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Two separate intromittent organs in *Zorotypus caudelli* (Insecta, Zoraptera): a seemingly paradox coexistence of an extremely long and narrow tube and a large spermatophore

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A short running title: Two intromittent organs in Insecta

Abstract

Extremely elongated intromittent organs have evolved independently in different groups of insects. Males have to accommodate these unwieldy structures in the limited spaces of the abdomen and manipulate them acutely during copulation. A crucial question is how species with an elongated penis cope with these requirements? To elucidate this, we investigated key features enabling storage, insertion and withdrawal of the elongated organ in *Zorotypus caudelli*. The genital anatomy and fitting during copulation of the tiny insects (ca. 2 mm) was reconstructed. An elongated, very narrow intromittent tube is present, despite the production of a large spermatophore. The co-existence of a narrow and elongated tube and a bulky spermatophore appears paradox, but it turned out that the tube is not involved in the sperm transmission. The spermatophore is transferred to the female genital tract by a membranous sac-like intromittent organ. The movements of the spermatophore and the two intromittent organs are apparently promoted by the same mechanism. A comparison with the genital anatomy and reproductive mode in related groups suggests that the elongated spiral-shaped tube is a de novo structure of some zorapteran species, and that sperm transport via spermatophore is a preadaptive condition for the acquisition of this unusual structure.

Key words: copulation, insertion, novelty, penis, preadaptation, sexual selection, withdrawal, Zoraptera

INTRODUCTION

The structure of animal genitalia is often very complex despite of their seemingly simple function, i.e. the transportation of sperm (Eberhard, 1985). The “extreme elongation” of the intromittent organ is a conspicuous novelty observed in males of different groups. This condition occurs only sporadically in the animal kingdom, but has apparently evolved independently in a considerable number of insect groups (summarized in Matsumura & Yoshizawa, 2012). This phenomenon has attracted the attention of evolutionary biologists and it was suggested that sexual selection is the primary driving force in this context (e.g. Tadler, 1999; Gschwentner & Tadler, 2000; Rodriguez et al., 2004; Kamimura, 2005; van Lieshout & Elgar, 2011). The behavioural ecological issue usually attracts most of the attention, but a different perspective reveals another aspect of the evolution of elongated penises. Intromittent organs in repose have to be stored within a limited space of the abdomen in pterygote insects (e.g. Snodgrass, 1935), even in species with a penis as long as the entire body (summarized in Matsumura & Yoshizawa, 2012). Males have to accommodate it and move it during copulation without tangling and breakage. These requirements appear problematic, and lacking the abilities for accommodation and efficient handling could represent an impeding factor for the evolution of extreme elongation of penises (Eberhard, 2005; Gack & Peschke, 2005; Matsumura & Yoshizawa, 2010, 2012; Briceño et al., 2011). This

raises the question how the apparent constraints could be overruled several times in insects, resulting in the formation of very long intromittent organs?

To answer the issue it is necessary to understand key features enabling insects to store, insert and withdraw an extremely elongated (part of an) intromittent organ without tangling and breakage of the fine elongated structures. However, mechanisms of movement of elongated penises are very insufficiently known, with scattered and partly incomplete information on only a few taxa (Eberhard, 2005; Gack & Peschke, 2005; Matsumura & Yoshizawa, 2010; Briceño et al., 2011). Eberhard (2005) showed how the specifically modified anatomy of males of a medfly enables them to insert the elongated intromittent organ quickly and completely (Fig. 1A). Possible insertion mechanism in another fly is discussed, suggesting stiffening and straightening of the intromittent organ by rhythmic cycles of inflation and deflation of a membranous region (Briceño et al., 2011). In a very distantly related insect, a leaf beetle, Matsumura & Yoshizawa (2010) suggested a specialized structure of the intromittent organ and mechanisms allowing efficient insertion and withdrawal just by increased hemolymph pressure and the contraction of muscles (Fig. 1B). Gack & Peschke (2005) showed that a highly specialized behaviour enables a male rove beetle to withdraw the elongated part of the intromittent organ from a female genital tract without tangling. These previous studies suggest that a similar combination of these features also occurs in other groups: specialized structures and the corresponding behaviour make it possible to insert the elongated intromittent organ just by increased hemolymph pressure and to withdraw it by contractions of muscles as in other insects without elongated parts.

Even though the specific structure of extremely elongated intromittent organs is highly variable, our literature survey (mainly covering taxonomic works) suggests that a spiral-shaped structure is widespread in insects (Fig. 1C). Of them, the zorapteran species *Zorotypus caudelli* Karny, 1927 appeared ideal for the present study for several reasons. It was successfully reared in the lab (Mashimo et al., 2011), the internal reproductive structures were investigated at the ultrastructral level by Dallai et al. (2011, 2012b), and the simple mating behaviour was documented in detail by Dallai et al. (2013). This favourable situation for functional, morphological and behavioural investigations motivated us to study the accommodation and the insertion and withdrawal mechanisms of the seemingly widespread type using the zorapteran species. Comparing the genital structures, mechanical explanation, and reproductive strategies with those found in other zorapterans and potentially related groups (polyneopteran orders), we discuss the background which may have enabled *Zorotypus caudelli* to acquire its extremely elongated genital structure and possible mechanisms facilitating the evolution of similar configurations in other groups of insects.

