



Sex role alternation in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis* is determined by the availability of seminal fluid

JORIS M. KOENE & ANDRIES TER MAAT
Faculty of Earth and Life Sciences, Vrije Universiteit

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Encounters between potential mating partners are usually accompanied by sexual conflict. In the case of internally fertilizing simultaneous hermaphrodites that perform one sexual role at a time, this conflict arises over the sexual roles. If both animals prefer to mate in one role, and simultaneous reciprocal insemination is not possible, the conflict can be resolved by sex role swapping after the first insemination, potentially resulting in a form of sperm trading called conditional reciprocity. Although sequentially reciprocal insemination has been reported in several hermaphroditic species, we investigated whether conditional reciprocity is the only possible explanation for such mating patterns. We studied the pond snail *Lymnaea stagnalis* in which sexual isolation increases male sexual drive. Sex role alternation occurred only in pairs where both animals had been isolated and may be conditional. Previous studies in freshwater molluscs have interpreted the occurrence of role alternations per se as conditional reciprocity. However, based on our finding and a review of previous studies, we conclude that future experiments need to be designed very carefully to prove the existence of conditional reciprocity.

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Sexual conflicts arise when reproductive interests of mating partners are not compatible. Simultaneous hermaphrodites seem especially liable to such conflicts, because they can perform both sexual roles and can gain male and female reproductive fitness through mating (Charnov 1979). When the sexual roles must be performed separately, simultaneous hermaphrodites encounter a specific problem. Namely, they need to choose which sexual role to perform during a mating encounter. When both mating partners decide to perform the same role, a conflict may ensue, indicating that conflicting preferences can be important in shaping the mating behaviour of simultaneous hermaphrodites (Leonard 1991).

Several hypotheses have been proposed to explain what determines the choice of a sexual role by a simultaneously hermaphroditic individual. For externally fertilizing hermaphrodites, such as the black hamlet, *Hypoplectrus nigricans*, and the polychaete worm *Ophryotrocha diadema*, egg trading seems to resolve the conflict over sexual roles. By alternating fertilizations of egg packages repeatedly,

both mating partners are assured of having their eggs fertilized and fertilizing eggs with their sperm (Fischer 1980; Sella 1985). Leonard & Lukowiak (1984) extended this trading mechanism to male and female gametes. In their gamete-trading hypothesis they assumed that the sexual role with the highest certainty of parentage is preferred, which is the female role in internally fertilizing animals (Leonard & Lukowiak 1984). However, Greeff & Michiels (1999) have argued that the preference could also go to the male role, because this allows animals to obtain additional paternity (see also Michiels et al. 2003: opportunistic male hypothesis). Distinguishing between these two hypotheses experimentally and demonstrating a general preference for one sexual role has proved very difficult, partly because the predicted behavioural outcome is similar. Furthermore, recent observations suggest that an individual's sexual preference may change, even within a mating sequence (Michiels et al. 2003).

Besides having different reasons for trading sperm per se, hermaphrodites can use different degrees of trading. Conditional reciprocity is defined as the trading of the opportunity to fertilize the partner's eggs (e.g. Michiels & Streng 1998) or the opportunity to get one's own eggs fertilized (Leonard & Lukowiak 1984). Trading by amount is defined as the matching of the amount of donated sperm

Correspondence: J. M. Koene, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands (email: joris.koene@falw.vu.nl).

(e.g. Vreys & Michiels 1998). In both cases, for simultaneous hermaphrodites that perform the sexual roles separately, alternation of sexual roles within a pair should be common, whereas unilateral inseminations in which one animal acts only as male and the other only as female should be rare. Research on sperm trading in sea slugs has provided some support for trading by amount in *Navanax inermis* (Leonard & Lukowiak 1984; Michiels et al. 2003), but not in *Chelidonura sandrana* (Anthes & Michiels, in press).

Despite the above-mentioned studies, in a wide range of simultaneous hermaphrodites the existence of conditional reciprocity has simply been deduced from observations of swapping of sexual roles within mating pairs (Leonard 1991). Sex role alternation has, for example, been reported in *Partula suturalis* and *P. taeniata* (Lipton & Murray 1979), *Stagnicola elodes* (Rudolph 1979a), *Lymnaea stagnalis* (Van Duivenboden & Ter Maat 1985), and *Alderia modesta* (Angeloni 2003). However, unequivocal evidence demonstrating that role alternation in these internally fertilizing hermaphrodites reflects conditional reciprocity is lacking. Although the deduction may be correct, conditional reciprocity may not be the only explanation for the observed role alternation. For example, sperm donors could stimulate their partners to change sexual role after having been inseminated (Rudolph 1979a). Alternatively, the motivational state of the mating partners can result in mating patterns that appear conditionally reciprocal, but that could actually represent unconditional reciprocity. We used the hermaphroditic pond snail *Lymnaea stagnalis* to address these alternatives.

