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RESEARCH ARTICLE

Common effect of the mucus transferred during mating in two dart-shooting snail species from different families

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ABSTRACT

Several taxa of pulmonate land snails exhibit a conspicuous mating behaviour, the shooting of so-called love darts. During mating, such land snail species stab a mating partner with a mucus-coated dart. It has previously been shown that the sperm donor physiologically influences the sperm recipient via the mucus covering the dart and thereby decreases the number of sperm digested by the recipient. However, the generality of this effect of the dart's mucus is unclear, because almost all the previous studies on the effect of the mucus used the brown garden snail *Cornu aspersum* from the family Helicidae. Therefore, the relationship between the acquisition of the mucus effect on the recipient and the evolution of the dart itself, and its mucus, is still open to debate. To test the commonality of the physiological effect of the dart mucus, we examined this in *Euhadra peliomphala*, a species from the Bradybaenidae family, and compared our findings with the results of previous work using *C. aspersum*. Our experiments showed that in *E. peliomphala*, the dart mucus had a physiological effect and lowered the accessibility of the gametolytic organ, as found in *C. aspersum*. This indicates that in various dart-bearing species the mucus from the dart glands targets the same organ and that the inhibition of sperm digestion has played a crucial role in the evolution of the dart and its mucus.

KEY WORDS: Mate manipulation, Sperm digestion, Allohormone, Pulmonate land snails, Simultaneous hermaphrodites

INTRODUCTION

Simultaneous hermaphroditism is a common reproductive system in nature. In the last few decades, there has been increasing attention on sexual selection in such hermaphroditic organisms (reviewed in Anthes, 2010; Leonard, 2013; Nakadera and Koene, 2013). Theoretical studies have suggested that the influences of sexual selection are significant in simultaneous hermaphrodites, as they are in species with separate sexes (Charnov, 1979; Michiels and Koene, 2006). Also, various conspicuous reproductive behaviours have been reported in hermaphroditic animals (e.g. Michiels and Newman, 1998; Koene et al., 2005; Chase, 2007). However, because of the relatively limited literature on sexual selection in hermaphrodites, it is still unclear how important sexual selection has been for the evolution of their reproductive behaviours.

Here, we focus on the shooting of so-called love darts, which is one of the best known examples of conspicuous reproductive traits in simultaneous hermaphrodites. Dart shooting is a behaviour during which a snail drives its love dart(s) into its mating partner (i.e. through its partner's body wall). Such darts are hard and sharp objects, made of a crystalline form of calcium carbonate called aragonite, and are everted using a muscular dart sac. Dart-bearing species are known from at least nine families of stylommatophoran land snails (Davison and Mordan, 2007). Nonetheless, most of the research on dart shooting has been done on species from the Helicidae, in particular *Cornu aspersum*. For *C. aspersum*, a surgical experiment has shown that penetration of the dart transfers the mucus from the glands associated with the dart into the recipient's blood (Adamo and Chase, 1990). It has subsequently been demonstrated that this mucus causes conformational changes in the female reproductive system and that, as a result, the route to the bursa copulatrix, which is a gametolytic organ, is closed off (Koene and Chase, 1998). Furthermore, the mucus induces peristaltic movements in the bursa tract diverticulum, which is a dead-end duct branching off from the tract to the bursa copulatrix. As the spermatophore is transferred into this bursa tract diverticulum in *C. aspersum*, it was proposed that the conformational change and the peristalsis would delay digestion of donated sperm and promote sperm storage. Indeed, follow-up studies found that successful dart shooting, or experimental injection of only the mucus, resulted in greater sperm storage and paternity of the sperm donor (Landolfa et al., 2001; Rogers and Chase, 2001; Chase and Blanchard, 2006). However, it has been reported that, contrary to the results in *C. aspersum*, dart shooting has no influence on sperm storage in *Arianta arbustorum*, which also belongs to the Helicidae just like *C. aspersum* (Bojat and Haase, 2002). Moreover, a more recent study revealed a novel effect of dart shooting, a suppression of remating by dart recipients, in *Euhadra quaesita*, which belongs to the Bradybaenidae (Kimura et al., 2013). Although the effects of dart shooting have been well studied, there is limited understanding of the generality of these effects among dart-bearing land snail species. To better understand how and why this conspicuous reproductive trait has evolved, it is important to investigate the generality and variations in the function of dart shooting and the dart mucus using various dart-bearing species.

