ZOOLOGY

Cutaneous application of an accessory-gland secretion after sperm exchange in a terrestrial slug (Mollusca: Pulmonata)

Mandy Benke [a,b,c], Heike Reise [a,*], Kora Montagne-Wajer [b], Joris M. Koene [b]

[a] Senckenberg Museum of Natural History Görlitz, Am Museum 1, D-02826 Görlitz, Germany
[b] Department of Animal Ecology, Faculty of Earth and Life Sciences, VU University Amsterdam, De Boelelaan 1085, NL-1081 HV Amsterdam, The Netherlands
[c] Department of Animal Ecology & Systematics, Justus Liebig University, Heinrich-Buff-Ring 26–32, D-35392 Giessen, Germany

ARTICLE INFO

ABSTRACT

Competition for fertilisation in hermaphroditic animals seems to have led to many odd behaviours and complex morphologies involved in the transfer of accessory-gland products to the partner. Terrestrial slugs of the genus *Deroceras* show remarkably elaborate and interspecifically diverse penis morphologies and mating behaviours. Most species have an appending penial gland, which in *Deroceras panormitanum* consists of a few long fingers that are everted after sperm exchange and laid onto the partner's back. To investigate whether this gland transfers a secretion onto the partner's skin, we killed slugs at different mating stages and studied their penial glands and skin histologically. Two types of secretion granules appeared at a very early stage of courtship, and the penial gland was already filled 15 min into the courtship. At copulation, the gland everted this secretion onto the partner's body, where it remained for at least 50 min. No lysis of skin tissue or other effects on the skin were observed. The slugs tried to lick the received secretion off their own body, and some droplets were observed to be shed with the body mucus. Our results indicate the external application of a glandular substance that could function as either a pheromone or allohormone. The behaviours of the recipients suggest sexual conflict, although mutual interest cannot be ruled out.

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Introduction

In species with separate sexes, competition between males or their sperm for fertilisation of eggs can considerably influence the evolution of reproductive physiology and behaviour as well as associated morphological characters (reviewed in Arnqvist and Rowe, 2005). This selection pressure is starting to appear equally important in simultaneous hermaphroditic animals. Most research on sexual selection involving transfer of accessory-gland products in hermaphrodites has focused on bizarre stabbing behaviours. Examples include dart shooting in land snails (Koene and Schulenburg, 2005), body piercing in earthworms (Koene et al., 2005), and stylet piercing in sea slugs (Anthes and Michiels, 2007). In these examples, stabbing devices are used to transfer substances into the mating partner. However, many hermaphrodites that have evolved elaborate accessory reproductive glands do not possess organs with which they can pierce the partner's skin. Terrestrial slugs of the genus *Deroceras* can have particularly striking accessory glands, and this paper examines the hypothesis that these glands transfer a secretion onto the partner's skin (reviewed in Reise, 2007).

Such an accessory-gland product might be of importance in terms of competition for the fertilisation of eggs. There is scope for such competition in *Deroceras*, because individuals of at least some species mate repeatedly within a breeding season, can store sperm and use sperm from different mating partners for egg fertilisation within clutches, and can self-fertilise (Reise, 2007). It thus seems plausible that these slugs might have evolved penial glands as a mechanism to transfer a pheromone or allohormone that somehow improves the chances that their sperm fertilise the recipient's eggs. A manipulation of a postmating process affecting paternity is also suggested by the fact that the gland is everted after sperm exchange in *D. panormitanum* (Reise and Hutchinson, 2001).

However, other hypotheses have been proposed to explain the function or evolutionary origin of the penial gland. Webb (1961) assumed that it functions as a "semen-securing and retaining" organ during retraction of the penis, but no evidence has been found for this (Nicholas, 1984; Benke, 2006). For Nicholas (1984), the function remained unclear, but she likened the penial gland to the flagellum of spermatophore-producing snails and postulated that the gland's secretion in *Deroceras reticulatum* (Müller, 1774) might represent the "rudimentary tail of the spermatophore" (based on the observed accumulation of a secretion in the penial

* Corresponding author. Tel.: +49 3581 4760410; fax: +49 3581 4760499.
  E-mail address: heike.reise@googlemail.com (H. Reise).

gland during courtship and transfer of this secretion onto the partner during copulation). Our study leaves aside the issue of evolutionary origin but aims to learn more about the workings of the gland in order to shed light on its current function.

