* did not respond to the interspecific female moulting water, and the number of male *L. wurdemanni* responded to interspecific female moulting water was markedly reduced, suggesting there might be other specific components in the sex pheromone bouquet of individual species that in combination with UTP to make a species-specific blend. Animal sex pheromones are generally not singular. Individual
components of a pheromonal blend may lack behavioural activity, but mixtures of 
individual components in different ratios may lead to the blend highly specialized 
(Sorensen 1996). Consequently sex pheromones of many species are a species-specific 
blend, such as in insects (e.g., Glover et al. 1987, Christensen et al. 1989, Danci et al. 
2006, Geiselhardt et al. 2008) and goldfish (Poling et al. 2001). Although mixing UTP 
and UDP is the simplest way to form a blend, in our study the mixtures of UTP and UDP 
did not show stronger combined effect on eliciting the male’s approach behaviour in the 
two shrimp species. Alternatively, UTP might be only one compound of the sex 
pheromone bouquet of the two species, or similar to the natural cue in one or the two 
species and bioactive through structural similarities activating a pheromone receptor if 
they use single compound as sex pheromone. Whether there are more than one 
component in the sex pheromone bouquet of the two species needs to be investigated in 
the future. Our current data represent the first identification of a sex pheromone in marine 
shrimps and supports the theory that simple nucleotides can function as infochemicals in 
marine invertebrates, especially if they are reaction specific cues that enable a receiver to 
detect a physiological state of the sender, here moulted shrimp. As such our study will 
open opportunities to investigate the mechanisms how such cues work, weather the 
release of physiologically expensive nucleotides as signals qualifies these as honest 
signals and make progress towards an understanding how marine chemical signals have 
evolved.

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component close-range sex pheromone in the parasitic wasp *Glyptapanteles*


Fig. 1 Number of male *Lysmata wurdemanni* (LW) and *L. boggessi* (LB) out of 30 responded to UTP, UDP, the mixtures of UTP and UDP, conspecific and interspecific female moult water. 1 = UTP, 2 = UDP, 3 = 80% UTP+20% UDP, 4 = 50% UTP+50% UDP, 5 = 20% UTP+80% UDP, 6 = LW female moult water, 7 = LB female moult water, 8 = regular seawater (control). *: P < 0.05 (Fisher’s test, compare to the control).
Fig. 2 Number of male *Lysmata wurdemanni* (LW) and *L. boggessi* (LB) out of 30 responded to UTP of different concentrations. 1 = $10^{-4}$ M, 2 = $10^{-5}$, 3 = $10^{-6}$ M, 4 = $10^{-7}$ M, 5 = Control (regular seawater). *: P < 0.05 (Fisher’s test, compare to the control).
Chapter 4

Primary components of a contact pheromone bouquet in the simultaneous hermaphroditic shrimp, *Lysmata boggessi*
Abstract

It is known from behavioral evidence that for reproduction some male caridean shrimp identify conspecific females via contact pheromones on the female body surface. In this study, we demonstrate direct chemical evidence for the presence of contact pheromones in a decapod crustacean, *Lysmata boggessi*, a protandric simultaneous hermaphroditic shrimp. Extracts from the exoskeleton of newly molted and intermolt euhermaphrodite-phase (EP) and male-phase (MP) shrimp were analyzed with gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). EP and MP shrimp share a series of extractable cuticular compounds. Two major chromatographic peaks exist in the extracts of newly molted EP shrimp that are absent in those of intermolt EP, MP, and newly molted MP shrimp. One of the identified compounds present in the cuticular extracts of newly molted EP shrimp is (Z)-9-octadecenamide. Behavioral assays indicate that male-role shrimp (MP or EP) respond to cuticular extracts of newly molted EP shrimp, of which (Z)-9-octadecenamide is the major active component with hexadecanamide and methyl linoleate enhancing the efficacy of the bioactivity. Our results confirm that contact pheromones, in addition to distance pheromones, are involved in mediating the mating behavior of *L. boggessi* and provide the first evidence of the primary components’ molecular structures.
Introduction

Sex pheromones play important ecological roles in animal behavior, such as for mate and kin recognition, sexual selection, and preventing gene exchange among individuals from different populations and species (e.g. Higgin et al. 2000; Shine et al. 2002; Howard et al. 2003). The role of sex pheromones in sex recognition of many invertebrates, including crustaceans (e.g. Dunham 1978, 1988), has been well documented. Two principle classifications of sex pheromones, distance and contact, exist, and examples have been identified in an ever growing number of species (see review in Wyatt 2003). In terrestrial animals, distance pheromones are usually air transmitted and, as such, are volatile compounds, while contact pheromones are coated on the body surface (see review in Wyatt 2003). In aquatic animals, such as crustaceans, distance pheromones are required to be water soluble (polar compounds) for transmission and contact pheromones need to be relatively insoluble in water (non-polar) to remain on the exterior surfaces. In decapod crustaceans, such as crabs (Ryan 1966; Gleeson 1980; Seifert 1982; Hardege et al. 2002, Bublitz et al. 2008), lobsters (Atema 1984 for a review), and crayfish (Ameyaw-Akumfi & Hazlett 1975; Tierney et al. 1984), the urine of females contains a pheromone that acts over a distance to attract male mating partners. Some caridean shrimp (Burkenroad 1947; Kamiguchi 1972; Bauer 1979; Caskey & Bauer 2005) and copepods (Snell et al. 1995) use contact pheromones in mate recognition. Recently, behavioral evidence suggests the existence of both water soluble (distance) and insoluble (contact) pheromones in *Lysmata wurdemanni* and *L. boggessi* (Zhang & Lin 2006).

The reproductive system of *Lysmata* species, a protandric simultaneous hermaphrodite, is rare among the decapod crustaceans (Bauer 2000). A shrimp first matures as a male (male phase - MP), but with growth may change into a
euhermaphrodite. These euhermaphrodite-phase (EP) shrimp have both male and female functions and can mate as a female during post-molt and as a male during inter-molt (Bauer 2000). Male-role *L. boggessi* (MP or EP) have an active pre-copulatory behavior that indicates these shrimp use distance pheromones to track and locate receptive females (Zhang & Lin 2006). However, male-role shrimp use contact pheromones to recognize newly molted EP (reproductively female) shrimp (Zhang & Lin 2006), thus playing a key role in intra- and inter-specific mate recognition in this species of *Lysmata*. It remains to be investigated in other members of the genus.

Contact sex pheromones are well known and have been identified as cuticular hydrocarbons in a variety of insect species including *Drosophila* and a variety of beetles (e.g., Linn & Roelofs 1995; Etges & Jackson 2001; Zhang et al. 2003; Sugeno et al. 2006); they have not been well investigated in crustaceans. Currently, a contact pheromone found to be a glycoprotein has been identified in the harpacticoid copepod, *Tigriopus japonicus* (Ting et al. 2000). However, glycoproteins are not the contact pheromones in *Lysmata* shrimp (Zhang et al. 2010). Here we hypothesize that the contact pheromones of *Lysmata* shrimp are cuticular hydrocarbons as found in insects (Etges & Jackson 2001). In our study, we analyzed cuticular extracts of *L. boggessi* using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) to identify the active components providing direct evidence for contact pheromones in the shrimp.

**Methods and Materials**

*Lysmata boggessi* shrimp were collected from Hernando Beach, Florida, U.S.A. The male phase (MP) and euhermaphrodite-phase (EP) shrimp (2.6 - 4.4 cm) were housed in 20-L tanks with flow-through seawater (35‰; 26 - 28 °C) and photoperiod of 14 h L/10 h D, and were fed frozen adult *Artemia* once daily.
Extraction

From our hypothesis that the contact pheromones are non-polar hydrocarbons, HPLC grade hexane (Sigma Chemical Company) was chosen as the extraction solvent as has been previously described for insects (e.g. Etges & Jackson 2001; Ginzel et al. 2003; Zhang et al. 2003; Sugeno et al. 2006). Cuticular compounds from the abdominal region of the exoskeletons of both newly molted and intermolt MP (IMP) and EP (IEP) shrimp were extracted through immersion in 1.5 mL hexane for 30 seconds. Ten newly molted MP (NMMP) or EP (NMEP), and 20 – 30 IMP or IEP shrimp were used for extraction. The pooled extracts (based on molt stage and phase) were stored in 20-mL glass vials with polyethylene caps at –20 ºC. Prior to GC analysis, the solvent in the samples was removed by rotary evaporation at 30 ºC, and the residue was re-dissolved with 1 mL hexane. The same extraction procedure was also carried out using HPLC grade ethanol (Sigma Chemical Company).

Results from the hexane extract indicate that (Z)-9-octadecenamide is the major component of the contact pheromones of *L. boggessi*. However, synthetic solid (Z)-9-octadecenamide is not readily soluble in hexane. Although (Z)-9-octadecenamide in the extract of IEP shrimp is absent (see Results), we assume that (Z)-9-octadecenamide still exists in cuticle of the shrimp. To examine the hypothesis, we extracted the pheromones using ethanol as well to compare to the hexane extracts. The same procedures were applied as that in hexane extract.
Chemical analysis

GC analysis was accomplished with a HP 6890 system. Components were detected using a flame ionization detector. A 2 μL aliquot of the extract was injected onto a fused-silica capillary column (HP-5; 30 m × 0.25 mm I.D. × 0.25 μm film thickness; Agilent Technologies) in split-less mode. Helium was used as the carrier gas (1.5 mL/min). Oven temperature was initially held at 150 ºC for 2 min, increased to 300 ºC at 10 ºC/min and finally held at 300 ºC for 20 min. GC-MS was conducted with a HP 6890 system using the same chromatographic protocol described above coupled to a HP 5973 mass selective detector (EI, 70eV). Peaks were identified by comparison with NIST-library spectra.

Behavioral bioassay

The purpose of the behavioral assay was to test the bioactivity of the hexane-extractable cuticular compounds. Only MP shrimp were used and no individual was used more than once. Bioassays were conducted on single MP shrimp in 10-L containers with seawater (35‰; 26 - 28 ºC) under fluorescent illumination. Plastic tubes 3 mm in diameter and 2 cm in length were treated with extracts from NMEP shrimp, NMMP shrimp, or hexane (as control) by immersion and then allowed to dry for 15 seconds. One tube (randomly selected) was placed in a container described above. Thirty replicates were carried out for each treatment and the control. The behavioral response of the MP shrimp to the treated tube was observed using fluorescent illumination and recorded with a Sony camcorder. A positive response was scored if the MP shrimp grasped the extract treated tube, as it does when encountering a NMEP shrimp (Zhang et al. 2009). The number of positive responses
to extract-treated and hexane-treated (control) tubes were compared using $2 \times 2$ G-tests with William’s corrections for significance analysis (Sokal & Rohlf 1995).

Synthetic analogues of candidate compounds identified through GC and GC-MS of hexane and ethanol cuticular extracts (e.g. (Z)-9-octadecenamide (Cayman Chemical Co.), methyl linoleate, hexadecanamide, and squalene (all from Sigma Aldrich)) were also tested both singly as well as in blends using the above bioassay. Because of low solubility of (Z)-9-octadecenamide in hexane, 0.5 mg was first dissolved in 0.1 mL ethanol and 1.4 ml hexane was then added. Blank samples containing the same ratio of ethanol and hexane were used as controls.

**Results**

**Behavioral response to hexane extracts**

After the extract-treated tubes were placed into the test container, shrimp did not display any search behavior. The extract treated tubes were recognized only after being touched by the antennae or antennules. After detecting the extract-treated tubes, MP shrimp approached slowly, touched and then attempted to grasp them. For tubes treated with hexane, most MP shrimp did not show the grasping behavior even if they swam across the tubes. Twenty-two of the 30 MP shrimp displayed the grasping behavior to the NMEP-extract-treated pipes, significantly higher than the numbers of the MP shrimp grasping the NMMP-extract-treated (4 of 30, $G_{adj} = 23.160$, $P < 0.01$) and the control pipes (3 of 30, $G_{adj} = 26.514$, $P < 0.001$). There was no significant difference between NMMP-extract and the control ($G_{adj} = 0.151$, $P > 0.05$) (Table 1).
Gas chromatograms and chemical identification

Gas chromatograms of cuticular hexane-extracts of newly molted euhermaphrodite-phase shrimp (NMEP) were different from those of (IEP), IMP and NMMP shrimp, whereas there were no differences among the latter three (Fig. 1). EP and MP shrimp shared some components, but differed in others (Table 2, Fig. 1) and have six major components in the cuticular extracts. Of which, three were identified as lipophilic esters (Table 2).

