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CHANGES IN THE REPRODUCTIVE SYSTEM OF THE SNAIL *HELIX ASPERSA* CAUSED BY MUCUS FROM THE LOVE DART

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Summary

The function of the love dart in certain species of terrestrial snails is unknown. In *Helix aspersa*, the dart is a sharp calcareous structure that is used to pierce the partner's skin during courtship. When expelled, the dart is covered with a thick mucus. The hypothesis tested here is that the mucus contains a biologically active substance. Extracts of the digitiform glands that produce this mucus were applied to parts of the reproductive system *in vitro*. The extracts triggered an initial reconfiguration of the copulatory canal that caused the bursa tract diverticulum to become more accessible to the spermatophore. The reconfiguration of the copulatory canal also closed off the tract leading to the bursa copulatrix, a sperm-digesting organ. A few minutes after the initial contraction, the peristaltic contractions in the diverticulum became significantly more frequent. This latter effect continued for at least 1 h, provided that the mucus extract remained in the saline bath. The minimum effective dosage was less than the 2.2 mg of mucus transferred with the dart.

Sperm competition is expected in *Helix aspersa* since multiple matings occur before eggs are laid. By influencing the female organs involved in the processing of foreign sperm, the dart shooter may increase the chance that his sperm will fertilise eggs.

Key words: love dart, mollusc, snail, *Helix aspersa*, reproduction, sperm competition, mucus.

Introduction

Nature is replete with examples of bizarre mating behaviours. Among these is the shooting of a love dart in several species of land snails. The function of dart shooting has bewildered investigators since at least the time of Swammerdamm (1637–1680). Numerous hypotheses have been proposed (reviewed by Kothbauer, 1988), but none is yet proven. In the garden snail *Helix aspersa* dart shooting is expressed at the end of courtship behaviour (Adamo and Chase, 1988). The dart sac, which produces and stores the dart, is forcefully everted from the genital pore, causing the dart to be expelled. Typically, but not invariably, the shot dart perforates the skin of the mating partner. We use the term 'shot' loosely. Since the courting partners are in close contact, the dart does not fly freely.

One type of explanation for the dart derives from the fact that the dart is made of calcium, formed as the crystal aragonite (Tompa, 1980). Since calcium is important for the development of snails (Crowell, 1973; Tompa, 1980), it has been thought that the dart might be a nuptial gift of calcium for the production of eggs (Charnov, 1979). We recently refuted this hypothesis by demonstrating that an insignificant amount of calcium is transferred with the dart relative to the amount present in the eggs and, moreover, that the dart is seldom internalised by the recipient. We also found no experimental support for the related ideas that the dart might induce egg-laying in the recipient or that it might signal the readiness of the shooter to lay eggs (Koene and Chase, 1998). It remains possible that the dart serves some other signalling function (Leonard, 1992), but in our view this is unlikely (Adamo and Chase, 1996).

Another type of explanation assumes that the dart directly influences either the behaviour or the reproductive physiology of the mating partner. This idea has been suggested in several different forms. Many authors (Dorello, 1925; Börnchen, 1967; Chung, 1986; Adamo and Chase, 1990) have sought to detect an effect of the dart on sexual arousal, thus echoing the legends of Eros/Cupid from classical mythology (Kothbauer, 1988). Observations indicate, however, that the receipt of a dart has only a small behavioural effect on sexual arousal, as measured by the degree of genital eversion, and it only slightly shortens the duration of courtship (Chung, 1986; Adamo and Chase, 1990). The effect seems to be too small to account for such a costly behaviour.

It is important to note that the dart could stimulate the recipient either mechanically or chemically. As the dart is expelled from its storage sac, it is covered by a mucus that derives from a pair of digitiform glands. Earlier experiments have demonstrated the feasibility of the dart acting as a hypodermic device to deliver the mucus to the interior of the recipient. In fact, the arousing effects noted above were shown

to be equal whether the dart was shot normally or the mucus of the dart was injected by a method that minimised mechanical stimulation (Chung, 1986; Adamo and Chase, 1990). Furthermore, in the latter case, the stimulatory effect was lost when the mucus extract was treated with pronase (Chung, 1986).

