

## On the function of body piercing during copulation in earthworms

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### Summary

In many organisms, including hermaphroditic species, received sperm can be either digested or stored. If they do not get digested, the sperm are often stored for prolonged periods of time during which copulations with several partners occur. Due to these processes, sexual selection favours tactics that increase the chances of the donated sperm fertilizing the partner's eggs. One way to achieve this is by transferring a bioactive substance (an all hormone) that affects the reproductive processes of the mating partner. The hermaphroditic earthworm *Lumbricus terrestris* uses 40 needle-like setae (chitinous hairs) to inject a substance into the mating partner. During mating, these copulatory setae are pierced through the partner's skin. Compared to the normal (crawling) setae, these setae are longer and grooved; they are situated ventrally on segments 10, 26, and 31 to 38 (the clitellar segments). This body piercing results in the injection of the product from the setal glands which accompany these setae. We propose that the setal glands may produce an all hormone that manipulates the reproductive physiology of the mating partner. The damage and possible loss of control over fertilization as a result of body piercing hint at the existence of a sexual conflict in simultaneous hermaphrodites.

**Key words:** All hormone, mate manipulation, setae, sexual conflict, simultaneous hermaphrodite

### Introduction

In promiscuous species, sexual conflicts occur when the reproductive interests of mating partners do not coincide. This is usually the case because males will compete among each other for the fertilization of eggs in order to increase their own reproductive success. Sexual selection will, therefore, favour tactics

that increase the fertilization chances of the male's sperm. However, such tactics can at the same time negatively affect the female's reproductive success. Examples of such sexual conflicts are abundant for species with separate sexes (e.g., Rice, 1996). Few clear examples are known for hermaphroditic animals except for dart shooting in the garden snail *Helix aspersa* (Koene and Chase, 1998; Rogers and Chase,

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2001) and penis fencing in the tropical flatworm *Pseudoceros bifurcus* (Michiels and Newman, 1998).

Nonetheless, sexual conflicts are expected to be common in hermaphroditic animals because they possess specialized organs for long-term storage as well as organs for digestion of allosperm (reviewed in Michiels, 1998). Additionally, hermaphrodites copulate with several partners during a mating season and often have elaborate pre-copulatory behaviours to assess the quality of these partners (e.g., Nuutinen and Butt, 1997; Vreys and Michiels, 1997; Michiels et al., 2001). Hence, just as in species with separate sexes, successful copulation with a partner does not imply successful fertilization of that partner. As a consequence, sexual selection will favour strategies that help assure paternity. Such strategies potentially result in sexual conflicts between the male and female function in hermaphrodites.

One tactic that can help to assure paternity is the introduction of biologically active substances (allohormones: Koene and Ter Maat, 2001, 2002) into the partner. Such allohormones can have various effects that help to increase the chances of the sperm fertilizing the eggs. By introducing bioactive substances, the reproductive system of the recipient can be influenced in different ways; and these allohormones can act either directly on the target organs, via the blood, or via the central nervous system (Koene and Ter Maat, 2001). Such effects include induction of oviposition (e.g., the fruit fly *Drosophila funebris*: Baumann, 1974a, 1974b), inhibition of remating (e.g., the adder *Vipera berus*: Andrén and Nilson, 1987), and elevation of survival and storage of sperm (respectively, the “assassin bug” *Rodnius prolixus*: Davey, 1958; the fruit fly *Drosophila melanogaster*: Harsmann and Prout, 1994).

These examples reflect the fact that such chemical substances have been investigated in many species with separate sexes (reviewed by Eberhard and Cordero, 1995), but the presence of allohormones has remained largely unexplored in hermaphrodites. One exception is the so-called love dart of hermaphroditic land snails. In the garden snail *Helix aspersa* this dart is forcefully stabbed through the skin of the mating partner during courtship. This dart shooting serves to transfer an allohormone, which is present in the mucus on the dart, into the blood of the mating partner. As a result of successful dart shooting, the subsequently donated sperm are less likely to be digested and have a higher chance of being stored for later use in fertilization (Koene and Chase, 1998; Rogers and Chase, 2001; Landolfi et al., 2001).

