



Mating behaviour, dart shape and spermatophore morphology of the Cuban tree snail *Polymita picta* (Born, 1780)

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ABSTRACT

Hermaphroditic animals display a remarkable range of complex mating behaviours that are frequently related to the transfer of accessory-gland products. Here, we describe the use of the dart, an accessory reproductive device, and the mating behaviour of the hermaphroditic Cuban tree snail *Polymita picta*. Mating can be divided into three stages: courtship, copulation and post-copulation. *Polymita picta* has the longest mating duration of all *Polymita* species investigated so far. During courtship, a partial genital eversion exposes the sensitive zone, genital lobes and dart apparatus. During all mating stages, three uses of the dart apparatus can be distinguished: wiping, rubbing and stabbing, all of which mainly target the anterior region of the body, usually without loss of the dart. Morphological variation in the dart involves the extent of curvature, irregularities along the length and total length. These characteristics possibly provide a larger contact surface and deeper penetration through the body wall, which could result in increased sperm storage and paternity. The spermatophore is equipped with spines and we suggest that these might slow down spermatophore uptake into the bursa copulatrix. Overall, the general mating pattern, the presence of the sensitive zone, repeated use of the dart and its shape diversity support the idea that in more ancestral dart-possessing snails the dart apparatus is used to transfer accessory gland secretions, not only by stabbing but also through wiping and rubbing.

INTRODUCTION

Sexual selection and sexual conflict play a crucial role in shaping both mating behaviour and the associated morphology of genitalia, gametes and accessory gland products. This has been convincingly demonstrated in species with separate sexes (Chapman *et al.*, 2003; Arnqvist & Rowe, 2005). Similar selection processes can take place in hermaphroditic organisms (Koene & Chase, 1998a, b; Koene & Ter Maat, 2005; Leonard, 2006; Bedhomme *et al.*, 2009; Anthes *et al.*, 2010; Hoffer, Ellers & Koene, 2010). Conversely, in simultaneous hermaphrodites, only a small number of species have been sufficiently investigated to put sexual selection theory to the test, even though a theoretical basis for the understanding of hermaphroditic mating systems is available (Charnov, 1982; Michiels, 1998; Michiels & Koene, 2006; Leonard, 2013; Schärer & Pen, 2013). For example, for land snails this only became a topic for

research from the late 1990s onwards (Michiels, 1998; Koene & Schulenburg, 2005; Koene, 2012; Nakadera & Koene, 2013).

A remarkable range of complex, elaborate, often traumatic, and probably sexually selected mating behaviours are displayed by hermaphroditic molluscs (Schilthuizen, 2005; Koene *et al.*, 2013; Lange *et al.*, 2013; Lange, Werminghausen & Anthes, 2014). Stylommatophoran gastropods are simultaneous hermaphrodites and many of them are equipped with an accessory copulatory apparatus, which includes a gland that produces accessory male substances (Zizzari, Smolders & Koene, 2014). In several helicoids this 'dart apparatus' produces one or more sharp, calcified or chitinous devices. Strictly speaking, only a calcareous dart is usually referred to as a so-called 'love dart' (gypsobelum) (Tompa, 1984; Gómez, 2001), but this does not imply that chitinous darts cannot have a similar function and/or evolutionary origin.

Dart shooting is perhaps one of the most prominent examples of presumed sexually selected behaviours in hermaphrodites and has been most studied in stylommatophorans (Chase, 2007; Baur, 2010; Koene, 2012). The behaviour occurs when a snail everts the dart sac via the genital pore and stabs the dart through the body wall of its mating partner. Dart shooting is usually reciprocal and followed by copulation and sperm exchange (Lind, 1973; Jeppesen, 1976; Chung, 1987; Adamo & Chase, 1988; Landolfa, 2002). The function of stabbing a 'love dart', mainly studied in helioid and bradybaenid species, appears to be the injection of accessory gland products into the partner to enhance sperm survival (Chase & Blanchard, 2006; Kimura, Chiba & Koene, 2014). Although the well-known northern European helioid, *Cornu aspersum* (formerly *Helix aspersa*), shoots its dart once and leaves it behind in its partner's body wall (Adamo & Chase, 1988; Koene & Chase, 1998a, b), some helminthoglyptid, bradybaenid and cepolid species seem to stab their partner repeatedly with the same dart, not only during courtship but also afterwards (Webb, 1942, 1952; Koene, 2006; Koene & Chiba, 2006; Reyes-Tur & Koene, 2007; Kimura & Chiba, 2013).

Antillean land snails provide unexplored prospects for studies on the evolution of the dart apparatus. Cuban dart-possessing land snails are restricted to the family Cepolidae Ihering, 1909 (Bouchet *et al.*, 2005; Nordsieck, 2013), represented by 61 species in nine genera, including *Polymita* Beck, 1837. This genus consists of six species endemic to eastern Cuba, all reported on the Cuban Red List for endangered species (Amaro-Valdés, 2012).