Lymnaea stagnalis is a simultaneous hermaphrodite that can mate in the male and female role, but within one copulation only one sexual role is performed at a time. Individuals usually seem receptive as females and are impervious when copulating in this role (Van Duivenboden & Ter Maat 1985). Investment in female behaviour mainly consists of producing eggs (Ter Maat et al. 1983, 1986, 1989). The male behaviour entails a fixed sequence of events ending with penis intromission and transfer of a copious amount of semen (De Visser et al. 1994). However, these snails are not always prepared to mate in the male role. Their male sexual drive increases when they have not mated for several days (De Boer et al. 1997). This increased eagerness to mate after sexual isolation seems to be a common phenomenon in simultaneous hermaphrodites (*Aplysia fasciata*: Ziv et al. 1989; *Helix aspersa*: Adamo & Chase 1990; *Dugesia polychroa*: Peters et al. 1996). The advantage of using *L. stagnalis* is that the mechanism regulating male mating behaviour has been largely unravelled. Namely, sexual isolation causes an increase in the size of the prostate gland, which produces the seminal fluid. This increase in size is detected by the brain via a small branch of the penial nerve (De Boer et al. 1997). The brain area that receives this information controls male reproductive behaviour and is evolutionarily conserved in gastropods (Koene et al. 2000).

Based on current knowledge, we predicted that role alternation would take place only when both partners are eager to mate as males (i.e. have a full prostate gland). In addition, if role alternation takes place even when the

sperm recipient is not motivated to become male, this would imply that the sperm donor somehow induces its partner to reverse its sexual role after the first insemination. For example, an individual may be willing to donate sperm once the partner has shown its good intentions by donating sperm first (honest signal hypothesis: Landolfa 2002). Alternatively, role alternation could also be the result of physiological manipulation by an all hormone transferred in the semen (Koene & Ter Maat 2001, 2002).

METHODS

We randomly divided 400 adult *L. stagnalis* into two equal groups and marked them with nail polish for identification. The snails from one group were isolated in small perforated polythene jars (sexually isolated, I). The snails in the second group were divided equally between three large perforated polythene boxes (nonisolated, N). The snail boxes were all kept in the same water tank with continuous water exchange, at 20°C with a light:dark cycle of 12:12 h (Van Der Steen et al. 1969). Snails received lettuce every day.

Two weeks after the initial division of the snails, during which one isolated animal had died, we randomly divided the remaining 399 snails into pairs. Three types of pairs were formed: pairs composed of two sexually isolated snails (I × I), pairs composed of one isolated and one nonisolated snail (I × N), and pairs composed of two nonisolated snails (N × N). This resulted in 79 I × I pairs, 80 I × N pairs and 40 N × N pairs. The snails were paired in the morning and all mating behaviour was observed for the following 8 h.

Spontaneous mating behaviour was observed in 800-litre tanks, which are used for breeding the snails in our animal facility. Each tank contained approximately 700 snails. These snails were fed alternately lettuce and fish food (Tetraphyll, Tetrawerke A.G., Melle, Germany) three times a week but were otherwise kept under the same conditions.

RESULTS

The 95 mating sequences observed in the 199 pairs occurred only in the I × I and I × N pairs. No copulations were seen in the 40 N × N pairs. The copulation rate was significantly higher in I × I pairs (58/79) than in I × N pairs (37/80; Fisher's exact test: $N = 159$ pairs, $P = 0.0004$; Fig. 1).

We observed 47 role alternations (Fig. 1). Most of these (36) took place in I × I pairs; only 11 were observed in I × N pairs. The difference in role alternation frequency was significant when compared over all I × N and I × I pairs (Fisher's exact test: $N = 159$, $P < 0.0001$). When we compared only the mating pairs, role alternations also occurred significantly more often in I × I pairs than in I × N pairs (Fisher's exact test: $N = 95$, $P = 0.002$). In I × N pairs the observed frequency of inseminations showed only a marginally significant difference from the frequency expected by chance, based on random insemination (chi-square test: $\chi^2_1 = 4.093$, $N = 80$, $P = 0.043$;