Here we report that a similar introduction of an

allohormone may occur in the hermaphroditic earthworm *Lumbricus terrestris*. During copulation, these worms use specialized setae (chitinous hairs) to pierce through the skin of the mating partner in order to introduce a product from the setal glands. This body piercing was reported in the 1920s (Feldkamp, 1924; Grove, 1925) and subsequently fell into oblivion. Here we present some new results on this bizarre behaviour and discuss them in a sexual selection context.

## Methods

Specimens of the common earthworm, *Lumbricus terrestris* L., were either collected on a sports field in Münster (Germany) or obtained from an angler shop. They were kept in isolation in the laboratory in small jars containing several centimetres of moist earth. They were fed frozen lettuce twice a week.

For scanning electron microscopy, the ventral setae of three worms were removed from segments 1 to 40. These worms had been fixed in 95% ethanol several days prior to setae removal. The setae were glued on small aluminium plates with an electrically conducting adhesive (Leit-Tab, Plano) and air-dried. Subsequently, they were coated with gold using a Metalloplan (Leitz). The setae were then placed under a scanning electron microscope (S-530 SEM, Hitachi) and photos were taken. The different types of setae were determined and a map of the setae was constructed. Whenever we refer to the size of the different setae, we indicate the mean length of 10 setae as well as the standard deviation.

For histological observations, worms were fixed in aqueous Bouin's solution and subsequently embedded in Paraplast (Sherwood). They were then cut in 10 µm sections and stained using Goldner's trichrome staining. The same procedure was used for worms that were collected *in copula*. The latter were collected by simultaneously cutting the worms behind the clitellum during copulation (Grove, 1925). The two anterior parts, which remained attached, were immediately placed in the fixative.

For the behavioural experiments, recordings were made with time-lapse video recorders. Because earthworms are active at night, these recorders were attached to infrared-sensitive cameras and the set-ups were irradiated with infra-red diodes. Earthworms are insensitive to the red and infrared 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 14h:10h. 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 1 month each, the burrows were checked for cocoons. Whenever we refer to behavioural events we indicate the mean and standard deviation.

To test whether setae regenerate and whether the absence of the setae on the clitellum affects reproductive behaviour, the ventral setae of the clitellum were removed from live animals. Before removal of the setae, worms were anaesthetized by placing them in 2.0 N Chloretone for 10 to 15 min. The setae were removed with the help of two pairs of fine forceps after which the worms were rinsed with 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 each other.

To test the effect of the setal gland product on cocoon production and mating behaviour, extracts of the setal glands were injected into worms. For these injections, the setal glands from the clitellar region were removed from the skin of eight worms, placed in 0.5 ml earthworm saline solution (Prosser and Zimmermann, 1943), and homogenized in a hand homogenizer. A homogenate of the dorsal side of the clitellum was also made. The homogenates were spun at 13000 rpm for 1 min in a centrifuge to remove larger pieces of tissue from the homogenate; the supernatants were used for injections. The homogenate of the body wall from the dorsal side of the clitellum and saline solution were used as controls. The extracts were injected into segment 10, which is one of the segments where the copulatory setae of the clitellum would normally pierce the partner. Each worm was only injected with one type of test substance, resulting in three groups of 16 worms. For the injections, worms were placed on a smooth surface which had a temperature of -5°C. The result was that, during the injection, the worm briefly froze to the surface which prevented it from reacting too violently to the injection. Immediately after injection the behaviour was observed for several minutes. However, none of the injections induced any overt behaviours; for example, behaviours associated with cocoon production — which can be induced within minutes (Oumi et al., 1996) — were not observed. Therefore, the worms were placed in individual burrows where they were allowed to come to the surface to mate and to produce cocoons in their burrows. We found that a total of four worms had died at the end of the experiment; three gland-injected individuals and one saline-injected individual died, resulting in N=13 and N=15 for the respective groups.

