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ORIGINAL ARTICLE

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Determinants of paternity in the garden snail *Helix aspersa*

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Abstract Despite the likely importance of post-copulatory sexual selection in simultaneous hermaphrodites, the factors influencing sperm competition in these organisms are generally unknown. We have investigated the effects of dart-shooting, mating order, and several other predictors on the proportion of offspring fathered by penultimate ($P_{n\text{-}1}$) and ultimate (P_n) sperm donors in multiply mated garden snails, *Helix aspersa*. While paternity ratios were biased towards the penultimate donor (mean $P_{n\text{-}1}$=0.61), the magnitude of this advantage was dependent upon which of the two donors successfully darted the recipient. Mean P_n-values increased from 0.17 when the recipient was hit by the penultimate donor to 0.39 when it was hit by the ultimate donor. Furthermore, the effect of the dart was more pronounced in the clutches of smaller recipients. From these results, and observations of live sperm in the storage organs, we propose a novel mechanism to explain the detected pattern of sperm utilization in helicid snails. Electronic supplementary material to this paper can be obtained by using the Springer LINK server located at http://dx.doi.org/10.1007/s00265-002-0519-6.

Keywords Sperm storage · Love dart · Sperm precedence · Simultaneous hermaphroditism · Sperm competition

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Introduction

Despite a strong theoretical basis (Charnov 1996; Greeff and Michiels 1999), empirical studies of sperm competition (Parker 1970) in simultaneous hermaphrodites are rare. Moreover, the reciprocal nature of copulation in many of these organisms has engendered a view of their mating systems as being largely cooperative (Leonard 1990). However, reciprocation does not imply an absence of conflict. It indicates that, rather than being restricted to the male half of the population, sperm competition engages every individual hermaphrodite in a contest for paternity. Although the determinants of success or failure in this conflict remain poorly understood, sperm competition between simultaneous hermaphrodites is likely a powerful selective agent shaping morphology, physiology, and behavior (Michiels 1998).

In the present study, we have attempted to identify factors influencing sperm utilization in the simultaneously hermaphroditic land snail *Helix aspersa*. While only intermating interval has been identified as a determinant of paternity in snails (Baur 1994), other factors are suggested by the literature. Several studies have provided evidence of first-male sperm precedence in gastropod molluscs. High paternity scores have been assigned to early sperm donors after multiple subsequent matings in both the nudibranch, *Phestilla sibogae* (Todd et al. 1997), and the marine prosobranch, *Littorina obtusata* (Paterson et al. 2001). Of more relevance to the current study, non-significant trends toward first-male precedence have been reported in the terrestrial snails *Arianta arbustorum* (Baur 1994; P_2=0.34) and *H. aspersa* (Landolfa et al. 2001; P_2=0.40).

The "love dart" has also been proposed as a factor promoting paternal reproductive success (Charnov 1979; Chung 1987; Adamo and Chase 1996; Koene and Chase 1998). During the final stage of courtship, helicid land snails attempt to push sharp calcareous darts into their mating partners. Once-mated snails struck by darts store roughly twice as many sperm as those avoiding their partners' shots (Rogers and Chase 2001). Mucus derived

from specialized digitiform glands coats the dart and induces conformational changes in the recipient's reproductive tract, likely closing off the entrance to the gametolytic bursa copulatrix where the vast majority of transferred sperm are digested (Koene and Chase 1998), allowing more sperm to escape to the storage organs in darted recipients. A preliminary study of the influence of dart-shooting on paternity ratios in the clutches of twice-mated snails found only a marginally significant effect of the second donor's dart and no significant effect of the first donor's dart (Landolfa et al. 2001).