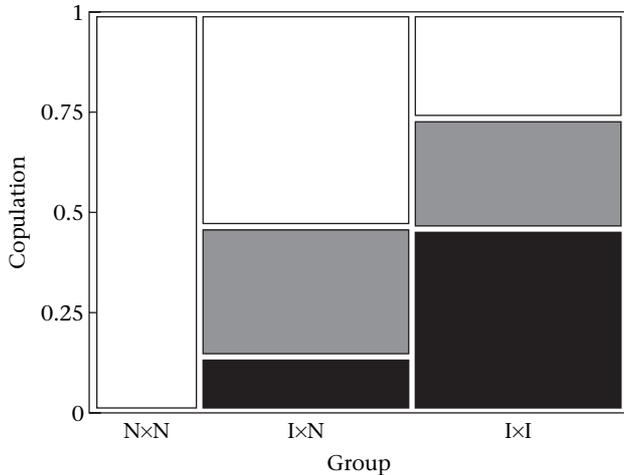


Figure 1. Contingency table for observed copulations and role alternations in the three different groups. N: snail not sexually isolated; I: snail sexually isolated. □: No copulations; ■: no role alternation; ■: role alternation.

Fig. 2). Because the number of inseminations in these pairs was close to random, this suggests that insemination is not based on conditional reciprocity. However, in the $I \times I$ pairs the observed numbers of no copulations and reversals were significantly higher than the expected values, whereas the number of unilateral matings was significantly lower ($\chi^2_1 = 14.082$, $N = 79$, $P < 0.0005$; Fig. 2). This suggests that when $I \times I$ pairs mate, they either both mate in both sexual roles or do not mate at all, which is indicative of a conditional component.

Within the $I \times N$ copulations without role alternation, it was always the I individual that inseminated the N individual. Similarly, in the $I \times N$ copulations with role alternation, in 10 of the 11 events the first insemination was by the I individual. After alternating roles, i.e. the second insemination within a pair, the snails separated and a third insemination was never observed within pairs. Alternation of sexual roles was never seen in the 74 spontaneous mating sequences that were observed in the large breeding tanks.

The snails often showed a stereotyped alternation behaviour. Before swapping sexual roles, during the first insemination the sperm recipient assumed a typical alternation position (Fig. 3). To achieve this position, the individual that was being inseminated stretched its body and reached back to the shell of the sperm donor (Fig. 3a) and firmly attached its foot to the partner's shell (Fig. 3b). In this position, the sperm recipient was ready to mount the donor's shell and started courtship (circling the partner's shell) as soon as the first insemination was finished. This resulted in a second insemination where the sexual roles of the two snails were reversed.

DISCUSSION

Our study shows that in the simultaneous hermaphrodite *L. stagnalis*, role alternation within a mating pair occurs only when both individuals are motivated to mate in the

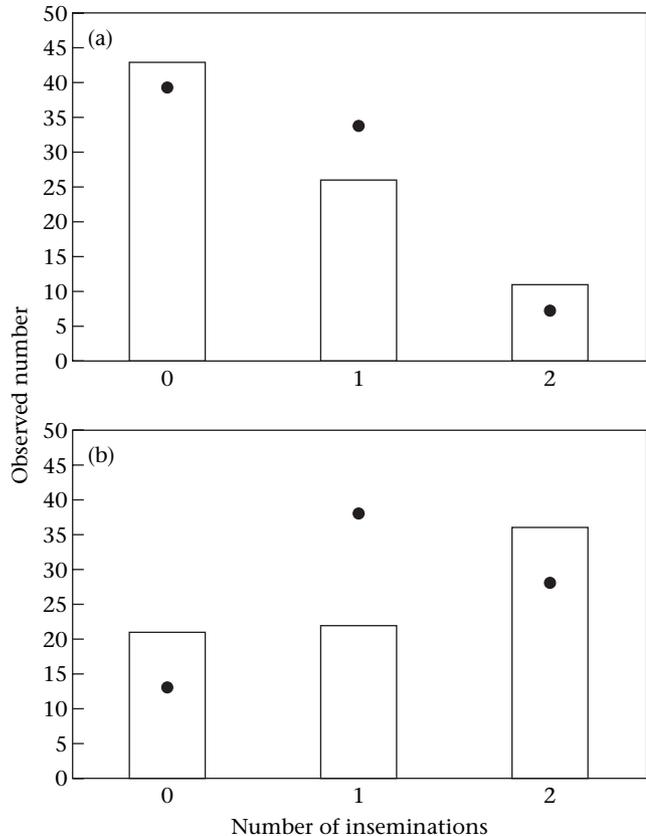
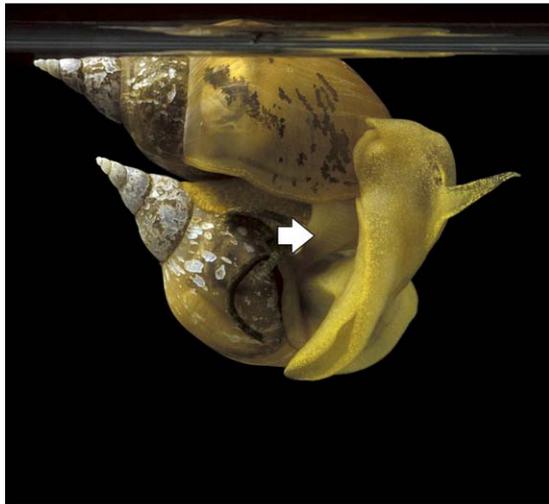


