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THE LOVE DART OF *HELIX ASPERSA* MÜLLER IS NOT A GIFT OF CALCIUM

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ABSTRACT

The phenomenon of dart shooting in several species of land snails has still not been explained. We were interested in whether the dart can function as a nuptial gift of calcium, as previously proposed. Donating calcium would increase the fitness of the offspring and thereby result in a higher reproductive success for the donor. We confirmed in *Helix aspersa* that the developing embryo takes up calcium from the egg shell for the formation of its embryonic shell. However, other results from behavioural observations and calcium measurements in various reproductive structures do not support the calcium hypothesis. We found that the dart penetrated the skin in 91.7% of the shootings, but it was internalized by the recipient in only 6.3% of the shootings. The amount of calcium in one dart is roughly equal to that of one egg, and thus it would not contribute significantly to an average clutch of 59 eggs. The spermatophore contains virtually no calcium, and therefore it is unlikely that the dart signals a donation of calcium with the sperm. The dart is also unlikely to influence egg laying since dart shooting does not predict either the latency or the productivity of egg laying in the shooter or the recipient. We conclude that the love dart of *Helix aspersa* is not a gift of calcium. Instead, we suggest that it is a vehicle to introduce a substance into the partner to influence the fate of the donated sperm.

INTRODUCTION

Several species of land snails make use of a dart during mating. The so-called love dart is a hard structure, made of the calcium crystal aragonite (Tompa, 1984), which is pushed through the skin of the partner during courtship. The reason for this extraordinary behaviour has still not been determined, although many hypotheses have been proposed (reviewed by Kothbauer, 1988). In this study we are interested in whether the love dart can serve as a nuptial gift of calcium for egg production (Charnov, 1979; Leonard, 1992).

One of the species expressing dart shooting behaviour is the brown garden snail *Helix aspersa* (Müller). The simultaneous, reciprocal mating behaviour of this snail can be divided into three phases, namely introduction, dart shooting and copulation (Adamo & Chase, 1988). At the end of the introductory phase one of the snails in a courting pair shoots its dart, and the second snail usually shoots its own dart within 30 minutes (Adamo & Chase, 1988). The term "dart shooting", while convenient, is inaccurate because the dart does not actually fly through the air. The dart is pushed into the skin of the partner by a quick and forceful eversion of the muscular dart sac, in which the dart is produced and stored. The outside of the dart is covered with mucus from the digitiform glands that empty into the dart sac. Treatments of this behaviour in the literature generally assume that once the dart is shot it detaches from the dart sac and remains in the skin of the partner. It is further assumed that the shot dart is incorporated into the recipient (Charnov, 1979; Leonard, 1992). The dart is usually shot into the partner's right side, a few millimetres behind the partially everted genital atrium (Giusti & Lempri, 1980; Chung, 1987).

Once both snails have shot their darts they attempt to achieve a simultaneous intromission. The first dart shooter will already be attempting intromission even before the second dart is shot, but the partner will not allow intromission until it itself has shot a dart and is ready to intromit. After successful intromission the pair becomes motionless and maintains reciprocal intromission for 4 to 6 hours. This time is required to exchange the spermatophores which package the sperm (Adamo & Chase, 1988).

After mating, the snails lay eggs within a variable period of time. The egg clutches of *Helix aspersa* comprise of 50 to 100 eggs (Tompa, 1984), and they have a semi-calcified shell, which means that calcium crystals are embedded in a jelly-like inner layer of the shell (Tompa, 1984). It has been observed for

several other species of snails that the embryo takes up this calcium from the egg shell during development and uses it for the formation of the embryonic shell (*Anguispira alternata:* Tompa, 1975; *Stenotrema leai*: Tompa 1979; *Veronicella ameghina*: Tompa, 1980).

Crowell (1973) showed that calcium is one of the limiting factors for development, growth and reproduction in *Helix aspersa*. This fact, together with those mentioned above, suggests that a large amount of calcium needs to be provided to the eggs for survival. Thus, donating calcium to the mating partner, as a nuptial gift, could be a way to increase the fitness of the offspring. The concept of a nuptial gift is well known from studies of insect reproduction, e.g. the sodium donation by the male moth to increase the fitness of its offspring (Smedley & Eisner, 1996). The main question that we ask here is whether the dart is such a gift of calcium. This idea was first suggested by Charnov (1979) and has remained an attractive explanation for the dart shooting phenomenon (Leonard, 1992). An alternative hypothesis is also tested here, namely that the dart is a signal that the animal possesses a large supply of calcium and that it will transfer some calcium with the spermatophore. This idea, that the dart may function to entice the partner before the actual nuptial gift is donated, also comes from insect biology. The male moth of the species *Neopyrochroa flabellata* donates a small precopulatory amount of cantharidin (Spanish fly) and transfers more of it with the sperm (Eisner, Smedley, Young, Eisner, Roach & Meinwald, 1996a). The cantharidin is then transferred to the eggs and protects them from predators (Eisner *et al.*, 1996b). In this case the spermatophore contains the actual gift. The possibility that a similar scheme is operative in snails is supported by the fact that the spermatophore wall is impregnated with calcium salts in some species (Tompa, 1984).