We have investigated the influence of dart-shooting, mating order, intermating interval, and several other factors on paternity ratios in the offspring of twice-mated snails. Based on the results of this analysis and observations of allosperm in the spermathecae, we propose a novel mechanism to explain the observed pattern of sperm utilization in *H. aspersa.*

H. aspersa is a simultaneous reciprocal hermaphrodite. During prolonged copulation (6–8 h; Adamo and Chase 1988), each snail transfers a spermatophore containing between 1×10^6 and 14×10^6 sperm (Rogers and Chase 2001) into its partner's bursa tract diverticulum. While the vast majority of these sperm are digested in the bursa copulatrix, a small number escapes into the spermoviduct via the long tail of the spermatophore and travel up to the fertilization pouch-spermatheca complex (FPSC; Tompa 1984). Roughly 0.025% of the transferred sperm are stored in the blind-ended spermathecal tubules of the FPSC (Rogers and Chase 2001). Fertilization coincides with oviposition, which general occurs only once per breeding season (Madec et al. 1998). The mating system is potentially subject to sperm competition since individuals mate with multiple partners (1–6; Madec et al. 1998) between oviposition events and can store viable allosperm for up to 4 years (Duncan 1975). Virtually all individuals possessing darts shoot them during courtship (>97%; Adamo and Chase 1988). The exact taxonomic status of this species is controversial; it is variously assigned to the genera *Cornu*, *Cantareus*, *Cryptomphalus* or *Helix*. Since none of these names has gained consensus status, we retain the use of *Helix* to be consistent with the existing literature.

Methods

General methods

Sexually mature garden snails, *H. aspersa*, were collected by commercial suppliers from three geographically distant natural populations near the Californian cities of Strathmore, Fresno, and Long Beach, as well as from one natural population in Argentina. The approximate linear distances between the Californian populations ranged from 96 km to 364 km.

Upon arrival in the laboratory, snails were isolated in small chambers (5×5×8 cm) and, in most cases, stored under hibernation-inducing conditions (24-h darkness at 10°C) for 3–6 weeks. Once aroused from hibernation, isolated snails were maintained at 18–21°C under a 16 h light:8 h dark photoperiod. They were cleaned and fed ad libitum a diet of chicken feed and powdered grains supplemented with calcium carbonate. Prior to use in mating trials, snails were isolated for a further 2 weeks and individually marked by gluing a small numbered plastic tag (queen marking kit, The Bee Works, Orillia, Canada) to the shell. Using different colored tags to identify each population, we marked a total of 96 snails from each collection site.

Mating trials

Trials were designed to obtain recipient snails mated with two different sperm donors. Appropriate matings met two requirements: (1) the two donors and the recipient all originated from different populations, and (2) the recipient was hit by the dart of one donor and missed by the dart of the other. Donors from different populations were selected for two reasons. First, since land snails exhibit very low levels of within-population genetic diversity and comparatively high levels of between-population genetic diversity (Arnaud et al. 2001), using potential fathers from genetically unrelated populations increased our ability to distinguish between the two donors in question, as well as any previous donors (which would be genetically similar to the recipient). Second, if the dart is subject to antagonistic co-evolution (i.e., if stronger effects of the dart are selected for in the male component and resistance to the dart is selected for in the female component) then we would expect females to be most resistant to the darts of sympatric donors (see Knowles and Markow 2001). By using allopatric shooters, we hoped to capitalize on reduced female resistance to observe a stronger effect of dart receipt.

Mating trials were conducted under a 4-day feeding/3-day starvation cycle to maximize sexual proclivity (Adamo and Chase 1991). During the feeding phase, approximately ten snails from each population were placed in each of several large Lucite boxes (36×36×8 cm), provided with sliced carrots, and allowed to court freely. Desired pairs were isolated following the initiation of courtship, and dart-shooting behavior was closely monitored. Courtships were interrupted if the courting snails were from inappropriate populations, or if either of the snails was shot inappropriately. Shot darts were assigned to one of two strictly defined categories (Rogers and Chase 2001): (1) miss – the dart was shot but failed to penetrate the recipient, or (2) hit – the dart penetrated over 50% of its length and remained embedded in the recipient throughout copulation. When a dart was shot inappropriately (i.e., in the same category as the recipient's first partner or in neither of the defined categories), the shooter was isolated for 10 days to produce a new dart (Tompa 1984) before it was allowed to court again. The recipient was isolated for 2 days to allow any effects of the received mucus to dissipate.