MATERIALS AND METHODS

SPECIMENS

In the very small order Zoraptera (39 extant species), males of species related to *Zorotypus caudelli* (e.g., *Z. hubbardi*, *Z. impolitus*) are known totransfer sperm packed in spermatophores which are large in relation to the body size (Dallai et al., 2012a, 2013, RD pers. obs.). As this is seemingly in conflict with the presence of a thin and elongated tube in the studying species *Z. caudelli* (New, 2000) we investigated the morphology and dynamics of the male and female genital apparatus with a focus on the sperm transmission by fixation of copulating pairs

We used specimens of *Z. caudelli* from the rearing stock of Mashimo et al. (2011) and followed the methods described by these authors. The initial population was collected in Malaysia. To obtain copulating pairs, we placed two males and two females into a plastic case (3.5 cm × 3.5 cm × 1.0 cm) and observed them at intervals of ca. 30 min. The copulation of this species starts quite abruptly, probably with a very short pre-copulatory behaviour (Dallai et al., 2013). The copula is completed within 11.5 - 23.2 min (Dallai et al., 2013). When we found a copulating pair, alcohol cooled in a normal household freezer was poured into the case, which was then kept in a freezer for more than ten minutes. Afterwards, all couples were preserved in 70% ethanol in a container for anatomical investigation. The exact stage of the copula was unknown prior to the anatomical investigation. Following the methods outlined above, we obtained 32 fixed pairs in copula, and additionally we used single males and females preserved in 70% ethanol for anatomical study.

ANATOMY

In addition to manual dissection we used histological sectioning, micro-computed tomography (μCT), computer-based 3D reconstruction, and confocal laser scanning microscopy (CLSM). Transverse and longitudinal semithin sections were made of two copulae and of one single male of *Z. caudelli*. All samples were embedded in araldite CY 212® (Agar ScientiWc, Stansted/Essex, England) and cut at 1 μm using a microtome HM 360 (Microm, Walldorf, Germany) equipped with a diamond knife. Sections were stained with toluidine blue and pyronin G (WaldeckGmbH and Co.KG/ Division Chroma, Münster, Germany). Pictures were taken of every second or every third section using a microscope (Zeiss Axioplan, Germany) equipped with a camera (PixeLink Capture OEM). The images were aligned using Amira 4.1.2 software (Visage Imaging, Berlin, Germany). Based on the aligned image stacks (cross sections of a single male: 518 images; cross sections of a pair in copula: 534; longitudinal sections of a pair in copula: 458) we evaluated the arrangement of internal structures and manually traced each element to reconstruct three dimensional images. For smoothing and coloring them we used MAYA7 (Alias Wavefront, Toronto/Ontario, Canada). As histological sections are

never completely free of deformations, we also used µCT (Skyscan1172) to obtain a perfectly aligned three dimensional image. The resolution is lower compared to the sections but this non-invasive technique is largely artifact free (e.g. Friedrich & Beutel, 2008). To examine the genital fitting using µCT, an alcohol preserved copula of *Z. caudelli* was dried at the critical point (Emitech K850 critical point dryer, Ingenieurbüro Peter Liebscher, Wetzlar, Germany) and scanned. Then we traced selected structures using Amira software to highlight genital structures.

We used scanning electron microscopy (SEM, Philips XL 30 ESEM) to observe surface structures of the male genitalia. We gradually dehydrated specimens preserved in 70% ethanol with ethanol-hexamethyldisilazane series and dried them in a draft chamber. The samples were mounted on the tip of a fine needle and fixed on the rotatable specimen holder developed by Pohl (2010).

The material composition of the male genitalia was analyzed applying CLSM without staining. The preparation and visualization methods described by Michels & Gorb (2012) were used, with the only difference that instead of a longpass emission filter transmitting light with wavelengths ≥560 nm we applied wavelengths of ≥640 nm to detect the autofluorescence excited by 639 nm. However, the filter differences did not have any influence on the results.

We describe the genitalic anatomy briefly in the results section. A detailed description of the complex genital morphology is provided in an appendix. As Zoraptera are arguably the most enigmatic order in insect systematics (e.g. Beutel & Weide, 2005), our anatomical data on genital morphology, which are presented here for the first time, may turn out as useful in a phylogenetic context in the future.

