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Sperm transfer is affected by mating history in the simultaneously hermaphroditic snail *Lymnaea stagnalis*

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Abstract. Males are predicted to strategically allocate sperm across mating partners in order to maximize their chances of paternity. This requires that males have the ability to detect aspects of their partner's mating history or the number of potential mates. We investigated whether simultaneous hermaphrodites mating in the male role strategically adjust sperm transfer depending on rearing conditions. The pond snail *Lymnaea stagnalis* (Basommatophora) is known to donate sperm repeatedly to different partners during a breeding season and store received sperm for >3 months. The rearing conditions of the donor as well as the recipient affect the amount of sperm transferred. Sperm donors raised in isolation transfer more sperm than those raised in groups. Furthermore, isolated sperm donors transfer less sperm to partners that were raised in groups than to those raised in isolation, i.e., virgins. These findings suggest that snails raised in isolation shift their sex allocation toward the male function and indicate that they can somehow assess the mating status of their partner.

Additional key words: pulmonata, sex allocation, sperm competition

Since the seminal work by Dewsbury (1982), it is now generally accepted that sperm and seminal fluids can be costly to produce. Especially when animals mate promiscuously and can store received sperm for prolonged periods, donors will be selected that optimally allocate their sperm (to different partners). Such widespread allocation also requires that sperm donors can assess the number of potential mates or female mated status. In species in which females store received sperm, males might be able to detect the level of sperm competition via sperm storage cues or other evidence of recent mating activity (e.g., Simmons & Kvarnemo 1997). With elevated sperm competition, theory predicts an increase in investment in sperm numbers (e.g., Parker 1990a,b; but see Snook 2005). There are many examples of research supporting this idea (e.g., Raimondi & Martin 1991; Wedell et al. 2002; Schärer & Ladurner 2003).

It is important to note that these theoretical models are divided between those investigating sperm competition risk (involving matings that either are free of competition or have two competing ejaculates, e.g., Parker 1990a,b) and those investigating sperm competition intensity (involving two or more competing ejaculates, e.g., Parker et al. 1996). In general, they all predict larger ejaculates in situations of sperm competition between two ejaculates. However, the latter model predicts smaller ejaculates when more than two competing ejaculates are involved. Under such circumstances it may pay to invest less in high-risk copulations and instead conserve sperm for less competitive matings (Parker et al. 1996).

In a recent study combining these two types of models, Engqvist & Reinhold (2006) showed that when the level of sperm competition is high and males can distinguish virgins from mated individuals, it pays to invest more in matings with the former. Ball & Parker (2007) support the finding that matings with virgins can result in higher sperm allocation to virgins, but in their sperm competition risk model this occurs when the level of sperm competition is low and females are sperm limited. Males of several species have indeed been found to provide a larger ejaculate in matings with virgins; examples include a mite (Yasui 1996), bumblebee (Sauter & Brown 2001), bedbug (Siva-Jothy & Stutt 2003), orbweaving spider (Bukowski & Christenson 1997), and bushcricket (Wedell 1992). The two models thus provide the basis

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for an explanation for these observations, which were so far contrary to the predictions of other sperm competition models.

All of the above-mentioned studies were done on species with separate sexes. However, hermaphrodites also experience sperm competition (e.g., Michiels 1998) and investment in the male role can be high (e.g., De Visser et al. 1994). Here we test the consequences of mating history on sperm transfer in the great pond snail, *Lymnaea stagnalis* Linnaeus 1758. This species is a simultaneous hermaphrodite, i.e., has functional male and female reproductive organs at the same time. Nonetheless, copulating individuals of *L. stagnalis* can only perform one sexual role at a time: one snail adopts the male role (sperm donor) while the other adopts the female role (sperm recipient). The male courtship behavior consists of a fixed set of elements (de Boer et al. 1997) followed by insemination. After copulation the sexual roles can be swapped (Koene & Ter Maat 2005). Although the bulk of the received ejaculate is digested in the bursa copulatrix, Cain (1956) showed that these pond snails are capable of storing and using received sperm (allosperm) for roughly 3 months. Field work has furthermore shown that this species has a strong preference for outcrossing over self-fertilization (Knott et al. 2003) and generations do overlap in spring (J.M. Koene et al., unpubl. data).