In this study, we tested the hypothesis that the dart mucus inhibits digestion of donated sperm in a species of the family Bradybaenidae, just as it does in *C. aspersum*. In a laboratory experiment, we investigated the effect of the dart mucus on the conformation of the female reproductive system and accessibility to the bursa copulatrix in *Euhadra peliomphala* (Pfeiffer 1850).

RESULTS

In all of the 15 snails injected with mucus, the Alcian Blue-coloured saline was transferred into the oviduct (Fig. 1B). In contrast, in 11 of 15 snails injected with the control, the coloured saline was found in the bursa tract and copulatrix (Fig. 1A), while in the remaining four snails it was found in both the bursa tract and the oviduct. Fisher's exact test showed that the proportions of the transferred position (oviduct or bursa tract) were significantly different between the two treatments ($P<0.01$).

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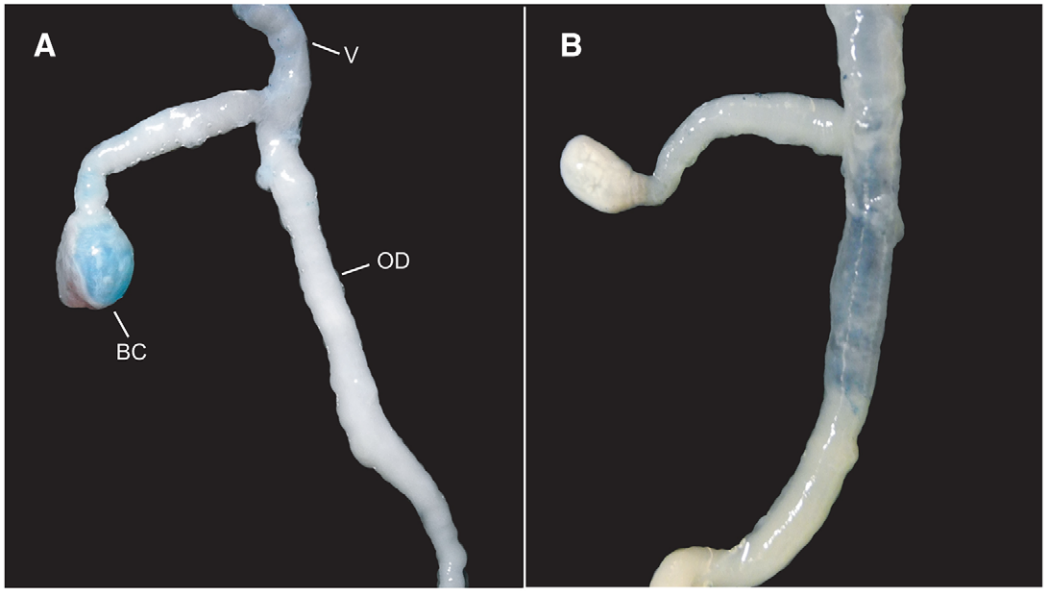

Fig. 1. The position of the coloured saline injected into *Euhadra peliomphala*. (A) For the individual that was injected with saline solution. (B) For the individual injected with the dart mucus extract. V, vaginal duct; BC, bursa copulatrix; OD, oviduct.