Similar to other pulmonate land snails, *Polymita* species are simultaneous hermaphrodites. Species of this genus have a single dart in their reproductive tract and not one, but two types of mucus glands associated with the dart apparatus (Moreno, 1950). The genus is classified in the Cepolidae because the structure of its pedunculate mucus gland suggests that it is not homologous with the mucus bulbs of Helminthoglyptidae. In addition, no homology of the bilobulate mucus gland with structures of the American Helminthoglyptidae has been demonstrated (Roth, 1996; Cuzzo, 1998; Nordsieck, 2013). Furthermore, molecular phylogenetic analysis has also shown that *Polymita picta* (Born, 1780) is not grouped within the Helminthoglyptidae (Koene & Schulenburg, 2005; Wade *et al.*, 2007).

Polymita picta is distributed in northeastern Cuba from Maisí to Baracoa, and is renowned for its colourful shell polymorphism (Torre, 1950; Berovides, Valdés & Milera, 1986). It lives mainly in coastal habitats on trees and shrubs, where it feeds on moss, lichen and fungal biofilms on leaves and bark. Its life cycle lasts around 15 months, with breeding during the wet periods, mainly between September and October. At the beginning of the dry season, in December, the snails become dormant (until the beginning of May) and are firmly attached to trunks and branches by their epiphragms (Valdés, Berovides & Milera, 1986; Bidart, Espinosa & Pérez, 1989).

Polymita snails are the only group known to use the dart apparatus after copulation and to possess a 'sensitive zone' as one of the dart target areas. Moreno (1950) did not describe this zone; nevertheless, he identified an area located in the epithelium between the atrium and the atrial sac characterized by leaf-like gland structures. At least for *P. muscarum*, the location of the sensitive zone is consistent with the position of these structures, suggesting that they could be part of the sensitive area when the partial eversion of genitalia takes place. Even though the contact of the dart apparatus with this zone seems related to a quiescent state of the mating pairs, there is no confirmed function of the zone (Reyes-Tur & Koene, 2007).

Our current understanding of *Polymita* mating behaviour is based on two species, *P. muscarum* and *P. venusta*, which both follow a pattern of three well-defined stages: courtship, from the

start of tactile contacts to the simultaneous penis intromission; copulation, from the end of courtship to the penis withdrawal; and post-copulation, which finishes with cessation of tactile contact (Bidart, Fernández & Iglesias, 1998; Reyes-Tur, Fernández & Ortiz, 2000; Reyes-Tur, 2004; Reyes-Tur & Koene, 2007). In contrast, little is known about *P. picta*. Additionally, there are few detailed descriptions of the mating behaviour of land snails in general (Davison & Mordan, 2007) and observing more matings in more species will help to discriminate between intraspecific variation and general patterns of mating behaviour (Jordaens, Dillen & Backeljau, 2009).

Furthermore, significant interspecific variation has been found both in number and design of the love dart. Such diversity includes species with several conical darts and others with more elaborate ones (Tompa, 1980; Fedoseeva, 1994; Byun *et al.*, 2004; Koene & Schulenburg, 2005). Dart morphology is often species-specific (Koene & Muratov, 2004) and research has focused on this interspecific variation to reveal phylogenetic patterns. Work on diversity in dart morphology within species remains very scarce (but see Andrei, 2000; J. Weyer & J.M. Koene, unpubl.). In this study, we address the following questions: What is the general pattern of mating behaviour and dart use in *P. picta*? Could the variation in dart shape of the studied species imply a hypothesis on the transfer of accessory gland products during mating? And how is the shape of the spermatophore involved in this process?

MATERIAL AND METHODS

Animal collection

Since *Polymita picta* is a protected species, destructive sampling was limited. Observations were performed in the laboratory (2006–2012) and in the wild (2004–2012) from July to December. Adult specimens (recognized by a reflected lip at the shell aperture) and immature ones were collected at six locations in Guantánamo, eastern Cuba: Maisí municipality: Pueblo Viejo (20°13'64"N, 74°13'91"W), La Asunción (20°10'4.1"N, 74°14'13"W); Baracoa municipality: Majayara (20°20'44.9"N, 74°27'33.1"W), Caguasey (20°20'38.5"N, 74°30'5.7"W), Maguana (20°28'33.8"N, 74°35'39.5"W) and Nibujón (20°30'37.2"N, 74°40'5.8"W).

Laboratory maintenance

We studied the reproductive biology of *P. picta* at the Animal Behavior Laboratory of the Universidad de Oriente, Santiago de Cuba. To exclude the influence of previous matings in the field, snails were individually isolated in plastic containers (1500 ml drinking water bottles) for 25–48 d before mating trials. They were fed twice a week with sooty mould fungi on leaves from two plant species, *Mangifera indica* (mango) and *Ixora coccinea* (jungle geranium). The leaves were carefully washed to eliminate arthropods prior to administration and the containers were cleaned once a week. All snails were maintained between 24 and 32 °C under the natural light/dark cycle (13 : 11 h).