Here, we test whether an effect can be observed in the reproductive system of *Helix aspersa* by applying extracts of the digitiform glands *in vitro*. Fig. 1 shows a schematic overview of the reproductive system. Although *Helix aspersa* is a simultaneous reciprocal hermaphrodite, we will occasionally refer to gender-specific roles. As can be seen from Fig. 1, all the organs have an opening into the genital atrium, which connects to the outside *via* the genital pore. The genital atrium is gradually everted during courtship and it becomes visible as a white bulge on the right side of the animal (Adamo and Chase, 1988). Following dart shooting, the penis is everted and each snail attempts to intromit its partner. Simultaneous intromission, required for successful copulation, is achieved when the penises of both snails are inserted into the copulatory canals of the partners (Tompa, 1984). At this point, the spermatophore is formed in the epiphallus and the flagellum, and it is filled with sperm from the hermaphroditic duct *via* the vas deferens. When the spermatophore is complete, it is transferred into the bursa tract diverticulum of the partner, after which the snails separate. The sperm escape from the spermatophore through its tail canal to enter the female tract. They travel up the spermoviduct to reach the spermathecal sacs, where they are stored prior to being used for fertilisation (Lind, 1973). The spermatophore and the remaining sperm are transported through the bursa tract into the bursa copulatrix, where they are digested (Lind, 1973).

Snails mate with several partners during a season before they lay eggs (Tompa, 1984). From each mating, it is estimated that only approximately 0.1 % of the donated sperm actually reach the spermathecal sac; the rest are digested in the bursa copulatrix (*H. pomatia;* Lind, 1973). For these reasons, it would be advantageous for a sperm donor to increase the survival and utilisation of his sperm relative to that of his competitors. The experiments described here suggest a possible role for the dart in sperm competition.

Materials and methods

Specimens of *Helix aspersa* Müller were obtained from Santa Barbara, California. The snails were kept at 20–25 °C with a light:dark cycle of 16 h:8 h. They were fed lettuce, carrots and crushed oyster shells. To increase sexual proclivity, the snails were isolated for at least 10 days prior to use. Thereafter, groups of 10–20 snails were occasionally placed in a small box (18 cm×18 cm×8 cm) for observation. Pairs of snails were selected for experimentation when they exhibited courtship behaviour (Koene and Chase, 1998).

To confirm that mucus is transferred with the dart, the masses of darts were measured before and after shooting. Shot darts were collected and weighed immediately after shooting (in cases where they missed the intended recipient) or later, after they had been sloughed off by the recipient. In the latter cases, we used only darts that had penetrated the skin for 25 % or less of their length, to avoid losing mucus inside the recipient. The masses of darts before shooting were determined by dissecting darts from the dart sacs of sexually active snails. After the wet mass had been measured, the darts were lyophilised to determine their dry mass.

The digitiform glands were dissected out of sexually active snails. Extracts of the glands were made in a hand-held homogeniser with 0.5 ml of saline solution. Extracts of the columellar retractor muscle were used as a control for muscle tissue, and extracts of the pedal gland were used as a control for non-specific effects of mucus. The quantity of control tissue homogenised was comparable to that of the digitiform glands. To test effects from soluble components of recently shot darts, these were collected immediately after shooting and incubated in saline for at least 1 h at room temperature (20–25 °C).

In initial experiments, the entire reproductive system (Fig. 1) was dissected out and tested. Later, the preparations were reduced to include only the genital atrium, the copulatory canal and the bursa complex. The bursa complex comprises the