Ten major components from ethanol extracts were identified as lipophilic esters (Table 2). GC chromatograms (Fig. 2) indicate there is no difference in chemical compositions in ethanol extracts of NMEP, NMMP, and IEP shrimp. Ratios among major peaks in NMMP and IEP are similar, however hexadecanamide (Peak 3), (Z)-9-octadecenamide (Peak 7) and methyl linoleate (Peak 2) ratios differed between NMEP and NMMP shrimp.

Behavioral response to possible bioactive components and the blends

For the treatments testing the bioactivity of single compounds, only (Z)-9-octadecenamide (14 of 30 MP shrimp) elicited positive responses significantly different from the control (4 of 30 MP shrimp) ($G_{adj} = 8.035, P < 0.01$). Both hexadecanamide (4 of 30 MP shrimp) and methyl linoleate (6 of 30 MP shrimp) were not significantly different from the control ($G_{adj} = 0.000, P > 0.05$ and $G_{adj} = 0.459, P > 0.05$, respectively) (Table 1).

All blends containing (Z)-9-octadecanamide resulted in positive responses (blend with squalene, 16 of 30 MP shrimp, $G_{adj} = 11.043, P < 0.01$; blend with
hexadecanamide and methyl linoleate, 19 of 30 MP shrimp, $G_{adj} = 16.448, P < 0.01$).
Interestingly, not only were the number of shrimp responding greater, but the average
time spent grasping the blend-treated tubes was also significantly longer than (Z)-9-
octadecenamide by itself with $2.60 \pm 0.40$ s for (Z)-9-octadecanamide, $2.69 \pm 0.31$ s
for the blend with squalene, and $6.05 \pm 1.71$ s for the blend with hexadecanamide and
methyl linoleate ($t$-test, $P = 0.033$, df = 31 data were log-transformed because of
heterogeneity of variances).

**Discussion**

To our knowledge, this is the first direct chemical evidence of a contact
pheromone in a decapod crustacean. Both behavioral bioassays and GC analysis
indicate the presence of contact pheromones in the shrimp, *Lysmata boggessi*. Our
results suggest that (Z)-9-octadecenamide is a major component of the contact
pheromone in EP shrimp of *L. boggessi*; additional compounds, such as methyl
linoleate and hexadecanamide also contribute to the bioactivity of the contact
pheromone bouquet.

The behavioral assays show that cuticular compounds on the surface of the
shrimp are hexane-extractable, and that there are active components (i.e. contact sex
pheromones) in these extracts. GC analysis indicates that some components of the
extracts are exclusively present in NMEP shrimp, but absent in NMMP and IEP and
IMP shrimp, suggesting the presence of sex-related contact pheromones. The number
of MP shrimp responding to a synthetic analogue of one of the components found
only in NMEP shrimp ((Z)-9-octadecenamide) is significantly higher than the control.
Interestingly, IEP shrimp shared the same chemical components with MP shrimp.
Whether this is related to euhermaphroditism is worth investigating in the future.
Although insect cuticles often contain a complex mixture of compounds, only a few may be involved in functioning as contact pheromones (Howard, 1993 for a review). Studies on insects indicate that both the components themselves and their ratios are known to vary between conspecific males and females (e.g., Linn & Roelofs 1995; Zhang et al. 2003; Lacey et al. 2008). Active compounds are sometimes present in both sexes, but may be more abundant on the cuticle of females. For example, of the more than 20 compounds present in the cuticle extract of Asian longhorned beetles, *Anoplophora glabripennis*, only five of them, all of which are more abundant in females than in males, are bioactive compounds (Zhang et al. 2003).

GC chromatograms of both hexane and ethanol cuticular extracts show a similar pattern in *L. boggessi*. In the hexane extracts of the shrimp species, there were only two major components present in the NMEP shrimp (female role) (Peak 4: (Z)-9-octadecenamide; Peak 8: unknown, Fig. 1); the corresponding chromatographic peaks were small or absent in IEP and IMP shrimp (male role). In the ethanol extracts, three major peaks (Peak 7: (Z)-9-octadecenamide; Peak 2: methyl linoleate; and Peak 3: hexadecanamide) were found in NMEP, but are small in NMMP and IEP shrimp. Additionally, the ratios of the three major components were different between NMEP and NMMP shrimp.

The bioactivity of (Z)-9-octadecenamide identifies it as a major component of the contact pheromone; however, greater behavioral responses resulted from blends of (Z)-9-octadecenamide and squalene, as well as blends of (Z)-9-octadecenamide, methyl linoleate and hexadecanamide. This suggests that the contact pheromone is a pheromone bouquet, which also consists of minor components strengthening the bioactivity, the use of a bouquet being a common phenomenon in insect pheromone systems. For example, (Z)-9-heptacosene is the most abundant component of cuticular extracts of female *A. glabripennis*, but does not elicit copulatory activity on its own.
Even when mixed with several other bioactive components, the number of males displaying responses to the synthetic mixture was lower than for the authentic female extract (Zhang et al. 2003).

9-Octadecenamide is a naturally occurring amide of oleic acid. It is found in both plants and animals having first been identified in the cerebrospinal fluid of sleep-deprived cats (Cravatt et al. 1995). While our study is the first to describe (Z)-9-octadecenamide as a pheromone in an invertebrate, the compound has been found in anogenital gland secretions of the giant panda, *Ailuropoda melanoleuca*, which is used to mark scent post (Yuan et al. 2004). Both the carboxylic acid ((Z)-9-octadecenoic acid, oleic acid) as well as the saturated methyl ester (methyl (Z)-9-octadecanoate) analogues is well known chemical cues in a variety of insect orders including Hymenoptera (Tentschert et al. 2002), Lepidoptera (Roelofs & Wolf 1988), and Coleoptera (Yasui et al. 2003).

In plants, (Z)-9-octadecenamide has been isolated from *Zostera marina* (Kawasaki et al. 1998), *Vetiveria zizanioides* (Hunag et al. 2004), and *Clausena lansium* (Zhao et al. 2004). (Z)-9-octadecenamide is known to be a volatile component in algae (Kawasaki et al. 1998; Huang et al. 2004; Zhao et al. 2004) such as the freshwater green alga *Rhizoclonium hieroglyphicum* (Dembitsky et al. 2000). These examples of the use of (Z)-9-octadecenamide make it reasonable to be a component of the contact pheromones since in the field *L. wurdemanni* shrimps eat such algae (Baeza 2006).

Hexadecanamide and methyl linoleate are also found in algae (Kawasaki et al., 1998; Nichols et al., 1968). Neither the individual compounds nor a mixture of the two elicit grasping behavior, however they do when they were blended with (Z)-9-Octadecenamide as minor components of the contact pheromone bouquet.
The results of this study show that the main component of the suite of active cuticular contact pheromones of *L. boggessi* (Z)-9-octadecenamide, is peculiar in its extractability using hexane. (Z)-9-Octadecenamide, is present in the cuticles of intermolt and newly molted EP and MP shrimp, when extracted with ethanol. However, hexane was effective in extracting the compound only from newly molted EP shrimp and not the others. This may be a result of either a chemical or physical difference in the body surface (i.e. chitin) that is associated with a recent molt. This change in the characteristics of the cuticle surface allows (Z)-9-octadecenamide to be extracted with ethanol but not hexane. At this point in time, we can only speculate that a co-secretion (or lack thereof) of an as yet unidentified compound is the more likely reason, thus drastically changing the chemical partitioning of (Z)-9-octadecenamide between the body surface of the different shrimp forms and the hexane extraction solvent. A change in the chitin porosity or density is less likely because of the continued ability of ethanol to extract the compound. Regardless of the reason, this mechanism may be the key to the ability of male-mating shrimp to distinguish between newly molted EP (reproducing as female) and other, non-sexually responsive forms.

Although we provide direct chemical evidence of a contact sex pheromone in *Lysmata boggessi*, additional specific components in the profile for mate recognition remain unknown (e.g. Peak 8 of hexane extract), as such, further identification of other minor active components is required in the future. So far, contact sex pheromones in crustaceans have only been found for copepods (e.g. Ting et al., 2000) and caridean shrimp (the present study). Interestingly, the contact pheromones are different between the two with glycoproteins serving as contact pheromones in the harpacticoid copepod *Tigriopus japonicus*, similar to that in the rotifer (Snell et al.,
and lipids in L. boggessi. Contact pheromones play a key role in intra- and inter-specific mate recognition of Lysmata shrimp (Zhang and Lin 2006). Future studies will aim to identify contact pheromones of other species of Lysmata in order to develop an understanding of the contact pheromone evolution and its role in speciation for this genus.

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Table 1 Number of MP shrimp (out of 30) displaying grasping and touching behavior toward tubes treated with cuticular extracts of newly molted euhermaphrodite-phase (NMEP), newly molted male-phase (NMMP) shrimp, and components of NMEP extract, including (Z)-9-octadecenamide, a blend of (Z)-9-octadecenamide and squalene (blend 1), and a blend of (Z)-9-octadecenamide and methyl linoleate (blend 2).

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
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<tr>
<td>NMEP</td>
<td>22*</td>
<td>8</td>
</tr>
<tr>
<td>NMMP</td>
<td>4ns</td>
<td>26</td>
</tr>
<tr>
<td>Control (hexane)</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>(Z)-9-Octadecenamide</td>
<td>14*</td>
<td>16</td>
</tr>
<tr>
<td>Hexadecanamide</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Blend 1</td>
<td>16*</td>
<td>14</td>
</tr>
<tr>
<td>Blend 2</td>
<td>19*</td>
<td>11</td>
</tr>
<tr>
<td>Control (ethanol-hexane)</td>
<td>4</td>
<td>26</td>
</tr>
</tbody>
</table>

*: Number of MP shrimp displaying touching and grasping in the NMEP, (Z)-9-octadecenamide, and blend treatments is significantly higher ($P < 0.01$) than in the NMMP and control); ns: No significant difference ($P > 0.05$) in number of MP shrimp displaying grasping between the NMMP and control
Table 2 Composition of hexane fractions (A, left column) and ethanol extracts (B, right column) from newly molted male-phase shrimp (NMMP) and newly molted euhermaphrodite (NMEP) of *Lysmata boggessi*.