In addition to the morphological investigation, to assess the movement of the elongated tube during copulation, we measured the length of the elongated tube and the spermatheca based on photographs of slide mounted specimens using a curvimeter (Koizumi COMCURVE-9 Junior, Japan). We followed the methods described by Matsumura & Yoshizawa (2010). Twenty three of 32 pairs fixed in copula were disconnected after fixation during a transportation process from Hokkaido University to Friedrich-Schiller-Universität Jena. They were used for an indirect assessment of the rates of the length of the elongated tube inserted into the female genital tract. The length of the part of the elongated tube remaining in the spiral-shaped pouch (of the male) was measured, and also the complete length of the elongated tube of the single males. Using the average length of the elongated tube of the single males (n=18) we calculated the length of the part inserted, before the couple was disconnected. The part of the elongated-tube remaining in the male abdomen (n=19) is significantly shorter than that of the single males (Wilcoxon singed rank test, Z=3.80, P<0.001, range: 220-1895µm), which is indirect evidence that a part must have been inserted in the female genital tract. At least in the connected pairs (n=9), we observed that some males had inserted the elongated tube into the female spermatheca (n=5, Fig. 2C).

TERMINOLOGY

We followed the terminology used by Gurney (1938) who studied the genitalia of different zorapteran species. However, the intromittent organs vary greatly between species (Gurney, 1938), greatly impeding the homologization of the parts. Consequently we used some general terms, also for some of the muscles. The musculature related to the male genitalia of *Zorotypus hubbardi* was described in an earlier study (Hünefeld, 2007), but a reliable homologization among zorapteran species turned out as unfeasible with the presently available information.

Males assume a supine position while females maintain the back oriented upwards during copulation (Mashimo et al., 2011, Fig. 2A). Consequently, in almost all figures we show males with the ventral side directed upwards. Even though this is unusual in morphological studies it facilitates the understanding of the configurations and movements during copulation.

RESULTS

THE MALE REPRODUCTIVE SYSTEM (Figs. 2, 3, 4, 6E, F)

The entire male reproductive apparatus fills out a very large part of the male's abdominal lumen (Fig. 2B), but the genitalia (copulatory apparatus) involved in copulation occupy only a relatively small posterior portion (Fig. 2A). The male reproductive system is composed of the paired testes, paired ducts connecting them with the accessory gland, and the genitalia. An additional short ejaculatory duct connects the accessory gland and the membranous part of the genitalia and has no connection with the other parts of the reproductive organs (Fig. 3A, B).

The male genitalia are composed of three main components, a basal plate, a spiral-shaped membranous pouch, and a largely membranous sac-shaped region (Fig. 3C). In addition to the basal plate two other sclerotized elements (strongly sclerotized areas of a membranous sheet) are present (Figs. 3, 4A): a bifurcated sclerite placed on the spiral-shaped pouch and an elongated tube inside of this structure (Figs. 3E, 4C, D). The spiral-shaped pouch is a rolled up membranous sheet containing the elongated tube (Figs. 3, 4). Therefore the tube can only be released when the membranous sheet is uncoiled. Relatively long hairs (probably setae) are arranged on the interior surface of the pouch (Fig. 3E, F: on the blue line). The pouch is apparently formed as a deep invagination of the ventral genital membrane (Fig. 3E, F). After we extracted the elongated tube from the spiral-shaped pouch of anesthetized males, it maintained a helical or at least sinuate shape (appendix Fig. 10D). The tip of the elongated tube widens (Fig. 6E, F).

The CLSM results show that the elongated tube, the bifurcated sclerite, and the basal plate exhibit some green autofluorescence and large proportions of red autofluorescence. This indicates that these structures consist mainly of sclerotized chitinous material (Fig. 4A, B).

MUSCULATURE OF MALE GENITALIA (Fig. 3D)

Five pairs of muscles and one unpaired muscle are directly connected to the male genitalia (appendix). Two of them play a major role in the mechanisms of movements of the male genitalia as discussed below.

The first muscle is unpaired and transverse; it is broad and encloses the membranous region and the ejaculatory duct (Fig. 3D, no. 2); the sac-shaped region is connected with the anterior tip of the basal plate by this muscle. The second muscle is paired and stout; it connects a part of the body wall (sternite VIII) and the spiral-shaped pouch (Fig. 3D, no. 6).

GENITAL FITTING DURING COPULATION (Figs. 5, 6, 7, tables 1, 2)

Observations of the reproductive organs of fixed pairs at different stages of the copulation (n=30) suggest four stages after males and females connect (stages 1-4 in the following). Even though the exact timing of the initiation of the copula could not be assessed in the fixed couples, the structural configurations and the position of the spermatophores (see below) suggest a specific sequence of genital movements during copulation (Fig. 5, table 1). Throughout the copulation, the male intermittent organs are in contact with a primary receptacle, the vagina, and with a spermathecal duct connecting the dorso-basal vaginal area with a spherical spermatheca (sperm receptacle, receptaculum seminis) (see Fig. 2B for the terms). The spermathecal duct opens on the posterior dorsal region of the vagina, and the exit of the male elongated tube is placed in front of the entrance during copulation (Figs. 2B, 5F)

In stage 1 the posterior part of the basal plate is inserted into the female vagina and a part of the elongated tube is inserted into the spermathecal duct (Fig. 5A, B, table 1, n=9). Amorphous jelly-like objects are present in the accessory gland (Fig. 5B).