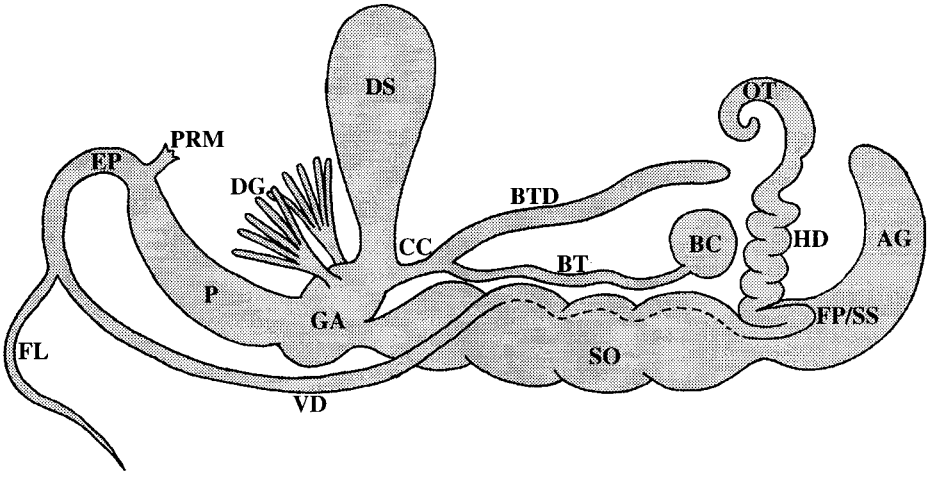

Fig. 1. Schematic drawing of the reproductive system in *Helix aspersa*. AG, albumen gland; BC, bursa copulatrix; BT, bursa tract; BTD, bursa tract diverticulum; CC, copulatory canal; DG, digitiform glands; DS, dart sac; EP, epiphallus; FL, flagellum; FP/SS, fertilisation pouch/spermathecal sacs; GA, genital atrium; HD, hermaphroditic duct; OT, ovotestis; P, penis; PRM, penis retractor muscle; SO, spermoviduct; VD, vas deferens.

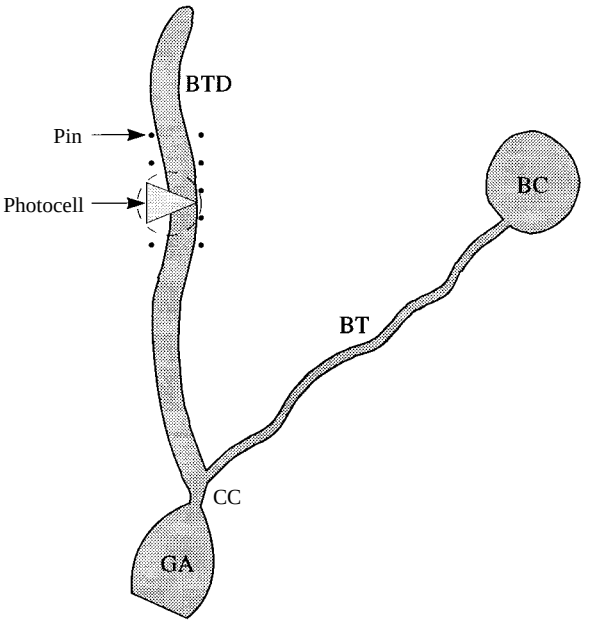

Fig. 2. Arrangement for recording contractions in the reproductive organs. The preparation was placed in a small dish and covered with 2 ml of saline. A triangular photocell was placed over the organ of interest, with a small light source coming from underneath, as indicated by the broken circle. Several pins were placed around the organ to prevent it from moving out of the field of measurement. For abbreviations, see Fig. 1.

bursa tract diverticulum and the bursa tract leading to the bursa copulatrix. These tissues were placed in a dish containing 2 ml of saline solution. Fig. 2 shows the experimental arrangement for measuring the responses of the copulatory canal and the diverticulum (shown here only for the diverticulum). A photocell was placed over the organ of interest, with a light source coming from underneath. When the organ contracted, there was a change in light intensity measured by the photocell and registered on a chart recorder. The photocell was preferred over a force transducer because the contracting diverticulum tended to pull in multiple, unpredictable directions. Movements of the organ were restricted by several pins inserted into the Sylgard base of the dish to prevent displacement of the organ out of the range of the measurement system.

For each trial, the maximum and minimum levels of the output of the photocell were determined, as shown in Fig. 3. Contractions of the organ were scored if their amplitude exceeded 25 % of the difference between the minimum and maximum levels. Peaks had to be separated by at least 30 s, otherwise they were counted as single occurrences. If the trace remained above the criterion level for more than 3 min, the event was not scored as a contraction and a new criterion level was established. Fig. 3 shows an example of these procedures; criterion-level contractions are labelled with an asterisk.

