Precopulatory stabbing, hypodermic injections and unilateral copulations in a hermaphroditic sea slug

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Reciprocity constitutes the prevalent mating mechanism among simultaneous hermaphrodites. Yet, when copulations in the female role confer fitness costs through male manipulation, it becomes advantageous sometimes to mate unilaterally in the male role only. In the sea slug *Siphotheron quadrispinosum*, acting males stab their partner with a bipartite penis, which not only hypodermically injects prostate fluids, but also apparently mechanically enforces unilateral male matings. Despite a pronounced male mating drive in both partners, unilaterality ensued when one slug stabbed more rapidly than its partner. The acting male may thus avoid the costs inflicted by traumatic injections and penial spines. While future studies need to elucidate the net fitness consequences of stabbing, our behavioural evidence is in line with the hypothesis that mating in *S. quadrispinosum* represents conflicting rather than complementary mating interests between mates.

Keywords: hermaphroditism; sexual conflict; *Siphotheron quadrispinosum*

1. INTRODUCTION

Reciprocity is the vastly predominant mating mechanism in simultaneous hermaphrodites and is considered essential for maintaining hermaphroditism against the invasion of pure sex individuals (Fischer 1980; Leonard 1990; Edlund & Korn 2007). The bidirectional exchange of sperm is achieved either simultaneously or through serial alternation of sexual roles. Strict reciprocity is even maintained when ejaculate or secondary sexual fluid compounds manipulate the receiver's reproductive physiology to the donor's benefit, rendering matings in the female role costly (Koene *et al.* 2005; Chase & Blanchard 2006). This pattern is surprising. Long-term allosperm storage is very efficient in most species and should make frequent sperm receipt dispensable. Hence, individuals that occasionally mate in the male function only should have a clear fitness advantage (Anthes *et al.* 2006; Michiels & Koene 2006). Yet, the few known systems with substantial unilaterality do not appear

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to be driven by costs associated with the female role, but rather reflect asymmetrical and thus complementary mating drives between mates (e.g. Jordaens *et al.* 2005; Koene & Ter Maat 2005).

Here, we describe the unique mating mechanism of the sea slug *Siphotheron quadrispinosum*, where an exceptionally high incidence of unilateral copulations is associated with asymmetrical outcomes of a precopulatory stabbing contest. Unilateral copulations may follow from sexual antagonism between mates, asymmetrical mating drives or female choice of particularly persistent partners. The following predictions, being exclusive to a sexual antagonism scenario, serve to evaluate our observations: (i) in unilateral matings, the acting female shows a strong incentive to perform the male role; (ii) the first 'male' strictly refuses alternation of sexual roles; (iii) hypodermic injection does not occur in a 'controlled' secondary receptive organ of the partner, but at a 'random' location; (iv) more precopulatory struggle; and (v) more penial eversions in unilateral than in reciprocal copulations. The opposite is expected under the alternative scenarios.

2. STUDY SPECIES AND METHODS

The small (less than 5 mm) opisthobranch *Siphotheron quadrispinosum* (figure 1a; electronic supplementary material, figure S1, Gosliner 1989; Klusmann-Kolb & Klusmann 2003) has a peculiar reproductive anatomy (details in electronic supplementary material). Importantly, the male copulatory organ consists of two, not one, eversible structures (figure 1b; electronic supplementary material, figure S2). The muscular 'penial bulb' ('penis' from here onwards) is equipped with two to five large spines and a series of small spines. The second branch diverges from the prostate and ends in the 'penial papilla' with an eversible terminal stylet (figures 1b and 2a; 'stylet' from here onwards).

We collected 138 slugs at Lizard Island (Queensland, Australia) in November 2003, January–February 2005 and December 2005. Animals were kept isolated in 75 ml vials at 26°C and natural day length. Once per day, containers were rinsed and supplied with fresh seawater. Wet weight was measured ± 0.1 mg immediately after collection. We observed matings under a dissecting microscope in a 5 ml glass dish. Up to four individuals were placed together to increase encounter rates. If no mating commenced within 60 min, observations were terminated and repeated the next day with the same individuals. When two individuals initiated mating, the remaining (unmated) animals were removed and reused in a single repeat trial. We completed 54 initial trials plus 40 repeat trials. Relevant behaviour was recorded or timed as applicable (± 1 s). We also noted if and where the penial stylet pierced the partner and estimated fluid transfer rates as pulses min^{-1} . Statistical comparisons between unilateral and reciprocal mating types were performed using general linear models with mating type and season as fixed and random factors, respectively.

3. RESULTS

Mating in *Siphotheron quadrispinosum* involves four phases.

Figure 1. (a) Reproductive anatomy of *Siphopteron quadrispinosum* (dorsal view). (b) Retracted and everted male copulatory organ. Grey shading indicates the interior body cavity. (c) Unilateral copulation sequence: precopulatory struggle with parapod flapping (arrows), reciprocal stabbing attempts during rapid clockwise circling, unilateral injection and insemination, and withdrawal of the penial bulb (life images in electronic supplementary material, figure S1).