Deroceras slugs are simultaneous hermaphrodites with a mating system involving external mutual sperm exchange from penis to penis; this mode of sperm transfer is unique to a few groups of pulmonate gastropods (Emberton, 1994). Copulation in *Deroceras* starts with a rather sudden eversion and entwinement of both partners' penes. The ejaculates are transferred mutually and simultaneously, each in a single amorphous package. Sperm exchange can be very fast in some species, for instance 1 s after the start of penis eversion in *Deroceras gorgonium* Wiktor, Vardinoyannis and Mylonas, 1994, in which copulation lasts for 18–25 s (Reise et al., 2007). In other species, copulation can take much longer, for instance up to 15 min in *Deroceras panormitanum* (Lessona and Pollonera, 1882), even though in this species we know that sperm is exchanged early in copulation (Benke, 2006).

Deroceras shows great diversity in mating behaviour both at copulation and in the extensive precourtship and courtship phases that precede it (Reise, 2007). This is accompanied by an enormous diversity in penis morphology. Usually the penis has a more or less sac-like shape, but it can consist of one or more chambers, and may have side pockets and diverse appendages (Wiktor, 2000; Reise, 2007). In *D. panormitanum*, the focal species of this study, the penis has two side pockets (diverticulae), one longer than the other (Fig. 1). Somewhere near the proximal end of the penis of most *Deroceras* species there is an appending, more or less digitiform, gland, also called the trifid or penial appendage (e.g., Quick, 1960; Nicholas, 1984) or flagellum (e.g., Simroth, 1885; Gerhardt, 1933, 1935; Wiktor, 1960; Webb, 1961). We here refer to this organ as the penial gland. Like other penial structures, it varies widely in shape and size between species and can be unlobed or lobed, unbranched or highly branched (Wiktor, 2000). There is also some degree of intraspecific variability (Wiktor, 2000). The penial gland in *D. panormitanum* inserts between the two penis diverticulae and consists of three to seven long, usually unbranched fingers (Quick, 1960; Wiktor, 2000; Benke, 2006). In species in which it is present, the penial gland is everted during copulation and laid onto or under the partner's body (Reise, 2007) (Fig. 2).

The aims of our study were to investigate (i) whether and when the penial gland manufactures or accumulates a secretion, (ii) whether this secretion is transferred onto the partner, and (iii) whether there is any lysis of the skin tissue that might facilitate the secretion's entry into the body. Additionally, we provide the first detailed description of gland eversion and associated patterns of mating behaviour.
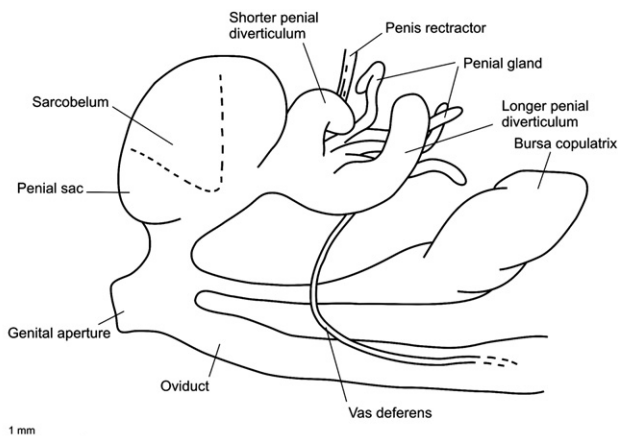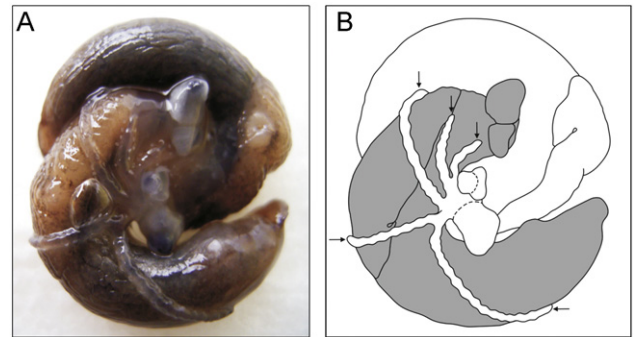
Fig. 2. (A) Photograph and (B) drawing of the full eversion of the penial gland of one slug (white) onto the body of its partner (grey). Arrows indicate the fingers of the penial gland.