Basal (control) activity was recorded for 10 min before a test solution was added to the bath (Fig. 3). After the test solution had been added, the extract was allowed to take effect for 5 min, then activity was recorded for another 10 min (response period). The number of contractions was counted during the 10 min control period and during the 10 min response period. To calculate the overall response, the number of contractions in the control period was subtracted from the number in the response period. For the dose–response curve, each of 10 preparations was tested using six different test solutions. At the end of each trial, the contents of the saline bath were exchanged twice with fresh saline to remove the test solution. There was a 5 min rest period between each trial. Some preparations were tested with digitiform gland extract as well as with control substances. When single preparations were tested with either multiple doses or with multiple types of material, the order of testing was varied systematically. To avoid pseudoreplication, each dose or type of extract was used only once in any single preparation, and no test result was included in more than one figure or more than one statistical analysis. All records were coded before analysis.

Several other preparations were recorded for a longer time. In these cases, the control level activity was recorded for 10 min before the extract was added, followed by 60 min of response time. The recording was then interrupted for 1 min to wash out the extract, and the recording was continued for another 20 min. The number of contractions was counted in 10 min bins.

To test whether there is an effect of dart shooting on the success of spermatophore transfer, observations were made of dart shooting events during courtship. Particular attention was paid to whether the dart penetrated the skin. At 16–20 h after the beginning of copulation (10–16 h after the end of

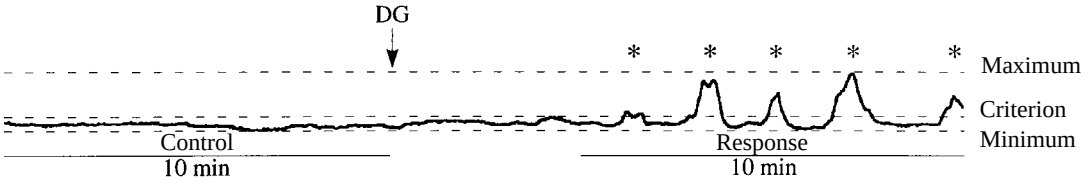

Fig. 3. Procedure for scoring contractions of the bursa tract diverticulum. The criterion level was set at 25 % of the difference between the minimum and maximum levels. Additional criteria were that individual contractions of the appropriate amplitude had to be separated from one another by at least 30 s and should not exceed 3 min in duration. The asterisks indicate contractions that were counted in this record. For each trial, the response to the application of a test substance (here, digitiform gland extract, DG) was measured as the number of contractions counted in the response period minus the number counted in the control period.

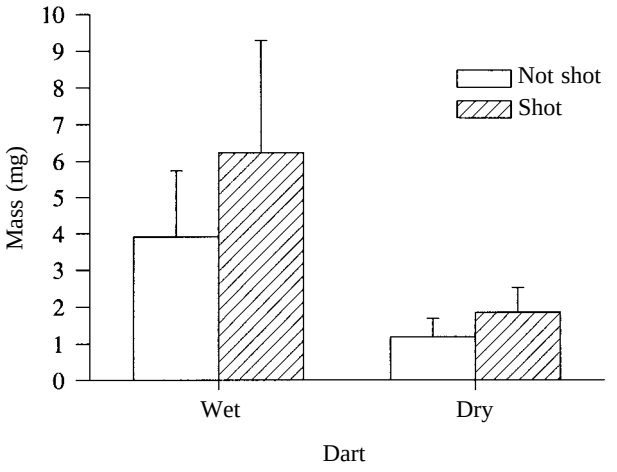

Fig. 4. Masses of the dart before and after shooting. The darts collected before shooting (not-shot, N=14) were obtained by operating on sexually active animals. The shot darts (N=15) were collected from snails of the same group after they had been expelled. The mean masses, wet and dry, are shown with their standard deviations. In both cases, shot darts were significantly heavier than not-shot darts (P<0.05; two-way ANOVA with Bonferroni correction).

copulation), the snails were examined to determine the extent to which the spermatophore had been transferred.

Results

Shot darts weigh significantly more than not-shot darts (Fig. 4), in both wet and dry conditions (P<0.05; two-way analysis of variance, ANOVA, with Bonferonni correction). The difference between the mean wet masses is 2.2 mg.