Following successful copulation, mated snails were isolated for 8 days to allow allosperm to reach the spermathecae (Lind 1973) and to restore depleted autosperm reserves (Locher and Baur 1999). Mated snails were subsequently either returned to the pool of courting snails (after their first mating) or placed in egg-laying chambers (after their second mating). Isolated snails were prevented from laying prior to completion of their second mating by depriving them of soil substrate. Dead and moribund snails discovered during the course of the mating trials were immediately frozen at –20°C, as were successful egg-layers.

Egg-laying

Appropriately twice-mated snails were placed in individual chambers (8×14 cm) lined with 5 cm of a 3:1 mixture of potting soil and powdered oyster shell. These chambers were maintained under a 14 h light:10 h dark photoperiod with a "light" temperature of 22–23°C and a "dark" temperature of 18–19°C. High humidity was maintained at all times. Candidate egg-layers were subjected to a 2-day feeding/5-day starvation cycle to promote oviposition.

Chambers were checked daily for eggs, which were allowed to hatch. Offspring were fed and kept active until the shell lengths of the largest animals reached 1 cm, at which point they were forced to aestivate. All offspring were starved for at least 7 days prior to genotyping.

The heights, lengths, and widths of the shells of all successful egg-layers and their mating partners were measured to the nearest 0.1 mm using vernier calipers. These measurements (in millimeters) were used to calculate shell volume according to the following formula (D. Rogers, unpublished data): shell volume (cm^3)=3.00×10^{-4}(height×length×width)–0.46.

Paternity determination

Following egg-laying, samples of the hepatopancreases of the parental snails were prepared for electrophoretic analysis. After rinsing in distilled water, samples were homogenized in 750 µl distilled water and slowly frozen at –20°C to encourage cell disruption, then stored at –85°C. Randomly selected offspring (n=30) from each clutch were prepared using a similar protocol, except entire snails were each homogenized in only 300 µl distilled water. Immediately prior to electrophoresis, samples were thawed, briefly vortexed and centrifuged at 10,000 rpm for 15 min. The resulting supernatants were run on horizontal starch gels as described by Murphy et al. (1996). All 3 parents and 23–27 offspring were included on each gel. Based on previous results (Landolfa et al. 2001), we used the amine citrate (morpholine) pH 6.1 buffer system described by Clayton and Tretiak (1972). We initially tested 12 loci: ADH, MDH, LDH, IDH, EST, SOD, PER, ALP, ACP, CAP, AAT, and CAT but obtained the best combination of staining quality and allelic diversity using CAP (EC 3.4.11.1; two distinct loci), AAT (EC 2.6.1.1; single locus), and CAT (EC 1.11.1.6; single locus).

Observations of stored allosperm

Fertilization pouch spermatheca complexes were removed from snails 7–72 days after mating by cutting the ducts connecting to the seminal vesicle and the spermoviduct. Prior to dissection, snails were either anesthetized by injection of 2 ml isotonic $MgCl_2$ (97.4 mM $MgCl_2$, 5 mM Tris HCl) or left unanesthetized. Each FPSC was placed in a depression slide with a drop of either snail saline (Kerkut and Meech 1966), phosphate-buffered saline, or snail hemolymph, and viewed under oil immersion at ×1,000 total magnification. Observations were initiated within 5 min of the start of dissection. Video sequences were captured with a CCTV camera connected to a frame grabber (LG-3, Scion) and recorded on VHS tape (see electronic supplementary material). Snails used for videomicroscopy were not involved in the mating trials described above.

Statistical analysis

Statistical analyses were performed using SPSS 10. Paternity data were analyzed using generalized linear model multiple regression. The categorical variable "dart-shooting order" was included in the model through effects coding. Backward elimination of statistically non-significant factors (P>0.3) was employed to obtain the most appropriate model. All reported P-values are two-tailed. To normalize the distribution of paternity proportions, P-values were transformed using the arcsine transformation ($p'=\sin^{-1}p^{1/2}$; Zar 1996). The arcsine transformation was preferred over the logit transformation as the data set contains several 0 values. All statistical analyses were performed using transformed data and all reported P-values are two-tailed.