We chose *D. panormitanum* for this study because copulation is comparatively slow (up to 15 min; Reise, 2007) and thus relatively easy to observe. Also, the appending penial gland is large and everted only after sperm exchange. Finally, the species is easy to keep and breed, and many years of culturing in our laboratory provide a broad knowledge about its biology, including mating and reproduction.

Material and methods

Animals

Most slugs were collected in Nork Park, Banstead (Surrey, England) as adults or subadults. The latter were raised prior to the study. Additionally, we used two offspring of slugs originating from the same locality. All 53 individuals from England (and also the other 12 individuals) were isolated for at least 3 days (maximum 29 days) prior to the experiments. They were kept in transparent Petri dishes (95 mm diameter, 15 mm high) in temperature- and light-controlled chambers (15 °C and 12L:12D). The Petri dishes contained several layers of wet tissue paper and some beech leaves; for food, pieces of carrots, lettuce, oat flakes, and cat-food pellets were provided in excess of requirements. Containers and food were renewed every three or four days. Some additional slugs for histological observations were collected near Uilenstede, Amstelveen (The Netherlands). These were kept in transparent plastic boxes slightly larger than the Petri dishes with wet tissue paper at 18 °C and 12L:12D and fed lettuce only. Finally, for some additional behavioural observations, a few slugs were collected in Görlitz, Germany. All these populations are believed to originate from the twentieth-century invasion from the native range of the species around the Mediterranean (Kerney, 1999).

Experiments

In the first study the penial gland was investigated histologically at five different mating stages: (1) sexually inactive; (2) during courtship (15, 30 or 45 min after sarcobelum extrusion); (3) during copulation, before penial gland eversion; (4) during copulation, after penial gland eversion; and (5) immediately after full retraction of genital organs. All slugs, except for stage 1, were placed in pairs into transparent plastic boxes (115 mm × 115 mm × 37 mm) the floor of which was lined with wet tissue paper. The couples were kept under direct observation, and matings were interrupted at stages chosen in advance. Some matings were observed under a binocular microscope. Matings were set up in the morning and evening, when there is the highest chance of mating. Couples which did not show any mating behaviour within about 80 min were separated.

Fig. 1. Part of the reproductive morphology of *D. panormitanum*.

Table 1

Filling state of the penial gland (PG) of slugs fixed at different mating stages. The sum of the numbers of empty and filled PGs corresponds to the total number of individuals examined at each stage. The periods of pre- and post-eversion of the PGs during sexual activity are also indicated. The proportion of individuals with filled glands differs significantly between sexually inactive animals and those killed pre-eversion (Fisher's exact test: $p=0.0002$), and between the latter and those killed after eversion ($p < 0.0001$).

Mating stage number	1	2					3	4	5
Mating stage	Sexually inactive	Courtship					Copulation		Postcopulation
		Time after sarcobelum protrusion (min)							
		5	10	15	30	45			
Number of empty PGs	6	0	0	0	0	0	0	7	6
Number of filled PGs	1	1[a]	1[a]	2	3	3	3	1	2[b]
		Pre-eversion						Post-eversion	

[a] Only few secretion granules lining the epithelium.
[b] In one specimen only some branches were filled.

In total, 49 slugs from England were killed at one of the five mating stages. However, failures in preparation or fixation prevented the use of 15 of these for histological investigation of the penial gland (Table 1); for instance, seven out of ten slugs fixed at stage 3 everted the penial gland when being killed. Since our initial findings indicated that the secretion appeared in the penial gland early during stage 2, we also included an additional pair (from The Netherlands) killed 5 and 10 min after extrusion of the sarcobelum (one slug protruded its sarcobelum 5 min earlier than the other).