Recent research has revealed that populations of *L. stagnalis* with increasing group size but constant density will mate more frequently (J.M. Koene & A. Ter Maat, unpubl. data). Interestingly, animals with a high mating frequency produce more eggs, develop smaller prostate glands, and do not seem to increase sperm production (Koene et al. 2006). Previous studies have also shown that animals that were raised in isolation delay the onset of female function (Van Duivenboden 1983) and grow larger (Koene & Ter Maat 2004). These differences in the allocation of resources to growth, male reproduction, and female reproduction are contrary to the predictions made by sperm competition and sex allocation theories (Charnov 1982, 1996; Parker 1990a,b). Under high mating frequencies, sex allocation theory generally predicts an increased investment in sperm and seminal fluids, and a decreased investment in egg production, in simultaneous hermaphrodites.

The above-mentioned studies indicate that mating frequency may play an important role in resource and sex allocation of these simultaneous hermaphrodites. Because the foregoing studies have mainly focused on egg laying, we wanted to know whether the observed feminization is also reflected in the amount of sperm that is transferred during copulation. In order to answer this question we studied the effect of different social isolation periods on the amount of transferred sperm. Moreover, we tested whether the number of sperm transferred is adjusted depending on the mating history.

Methods

All specimens of *L. stagnalis* were obtained from our laboratory culture. The water was kept at 20°C in the breeding facility and the experimental tank. The light:dark cycle was 12:12 h. Snails were each fed with a circular disc (19.6 cm^2) of lettuce per day. This amount is slightly below their maximum food intake and thus completely consumed. Hence, the total energy intake of individuals was equal. Shell height and body weight are tightly correlated (third power fit: $r = 0.988$, $N = 47$, $p < 0.0001$; see also Zonneveld & Kooijman 1989; Koene et al. 2007).

For the experiments, immature specimens of *L. stagnalis* with a shell height of ~9 mm were obtained. The immature animals were randomly assigned to either isolated housing (I, i.e., virgins) in perforated polythene jars (460 mL) or in groups (G, i.e., mated) of 25 animals per large perforated polythene box (5600 mL). All jars and boxes were placed in the same tank. Under these circumstances, the animals were raised to maturity with an average weight of 2.60 ± 0.42 g (31.4 ± 2.2 mm) in 9 weeks. Each individual was used only once.

To increase their willingness to mate in the male role, these animals are usually isolated 1 week before copulation experiments (De Boer et al. 1997). To evaluate the effect of longer isolation durations on the number of sperm transferred, we used 72 mature G snails that were isolated on the same day. After 1 week of isolation, 24 randomly selected snails were placed together in pairs, after 2 weeks another 24 snails were paired, and after 3 weeks the final 24 snails were paired. Of these 12 pairs within each treatment, respectively 10, 9, and 9 pairs mated unilaterally.

To investigate the effect of rearing condition on sperm transfer, 50 I and 50 G snails were used. The G animals were isolated 1 week before copulation. The copulations in this experiment were set up between pairs of individuals from the same treatment (12 I×I and 12 G×G, where the notation indicates donor × recipient) as well as pairs from different treatments (24 mixed pairs). Because we could not predetermine the direction of sperm transfer in the mixed pairs, these were left to chance. Fortunately, as can be seen in the results, the number of G animals (G×I pairs) was nearly the same as the number of I animals donating sperm (I×G pairs).

Before copulation, the animals were weighed, measured, and marked. The animals were first placed on a tissue to dry off the excess water and then weighed on a closed scale (Sartorius, model 1712 MP8, Gottingen, Germany). Subsequently, shell height was measured with a caliper. The snails were then marked with nail polish for individual recognition and randomly paired in polythene jars (460 mL) with fresh low-copper water. The behavior of the individuals was noted every 10 min until the end of copulation.

The transferred sperm was extracted from the sperm recipient using the following protocol. Immediately following copulation ~2 mL of 50 mmol L^{-1} $MgCl_2$ was injected to anesthetize the recipient snail. The shell was peeled off and the skin of the animal was carefully cut open with small surgical scissors, continuously taking care not to damage any of the underlying, sperm-filled ducts. Within the first minute after copulation, the transferred sperm are located in these ducts (vaginal duct, truncus bursae/pedunculus, and oviduct) and not yet transported to the bursa copulatrix. The ducts containing the mass of transferred sperm were cut out and placed in an Eppendorf tube containing 200 μL of saline (5.83 mmol L^{-1} $CaCl_2 \cdot 2H_2O$, 3.76 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 42.69 mmol L^{-1} NaCl, 37.53 mmol L^{-1} KCl). The tube was vortexed for 30 s. The female tissue was then removed from the tube with a fine forceps and placed in a second tube containing another 200 μL of saline and vortexed for another 30 s. This procedure was repeated once more and the contents of the three Eppendorf tubes were then pooled (without the female tissue) and again vortexed for 30 s. Next, 30 μL was added to each counting chamber, and then covered with a coverslip. This procedure prevented the sperm from clumping. We used a Neubauer counting chamber (depth, 0.1 mm; area, 0.0025 mm^2). To avoid counting sperm twice, we counted sperm within each square and those sperm heads overlapping with the top and right margins, and ignored those on the left and bottom margins. Sperm were counted in five squares of each counting chamber and added up. Counting was performed in quadruple. With the following function we calculated the total number of sperm from the counts:

$$\text{No. of sperm} = (\text{average count} \times \text{total volume of solution}) / (\text{number of squares counted} \times \text{area} \times \text{depth})$$

The data were tested for normality and homogeneity of variance. The statistical analyses were performed with JMP version 5.0.1 (SAS Institute Inc., Cary, NC).