In stage 2, the membranous fold of male genitalia is everted posteriorly and inserted into the vagina (Fig. 5C, D, table 1, n=13). The size of the membranous fold varies among individuals. The lumen of the membranous fold contains hemolymph, but this space is relatively narrow and the entire surface is covered with wrinkles (Fig. 6A, B, C). The jelly-like object is located in the male genital chamber at this stage (Fig. 5C, D).

At stage 3 we found the jelly-like object including sperm (Fig. 7B, C) within the female vagina (Fig. 5E, F). The transfer was not completely accomplished as it was still enclosed by the male membranous fold forming a pocket (Fig.

5E, F, table 1, n=5). Histological sections of a pair at this stage revealed free sperm in a posterior area of the opening of the spermathecal duct tangled up with hairs on the spiral-shaped pouch membrane (Fig. 7E, F, compare with an image of sperm in a spermatophore in Fig. 7B, C and a spermatheca shown in Fig. 7D). The sperm of this male are still packed in an intact spermatophore, but free sperm, which have likely been deposited in a previous copulation, were observed in the spermatheca of the female.

At stage 4 (table 1, n=2), we observed males with the tube still partly inserted in the female spermathecal duct but with the membranous intromittent organ retracted into the own body cavity. No spermatophore was present in these male specimens.

The elongated tube is slightly shorter than the spermathecal duct (table2). In most fixed specimens (n=19/21) where we measured the length of the elongated tube, we found this structure inserted into the spermathecal duct throughout the entire copulation (Figs. 2C, 7G). In some cases, the most part of the elongated tube is inserted into the spermathecal duct during copulation (table 1). The ratio of the inserted portion to the entire length of the tube varied greatly among the couples within each stage (Table 1). When the elongated tube is inserted into the female spermathecal duct the membrane of the spiral-shaped pouch is released from the spiral. At this stage the morphological interior of this released membrane is not filled with body fluid, and we observed that the surface membrane of the spiral is uncoiled and folded regularly and the hairs on the surface are tightly aligned around the bifurcated sclerite (Fig. 6 C, D).

DISCUSSION

Elongated tube-like genitalia are almost automatically considered as sperm transporting organs. In contrast to such an intuitive assessment, the current study clearly shows that males of *Z. caudelli* transport their sperm packed in a large spermatophore, and that the elongated tube is not directly involved in this process. This is evident as the ejaculatory duct is not connected to the base of the tube, but opens directly into the sac-shaped region (Fig. 3E, F). In addition, it is shown that the males insert the membranous fold into the vagina, but the elongated tube into the spermathecal duct (Figs. 5, 7G). This means that two intromittent organs are inserted during copulation in this species and have different functions. Such a highly unusual condition is known in sea snug species which have a branched penis and transfer sperm and prostate secretion separately into female genital tract and body cavity (Anthes & Michiels, 2007a, b). The odd penis shown here is the second example in animals as far as we know.

THE POSSIBLE FUNCTION OF THE ELONGATED ORGAN

Different functions of elongated intromittent organs are known in different groups of insects. It is a guiding device involved in the transfer of spermatophores in staphylinid beetles (the elongated organ of this group is relatively thick; see summary in Naomi, in press.), it widens the spermatheca in a lygaeid bug (Gschwentner & Tadler, 2000), and it is used to remove rival sperm in an anisolabidid earwig (Kamimura, 2000). In chrysomelid beetles it was identified as an indicator of cryptic female choice (Rodriguez, 1995; Rodriguez et al., 2004), which is a part of female choice occurring after the initiation of the copulation (Thornhill, 1983; Eberhard, 1996).

As the elongated tube in *Z. caudelli* is definitely not involved in transferring sperm or a spermatophore, one can exclude a guiding function. The second option, widening the spermatheca, is also highly unlikely. The spermathecal duct of *Z. caudelli* is "*a long canal with swellings in some regions and a regular cylindrical shape in others*" even in inactive females (Dallai et al., 2012b). The diameter of the tube is definitely too small to widen it (ca. 20 µm in diameter, Dallai et al., 2012b) (see also Fig. 7G). An active choice whether females store the sperm in the spermatheca or reject it using a spermathecal muscle is also very unlikely. Such a muscle is described in a leaf beetle and a weevil (Villavaso, 1975; Rodriguez, 1994) but is entirely absent in *Z. caudelli* (Dallai et al., 2012b). However, it is conceivable that females decide acceptation or rejection of sperm by controlling the spermatophore before sperm enters the spermatheca. The length of the elongated tube may have an influence on the decision of the female, or possibly affect further mating attempts, ovulation, ovipositon, etc.

