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Reproductive isolation between two sympatric simultaneous hermaphroditic shrimp, *Lysmata wurdemanni* and *L. boggessi*

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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) specific mate choice or preference where a female had a choice between conspecific and heterospecific males. Behavioural studies reveal that female *L. wurdemanni* accepted only conspecific male shrimp, whereas *L. boggessi* females would mate with an interspecific male if there was no conspecific male present. When males of both species were present, *L. boggessi* females 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 newly moulted female *L. wurdemanni*. On the other hand, some male *L. wurdemanni* responded to female *L. boggessi*. Although mating was successful between male *L. wurdemanni* and female *L. boggessi*, 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 most likely responsible for pre-zygotic isolation.

Key words: Chemical cues, mating behaviour, reproductive isolation, shrimp, sympatric

Introduction

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

Several factors, including behaviour, genetic, habitat, and morphological characteristics, are generally associated with the natural hybridization process (see Gardner 1997 for a review). Barriers to hybridization have been explored in many taxa (see Howard & 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 behavioural (Smith 1981; Tierney & Dunham 1984; Knowlton et al. 1993; Mathews et al. 2002) or gametic isolation (Misamore & Browdy 1997), i.e. sperms of one species being prevented from entering eggs of another species for fertilization (Coyne & Orr 2004).

Shrimp of 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 & Holt 1998; Fiedler 1998; Lin & Zhang 2001; Calado & Narciso 2003; Baeza & Bauer 2004; Zhang & Lin 2005a, 2006). To date, all the studies

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indicate that individuals in the genus first develop into a male phase (MP) and then may change sex to a euhermaphroditic phase (EP) [termed female-phase by Bauer and his colleagues (e.g. Bauer & Holt 1998), or simultaneous hermaphroditic phase by Calado (e.g. Calado & Narciso 2003)] with both male and female functions (see Bauer 2000 for a review; Zhang & Lin 2005b).

Recently, _Lysmata_ from the western Atlantic Ocean have been revised taxonomically (Rhyne & Lin 2006). Of the six taxonomically related species studied, _Lysmata wurdemanni_ (Gibbes, 1850) and _L. boggessi_ Rhyne & Lin, 2006 show a partially overlapping distribution (Rhyne & Lin 2006). The two species have similar breeding seasons, reproductive behaviour, and morphology (Rhyne & Lin 2006), and molecular data also suggest that the two are very close in phylogeny (Fiedler et al. 2007). Hence, a 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 viable hybrids were produced (Rhyne & Lin 2006; Zhang & Lin 2006). This suggests that they are probably post-zygotically reproductively isolated. However, previous studies did not focus on the isolation issue; pre-zygotic isolation may also be involved in speciation process of _Lysmata_ shrimp. It has been suggested that pre-zygotic isolation, particularly behavioural isolation, may be more important than other isolation barriers in causing a rapid speciation (see Coyne & 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 cues are involved in behavioural isolation, of which chemical cues are often predominant in many taxa, such as insects (e.g. Collins & 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 & Roelofs 1995 for a review; Shine et al. 2002) and pheromonal differences 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 & Vitt 1984, 1987), snakes (e.g. Shine et al. 2002), insects (e.g. Collins & Tuskes 1979) and decapod crustaceans (see 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. wurdemanni_; (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 interspecific and intraspecific pairs, thereafter tested whether heterospecific males could compete with conspecific males for mating. Furthermore, we tested the role of sex pheromones (soluble pheromone using water in which females had moulted) in 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 F$_1$ 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, USA. 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 interspecific mating behaviour between wild-collected and F$_1$ shrimp.

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 26.5–27.0°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 4 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 & 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 20-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 males
Mating behaviours were videotaped with a Sony camcorder under fluorescent illumination unless stated otherwise. Mating behaviours were recorded under light for two reasons: many female shrimp moult and mate during daytime in the laboratory (personal observation), and males’ pre-copulatory behaviour under light does not differ from that at night (e.g. Bauer & Holt 1998). The recorded behaviours were analysed according to the criteria established by Zhang & Lin (2004). A complete mating process includes five stages: three stages of pre-moult and two of post-moult. Because of the variations in the frequency that males ‘flirted’ with females (approach, approach and follow, and chase) is high and males in interspecific mating may not display all five stages as in intraspecific 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 a heterospecific male; and (2) male competition/female preference, in which a female was housed with a conspecific and a heterospecific male.

