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ORIGINAL ARTICLE

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Piercing the partner's skin influences sperm uptake in the earthworm *Lumbricus terrestris*

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Abstract Sexual conflict between mating partners can give rise to strategies that are advantageous for one sex but harmful to the opposite sex. Usually, sperm donors develop (offensive) traits to enhance their chances in sperm competition, while sperm recipients evolve (defensive) traits that allow them to stay in control of who fathers their offspring. Here, we demonstrate that these processes are also at work in simultaneous hermaphrodites. The hermaphroditic earthworm *Lumbricus terrestris* uses 40 to 44 copulatory setae to pierce into its partner's skin, causing damage and injecting a substance from its setal glands. Experimental injection of the gland substance indicates that a refractory period may be induced. More importantly, removal of the copulatory setae shows that they influence the partner's sperm uptake. When the setae are present, more sperm are taken up and sperm are distributed more equally over the four spermathecae. We interpret this as a strategy that stacks the odds for the donor's sperm in fertilizing cocoons.

Keywords Hermaphrodite · Sexual conflict · Sperm competition · Allohormone · Manipulation

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Introduction

Copulatory behaviors that impose a cost on the partner or manipulate its reproductive processes often hint at the existence of sexual conflicts. Multiple matings with different partners as well as sperm storage and digestion intensify such conflicts and allow for (cryptic) female choice to take place. Such choice is often not in the interest of a single sperm donor. Hence, sexual conflict may exist over the utilization of the donated sperm (Ward 2000).

Darwin believed that sexual selection, which drives sexual conflict, could not act in hermaphroditic organisms, mainly because the sexes are combined within one individual (Darwin 1871). However, it is now long accepted that all the necessary ingredients for sexual selection are present in simultaneous hermaphrodites (Morgan 1994). For example, multiple matings are common (Michiels 1998), as in separate sex species (e.g., Chapman et al. 2003). Sperm can be selectively used (e.g., Bishop et al. 1996), as in species with separate sexes (e.g., Olsson et al. 1996) and many hermaphroditic snails have compartmentalized sperm storage organs (e.g., Haase and Baur 1995; Rogers and Chase 2002) like many insects do (Hellriegel and Bernasconi 2000). Such compartmentalization can be used by the sperm recipient to influence the paternity of the offspring (Fedina and Lewis 2004).

Hence, in hermaphrodites, sexual selection may also drive the evolution of strategies that increase the fertilization chances of the donated sperm, even though this may conflict with the sperm recipient's interests. A recent model suggests that in order to increase their paternity, hermaphrodites may be more prone to accept and develop harmfulmating mechanisms than females and males would (Michiels and Koene, unpublished). An alternative method of increasing paternity is to transfer a bioactive substance (allohormone) that physiologically affects the reproductive processes of the partner (Koene and Ter Maat 2001, 2002; Koene 2004). For example, in *Lymnaea stagnalis* the semen initiates the female function of the partner, and an early onset of oviposition can occur at the expense of

both the growth and male function (Koene and Ter Maat 2004). Another well-known adaptation that influences the sperm storage process is the love dart of land snails. In *Helix aspersa* (*Cantareus aspersus*) this calcareous dart is used to penetrate the partner's skin. This bizarre behavior results in the transfer of an allohormone that inhibits sperm digestion, and thus, increases sperm storage and ultimately paternity (resp. Koene and Chase 1998; Rogers and Chase 2001, 2002; Landolfa et al. 2001). Recently, it has also been shown that this behavior causes counter adaptive co-evolution that plays a key role in the evolution of the reproductive morphology and mating behavior of these snails (Koene 2005; Koene and Schulenburg, 2005; Koene and Chiba, unpublished).

Analogous to dart shooting in land snails, earthworms of the species *L. terrestris* L. may experience sexual conflict because they use 40 to 44 specialized setae to pierce the partner's skin during copulation and inject a substance into the body wall (Feldkamp 1924; Grove 1925; Koene et al. 2002). These copulatory setae are located ventrally on segments 10, 26 and 31–38, two pairs per segment (and in some animals also on segment 25). In *L. terrestris*, copulation lasts for an average of 3.63 h (Michiels et al. 2001), is preceded by extensive courtship (Nuutinen and Butt 1997), and occurs repeatedly during a mating season, presumably also with different partners (Michiels et al. 2001). During copulation, sperm are exchanged simultaneously reciprocally and stored in two pairs of spermathecae, located in segments 9 and 10 (Feldkamp 1924; Grove 1925). Before fertilization, the cocoon is formed outside the clitellum and is peristaltically moved forward, passing over the two female gonospores where one or more eggs are released into the cocoon. Subsequently, it passes over the four spermathecal pores where the sperm are released from one or more spermathecae. The cocoon is finally closed-off after passing over the head. Interestingly, the two segments containing the four spermathecae, are also the region of the partner into which most of the copulatory setae are pierced. All these aspects hint at the possibility of sexual conflict over sperm storage and use in earthworms. Here we investigated whether the copulatory setae are used to manipulate sperm storage. In two injection experiments, we looked at the effect of setal gland homogenates on cocoon production and mating behavior. In a third experiment, sperm uptake was compared between individuals mated to a partner with or without copulatory setae.