## Results

By creating an overview of all the ventral setae of three worms, we were able to reconstruct the position of the copulatory setae as well as any other setae that had a different shape than the crawling setae. In this way we were able to confirm and extend earlier reports on the position of differently shaped setae (Stephenson, 1921; Feldkamp, 1924; Grove, 1925).

Four ventral setae are present on each segment and three different types of setae occur. We found that the ventral setae of segments 6 to 9 are enlarged crawling setae with an average length of 1.51 mm (Fig. 1A). They are approximately one and a half times as long as the similarly-shaped crawling setae located on other segments. Most crawling setae are usually worn-off at the tips, confirming that they are extensively used during crawling through the earth (see Fig. 1A). On its side this first type of seta has a typical pattern of indentations. The second type are the copulatory setae which are located on segments 10, 26 (sometimes also 25) and 31 to 38; they are nearly twice as long as the crawling setae (Fig. 1B). Copulatory setae are very slender and end in a sharp tip. Along most of their length they have four grooves. Within these grooves a hair-like structure is present; the hairs in these grooves point forward. The third type of seta is found on segment 15 and is referred to as penial seta (Fig. 1C) because it occurs next to the male gonopore. These four setae have the same indented structure as crawling setae along their base, but have short grooves at the tip. Due to the shape of these grooves the tip is spoon-shaped.

Note that the complete length of a seta does not correspond to its functional length because the basal part of each seta, which is about half of the complete length, remains embedded in the skin. The functional portion can be extended and retracted with the help of muscles. The tips of crawling setae usually protrude from the skin via the setal pore (Fig. 2A). The copulatory setae are also controlled by muscles but their tips are usually not visible in the live animal; they only come out of the skin via the setal pores during copulation. In contrast to the crawling setae, the copulatory setae are accompanied by glands (Fig. 2B). The lumen of the setal gland forms a circular duct surrounding the seta near the epidermis. In Fig. 2C it can be seen that the lumen of the setal gland connects via the setal pore to the outside of the worm.

The histological sections made of worms *in copula* reveal that the copulatory setae are used to pierce through the skin of the mating partner and by doing so

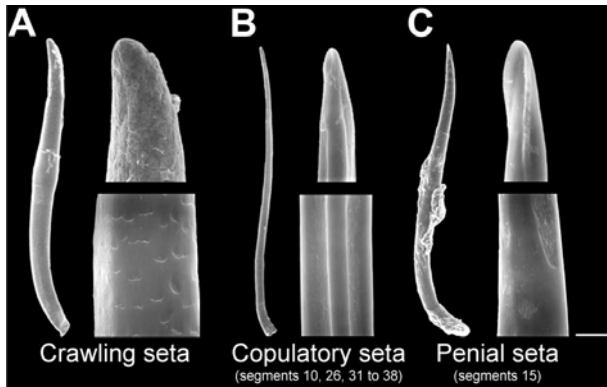


Fig. 1. Scanning electron microscopic photographs of the different types of ventral setae of the earthworm *Lumbricus terrestris*. A. The crawling setae. These setae are present on most segments, both ventrally and laterally, and have an average length of 1.06 mm ( $\pm 0.09$ , N=10). On segments 6 to 9 they are enlarged (1.51 $\pm$ 0.07 mm, N=10). Details of the side and the worn-off tip are shown. B. The copulatory seta (1.81 $\pm$ 0.17 mm, N=10). These setae are present on segments 10, 26, and 31 to 38 (i.e., the clitellum). In the enlarged images two of the four grooves as well as the sharp tip can be seen. C. The penial seta (1.00 $\pm$ 0.07 mm, N=10). These setae are present on segment 15 and are slightly grooved near the tip, resulting in a typical spoon-shaped ending. The scale bar (20  $\mu$ m) applies to the enlarged images only.

they inject a substance into the body wall, presumably originating from the setal gland (Fig 2D). This substance has mucus-like properties because histologically it is similar to mucus produced by the epidermis and PAS staining was positive. Due to the head-on position of the worms during copulation, each worm injects its partner at several different places. The setae of segment 10 pierce into the partner's clitellum; the setae of segment 26 pierce segment 15; the setae of the clitellar segments pierce around segments 9 and 10. In Fig. 2D "worm 1" injected its partner in segment 10, while "worm 2" injected its partner in the clitellar region. Fig. 2D also illustrates the impressive amounts that are injected into the tissue under the epidermis where also many small blood vessels are known to be present (Grove, 1925). The penial setae are not used for piercing the partner.