METHODS

Specimens of the garden snail *Helix aspersa* (Müller) were obtained from Santa Barbara, California. Snails were only selected for study if they had a reflected shell lip, indicative of sexual maturity (Chung, 1987). They were aroused from aestivation and kept in individual Lucite boxes (7 × 8 × 8 cm). In most cases, they were fed lettuce, carrot roots and crushed oyster shells (as a source of calcium) *ad libitum*. Some snails (no-calcium group) were deprived of calcium for 8 months, beginning immediately after they shot a dart. The boxes were kept moist by adding water every day and they were cleaned every day or every two days. Whenever the boxes were cleaned, the snails were given a shower to keep them moist and active. The temperature was maintained at 23 ± 2°C, with a light-dark cycle of 14/10h.

After 7 to 10 days of isolation, the snails were put in group boxes (18 × 18 × 8 cm) for observation during several hours every day, at the start of the dark period. Each group box contained 16 snails. The courtship and dart shooting behaviours were observed every 10 minutes. Whenever two snails formed a pair and were at a high level of genital eversion (Adamo & Chase, 1988) they were carefully removed from the group box and put into a pair box (7 × 8 × 8 cm) for closer observation. The transfer to another box did not affect their mating behaviour. Whenever the typical dart shooting posture was observed the snails were watched until the dart was shot. With this protocol no dart shooting events went unobserved.

Darts that were lost during the mating trials were collected for calcium measurements. To obtain spermatophores for calcium measurements, copulating snails were separated just prior to the transfer of the spermatophore. The spermatophore is formed immediately after intromission but it is not transferred until 4.5 hours after formation (Adamo & Chase, 1988). When snails are separated before sperm transfer they expel the untransferred spermatophore through the genital pore within an hour (Lind, 1973), thus allowed for collection. In a few cases the spermatophores were surgically removed from the bursa tract diverticulum after transfer.

Eggs were obtained by placing snails in individual boxes (7 × 8 × 8 cm) that were lined with 3 cm of moist sand. The eggs that were collected for analysis were either left intact or the egg shell was separated from the embryo and the albumen fluid. Separation was achieved by carefully peeling off the egg shell with a pair of fine forceps.

To measure calcium, a Perkin Elmer 3100 atomic absorption spectrophotometer was used. The spectrophotometer was fitted with a hollow cathode lamp with an operating current of 12 mA, and it was set for the calcium specific wavelength of 422.7 nm. All glassware was pre-treated with 15% nitric acid (HNO_3) in distilled water to prevent calcium contamination. The biological tissues were dissolved in 5 ml of 70% HNO_3. After 2 hours of digestion, a 0.1 ml sample was taken and diluted in distilled water to a resulting volume of 10 ml. Each sample was measured three times. A range of standards was made from a 100 ppm calcium stock solution in the same HNO_3 matrix as the biological samples.

RESULTS

Dart shooting

To examine the reliability of dart shooting, mating snails were categorized by their dart shooting behaviour and divided into those that shot a dart and those that did not shoot a dart.

In Table 1 the results are reported as absolute numbers, as percentages of the total darts available and as percentages of the total darts shot. From the snails that shot a dart (60%), a further categorization was based on the dart's ultimate destination. Darts that did not hit the partner and were either lost on the ground or retracted into the dart sac were defined as missed; these were observed in 8.3% of the dart shootings. A majority of the darts, 85.4%, were categorized as temporary penetrations. These darts were shot into the skin of the partner and stayed there for several minutes to several hours, but were eventually lost. The remaining 6.3% of the darts were categorized as internalizations because they were retained by the partner for more than 24 hours.

Of the darts that penetrated the skin most (73.1%) were shot in the right side of the animal just posterior to the genital pore. Other darts were shot in the foot (23.1%) or the head (3.8%). These results are similar to those reported earlier by Giusti and Lempri (1980), Chung (1986), and Adamo and Chase (1988). The position where the dart was shot did not correlate with internalization of the dart. Also, the depth of a dart's initial penetration did not seem to predict its ultimate destination.

To test the effect of the availability of calcium on dart shooting, snails were deprived of calcium for 8 months, beginning immediately after they shot a dart. This caused a decrease in shell strength and in increase in deaths due to shell failure, indicative of an overall lack of calcium. In this group dart shooting occurred in 62.1% (18 of 33 individuals) of the mating snails, which is not significantly different (χ^2-test) from the result reported above for snails that had unlimited access to calcium.