Another option is 'removal of rival sperm' by the elongated tube. Actually this species is polyandrous (Y. Kamimura pers. comm.). It is conceivable that the widened distal tip of the male elongated tube (Fig. 6E, F) interacts with the spermatozoa. The male elongated tube is as long as the female spermathecal duct on average and deeply inserted during copulation (table 1), and it is known that the duct itself can also function as a sperm receptacle (Dallai et al., 2012b). In addition, due to the relatively long sperm cells (770-800 µm) (Dallai et al., 2011), the elongated tube can interfere with rival sperm in the female sperm receptacle by wrapping the long spermatozoa around its distal part. We also found free sperm outside of the spermathecal duct in the vagina possibly pulled back by the tip of the elongated tube (Fig. 7E, F). Only the lumen of the spermatheca and spermathecal duct is filled with secretion and therefore suitable for sperm storage (Dallai et al., 2012b). These observations presented here suggest 'removal of rival sperm' is a hypothesis which should be tested with priority.

MANIPULATIONS OF GENITAL MOVEMENTS DURING COPULATION

The male reproductive system of *Z. caudelli* is characterized by a complex of two closely adjacent paired accessory glands which receive spermatozoa from the testes (Dallai et al., 2011). Our observations show that the spermatophore is formed within the accessory gland and then passes through the comparatively small genital tract (Fig. 5). Although the walls of this pathway are membranous and can be expanded when a spermatophore passes through, males apparently need considerable force to move it posteriorly. Muscle fibres were observed only between the paired accessory glands (Dallai et al., 2011), and it is unlikely that these fibres alone can accomplish this process. Alternatively, abdominal contraction is the most plausible primary transfer mechanism, because contraction and/or peristaltic movements of the abdomen can increase the hemolymph pressure on the abdominal walls (Fig. 8A, B). In fact, in the Malaysian species *Zorotypus magnicaudelli*, which is equipped with similar genitalia (Mashimo et al., in press; RD pers. obs.) and shows a similar mating behaviour (Dallai et al., 2013), males displayed peristaltic abdominal movements during copulation, probably related to spermatophore transfer (Dallai et al., 2013). After the spermatophore is transported to the male genitalia (Fig. 5C, D), contractions of the muscle surrounding the sac-shaped region of the genitalia (Fig. 3D, no. 2) likely move the spermatophore posteriorly. Finally, the spermatophore is inserted into the female vagina by the male membranous fold.

We did not identify movements of other parts which could be involved in the insertion of the elongated tube (table 1), and an extensor muscle inserted on the spiral-shaped pouch and/or the elongated tube is missing. When the elongated tube is inserted into the spermathecal duct, the lumen of the released spiral-shaped membranous pouch is not filled with hemolymph, and the membranous wall of the pouch is just uncoiled and folded (Fig. 6C, D). Repeated pressure pushing out the spiral-shaped pouch, caused by repeated abdominal contractions for transmission of a spermatophore, would likely result in such a condition with the pouch membrane released from the spiral (Figs. 6D, 8C, D). As the ventral part of the membranous genitalia is fixed to the basal plate by muscles (Fig. 8D, detailed anatomical information is available in the appendix), pressure generated by abdominal contractions consequently can cause uncoiling of the spiral-shaped pouch (Fig. 8D). At the same time, avoidance of rewinding movement of the elongated tube and uncoiled membrane is essential. The hairs arranged on the pouch membrane along the elongated tube (Fig. 6D) probably help to limit reverse movements of the membrane by interacting with each other. As the entrance of the spermathecal duct and the apex of the male elongated tube face each other (Figs. 2B, 5F), upon its release the distal part of the tube is forced to enter the spermathecal duct.

In contrast to the insertion mechanism, the withdrawal of the elongated tube and the membranous fold can be simply explained. One of the two intromittent organs, the membranous fold, is equipped with a paired retractor muscle (Fig. 3D,

no.6). The withdrawal of this structure is obviously effected by contractions of this muscle. The withdrawal of the elongated tube is also, at least partly, caused by muscle contraction, in this case of the paired muscles connecting the spiral-shaped pouch and the body wall (sternum VIII) (Fig. 3D, no. 6). The effect of the muscles probably supports the material properties of the elongated tube, which likely behaves as a spiral spring. Although the elongated tube mainly consists of relatively stiff, sclerotized chitinous material, it is deformed and uncoiled during the insertion process. In case the deformation forces are not active anymore, the elongated tube probably returns to its helical structure due to the stiffness of its material. This assumption is supported by the observation that the elongated tube largely maintains its helical structure after being extracted from the spiral-shaped pouch (Appendix Fig. 10D).