No specific mate choice

We investigated interspecific mating in the absence of conspecific males, and compared the copulation behaviour between interspecific and intraspecific 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 intraspecific 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 trial in the absence of light was conducted to test whether visual cues were also involved in mate discrimination. Twenty pairs for each of the two interspecific combinations were tested under dark conditions. The same number of intraspecific crossing replicates under dark conditions served as control. Mating successes under dark and light conditions were compared using a 2×2 Chi square test (Sokal & 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 & Lin 2006), so a competition between male *L. boggessi* and male *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.

The degree of pre-zygotic isolation was represented by an index (PII; pre-zygotic isolation index) that was calculated from the equation (Coyne & Orr 1989) for the two series of mating bioassays:

\[
PII = 1 - \left( \frac{\text{frequency of interspecific matings}}{\text{frequency of intraspecific matings}} \right)
\]

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

Post-zygotic 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 interspecific mating were mated intraspecifically first. After spawning, females were kept individually in a 20-L tank with flow-through seawater (26.5–27.0°C). Females with successful hatchings were then used in the interspecific matings. Females with fertilized eggs attached were placed individually in the same system to monitor development of the embryos. If interspecific 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 h after spawning. If more than 90% of the eggs were fertilized and developing, the mating was considered successful (Zhang & Lin 2004). The developing embryos were monitored until the female’s subsequent moult. *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 & Holt 1998; personal observation).

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

An assay following the method described by Zhang & 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 heterospecific 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 analysed. Positive responses of males to female moult water was defined as approach and follow: the male would approach the tube and stay seconds to tens 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) displaying different behaviour during the mating process between interspecific and intraspecific pairs; Yates’s correction was applied (Sokal & Rohlf 1995).

**Results**

**Pre-zygotic isolation**

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

**No mate choice**

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 s, but does not follow if the female moves away) which begins as early as 8 h 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 min prior to moulting). Two post-moult behaviours include post-moult chase (male chases the newly moulted female) and copulation. Under light and without the presence of male *Lysmata wurdemanni*, there was no interaction between male *L. boggessi* and pre-zygotic female *L. wurdemanni*. Although there was no typical post-moult behaviour (males chased newly moulted females) 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, 19 of the 20 *L. boggessi* males display pre-copulatory behaviour toward

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

<table>
<thead>
<tr>
<th>Mating combination</th>
<th>No. of replicate pairs</th>
<th>No. of copulations</th>
<th>Days of embryo development (mean ± s.d.)</th>
<th>Pre-zygotic isolation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>bM × bF (light)</td>
<td>20</td>
<td>20</td>
<td>10.6 ± 0.3</td>
<td>0.00</td>
</tr>
<tr>
<td>bM × wF (light)</td>
<td>20</td>
<td>1</td>
<td>5</td>
<td>0.95</td>
</tr>
<tr>
<td>bM × wF (dark)</td>
<td>20</td>
<td>1</td>
<td>5</td>
<td>0.95</td>
</tr>
<tr>
<td>wM × wF (light)</td>
<td>20</td>
<td>20</td>
<td>10.5 ± 0.4</td>
<td>0.00</td>
</tr>
<tr>
<td>wM × bF (light)</td>
<td>20</td>
<td>13</td>
<td>8.1 ± 1.1</td>
<td>0.35</td>
</tr>
<tr>
<td>wM × bF (dark)</td>
<td>20</td>
<td>19*</td>
<td>8.4 ± 1.3</td>
<td>0.05</td>
</tr>
<tr>
<td>1bF + 1bM + 1wM</td>
<td>20</td>
<td>0</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>1bF + 1bM + 3wM</td>
<td>20</td>
<td>0</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

\(b = Lysmata boggessi, w = L. wurdemanni; 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, \( \chi^2_{adj} = 3.906, P < 0.05 \)). a: indicates that there is no copulation between female *L. boggessi* and male *L. wurdemanni.*
Males displayed approach, approach and follow, and pre-moult chase behaviours sequentially when the conspecific female was about to moult, and pre-moult chase behaviour occurred typically within 2 min prior to female moult (Figure 1). All the female L. boggessi mated successfully with conspecific males (Table I).