Areas of the skin observed to be touched by the partner's penial gland (stage 4 or 5; $n=8$ animals) were prepared for histological investigation. Skin areas of slugs not hit by the gland (stage 1, 2 or 3; $n=5$) were used as controls. All skin samples were taken from English individuals.

In a second study, 14 additional animals were observed under a binocular microscope in order to investigate the fate of the received penial gland secretion. Three couples (two German, one Dutch) were left undisturbed, and four couples (two English, two German) were killed 30–120 min after the end of copulation. Because slugs had been observed to lick off the transferred secretion (see *Results*), the latter eight individuals were prevented from doing so by disturbing them with a spatula. We also investigated skin samples from the four English individuals (killed 45–50 min after copulation).

Tissue sampling and histological techniques

The slugs were injected with an overdose of the anaesthetic magnesium chloride through the foot into the haemocoel (0.5–1.0 ml of 0.06 M MgCl$_2$). Each animal was then immediately dissected, and the penis removed. Both the penis and the remains of the animal were fixed in Bouin's solution for 24 h and then transferred to 70% ethanol. The penes and skin tissue samples were later embedded in paraffin for serial sectioning (7 μm). One set of alternate sections was stained with haematoxylin and eosin, and mounted in Entellan (Merck, Darmstadt, Germany). The sections are deposited in the Senckenberg Museum of Natural History Görlitz (Germany), together with the remaining parts of the bodies.

Results

Secretory activity of the penial gland at different mating stages

The uneverted penial gland consists of an outer layer of connective tissue and the underlying epithelium (Fig. 3A). The series of slugs fixed at different mating stages showed that the
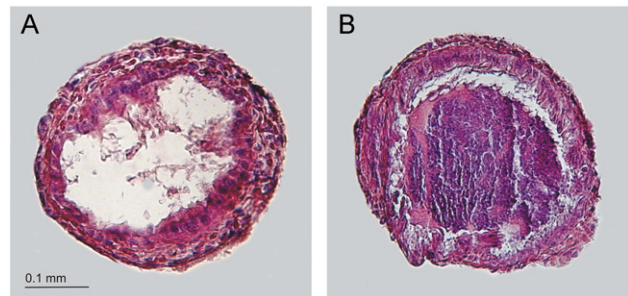

Fig. 3. Cross-sections through part of the penial gland of two different individuals, illustrating the difference between the empty (A) and filled (B) gland. The sections were stained with haematoxylin and eosin. The scale bar applies to both images.

lumen of the penial gland became filled with at least two different secretions during courtship: granules staining pink (basic) and others staining violet when stained with haematoxylin and eosin.

Penial gland filling began very soon after the start of courtship (it might start even during precourtship), and the lumen of the gland is full within the first 15 min of courtship. (The entire courtship takes 44–107 min; Benke, 2006; Reise, 2007.) However, our method did not allow us to examine whether the gland swells further thereafter as a result of continuing secretion. In the specimen killed 5 min after sarcobelum protrusion, a few pink granules were visible on the apical end of the epithelial cells; more were visible in its partner killed at the same time, which had protruded its sarcobelum 5 min earlier. In all animals killed 15 min after sarcobelum protrusion or later during courtship, the whole lumen contained violet granules surrounded by a thin layer of pink granules lining the epithelium (Fig. 3B). The origin of the two different penial-gland secretions remains partly unclear. The first appearance of the pink granules on the lumen side of the epithelial cells indicates apocrine secretion by the penial gland cells (as suggested for this species by Sirgel, 1973). Nicholas (1984) found secretions accumulating within the epithelial cells of the penial gland in early adult *D. reticulatum* but did not mention penial gland secretion during mating. The violet granules in the lumen of the gland fingers emerged later and were at that time already surrounded by a thin layer of pink granules. This indicates that the violet granules may have been produced elsewhere (e.g. by the prostate, as in *D. reticulatum*; Nicholas, 1984) and were subsequently transported into the penial gland, although it is also possible that the pink granules were modified after their release to form violet granules.

At the beginning of copulation, before gland eversion (mating stage 3), the penial gland was still filled, but in most cases it was

empty once the gland had been everted (stages 4 and 5). The gland was always completely empty if all branches had hit either the partner (eight cases) or the ground (three cases). However, in the four cases in which only some branches of the penial gland had hit the partner it was entirely (two cases) or partially filled (one case), or completely empty (one case). Table 1 provides a summary of these data and their statistical analysis.