Mating behaviour under laboratory conditions

For each observation session in the laboratory, 10 to 20 adult snails were placed together in a transparent plastic experimental container (50 × 25 × 20 cm) with moistened floor and walls. In 2006, homotypic matings were analysed with snails from the same location, Pueblo Viejo. From 2007 to 2012, the snails from the remaining locations were mixed together during each session in order to facilitate heterotypic mating between snails from different populations. Observations of isolated mating pairs were made between 07:00 and 24:00 h; after 17:00 h we used fluorescent ceiling lighting in the laboratory.

Laboratory sessions revealed 24 courtship attempts, with time measurements taken for 11 mating pairs. All measurements of mating stage duration were based on live observations of the complete mating behaviour (from the beginning of courtship to the end of post-copulation). Additionally, we took photographs and videos lasting between 5 and 10 min for five mating pairs. These were only used for illustrative purposes and were not used to determine the mating stage duration. Duration of courtship, copulation, post-copulation and penis withdrawal (time between penis withdrawal of each partner) were recorded in minutes (Reyes-Tur *et al.*, 2000).

Mating behaviour under field conditions

Each field locality was carefully searched for mating pairs between 09:00 and 14:00 h; data were recorded for 24 pairs. The substrates of the mating snails were recorded as branches, trunks or leaves. The duration since detection of each mating pair until the mating behaviour finished was quantified, as well as the duration of penis withdrawal and post-copulation.

For a more comprehensive analysis of mating and use of the dart apparatus, information was captured on digital video (or photographs) of 29 mating pairs (5 in the laboratory, 24 in the field).

Role of the dart apparatus

Whenever possible, mating pairs were observed for periods between 5 and 10 min to record use of the dart apparatus. The number of contacts, frequency (contacts/min) and types of use of the dart apparatus, target zone, stage of mating and duration (in s) were all recorded.

Spermatophore exchange and dart variation

Animals for dissection were drowned in distilled water for 24 h and then preserved in 75% ethanol for further analyses. Seven pairs (two from the laboratory and five from the field) were dissected after mating to check whether spermatophores had been exchanged and to remove the darts. We also collected and measured four spermatophores of the same set of pairs, which were photographed in lateral and cross-sectional view, using a digital camera coupled to a stereomicroscope (Leica Wild M8). These were collected immediately after observed matings to avoid them being digested. Each dart apparatus was carefully dissected from the reproductive tract and placed in NaOH (2 N) for 24 h to remove surrounding tissues and mucus. SEM photographs of darts were taken in cross-section and in lateral view (following Koene & Schulenburg, 2005). Additionally, at least two darts of snails from each sampled locality were photographed in lateral view, using a digital camera coupled to a stereomicroscope (Lavobal® Carl Zeiss Jena, 25×: 0.5 mm precision). Digital image processing was performed with Adobe Photoshop CS 3 v. 10.0 and Corel Draw v. 13.0. Measurements of the darts were made with the software Image Tool v. 3.0 from photographs. The ocular objective lens used was 4× with a precision of 0.01 mm. The morphometric variables analysed were:

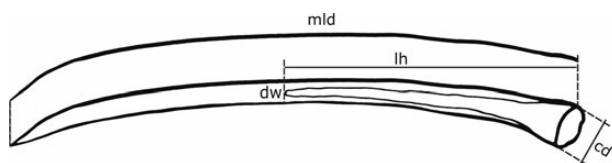


Figure 1. Dart morphometric variables in *Polymita picta*. Abbreviations: cd, corona diameter; lh, length of hollow core; dw, dart width at end of channel; mld, maximum length of dart.

corona diameter, length of hollow core, dart width at the end of the channel and maximum length of the dart (Fig. 1).

Statistical analyses

We compared pairs of means by Student *t*-test; mean values are given \pm standard error. All statistical analyses were performed using STATISTICA v. 8.0.

RESULTS

Mating behaviour

The data for the duration of courtship, copulation and post-copulation are given in Table 1. The complete mating behaviour lasted 264 ± 12 min. Two out of 11 fully observed mating pairs were excluded from calculation of means and standard errors, because no post-copulation times were recorded.

In the field, the mating pairs were detected from 09:30 to 13:55, always between October and November and normally on rainy days. The mating couples were observed generally on branches (64%). In the laboratory, couples were found mainly on the lateral walls of experimental containers (82%). Penis withdrawal was simultaneous in 20% ($n = 5$, field) and 27% ($n = 9$, laboratory) of the mating pairs. There were no statistically significant differences between the duration of penis withdrawal in field and laboratory ($t = 0.15$, $df = 12$, $P = 0.88$; Table 1). However, the duration of post-copulation in the field was shorter than in the laboratory ($t = 2.6$, $df = 11$, $P = 0.026$; Table 1). The same behavioural patterns were observed in the types of use of the dart apparatus in both laboratory and field observations.