Figure 2. Observed and expected numbers of pairs in relation to number of inseminations within pairs. (a) $I \times N$ pairs and (b) $I \times I$ pairs. N: snail not sexually isolated; I: snail sexually isolated. 0 denotes no copulation within the pair, 1 denotes unilateral insemination and 2 denotes role alternations (i.e. bilateral insemination). The bars indicate the observed values; the accompanying points represent the expected values based on random insemination. Statistics are reported in the text.

male role. The fact that nonisolated individuals that were inseminated only very rarely showed role alternation shows that insemination does not evoke a switch in the sexual role of the sperm recipient. Hence, this finding suggests that these snails will mate in the male role only when enough seminal fluid is present for successful fertilization. From this we conclude that, in general, copulation in *L. stagnalis* is based on unconditional reciprocity, although we did find evidence for a conditional component in pairs of isolated snails. As a consequence, sperm donors have no guarantee that they will receive sperm from a partner that they inseminate. On the contrary, sperm recipients seem able to ensure that they get to inseminate their partner by assuming the alternation position, from which the partner seems unable to escape. This implies that sex role alternation is driven entirely by the motivation to mate as a male and does not seem to be influenced by the potential attractiveness of a partner as a female (no sexy mother effect, see Michiels & Bakovski 2000).

These observations are consistent with the previous finding that the individual that has been sexually isolated

(a)



(b)



Figure 3. The typical sex role alternation position of *Lymnaea stagnalis*. The two photographs show the same pair of snails. (a) The snail that is being inseminated can be seen reaching back to the shell of its partner. The partner's preputium, which carries the penis, is still visible (see arrow). (b) The foot is firmly attached to the partner's shell and the snail is ready to mount the partner as soon as insemination is finished. Note that, to reach the sex role alternation position, the snail had to let go of the substrate which is why the pair floats at the water surface.

for longest will act as a male (Van Duivenboden & Ter Maat 1985). Here, we have shown that when both individuals have been isolated, role alternation will take place so that both individuals get to donate sperm. This swapping of roles occurs only once within a pair, which agrees with the finding that after insemination the prostate gland is partially depleted, resulting in a decrease in male motivation (De Boer et al. 1997). Our results indicate that, for the male role, having enough seminal fluid is essential for successful transfer and survival of the donated sperm, and probably ensures the eventual fertilization of the partner's eggs. Seminal fluid often contains

substances that aid the fertilization process and increase the chances that the donated sperm are used for fertilization of eggs, even in hermaphrodites (e.g. Koene & Ter Maat 2001, 2002, 2004; Koene 2004). Producing such substances can be very costly. As in species with separate sexes (e.g. Dewsbury 1982), in hermaphrodites investment in male reproduction can be as high as that in female reproduction (Greeff & Michiels 1999). The enormous amounts of seminal fluid that are pumped into the partner during copulation suggest that *L. stagnalis* indeed invests a lot in the male function, as has been shown by De Visser et al. (1994).

The general assumption has always been that *L. stagnalis* is always receptive as a female, because individuals seem to invest minimally in the female role during sperm receipt (Van Duivenboden & Ter Maat 1985) and potentially benefit from energy obtained through the digestion of received sperm and seminal fluid (Greeff & Michiels 1999). However, during our behavioural observations, it became apparent that these pond snails are not always prepared to be inseminated. Snails were often seen withdrawing upon attempts of penis intromission by the partner (unpublished data). Although we did not quantify this insemination avoidance behaviour in the current study, it is suggestive of conflict (and a topic that we intend to investigate in detail in the future). Our data already indicate that snails in the $I \times I$ pairs may resolve the conflict over sexual roles by either mating in both sexual roles (role reversal) or not mating at all, which indicates that these pairs do conditionally reciprocate mating. However, the sperm recipient cannot assure its role as a male in the second insemination prior to being inseminated, because the alternation position can be established only after the first insemination has started.