In sexually inactive animals (stage 1), the penial gland was almost always empty. The only exception, a gland filled with a few pinkish granules, was found in a specimen that had been involved in a courtship 26 h earlier but had broken off courtship about 2 min after sarcobelum extrusion. In order to determine whether these granules could be left over from this previous mating attempt, we investigated in a few additional Dutch slugs how long the secretion remained in the penial gland if it was not transferred. Five slugs whose courtship we interrupted 30 or 40 min after sarcobelum extrusion were killed 6, 12, 18, 24 and 30 h later. In the two specimens killed 6 and 18 h after interruption, the gland secretion was modified (brighter violet, interspersed with pink granules and with less sharp boundaries than in animals killed during mating). The quantity of secretion was strongly reduced in the two specimens killed after 12 and 24 h, and had disappeared almost entirely in the slug killed after 30 h. These results indicate that the gland secretion found in the supposedly sexually inactive individual had almost certainly been produced the day before during the short period of courtship.

Behavioural observations during and after secretion transfer and effect on the partner's skin tissue

Close observation of mating couples under a binocular microscope revealed that the everting fingers of the penial gland released small (ca. 0.5 mm diameter) whitish globules of secretion onto the partner's skin. In eight individuals closely watched after transfer, but prevented from licking themselves, droplets were seen to remain on the body surface for a while. They disintegrated on two slugs after ca. 30 and 45 min, and were still visible on all other slugs when they were killed after 30–120 min. In some cases these globules were observed to move on the body surface, at least partly along with the body mucus. In one case this was in a forward direction towards the mantle and in two cases backwards towards the tail, where in one of these latter two cases the secretion was observed to be shed with the body mucus.

The three couples closely watched after copulation and left undisturbed turned their heads backwards and licked off the received penial gland secretion from their own skin (Fig. 4). This licking behaviour was also attempted by the other eight animals that were closely watched and killed 30–120 min after copulation. However, we prevented them from doing so by disturbing them with a spatula each time they bent backwards.

When the skin tissue hit by the gland was examined histologically, in only one out of eight animals fixed at stage 4 or 5 was a violet mass (resembling the secretion within the penial gland) found on the skin surface (Fig. 5, arrow). In this animal the deposit had been covered by the edge of the mantle. In two out of the four animals killed later (45 or 50 min after copulation) and prevented from licking their own body, secretion was found on the skin. Thus, where the removal of the secretion on the skin was prevented, either by the experimenter or by the mantle, 3 out of 5 times the secretion was found in the histological sections. However, in no case did we find differences between skin tissue hit by the penial gland (fixed immediately after penial gland eversion or 45–50 min after copulation) and the skin of the five animals which had not been hit.

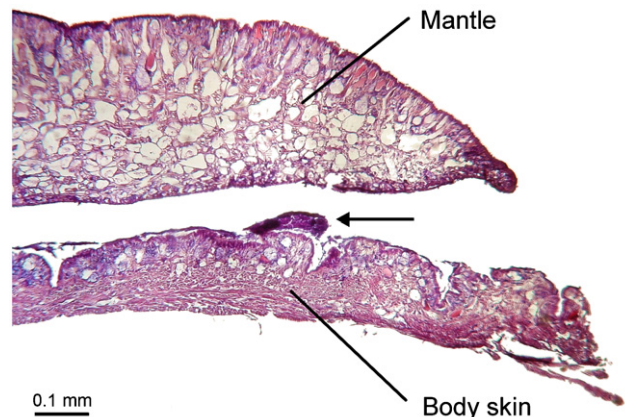**Fig. 4.** Slug licking off the transferred secretion from its own body.

Fig. 5. Cross-section of the skin of an individual killed at stage 4 showing received gland secretion (arrow). See text for explanation.