EVOLUTIONARY PERSPECTIVES

Direct insemination with free sperm occurs in the zorapteran species *Z. barberi* (Choe, 1995), but at least four of 39 described species form spermatophores (Dallai et al., 2012a, 2013, present study; RD pers. obs.) and this is likely a groundplan feature of the order. Spermatophores evolved several times in arthropods (e.g. Proctor, 1998), and they are also formed in polyneopteran insects (Blattodea: e.g. Chapman, 1998; Mantodea: e.g. Holwell, 2007; Orthoptera: e.g. Alexander & Otte, 1967; Embioptera: e.g. Ross, 1970; Phasmatodea: e.g. Bragg, 1991) which include Zoraptera (e.g. Yoshizawa, 2011), and in many other groups of lower Hexapoda. In contrast to this, the presence of a spiral-shaped elongated tube of *Z. caudelli* and some other zorapteran species is a highly unusual apomorphic condition (e.g. Gurney, 1938; Bolivar y Pieltain, 1940; New, 1978; Mashimo et al., in press). This is suggested by the absence of the elongated tube in the majority of zorapteran species and the absence in potentially related groups such as Embioptera (webspinners) and Phasmatodea (stick insects) (e.g. Yoshizawa, 2011). This does not completely exclude the presence of the elongated tube in the zorapteran groundplan (or the groundplan of a zorapteran subunit) and secondary loss in some of the species. However, considering the implied morphological modifications and the absence of potential precursors, it appears likely to us that combination of the spiral-shaped pouch and the elongated tube is de novo formations. For a reliable interpretation a species level phylogeny would be required. Moreover, detailed anatomical information on the genitalia of zorapteran species is still scant. It is noteworthy that an elongated rod (= the intromittent probe sense Choe, 1995) (Gurney, 1938) is present in *Z. barberi*. It is half as long as the entire body, inserted into the female spermathecal duct, and used as transporting organ of free sperm (see above) according to Choe (1995). Apparently the simple elongated tube in *Z. caudelli* is unlikely to be homologous with the rod of *Z. barberi*, which is composed of several

elements (see Gurney, 1938). Concerning the origin of the elongated tube itself, it is still necessary to re-evaluate this issue based on a broader morphological comparison in the future.

Our mechanical explanations suggest that the difficulty of storing and manipulating an elongated tube is overcome by a previously present mechanism for spermatophore transfer in *Z. caudelli* (i.e. hemolymph pressure caused by peristaltic movements of the abdomen). This suggests that spermatophore transfer is a preadaptation for the evolution of the spiral-shaped pouch in this case, although the coexistence of an extremely long and narrow tube and a large spermatophore initially appeared as a paradox. The mechanical explanation presented here suggests similar mechanisms for insertion and withdrawal of the elongated tubes in very distantly related taxa, Zoraptera (groundlice, the present study) and Coleoptera (beetles, Matsumura & Yoshizawa, 2010), i.e. increased hemolymph pressure and the contraction of muscles directly connected to the male copulatory organs. In the case of the beetle species, they transport sperm directly through the elongated organ, but the hemolymph pressure is probably used for the protrusion of the male copulatory organs like in other species without an elongated organ (Verma & Kumar, 1972; Matsumura & Yoshizawa, 2010). At least the explanation for the insertion process is identical with the mechanism suggested for a medfly (Diptera) too (Eberhard, 2005). Although the species of Coleoptera, Diptera, and Zoraptera have elongated parts in their genital apparatus, the function and morphology of these modified elements are very different. Nevertheless, we show here how the acquisition of a unique and complicated feature is possible in conjunction with an already available ancestral mechanism for manipulating penises, and this applies to groups widely separated phylogenetically.

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Figure legends

Figure 1. Schematic drawings of structures and the insertion mechanism of elongated intromittent organs. The focus is on areas which are actually inserted into the female genital cavity. A: male genitalia of the medfly *Ceratits capitata*. The phallus is elongated (pink), and an ejaculatory duct passes through it (Marchini et al., 2001); hemolymph pressure (blue regions and arrows), which is probably produced by an ejaculatory apodeme and sperm sac, pushes the elongated part; a folded site of the elongated part gradually moves distally, and then almost the entire elongated part is inserted (Eberhard & Pereira, 1995; Eberhard, 2005); grey regions show a kind of stopper of the elongated part, which is called phalloapodeme. B: the intromittent region of the male genitalia of the leaf beetle *Lema coronata*; a specialised pocket is present in their internal sac; the elongated sperm transmitting tube, flagellum (pink), is stored within it in the resting state; hemolymph pressure (blue regions and allows) that is probably generated by contraction of abdominal segments everts the pocket membrane and the flagellum (Matsumura & Yoshizawa, 2010). C: male intromittent organ with spiral-shaped elongated part; this type is widespread in pterygote insects, but morphological details and mechanisms of insertion and withdrawal remain largely unknown.

Figure 2. Outline of the male reproductive system of *Z. caudelli*. A: µCT scanned image of a couple; some organs were highlighted to visualize their relative volume in comparison with the remaining body; coloured objects indicate parts of genitalia, i.e. the basal plate (green), the bifurcated sclerite (purple), and the spiral-shaped pouch (yellow). B: 3D image of other couple in lateral view (sagittal plane), focusing on the male. C: elongated tube inserted into female spermatheca, light microscopic image.