Results

The prostate gland of *L. stagnalis* has been shown to increase in size during the first 8 d of sexual isolation and afterwards to stay at the same level (Koene 2006). We tested whether this was also the case for sperm transfer. We used a general linear model with two factors, isolation period and donor weight, nested for isolation period, with the dependent variable being number of sperm transferred. We found a difference in the number of sperm transferred between the three different isolation periods ($F_{2,22} = 5.18$, $p = 0.014$) while the model revealed no effect of donor weight ($F_{3,22} = 0.42$, $p = 0.743$). The Tukey–Kramer *post hoc* test revealed that the number of sperm transferred after 1 week of isolation was different from that transferred after 3 weeks of isolation ($p < 0.05$), while the amount transferred after 2 weeks was different from neither ($p > 0.05$). Interestingly, after 1 week of isolation more sperm were transferred ($N = 10$, $8.62 \pm 0.42 \times 10^6$ sperm) than after 2 ($N = 9$, $7.01 \pm 0.45 \times 10^6$ sperm) and 3 weeks ($N = 9$, $5.73 \pm 0.45 \times 10^6$ sperm).

Previous work has shown that similar rearing conditions affect growth and oviposition in *L. stagnalis* (Koene & Ter Maat 2004; Koene et al. 2006). To complete the picture, we investigated the effect of such conditions on sperm transfer. Data were analyzed with a full-factorial general linear model with donor weight, recipient weight, donor type, and recipient type as factors, and the amount of sperm transferred as the dependent variable. Both donor and recipient weight had no significant effect on the amount of transferred sperm (respectively, $F_{1,23} = 1.10$, $p = 0.304$; $F_{1,23} = 1.28$, $p = 0.269$). The analysis further revealed that both donor and recipient type significantly affect the amount of sperm that were transferred. Sperm donors raised in isolation transferred more sperm than donors raised in groups ($F_{1,23} = 46.16$, $p < 0.0001$). Sperm recipients raised in isolation received more sperm than recipients raised in groups ($F_{1,23} = 6.66$, $p = 0.017$). Because the interaction between donor- and recipient type was close to significance ($F_{1,23} = 3.42$, $p = 0.078$), we performed a Tukey–Kramer *post hoc* test (Fig. 1). All other interactions were insignificant ($p > 0.05$).

Discussion

When sperm are costly to produce and individuals mate promiscuously, sperm donors will be selected to be prudent with their sperm reserves to optimize their reproductive success. This is generally true for both gonochorists (e.g., Parker 1990a,b) and hermaphro-

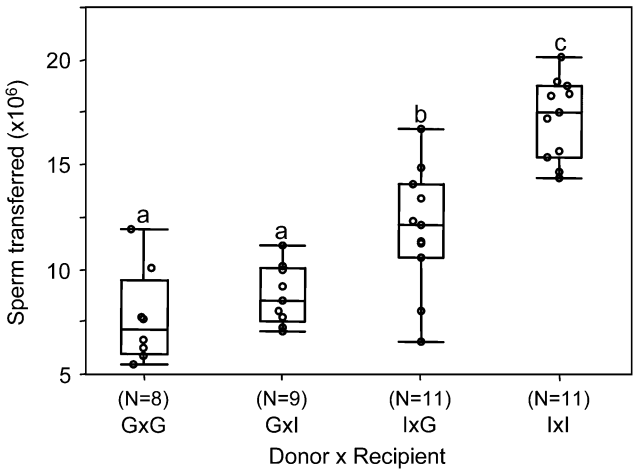

Fig. 1. The number of transferred sperm for each pair type. Box plots show the median, 25th quartile, 75th quartile, and total range. Individual data points are jittered. For each pair type, the first-mentioned letter indicated the sperm donor and the second letter the recipient. The different letters indicate significant differences based on a Tukey–Kramer *post hoc* test ($p < 0.05$). G, individual raised in groups; I, individual raised in isolation.

dites (e.g., Michiels 1998). Our results show that experimental rearing conditions, resulting in different mating histories (i.e., virgin or mated), affect the number of sperm that are transferred. Sperm donors raised in groups (i.e., mated) were found to transfer fewer sperm than those raised in isolation (i.e., virgins). In addition, sperm donors raised in isolation transferred more sperm to partners that were raised in isolation compared with those raised in groups.