Results

Mating trials

We observed approximately 500 courtships (roughly 1,000 separate dart-shooting events) resulting in a total of 108 appropriate reciprocal matings. From these observations, we obtained 64 appropriately twice-mated snails, 38 (59%) of which produced clutches of eggs. Since 3 clutches (8%) failed to hatch, 35 broods were available for paternity determination. Snails from each population were roughly evenly distributed between roles (i.e., successful vs unsuccessful shooter, first donor vs second donor, recipient hit by first donor's shot vs recipient hit by second donor's shot).

Each egg-layer was shot with a dart in only 1 of its 2 matings; in 19 cases the first donor shot successfully while in the remaining 16 cases the second donor was successful. The time interval between the two matings for each egg-layer ranged from 8 to 77 days with a mean±SD of 27.2±16.2 days.

We detected outside fathers (i.e., alleles not present in either of the focal donors) in 17 (49%) of the 35 clutches. The proportion of offspring assigned to outside fathers (donors mated to focal recipients prior to their collection from the wild) ranged from 0.00 to 0.52 with a mean±SD of 0.11±0.15. The proportion of offspring assigned to the first focal donor (penultimate donor, P_{n-1}) ranged from 0.05 to 1.00 with a mean of 0.61 (arcsine-transformed mean±SD=0.90±0.28). The observed value of P_{n-1} is significantly greater than 0.5 (one-sample t-test: t=2.20, df=34, P=0.035), indicating biased paternity in favor of the penultimate donor. In contrast, the proportion of offspring assigned to the second focal donor (ultimate donor, P_n) ranged from 0.00 to 0.72 with a mean of 0.27 (arcsine-transformed mean±SD=0.55±0.25). The observed value of P_n is significantly less than 0.5 (one-sample t-test on transformed data: t=–5.63, df=34, P<0.001), indicating biased paternity acting against the ultimate donor. If offspring assigned to outside fathers are excluded from the analysis (i.e., $P_{n-1}+P_n$=1.00), the mean P_n-value increases to 0.32 (arcsine-transformed mean±SD= 0.60±0.29) but remains significantly lower than 0.5 (one sample t-test on transformed data, t=–3.912, df=34, P<0.001). Exclusion of outside-fathered offspring from the analysis reduces the range in the number of offspring used to determine P_n scores to between 13 and 27 (mean±SD=21.0±3.7).

We employed backwards elimination to obtain the generalized linear model that best accounts for the observed variance in the transformed P-values. Since outside fathers were included in the determination P_{n-1} and P_n (such that $P_{n-1}+P_n\leq1$), the two paternity measures are not always complementary, necessitating separate analyses for the observed values of P_{n-1} and P_n (Table 1). Our preliminary models included the following candidate predictors: a constant, order of dart receipt, maternal shell volume, the interaction between order of dart receipt and maternal shell volume, intermating interval (inverse transformed to normalize the distribution), and the difference in shell volume between the first and second fathers. The difference in paternal shell volumes was calculated as the shell volume of the first donor minus the shell volume of the second donor. The inclusion of maternal shell volume and intermating interval failed to improve any of the three models more than would be ex-

Table 1 Selected generalized linear model for factors affecting the proportion of offspring fathered by the penultimate sperm donor (P_{n-1}) and the ultimate sperm donor (P_n) in multiply mated snails (n=35). The term "shooting order×maternal volume" indicates a statistical interaction. A constant was included in each model since the regression lines do not pass through the origin

Factor	*df*	*MS*	*F*	*P*
Penultimate sperm donors				
Constant	1	0.775	13.20	0.001
Dart-shooting order	1	0.795	13.54	0.001
Shooting order×maternal volume	2	0.308	5.26	0.011
Donor volume difference	1	0.123	2.09	0.159
Error	30	0.059		
Ultimate sperm donors				
Constant	1	0.441	12.21	0.001
Dart-shooting order	1	0.726	20.09	<0.001
Shooting order×maternal volume	2	0.293	8.11	0.002
Donor volume difference	1	0.238	6.60	0.015
Error	30	0.036		

pected by chance alone (in all cases P>0.3), so both candidate predictors were dropped. The improvement in the models associated with the inclusion of the difference between the first and second father's shell volumes was significant in the analysis of P_n (P=0.015; Table 1) but not P_{n-1} (P=0.159; Table 1). However, since our purpose is partially to explore the factors influencing paternity, we included this predictor in both final models.