Figure 3. Male reproductive organs of *Z. caudelli*. All figures except for B were drawn with the same direction of the indicator above A. A: male post abdomen, lateral view (testes removed). B: postabdomen, ventral view. C: habitus of genitalia in lateral view. D: muscles related to genitalia, lateral view. E: a schematic drawing of C. F: spiral-shaped sac region, simplified. Inner regions indicated by light grey in E, F; roman numerals refer to segments.

Figure 4. Spiral-shaped pouch of *Z. caudelli*. A, B: confocal laser scanning micrographs showing the autofluorescence composition of the copulatory organ in lateral view, enlargement of central region of the spiral-shaped pouch are shown in B. C, D: two cross sections of spiral-shaped pouch around central region.

Figure 5. Serial movements during copulation of *Z. caudelli*. Line drawings (A, C, E) and schematized illustrations (B, D, F), lateral view. A, B: male postabdomen, stage 1, female body removed. C, D: male postabdomen, stage 2, female body removed. E, F: couplings in stage 3. Inner regions indicated by light grey in B and D; roman numerals (males) refer to segments.

Figure 6. SEM micrographs of male genitalia of *Z. caudelli*. A: surface structure of the dorsal membrane in stage 2; B: enlargement of square in A. C: sagittal plane of postabdomen in stage 2. D: enlargement of square in C. E: elongated tube. F: tip of elongated tube enlarged.

Figure 7. Histological cross sections of a couple of *Z. caudelli* (all sections from one copula). A: positional diagram of sections shown below. B, C: female abdomen with spermatophore (B) and the sperm packing region enlarged (C). D: sperm in spermatheca. E, F: posterior region of male abdomen (E) with free sperm (F). G: male elongated tube in spermathecal duct.

Figure 8. Schema of insertion mechanisms of the spermatophore and the elongated tube. A-C: postabdomen. D: genitalia. Dotted areas in A and C indicate membranous regions. Red arrows in A indicate possible pressure acting on the accessory gland and subsequent movement of spermatophore, red arrows in C indicate possible pressure acting on the genitalia and subsequent movement of spermatophore and membrane of the spiral-shaped pouch and membranous fold; muscles inserted on genitalia (D) function as stoppers for the movement of structures except for the spiral-shaped pouch. Morphological inner regions indicated by light grey. Roman numerals refer to segments.

Table 1. Summarized data about copulation stages.

	stages			
	1	2	3	4
Numbers of samples observed.	9	13	5	2
Is the basal plate protruded?	Yes[a]	Yes	Yes	Yes
Is the membranous fold everted?	No	Yes	Yes	No
Where is a jelly-like object found?	Accessory gland	Male genitalia	Female genitalia enclosed by male membranous sac	Probably vagina[b]
Ratio of insertion to the whole elongated tube length in average (%).	18.3 (N = 7[c])	22.0 (N = 10[c])	34.0 (N = 2[d])	9.6 (N = 2)
Variation of the ratio within a stage.	0.6 – 70.5	0 – 74.1	40.3 – 87.7	0 – 24.8

[a] It was unclear in two cases. [b] Because we preserved all couples in a container and many of them separated during transportation, we lost data on who was whose partner. However as far as we observed separated female, some females had a jelly-like object, a spermatophore, in her vagina. [c] Some could not be used as slide specimens (n=5). [d] Some were used for histological sections (n=2) or observations by μCT (n=1).

Table 2. Length of the elongated tube and spermathecal duct.

	N	mean ± s.e.	range
Male elongated tube	18	1795 ± 38 μm	1470 - 2015 μm
Female spermathecal duct	7	2274 ± 87 μm	1810 - 2537 μm

APPENDIX (incl. Figs. 9, 10)

ANATOMY OF THE MALE REPRODUCTIVE SYSTEMS IN DETAIL

The reproductive organs fill out a very large part of the abdominal lumen (Fig. 2). The paired testes consist of two follicles. Paired ducts connect them with the accessory gland, which appears as an undivided (but internally divided into two compartments) and inflated very voluminous structure. The paired ducts merge into a short common ejaculatory duct just behind the accessory gland. The ejaculatory duct opens on the dorsal membranous region of the male copulatory apparatus (Fig. 3). This duct is almost round in cross section with a diameter of 113.9 μm at rest. The size of the phallotreme (the site where the genitalia open on the posterior tip of the abdomen) is 136.0 μm ×295.9 μm.

The male genitalia are composed of three main components, a basal plate, a membranous sheet, and a spiral-shaped pouch (Fig. 3). In the spiral region, two sclerites (strongly sclerotized areas of a membranous sheet) are present (Figs. 3, 4): a bifurcated sclerite positioned to the spiral-shaped pouch and the elongated tube inside of the pouch. The tube within the spiral sac (but morphologically outside of the body, Fig. 3E, F) is relatively long and stout hairs (probably they are setae) are arranged on its surface (Fig. 3E, F on a blue line). The spiral sac is formed by a deeply invaginated pouch of the ventral genital membrane (Fig. 3F). The elongated tube has no connection with the ejaculatory duct (Fig. 3), and the tip of the tube widens (Fig. 5E, F).