The tissue extracts were first tested on the entire reproductive system. We observed the induction of contractions in several organs including the penis, the spermoviduct and the genital atrium. However, with the exception of those in the copulatory canal and the bursa tract diverticulum, all the induced contractions were of short duration (<2 min). We therefore reduced the preparation to focus on effects in the copulatory canal and the bursa tract diverticulum. When monitored using a photocell, the copulatory canal shows an initial large movement in response to the application of digitiform gland extract followed by a prolonged change of position (Fig. 5A). From visual observations, we could see that the induced contractions closed off the entrance to the bursa tract while opening up the entrance to the diverticulum (Fig. 5B). This was confirmed by probing the two organs just before, and 5–10 min after, addition of the glandular extract. The probing was performed with a small piece of Plasticine shaped like the widest part, i.e. the body, of the spermatophore. Prior to the addition of the extract, the bursa tract was easily accessed from the copulatory canal while the diverticulum was obstructed. The situation was reversed after the addition of the extract, with the diverticulum now being more accessible than the bursa tract. The effect on the copulatory canal was confirmed by photocell recordings in 20 of 22 preparations.

In contrast to the early effect on the copulatory canal, the digitiform gland extract influenced the bursa tract diverticulum only after a latency of several minutes (Fig. 5A). The nature of the effect in the diverticulum was also different because, in this organ, the extract either initiated peristaltic contractions or increased their frequency. The potency of the extract was established using dose–response tests (Fig. 6). Doses of 0.03–0.05 ml caused increases in peristaltic rate that were significantly greater than those caused by the saline control (P<0.05; two-tailed Mann–Whitney U-test with Bonferroni correction), whereas lower doses were not significantly effective.

The duration of the effect was tested by recording from several preparations for a longer period. In these experiments, in which the digitiform gland extract remained in the saline bath for 60 min before being washed out, we found that the rate of peristalsis remained significantly elevated for at least 30 min (Fig. 7).

To test the specificity of the digitiform gland extract in producing changes in contraction rates, additional extracts were prepared from several tissues (Table 1). Responses to these extracts were determined in the same manner as previously described. Extracts of the columellar retractor muscle were consistently ineffective in increasing contraction rates, while extracts of a shot dart (which contained digitiform gland mucus) were always effective. These results suggest that the active material in the digitiform gland extract is mucus, not muscle or connective tissue. We tested this idea by using extracts of the pedal gland, which secretes mucus onto the sole of the foot as an aid to locomotion. In 68 % of the preparations tested, pedal gland extracts were effective in producing an increase in the contraction rate of the diverticulum. By comparison, extracts of the digitiform glands were effective in 84 % of the preparations. The difference in reliability of the two extracts is significant (P<0.005; χ^2-test). To compare the rate increases caused by pedal gland extracts and by digitiform gland extracts, the two substances were tested alternately in the same preparations (N=8). The mean increases (± S.D.) in the

Table 1. *Specificity of the substance inducing contractile changes in the bursa tract diverticulum*

		Percentage of preparations	
Extract	N	Rate increase	No rate increase
Digitiform glands	62	83.9	16.1
Pedal gland	22	68.2	31.8
Columellar retractor muscle	5	0	100
Shot dart (with mucus)	6	100	0

Rate changes were measured as described in Fig. 3. All rate increases, regardless of magnitude, are indicated here. In none of the preparations was a rate decrease observed.