DISCUSSION

Our knowledge about the function of the love dart in land snails was, up to now, largely based on one species of garden snail, *C. aspersum* (e.g. Chase and Blanchard, 2006). Here, we present the first physiological experiments using a different species, the endemic Japanese land snail *E. peliomphala*. Using its reproductive system, we show that the dart's mucus altered the organ by which the injected coloured saline was taken up from the bursa copulatrix to the oviduct. This result indicates that the mucus interrupts transfer of objects into that gametolytic organ in our study species in a similar way to that reported for *C. aspersum* (Koene and Chase, 1998). In our experiment we did not investigate transfer from the oviduct (the spermatophore-receiving organ) under the influence of the dart mucus or a control injection, because the coloured saline was difficult to inject into the oviduct without the presence of mucus. However, the change in accessibility of the bursa copulatrix indicates that transfer from the oviduct into the bursa copulatrix is also interrupted when the mucus is injected. While the amount of dart mucus transferred into a recipient has been investigated in *C. aspersum* (Koene and Chase, 1998), it is unknown for our study species. However, although further experiments are needed to examine the amount of mucus transferred via dart shooting in *E. peliomphala*, the experimental concentrations of the mucus (1.0–1.75 mg ml^{-1}) were similar to those in the snails stabbed with the dart in *C. aspersum* (Koene and Chase, 1998) and, therefore, do not seem to be an unnatural condition.

For the underlying mechanism of the position change, Koene and Chase reported conformational changes occurring in the reproductive system after the addition of mucus (Koene and Chase, 1998). Although further experiments are needed, our current findings suggest that similar conformational changes occur in *E. peliomphala*. Regardless of the mechanism, the change in accessibility of the bursa copulatrix probably affects the fate of the donated sperm in the female reproductive system of the sperm recipient. In *E. peliomphala*, the donated spermatophore is moved from the oviduct to the bursa copulatrix after mating (Kimura and Chiba, 2013). Once the sperm are transferred into the bursa copulatrix by peristaltic waves occurring in the spermatophore-receiving organ, they are rapidly and permanently immobilised (Lind, 1973); therefore, only the sperm that swim out of the spermatophore into the allosperm storage organ (the spermatheca) before removal of the spermatophore can contribute to fertilisation. Therefore, inhibition of removal of the sperm and the spermatophore is thought to result in an increase in the number of sperm that reach the spermatheca of the recipient. In the present study, while we found that the change in accessibility is rapidly induced, it is still unclear how long this change lasts. However, it has been revealed that the dart mucus has a long-term effect in the recipient in *Euhadra quaesita*, which is closely related to *E. peliomphala*, although this effect was not a change in the accessibility of the bursa copulatrix but a delay in remating (Kimura et al., 2013). Moreover, it has also been reported that sperm swim out of the spermatophore within 8 h of mating in *E. quaesita* (Emura, 1932). Therefore, it is expected that the low accessibility of the bursa copulatrix induced by the mucus promotes the storage of sperm donated by the dart shooter, and subsequently fertilisation, in *E. peliomphala* as in *C. aspersum*.

Among the Helicoidea, including Helicidae and Bradybaenidae, it is still controversial whether the love darts and the mucus have evolved once or multiple times (Wade et al., 2007). These organs of helicid and bradybaenid snail species may have different origins because they have different strategies of dart shooting (Koene and Chiba, 2006), although they seem morphologically rather similar. Whereas *C. aspersum* stabs a mating partner once with their dart during courtship, *E. peliomphala* stabs multiple times in a single mating event and it usually lasts more than 10 min. Moreover, *E. peliomphala* uses its dart during copulation (Kimura and Chiba, 2013). Regardless of their evolutionary origin, however, the commonality of the physiological effect of the dart mucus in the two dart-bearing species provides important information on the generality of the effect among the Helicoidea. Although an inconsistent finding has been reported in the helicid snail *A. arbustorum* that dart shooting has no effect on sperm storage (Bojat and Haase, 2002), it seems to be an exceptional example because it has also been suggested that sexual selection is weak in this species (Baminger and Haase, 1999; Baminger and Haase, 2000; Locher and Baur, 2000). Therefore, although further investigations using broad taxonomic groups are needed to confirm this, our finding suggests that the physiological control of the reproductive system of the recipient via the dart mucus is widespread in the dart-bearing