<table>
<thead>
<tr>
<th>Peak</th>
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<th>NMEP</th>
<th>Compound</th>
<th>Peak</th>
<th>NMMP</th>
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<tbody>
<tr>
<td>P1</td>
<td>+</td>
<td>+</td>
<td>Pentadecyl-2-propenoate</td>
<td>P1</td>
<td>+</td>
<td>+</td>
<td>Ethyl palmitate</td>
</tr>
<tr>
<td>P2</td>
<td>+</td>
<td>+</td>
<td>Unidentified</td>
<td>P2</td>
<td>+</td>
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<td>Methyl linoleate</td>
</tr>
<tr>
<td>P3</td>
<td>+</td>
<td>-</td>
<td>Unidentified</td>
<td>P3</td>
<td>+</td>
<td>-</td>
<td>Hexadecanamide</td>
</tr>
<tr>
<td>P4</td>
<td>-</td>
<td>+</td>
<td>(Z)-9-Octadecenamide</td>
<td>P4</td>
<td>+</td>
<td>+</td>
<td>Ethyl stearate</td>
</tr>
<tr>
<td>P5</td>
<td>+</td>
<td>+</td>
<td>Squalene</td>
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<td>+</td>
<td>+</td>
<td>Methyl eicosapentaenoate</td>
</tr>
<tr>
<td>P6</td>
<td>+</td>
<td>-</td>
<td>Dodecyl octadecanoate</td>
<td>P6</td>
<td>+</td>
<td>+</td>
<td>Methyl eicopentaenoate</td>
</tr>
<tr>
<td>P7</td>
<td>+</td>
<td>+</td>
<td>Unresolved acid ester</td>
<td>P7</td>
<td>+</td>
<td>+</td>
<td>(Z)-9-Octadecenamide</td>
</tr>
<tr>
<td>P8</td>
<td>-</td>
<td>+</td>
<td>Unidentified peak</td>
<td>P8</td>
<td>+</td>
<td>+</td>
<td>Methyl docohexanoate</td>
</tr>
<tr>
<td>P9</td>
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<td></td>
<td>P9</td>
<td>+</td>
<td>+</td>
<td>Squalene</td>
</tr>
<tr>
<td>P10</td>
<td>+</td>
<td>-</td>
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+ = present, - = absent; peak numbers correlate with those shown in Figures 1 (hexane extract) and Figure 2 (ethanol extract)
Fig. 1 Gas chromatograms of hexane extracts of newly molted euhermaphrodite phase (EP) shrimp (top) and intermolt EP shrimp, newly molted male phase (MP) shrimp, and intermolt MP shrimp (bottom, because no difference among the chromatograms, only NMMP’s chromatogram is presented). Peak 1 (pentadecyl 2-propenoate) at t = 7.44 min, peak 2 (unidentified) t = 10.26 min, peak 3 (unidentified) at t = 10.74 min, peak 4 ((Z)-9-octadecenamide) at t = 13.81 min, peak 5 (squalene) at t = 17.32 min, peak 6 (dodecyl octadecanoate) at t = 21.30 min, peak 7 (unresolved organic acid ester) at t = 28.06 min, peak 8 (unidentified, unresolved double peak) at t = 28.16 min.
Fig. 2 Gas chromatograms of ethanol extracts of newly molted euhermaphrodite phase (EP) shrimp and intermolt EP shrimp, newly molted male phase (MP) shrimp, and intermolt MP shrimp. A: represents the chromatograms of intermolt EP shrimp, newly molted MP shrimp and intermolt MP shrimp; B: enlargement showing detail of minor components in chromatograms of newly molted MP shrimp and intermolt MP shrimp; C: enlargement showing detail of minor components in chromatograms of intermolt EP shrimp; D: chromatograms of newly molted EP shrimp. Peak 1 (ethyl palmitate) at t = 10.43 min, peak 2 (methyl linoleate) at t = 12.05 min, peak 3 (hexadecanamide) at t = 12.10 min, peak 4 (ethyl stearate) at t = 12.25 min, peak 5 (methyl eicosapentaenoate) at t = 12.50 min, peak 6 (methyl eicopentaenoate) at t = 13.48 min, peak 7 ((Z)-9-octadecenamide) at t = 13.73 min, peak 8 (methyl or ethyl docohexanoate) at t = 14.98 min, peak 9 (squalene) at t = 17.27 min, peak 10 at t = 20.15 min (unidentified).
Chapter 5

Density-dependent effect on reproductive behaviour of *Lysmata amboinensis*

and *L. boggessi* (Decapoda: Caridea: Hippolytidae)
Abstract

We compared the reproductive behaviours of two protandric simultaneous hermaphroditic species (*Lysmata amboinensis* and *L. boggessi*) that belong to two groups of *Lysmata* shrimp with different morphology, geographical distribution, and density. *Lysmata amboinensis* occurs in tropical waters at low population densities, and *L. boggessi* is found in aggregation in sub-tropical and temperate areas. Reproductive behaviour of *L. boggessi* under two densities and *L. amboinensis* in different habitats were compared. Results show that *L. amboinensis* was much less active during mating than *L. boggessi*. Male shrimp of *L. amboinensis* did not display obvious pre-copulation behaviour. They also took significantly longer to transfer spermatophores and lay eggs after mating than *L. boggessi* shrimp did. For *L. boggessi*, moulting time of female shrimp, copulation time and the interval between moulting and mating were significantly shorter when three male shrimp were present than when only one male shrimp was present. Our study suggests that the reproductive behavioural differences in the two shrimp species are possibly the results of density-dependent effect.
Introduction

It has been well known that behavioural development of a species is determined by both genetic and environmental factors (e.g. reviewed by Alcock, 2001). Density plays a significant role in the evolution of characteristics within populations (reviewed by MacArthur & Wilson, 1967) and affects a number of processes, such as competition (e.g. reviewed by Brown, 1964), population regulation (e.g. Hopper & Crowley, 1996), territoriality (e.g. Elliott, 1994), and a variety of behaviours (e.g. Simmons, 1986; Woolbright et al., 1990; Hopper & Crowley, 1996; Moksnes, 2004). Many theoretical studies (e.g. Anderson, 1971; Anderson & Arnold, 1983; Charlesworth, 1971) have predicted, and empirical studies (e.g. Sokolowski et al., 1997) have verified that behavioural polymorphism caused by density-dependent selection is heritable. For example, two types of foraging behaviours in *Drosophila melanogaster* larvae are density-dependently selected (Sokolowski et al., 1997). On the other hand, many density-dependent behaviour changes may be due to phenotypic plasticity (e.g. Greenfield & Shelley, 1985; Woolbright et al., 1990; deRivera et al., 2003). For example, males of woodfrog (*Rana sylvatica*) are less active in the low-density aggregation than in the high density (Woolbright et al., 1990), and both male and female fiddler crab, *Uca beebei*, alter their search rate for mates in response to density change (deRivera et al., 2003).

A fundamental task in ecology and evolution is to understand the origin and maintenance of biological diversity. Although effect of density on intraspecific behaviour has been well known, information about whether interspecific variation in behaviour is density-related is limited. The primary aim of this study was to investigate whether the reproductive behaviours of two *Lysmata* species that usually occur at different densities is density-associated.
Shrimp in the genus *Lysmata* not only attract the interest of aquarium hobbyists because of their striking colour and functions, but also of biologists because they have a rare and unique reproductive system among the decapod crustaceans, protandric simultaneous hermaphroditism (PSH) (Bauer, 2000). A shrimp matures first as a functional male having male external characteristics and may later change to the euhermaphrodite-phase (EP, termed female-phase by Bauer and colleagues, or simultaneous hermaphrodite (SH) by Calado and collaborators) with both male and female functions. Intermoult EP shrimp that functions as a male is able to mate with newly moulted EP shrimp that plays the female role. In *L. wurdemanni* (Gibbes, 1850) and *L. boggessi* (a new species, previously referred to as *L. rathbunae*, ref. Rhyne & Lin, 2006), most male-phase (MP) shrimp pass through four transitional phases (i.e. four transitional moults) to become EP when they reach about 23.0 mm in total length (5.0 - 6.0 mm in carapace length), as external male characteristics gradually disappear (Zhang & Lin, 2005). *Lysmata amboinensis* is also a PSH species (Fiedler, 1998) that changes from MP to EP at about 36.0 mm total length (8.6 mm carapace length) (unpublished data).

The *Lysmata* shrimp may be an ideal model to examine whether a difference in reproductive behaviour associated with density because there is a distinct dichotomy in sociobiology of *Lysmata* species. Low density/pair-living species (e.g., *L. amboinensis* (De Man 1888), *L. debelius* Bruce 1983 and *L. grabhami* (Gordon 1935)) live in tropical waters and are specialized fish cleaners; while group-living species (e.g., *L. boggessi*, *L. seticaudata* (Risso, 1816), and *L. wurdemanni*) live mostly in sub-tropical and temperate areas and are unspecialized, facultative fish cleaners (Wirtz, 1997; Fiedler, 1998; Bauer, 2000). Mating behaviour of *L. wurdemanni* has been well studied (Bauer and Holt, 1998; Bauer, 2002; Zhang and Lin, 2004). Similar behaviour has also been observed in *L.*
Male mating tactics in *L. wurdemanni* and *L. boggessi* can be classified as “pure searching”, since they are continuously “on the prowl” for a receptive female (Correa & Thiel, 2003). When one is encountered, copulation occurs almost immediately after a brief interaction (Bauer & Holt, 1998; Bauer, 2002; Zhang & Lin, 2004). Several pre-copulatory behaviours of *L. amboinensis* have been described based on observations of one pair (Fiedler, 2000). It seems that male-role *L. amboinensis* do not actively search for female-role shrimp as *L. wurdemanni* and *L. boggessi* do. This behavioural difference might be the results of adaptation to their respective social environment (density), i.e. low male competition at low density causes inactive pre-copulatory behaviour. Within species, effects of density and/or operational sex ratio on reproductive activities have been demonstrated in many taxa, such as insects (e.g. Greenfield & Shelley, 1985; French & Cade, 1989; Cade & Cade, 1992), crustaceans (e.g. Debuse et al., 1999; deRivera et al., 2003), and fishes (e.g. Jirotkul, 1999a, 1999b).

In this study, two hypotheses were tested: (1) male *Lysmata amboinensis* do not display obvious pre-copulatory behaviour as *L. boggessi* do (approach, follow and chase); (2) reproductive behaviours of *L. boggessi* are different when a female-role shrimp was with three vs. one male-role shrimp.

**Materials and Methods**

This study was carried out at Florida Institute of Technology’s Vero Beach Marine Laboratory from December 2004 to April 2005. Juvenile *Lysmata amboinensis* shrimp were purchased from a marine life importer and were originally collected in Bali, Indonesia. The shrimp were kept in pairs in 75-liter flow-through maintenance tanks, because the shrimp display aggressive behaviour (Fiedler, 2000) that may lead to mortality when more than two shrimp are housed in a tank without shelter (personal
observation). They were fed with squid and frozen adult *Artemia* sp. once a day. The same water temperature (24.5 – 25.5 °C), salinity (35 ppt) and photoperiod (14 h light and 10 h dark) were maintained for both *L. amboinensis* and *L. boggessi* (see below). The shrimp were grown to EP before being used for the study. Sizes of EP shrimp used for the observation were 36.0 – 62.0 mm total length.

*Lysmata boggessi* shrimp (between 26.0 and 40.0 mm in total length) were collected from the Florida Keys, Florida, U.S.A. and housed in 20-liter maintenance tanks with flow-through seawater. The shrimp were fed in excess frozen adult *Artemia* sp. twice daily.

*Lysmata amboinensis* and *L. boggessi* spawn once every 15 and 12 days, respectively, under about 25 °C (personal observation). Eggs are attached beneath the abdomen of the EP shrimp that usually moult within 24 hours after releasing the larvae. Recently moulted EP shrimp are receptive to mating as female for several hours. EP shrimp function as male during inter-moult. To facilitate observation of mating behaviour, moult cycle of individual EP shrimp serving as female-role shrimp (simplified as female thereafter; MP or EP shrimp serving as male-role shrimp was simplified as male) that was used for mating assay was recorded in advance.

Our preliminary study found that within the size range used in the present study, size difference of male and female shrimp did not affect the mating behaviour. Therefore size effect was not considered in this study.

**Identification of mating behaviour**

Mating behaviour was identified according to the following criteria (Zhang & Lin 2004) and analyzed. The suitor (male) would approach and follow the pre-moult female shrimp. This behaviour begins as early as 8 hr before the female shrimp moult, and is the
most intense within 2 min of the moulting. A male shrimp would approach the female and explore her (via contact) repeatedly with his head/antennules. The behaviour usually lasts from several seconds to minutes. Some male shrimp stay beside the females and mate with them immediately after the females complete the moulting. Pre-copulatory behaviour of male could be divided into three phases: approach (male approach female, and stay aside and face her for a brief period, usually less than 15 s, but do not follow female if she moves away); approach and follow (male approach, and follow female, but may not follow female every time when she moves away); chase (male follow female closely and consistently, especially when female swim quickly within 2 min. prior to moulting).