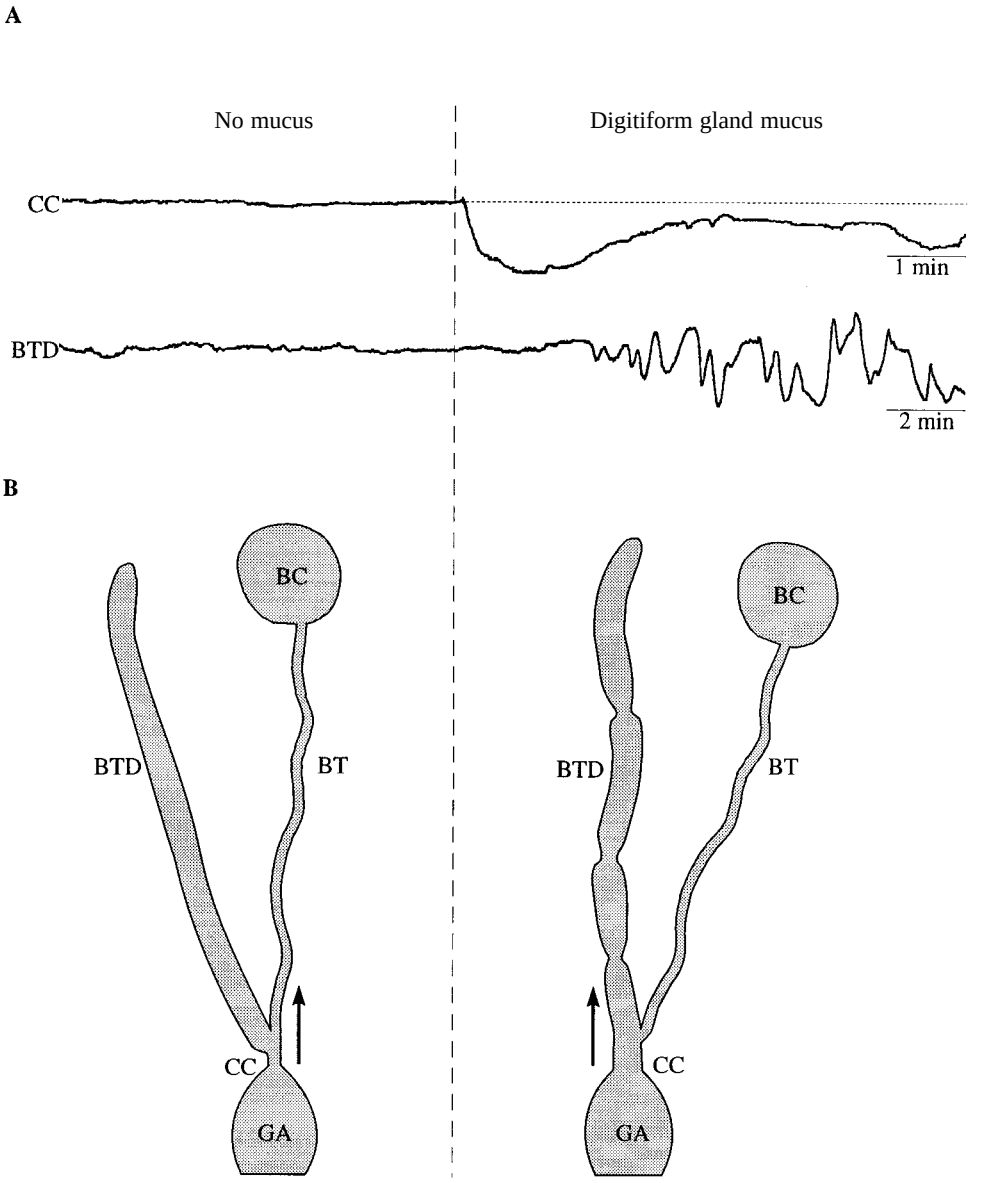

Fig. 5. Summary of the effects of digitiform gland mucus. (A) Sample traces for the copulatory canal (CC) and the bursa tract diverticulum (BTD). The dashed vertical line indicates the time of application of the extract. The dotted horizontal line provides a reference for the new configuration of the copulatory canal after application of the extract. Note the different time scales for the two traces. (B) Schematic representation of the observed effects. When no mucus is present, the copulatory canal connects to the bursa tract as indicated by the arrow. When the digitiform extract is added, the copulatory canal reconfigures to close off the bursa tract and make the bursa tract diverticulum more accessible (indicated by displacement of the arrow). Also, as indicated by crimping, peristaltic movements begin in the diverticulum or their rate increases. For other abbreviations, see legend to Fig. 1.

number of contractions per 10 min period were 2.88±1.96 with pedal gland extract and 3.31±2.46 with digitiform gland extract. Since these means are not significantly different (P=0.277; paired t-test), we conclude that the two extracts were equally potent, but unequally reliable.

