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# Dart shooting influences paternal reproductive success in the snail *Helix aspersa* (Pulmonata, Stylommatophora)

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Although animal courtship behaviors are generally understood within the context of sexual selection, the relevance of many sexual behaviors to sexual selection, and vice versa, remains unexplained. For example, the adaptive function of the "love dart" used in the precopulatory behavior of hermaphroditic land snails is only now becoming apparent. Contrary to previous assumptions, dart shooting is unlikely to function as a stimulus for copulation. In searching for a more ultimate explanation of the dart's function, we tested whether variation in dart shooting influences reproductive fitness in *Helix aspersa.* Individual mother snails were mated sequentially to two potential fathers. Dart shooting was closely observed and quantified for all pairings, and percentages of offspring sired by each potential father were determined using allozymes. The results indicate that snails that shoot darts effectively have significantly greater paternal reproductive success than snails that shoot poorly. In contrast, there was no significant effect of mating order on either dart shooting or paternal reproductive success. *Key words:* courtship, *Helix aspersa,* hermaphrodites, land snails, love dart, sexual behavior, sexual selection. *[Behav Ecol 12:773–777 (2001)]*

Dart shooting is a remarkable and unexplained feature of the biology of helicid land snails. In the brown garden snail *Helix aspersa,* each hermaphroditic member of a courting pair pushes a deciduous, 9 mm-long calcareous "love dart" toward its partner. The dart, which is coated by a gland-derived mucus, often penetrates the partner's body wall, transferring some of the mucus to the recipient's hemolymph (Adamo and Chase, 1990). After dart shooting by both snails, mutual intromission and bidirectional spermatophore transfers occur. Most allosperm is digested, but a variable quantity may be stored for up to 4 years (Duncan, 1975; Haase and Baur, 1995). Fertilization occurs at oviposition, which may or may not follow a given mating. Both mating and oviposition occur up to six times per season (Baur, 1998).

The dart's adaptive function has long been subject to speculation. Prior to about 1990, it was thought that the dart functioned to stimulate and/or synchronize sexual behavior, or otherwise to facilitate copulation (Baur, 1998; Meisenheimer, 1912; Tompa, 1984). Acceptance of this explanation was maintained, despite inconsistent evidence (Adamo and Chase, 1990; Börnchen, 1967; Dorello, 1925). In fact, several studies found no influence of successful dart shooting (i.e., penetration of the dart into the recipient) on the timing or likelihood of copulation (Chung, 1987; Giusti and Lepri, 1980; Lind, 1976). Receipt of a dart is not required for successful copulation or sperm transfer, although snails possessing a dart almost invariably shoot it before attempting intromission.

Recent studies have offered hypotheses for dart function that, without explicitly denying a stimulatory role, emphasize its more ultimate effects on reproductive fitness. Charnov (1979) hypothesized that the dart is a sexual signal used by dart recipients to exercise mate choice. Tompa (1980) proposed that dart receipt induces oviposition, thereby augmenting the shooter's paternity; however, Koene and Chase (1998b) found that dart receipt had no significant effect on either the timing or the number of eggs laid. Chung (1987) speculated that successful dart shooters coerce their partners to accept the shooter's sperm for fertilization of the recipient's eggs. Extending this hypothesis, Adamo and Chase (1996) proposed that the dart shooter manipulates its partner's reproductive physiology for its own benefit. This last idea led Koene and Chase (1998a) to discover that the dart mucus induces muscular contractions in the female reproductive tract. The contractions have two notable consequences: they facilitate spermatophore uptake, and they close off the duct leading to the sperm-digestive organ. Both effects might influence the proportion of transferred sperm escaping digestion and reaching the sperm storage organ, thereby providing the ultimate function of raising the paternal reproductive success of successful dart shooters.

To determine whether dart shooting influences reproductive success, by any mechanism, we tested the association between dart-shooting effectiveness and paternal reproductive success using a protocol in which two potential fathers were mated with the same mother. We found that snails that shot darts effectively had a reproductive advantage over snails that shot poorly.

## METHODS

### Snails

Seventy adult snails of unknown reproductive history were collected in Vienna, Austria, in October 1997. Immediately upon their arrival in Montreal the snails were individually marked and isolated in lucite boxes. Twice per week throughout the experimental period (November 1997–August 1998) they were washed and fed (carrot, lettuce or spinach, and crushed oyster shells). The culture was maintained at 21–24° on a reversed 16:8 h light:dark photocycle.