Methods

Animals and behavioral observations

Adult specimens of the common earthworm, *L. terrestris*, were obtained from a commercial supplier. Before use, they were kept in isolation for 5 weeks in the laboratory, in small jars containing several centimeters of moist earth. Throughout the experiments, the worms were fed twice a week with a few grams of frozen lettuce that was left to thaw in their jars. The earth in the jars and especially the surface of the experimental set-up were kept moist by spraying water daily.

For the behavioral experiments, earthworms were kept individually in 40 cm perforated PVC tubes with a diameter of 2.5 cm and a closed bottom. The tubes were tightly filled with a mixture of moist earth and cellulose, after which a long metal rod was inserted into the middle of the tube to prepare a burrow. The surface of the set-up was covered with a layer (approx. 1 cm) of moist cellulose. Behavioral recordings were made with time-lapse video recorders. Because earthworms are active at night, we used infra-red sensitive cameras, while the set-ups were irradiated with infra-red diodes. Earthworms are insensitive to the red and infra-red range of the light spectrum (Nuutinen and Butt 1997). These experiments were performed in a climate chamber where the light was set to a day:night cycle of 14 h:10 h. For the dark period, the temperature was gradually lowered from 15 to 10°C and the humidity was gradually raised from 70 to 85%. At the end of each experiment, which lasted for a month each, the burrows were checked for cocoons. Animals were fed on frozen lettuce daily.

Electron microscopy and histology

After copulation, one of the pairs was anaesthetized and the ventral skin of segments 7–16 was removed and prepared for scanning electron microscopy. After careful cleaning, the skin was fixed for 1 h in Bouin, dehydrated in an alcohol series, and dried using a critical point dryer. The tissue was then glued on a small aluminum plate with adhesive tape (Tempfix, Structure Probe, Inc.) and stabilized with conductive silver paint (Leitsilber, Degussa), then coated with gold using a Metalloplan (Leitz). A scanning electron microscope (S-530 SEM, Hitachi) was used to take photographs of the damage to the skin, caused by the copulatory setae.

For histology, worms were collected *in copula* by simultaneously cutting both the worms behind the clitellum (Grove 1925). The two anterior parts, which remained attached, were immediately placed in aqueous Bouin's solution and embedded in Paraplast (Sherwood), 24 h later. They were then cut into 10 μm sections and stained using Goldner's trichrome staining.

Injection of setal gland homogenate

To test the effect of the setal gland product on cocoon production and mating behavior, homogenates of the setal glands were injected. Setal glands from the clitellar region were removed from the skin of donor worms, but because the glands are completely embedded in the skin, some surrounding tissues were also included. The tissues were then placed in 0.5 ml earthworm saline (Prosser and Zimmermann 1943) and homogenized in a hand homogenizer. A homogenate of the dorsal side of the clitellum was also made. Homogenates were spun at 13,000 rpm

for 1 min to remove larger pieces of tissues and the supernatants for each treatment were pooled before injection. About 10 μl of each substance was injected via a single injection, using a needle with a diameter of 0.6 mm. The injected amount was equivalent to half of the gland contents of one worm. Equal volumes of homogenate of the body wall from the dorsal side of the clitellum and saline alone were used as negative and treatment controls, respectively. The substances were injected into segment 10, which is one of the segments where the copulatory setae of the clitellum would normally be pierced. Each worm was injected only with one type of test substance. The different substances were injected alternatingly and the depth of injection was standardized as much as possible.