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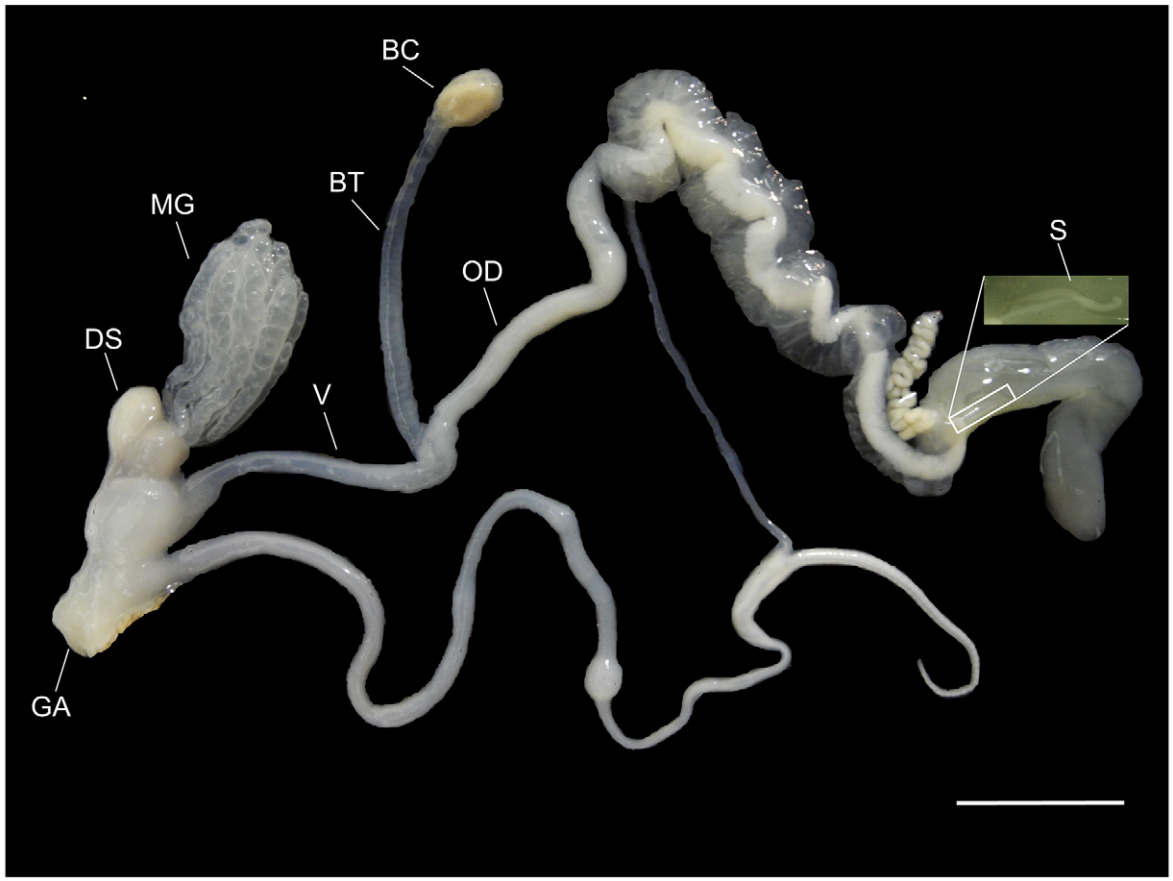

Fig. 2. Reproductive system of *E. peliomphala*. Dart sac (DS), mucus glands (MG), genital atrium (GA), vaginal duct (V), bursa tract (BT), bursa copulatrix (BC), oviduct (OD) and spermatheca (S) are shown. DS includes a love dart; MG secretes the mucus covering the love dart; BC is the gametolytic organ; S is the allosperm storage organ. Scale bar, 10 mm.

species. The commonality of the mucus effect in *E. peliomphala* and *C. aspersum* also suggests that selection for avoiding the digestion of the donated sperm by the recipient has an important role in the evolution of dart shooting in pulmonate land snails. Moreover, because many hermaphroditic animals, including such land snails, possess a gametolytic organ (Michiels, 1998), lowering of the accessibility to the organ may be a common strategy against such sperm digestion.

Besides the reported effect on the accessibility of the sperm-digesting organ, it was recently shown that dart shooting has another effect: suppression of remating by mating partners (Kimura et al., 2013). This remating suppression was reported for *E. quaesita*. The generality of the effect, however, is unknown and has also not been tested in *C. aspersum*. Therefore, this remains to be resolved in order to understand the general effects of the dart mucus.