### Matings

Mating trials were initiated 5 months after the snails were isolated. We preselected individuals for inclusion in a given triad

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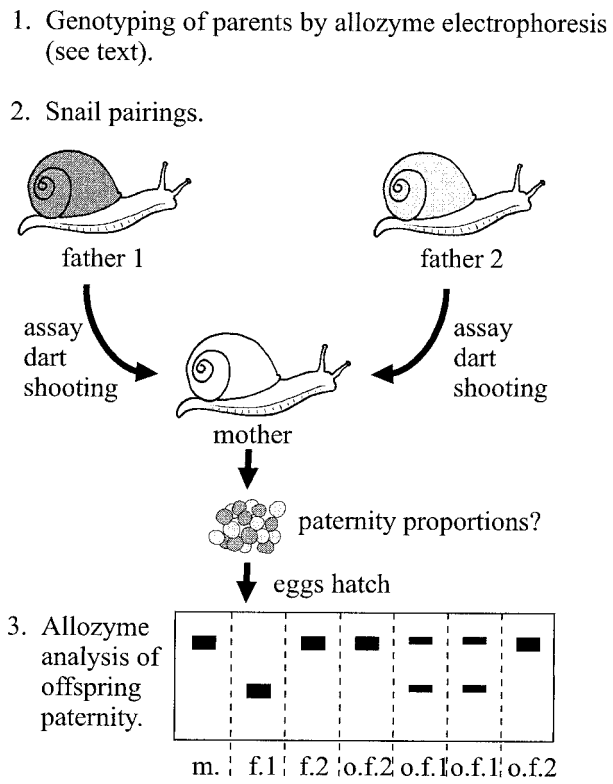


**Figure 1**
Experimental design to test for a relationship between dart-shooting effectiveness and paternal reproductive success in *Helix aspersa*. m, mother; f1, father 1; f2, father 2; of1, offspring of father 1; of2, offspring of father 2.

(one mother and two potential fathers) based on their genotypes such that offspring paternities could later be determined (see below; Figure 1). Each mother snail was mated sequentially to the two potential fathers, with time intervals between first and second matings of 14–71 days ($n = 23$, mean 37.2 days, SD 18.5 days). As snails oviposit only when provided with a soil substrate, mothers were prevented from laying eggs between matings by maintaining them in soil-less lucite boxes. Three days after a mother's second mating, that snail was allowed to oviposit by transferring it to a 1-l plastic cup containing 5 cm of moist soil. Potential egg layers were given 10 days in the oviposition chambers to oviposit. If oviposition occurred, the mother was removed and the buried eggs were left to develop. If a snail failed to oviposit within 10 days, it was returned to its isolation box for 1 week before being given a second opportunity. Hatching occurred 2–3 weeks after oviposition. The offspring grew for 3 weeks before being processed for genotyping.

### Genotyping

Before the mating trials, we genotyped all parental snails according to the following protocol. A tissue sample was obtained by removing 7–8 mm of the foot with a clean razor blade as the snail crawled across a glass plate. The tissue was placed in 4 drops of distilled water in an Eppendorf vial and crushed. The samples were slowly frozen at −15°C to encourage cell disruption, then stored at −80°C. Immediately before processing, samples were defrosted, recrushed, and centrifuged at 13,000 rpm for 6 min. Supernatants were processed by horizontal starch gel electrophoresis according to protocols given in Murphy et al. (1996). The buffer system was amine citrate (morpholine), pH 6.1. The allozyme loci tested initially were *PGM, PGDH, MDH, LDH, IDH, G6PDH, EST, CAP,* and *AAT*. The combination of best staining quality and highest genetic variability was achieved using *CAP* (two distinct loci) and *AAT,* so these three loci were used for genotyping. The number of distinct alleles at the $CAP_1$, $CAP_2$, and *AAT* loci were three, four, and two, respectively.

### Behavioral observations

Snails selected for mating on a given day were washed by showering and placed together in a box containing 2–3 mm of water. Mating trials were begun in a lighted room approximately 1 h after the onset of subjective night. If two appropriate snails displayed signs of sexual activity, these were removed from the group box and placed together in a smaller box where courtship and copulation could proceed. We observed the pair's behaviors continuously until the snails either became unreceptive, achieved mutual copulation, or were separated before copulating. We recorded the depth and duration of received darts.