Courtship

Whitish tissue around the genital pore is the distinctive sign of proclivity to courtship, and is the first stage of the progressive eversion of the genital pore region. This process is associated with continued tactile contact and involves four stages leading to the externalization of the sensitive area (zone), vaginal lobe, penial lobe and the dart apparatus, in the anterior–posterior axis (Fig. 2).

In 80% of the mating pairs, three types of tactile contact would start courtship, all involving tentacles: ‘tentacle–tentacle’, ‘tentacle–foot’ and ‘tentacle–partially everted genitalia’. After genital eversion reaches stage 4 (Fig. 2), the juxtaposition of both genital pore regions occurs, with the vaginal pore and the penial lobe in contact.

Copulation

Courtship ends and copulation starts when the partners simultaneously intromit their penial lobes. After intromission, the anterior region of the body thickens, the cephalic region turns upward and tentacles are withdrawn, usually completely or at

Table 1. Duration of mating behaviour stages in *Polymita picta* in laboratory and field conditions.

Parameter	Laboratory	Field
Courtship	30 \pm 7 (9)	Not quantified
Copulation	192 \pm 12 (9)	Not quantified
Penis withdrawal	10 \pm 5 (9)	9 \pm 3 (5)
Post-copulation	40 \pm 6 (9)	18 \pm 6 (4)
Mating	264 \pm 12 (9)	30–190 (14)

Values express the mean \pm standard error (sample size), in minutes.



Figure 2. Stages of partial genital eversion in *Polymita picta*. **A.** During stage 1 the start of genital eversion occurs, tissue around the genital pore region turns whitish, clearly contrasting with surrounding tegument. **B.** At stage 2, a white bulge is observed protruding from genital pore region. **C.** At stage 3, as shown by photograph of a courting pair, the left snail's genitalia are partially everted and the sensitive zone (b) and dart apparatus (e) are visible. The right snail's dart apparatus (a) is stabbing the mantle edge of its partner. **D.** During stage 4, reciprocal copulation occurs, here shown for same couple as in **C.** Both snails have genitalia partially everted in stage 4. The sensitive zone (b) and vaginal pore (d) of the left snail and dart apparatus (a) and penial lobe (c) of right one are indicated. Right snail is in head-on position. Maximum shell length of these animals is 24 mm.

least to half their total length. Such retraction is associated with cessation of circling. Sixty minutes after the initiation of copulation, the penial lobes are more dilated than they were at the beginning of intromission. On several occasions it was possible to observe the spermatophore's transfer through the body wall. Only under laboratory conditions, two of 11 mating pairs had non-reciprocal spermatophore transfer, one homotypic and the other heterotypic.

The spermatophore of *Polymita picta* (about 25–36 mm long) consists of a distinctive neck, body and tail (Fig. 3A). The body (3–5 mm long) contains the sperm and is preceded by a long neck (16–30 mm) and followed by a needle-shaped tail (3–6 mm long). The tail is round and hollow (Fig. 3B) and the body has a triangular shape (Fig. 3C). The neck of the spermatophore is a spiral with two lateral ridges of which only one possesses spines (Fig. 3D, E). The spines are oriented forwards (Fig. 3F) and their length is *c.* 100 μm right towards the anterior region of the neck (Fig. 3G), and increases to a maximum of 200 μm in the last portion near the beginning of the body.

Post-copulation

Penis withdrawal sets the beginning of post-copulatory activity. This stage is characterized by circular movements, a gradual decrease in the interaction between the genital pore regions and a gradual extension of the tentacles. At the end of post-copulation both partners move off in opposite directions, but cessation of touching is the unequivocal criterion that this stage has ended.

Mating frequency

During the observation sessions of 2006, eight homotypic mating pairs involving 11 individuals from Pueblo Viejo were recorded. When given the chance, four individuals (36%) copulated twice, of which two were with different partners and the rest with the same partner. The time interval between successful mating was 9–15 d, but it should be noted that in all cases snails had been separated for at least a week after they had mated and thus there had been no opportunity for them to re-mate in between.