(a) Precopulatory phase

Following apparently accidental encounters, slugs responded by head retraction, vigorous parapod flapping and swimming. Individuals also attacked their partner with their eversible pharynx (figure 1c, table 1). Sexually motivated individuals continued by clockwise circling and everting the male organ (62 out of 94 trials, figure 1c), probing the partner's ventral side with their stylet and the female gonopore with their penis. Both kept their front turned away from their mate and laterally shook their parapods, which held off the partner's stylet and penis. Consequently, it took on average 11 unsuccessful precopulatory contacts for a single successful mating (table 1).

(b) Style and penis insertion

Precopulatory interactions led to both stylet and penis insertion in 41 mating trials (24 initial trials (44.4%) and 17 repeat trials (42.5%)). All 22 cases with almost synchronous stylet piercing (average delay between partners 0.2 min; table 1) resulted in reciprocal insemination, which commenced without further movement. In contrast, matings proceeded unilaterally when only one partner successfully stabbed while the other was still probing ($n=19$, figure 1c; Fisher's exact test on the frequencies of reciprocal and unilateral stabbing versus mating type, $p<0.0001$). The latter partner was then kept at distance by the inserted and inflated male apparatus of its mate and failed to insert. The acting male also continued to shake its right parapod whenever approached by the partner's male organ. In all

unilateral matings, the acting female attempted stylet and penis insertion for another 1.60 ± 1.57 min (0.1–6 min, $n=19$) before these attempts gradually ceased. Stylets were thrust at various locations in the ventral head region without any detectable female receptive structure (59 out of 63 cases; posterior foot and right parapod in the remaining cases).

(c) Copulation

Copulation refers to the total insertion period of either penial branch and consists of two successive phases. During the first and longer phase ('hypodermic injection', approx. 12.5 min, table 1), animals injected an almost transparent fluid through the stylet, but not through the simultaneously inserted penis. Histological sections showed that prostate secretions, but no sperm are present in the penial appendage prior to and after copulation, indicating that hypodermic injection is restricted to prostate fluids. During the second phase ('insemination', approx. 6.5 min, table 1), animals transferred yellowish parcels exclusively through the 'true' penis into the receiver's gonopore. During this time, the stylet was already withdrawn in 40% of all cases. In histological sections, we found both sperm and prostate secretions in the penis, indicating that this second phase serves sperm transfer.

(d) Withdrawal and separation

Withdrawal required considerable pulling (figure 1c) to disengage the penial spines anchored in the genital

Figure 2. Penial morphology of *Siphopteron quadrispinosum*. (a) Light microscopy images of (i) the penial papilla and (ii) the penial bulb. (b) Serial sections of the inserted penial bulb showing two serial sections (i) anchored in the receiver's genital tract and (ii) the deeply inserted penial bulb plus the tip of one spine. Tissue of the acting female is shaded to facilitate orientation.

chamber (figure 2b). Following reciprocal copulations, both partners glided apart without further mating attempts. Following unilateral copulations, however, the acting female attempted penis insertion in 11 out of 19 cases (Fisher's exact test on mating type versus the presence of subsequent mating attempts, $p < 0.0001$). None of these attempts was successful because the acting male deserted immediately. Although total mating duration (hypodermic injection + insemination + withdrawal) tended to be shorter in unilateral than in reciprocal matings (table 1), we found no indication that the time spent inseminating or injecting prostate fluid varied between these two mating types (table 1).

4. DISCUSSION

Consistent with the scenario that unilateral matings follow from a conflict over sexual roles (sexual antagonism, Arnqvist & Rowe 2005), we found that during or after all unilateral copulations, acting females vigorously attempted stabbing and penial intromission and thus showed a strong drive towards the male sexual role. Acting males thwarted those attempts by parapod shaking, penial inflation, rapid circling and minimal exposure of their ventral side. They always departed without showing an incentive to be female themselves, foregoing sex role alternation. Hypodermic injections occurred at an unspecific location as expected under sexual antagonism. Neither the amount of precopulatory struggle nor the number of penial eversions prior to copulation differed significantly between unilateral and reciprocal matings, lending no exclusive support for any of the scenarios under discussion. As further corroborating evidence, we found that the reluctance to mate in *S. quadrispinosum* exceeds that of close relatives (Anthes & Michiels in press). Under comparable laboratory conditions, members of the family Gastropteridae and the closely related family Aglajidae with apparently more modest mating mechanisms typically initiated mating within 20 min and after fewer than five body contacts, predominantly leading to reciprocal copulations. In summary, our observations provide a poor match with the mate choice and complementary mating interest scenarios, but are consistent with sexual antagonism.