We observed mated pairs to determine whether the spermatophores had been successfully transferred. In the majority of cases no part of the spermatophore was visible 16–20 h after the start of copulation, and it was assumed to have been incorporated. However, in some cases the tail of the spermatophore was still visible protruding from the genital pore. This was observed in only 2.5 % of the snails that received a dart (N=118), but in 13 % of the matings in which the dart did not penetrate the skin (N=92). Thus, complete transfer of the spermatophore is associated with successful dart shooting significantly more often than it is with unsuccessful dart shooting (P<0.001; χ^2-test).

Discussion

The mucus of the digitiform glands causes two important changes in the female portions of the reproductive system, as illustrated in Fig. 5. First, there is an early reconfiguration of the copulatory canal that begins immediately after the digitiform gland extract is added. Second, there is a delayed induction or potentiation of peristalsis in the bursa tract diverticulum that begins after a latency of several minutes.

The contractions in the copulatory canal seem to close off the entrance to the bursa tract and make the bursa tract diverticulum more accessible. Since the spermatophore is transferred into the diverticulum, transfer could be facilitated when the entrance to the diverticulum is widened. More importantly, the bursa tract leads to the bursa copulatrix, where excess sperm is digested. When the route to the bursa copulatrix is closed off, digestion of sperm would be delayed

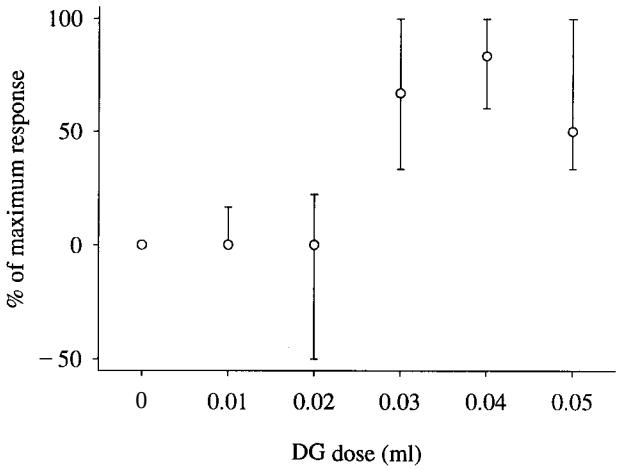

Fig. 6. The dose–response curve for the effect caused by the digitiform gland (DG) extract on the bursa tract diverticulum. A measure of response was calculated for each dose by taking the difference between the number of contractions observed in the control period and the response period (see Fig. 3). Ten preparations were tested with five doses. For each preparation, the response at a given dose was calculated as a percentage of the maximum response for that preparation regardless of dose. The graph shows the medians of these normalised responses, with error bars indicating the twenty-fifth and seventy-fifth percentiles. Maximum response values range from 2 to 9, with a mean of 5.0. Doses of 0.03–0.05 ml caused responses significantly greater than the saline control (0 ml), but doses of 0.01 and 0.02 ml had no significant effect (P<0.05; two-tailed Mann–Whitney U-test with Bonferroni correction).

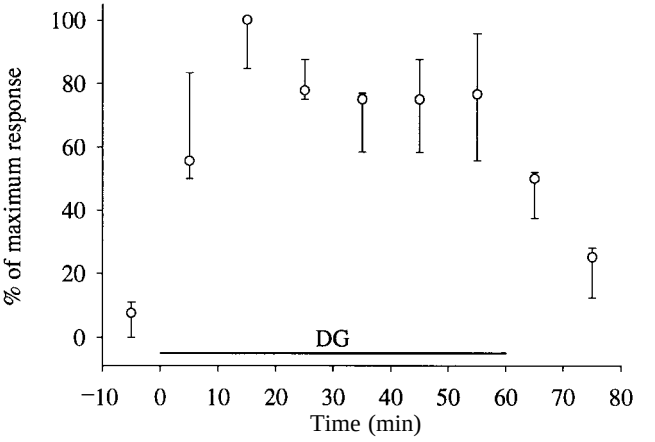

Fig. 7. Persistent influence of digitiform gland (DG) extract on contractile activity in the bursa tract diverticulum. Digitiform gland extract (0.05 ml) remained in the saline bath for 60 min as indicated by the horizontal bar. Response values were calculated as in Fig. 6. The bin width is 10 min, with medians and error bars (twenty-fifth and seventy-fifth percentiles) from five preparations plotted in the middle of the bins. Maximum response values range from 4 to 8, with a mean of 5.8. The responses recorded in the period 10–30 min following application of the extract are significantly greater than those recorded in the period from −10 to 0 min, i.e. before application (P<0.05; two-tailed Mann–Whitney U-test with Bonferroni correction).

and more sperm would have the opportunity to reach the spermathecal sacs.