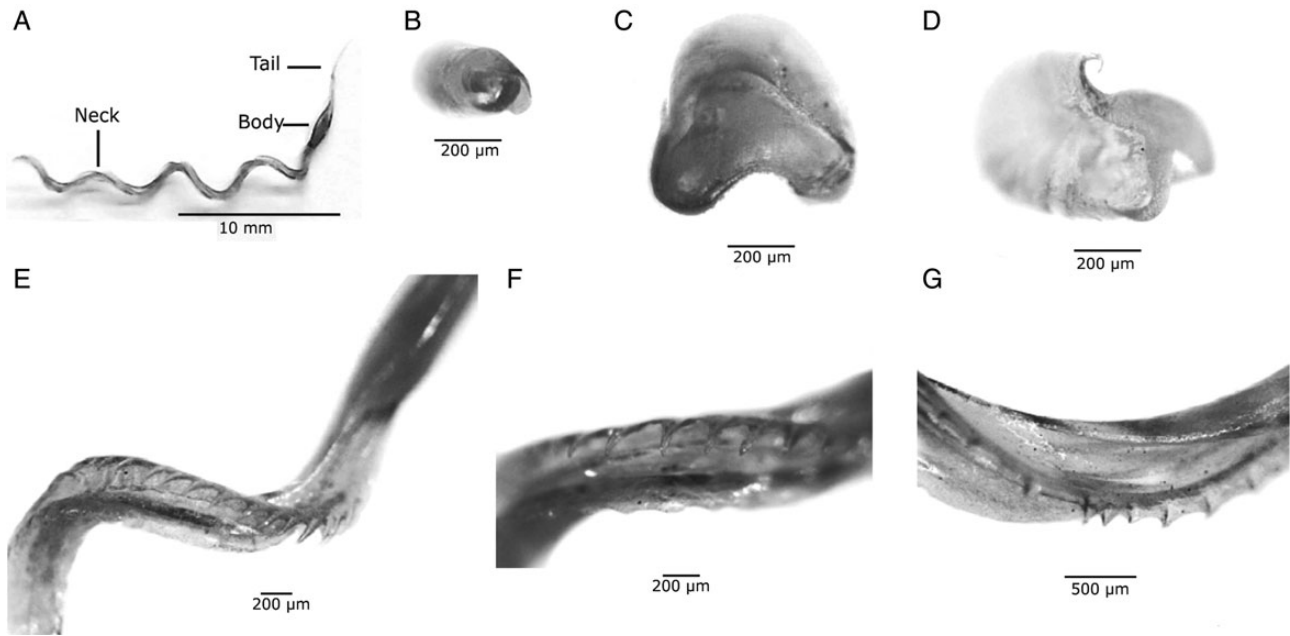


Figure 3. Spermatophore of *Polymita picta*. **A.** Tail, body and neck portion of spermatophore. **B.** Cross section of tail. **C.** Cross section of body. **D.** Cross section of neck. **E.** Final portion of neck before body of spermatophore. Neck has two lateral wings and only one is equipped with spines. The spines are oriented forwards. **F.** Detail of forwards orientation of spines on one wing of neck. **G.** First part of neck with short spines.