Male shrimp may follow newly moulted female shrimp consistently after touching the females with antennae/antennules, and grasp the females and bring their ventral surfaces into contact. Normally there is no interaction between male and female shrimp if they encounter each other more than 8 hours before the female shrimp mouls (Zhang & Lin, 2004).

**Mating behaviour of Lysmata amboinensis and L. boggessi**

For each species, two EP shrimp were housed in a 10-liter white bucket with diameter of 23 cm, one about to moult served as female, and the other as male. Mating behaviour in 15 replicate buckets for each species was recorded with a Sony video camcorder using fluorescent illumination and analysed. Behaviour from 10 hours before the female moulting to mating completion was recorded. We observed reproductive behaviour under light, because both species can moult and mate during daytime (personal observation). Also, mating behaviour of a related species, *Lysmata wurdemanni* under light (Zhang & Lin, 2004) does not differ from that observed in the dark under infrared
light (Bauer & Holt, 1998). No food was supplied during the observation because mating shrimp stop feeding several hours before the female moulted (personal observation).

Effect of density/habitat size

For *Lysmata amboinensis*, because of aggressive behaviour and pair-bonded living (Fiedler, 2000; personal observation), density effect was not tested. Instead, mating behaviour in the 75-liter tank was observed and compared with that in the 10-liter bucket to examine the behavioural difference in habitats of different sizes.

For *Lysmata boggessi*, mating behaviour at two densities was observed. Three male were housed with one female shrimp in each of the 15 10-liter white buckets. The mating behaviour was taped with a Sony video camcorder using fluorescent illumination and analysed. The results were compared with those of the 1 male : 1 female ratio (see above).

Statistics

Moulting time of female shrimp, copulation time, interval between moulting completion and mating initiation, interval between mating completion and spawning, and moult cycle (interval between two complete moultings) were measured (mean±s.d.). Student’s *t* test was employed to compare these measurements between the two habitats within *Lysmata amboinensis*, and the two densities within *L. boggessi*. All measurements between *L. amboinensis* and *L. boggessi* in 10-liter bucket are also compared. If the homogeneity of variance assumption was violated, Welch’s approximate *t*-test was used (Sokal & Rohlf, 1995).
Results

*Mating behaviour of Lysmata amboinensis*

In the 10-liter buckets, male shrimp of *Lysmata amboinensis* did not display any obvious searching behaviour (approach, follow and chase) before or after the female shrimp moulted (Table 1). Some male and female shrimp stayed out of reach of each other’s antenna/antennule, and some stayed together without the contact behaviour as shown in *L. boggessi* (male shrimp face female shrimp). Moulting took 45-70 seconds (55.9±6.5 s, n=15) for the female shrimp (Table 2). Copulation lasted 6-20 seconds (10.8± 4.8, n=15) (Table 2) and took place within 0-280 seconds (77.0±78.8 s, n=15) after the female shrimp moulted. Of the 15 replicates, only in one case mating took place immediately (<1 second) after the female shrimp moulted; for the rest, mating occurred within 35-280 seconds after the female shrimp moulted.

In the 75-liter tanks, male and female shrimp generally stayed together on the outlet pipe. Within 30 minutes before the female shrimp moulted, the male shrimp would normally search for the female shrimp if the female shrimp moved away. Male shrimp started searching 1-5 minutes after the female shrimp moved. After catching up with her, the pair would just stay together. The male shrimp never displayed active approach and follow as *L. boggessi* did (see below) before the female shrimp moulted, and the female shrimp did not flee before moulting. Moulting of the female shrimp took 45-64 seconds (56.8±6.1 s, n=15) (Table 2). Mating took place within 32-292 (79.6±82.8, n=15) seconds after the female shrimp moulted. Before copulating, some male shrimp stayed aside the female shrimp for a while, and some even wandered away after antennular contact before coming back. Copulation lasted 5-15 seconds (9.3± 3.8, n=15) (Table 2). All the reproductive behavioural parameters measured were not significantly (for individual *t* value see Table 2; df=28; P > 0.05) different from those observed in the 10-litre buckets.
Mating behaviour of Lysmata boggessi

Pre-copulatory behaviour of *Lysmata boggessi* could be divided into three phases (approach, follow and chase). Male shrimp might approach and follow the female shrimp as early as 8 hours before the female shrimp moulted, but most shrimp did so beginning 2 hours before the moult. The male shrimp swam close to the female shrimp, and touched it with antennules/head repeatedly. Within 30 minutes before the female shrimp moulted, most male shrimp always approach and follow the female shrimp. Within 1-2 minutes before moult, the female shrimp actively swam forward or backward to escape the following male shrimp. Some male shrimp caught up with the female shrimp and waited beside them. They would mate as soon as the female shrimp moulted. Sometimes mating took place even before the female shrimp completed the moult. In addition to approach/follow, some male shrimp would mount on the dorsum of the female shrimp for a few seconds (perching – Bauer, 2002), as observed in other caridean shrimp (Kamiguchi, 1972; Bauer, 1976). If male shrimp did not catch up with the female shrimp before the female shrimp moulted, they may chase the female shrimp after the moult. After moult, the female shrimp swam around. Once male shrimp detected the newly moulted female shrimp with antennules/antennae, they immediately swam close to the female shrimp and tried to grasp them to mate. However, some female shrimp fled, followed by the chasing male shrimp. Mating occurred from 0 to 6 seconds (1.9±1.9, n=15) after the female shrimp moulted under 1 male : 1 female ratio, significantly (*t* = 2.505; df=28; *P*<0.05) longer than that under 3 male : 1 female ratio (0 – 2 s, 0.5±0.7 s, n=15) (Table 2). Under 1 male : 1 female ratio, most (11/15) male shrimp grasped the female shrimp and turned their body under the female shrimp to position their thoraco-abdominal junction beneath the female shrimp’s first abdominal sternite. The other male shrimp (4/15) positioned themselves beside or above the female shrimp. The process of
spermatophore transfer took 4 – 16 seconds (7.4±3.3 s, n=15) under 1 male : 1 female ratio, significantly (t=2.691; df=28; P<0.05) longer than that under 3 male : 1 female ratio (4.8±1.7 s, n=15) (Table 2).

Some female shrimp actively fled to escape the chasing male shrimp within 2 minutes before moulting. Under 1 male : 1 female, the female shrimp spent 20-52 seconds (37.9±8.7 s, n=15) in moulting, significantly (t=3.614; df=28; P<0.001) longer than that under 3 male : 1 female ratio (range of 19–43 s, 27.4±7.1 s, n=15) (Table 2). When the male shrimp were very active or more than one male shrimp interacted with the female shrimp during moulting, the female shrimp often quickly got out of the old exoskeleton by tail-flipping. The female shrimp would refuse to mate more than once, even when multiple male shrimp were presented.

Comparisons of mating behaviours of two species

Major differences of mating behaviour between the two species are summarized in Tables 1 and 2. Male *Lysmata boggessi* displayed the distinct three phases of pre-copulatory behaviour (see Materials and Methods) and chased newly moulted EP shrimp. In contrast, male *Lysmata amboinensis* did not display any obvious pre-copulation behaviour and did not chase newly moulted EP shrimp when they were held in the small habitat (10-L bucket). In the large habitat (75-L tank), follow behaviour of the males was only detected within 30 minutes before female moulting. However, male shrimp did not follow female shrimp immediately after she moved, only started following 1-5 minutes after female shrimp moved away and not always approached and followed female shrimp.
Post-mating

Female shrimp of *Lysmata amboinensis* had significantly (*t*=31.254; df=28; P<0.001) longer intervals from mating to egg laying (9.3±0.6 hr. in 10-liter tank, n=15; 9.4± 0.5 hr. in 75-liter tank, n=15) than those of *L. boggessi* (3.3±0.5 hr. under 1 male : 1 female, n=15; 3.4± 0.5 hr. under 3 male : 1 female, n=15) (Table 2).

Discussion

Results from this study show that *Lysmata amboinensis* and *L. boggessi* displayed different reproductive behaviours. *Lysmata amboinensis* shrimp were much less active during mating than *L. boggessi*. Male *L. amboinensis* did not display obvious pre-copulatory searching behaviour as *L. boggessi* did, and seldom immediately grasped newly moulted female shrimp for copulation even after physical contact by antennules/antennae. *Lysmata boggessi* displayed different reproductive behaviours when one or three male shrimp were present, suggesting that density is a force shaping the reproductive behaviours.

One of the causes for behavioural difference between *Lysmata amboinensis* and *L. boggessi* might be their different living habits and environments. *Lysmata boggessi* and *L. wurdemanni*, a closely related species which displayed similar mating behaviour to *L. boggessi* (Bauer & Holt, 1998; Bauer, 2002; Zhang & Lin, 2004), aggregate in small tide pools and rock jetties in the wild (Bauer, 2000; personal observation), and males vigorously search (follow and chase) the pre- and post-moult female shrimp. *Lysmata amboinensis* is often found in isolated pairs in discrete cavities shared with eels and other cleaning hosts in the wild (Fiedler, 2000). Similarly, a closely related species, *L. grabhami*, also live in pairs with sea anemone or moray eel to reduce predation risk (Wirtz, 1997). Male *L. amboinensis* did not display any pre-copulatory behaviour in the
10-liter buckets. This may be a reflection of the long-term pair-bonded living in cavities where there is no competition for mate and shrimp are confined in close proximity, thus no need to search for the mate. This may be supported by the behaviour in a larger habitat (75-liter tank) in which male *L. amboinensis* did display following behaviour. Moreover, no search or reduced search rate may also be an adaptive response to living at high levels of predation risk. This phenomenon has been demonstrated in the fiddler crab, *Uca bbeebei*, that reduced their search rate for mate when exposed to high levels of predation risk (deRivera et al., 2003).

Although male *Lysmata amboinensis* in the 75-liter tanks did follow the female shrimp within 30 minutes before the female shrimp moulted, the behaviour differed from that of *L. boggessi*. Male *L. boggessi* always approach and follow the female shrimp, especially within 30 minutes before the female shrimp moulted (Table 1). Moreover, male- role *L. amboinensis* in both 10-liter buckets and 75-liter tanks did not mate with newly moulted female shrimp immediately after detecting their presence. This is also different from *L. boggessi* and *L. wurdemanni*. These behavioural differences between the species may be at least partly caused by different social environments. Population density is an important variable that can affect the intensity of competition for resources (e.g. Brown, 1964). Effects of density on reproductive behaviour have been reported in many taxa, such as insects (e.g. Greenfield & Shelley, 1985), decapod crustaceans (e.g. deRivera et al., 2003), frog (Woolbright et al., 1990), and fish (e.g. Jirotkul, 1999b). For example, males of woodfrog are less active in the low-density aggregation (Woolbright et al., 1990). In this study, we found that group size significantly affected the mating behaviour of *L. boggessi*, suggesting that density (therefore competition) has a strong influence on the mating behaviour. The phenotypic plasticity of mating behaviour of *L. boggessi* might predict that mating behaviour of *L. amboinensis* is a result of a long-term
enforcement under low density. Even if we do not know whether the mating behaviour of 
*L. amboinensis* is heritable or phenotypically plastic, and if the ancestors of *Lysmata* 
shrimp were living at low or high density, our results suggest that interspecific variation 
in mating behaviour is density-dependent, i.e. density plays a key role in mating 
behavioural development in *Lysmata* shrimp. Furthermore, these results predict that 
species of *Lysmata* shrimp living at low density probably diverged from an ancestor 
living at high density. When a species that originally lives at high density changed to low 
density living, over time it could be expected that pre-copulatory behaviour of the species 
gradually changed. It is difficult to imagine that *L. boggessi* comes from a low-density 
living ancestor like *L. amboinensis* that fights intensively when more than two shrimp 
live together. Latest molecular evidence supports our suggestions (A. Rhyne, in prep.). 