For injection, worms were rinsed and placed on a smooth surface that had a temperature of −5°C. The result was that the wet worm briefly froze to the surface, thus preventing it from reacting too violently to the injection. The worm was released by rinsing with water at room temperature. Immediately after injection, the behavior was observed for several minutes. However, none of the injections induced any overt reproductive behaviors; for example, behaviors associated with cocoon production—which could be induced within minutes in other earthworm species (Oumi et al. 1996) were not observed. Worms were subsequently placed in individual burrows and could come to the surface to mate with one identically treated partner (to avoid sperm depletion). The above technique was used in two different experiments. In the cocoon production experiment the animals' burrows were opened 30 days after injection (N=8 worms for each group). In the mating behavior experiment (N=12 pairs in each group), some worms died, resulting in sample sizes of 10, 6, and 11 pairs for skin tissue, saline, and setal gland injections, respectively. The lower mortality in the skin and setal gland treatments, though not significant, may be explained by the presence of antimicrobial substances in the skin (Wang et al. 2003).

Setae removal

The 32 copulatory setae of the ventral clitellum region (segments 31–38) were removed from 27 animals. *L. terrestris* also has copulatory setae on segments 10, 26, and sometimes 25, but these are not inserted into the spermathecal region and were left untouched in this experiment. In 27 control animals an equal amount of crawling setae was removed from the ventral region, anterior (segments 28–30) and posterior (segments 39–43) to the clitellum. Before the removal of setae, worms were anaesthetized by placing them in 2.0 N chlorotone for 10 to 15 min. Setae were removed with two pairs of fine forceps, after which worms were rinsed with tap water and allowed to recover for several days. Subsequently, they were placed in individual burrows in the climate chamber and were allowed to interact with one partner, which had received the opposite treatment. For a comparison between the effects of injection versus non-injection, partners of one pair were treated as paired data because they mated simultaneously reciprocal and therefore had equal copulation durations. After mating, the pair was immediately frozen for later use in sperm counting.

Sperm counting

The frozen worm was thawed with warm water and dissected from the dorsal side to collect the four spermathecae. The organs were carefully blotted dry on a tissue. Each spermatheca was put in a separate Eppendorf tube filled with 500 µl saline solution (Prosser and Zimmermann 1943). The sequence of counting of different spermathecae was randomized within an individual. Each spermatheca was homogenized with a plastic hand homogenizer and sperm were immediately counted in four areas (depth: 0.02 mm; area: 0.0025 mm^2) of a Thoma counting chamber. Sperm were clearly visible without labeling and did not clump. The chamber was cleaned and filled for the second time with the same sample to obtain a duplicate count. For each spermatheca, both counts were averaged. The total amount of sperm in each spermatheca was determined using the following formula:

$$\text{No. of sperm}/\mu\text{l} = \frac{\text{count} \times 500\,\mu\text{l}}{4 \times 0.0025 \times 0.02}$$

Statistical tests

In five individuals, one of four spermathecae was lost during dissection. In order to rescue the pair as a data point (with available counts for the remaining 3+4 spermathecae), we estimated the missing sperm count using multiple linear regression analysis. Data of complete individuals showed that counts from three spermathecae can predict the contents of the fourth with high confidence (R^2=0.67, 0.85, 0.85 and 0.90 for each spermatheca). Using this approach, we replaced the missing values with the values predicted by the appropriate regression model. In another 19 individuals, at least one spermatheca contained no sperm (sperm number = 0). During analysis, we checked whether these outliers had a strong effect by comparing results with and without them. Non-parametric tests were used whenever data failed to fulfill the usual assumptions. Statistical tests were performed using SPSS 12.0.1. Means are given ± standard deviation.

Results

Tissue damage

The damage that the copulatory setae caused to the partner's skin during copulation can be detected with electron microscopy. Figure 1A shows an electron microscopy photograph of such a piercing. Moreover, the histological sections clearly show that the copulatory setae inject a substantial amount of gland product (from the setal glands) into the muscle layer under the epidermis (Fig. 1B).