Our findings have several important implications for research on role alternation and gamete trading in hermaphrodites that perform the sexual roles separately. Most importantly, valuable information about the mating system can be obtained by a thorough understanding of the underlying mechanism that regulates the behaviour. The fact that in *L. stagnalis*, male motivation is determined by the amount of stored seminal fluid explains why role alternation takes place only between sexually isolated partners. Thus, if we had used only sexually isolated *L. stagnalis*, the resulting mating patterns would have been consistent with patterns predicted by conditional reciprocity. Obviously, our data provide a different explanation for the occurrence of sex role alternation.

Sexual isolation is often used in studies of mating behaviour to increase the chance of observing mating individuals. Although a very useful tool, one has to be careful with conclusions about conditional reciprocity. As we have shown here, the mating history of the animals used in studies investigating reciprocity can be of fundamental importance. Therefore, this calls for a close examination of studies that have reported reciprocal insemination patterns. For example, the studies on the copulation strategy of *S. elodes* were carried out with sexually isolated individuals (Rudolph 1979a). Similarly, the freshwater snail *Biomphalaria glabrata*, the land snails *P. suturalis* and *P. taeniata*, and the sea slug *A. modesta* were

kept in isolation before mating trials (Lipton & Murray 1979; Vernon & Taylor 1996; Angeloni 2003).

The study that originally reported sperm trading also used sexually isolated *N. inermis* (Leonard & Lukowiak 1991), but in recent work the effect of isolation was taken into account and the results confirmed that these hermaphrodites do indeed trade sperm (Michiels et al. 2003). However, it also became clear that isolated individuals were more eager to mate as males than nonisolated individuals in this sea slug species (Michiels et al. 2003). Similarly, in the flatworm *Schmidtea (Dugesia) polychroa* isolated individuals are more willing to donate sperm than nonisolated worms (Michiels & Streng 1998; Michiels & Bakovski 2000). Looking at the study of Wethington & Dillon (1996) on *Physa heterostropha pomilia* in more detail also shows that isolated individuals tended to mate more often as males than did mated individuals (Fisher's exact test: $N = 88$, $P = 0.026$). In addition, a reanalysis of their data reveals that role alternations took place predominantly between isolated individuals ($\chi^2 = 12.856$, $N = 121$, $P = 0.002$), just as in our study. Again, this shows that sexual isolation can be an important determinant for the occurrence of sex role alternation. Finally, in agreement with our observations, in *P. h. pomilia*, *Bulinus globosus* and *L. stagnalis* reversals have been reported to be very rare in 'spontaneous' copulations, i.e. copulations between nonisolated snails (Rudolph 1979b; Van Duivenboden & Ter Maat 1988; Wethington & Dillon 1996). How the experimentally controlled sexual isolation relates to the natural situation is still unclear, but we do know that population densities of *L. stagnalis* can fall well below the densities typically maintained in our laboratory cultures (unpublished data), which indicates that sexual isolation could also be important in the field.

A recent model (Puurtinen & Kaitala 2002) showed that reciprocal sperm exchange can evolve as a result of mutual willingness to donate sperm when individuals are generally willing to receive sperm. For the willingness to donate sperm, two important assumptions in this model are poor mate-searching efficiency and low population density. Such conditions essentially lead to sexual isolation. Under those assumptions, the model predicts that all individuals are willing to mate in the male role. Our finding that under such conditions *L. stagnalis* often alternates sexual roles fits the model's prediction.

In conclusion, although observed mating patterns may seem consistent with conditional reciprocity and can be interpreted as such (e.g. Leonard 1991), conclusive evidence is often lacking and alternative explanations such as unconditional reciprocity are possible. This does not mean that conditional reciprocity cannot be a solution to the conflict between mating partners over sexual roles (for examples see Michiels & Streng 1998; Michiels & Bakovski 2000), and conditional reciprocity provides an explanation for the high occurrence of role alternations in pairs of isolated *L. stagnalis*. Nevertheless, based on current knowledge about many simultaneously hermaphroditic species that can perform the sexual roles separately, unconditional reversal of sexual roles provides an equally likely explanation with the same behavioural outcome. As we have shown here, such unconditional reciprocity can

simply result from both mating partners being eager to donate sperm because of sexual isolation. Therefore, future experiments aimed at demonstrating conditional reciprocity will have to be designed very carefully, preferably taking into account knowledge of the underlying mechanisms.

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