### Statistics

All data sets were tested for normal distributions using the Kolmogorov-Smirnoff one-sample test; none was significantly different from normal ($p > .05$). We used a two-factor analysis of variance (ANOVA) to test the associations between each of the two fathers' dart shootings and the reproductive success of the second father. Because the data were not equally replicated, a general linear model was used. All statistical tests were performed using Systat software, version 8.0.

## RESULTS

### Paternity determination

Because some snails probably received sperm from matings in the wild before their collection, our ability to assign offspring paternity to either of the two experimental fathers was potentially compromised. There is a tendency, however, for snails not to use very old allosperm. Baur (1994) found, in *Arianta arbustorum,* that sperm received during a previous mating season (more than 70 days previously) was only half as likely to be used for fertilization as sperm received in the same mating season. Snails used in the present study were isolated for at least 150 days before their participation in pairings.

Notwithstanding the above, we estimated the degree to which offspring sired by unknown fathers contaminated the data. We first looked for offspring having genotype combinations that could not possibly have arisen from a mating between the mother and either experimental father; these offspring could only have come from unknown snails mating with the mother before she was collected in the field. The multiple alleles at each of the three loci used for paternity testing made it likely that some third-party fathers would be revealed by the allozyme analysis, and, indeed, we found 33 of 1954 offspring (1.7%) having genotypes that the experimental parents could not have produced. These offspring were present in 6 of the 22 clutches (27%; identified in Table 1), where they constituted 1–12% (mean 5.7%) of the clutch sizes. These positively identified third-party-sired offspring were excluded from the data reported in Table 1.

Apart from the third-party–sired offspring identified by the procedure described above, additional third-party–sired offspring would have escaped detection if their fathers had even one allele in common, at the tested loci, with either of the experimental fathers. We estimated the percentage of off-

**Table 1**
**Measures of dart-shooting effectiveness and paternal reproductive success**

| Snail | Int | De1 | Du1 | DD1 | DS1 | No. 1 | P1 | De2 | Du2 | DD2 | DS2 | No. 2 | P2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 101 | 69 | 4 | 7 | 28 | 0 | 53 | 0.52 | 0 | 0 | 0 | 0 | 49 | 0.48 |
| 107 | 43 | 7 | 35 | 245 | 1 | 0 | 0.00 | 4 | 410 | 1640 | 1 | 133 | 1.00 |
| 109 | 14 | 2 | 20 | 40 | 0 | 91 | 0.83 | 2 | 0.5 | 1 | 0 | 19 | 0.17 |
| 110 | 38 | 9 | 1440 | 12960 | 1 | 69 | 1.00 | 0 | 0 | 0 | 0 | 0 | 0.00 |
| 112 | 24 | 7 | 180 | 1260 | 1 | 102 | 0.99 | 0 | 0 | 0 | 0 | 1 | 0.01 |
| 113 | 22 | 7 | 300 | 2100 | 1 | 106 | 1.00 | 5 | 360 | 1800 | 1 | 0 | 0.00 |
| 117[a] | 22 | 0 | 0 | 0 | 0 | 66 | 0.57 | 5 | 360 | 1800 | 1 | 50 | 0.43 |
| 121[a] | 21 | 0 | 0 | 0 | 0 | 66 | 0.80 | 6 | 23 | 138 | 1 | 16 | 0.20 |
| 125 | 58 | 1 | 0.5 | 0.5 | 0 | 60 | 0.57 | 4 | 360 | 1440 | 1 | 46 | 0.43 |
| 126 | 19 | 6 | 20 | 120 | 1 | 57 | 0.95 | 7 | 19 | 133 | 1 | 3 | 0.05 |
| 129[a] | 21 | 8 | 1440 | 11520 | 1 | 21 | 0.29 | 8 | 1200 | 9600 | 1 | 51 | 0.71 |
| 133 | 42 | 8 | 18 | 144 | 1 | 7 | 0.78 | 0 | 0 | 0 | 0 | 2 | 0.22 |
| 134 | 55 | 6 | 1140 | 6840 | 1 | 81 | 1.00 | 0 | 0 | 0 | 0 | 0 | 0.00 |
| 141 | 24 | 1 | 0.2 | 0.2 | 0 | 0 | 0.00 | 2 | 0.25 | 0.5 | 0 | 27 | 1.00 |
| 142[a] | 54 | 0 | 0 | 0 | 0 | 75 | 0.78 | 1 | 0.75 | 0.75 | 0 | 21 | 0.22 |
| 144 | 71 | 2 | 1 | 2 | 0 | 33 | 0.32 | 6 | 11 | 66 | 1 | 71 | 0.68 |
| 145 | 27 | 0 | 0 | 0 | 0 | 52 | 1.00 | 2 | 0.2 | 0.4 | 0 | 0 | 0.00 |
| 149 | 23 | 1 | 0.1 | 0.1 | 0 | 19 | 0.13 | 5 | 16 | 80 | 1 | 124 | 0.87 |
| 151 | 19 | 1 | 0.25 | 0.25 | 0 | 0 | 0.00 | 7 | 8 | 56 | 1 | 105 | 1.00 |
| 157 | 32 | 6 | 360 | 2160 | 1 | 5 | 0.07 | 9 | 1260 | 11340 | 1 | 71 | 0.93 |
| 165[a] | 57 | 6 | 30 | 180 | 1 | 58 | 0.71 | 2 | 8 | 16 | 0 | 24 | 0.29 |
| 167[a] | 63 | 8 | 59 | 472 | 1 | 69 | 0.79 | 5 | 15 | 75 | 1 | 18 | 0.21 |
| Mean | 37.2 | 4.1 | 230 | 1731 | 0.50 | 49.5 | 0.60 | 3.6 | 184 | 1281 | 0.59 | 37.8 | 0.40 |
| SD | 18.5 | 3.3 | 465 | 3733 | 0.51 | 33.8 | 0.37 | 2.9 | 368 | 3055 | 0.59 | 40.9 | 0.37 |