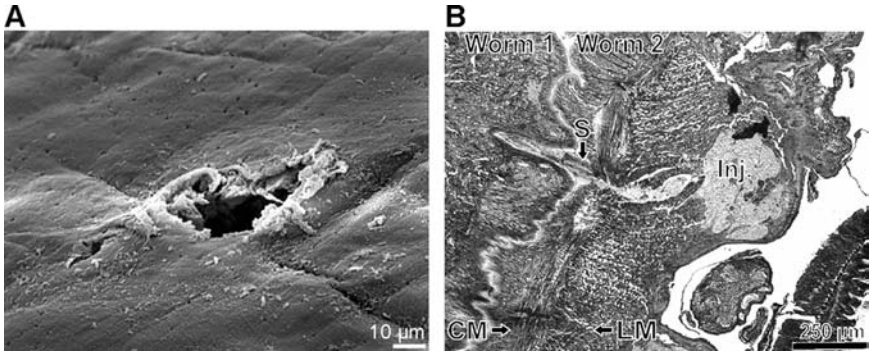

Fig. 1 **A.** Electron microscopic photograph of the skin damage caused by one of the copulatory setae. The diameter of the hole roughly corresponds with the diameter of a copulatory seta (see Koene et al., 2002). **B.** Histological section from a pair of earthworms fixed *in copula*. Worm 1 can be seen injecting the product from the setal gland into the partner's skin (Worm 2). Abbreviations: CM, circular muscle layer; Inj., injected gland product; LM, longitudinal muscle layer; S, copulatory seta

Effect of injections of setal gland extracts

Cocoon production of pairs was not affected by the injections of the setal gland extracts compared with the two control injections (Kruskal–Wallis test (K–W): χ^2=0.55, *df*=2, *p*=0.76). The average numbers of cocoons per individual was 0.69±0.27 for saline injection, 0.75±0.26 for skin injection and 0.94±0.26 for setal gland injection. As in a previous study (Koene et al. 2002), and despite the larger sample size in this study, we found no significant differences in the courtship duration (K–W: χ^2=0.63, *df*=2, *p*=0.73) or copulation duration (K–W: χ^2=2.17, *df*=2, *p*=0.34). However, we found an effect, though statistically not significant, of the injections on the time it took for pairs to copulate after the injection, which could be interpreted as a refractory period (One-way ANOVA: $F_{2,24}$=3.33, *p*=0.053). The differences on the overall ANOVA are due to a significant difference between the injections of extracts from the skin and setal glands, the latter being higher (Tukey HSD: $p<0.05$). The saline injection was not significantly different from either of these two (Tukey HSD: $p>0.05$) (Fig. 2).

Removal of setae

A surprising 19 out of 54 individuals had at least one empty spermatheca after mating. This occurred significantly more often in pierced individuals than in non-pierced individuals (14 out of 27 versus 5 out of 27; Fisher's exact test: $p = 0.021$). Despite this effect, the pierced individuals had significantly more sperm (sum of all spermathecae) than their non-pierced partner (paired *t*-test: $t_{27} = 2.57$, $p = 0.016$, Fig. 3). This effect is still present when ignoring pairs in which at least one individual had an empty spermatheca (paired *t*-test: $t_{12} = 2.96$, $p = 0.013$).

Comparing the distribution of sperm across the four different spermathecae also shows interesting patterns.

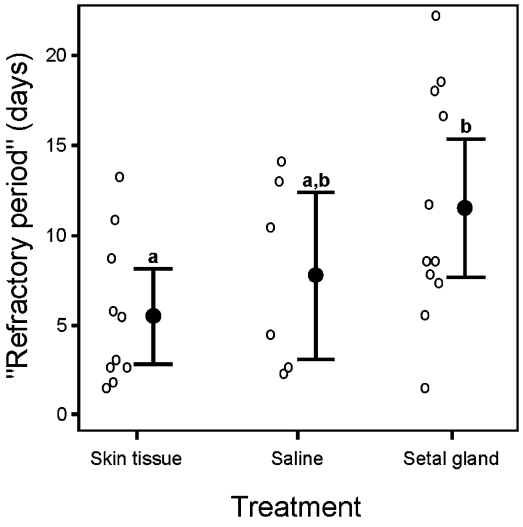

Fig. 2 Scatter plot of time until mating after treatment ("refractory period"). The individual data points (*open circles*) are jittered horizontally within treatments and the means and 95% confidence intervals are indicated. Treatments with different letters (a or b) differ significantly from each other

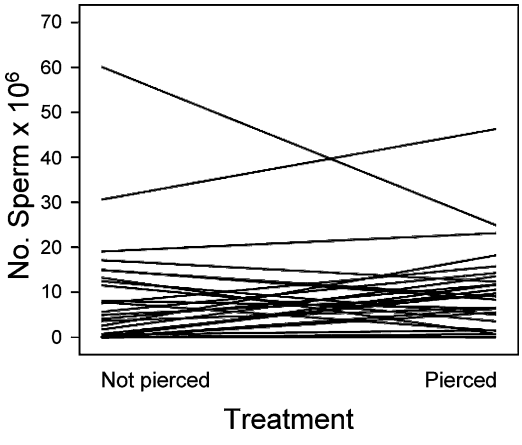

Fig. 3 Total number of sperm collected from individuals which were pierced by copulatory setae and injected with setal gland product during copulation versus individuals that were not. Lines connect the points for the two individuals in the same pair. 'Not pierced' represents the sham-treated animals that were able to pierce their partner; 'Pierced' represents the animals from which the copulatory setae were removed and that were therefore unable to pierce their partner