The most striking finding that emerges from this study is that the mating history of both the sperm donor and recipient affects the amount of sperm that is transferred during copulation. Donors transfer more sperm and recipients receive more sperm when raised in isolation than when raised in groups, irrespective of their weights. This result implies two things. Firstly, sperm donors raised in isolation shift their sex allocation toward the male function. This is in agreement with the lower egg production found under similar rearing conditions as those used in this experiment (Koene & Ter Maat 2004; Koene et al. 2006). Secondly, the finding that more sperm were transferred to partners that were raised in isolation (i.e., virgins) could suggest that donors can assess the mating history and donate sperm accordingly. Similarly, previous work showed that these snails are able to detect whether a partner is familiar or novel (Koene & Ter Maat 2007). Obviously, an alternative explanation for our findings could be that sperm recipients can selectively prevent or encourage receiving excess sperm.

In general, in studies on other species, an opposite sperm allocation pattern is predicted and found (Parker 1970). For example, members of *Macrostomum lignano* receive more sperm when in larger groups (Schärer & Ladurner 2003). However, in the land snail *Arianta arbustorum* such effects were not found (Baur et al. 1998), and pairs of the flatworm *Schmidtea polychroa* transfer more sperm to each other when they have not mated recently (Michiels & Bakovski 2000). Such discrepancies might be explained by differences in experimental set-ups or life history traits of the study species. Several insect studies did find comparable results to the ones we report here for *L. stagnalis,* i.e., more sperm being transferred to virgin partners (e.g., Wedell 1992; Siva-Jothy & Stutt 2003). Engqvist & Reinhold (2006) recently presented a theoretical model providing an explanation for such counterintuitive findings. They find that when sperm donors can detect mating status and have equal paternity chances, and sperm recipients have high re-mating rates, more sperm should be allocated to virgin mates. In contrast, Ball & Parker (2007) find higher sperm allocation to virgins when second males' sperm are disfavored and when females are sperm limited; these conditions are not met for *L. stagnalis,* while the Engqvist and Reinhold conditions are (Koene 2006). Strategic sperm allocation should pay off in *L. stagnalis,* especially given that earlier research has shown that acting as a sperm donor is costly in this species (De Visser et al. 1994).

To exclude the possibility that the difference in sperm transfer between the two treatments is caused by different isolation periods, we looked at sperm donors that were raised in groups and then kept in isolation for 1, 2, or 3 weeks. If sperm keeps accumulating during isolation, an increase in the amount transferred with time in isolation would be expected. On the contrary, we find that snails that have been isolated for 1 week transfer more sperm than those isolated for more weeks. Hence the differences we find in this study cannot be explained by isolation duration. The lower numbers transferred after 2 and 3 weeks of isolation is probably a reflection of the resorption of autosperm in the seminal vesicles, as reported by Joosse et al. (1968). The fact that the amount of sperm transferred is reduced in later weeks could indicate that sex allocation is affected by these social conditions. Interestingly, Van Duivenboden et al. (1985) found, in a very similar experiment where snails were transferred from group to isolation jars, that egg laying increased significantly during the

weeks after isolation (and especially after 6 d). This is complemented by our findings, which reveal the opposite effect on the male side. This reduced allocation to the male function is consistent with previous research showing that pond snails raised in groups invest much more in the female function and display reduced growth (Koene & Ter Maat 2004; Koene et al. 2006). Taken together, these results indicate that the sex allocation of *L. stagnalis* can be flexibly altered according to differences in social conditions. Another example of such phenotypically flexible sex allocation was recently reported in *M. lignano* (Brauer et al. 2007). As they also note, it remains to be shown how such flexibility relates to situations in the field, but given that generations overlap in *L. stagnalis,* encounters between virgins and mated individuals are not unlikely.

In conclusion, sperm donors raised in isolation transfer more sperm than those raised in groups. Furthermore, isolated sperm donors transfer less sperm to partners that were raised in groups than to those raised in isolation. This provides the first experimental evidence for the distribution of sperm based on mating history in a hermaphrodite. Moreover, it ties in with previous work demonstrating that animals distribute their sperm prudently by preferentially inseminating novel rather than familiar partners (Koene & Ter Maat 2007). It remains unclear whether these animals assess such aspects of their partners via chemical or tactile cues—a promising topic for future research.

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