Snail, identity of mother snail; Int, interval (days) between first and second matings. De1, De2, dart penetration depths (mm) of fathers 1 and 2. Du1, Du2, dart penetration durations (min) of fathers 1 and 2. DD1, DD2 = De*X* × Du*X*; DS1, DS2, dart shoot scores of first and second fathers. No. 1, No. 2, number of offspring of fathers 1 and 2. P1, P2, proportion of offspring of fathers 1 and 2.

[a]Clutches containing offspring positively identified as not belonging to either experimental father (these offspring are not included in the data set).

spring sired by hidden third-party fathers by first calculating the genotype frequencies for every potential third-party father at each locus, based on the allele frequencies in the experimental population; then we calculated the product of these sums across all three loci. Assuming a single mating by the mother before she was collected, the final values represent the probabilities, one for each clutch, of the father having a genotype that could be mistaken for one of the experimental fathers. In most cases these probabilities are $< 1.00$, and the mean probability for 22 clutches is 0.60. For those clutches with detected third-party–sired offspring, we calculated similar probabilities from the genotype frequencies of all possible third-party fathers; the mean probability for the six clutches is 0.27. If we now assume equal reproductive success for fathers of hidden third-party–sired offspring and fathers of revealed third-party–sired offspring, the following equation can be used to estimate the percentage of hidden third-party–sired offspring in the total sample:

$$p_{\text{hid}}/p_{\text{rev}} = f_{\text{hid}}/f_{\text{rev}},$$

where $p_{\text{hid}}$ = percentage of hidden third-party–sired offspring; $p_{\text{rev}}$ = percentage of revealed third-party–sired offspring = 1.7; $f_{\text{hid}}$ = mean probability of hidden third-party fathers = 0.60; and $f_{\text{rev}}$ = mean probability of revealed third-party fathers = 0.27.

By solving for $p_{\text{hid}}$ in the above equation, we estimate the percentage of hidden third-party–sired offspring to be 3.8%. Because the identities of these offspring could not be determined, they could not be eliminated from the data set.

With regard to the possibility that our data set was corrupted by offspring arising from third-party fathers, we again note that all revealed third-party–sired offspring were eliminated. As for the hidden third-party–sired offspring, it is important to realize that these would be randomly associated with the two experimental fathers (i.e., the good dart shooters and the poor dart shooters). Because the percentage of hidden third-party–sired offspring is relatively small (3.8%), their presence is unlikely to have biased the results with respect to the main questions tested in this study.

### Dart shooting and paternal reproductive success

We arranged 56 snail triads to allow each hermaphroditic snail to be used as both a mother and a potential father. Fifty-seven bilateral matings (114 total copulations) were documented. Of the 39 mothers that mated twice, 23 (59%) oviposited. The clutch of one mother failed to produce hatchlings, thus yielding a data set of 22 viable clutches. Table 1 shows the dart-shooting effectiveness and paternal reproductive success (PRS) for each of the two potential fathers. PRS is given in terms of both offspring number and proportion. We quantified a potential father's dart-shooting effectiveness as the product (DD) of the maximum depth of penetration (De) and the duration of penetration (Du) of its dart into the mother (Table 1, $n = 44$ for each variable). These measures of dart shooting are, in fact, properties of dart receipt, but we speak of shooting effectiveness because of the likely relevance of shooting to the shooter's PRS.