In conclusion, the effect of the dart mucus in lowering the accessibility of the gametolytic organ in the reproductive system of the sperm recipient is now known for two species, one from the Bradybaenidae and one from the Helicidae. This result supports the idea that this conspicuous reproductive trait of land snails, dart shooting, has evolved through a conflict over sperm digestion between sperm donors and recipients.

MATERIALS AND METHODS

Study species

We used the simultaneously hermaphroditic land snail *E. peliomphala* in this study. Its reproductive system, shown in Fig. 2, includes a dart sac, mucus glands, genital atrium, vagina, bursa tract, bursa copulatrix, oviduct and spermatheca. The dart sac contains a love dart (Fig. 3), while the mucus glands produce and store the secretion that coats the love dart when the dart is pierced into a mating partner. The bursa copulatrix is a gametolytic organ that digests the excess of sperm as well as the casing of the spermatophore, while the spermatheca stores some of the allosperm (received sperm). Contrary to *C. aspersum*, our study species does not have the bursa tract diverticulum. Copulation of this species lasts 100–160 min. During copulation but prior to spermatophore exchange, the two mating partners stab each other repeatedly with their dart. At this time, they also reciprocally

Fig. 3. Electron microscopic photographs of the love dart of *E. peliomphala*. (A) The side view. Scale bar, 1 mm. (B) The cross-section. Scale bar, 100 µm.

insert their penises. *Euhadra peliomphala* donates a spermatophore into the oviduct of a mating partner (Kimura and Chiba, 2013), while *C. aspersum* donates a spermatophore into the bursa tract diverticulum. Matings of *E. peliomphala* are observed throughout their active period (i.e. except winter) in the wild. Therefore, *E. peliomphala* is assumed to have the dart and synthesise its mucus throughout this period. In our preliminary investigation, visual inspection of the glands revealed that these always contained stored secretions.

Adult snails were collected in the summer of 2010 and 2011 in Sendai, Japan, and kept individually in plastic pots (450 ml) at 22°C and ~65% relative humidity. All snails were maintained under the same photoperiod. These snails were fed cucumbers coated with powder including proteins and calcium *ad libitum* and the housing was cleaned every 2 weeks. To exclude the influence of matings already experienced in the field, laboratory experiments took place at least 8 weeks after collection.

Accessibility to the gametolytic organ

Adult snails were anaesthetised with an injection of 50 mmol l^{-1} $MgCl_2$ via the skin under the back of the shell. Subsequently, the snail was left intact (to maintain the animal's hydrostatic pressure) and a small plastic tube (0.50 mm diameter) was carefully inserted into the genital atrium until ~7 mm of the tube was inside. Our preliminary investigation revealed that with this length of insertion we reached the vaginal duct (N=5). The mucus glands were dissected out of another adult snail and an extract of these glands was made in a hand-held homogeniser with 0.5 ml of saline solution (for *C. aspersum*) (Chiarandini, 1964). The supernatant of the extract of the mucus glands (0.1 ml) was then hypodermically injected around the genital atrium (where the dart of a mating partner stabs) with a syringe (0.45 mm diameter), imitating a successful dart shooting; 0.1 ml of this supernatant contained 2.0–3.5 mg of the mucus gland extract. Then, ~0.05 ml of saline coloured with Alcian Blue was injected through the inserted tube and the position of this saline was subsequently examined by dissecting the individuals within 1 min of the injection. Our preliminary investigation suggested that the amount of coloured saline injected was adequate to assess accessibility.

Given that the blood volume is estimated to be 40.3% of the wet body mass without the shell in our study species, as in *Achatina fulica* (Martin et al., 1958), our preliminary investigation suggested that the mean volume of blood is 2.0 ml in *E. peliomphala* (N=8). Therefore, the concentration of mucus gland extract reaches 1.0–1.75 mg ml^{-1} in the individuals after the experimental injection. As a control, trials in which 0.1 ml saline instead of mucus extract was injected were conducted for another group of adult snails. The sample size of each treatment was 15 snails.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.K. designed the study, carried out the physiological experiments and data analyses, and drafted the manuscript. J.M.K. and S.C. participated in the design of the study and drafted the manuscript.

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