Discussion

The histological and behavioural results clearly demonstrate that an accessory gland, the penial gland, of the simultaneously hermaphroditic *D. panormitanum* transfers a secretion onto the partner's skin during copulation. This refutes previous suggestions that the gland produces a "rudimentary spermatophore tail" (Nicholas, 1984) or is a semen-securing organ (Webb, 1961). The latter is also disproven by the observation that the received ejaculate sticks to the base of the sarcobelum rather than to the penial gland (Benke, 2006). Furthermore, the elaborate morphology and behaviour related to the penial gland imply some purpose for secretion transfer onto the partner. For the time being, the function of this substance remains elusive and could serve the mutual interests of both mating partners or reflect a sexual conflict.

The fact that the penial gland is everted only after sperm exchange, not only in *D. panormitanum* but also in at least one other species (*D. gorgonium*; Reise et al., 2007) implies that this organ does not assist sperm transfer in a direct or indirect way but rather affects some postmating process. For example, Reise and Hutchinson (2001) hypothesised that it might increase the donor's paternity. Male manipulative gland secretions are often transferred directly into the female genital tract (probably most often with the ejaculate; e.g. Eberhard, 1996; Arnqvist and Rowe,

2005). However, transfer by peripheral routes may be more efficient because it evades ejaculate digestion or other female adaptations in the genital tract to resist manipulation (Arnqvist and Rowe, 2005). The method of application found in D. panormitanum is unique, since such peripheral routes of secretion transfer usually involve some kind of hypodermic injection, such as by love darts in helicid snails (Chase and Blanchard, 2006), setae in lumbricid earthworms (e.g., Koene et al., 2005), and mental glands in plethodontid salamanders (e.g., Picard, 2005). Without hypodermic injection, lytic enzymes can play an important role in improving secretion uptake through the skin. Lysis, for example, allows sperm to enter the body from sperm packages deposited onto the partner's body in some marine flatworms (reviewed in Arnqvist and Rowe, 2005) and sea slugs (Haase and Karlsson, 2000). Although we did not find any evidence for lysis in the present study, the active ingredients of the penial gland secretion might nonetheless enter the recipient's body through the integument without causing damage (Ryder and Bowen, 1977; Prior and Uglem, 1984; Uglem et al., 1985).

There are a number of possible mechanisms by which penial gland secretion might increase the donor's paternity. (1) Analogous to the effect of the love dart in C. aspersum, the secretion might inhibit sperm digestion in the bursa copulatrix and increase the amount of sperm reaching the sperm storage organ, thus increasing paternity under conditions of sperm competition (reviewed in Chase, 2007). (2) The secretion could function as an anti-aphrodisiac. It is well known from arthropods that male accessory-gland products can induce a refractory period in the female or even completely prevent her from remating (Eberhard, 1996; Chapman et al., 2003b; Gillott, 2003; Arnqvist and Rowe, 2005; Estramil and Costa, 2007). Alternatively, they may mark the recipient as already mated to make it (temporarily) less attractive to future mates. Mated individuals may, for example, be less attractive as female mating partners because they are recognised as having received sperm (Andersson et al., 2003; Haase and Karlsson, 2004) and less attractive as male mating partners because they are recognised as being potentially sperm-depleted. Of course, recognising the partner as already mated need not deter a future partner completely but may cause it merely to adapt its mating strategy (Cigliano, 1995; Michiels et al., 2003; Uhía and Cordero Rivera, 2005; Anthes et al., 2006; Friberg, 2006; Loose and Koene, 2008). (3) The secretion might stimulate egg production or oviposition, thus curtailing the risk of sperm competition. Such stimulation has been shown in many gonochorists (Gillott, 2003; Arnqvist and Rowe, 2005), but there are only indirect indications in hermaphrodites (Bride and Gomot, 1991; Saleuddin et al., 1991; Baur and Baur, 1992). We expect the short-term increase in fecundity to be costly to the recipient by reducing its survival, subsequent egg production, or paternity (Michiels, 1998; Koene et al., 2006; Bedhomme et al., 2009; Schärer 2009).