Figure 2 shows the distribution of dart shooting depth × duration products (DD). Most dart shootings are either poor (DD $< 10$; 41%), indicating shallow and brief dart receipts, or good (DD $> 100$; 43%), indicating deep and durable penetrations. Given the bipolar distribution of DD values, and no obvious way to translate the scale of DD values into degrees of biological effectiveness, we chose to use a binary classification in which a dart shoot score (DS) of 0 (poor) or 1 (good) was assigned depending on whether or not DD reached a critical, or threshold, value—namely, 50. This

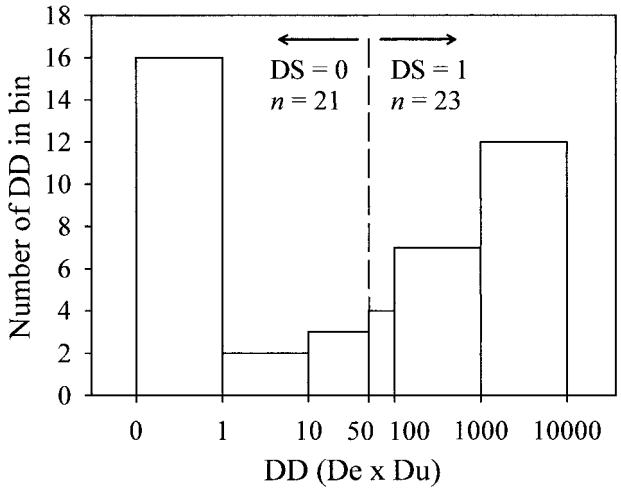


**Figure 2**
Distribution of DD (depth × duration) plotted on a $\log_{10}$ scale. The dashed line at DD = 50 marks the threshold for assigning dart shoot scores. Note that bins 10–50 and 50–100 are narrower than the others; if a single bin occupied the range 10–100, its height would be 7. De, dart penetration depth; Du, dart penetration duration; DS, dart shoot score; $N = 44$.

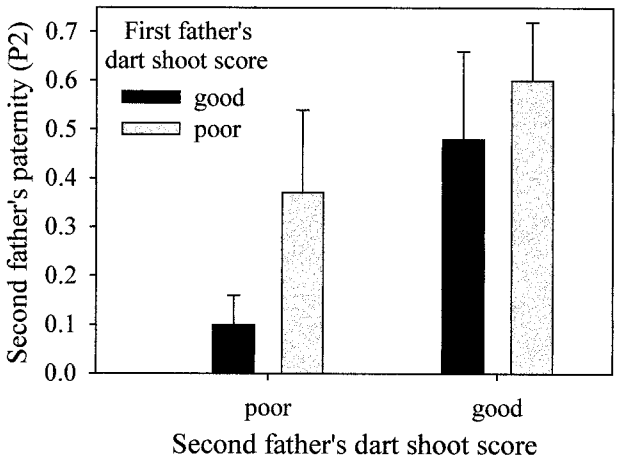


**Figure 3**
Second father's paternal reproductive success (P2) as a function of its own dart-shooting effectiveness and that of its competitor. Means and standard errors are shown. P2 was greatest when father 2 shot well and father 1 shot poorly (rightmost bar), and P2 was least when father 2 shot poorly and father 1 shot well (leftmost bar). The difference between P2 values in these two cases is significant ($p < .05$, $t$ test).

threshold value is near both the midpoint of the DD range (Figure 2) and the median DD value of 56.

We first examined whether mating order had any explicit effect on either dart-shooting effectiveness or PRS. The differences between the first and second fathers' dart penetration depths (De), durations (Du), De × Du products (DD), and dart shoot scores (DS) were all statistically insignificant (means are listed in Table 1; for all comparisons, $p > .05$, Mann-Whitney). Further, there were no significant differences between first and second fathers in the two measures of PRS, mean number and proportion of offspring ($n = 22$, $p > .05$, $t$ tests). Although our sample size was small, these analyses indicate that the order of mating had no significant effect on a snail's dart-shooting effectiveness or its PRS. Also, the interval between the first and second matings had no influence on relative PRS (Spearman correlation, $r_s = .06$, $p > .05$).