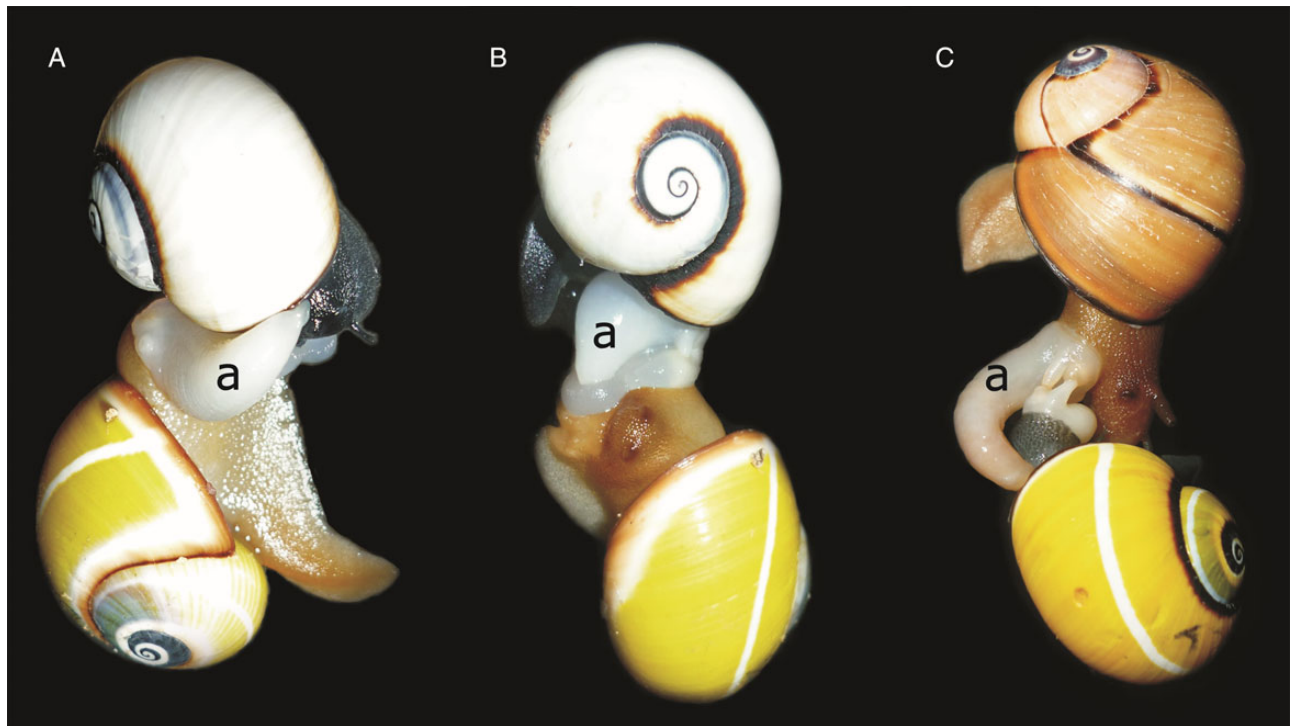


Figure 4. Dart apparatus use in mating pairs of *Polymita picta*. **A.** A pair of courting snails. Individual at bottom is using its partially everted dart apparatus (a) in a rubbing fashion with tip of dart sheath in contact with cephalic region. **B.** In a copulating pair the snail at top is wiping its partially everted dart apparatus (a) on centre of the sensitive zone. The snail below is in head-on position. **C.** In same pair as in Figs 2C, D, the uppermost snail is stabbing the dart apparatus (a), which is maximally everted, dorsally on cephalic region near the shell lip. Maximum shell length 24 mm; fully extended body length 40 mm.

Role of the dart apparatus

The beginning of eversion of the dart apparatus was usually associated with the probing of different parts of the partner's body. It occurred reciprocally or unilaterally during courtship,

copulation and post-copulation and the dart apparatus could be either partially or maximally extended. Mostly (61–70%), the probing with the dart apparatus targeted the anterior region, which included the partially everted genitalia (mainly sensitive zone) and cephalus (mouth, tentacles and the zone near the

Table 2. Quantitative summary of the uses of the dart apparatus in *Polymita picta*.

	Wiping	Rubbing	Stabbing
Frequency (min^{-1})	0.21 ± 0.06 (9) 0.09–0.29	0.29 ± 0.07 (9) 0.15–0.4	0.43 ± 0.13 (9) 0.19–0.64
Duration (s)	29 ± 4 (138) 2–480	19 ± 1 (322) 2–225	22 ± 4 (207) 2–754
Reciprocally (%)	73–100	59–62	21–49

Values express mean duration \pm standard error (sample size), and range.

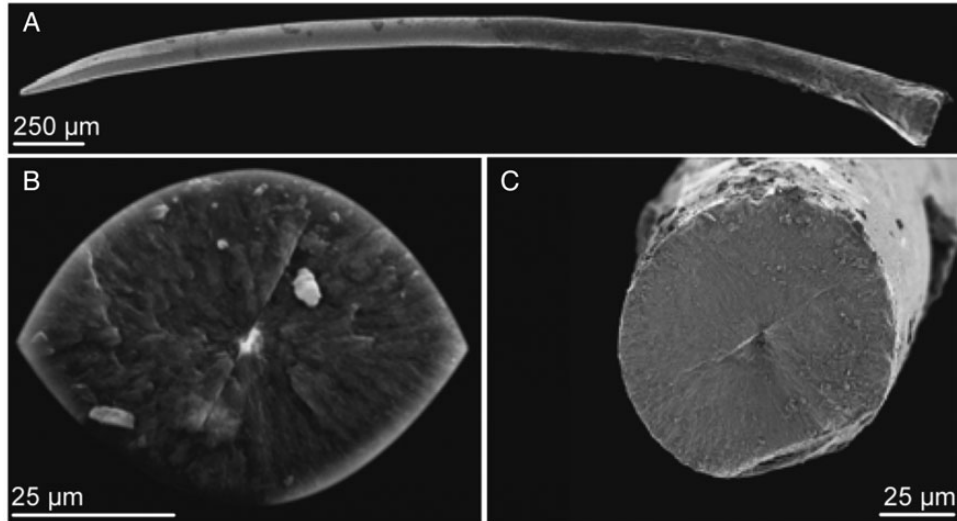


Figure 5. SEM images of dart from *Polymita picta*. **A.** Side view. **B.** Cross section at one-quarter of total length of dart measured from tip. **C.** Cross section in middle of dart.

mantle edge). In addition, there were contacts with the shell and posterior region of the body.

Three types of use of the dart apparatus could be distinguished: ‘rubbing’, ‘wiping’ and ‘stabbing’. Rubbing occurred when the surface of the dart apparatus, partial or maximally everted, rubbed any region of the partner’s body, which did not cause retraction of the target surface (Fig. 4A). It was usually associated with the transfer of abundant white mucus onto the body wall.

Wiping took place when the tip of the dart apparatus, usually partially everted, made contact with the sensitive zone, transferring white mucus (Fig. 4B). Wiping duration was between 7 and 10 min longer than the other modes of dart use (Table 2). This event was related to the quiescent state of the couple during spermatophore transfer. At this point, almost complete immobility was achieved and the sensitive zone gradually increased its size, seemingly in response to the wiping.

During mating the dart apparatus was frequently used for stabbing any region of the body, with the tip of the apparatus maximally everted. This occurred with a frequency 50% higher than that of wiping (Table 2). This event was associated with the immediate retraction of the target area and with the transfer of abundant white mucus to the stabbed surface (Fig. 4C). Most rubbing and wiping events occurred reciprocally, but stabbing in most cases took place alternately (Table 2).