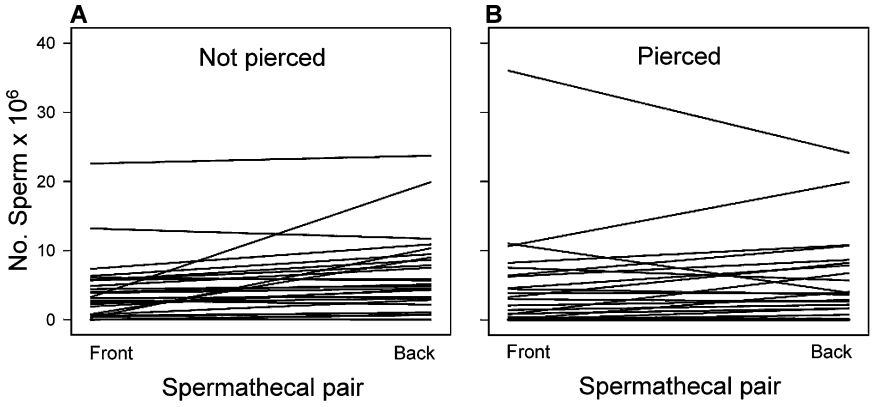

Fig. 4 Distribution of sperm in the spermathecae. Sperm counts in the front and back spermathecae are shown for Not pierced (A) and Pierced (B) individuals. Lines connect the points for the front and back of an individual. 'Not pierced' represents the sham-treated animals that were able to pierce their partner; 'Pierced' represents the animals from which the copulatory setae were removed and that were therefore unable to pierce their partner

In non-pierced animals, the distribution of sperm was unequal over the four spermathecae. The two posterior spermathecae contained significantly more sperm than the anterior ones (paired *t*-test: t_{27}=3.043, p=0.005; Fig. 4A). Animals which had been pierced with copulatory setae, had an equal distribution between anterior and posterior spermathecae (paired *t*-test: t_{27}=0.956, p=0.348; Fig. 4B). The equalizing effect of piercing on sperm distribution is even more explicit when individuals with at least one empty spermatheca (no. of sperm = 0) are ignored (not injected: t_{22}=2.74, p=0.012; injected: t_{13}=0.024, p=0.98).

Discussion

Evolutionary conflicts of interest between the sexes can give rise to traits that are advantageous to one sex but harmful to the other (Chapman et al. 2003). Usually, males develop strategies to enhance their chances in male–male or sperm competition. In contrast, females may be choosy, sometimes cryptically, about which partner(s) should father her offspring. The interplay of these two processes determine the outcome of the conflict between the sexes. Here, we demonstrate that these processes may also be at work in simultaneous hermaphrodites, namely, the 40 to 44 copulatory setae that the hermaphroditic earthworm *L. terrestris* uses to pierce its partner's skin, affect the partner's sperm storage process and possibly induce a refractory period.

The piercing of the copulatory setae into the partner causes substantial damage to the skin and results in the introduction of a product from the setal glands (Feldkamp 1924; Grove 1925; Koene et al. 2002). Although this damage itself could cause a conciderable cost that inhibits remating (Johnstone and Keller 2000; Michiels and Koene, unpublished), here we used a single experimental injection of gland extract to investigate the involvement of a chemical component, thus minimizing physical damage by inflicting only one skin injury instead of 40 to 44. The injection experiments show that the chemical component of body piercing does not influence cocoon production, confirming previous experiments (Koene et al. 2002), but suggest that the time until the next mating may be increased. This result can be interpreted as the induction of a refractory period by an allohormone from the setal gland. However, it should be noted that significant differences were between the gland and skin extract treatments, and that the saline extract treatment was not significantly different from either of these two. Thus, although it seems more likely that the setal glands induce a refractory period, we cannot exclude the possibility that the skin tissue decreases this period. Clearly, this finding requires further investigation. However, the observed effect suggests that the injected substance acts systemically, which is also supported by the presence of many small blood vessels in this skin area (Grove 1925). Hence, the impressive amounts that are injected into the tissue under the epidermis (Fig. 1A) can potentially act via the brain or via a raised immune response (which could diverge resources away from reproductive behavior).