The effects of both dart-shooting order and the interaction between maternal shell volume and dart-shooting order on the transformed P-values were significant in both analyses (Table 1). As depicted in Fig. 1, successful dart-shooting increased the proportion of offspring fathered by the successful shooter. When the penultimate donor shot successfully (and the ultimate donor missed) it fathered, on average, 71% of the offspring (least-squares mean±SE of transformed P_{n-1}-values=1.00±0.06) while the ultimate donor fathered only 17% (least-squares mean±SE of transformed P_n-values=0.43±0.04). Conversely, when the ultimate donor shot successfully (and the penultimate donor missed) it fathered, on average, 39% of the offspring (least-squares mean±SE of transformed P_n-values=0.68±0.05) while the penultimate donor fathered 50% (least-squares mean±SE of transformed P_{n-1}-values=0.78±0.06). If outside-fathered offspring are dropped from the analysis, successful penultimate donors fathered a mean of 79% of the remaining offspring (least-squares mean±SE of transformed P_2-values=0.47±0.05) while successful ultimate donors fathered a mean of 45% (least-squares mean±SE of transformed P_2-values=0.74±0.06). However, the effect of the dart was dependent on the size of the recipient, with the greatest advantage of successful shooting associated with the smallest recipients (Fig. 1).

The distribution of outside-fathered offspring was highly skewed and could not be normalized, thereby preventing generalized linear modeling. However, a non-parametric Mann-Whitney test revealed no significant effect of shot success order on the proportion of offspring fathered by

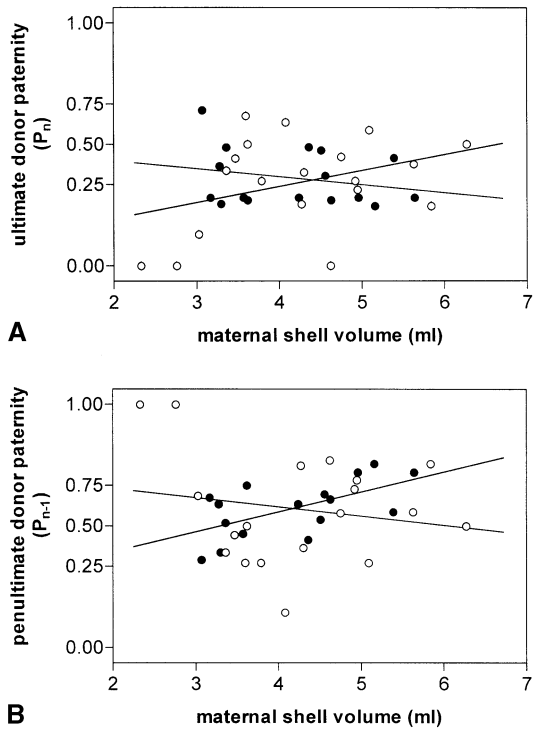

Fig. 1 Effect of dart-shooting order and maternal shell volume on the proportion of offspring fathered by: **A** the ultimate sperm donor (P_n), and **B** the penultimate sperm donor (P_{n-1}) in multiply-mated snails (n=35). The *Y-axis scale* is arcsine square-root transformed, but the values indicated are untransformed. Regression lines indicate the dependence of paternity ratios on maternal shell volume for each dart receipt category (*unfilled circles* only penultimate donor's shot hit recipient, *filled circles* only ultimate donor's shot hit recipient)

outside donors (U=129.5, n_1=19, n_2=16, P=0.428). The distribution of a subtracted data set including only those clutches where outside-fathered offspring were detected (n=17) was normal. A generalized linear model analysis of this subtracted data set failed to reveal any significant effect of paternal shell-volume difference, shooting success order, or the interaction between shooting success order and maternal shell volume (all P>0.3). When these candidate predictors were dropped from the model, regression of the proportion of outside-fathered offspring on maternal shell volume was not significant (b=–0.085, $F_{1,15}$=3.334, P=0.088) for the contribution of outside fathers to a clutch to be higher in small mothers than in large mothers.