Dart shape and variation

Dissections of pairs, immediately after laboratory and field matings, revealed that the dart is not lost during mating. Its shape is slender, with a round base, a wide crown and a sharp

Table 3. Quantitative summary of morphometric variables of darts in *Polymita picta*.

Corona diameter (mm)	0.24 ± 0.008 0.2–0.33
Length of the hollow core (mm)	1.7 ± 0.06 1.2–2.2
Dart width at the end of the hollow core (mm)	0.1 ± 0.005 0.08–0.14
Dart maximum length (mm)	3.4 ± 0.1 2.6–4.2

Values express the mean \pm standard error, including the range. Sample size was 15 darts for all variables.

end. The dart has a hollow core that extends through about half the total length (Fig. 5A), hence the tip of the dart is closed. The cross section is a sharp ellipse near the tip, but circular in the middle (Fig. 5B, C). The dart is covered by a membranous sheath, housed inside the dart sac, and protected by strong muscles that leave only the dart tip exposed. In some cases, during rubbing, it is possible to see the dart’s membranous sheath tip (Fig. 4A).

For the sampled specimens ($n = 15$), the dart morphometric results are summarized in Table 3. Morphological analyses of darts revealed at least three sources of variation: dart length, degree of curvature and contour irregularities (Fig. 6). Broadly, the dart’s maximum length ranged from 2.6 to 4.2 mm (Table 3). Additionally, darts may be classified into two groups

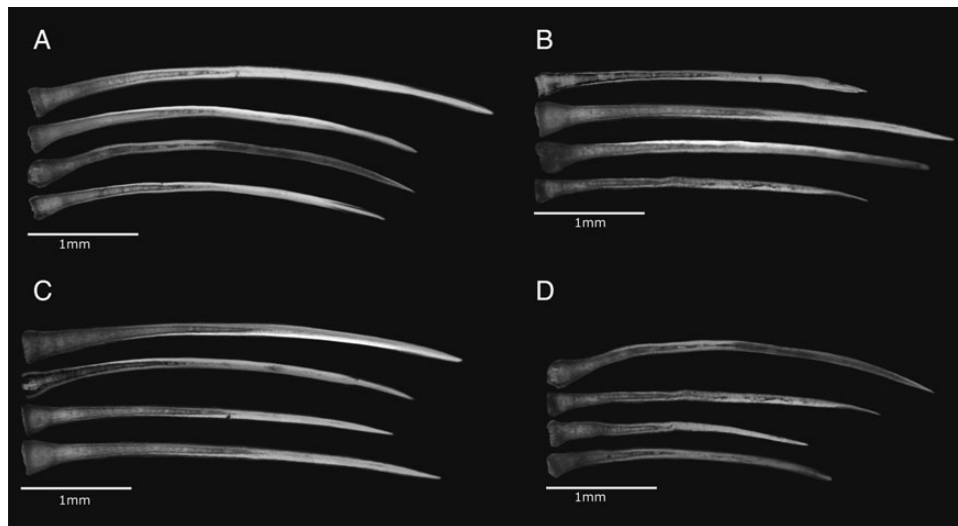


Figure 6. Stereomicroscopic photographs of *Polymita picta* darts in side view showing shape variation. **A.** Curved. **B.** Straight. **C.** Surface without abrupt changes in width. **D.** Surface irregular. Scale bars = 1 mm.

according to the degree of curvature: curved (Fig. 6A) and straight (Fig. 6B). Regarding the contour irregularities, darts can be classified as having either a rather regular surface without abrupt changes in width (Fig. 6C) or an irregular surface with frequent changes in width (Fig. 6D).

DISCUSSION

The mating behaviour of *Polymita picta* follows a pattern that is similar to other *Polymita* species and has three well-defined stages: courtship, copulation and post-copulation. For *P. muscarum*, Reyes-Tur & Koene (2007) reported a mean of 142 min for the duration of mating and for *P. venusta* Reyes-Tur (2004) gave a mean of 86 min. In contrast, as one of the unique features of *P. picta* mating is that it is about twice as long as in its two congeners, with copulation time occupying 63–83% of *c.* 4 h of mating duration. The data suggest differences between *Polymita* species in this variable, which may be determined by their different natural histories. *Polymita picta* has a geographical distribution restricted to a zone with the highest annual precipitation in Cuba, while the other two species are distributed in less favourable climates (Izquierdo, 1989). As a consequence, populations of *P. picta* may have been historically less exposed to stressful climatic conditions such as extreme drought. This seems to be reflected in their mating behaviour, given that they are under less selective pressure to save energy and/or reduce the risk of dehydration during copulation and mating than other *Polymita* species.

Our examination of spermatophore morphology in *P. picta* also revealed some interesting features. Based on the cross sections of the spermatophore, we suggest that sperm might exit the spermatophore of *P. picta* through the channel of the tail. In this species the spermatophore is delivered directly to the recipient's spermatophore-receiving organ and the tail is the last portion that enters it. If so, the site of exit of the sperm is the same as that in *Helix pomatia* (Lind, 1973); in both species sperm escape through the side of the spermatophore tail, which is shaped as a long gutter and is closest to the spermatheca.