The sperm storage data provide more conclusive evidence for the hypothesis that the copulatory setae manipulate sperm uptake and subsequent storage in the partner. Animals that were pierced by copulatory setae had more sperm in their spermathecae than their not pierced counterparts. Besides increasing the total number of stored sperm, the setae also affect the distribution of sperm over the four spermathecae. In the absence of the copulatory setae, sperm are predominantly stored in the two posterior spermathecae. When the copulatory setae are present, sperm are equally represented in each of the four spermathecae of the mating partner. Interestingly, the pierced worms more often had at least one empty spermatheca than not pierced worms. This finding may indicate that the setae also play a role in the removal of the rival sperm in the spermathecae by inducing the partner to empty its spermathecae at the start of copulation and then taking up the offered sperm (analogous to sperm displacement in some insects, Simmons 2001). In contrast to the injection experiment, the observed effects on sperm storage are most likely caused locally, given that the copulatory setae pierce exactly those segments where the partner's spermathecae are located.

The advantage of inducing the uptake of more sperm and the displacement of rival sperm are obvious for the sperm donor. But why would an equal distribution of sperm over all the spermathecae be beneficial? Many animals have several spermathecae or one compartmentalized spermatheca. Examples are the hermaphroditic land snail *Arianta arbustorum* (Haase and Baur 1995) and the yellow dung fly, *Scathophaga stercoraria* (e.g., Hellriegel and Bernasconi

2000). Females of this latter species have been shown to store sperm from different males in different compartments (Hellriegel and Bernasconi 2000). This discrete storage system may allow females to actively select sperm from certain partners to optimize paternity of their offspring, as has been demonstrated in the red flour beetle (Fedina and Lewis 2004). In contrast, males may aim at equal representation of their sperm in all compartments to stack the odds of their sperm fertilizing the eggs (Ward 1993). Hence, the selective use and storage of sperm can give rise to adaptations that manipulate this process.

For the sperm donor, an equal distribution of sperm can be important to assure fertilization of the partner's eggs. This can be beneficial in that earthworms becomes apparent when we consider the process of cocoon fertilization. After a cocoon is formed on the clitellum, it is peristaltically moved forward like a ring along the skin, passing over the segments that contain the spermathecae. Fertilization of the egg(s) inside the cocoon takes place by releasing stored sperm from the spermathecae. The cocoon is then further moved forward and closed-off once it passes over the head. Whether sperm from one or several spermathecae are used, remains unknown. Irrespectively, having its sperm stored in all the four spermathecae of its partner, most likely increases the donor's chance of fertilizing the egg.

We conclude that body piercing in earthworms represents a manipulation of the partner's sperm storage process and that the storage of equal amounts of sperm in each spermatheca can be seen as a bet–hedging strategy. The sperm recipient may control sperm storage, which is supported by the fact that sperm are stored differently when the copulatory setae are absent. Additionally, muscle fibers are present in the spermathecal wall (Breidenbach 2002), which suggests that they can potentially be controlled to take up and release sperm. This has also been proposed to occur in other species with compartmentalized sperm storage organs (e.g. *A. arbustorum*: Haase and Baur 1995; *S. stercoraria*: Ward 1993).

This type of manipulation of the partner's sperm storage may be beneficial for the sperm donor because it increases its chances of fertilization. However, the recipient can experience a cost due to partial loss of control over its reproductive processes and physical damage (40 to 44 holes are pierced into the partner, Fig. 1A). Obviously, because the interests of the two mating partners in terms of sperm storage differ, this creates a sexual conflict within this simultaneous hermaphrodite. This conflict can cause counter-adaptations at different levels. One way to remain in control over sperm storage is by increasing the complexity of the sperm receiving organs (Hellriegel and Ward 1998; Koene and Schulenburg, 2005). Interestingly, different species of earthworms have different numbers of spermathecae (Sims and Gerard 1999). For example, *Microscolex phosphoreus* has one pair of spermathecae, *L. terrestris* has 2 pairs, *Allolobophora chlorotica* has 3 pairs, and *Amynthas corticis* has 4 pairs. These differences may be the result of an arms race for the control over sperm storage. Finally, our finding that earthworms use their copulatory setae to manipulate the sperm storage process of their partner may also shed a new light on the function of setae in other species of worms (e.g. tubificids: Cuadrado & Martínez-Ansemil 2001).

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