The lack of competition from other males may eliminate the need for pre- 
copulatory behaviour in *Lysmata amboinensis*. The adaptive value of no pre-copulatory 
behaviour is significant as the behaviour can be costly. In the simultaneous 
hermaphroditic pond snail, *Lymnaea stagnalis*, copulation significantly reduces the egg-
laying rate (Visser et al., 1994). Pre-mating struggling (a pre-copulatory behaviour) of the 
female water striders, *Aquarius remigis*, consume an average 126% more energy 
compared to non-struggling females (Watson et al., 1998). Aggression and calling is 
diminished at high population densities in the field cricket *Gryllus bimaculatus*, because 
males would benefit by silently searching for females (Simmons, 1986). 

Pre-copulation behaviour of male *Lysmata wurdemanni* and *L. boggessi* is 
mediated by distance pheromone (Zhang & Lin, 2006). Similar pheromone may also exist 
in *L. amboinensis* (Fiedler, 2000). Behavioural difference between *L. boggessi* and *L. 
amboinensis* may be caused by the difference in olfactory sensitivity. Number of 
aesthetascs, sensory hair on the outer flagella of the antennules, in *L. amboinensis* is
significantly lower than in the high-density species *L. boggessi* (unpublished data). Number of aesthetascs may be associated with the sensitivity of shrimp to distance pheromone (Beltz et al., 2003). Males of *L. boggessi* with high number of aesthetascs displayed pre-copulatory behaviour earlier, suggesting that sensitivity of olfactory system is associated with number of aesthetascs (unpublished data).

Behavioural evidences indicate that both distance and contact pheromones are involved in the mating of *Lysmata wurdemanni* (Zhang & Lin, 2006). Mating behaviours of *L. boggessi* are similar to those found in *L. wurdemanni*, suggesting that both pheromones are involved as well. Pre-copulatory behaviour (searching) is triggered by distance pheromone, and male shrimp recognize recently moulted female shrimp by contact pheromone. Contact pheromone may also be present in *L. amboinensis*, although only one male shrimp immediately responded to newly moulted female shrimp after contacting by antennules/antennae. That male shrimp of *L. amboinensis* did not grasp the newly moulted female shrimp to mate immediately after physical contact may also be an adaptive response to low density/competition.

Copulated female shrimp did not spawn immediately, possibly because the egg extrusion requires a calcified exoskeleton. In *Lysmata wurdemanni*, when female shrimp copulated three hours after moulting, the eggs would be extruded immediately (personal observation). Female shrimp of *L. amboinensis* had significantly (*t*=31.254; df=28; P<0.001) longer intervals from mating to egg laying than those of *L. boggessi*, suggesting that *L. amboinensis* might take longer to calcify its exoskeleton than *L. boggessi*.

**Acknowledgements**

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the study. Drs. Martin Thiel, Patricio Bravo and P. Koeller, and anonymous reviewers have provided valuable comments on a draft of this manuscript. This project is partially supported by the National Sea Grant (E/INDST-2), NOAA, Department of Commerce, USA, the E-Institute of Shanghai Municipal Education Commission (Project Number: E03009), and the project 5010 of Wenzhou Medical College.

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shrimp genera: Hymenocera and Lysmata. PhD thesis, University of Hawai‘i, Manoa, USA.


**Table 1.** Summary of differences in mating behaviour of male shrimp toward female shrimp between *Lysmata amboinensis* and *L. boggessi*. See the text for detail description of the behaviours.

<table>
<thead>
<tr>
<th></th>
<th>Pre-moult female shrimp</th>
<th>Post-moult female shrimp</th>
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<tr>
<td><strong>L. amboinensis</strong></td>
<td></td>
<td></td>
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<tr>
<td>10-liter bucket</td>
<td>No pre-copulation behaviour.</td>
<td>No chase; male and female shrimp may stay close but male shrimp seldom copulated with female shrimp immediately after female moult.</td>
</tr>
<tr>
<td>75-liter tank</td>
<td>Followed within 30 min before female moult. Male shrimp started following 1-5 min after female shrimp moved away. Not always approached and followed female shrimp.</td>
<td></td>
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<tr>
<td><strong>L. boggessi</strong></td>
<td></td>
<td></td>
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<tr>
<td>1 male : 1 female</td>
<td>Pre-copulatory could be divided into three phases. Pre-copulatory behaviour begins as early as 8 hr before the female shrimp moult. Within 30 min before moulting, male always approached, followed and contacted the female; and chased female 1-2 min before female moult.</td>
<td>Chase or stayed aside female shrimp</td>
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<tr>
<td>3 male : 1 female</td>
<td></td>
<td>Male shrimp copulated with female shrimp as soon as female shrimp completed moult, and finished copulation sooner when 3 male shrimp were present than when on 1 male shrimp was present.</td>
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</table>
**Table 2.** Quantitative comparison of reproductive behaviours (mean±s.d., n=15) of *Lysmata amboinensis* and *L. boggessi*, and of *L. boggessi* at different densities. MT (s): moulting time; CT (s): copulation time; IMM (s): interval between moulting and mating; IMS (hr.): interval between mating and spawning. M refers to euhermaphrodite-phase shrimp functioning as male and F refers to euhermaphrodite-phase shrimp functioning as female.

a: Student’s t-test *t* values for statistic comparison between *L. amboinensis* and *L. boggessi* (1 and 3) at the same sex ratio (1:1) in 10-liter container (IMM were compared using Welch’s approximate *t*-test).

b: Student’s t-test *t* values for statistic comparison between the two densities (1 M : 1 F vs. 3 M : 1 F) within *L. boggessi* (3 and 4).

c: Student’s t-test *t* values for all parameter comparisons within *L. amboinensis* between 10-liter buckets and 75-liter tanks (1 and 2).

DF=28 for all comparisons, compare calculated *t* values with *t*0.05, 28 = 2.048, *t*0.01, 28 = 2.763, *t*0.001, 28 = 3.674 for significance level.

<table>
<thead>
<tr>
<th></th>
<th>MT</th>
<th>CT</th>
<th>IMM</th>
<th>IMS</th>
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<tbody>
<tr>
<td>1. <em>L. amboinensis</em> (10-liter)</td>
<td>55.9±6.5</td>
<td>10.8±4.8</td>
<td>77.0±78.8</td>
<td>9.3±0.6</td>
</tr>
<tr>
<td></td>
<td><em>(t=6.454)a</em></td>
<td><em>(t=2.249)a</em></td>
<td><em>(t'=3.692)a</em></td>
<td><em>(t=31.254)a</em></td>
</tr>
<tr>
<td>2. <em>L. amboinensis</em> (75-liter)</td>
<td>56.8±6.1</td>
<td>9.3±3.7</td>
<td>79.6±82.8</td>
<td>9.4±0.5</td>
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<td></td>
<td><em>(t=0.406)c</em></td>
<td><em>(t=0.934)c</em></td>
<td><em>(t=0.089)c</em></td>
<td><em>(t=0.524)c</em></td>
</tr>
<tr>
<td>3. <em>L. boggessi</em> (1M and 1F)</td>
<td>37.9±8.7</td>
<td>7.4±3.3</td>
<td>1.9±1.9</td>
<td>3.3±0.5</td>
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<td></td>
<td><em>(t=3.614)b</em></td>
<td><em>(t=2.691)b</em></td>
<td><em>(t=2.505)b</em></td>
<td><em>(t=0.412)b</em></td>
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<tr>
<td>4. <em>L. boggessi</em> (3M and 1F)</td>
<td>27.4±7.1</td>
<td>4.8±1.7</td>
<td>0.5±0.7</td>
<td>3.4±0.5</td>
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Chapter 6

Reproductive isolation between two sympatric simultaneous hermaphroditic shrimp, 

*Lysmata wurdemanni* and *L. boggessi*
Abstract

To investigate pre- and post-zygotic isolation between two sympatric and phylogenetically related species of *Lysmata* shrimp, two assays were conducted in the laboratory: (1) no specific mate choice where mating between the two species was “forced”; (2) male competition/female preference where a female had a choice between conspecific and interspecific male. Behavioural studies reveal that female *L. wurdemanni* accepted only conspecific male shrimp, whereas *L. boggessi* female could mate with an interspecific male if there was no conspecific male present. When males of both species were present, *L. boggessi* female always mated with the conspecific male. Male *L. boggessi* in general did not respond to the sex pheromones secreted by female *L. wurdemanni* and did not display any pre-copulatory behaviour to the newly moulted female *L. wurdemanni*. On the other hand, some male *L. wurdemanni* responded to female *L. boggessi*. Although mating was successful between *L. wurdemanni* males and *L. boggessi* females, the resulting embryos lived at most for 10 days and failed to hatch. The results indicate that the two species are both pre-zygotically and post-zygotically isolated. Behavioural observation suggests that chemical cue is mainly responsible for pre-zygotic isolation.
Introduction

Speciation in animals is often characterized by the presence of pre-zygotic (ethological barriers to inter-specific mating) and post-zygotic isolation (infertility and/or non-viability of inter-specific hybrids). Pre-zygotic factors include mating recognition and morphological constraints (e.g. Collins and Tuskes 1979; Gardner 1997; Coyne and Orr 2004). Genetic incompatibility is referred to as the post-zygotic element (Dobzhansky 1937, 1940; Coyne and Orr 2004).

Several factors, including behaviour, genetics, habitat, and morphological characteristics, are generally associated with the natural hybridization process (Gardner 1997 for a review). Barriers to hybridization have been explored in many taxa (Howard and Berlocher 1998 for a review), but little is known in decapod crustaceans. From the limited number of studies it appears that reproductive isolation among decapod crustaceans is generally achieved by pre-zygotic mechanisms, such as behaviour (Smith, 1981; Tierney and Dunham1984; Knowlton et al. 1993; Mathews et al. 2002) and maybe gametic isolation (Misamore and Browdy 1997), i.e. sperms of one species being prevented from entering eggs of another species for fertilization (Coyne and Orr 2004).

Shrimp in the genus *Lysmata* have attracted much attention because they have an unusual reproductive system, protandric simultaneous hermaphroditism (see Bauer 2000 for a review). Several studies have been conducted on their reproductive biology (e.g. Bauer and Holt 1998; Fiedler 1998; Lin and Zhang 2001; Calado and Narciso 2003; Baeza and Bauer 2004; Zhang and Lin 2005a, 2006). To date, all the studies indicate that individuals in the genus first develop into a male-phase (MP) and then may change sex to a euhermaphrodite-phase (EP) [termed female-phase by Bauer and his colleagues (e.g. Bauer and Holt 1998), or simultaneous hermaphrodite by R. Calado (e.g. Calado and
Narciso 2003]) with both male and female functions (Bauer 2000 for a review; Zhang and Lin 2005b).

Recently, *Lysmata* shrimp species living in the western Atlantic waters have been re-defined taxonomically (Rhyne and Lin 2006). Of the six taxonomical sister species, *Lysmata wurdemanni* (Gibbes 1850) and *L. boggessi* Rhyne and Lin 2006 show a partially overlapping distribution in the lower Florida Keys, around the Key West Lakes (Rhyne and Lin 2006). The two shrimp species have similar breeding seasons, reproductive behaviour, and morphology (Rhyne and Lin 2006), and molecular data also suggest that the two are very close in phylogeny (Rhyne et al. unpublished data). Hence, study on the reproductive isolation, pre- and post-zygotic isolation patterns of the two sympatric species would provide important information to better understand speciation patterns in the genus *Lysmata*. A laboratory “forced” inter-breeding assay (where only MP shrimp of one species and EP shrimp of the other species are housed together) indicates that mating can occur between the two species, but no live hybrids are produced (Rhyne and Lin 2006; Zhang and Lin 2006). This suggests that they are probably post-zygotically reproductively isolated. However, previous studies did not focus on the isolation issue, and there were no conspecific hatchings for controls. Not every EP shrimp would be successful in hatching larvae because embryos may be lost before developing to the final stage (personal observation). On the other hand, pre-zygotic isolation may also be involved in speciation process of *Lysmata* shrimp, as pre-zygotic isolation, particularly behavioural isolation, has been suggested being more important than other isolation barriers in causing a rapid speciation (Coyne and Orr 2004 for a review). Because the two *Lysmata* species could copulate with each other and fertilize each other’s eggs, morphological constraints and gametic isolation can be excluded, and
therefore only behavioural mechanisms for pre-copulatory isolation between the two species were considered in this study.