There were differences, however, in the proportions of offspring sired by good versus poor dart shooters. Figure 3 shows that the mean PRS of father 2 (P2) was greatest when that father shot well and its competitor (father 1) shot poorly ($n = 6$), and P2 was least when father 2 shot poorly and its competitor shot well ($n = 5$). The P2 values in these two cases, 0.60 and 0.10, respectively, are significantly different ($p < .05$, $t$ test). When the two fathers shot darts equally well, either both shooting poorly ($n = 5$) or both shooting well ($n = 6$), their PRS values were not significantly different (in both cases $p > .05$, $t$ test). The relationship between dart-shooting effectiveness and PRS is further supported by an ANOVA. The first run of the ANOVA generated $p = .62$ for the interaction between the first and second dart shootings. The interaction term was therefore dropped and the ANOVA was rerun. The second run indicated that the effect of the second father's dart shooting on its own reproductive success (P2) was significant ($p = .05$), whereas the effect of the first father's dart shooting on P2 was insignificant ($p = .21$).

ANOVAs were also performed using dart shoot scores assigned with different threshold DD values. The effect of father 2's dart shooting on its own PRS was significant ($p = .05$) for threshold DD values in the range 16–55, but insignificant ($p > .05$) for DD thresholds $< 16$ or $> 55$. DD values in the range 16–55 represent dart shootings in which the dart penetrates 2–5 mm for about 10 min.

## DISCUSSION

The results suggest that dart shooting influences paternal reproductive success. When a snail's dart-shooting effectiveness was good and its competitor's was poor, the paternal reproductive success of the better shooter was greater than that of the worse shooter (Figure 3). Furthermore, we found a significant effect of the second father's dart shooting on its own PRS. No such effect of the first father's dart shooting was found. This asymmetry may derive from the shorter delay between the second father's mating and the mother's oviposition, and/or from factors discussed below. We additionally found that, although the mean dart-shooting effectiveness and the PRS of the first father were greater than those of the second father, these differences were not significant. A similar insignificant advantage of first fathers was reported by Baur (1994). Mating order and dart shooting likely both affect PRS, and their interaction bears further investigation.

The positive result obtained in this study is consistent with the previously reported effect of the dart in promoting sperm storage in once-mated virgins (Rogers and Chase, 2001). If fertilization occurs by the random selection of allosperm, so that the reproductive outcomes for competing males are determined by the proportional representation of their sperm in the storage organ, then the uniquely successful dart shooter should father the most offspring because he will have the greatest number of sperm stored.

The conclusions are constrained by the procedures used. First, the sample size was small, as the data were derived from only 22 clutches; the power of the statistical procedures was therefore less than desirable. The data were likely also compromised by our use of nonvirgin snails as mothers; the mothers' possible use of sperm stored from mates other than the two experimental fathers probably added noise, estimated as 3.8%, to the data. Using virgin mothers would have simplified the paternity determinations, but would have necessitated raising snails from immature stages. The combined effects of unidentified fathers and a small sample size may have contributed to the marginal significance of the ANOVA results.

Our assignment of dart shooting scores using a binary classification is only a rather rough approximation of the real biology. More reasonably, dart-shooting effectiveness is a graded signal parameter, potentially eliciting graded responses in dart recipients. However, because the exact nature of the signal is unknown, our binary classification required the fewest assumptions while offering the greatest analytical power.

Most previous studies on the helicid mating system have focused on the immediate effects of dart shooting, rather than on the possibility that a snail's ability to shoot a dart might influence its reproductive fitness. Our assertion that the dart plays a more ultimate role does not exclude its having proximate effects; indeed, ultimate functions require proximate mechanisms (Alcock and Sherman, 1994). However, our results provide a link between the proximate consequences of dart shooting (induced contractions in the recipient's reproductive tract) and a possible ultimate effect (increased PRS).

An unresolved question is whether the dart's effect on paternal reproductive success is best viewed as mate manipulation or mate choice. If only the dart shooter benefits from the event while the dart recipient suffers a fitness cost, then dart shooting would be an example of mate manipulation (Adamo and Chase, 1996; Krebs and Dawkins, 1984). Alternatively, if dart-shooting ability correlates with mate viability and/or attractiveness, then the dart recipient would benefit by using the information contained in dart receipt to select sperm from different mates (Charnov, 1979). Dart shooting would then represent conventional sexual signaling and mate choice (Andersson, 1994).

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