Especially if the gland secretion has a manipulative function and causes some cost to the recipient (either directly, or indirectly via loss of control over fertilisation), such a manipulation could cause a conflict between the mating partners about secretion transfer (Chapman et al., 2003a; Arnqvist and Rowe, 2005; Chase and Vaga, 2006; Koene and Chiba, 2006). As a result, an evolutionary arms race over morphological, physiological or behavioural patterns may arise, with one side increasing the efficiency of secretion transfer and the other side counteracting this (Rice, 2000; Koene and Chiba, 2006; Parker, 2006). Such an arms race is probably involved in the evolution of genital structures in helicid snails (Koene and Schulenburg, 2005). An arms race (over the use of transferred sperm) has also been suggested to drive the evolution of genital organs and mating behaviour in Polygyridae, Limacidae (Emberton, 1994), other

stylommatophoran groups (Beese et al., 2009), and in sea slugs (Anthes et al., 2008). The elaborate mating behaviour and penial structures, and particularly the large penial glands, of some Deroceras species seem very likely candidates to be subject to a similar arms race, which might have strongly contributed to the radiation of this speciose genus (Reise, 2007; Reise et al., 2007).

However, it cannot be ruled out that the penial gland secretion is not a product of conflict but rather serves mutual interests. The recipient might not lick off the secretion in order to remove it, but rather because the substance acts via receptors in the mouth or has to be absorbed by the digestive tract. One possible function involving mutual interest is mutual marking in order to prevent remating with the same partner (e.g., Ivy et al., 2005), but for this function uptake through the digestive tract seems implausible. Another benefit to the recipient would be if the secretion acts as a nuptial gift. However, mutual exchange of nuptial gifts is not expected to evolve in mating systems of simultaneous hermaphrodites (Michiels, 1998). In the case of Deroceras, it seems particularly unlikely because the gland content is rather small, and there is no obvious reason why the gift should be donated in such a complex manner with an elaborate organ rather than with the sperm mass. Another possibility is that a successful hit with the gland could indicate partner quality. This has been proposed for the function of the love dart in C. aspersum (Landolfa, 2002; but see Chase, 2007).

Substances used for mutual marking or other functions serving mutual interest would probably involve a pheromone detected by the partner (or, in the case of an anti-aphrodisiac reducing attractiveness, by potential other mates) via the air or via contact. In contrast, direct paternity manipulation would require an allohormone that enters the recipient's body in order to change internal female reproductive processes (Koene and Ter Maat, 2001).

Three behavioural observations during or after copulation may be interpreted as avoidance of the secretion by the recipient and thus indicate sexual conflict. (i) In D. panormitanum, the appending penial glands are not everted simultaneously, and the first partner often starts crawling away before the second one everts its gland (Benke, 2006; Reise, 2007). This may lead to some or all fingers of the second slug's gland missing the target, and the first slug may thus escape some or all of the secretion. However, there is so far no evidence that the slug everting second misses its partner more often (Benke, 2006). (ii) Secretion which had been put onto the body surface was sometimes observed to be partially licked off by the recipients. (iii) The secretions were sometimes observed to be transported and shed off together with body mucus. However, it is unclear whether this is a side effect (the body mucus provides a protective layer that is formed continuously and transported backwards over the body surface; Deyrup-Olsen et al., 1989) or whether the gland secretion is removed deliberately by increased mucus secretion (a common response to mechanical or chemical irritation; Triebskorn and Ebert, 1989).

Considering that all slugs that were closely observed through a microscope transferred secretion during gland eversion, it may seem strange how rarely secretion was found on the skin samples investigated histologically. No secretion was found on slugs killed immediately after penial gland eversion, with the exception of one specimen where some secretion was protected under the mantle. In contrast, in two out of four slugs killed later and prevented from licking themselves, secretion was found on the skin sections. A possible explanation is that the secretion may be washed off during fixation if it has not already been on the skin for some time. It would also be worth investigating whether the recipients indeed lick off the secretion and how efficiently they do this, but we did not examine the skin of slugs killed later but left undisturbed and observed to lick themselves.

In conclusion, our data reveal that, besides stabbing devices, simultaneously hermaphroditic animals have also evolved another method for transferring substances to their mating partners. This could be as a means to increase their paternity. Our investigation of the penial gland will be followed up with a series of manipulation and paternity experiments aimed at testing the different hypotheses about the function of the secretion transfer. Once the function has been determined, we will investigate the underlying mechanism. It would also be worthwhile to use a marking technique in order to investigate the fate of the secretion once it has been transferred onto the partner's skin.

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