An important component for studying behavioural isolation is identifying the traits involved. It has been realized that chemical and visual, and other cues are involved in behavioural isolation, of which chemical cues are often predominant in many taxa, such as insects (e.g. Collins and Tuskes 1979), reptiles (e.g. snake, Shine et al. 2002), and amphibians (e.g. salamander, Rollmann et al. 2000). Pheromones have been demonstrated to be associated with speciation (e.g. Linn and Roelofs 1995 for a review; Shine et al. 2002) and pheromonal difference among sympatric species may provide the basis for specific recognition and avoidance of inter-specific mating in salamander (Rollmann et al. 2000), lizards (e.g. Cooper and Vitt 1984, 1987), snakes (e.g. Shine et al. 2002), insects (e.g. Collins and Tuskes 1979) and decapod crustaceans (Dunham 1978, 1988 for reviews). In this study, we focused on the role of chemical cues in behavioural isolation.

The primary goals of this study are to answer three questions: (1) whether behavioural mechanisms contribute to reproductive isolation between *Lysmata boggessi* and *L. wurdemani*, (2) if so what kinds of cues were involved, and whether pre-zygotic isolation is complete, and (3) whether post-zygotic isolation is complete. We first compared the mating behaviour between inter-specific and conspecific pairs, thereafter tested whether inter-specific male could compete with conspecific male for mating. Furthermore, we tested the role of sex pheromone (for soluble pheromone using water in which females had moulted; for contact pheromone based on behaviour) in a possible reproductive isolation. For post-zygotic isolation, fertilization and development of embryos after inter-breeding was observed to determine hybrid viability.
Materials and Methods

Animal maintenance and observation

The F1 shrimp of both Lysmata wurdemanni and L. boggessi used in this study were raised in Vero Beach Marine laboratory, Vero Beach, Florida, from broodstock originally collected from Key West Lakes, Florida, U.S.A. The larvae were reared following the procedures described in Calado et al. (2003) and grown to sexual maturity following the protocols described by Zhang et al. (1998). Results of a preliminary assay series indicate that there was no difference in inter-specific mating behaviour between wild-collected and F1 shrimp.

The shrimp, between 2.2 and 3.8 cm in total length (TL), were housed in 20-L buckets with a flow-through system, and were fed in excess with frozen Artemia sp. once a day. Water temperature was maintained at 26.5 to 27.0 °C, salinity at 35 ppt, photoperiod on a 14 h light : 10 h dark cycle with an artificial light source. MP shrimp were used to serve the male role in this study and housed individually for at least four days prior to the test to ensure that they did not have an opportunity to mate with other shrimp. MP and EP shrimp were identified according to Zhang and Lin (2005a).

EP shrimp moult about 12 – 24 h after larval hatching under 26 - 27 °C. When female-role shrimp were about to moult (parturial female), they were moved to a 10-L bucket for behavioural assays. No individual shrimp was used more than once. To simplify, male-role and female-role shrimp are referred to as male and female, respectively, throughout the paper hereafter.

The mating behaviours were videotaped with a Sony camcorder under fluorescent illumination unless stated otherwise. Mating behaviour was recorded under light for two reasons: many female shrimp moult and mate during daytime in the laboratory (personal
observation), and male’s pre-copulatory behaviour under light we observed does not differ from that at night (e.g. Bauer and Holt 1998). The recorded behaviours were analyzed according to the criteria established by Zhang and Lin (2004). A complete mating process includes three pre-moult and two post-moult stages. The three stages of pre-moult behaviour include approach (male approaches female, and stays aside and faces her for a brief period, usually less than 15 seconds, but does not follow if the female moves away) which begins as early as from 8 hr before the female shrimp moults; approach and follow (male approaches and follows female, but may not follow female every time when she moves away); and pre-moult chase (male follows female closely and consistently, especially when female swims quickly around within 2 minutes prior to moulting). Two post-moult behaviours include post-moult chase (male chase the newly moulted female) and copulation.Because of the variations in the frequency that males “flirted” with females (approach, approach and follow, and chase) is high and males in inter-specific mating may not display all five stages as in intra-specific mating, inter- and intra-specific mating behaviour was distinguished by presence or absence of each of the five stages.

Pre-zygotic isolation

Female and male shrimp were housed together in 20-L buckets and acclimated for one day before the mating observation. Male shrimp were always smaller than the females (difference <0.9 cm TL) in the study.

Mating isolation between the two species was tested with two assays: (1) no specific mate choice, in which a female was placed with an inter-specific male, (2) male competition/female preference, in which a female was housed with a conspecific and an inter-specific male.
No specific mate choice

We investigated whether interspecific mating occurred in the absence of conspecific male, and to compare the copulation behaviour between inter-specific and conspecific pairs (controls) in this bioassay. One male shrimp and one parturial female shrimp were placed in each replicate bucket. Twenty replicates of each male-female combination, *Lysmata wurdemanni* male × *L. boggessi* female and *L. wurdemanni* female × *L. boggessi* male, were observed. Twenty replicates of intra-specific combination for each species served as control. The behaviour of male shrimp during pre- and post-moult periods of the females was videotaped.

Because female *Lysmata boggessi* often refused to mate with male *L. wurdemanni* under light (see Results), an additional experiment was conducted to test whether visual cues were also involved in mate discrimination. Twenty pairs for each of the two inter-specific combinations were tested under dark. Same number of intra-specific crossing replicates under dark served as control. Mating successes under dark and light conditions were compared using $2 \times 2$ G-test (Sokal and Rohlf 1995).

Male competition/female preference

We tested whether *Lysmata wurdemanni* males could compete with male *L. boggessi* to mate with female *L. boggessi* in this bioassay. Mating between male *L. boggessi* and female *L. wurdemanni* was largely unsuccessful (Zhang and Lin 2006), so competition of male *L. boggessi* and *L. wurdemanni* for female *L. wurdemanni* was not conducted. Two combinations, first, a female *L. boggessi* with a male *L. boggessi* and a male *L. wurdemanni*, and second, a female *L. boggessi* with a male *L. boggessi* and 3 male *L. wurdemanni*, were conducted. Twenty bioassays were conducted for each combination. The assays were undertaken under light, and behaviours were video-taped.
Degree of pre-zygotic isolation was represented by an index (PII) that was calculated from the equation (Coyne and Orr 1989) for the two series of mating bioassays:

\[ \text{PII} = 1 - \left( \frac{\text{frequency of inter-specific matings}}{\text{frequency of conspecific matings}} \right) \]

This index ranges from 0 (no isolation) to 1 (complete isolation).

Post-zygotic isolation includes two components: hybrid non-viability and hybrid sterility. In this study, we only tested the first component as the hybrids were not viable. All females used for inter-specific mating were mated conspecifically first. After spawning, females were kept individually in a 20-L tank with flow-through seawater (26.5 –27.0 °C). Females with successful hatching were then used in the inter-specific matings. Females with fertilized eggs attached were placed individually in the same system to monitor development of the embryos. If inter-specific mating occurred, sub-samples of the eggs (at least 30 from each shrimp) were removed with forceps and examined under a compound microscope about 6 hr after spawning. If more than 90% of the eggs were fertilized and developing, the mating was considered successful (Zhang and Lin 2004). The developing embryos were monitored until the female’s subsequent moult. The two *Lysmata* species do not self-inseminate; unfertilized eggs are either not attached or are attached briefly to the abdomen and generally lost within a day (Bauer and Holt 1998; personal observation).

**Soluble sex pheromone in pre-copulatory isolation**

An assay followed the method described by Zhang and Lin (2006) was conducted to test the role of soluble (distance) sex pheromone in pre-copulatory isolation. One day
before observation, two male shrimp of different sizes were placed in a rectangular tank (20×40×24 cm) containing 6 L of regular seawater. Then the water in which the conspecific female shrimp (controls) had moulted was introduced. In another tank, two male shrimp of different sizes were exposed to the regular seawater, followed by the addition of water in which the inter-specific female shrimp had moulted. The “moult water” was added (3 drops/second) near (2 – 3 cm away) the tested male through a tube of 3.0 mm inside diameter. If the male approached the tube, then the tube was moved by hand slowly around the male to see whether the male would follow the movement. Ten replicates for each treatment were conducted and responses displayed by the shrimp were recorded with a Sony camcorder and analyzed. Positive response of male to female moult water was defined as approach and follow: male would approach the tube and stay seconds to 10’s of seconds, and some males may follow the movement of the tube.

Statistics

Chi-square test of independence (2 × 2 table) was used to compare the number of male shrimp (out of 20) displayed different behaviour during the mating process between inter-specific and intra-specific pairs, Yates’s correction was applied (Sokal and Rohlf 1995).

Results

Pre-zygotic isolation

*Lysmata boggessi* male largely failed to copulate with *L. wurdemanni* female (Table 1). *Lysmata wurdemanni* male was able to copulate with *L. boggessi* female if *L.
boggessi male was not present. However, L. wurdemanni male(s) was (were) not able to copulate with L. boggessi female if a L. boggessi male was present (Table 1).

No mate choice

Under light and without the presence of male Lysmata wurdemanni, there was no interaction between male L. boggessi and pre-moult female L. wurdemanni. Although there was no typical post-moult behaviour (males chased newly moulted female) as in conspecific mating, male L. boggessi may suddenly grasp the newly moulted female L. wurdemanni when they were in close proximity. Only 2 of the 20 male L. boggessi responded to, and only 1 copulated with, a newly moulted L. wurdemanni female. In contrast, Only 1 of the 20 L. boggessi males did not display pre-copulatory behaviour toward conspecific females. All the others displayed approach, approach and follow, and pre-moult chase behaviours sequentially when the female was about to moult, and pre-moult chase behaviour occurred typically within 2 min prior to female moulting (Figure 1). All the female L. boggessi mated successfully with conspecific males (Table 1).

Under light and without the presence of male Lysmata boggessi, most (16/20) female L. boggessi consistently repelled (female suddenly attacked or chased away male) the male L. wurdemanni when they were in close proximity, and the males did not display the typical approach and follow behaviour until 2 minutes prior to moult of the females, when the females moved around quickly and did not pay attention to the males. Of the remaining 4 pairs, 2 males displayed approach and pre-moult chase behaviour within 2 minutes prior to the female moult when the females did not repel them. Males in the other 2 cases did not display any pre-copulatory behaviour, although the female did not repel them. Forty percent (8/20) of the male L. wurdemanni followed or chased the females within 2 minutes before the parturial females moulted. Thirteen pairs mated
successfully (Table 1). Both inter- and intra-specific males may chase the newly moulted females. Only 2 of the 20 male _L. wurdemanni_ did not display any pre-copulatory behaviour toward conspecific females. Pre-copulatory behaviour of conspecific male was the same as in _L. boggessi_ (Figure 2).

In the dark, 19 of the 20 male _Lysmata wurdemanni_ and female _L. boggessi_ pairs mated successfully, significantly higher than that (13/20) under light (2 × 2 Chi-square test, $X^2_{adj} = 3.906, P < 0.05$) (Table 1). However, there was only 1 (out of 20) successful copulation between male _L. boggessi_ and female _L. wurdemanni_ under both light and dark (Table 1). All conspecific matings were successful and embryos fully developed to hatching in 10-11 days (Table 1).

**Male competition/female preference**

When a male _Lysmata boggessi_ was present, male _L. wurdemanni_ did not display the typical pre-copulatory behaviour toward female _L. boggessi_, because the female _L. boggessi_ repelled male _L. wurdemanni_ (even occasionally male _L. boggessi_). All the 20 female _L. boggessi_ mated with the conspecific males, even though more male _L. wurdemanni_ than male _L. boggessi_ were present. Reproductive isolation was complete (PII = 1) when conspecific males were present (Table 1).