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 min prior to mouling of the females, when the females moved around quickly and did not pay attention to the males. Of the remaining 4 pairs, females did not repel the males; 2 males displayed approach and pre-moult chase behaviour within 2 min prior to the female mouling whereas the other 2 males did not display any pre-copulatory behaviour. Forty percent (8/20) of the male L. wurdemanni followed or chased the females within 2 min before the parturial females moulted. Thirteen pairs mated successfully (Table I). Both conspecific and hetero-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 pre-copulatory behaviour toward conspecific females moulted. Thirteen pairs mated successfully (Table I). Both conspecific and hetero-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 males 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 occurring (13/20) under light (2 × 2 Chi-square test, \( \chi^2_{adj} = 3.906, P < 0.05 \)) (Table I). However, there was only 1 (out of 20) successful copulation between male L. boggessi and female L. wurdemanni under both light and darkness (Table I). All intraspecific matings were successful and embryos fully developed to hatching in 10–11 days (Table I).

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). Female L. boggessi always mated with the conspecific males, even though a larger number of male L. wurdemanni (3) than male L. boggessi (1) were present. Reproductive isolation was complete (\( \text{PII} = 1 \)) when conspecific males were present (Table I).

Post-zygotic isolation

The eggs of both intraspecific pairs were fertilized and developed to hatching in 10–11 days, whereas none of the intraspecific matings resulted in successful hatching although the eggs were fertilized (Table I).

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 (con-
trols). Responses of the males to interspecific and intraspecific female moult water were significantly different ($2 \times 2$ Chi square test, $\chi^2_{adj} = 29.192, P < 0.001$). 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, $\chi^2_{adj} = 8.025, P < 0.01$) than the positive response to conspecific female moult water (19 out of 20), but significantly higher ($2 \times 2$ Chi square test, $\chi^2 = 8.025, P < 0.01$) than the positive response to the filtered seawater control (1 out of 20).

**Discussion**

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

Behavioural incompatibility is one of the mechanisms of reproductive isolation in decapod crustaceans and has been reported in snapping shrimps (e.g. Knowlton et al. 1993; Mathews et al. 2002) where species failed to respond each other with higher rate, but the later species can still recognize the former. All these interesting topics deserve further investigation.

Although sex pheromones did not cause complete reproductive isolation between these closely related species, our bioassays demonstrated that presence of $Lysmata boggessi$ males totally prevented (PII = 1, Table I) male $L. wurdemanni$ from mating with female $L. boggessi$. Some male $L. wurdemanni$ copulated successfully with female $L. boggessi$ only when male $L. boggessi$ was not present (PII = 0.35 and 0.05 for under light and dark conditions, respectively; Table I). Pre-copulatory behaviour of male $L. wurdemanni$ towards female $L. boggessi$ seems to be restrained by the latter, as male $L. wurdemanni$ displayed obvious pre-copulatory behaviour towards female $L. boggessi$. The presence of male $L. boggessi$ totally suppressed the pre-copulatory behaviour of male $L. wurdemanni$, although there was little interaction between the males of the two species. These differences suggest that mate competition or species preference may be controlled by both chemical and visual stimuli. Male $L. boggessi$ did not display any pre-copulatory behaviour towards female $L. wurdemanni$, even though female $L. wurdemanni$ did not repel male $L. boggessi$, further suggesting that chemical cues are involved in reproductive isolation in shrimp.
interspecific mate recognition. In addition, female *L. boggessi* consistently and actively repelled male *L. wurdemanni*, and mating success between female *L. boggessi* and male *L. wurdemanni* was higher in the dark than under light. This indicates that female *L. boggessi* maybe capable of discriminating between the species utilizing visual cues when mating occurs under light, as found in other decapod crustaceans such as crayfish *Procambarus clarkii* (Dunham & Oh 1996) and *Austropotamobius pallipes* (Acquistapace 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 interspecific 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 demonstrate that post-zygotic reproductive isolation between the two shrimp species is complete. Even if cross-mating between male *Lysmata wurdemanni* and female *L. boggessi* as observed in our laboratory assay system would occur occasionally in the field, genetic incompatibility between the two species would ensure that 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 & Orr's (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 than post-zygotic barriers in sympatric species (Coyne & Orr 1989, 1997). Complete post-zygotic and incomplete pre-zygotic isolation suggest that the incipient speciation between the two shrimp species may have occurred before they lived 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.

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