Observation of stored allosperm

Allosperm in the storage organs were observed to be active at all post-copulatory intervals examined (7–72 days after mating). Most heads were embedded in the epithelium at the blind ends of the tubules although some were present in the lumen amidst the tails. The tails beat in a synchronous wave that was directed towards the entrance of the tubule (Fig. 2; see also electronic supplementary material). Individual sperm heads could be seen moving

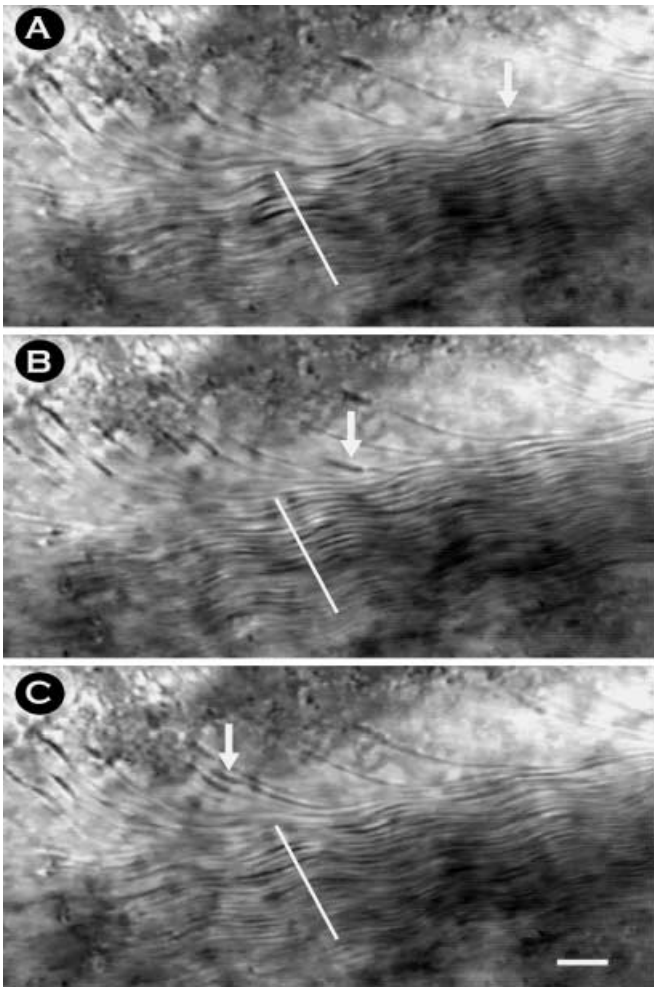

Fig. 2A–C Activity of allosperm within the spermatheca. Three still frames captured from a video of a storage tubule (near the blind end) at t=0.0 s (**A**), t=4.3 s (**B**), and t=8.5 s (**C**). Note the progress of the sperm head indicated by the *white arrow* as it moves through the beating tails in the lumen against the direction of wave propagation (**A**, **B**) and into the spermathecal epithelium (**C**). The *solid white line* is located in the same position in each frame and provides a reference for observing the synchronous beating of the tails. In **A**, the line is situated on a maximum of the sinusoidal wave formed by the beating tails. In **B**, the line is near the minimum, and in **C** it is once again closer to the maximum. The frequency of tail beating is approximately 0.7 s^{-1}. Scale bar=12 µm

within the tubules (Fig. 2) indicating that flagella are not passively undulating in response to the beating of cilia lining the tubule walls.

We adopted several measures to minimize the possibility that manipulation of the FPSC was activating the sperm. The observed activity was dependent neither on the medium bathing the FSPC (snail saline, phosphate-buffered saline, or snail hemolymph) nor on the use of an anesthetic prior to dissection. Moreover, physical manipulation of the FPSC was limited to freeing the organ from the snail and placing it in a depression slide. Similar actions failed to activate autosperm in the seminal vesicle or allosperm in spermatophores.