Calcium contents

Table 2 shows the mean calcium content (± standard deviation) of the different tissues determined with atomic absorption spectrophotometry. The eggs for the calcium measurements were taken from 4 different clutches (10 eggs per clutch). The results revealed that, on average, one dart contains nearly the amount of calcium of one egg. A spermatophore only contains trace amounts of calcium. From the observation that the average egg clutch consisted of 59 ± 22 eggs, the total amount of calcium per clutch was calculated to be 24.25 mg. On average, the dart contains only 1.5% of this amount and the spermatophore contains even less (0.07%).

Measurements of the egg shell and the embryo were made from freshly laid eggs and from eggs that had been developing for 12 days and were close to hatching. The total amount of calcium per egg was calculated by adding up the measured amounts for the shell and the embryo. The calcium contents of the egg shell and the embryo are reported in Table 3 as mean percentages (± standard deviations) of the total calcium content. The eggs came from the same 4 clutches that were used in Table 2. The data in Table 3 show that a significant amount of the calcium of the egg shell is taken up by the developing embryo. When the eggs are freshly laid most of the calcium is located in the jelly-like inner layer of the egg shell in the form of calcium crystals. During development of the embryo there is a gradual uptake of calcium (data not shown). By the time of hatching, most of the calcium has been taken up by the embryo for use in the production of the embryonic shell.

Table 1. Reliability of dart shooting in *Helix aspersa*.

Snails	Not shot	Shot	Missed	Temporary penetration	Internalization
80	32	48	4	41	3
100%	40%	60%	5%	51.2%	3.8%
		100%	8.3%	85.4%	6.3%

Table 2. Calcium contents of various reproductive structures.

Dart (N=32)	Spermatophore (N=22)	Egg (N=40)	Clutch (59 eggs)
0.369 ± 0.131	0.018 ± 0.041	0.411 ± 0.139	24.25 (calculated)

Means ± standard deviations, in mg.

Table 3. Distribution of calcium in the egg during development.

Egg part	Fresh eggs (n=32)	Hatching eggs (n=34)	T-test
shell	80 85% ± 15.31%	32.58% ± 30.48%	P<0.001
embryo	19.15% ± 15.31%	67.42% ± 30.48%	P<0.001

Means ± standard deviations expressed as percentages of the total calcium content of the egg.

Table 4. Egg laying in relation to dart shooting and dart reception.

	Shot a dart		Did not shoot a dart		Total	No calcium	Control
	Received (N=18)	Not received (N=12)	Received (N=3)	Not received (N=10)	(N=43)	(N=11)	(N=12)
eggs	66±22	57±24	42±3	55±19	59±22	66±23	59±28
latency	22±21	28±25	11±7	16±30	22±24	6±5	11±8

Eggs: mean number of eggs per clutch ± standard deviation; latency: mean number of days from mating to egg laying ± standard deviation; total: grand mean of the experimental groups; no calcium: mated snails deprived of calcium; control: unmated snails.

Egg laying

The mean latency from mating to egg laying for all the experimental groups was 22 ± 24 days, with an average clutch size of 59 ± 22 eggs (N=43). Table 4 shows the mean number of eggs and the mean latency to egg laying for snails grouped according to whether they shot and/or received a dart. There was no significant difference (two-way ANOVA) between any two groups for either the number of eggs laid or the latency to egg laying. Also, comparison of the grand mean of all experimental groups with the controls showed no significant difference (t-test). In the no-calcium group 11 snails laid eggs, with an average clutch size of 66 ± 23 eggs and a latency of 6 ± 5 days (Table 4). There was no significant difference (t-test) for either the number of eggs laid or the latency to egg laying compared to the unmated controls. Also, comparison to mated controls (not shown in Table 4), which laid 63 ± 26 eggs with a latency of 9 ± 8 days, revealed no significant difference.

DISCUSSION

Calcium is one of the limiting factors for growth and reproduction in snails (Crowell, 1973). To test whether calcium could be donated during mating we made behavioural observations and measured calcium levels in different reproductive structures. We confirmed that the developing embryo of *Helix aspersa* takes up calcium from the shell, as had been reported for other snail species (Tompa, 1975, 1979, 1980). However, additional results do not support the calcium hypothesis.

Contrary to general assumptions in the literature, our observations of dart shootings show that most of the darts are not internalized by the recipient. Chung (1987) and Adamo and Chase (1988) also found that many dart shootings do not result in internalization. From the published data of these authors, the percentage of darts that were, and were not, internalized was calculated for comparison to our own data. Table 5 shows these comparative data, and it is evident that all three investigations reveal the same trend. The totals show that, although 82.6% of the darts pierce the skin, only 22.1% are internalized. Some of the differences in percentages between the previous studies and the present one may be due to different definitions of missed and internalized darts. For example, in the present study some darts were found to be expelled a day after mating; these may have been considered by previous authors as internalized because of shorter observation periods. Nevertheless, the inference that can be drawn from these data is that the dart is unlikely to be a gift of calcium since it is seldom internalized.