**Post-zygotic isolation**

The eggs of both conspecific pairs were fertilized and developed to hatching in 10-11 days, whereas none of the inter-specific matings resulted in successful hatching although the eggs were fertilized (Table 1).
Soluble sex pheromone

Male *Lysmata boggessi* did not display any response to the moult water of female *L. wurdemanni*. However, 18 of the 20 male *L. boggessi* approached, and even followed the movement of the tube that delivered the conspecific female moult water (controls). Responses of the males to inter-specific and intra-specific female moult water were significantly different ($2 \times 2$ Chi square test, $X^2_{adj} = 29.192, P < 0.001$). In contrast, 10 of the 20 male *L. wurdemanni* responded positively (approach and follow) to the water in which female *L. boggessi* had moulted, significantly lower ($2 \times 2$ Chi square test, $X^2_{adj} = 8.025, P < 0.01$) than that (19 out of 20) displayed positive response to conspecific female moult water, but significantly higher ($2 \times 2$ Chi square test, $X^2_{adj} = 8.025, P < 0.01$) than that (1 out of 20) displayed positive response to regular seawater.

**Discussion**

This study indicates that gene flow between the closely related species *Lysmata wurdemanni* and *L. boggessi* is completely prevented pre- and post-zygotically, and that chemical cues are mainly responsible for the observed pre-zygotic isolation. When conspecific males were present, mate preference completely prevented the inter-specific mating. Even when mating occurred between the two shrimp species, embryos did not develop beyond 10 days, i.e. no viable hybrids were produced.

Behavioural incompatibility is one of the causes resulting in reproductive isolation in decapod crustaceans, such as snapping shrimps (e.g. Knowlton et al. 1993; Mathews et al. 2002) due to not recognizing of each other’s sex signals as the Recognition Concept (Paterson 1985) suggests. Pheromones are among the most important sex signals in animal communication, which has been observed to prevent interbreeding between two species in many animal groups, such as salamanders.
(Rollmann et al. 2000), lizards (e.g. Cooper and Vitt 1984, 1987), snakes (e.g. Shine et al. 2002), crayfishes (e.g. Tierney and Dunham 1982, 1984), as well as insects (e.g. Collins and Tuskes 1979). Differences in pre-copulatory behaviour of inter- and intra-specific shrimps, and test of the female moult water suggests that soluble and contact sex pheromones of *Lysmata boggessi* and *L. wurdemanni* have differentiated during speciation process, and the contribution of the soluble and contact sex pheromones to the reproductive isolation differs between these two species. Although mating behaviours of *L. wurdemanni* and *L. boggessi* are mediated by both distance and contact sex pheromones (Zhang and Lin 2006), soluble pheromones might be more important than contact pheromones in preventing inter-mating between the two species. For example, male *L. boggessi* did not display pre- and post-moult chase of newly moulted female *L. wurdemanni*, suggesting that male *L. boggessi* did not recognize the soluble sex pheromone secreted by female *L. wurdemanni* (Table 1, Figs. 1 and 2). Lower recognition of *L. boggessi* to the contact sex pheromone of *L. wurdemanni* further reduces the possibility of inter-specific mating. Contact sex pheromone may be more important in other caridean shrimp. It has been suggested that only contact chemical cues exist for species recognition during reproduction in caridean shrimp, such as *Palaemonetes pugio* (Burkenroad 1947; Caskey and Bauer 2005), *Palaemon paucidens* (Kamiguchi 1972), *Heptacarpus sitchensis* (Bauer 1979), *Rhynchocinetes typus* (Diaz and Thiel 2004).

Although pheromone recognition between *Lysmata boggessi* and *L. wurdemanni* has been reduced, response of male *L. boggessi* and *L. wurdemanni* to inter-specific females was different. Male *L. wurdemanni* was more active than male *L. boggessi* in searching for inter-specific females (Figs. 1 and 2), suggesting there might be a difference in sensory spectra or response threshold to pheromone between the two species.
(Pfaffmann 1971), i.e. *L. wurdemanni* might have wider sensory spectra than *L. bogessi* does or *L. bogessi* has higher threshold in eliciting behavioural response to chemical signals than *L. wurdemanni* does. Another possibility for the asymmetrical behaviour is that the evolution rate of sex pheromones is different between the two species as that has been found in moth species (Roelofs et al. 2002), i.e. sex pheromones of one species have changed much more than the other one from their ancestor. Therefore the species with lower pheromonal evolution rate might not recognize the other one with higher rate, but the later one can still recognize the former one. All these interesting topics need to be investigated in the future.

Although sex pheromones did not cause complete reproductive isolation between these closely related shrimp species, our bioassays show that presence of *Lysmata bogessi* males totally prevented (PII = 1, Table 1) male *L. wurdemanni* from mating with female *L. bogessi*. Some male *L. wurdemanni* copulated successfully with female *L. bogessi* only when male *L. bogessi* was not present (PII = 0.35 and 0.05 for under light and dark conditions, respectively; Table 1). Pre-copulatory behaviour of male *L. wurdemanni* towards female *L. bogessi* seems to be restrained by the latter, as male *L. wurdemanni* displayed obvious pre-copulatory behaviour towards female *L. bogessi*. The presence of male *L. bogessi* totally repressed the pre-copulatory behaviour of male *L. wurdemanni*, although there was little interaction between the males of the two species. These suggest that mate competition or species preference may be controlled by both chemical and visual stimuli. Male *L. bogessi* did not display any pre-copulatory behaviour towards female *L. wurdemanni*, even though female *L. wurdemanni* did not repel male *L. bogessi*, further suggesting that chemical cues are involved in inter-specific mate recognition. In addition, female *L. bogessi* consistently actively repelled male *L. wurdemanni*, and mating success between female *L. bogessi* and male *L.*
wurdemanni was higher in the dark than under light. These indicate that female *L.
boggessi* might be capable of discriminating between the species utilizing visual cues
when mating occurs under light, as found in other decapod crustaceans such as crayfishes
*Procambarus clarkii* (Dunham and Oh 1996) and *Austropotamobius pallipes*
(Aquistapace et al. 2002), where visual cues are used in short range communication
during mating. Results of male to male competition and mate preference in the present
study also suggest that *L. wurdemanni* and *L. boggessi* had not only developed a set of
signals to prevent inter-specific breeding, but also to enhance the ability of individuals to
maximize their own reproductive success by locating a prospective conspecific mate.

Results from this study indicate that post-zygotic reproductive isolation between
the two shrimp species is complete. Even if inter-crossing between male *Lysmata
wurdemanni* and female *L. boggessi* observed in our laboratory assay system would occur
occasionally in the field, genetic incompatibility between the two species would ensure
no viable hybrids are produced. Hybrids from male *L. wurdemanni* and female *L.
boggessi* crossings stopped developing at 8.3 ± 1.2 (mean ± s.d.) days, and the longest
hybrid embryos lived for 10 days. Post-zygotic isolation seems to be stronger than pre-
zygotic isolation between the two shrimp species. The study of pre- and post-zygotic
isolation patterns would help to understand speciation of the shrimp species. Coyne and
Orr’s (1989, 1997) classic studies of *Drosophila* speciation suggest that different
isolation patterns may represent different speciation processes. Conclusions drawn from
*Drosophila* studies indicate that both pre- and post-zygotic isolation will increase with
divergence time between taxa, and that pre-zygotic barriers evolve faster and than post-
zygotic barriers in sympatric species (Coyne and Orr 1989, 1997). Complete post-zygotic
and incomplete pre-zygotic isolation suggests that the incipient speciation between the
two shrimp species might occur before they live sympatrically. Current overlapping
distribution of the two species may be the secondary contact after pre-zygotic isolation had developed.

Although we cannot be completely certain whether speciation in the shrimp species is sympatric or allopatric, behavioural evidence from this study suggests that the current reproductive isolation between the two species is maintained by species-specific chemical cues (sex pheromones) that elicit courtship of males, and is enforced by the genetic incompatibility between the two species. Future studies should focus on characterization of the chemical nature of the sex pheromones as well as their function in pre-zygotic isolation, which will improve our understanding of the reproductive isolation and speciation in these shrimps.

Acknowledgements

The experiments comply with the current laws of the United States in which they were performed. We thank Chad Zhang for assistance in the laboratory. This study was partially supported by Project 5010 of Wenzhou Medical College.

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Table 1 Mating success, hybrid development, and pre-zygotic isolation.

b = Lysmata boggessi, w = L. wurdemannii; M = male, F = female; * indicates that interspecific mating success is significantly higher in the dark (19/20) than under light (13/20) (2 × 2 chi-square test, $X^2_{adj} = 3.906$, $P < 0.05$). a: indicates that there is no copulation between female L. boggessi and male L. wurdemannii.

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<th>Mating combination</th>
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Fig. 1 Number of shrimp (out of 20) displayed different behaviour during the mating process between interspecific (*Lysmata boggessi* male × *L. wurdemanni* female) and intraspecific pairs (*L. boggessi*). NP = no response, A = approach, F = follow, PreC = pre-moult chase, PostC = post-moult chase, Cop = copulation, FRM = female repelled male; Letters (a, b, c, d, e, k) above the bars represent significance of 2 × 2 Chi square test on behavioural difference of male or female during inter- and intra-specific mating, a: $X^2_{adj} = 324.812, P < 0.001$; b: $X^2_{adj} = 324.812, P < 0.001$; c: $X^2_{adj} = 324.812, P < 0.001$; d: $X^2_{adj} = 324.812, P < 0.001$; e: $X^2_{adj} = 324.812, P < 0.001$; k: $X^2_{adj} = 22.576, P < 0.001$. 
Fig. 2 Number of shrimp (out of 20) displayed different behaviour during the mating process between interspecific \((Lysmata\ wurdemanni\ male \times L.\ boggessi\ female)\) and intraspecific pairs \((L.\ wurdemanni)\). NP = no response, A = approach, F = follow, PreC = pre-moult chase, PostC = post-moult chase, Cop = copulation, FRM = female repelled male; Letters (a, b, c, d) above the bars represent significance of \(2 \times 2\) Chi square test on behavioural difference of male or female during inter- and intra-specific mating, a: \(X^2_{adj} = 8.901, P < 0.005;\) b: \(X^2_{adj} = 8.901, P < 0.005;\) c: \(X^2_{adj} = 6.234, P < 0.005;\) f: \(X^2_{adj} = 23.438, P < 0.001.\)
General Discussion

Results from the present study demonstrate that both distance and contact pheromones are involved in the mating process of *Lysmata wurdemanni*. Diffusible distance pheromones that are used as attractants have been reported in many decapod crustacean species (e.g. Dunham 1978, 1988 for reviews). Distance pheromones trigger the pre-copulatory behaviours in *L. wurdemanni*, as the male approached and followed the female before the female moulted. But the male shrimp depends on contact pheromone to identify the receptive females. Even if the male did not display any pre-copulatory behaviour toward the pre-moult female, it could eventually detect the newly moulted female shrimp via contact pheromone and mated successfully (Zhang and Lin 2004, 2005; present study). Male shrimp did not respond to the pre-moult males and attacked the newly moulted males, suggesting that both distance and contact pheromones are secreted only by female shrimp.