The increased peristaltic contractions of the diverticulum should produce consequences that are synergistic with those noted above. Because the bursa tract is initially closed off by the reconfiguration of the copulatory canal, the faster transfer brought about by increased peristalsis could allow more time for the sperm to escape from the spermatophore and reach the female tract before the copulatory canal resumes its normal configuration.

The effect of digitiform gland extract on peristalsis in the diverticulum was persistent. We found that there was a statistically significant increase in contraction rate for 30 min following application of the extract, and a substantial increase, albeit statistically insignificant, for an additional 30 min, provided that the extract remained in the bath (Fig. 7). Normally, when the mucus is introduced into the blood by the dart, it will remain there until broken down or sequestered. Our results suggest that the mucus retains its biological activity throughout the period of circulation in the blood. A long-lasting effect would be needed to influence spermatophore transfer because complete transfer requires 4.5–6 h (Adamo and Chase, 1988). The idea that dart shooting influences transfer of the spermatophore is supported by our observations of animals after copulation. Most of the animals that were found to have a spermatophore tail still external to the genital pore many hours after the end of copulation were animals that had not been penetrated by a dart. Successful dart shooting, here defined as penetration, reduced by more than fivefold the percentage of matings wherein transfer of the spermatophore was incomplete.

There is reason to think that the concentrations of extract that were effective in our experiments are biologically significant. The blood volume of *Helix aspersa* is estimated to be 2.0–2.5 ml (Martin *et al.* 1958; Chung, 1985). This is equivalent to the volume of the saline bath used in the experiments (2 ml). Since the pair of donor glands from a single snail was diluted in 0.5 ml of saline, the highest dose (0.05 ml) represents 10 % of the digitiform glands. Given that the mean mass (± S.D.) of the glands is 22.7±8.5 mg (N=39), the highest dose contained 2.27 mg of gland material, not all of which consisted of mucus. From the data shown in Fig. 4, we conclude that the dart carries approximately 2.2 mg of mucus (see also Chung, 1986). Thus, the amount of mucus contained in our highest dose is approximately equal to, or even less than, the amount transferred by the dart. Consistent with this conclusion, extracts made from shot darts (with mucus) caused effects that were statistically indistinguishable from those caused by extracts of the digitiform glands (Table 1).

It is noteworthy that a difference in mass between shot darts and not-shot darts is apparent regardless of whether the measurements are made before or after dehydration (Fig. 4). This result is surprising because in both cases the difference

must be attributed to the mucus. Other molluscan mucuses are reported to contain at least 90 % water (Denny, 1983). Our results suggest that mucus from the digitiform glands might be exceptional in containing less than the usual amount of water, but presumably more of a biochemical product that has the allohormonal function implicit in our observations.

The active substance may be a general constituent of mucus since extracts of the pedal gland also increase the rate of contraction of the diverticulum (Table 1). Alternatively, it is possible that the two extracts cause the same effect through different mechanisms. While both extracts cause rate increases, they may differ in variables that were not measured, such as the amplitude or shape of contractions. Regardless, it is clear that a priority for future research is the identification of the active substance(s) in the mucus of the digitiform glands and the pedal gland. Chung (1986) reported data suggesting that the active substance in the mucus is a polypeptide with a molecular mass of approximately 5000.

We conclude that the dart functions as a vehicle to transfer mucus from the digitiform glands into the mating partner. Further experiments will have to be performed to test our hypothesis that the net effect is to increase the survival of sperm transferred by the shooter to the female tract of the recipient. Our hypothesis implies that the dart shooter manipulates his partner to increase his own reproductive success. By influencing the female organs involved in the receipt and transport of foreign sperm, the shooter can increase the chances that his sperm will fertilise the eggs of the recipient. Since multiple matings occur before eggs are laid, sperm from different males are expected to compete for fertilisation of the eggs. According to this idea, the dart evolved as a result of sperm competition.

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