The calcium measurements are also inconsistent with the love dart being a gift of calcium because the results indicate that the dart does not contain enough calcium to contribute significantly to an egg clutch. On average, one dart

Table 5. Fate of the love dart.

	Shot (N)	Skin penetration	Internalization
Chung (1987)	42	66.6%	14.3%
Adamo & Chase (1988)	48	89.6%	45.8%
This paper	48	91.7%	6.3%
Total	138	82.7%	22.1%

Skin penetration: temporary penetrations plus internalizations (excludes misses).

(0.369 mg calcium) contains roughly the same amount of calcium as one egg (0.411 mg calcium). For an average egg clutch this would only be a contribution of about 1.5%. Also, the spermatophore contains virtually no calcium, indicating that the dart is unlikely to be a precopulatory gift with the spermatophore as the actual gift of calcium. In addition to these empirical arguments, it should be noted that a gift of calcium delivered via either the dart or the spermatophore would usually be reciprocal. Thus, a snail would receive and donate an equal amount of calcium. Neither of the mating partners would benefit from such a reciprocal donation.

It seems unlikely that the dart is a signal from the shooter indicating that it will soon lay eggs or that it has enough calcium for egg laying. If this were the case, a short latency from mating to egg laying might be expected. Instead, the latency to egg laying was long and variable. Nor did we find any difference in clutch size for snails that shot a dart compared to those that did not shoot. Lastly, the dart did not affect either the latency to oviposition or the number of eggs laid by the recipient, indicating that receipt of a dart does not trigger or influence egg laying. However, caution should be taken in interpreting these results because the sample sizes are small.

Any transmission of information about the size of a dart shooter's calcium store is irrelevant when calcium is freely available in the environment. Since egg production and oviposition only start once a successful nest has been excavated, which may require several attempts over several days (Tompa, 1984), the period between mating and egg laying allows ample time for the ingestion of calcium. This indicates that a snail does not need to ingest and store all the calcium necessary for egg production before mating. Therefore, there is no use for a dart to inform a potential mate of the shooter's calcium content.

In these experiments most of the factors that are thought to influence calcium uptake and egg laying (e.g. the availability of calcium, humidity, temperature and nutrition) were kept optimal (Daguzan, 1981). Conceivably, in some natural environments where calcium is not plentiful, and thus where post-copulatory calcium ingestion would be limited, the dart might convey useful information about the shooter's readiness to produce viable eggs. To test this possibility, the calcium deprivation experiment was performed. Since the results showed that the availability of calcium affected neither dart shooting nor egg laying, it seems unlikely that the dart functions differently in natural environments and laboratory experiments.

Why then would a snail produce and shoot a calcium dart if it is not used for the production of eggs or as a signal of fitness? The reason to produce such an expensive signal might be found in the conflict of interest between the male and female functions (Charnov, 1982). We favour the pheromone hypothesis that was suggested earlier by several authors (Dorello, 1925; Börnchen, 1967; Chung, 1986; Adamo & Chase, 1990, 1996). Accordingly, the dart might be used as a vehicle to introduce a substance into the partner that increases the chance that the shooter's sperm will be used to fertilize the recipient's eggs. This would explain why both snails shoot a dart, since both snails will benefit from influencing the fate of their sperm. Such a function for the dart would only require penetration of the skin, instead of full internalization, in order to transfer the substance to the partner. The economical construction (double-H beam construction; Hunt, 1979), which makes use of minimal material for maximum strength, is consistent with this idea.

Snails can store received sperm for long periods of time (Tompa, 1984), which increases sperm competition. A snail would have an advantage if it could get more of its donated sperm to escape from the spermatophore and reach the female system. Lind (1973) surmised that only 0.1% of the donated sperm reaches the female tract in *Helix pomatia*. We propose

that a substance in the mucus of the digitiform glands is transferred to influence the fate of the donated sperm. Lind (1973) reported that the mucus of the dart caused a dilation of the bursa copulatrix in *H. pomatia* where the spermatophore is received. In *Helix aspersa* the spermatophore is received in the bursa tract diverticulum, which contracts to pull in the spermatophore. If a pheromone in the mucus were to act, either directly or indirectly, to slow down the contractions of the bursa tract diverticulum, more sperm could escape through the tail canal of the spermatophore into the spermoviduct before the remainder was degraded in the bursa copulatrix. Another site where a factor in the mucus could have an effect is in the spermoviduct, where contractions occur to transport the received sperm to the spermatheca, the site where foreign sperm is stored. These ideas can be tested by experiments *in vitro*.

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