When the setae were removed, from the clitellar region or elsewhere, they had not regenerate after two months (N=10 worms). More importantly, the removal of the ventral setae on the clitellum affected neither the ability to produce cocoons (N=3 worms), nor the ability to mate (N=10 pairs). The injections of the extract from the setal glands of the clitellum did not significantly increase cocoon production (2.14  $\pm$  1.07

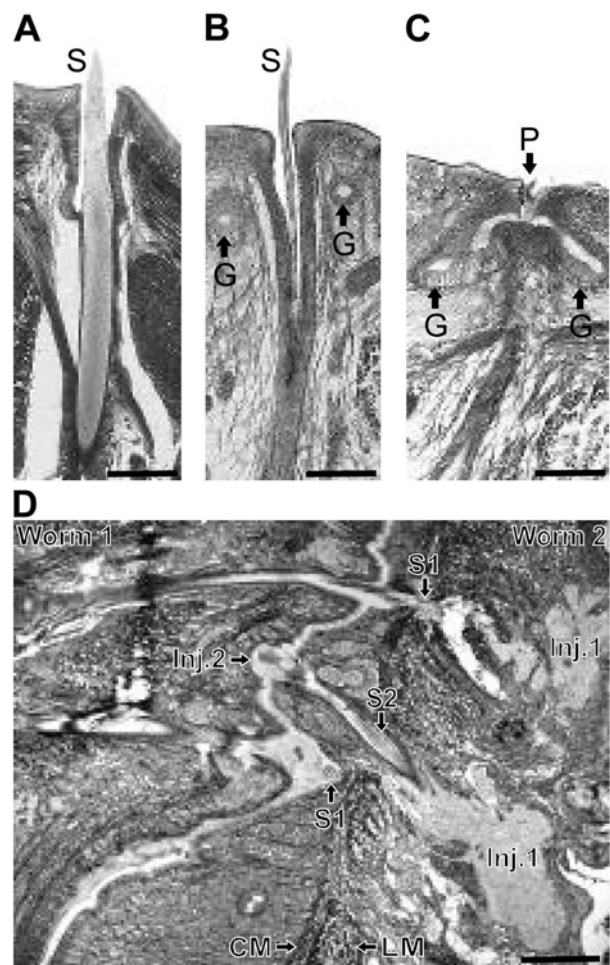


Fig. 2. Histological sections illustrating body piercing in the earthworm *Lumbricus terrestris*. A. Section of a normal seta. B. Section of a copulatory seta showing that such setae are accompanied by glands (G, glandular lumen). C. Section of the setal gland showing that its lumen connects to the outside via the setal pore (P). D. Section showing reciprocal body piercing in worms *in copula*. The worms were fixed 90 min after the start of copulation; copulation normally lasts around 180 min. Worm 1 injects (Inj.1) its partner in segment 10 with two setae (S1). Worm 2 injects (Inj.2) its partner in the clitellar region with one seta (S2). Note the impressive amounts that have been injected into the tissue under the epidermis of Worm 2 by Worm 1 (Inj.1). CM, circular muscle layer; LM, longitudinal muscle layer. All scale bars: 250  $\mu$ m.