We also report here that the spermatophore of *P. picta* is equipped with spines. On one side of the neck of the spermatophore, anteriorly oriented spines are present and cover the entire length of the neck. Spines have also been found in other dart-bearing species (Sauer & Hausdorf, 2009; Kimura & Chiba,

2013). These previous authors suggested that the spines could function to delay the removal of the spermatophore from the recipient's oviduct. This benefit might also occur for *P. picta*, given that the spines point in the direction of uptake, which might be particularly important since the spermatophore's tail is relatively short and without spines (in comparison with *Euhadra*; Kimura & Chiba, 2013). Further studies are necessary to determine the function of the spermatophore's spines as well as a possible evolutionary trade-off between the morphology of dart, spermatophore and the female reproductive system, especially the spermatophore-receiving organ (but see De Winter, 2007).

As a second peculiar feature in *P. picta* mating behaviour, we found a lower frequency of the use of the dart apparatus (0.04–1.05/min) than in another land snail that uses it repeatedly, *Euhadra subnimbosa* with a frequency of 2.52 stabs/s (Koene & Chiba, 2006).

A third notable feature is that in most helicoidean families the dart apparatus is associated with a single mucus gland (Nordsieck, 2013). On the contrary, *P. picta* bears two types of mucus glands (Moreno, 1950) and it is possible that the products of both glands are transferred via the dart apparatus. During rubbing, the large area of the dart apparatus and its elastic movements ensure spreading of the accessory gland secretion onto the surface of the anterior region (including the partially everted genitalia) of a mating pair. At first sight, this is similar to the use of the sarcobellum by *Deroceras gorgonium* (Limacidae) during courtship (Reise, Visser & Hutchinson, 2007).

Reyes-Tur & Koene (2007) did not mention wiping in *P. muscarum*. In the present work, we described wiping as a separate event from rubbing, because the dart apparatus contacts a specific zone of the partner, the sensitive area. In *Euhadra* the dart is used only during copulation and usually stabs around the posterior side of the partially everted genitalia (Koene & Chiba, 2006). However, with the exception of other *Polymita* species there have been no reports of a specific anatomical structure as target of the dart apparatus. In the studied species we confirmed that the location of the sensitive area is consistent with the position of the leaf-like structures described by Moreno (1950) in the non-everted atrial sac epithelium, which forms the sensitive area when the partial eversion of genitalia takes place. In contrast with stabbing, wiping and rubbing might avoid sexual conflict between partners, because they do not inflict direct harm (i.e. they are not traumatic).

Although the dart itself is difficult to observe during mating, it can be assumed to pierce the target surface (at least during stabbing). In addition, when the foot is stabbed, holes can be seen as a result of the dart's impact in the body wall. Therefore stabbing on the mantle cavity edge seems to be a way to transfer gland secretion to a highly vascularized area, thus securing its incorporation into haemolymph (for a review of similar strategies in different animal groups, see Zizzari *et al.*, 2014).

For helicids, the only hypothesis about mucus transfer by the dart apparatus that has received strong empirical support is that successful stabbing enhances the biological efficiency of 'males', since the best shooter has better odds of getting 'his' sperm stored in the partner's spermatheca and having it used in fertilization of eggs (Chung, 1987; Adamo & Chase, 1996; Koene & Chase, 1998a, b; Chase & Blanchard, 2006; Chase, 2007). The fact that the dart apparatus is used before, during and after spermatophore exchange in *P. picta*, implies that it could also affect sperm transfer and mating.

Finally, we found considerable variation in dart shape of *P. picta*. However, there were two extreme combinations of this variation, individuals with curved, irregular and long darts, as well as those with straight, regular and short ones. The observed variation in dart morphology suggests a hypothesis to be explored in future experiments. One prediction could be that the greatest efficiency in the transfer of accessory gland products during mating is achieved with the curved, irregular and long darts. These would produce the largest contact surface, deepest penetration in the body wall and could better increase the amount of stored sperm and the probability of producing offspring in the next generation. As an alternative explanation, the observed dart variation could be related to repair after being broken or lost during dart shooting. In the present study the seven pairs dissected after mating had undamaged darts. However, for the studied species, a recent study revealed that only 69% of individuals have a dart after mating (B. Reyes-Tur & L. Cepero-Benítez, unpubl.).

In sum, the general pattern of mating behaviour, the presence of the sensitive zone, repeated use of the dart and its shape variation in *P. picta* support the idea that in more ancestral dart-possessing snails the dart apparatus is used to transfer accessory gland secretions, not only by stabbing but also through wiping and rubbing (of which the function remains to be determined). All this indicates a structural and functional complexity of the genitalia that is in line with the idea that sexual selection could potentially influence the evolution of such reproductive traits.

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