Waterborne sex pheromones eliciting male searching behaviour in shrimps are not as commonly described as in crabs, lobsters and crayfishes. It has been hypothesized that the coordination of the reproduction in most of the shrimps studied (mostly in carideans) relies on contact chemical cues to complete copulation (e.g. Bauer 1979, Cakesy and Bauer 2005). *Lysmata* species are the only known shrimp to depend on both soluble and contact pheromones in controlling their reproductive process (Zhang and Lin 2006, Zhang et al. 2007). Unlike olfactory pheromones, contact pheromone is not common in aquatic animals. So far they have only been demonstrated in rotifers (Gilbert 1963; Snell et al. 1995), a squid (Buresch et al. 2003), and copepods (Ting et al. 2000). Decapod crustaceans with both distance and contact pheromones have not been reported before. Why the shrimp possesses both kinds of pheromones to mediate mating behaviour is unknown. This may be the result of environmental adaptation, as ecology influences the evolution of pheromone communication (Theissen 1977) and mating systems (Emlen & Oring 1977).
Although efforts to chemically characterize pheromones in decapod crustaceans have been made for more than 20 years, the progress is limited. The only identified sex pheromones are ceramides that are thought to be sex pheromones of the hair crab *Erimacrus isenbeckii* (Asai et al. 2000). However, bioassay results do not solidly support this hypothesis and further tests are required to confirm their biological functions. In this study, we partially characterized the soluble sex pheromone for the first time and further confirm the existence of such pheromones in the shrimp *Lysmata wurdemanni*. Bioassay result indicates that the molecular weight of the pheromone molecule is between 500 and 1000 Dalton. This is in accordance with what has been found in decapod crabs (Hardege et al. 2002, Kamio et al. 2002). Molecular size of the pheromone in *Carcinus maenas* (Hardege et al. 2002) and *Telmessus cheiragonus* (Kamio et al. 2002) is less than 1000 Dalton. Recently, uridine-di-phosphate (UDP) was identified to be the sex pheromone of the shore crab, *C. maenas* and was shown to induce mating in a number of decapod crustaceans (Bublitz 2007, Fletcher and Hardege 2009). Further test demonstrates that uridine-5’-tri-phosphate (UTP) is also a component of the pheromone in the crab *C. maenas* (Fletcher 2007). However, *L. wurdemanni* and *L. boggessi* did not show positive response to UDP. The results presented in this study suggest that UTP may be a major component of the soluble sex pheromone in the two shrimp species. UTP attracts male *L. wurdemanni* and *L. boggessi* and leads to courtship behaviour, approach and follow, which is the same as the behaviour that female moulting water elicits.

Male *Lymata boggessi* did not respond to the female moulting water of *L. wurdemanni*, and the number of male *L. wurdemanni* responded to the female moulting water of *L. boggessi* was markedly reduced, suggesting that there might be other specific components in the sex pheromone bouquet of individual species that in combination with UTP to make a species-specific pheromone blend. Animal sex pheromones are generally not singular. Individual components of a pheromonal blend may lack behavioural activity, but mixtures of individual components in different ratios may lead to the highly
specialized blend (Sorensen 1996). Consequently sex pheromones of many species are a species-specific blend, such as in insects (e.g., Glover et al. 1987, Christensen et al. 1989, Danci et al. 2006, Geiselhardt et al. 2008) and goldfish (Poling et al. 2001).

So far, contact sex pheromones in crustaceans are only identified in copepods as glycoproteins (e.g. Ting et al., 2000). Recently, we found *Lysmata* shrimp do not use glycoproteins as contact pheromones (Zhang et al. in press). In this study, both behavioural bioassays and GC analysis indicate the contact pheromones in the shrimp, *L. boggessi*, are hydrocarbons. GC analysis results indicate that some chemical components of the extracts are exclusively present in newly moulted EP shrimp, but absent in newly moulted MP and intermoult EP and MP shrimp, suggesting the presence of sex-related contact pheromones in *L. boggessi*. (Z)-9-Octadecenamide is the major component of the contact pheromones.

(Z)-9-Octadecenamide, the amide of oleic acid, was first identified in the cerebrospinal fluid of sleep-deprived cats, and has also been detected in the cerebrospinal fluid of rats and humans. The analog cis-9-Octadecenamide is a physiological sleep-inducer of rats (Cravatt et al. 1995). (Z)-9-Octadecenamide occurs naturally in plants and has been isolated from *Zostera marina* (Kawasaki et al. 1998), *Vetiveria zizanioides* (Hunag et al. 2004) and *Clausena lansium* (Zhao et al. 2004), and the freshwater green alga *Rhizoclonium hieroglyphicum* (Dembitsky et al. 2000). It also has been identified as a component of anogenital gland secretions of the giant panda, *Ailuropoda melanoleuca*, which is used for marking scent post (Yuan et al. 2004). This is the first report of (Z)-9-Octadecenamide being used as sex pheromone in an invertebrate.

Two mating systems (pure searching and mate guarding) may have been evolved in *Lysmata* shrimp. Results from this study show that *L. amboinensis* (low density/pair living species) and *L. boggessi* (high density species) displayed different reproductive
behaviours. *Lysmata amboinensis* shrimp were much less active during mating than *L. boggessi*. Male *L. amboinensis* did not display obvious pre-copulatory searching behaviour as *L. boggessi* did, and seldom immediately grasped newly moulted female shrimp for copulation even after physical contact by antennules/antennae. This behavioural difference suggests that chemical communication between the two groups of species has diverged.

One of the causes for behavioural difference between *Lysmata amboinensis* and *L. boggessi* might be their different habits and environments. *Lysmata boggessi* and *L. wurdemanni*, closely related species which displayed similar mating behaviour (Bauer and Holt 1998; Bauer 2002; Zhang and Lin 2004), aggregate in small tide pools and rock jetties in the wild (Bauer, 2000), and males vigorously search (follow and chase) the pre- and post-moult female shrimp. *Lysmata amboinensis* is often found in isolated pairs in discrete cavities shared with eels and other cleaning hosts in the wild (Fiedler 2000). Male *L. amboinensis* did not display any pre-copulatory behaviour in the 10-liter buckets. This may be a reflection of the long-term pair-bonded living in cavities where there is no competition for mate and shrimp are confined in close proximity, thus no need to search for the mate. Moreover, no search or reduced search rate may also be an adaptive response to living at high levels of predation risk. This phenomenon has been demonstrated in the fiddler crab, *Uca beebei*, that reduced their search rate for mate when exposed to high levels of predation risk (deRivera et al., 2003).

Pre-copulation behaviour of male *Lysmata wurdemanni* and *L. boggessi* is mediated by distance pheromone (Zhang and Lin 2006). Similar pheromone may also exist in *L. amboinensis* (Fiedler 2000). Behavioural difference between *L. boggessi* and *L. amboinensis* may be caused by the difference in olfactory sensitivity. Number of aesthetascs, sensory hair on the outer flagella of the antennules, in *L. amboinensis* is
significantly lower than in the high-density species *L. boggessi* (Zhang et al. 2008). Number of aesthetascs may be associated with the sensitivity of shrimp to distance pheromone (Beltz et al. 2003). Male *L. boggessi* with high number of aesthetascs displayed pre-copulatory behaviour earlier, suggesting that sensitivity of olfactory system is associated with number of aesthetascs (Zhang et al. in press). Another possibility is that EP shrimp of *L. amboinensis* release low quantity of distance pheromones to attract male role shrimp.

Pheromones are among the most important sex signals in animal communication, which has been observed to prevent interbreeding between two species in many animal groups, such as salamanders (Rollmann et al. 2000), lizards (e.g. Cooper and Vitt 1984, 1987), snakes (e.g. Shine et al. 2002), crayfishes (e.g. Tierney and Dunham 1982, 1984), as well as insects (e.g. Collins and Tuskes 1979). This study indicates that gene flow between the closely related species *Lysmata wurdemanni* and *L. boggessi* is completely prevented pre- and post-zygotically, and that chemical cues are mainly responsible for the observed pre-zygotic isolation. When conspecific males were present, mate preference completely prevented the inter-specific mating. Even when mating occurred between the two shrimp species, embryos did not develop beyond 10 days, i.e. no viable hybrids were produced.

Differences in pre-copulatory behaviour of inter- and intra-specific shrimps, and test of the female moult water suggest that soluble and contact sex pheromones of *Lysmata boggessi* and *L. wurdemanni* have differentiated during speciation process, and the contribution of the soluble and contact sex pheromones to the reproductive isolation differs between these two species. Although mating behaviours of *L. wurdemanni* and *L. boggessi* are mediated by both distance and contact sex pheromones (Zhang and Lin 2006), soluble pheromones might be more important than contact pheromones in
preventing mating between the two species. For example, male *L. boggessi* did not display pre- and post-moult chase of newly moulted female *L. wurdemanni*, suggesting that male *L. boggessi* did not recognize the soluble sex pheromone secreted by female *L. wurdemanni*. Lower recognition of *L. boggessi* to the contact sex pheromone of *L. wurdemanni* further reduces the possibility of inter-specific mating. Contact sex pheromone may be more important in other caridean shrimp. It has been suggested that only contact chemical cues exist for species recognition during reproduction in caridean shrimp, such as *Palaemonetes pugio* (Burkenroad 1947; Caskey and Bauer 2005), *Palaemon paucidens* (Kamiguchi 1972), *Heptacarpus sitchensis* (Bauer 1979).

**Summary**

1. Behavioural evidence indicates that both distance and contact pheromones are involved in the mating process of *Lysmata* shrimp. Pre-copulatory behaviour (i.e. searching) is triggered by distance pheromone, and male shrimp recognize recently moulted EP shrimp by contact pheromone.

2. Molecular weight of the pheromone molecule is between 500 and 1000 Dalton. HPLC fractions between 2-3 min are bioactive. UTP may be a major component of the soluble sex pheromone in *Lysmata wurdemanni* and *L. boggessi* because UTP attracts male of the two shrimp species and leads to courtship behaviour, approach and follow, which is the same as the behaviour that female moulting water elicits.

3. Contact pheromones of *Lysmata boggessi* are hydrocarbons. (Z)-9-Octadecenamide is the major component of the contact pheromones.

4. Chemical communication behaviour between low density/pair living species (*Lysmata amboinensis*) and high density species (*L. boggessi*) has diverged. *Lysmata amboinensis* shrimp were much less active during mating than *L. boggessi*. Male *L. amboinensis* did
not display obvious pre-copulatory searching behaviour as *L. boggessi* did, and seldom immediately grasped newly moulted female shrimp for copulation even after physical contact by antennules/antennae.

5. Pheromones play key role in pre-copulatory reproductive isolation in two sympatric species, *Lysmata boggessi* and *L. wurdemanni*. Soluble and contact sex pheromones of *L. boggessi* and *L. wurdemanni* have differentiated during the speciation process, and the contribution of the soluble and contact sex pheromones to the reproductive isolation differs between these two species.

6. This study provides direct evidence for the first time the use of both distance and contact sex pheromones by *Lysmata* shrimp, and the contact pheromones are characterised for the first time in decapod crustaceans. Methodology established in this study would provide protocols to study sex pheromones of other decapod crustaceans. More data collected from other species in the future would be helpful for understanding of sex pheromone evolution in decapod crustaceans.

**Future studies**

1. Identifying both the distance and contact sex pheromones of *Lysmata wurdemanni* and other *Lysmata* species. This would be helpful to understand pheromone evolution, reproductive isolation and speciation in *Lysmata*, an important group with a rare unusual reproductive system, protandric simultaneous hermaphroditism.

2. Secretion process of distance and contact pheromones in *Lysmata* shrimp should be investigated. In crabs (Hardege et al., 2002, Kamio et al. 2002), lobsters (Atema 1984 for a review), and crayfishes (Stebbing et al. 2003), females emit distance waterborne sex pheromones with the urine to attract mating partners. It is reasonable to believe that *Lysmata* shrimp also use the same way to release the distance sex pheromones. A
technique has recently been introduced to mark the chemical signal in urine by injecting dye to the heart of crayfish (Breithaupt and Eger 2002) and this may be applied to reveal the source of distance sex pheromones of *Lysmata* shrimp.

In insects, such as moths (Raina et al. 2000), there are specialized glands that produce contact pheromones. Whether there are similar glands in *Lysmata* shrimp needs to be investigated in the future. Alternatively, the contact pheromone might be part of the substances normally released during moulting process in crustaceans.

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a protandric simultaneous hermaphrodite (Crustacea: Decapoda: Caridea).

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