Our findings indicate that stabbing serves to mechanically bias copulations in favour of the animal's own male function. Although sperm receipt is obligatory for this outcrossing hermaphrodite, sperm storage allows it to skip sperm receipt occasionally without compromising female fertility. This may even enhance female fitness by avoiding physical and potential physiological costs through stabbing, hypodermic injection and penial anchorage. To elucidate these, the exact function of injections warrants further experimental assessment.

A previous example of enforced unilateral matings comes from polyclad flatworms, where rapid hypodermic inseminations (hit-and-run) prevent the sperm donor from being stabbed and having to pay for tissue loss (Michiels & Newman 1998). As 'hit-and-run' inseminations necessarily remain brief, they may result in suboptimal ejaculate sizes. In this context, hypodermic stabbing in *S. quadrispinosum* may be a mechanism that enables individuals to maintain the ability to transfer large ejaculates without facing the risk of being stabbed themselves. In agreement with this hypothesis, we found no difference in injection and insemination durations between unilateral and reciprocal copulations.

Enforced unilateral matings as described here represent an intriguing consequence of sexual antagonism in simultaneous hermaphrodites: following the spread of an antagonistic male trait, any trait bearer benefits when copulating as a male, but suffers the costs imposed by exactly the same trait when performing the female role to another trait carrier. Owing to the resulting asymmetry in fitness

Table 1. Body size, precopulatory behaviour and copulatory phases in *Siphopteron quadrispinosum* (data measured or averaged per pair).

	mating types						general linear model			
	unilateral matings ($n=19$)			reciprocal matings ($n=22$)			season		mating type	
	mean $\pm$ s.d.	min-max	n^a	mean $\pm$ s.d.	min-max	n^a	F	sig.	F	sig.
mean body weight (mg)	2.88 $\pm$ 0.96	1.6–5.1	19	3.27 $\pm$ 0.78	1.5–5.3	22	0.541	0.587	1.234	0.274
<i>precopulatory phase</i>										
n contacts	11.5 $\pm$ 10.3	2–36	14	11.1 $\pm$ 7.6	4–33	19	2.022	0.151	0.133	0.718
n 'bites'	4.0 $\pm$ 4.2	0–15	14	3.6 $\pm$ 3.7	0–15	19	5.011	0.014	0.082	0.777
n penis eversions	1.7 $\pm$ 1.9	0–6	14	0.9 $\pm$ 1.4	0–6	19	1.174	0.323	2.015	0.166
n parapod shaking	5.6 $\pm$ 4.2	1–13	14	5.0 $\pm$ 3.7	1–16	19	0.911	0.413	0.300	0.588
delay until copulation (min)	28.7 $\pm$ 19.3	2–60	19	25.2 $\pm$ 20.2	1.5–60	22	7.034	0.003	0.215	0.646
<i>copulatory behaviour</i>										
stabbing delay (min)				0.2 $\pm$ 0.4	0–2	22				
total mating duration (min)	18.1 $\pm$ 5.9	8.5–31.7	19	21.5 $\pm$ 4.5	12.5–30.4	22	4.977	0.012	4.220	0.047 ^b
hypodermic injection (min)	12.2 $\pm$ 4.6	4.8–21.0	15	13.1 $\pm$ 2.7	7.1–17.0	18	1.774	0.193	0.327	0.571
insemination (min)	5.9 $\pm$ 1.7	3.5–9.3	15	7.2 $\pm$ 3.2	2.8–14.8	18	11.638	0.002	1.077	0.308
penis withdrawal (min)	1.0 $\pm$ 0.4	0.2–1.8	17	2.0 $\pm$ 1.3	0.4–5.0	22	1.037	0.365	6.798	0.013 ^b
sperm transfer (parcels min ⁻¹)	21.2 $\pm$ 8.3	8.6–36.0	14	21.8 $\pm$ 4.4	16.0–30.4	15	2.763	0.082	0.124	0.727
hypodermic injection (strains min ⁻¹)	20		1	20.2 $\pm$ 5.3	12–26.7	5				

^a Copulatory position of some pairs did not allow measurement of the respective parameter, generating variation in sample sizes.

^b Bonferroni-adjusted significance level $\alpha < 0.0045$.

pay-offs per mating (Anthes *et al.* 2006), optimal mating rates for the two sexual functions within a single individual now diverge. In hermaphroditic species that are capable of performing unilateral copulations (i.e. no morphological necessity to mate reciprocally), selection should then favour inventions (such as the penial stylet) that help enforce unilateral matings and thus optimize male and female mating frequencies independently. Females, in turn, not only have to accept the less desirable role, but also miss an opportunity to achieve paternity, thus departing from both their male and female mating optima (Parker 2006). This unique interplay between inter- and intra-sexual conflict provides scope for models that more precisely define the battleground for sexual conflict in hermaphrodites, including three rather than traditionally two outcomes of mating contests: win (act as male, only), intermediate (male and female) and lose (female only).

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