cocoons per individual, N=7 worms) compared to the tissue-control (1.57 $\pm$ 0.79, N=7) or saline-control injections (1.71 $\pm$ 0.76, N=7) (one-way ANOVA:  $F = 0.448$ ,  $df = 2$ ,  $p = 0.648$ ). The duration of copulation of the worms was also not significantly affected by the injection (one-way ANOVA:  $F = 0.780$ ,  $df = 2$ ,  $p = 0.477$ ). However, we did observe a non-significant trend in the duration of pre-copulatory behaviour

where the gland-injected animals (N=4 pairs) interacted longer than the worms from the control groups (tissue control: N=6 pairs; saline control: N=7 pairs) (one-way ANOVA:  $F = 2.00$ ,  $df = 2$ ,  $p = 0.172$ ). As a measure of mating delay we looked at the time between injection and the first mating; we found no significant increase in this time for the experimental group ( $14.75 \pm 8.42$  days, N=4 pairs) compared to the tissue ( $13.83 \pm 6.85$ , N=6) and saline ( $12.00 \pm 5.54$ , N=7) controls (one-way ANOVA:  $F = 0.243$ ,  $df = 2$ ,  $p = 0.787$ ).

### Discussion

The earthworm *L. terrestris* has 40 copulatory setae that pierce through the skin of the mating partner during copulation. These setae are present on segments 10, 26, and 31 to 38 (i.e., the clitellum). By piercing through the skin of the partner, they transfer a large quantity of a substance, which most likely originates from the setal glands. The copulatory setae cause substantial damage to the body wall of the mating partner and it is possible that this damage itself inflicts a cost on the mating partner that serves to decrease the partner's tendency to remate, thereby increasing the male reproductive success of the actor (Johnstone and Keller, 2001). However, it is also possible that a bioactive substance (i.e., allohormone: Koene and Ter Maat, 2001, 2002) is transferred, which manipulates reproductive processes in the partner.

Due to the small sample size, the behavioural data do not allow us to rule out either of the above-mentioned hypotheses. We found no significant effects on cocoon production or the duration of mating behaviours, and we found no indication of remating inhibition. This latter effect would be expected if the setal glands produce an allohormone that is toxic for the recipient (Rice, 1996). However, it is also possible that the extensive physical damage caused by the setae itself inflicts a cost on the recipient that causes such an inhibition of remating (Johnstone and Keller, 2001). This was not considered in our present experiments because, by directly injecting the setal gland product, we minimized the physical damage.

That the observed piercing of the partner's skin occurs in order to manipulate the mating partner is supported by our finding that the setae are not necessary for holding on to the partner during copulation, as was initially proposed (Feldkamp, 1924). Matings were observed in pairs where either one or both animals had the copulatory setae on the clitellum removed. This now allows us to test in future experiments whether sperm storage processes are

affected by the absence of copulatory setae. One could imagine that an injected allohormone could affect the uptake of sperm into the spermathecae, especially given that the copulatory setae of the clitellum inject into the partner's segments where the sperm are taken up (the spermathecae are located in segment 9 and 10).

The forward-pointing hairs within the grooves of the copulatory setae may function to drive the injected secretion deeper into the partner's body wall. Due to the presence of four grooves in between four blades and a sharp tip, the slender copulatory setae show a striking resemblance with love darts of some species of land snails. The dart is also used to pierce through the skin of the mating partner in order to introduce an allohormone that manipulates the female reproductive organs (Koene and Chase, 1998; Rogers and Chase, 2001). Examples of the direct injection of allohormones are also found in animals with separate sexes. For example, the sexual sting of some scorpion species, e.g., the giant hairy desert scorpion *Hadrurus arizonensis* (Tallarovic et al., 2000) is thought to be used to increase the probability that the female will accept the male's spermatophore (Weygoldt, 1977). The male dusky salamander *Desmognathus fuscus* injures the female's skin with specialized teeth to inject an allohormone which also increases the female's willingness to accept the subsequently donated spermatophore (Arnold and Houck, 1982; Houck and Reagan, 1990; Houck, 1998).

All these examples of allohormone introductions result in an increase in male reproductive success. However, female reproductive success may be negatively affected by these allohormones. In earthworms, as well as in several of the other examples given, the method of introduction of the allohormone causes substantial damage to the body wall of the mating partner. Besides the cost of repairing this damage, there would be an additional cost if manipulation of the recipient's reproductive processes occurs. Such a manipulation could cause the recipient to partly lose control over its fertilization processes. The foregoing, therefore, suggests that body piercing could cause a sexual conflict in this simultaneous hermaphrodite.

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