Discussion

We have identified dart-shooting success and mating order as determinants of paternity in the garden snail *H. aspersa*. While the penultimate donor exhibited a competitive advantage, the magnitude of this advantage was contingent upon which snail successfully darted the recipient. Indeed, successful shooting by the ultimate donor negated the advantage of mating first, particularly in smaller recipients. The degree of bias in paternity effected by the dart reflects the dependence of the number of sperm stored on the interaction between dart receipt and recipient size. If we assume that paternity ratios simply reflect the proportional representation of each donor's sperm in the recipient's storage organ, then we would expect the effect of the dart on P_n to be stronger in smaller recipients since the increase in sperm storage associated with dart receipt is stronger in small recipients than in large recipients (Rogers and Chase 2001). While the mechanism underlying the interdependence of dart efficacy and recipient body size remains unknown, it might be associated with dilution of the digitiform mucus in the hemocoel of larger recipients. If the effect of dart mucus is dose-dependent, then we would expect dilution of the mucus in large recipients to result in a weaker effect on the number of sperm stored and paternity.

Despite the observed association between dart receipt and paternity, two factors limit the strength of our conclusions. First, since the results described herein are correlative, it remains possible that the observed bias in paternity results not from the action of the dart or the digitiform mucus but instead from an unidentified predictor associated with dart-shooting ability. The most likely candidate, a positive correlation between shooting ability and spermatophore size, is not supported by the available evidence (Rogers and Chase 2001). Experimental manipulation of dart receipt will be required to rule out other candidates, such as the reduced viability or competitive ability of sperm donated by poor shooters. Our utilization of donors and recipients from different populations presents a further confounding variable. Variation in dart efficacy and sperm competition success between populations likely added noise to the data, but our limited sample size prevented testing for such interactions.

Second, we cannot conclude from these data alone whether the shots of both donors affect paternity or if the observed effect is mediated by the dart of either the first or second shooter alone. However, previous studies suggest active roles for both. Rogers and Chase (2001) demonstrated a large increase in the number of sperm stored by once-mated snails struck by their partners' darts. Thus, the first donor stands to fertilize a higher proportion of the recipient's eggs if its shot is successful. Landolfa et al. (2001) reported a small increase in the proportion of eggs fathered by the second donor when its shot hit, but no effect of the first donor's dart-shooting success. Furthermore, when the shooting success of both donors was equal (i.e., both shot successfully or unsuccessfully), the second donor's paternity was not significantly different from 50%. The failure of Landolfa et al. to detect a signif-

icant effect of the first donor's dart or mating order on P_2 can likely be attributed to a lack of statistical power resulting from small sample sizes. Moreover, the classification of shots as "good" or "poor" was somewhat arbitrary, increasing the level of noise in the data. Sample sizes in the current study were 3–4 times larger than those in Landolfa et al.'s analysis, and the difference between successful and unsuccessful shots was maximized.

First-male sperm precedence is uncommon across most taxa and the underlying mechanisms are poorly understood. Consistently low P_2 values are generally attributed to one of four causes: post-copulatory mate guarding, the use of mating plugs, sperm stratification, or spermathecal filling. While some gastropods guard their mates following successful copulation (Bradshaw-Hawkins and Sander 1981) and others produce mating plugs (van Duivenboden and ter Maat 1988), helicids engage in neither activity. Stratification of stored allosperm from different donors in "conduit" spermathecae, which provide a direct connection between the sites of insemination and fertilization, is thought to result in first-male precedence (Austad 1984). However, the sperm-storage tubules of helicid snails are blind-ended (which, according to this model, would promote last-male precedence) and there is no evidence of sperm stratification in these animals.

The fourth hypothesis – spermathecal filling (Retnakaran 1974) – seems initially plausible as the capacity of any sperm-storage organ is finite. Individual snails possess relatively few thin, tubular spermathecae (4–6 in *H. aspersa*; Brisson et al. 1977) and the majority of sperm are stored in a single tubule (Baminger and Haase 1999). Allosperm are stored with their heads in tight contact with the spermathecal epithelium, usually at the bulbous blind ends of the tubules (Bojat et al. 2001). Todd et al. (1997) argued that the limited availability of these epithelial "slots" might result in first-male precedence. The first donor's sperm would fill the majority of slots, leaving only a small number of storage positions available to the second donor, resulting in biased storage favoring the first male. However, this hypothesis predicts that the spermatheca would fill to capacity after only two matings and subsequent partners would not contribute to the allosperm reserves. In contrast to this prediction, natural helicid snail populations exhibit high levels of multiple paternity (two to five fathers per brood; Murray 1964). Additionally, the number of sperm stored after a single mating, particularly by large snails, is insufficient to fill the majority of epithelial slots (Rogers and Chase 2001).

We propose a novel mechanism to account for the observed pattern of sperm utilization in *H. aspersa* based on the activity of allosperm in the spermathecae. Activity of stored allosperm has been reported in many taxa (tardigrades, De Zio and Gallo 1975; gastrotrichs, Ruppert 1978; insects, Parker 1970; Thibout 1979; turkeys, King et al. 1999; marine sculpin, Koya et al. 2002; prosobranch snails, Buckland-Nicks and Chia 1981; aplysiid sea slugs, Thompson 1976). Under our hypothesis, the unified beating of the flagella of resident sperm would generate resistance to incoming sperm entering the tubules; the higher the number of resident sperm, the stronger the resistive force. Thus, the probability of any sperm gaining entrance to the storage organ would decrease with each successive mating, and the advantage enjoyed by the penultimate donor would depend on the number of allosperm stored prior to its mating. This hypothesis might also explain why pulmonate sperm are longer than those of all other molluscs (~660 µm in *H. aspersa*; Maxwell 1977), as the beating of longer sperm should generate a greater force.

The ability of sperm from earlier matings to resist incoming sperm might also depend on their condition. If old allosperm provide less resistance than more recent arrivals, we would expect first-male precedence to decrease with increasing mating interval. Indeed, Baur (1994) found that mean P_2 scores increased from 0.34 when both matings took place during the same breeding season (within 70 days of each other) to 0.76 when the interval between matings exceeded 300 days. We found no evidence of an effect of intermating interval on paternity, but the range of time intervals in our data was extremely limited. However, the small contribution of outside fathers to most of the clutches we examined could be attributed to the reduced competitiveness of allosperm after prolonged storage.

The number of sperm reaching the entrance of the storage organ has importance for both first and second donors beyond the absolute quantity of eggs they can fertilize. Since the first donor's sperm enjoy unrestricted access to the spermatheca, larger numbers provide paternity assurance through increased resistance to the incoming sperm from subsequent donors. Since smaller snails store more sperm after a single mating than do larger snails (Rogers and Chase 2001), we would expect the contribution of prior fathers to be highest in the clutches of small snails, since the greater number of sperm stored from early matings would provide greater resistance to incoming sperm from subsequent matings.

If each of the ultimate donor's sperm has an equal probability of overcoming the resistance of the resident sperm, larger numbers reaching the entrance of the spermatheca translates into a greater representation of the ultimate donor's sperm in the storage organ. The number of sperm reaching the entrance can be augmented by preventing the recipient from digesting transferred allosperm through dart-shooting, or by transferring larger ejaculates. Since larger animals produce larger spermatophores (Rogers and Chase 2001), we would expect donor shell volume to influence paternity scores. Indeed, our results indicate that larger sperm donors enjoy higher paternity, but only when they are the last to mate (ultimate donor). While we detected no significant effect of paternal shell-volume difference of penultimate donor paternity, there is insufficient statistical power to conclude that no such effect occurs.

The described mechanism of sperm utilization in helicid snails remains to be conclusively demonstrated and we propose it only as the best current explanation of the observed paternity distribution. Moreover, it is impossible to definitively state that manipulation of the FPSC did not stimulate activity in sperm that are normally qui-

escent in the storage organs. However, we feel that all available evidence supports this mechanism. While largely neglected in the literature, the activity of sperm in storage organs may have consequences for the study of sperm competition in a wide range of animals.

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