MUSCULATURE OF MALE GENITALIA IN DETAIL

Five pairs of muscles and one unpaired muscle are directly connected to the male copulatory organ (Fig. 9). We assign a number to each muscle (1-6) and describe them in the following.

The first muscle (1) is paired, originates on the antero-lateral region of the subgenital plate and is inserted on the antero-ventral region of the basal plate (Fig. 9C). The second muscle (2) is unpaired and transverse; it is broad and encloses the membranous region and the short ejaculatory duct (Fig. 9C); the anterior part of the membranous region is connected with the tip of the basal plate by this muscle. The third muscle (3) is paired and connects the antero-dorsal region of the basal plate and the antero-lateral surface of the membranous region (Fig. 9B, 3). The fourth muscle (4) is also paired; it is very thin and elongated with a longitudinal orientation and connects the antero-dorsal region of the basal plate and the postero-ventral area of the membranous region (Fig. 9B, C, D). The fifth muscle (5) is paired and connects the anterior area of the membranous region and the spiral-shaped pouch (Fig. 9B, C, D). The paired sixth muscle (6) is stout and connects the antero-lateral area of sternite VIII and several attachment sites around the spiral-shaped pouch (Fig. 9C, D); they are inserted on a part of the bifurcated sclerite (Fig. 9C-a), the centre of the spiral-shaped pouch (Fig.

9C-b), and a ventral area close to the posterior end of the membranous region (Fig. 9C-c). The last insertion point is close to that of the fourth muscle (4) (Fig. 9D).

SPERMATOPHORE ANATOMY

We describe the morphology of the spermatophore with reference to its position in the male body. The spermatophore at the stage 2 has a rounded, slightly elongated main body (Fig. 10A, B). The length of its longitudinal axis is ca. 260 µm. A pair of elongated filaments extends from the postero-dorsal area of the main body, and only this part is still in the ejaculatory duct at this stage. A corn-shaped anteriorly directed projection is present on the ventral side of the main body.

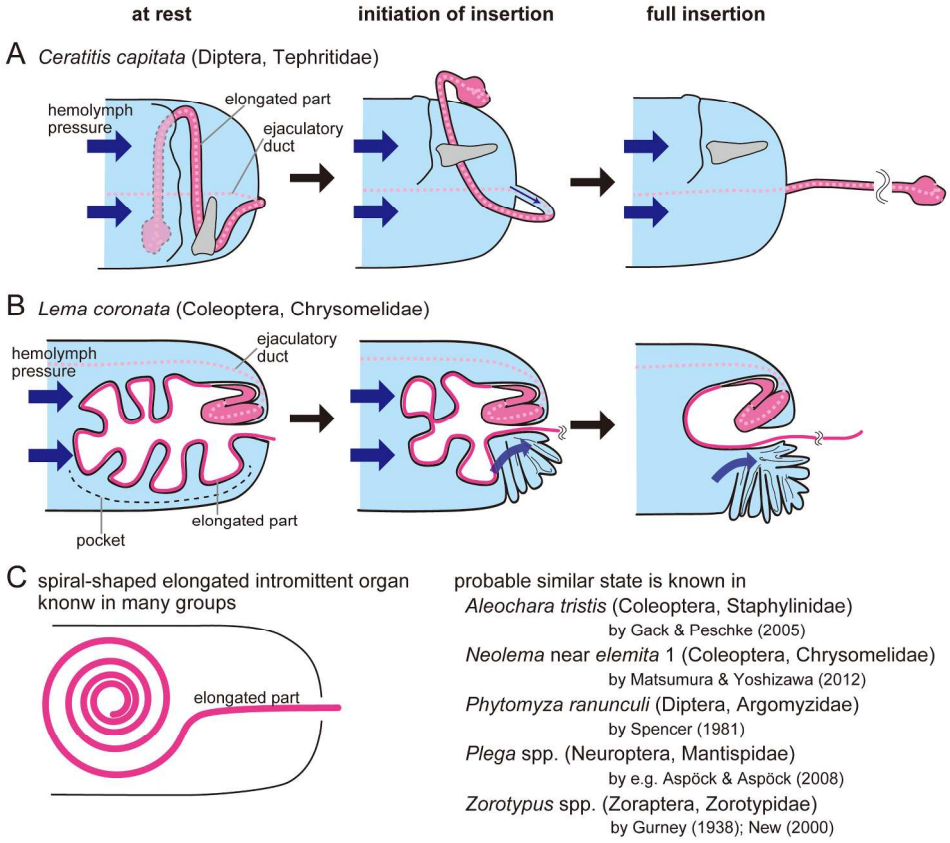
The shape has distinctly changed after the transfer to the female. The elongated filaments are tangled and clinging to the ventral surface of the main body, and the projection of the ventral surface directs in the opposite direction compared to the second stage (Fig. 10).

The sperm contained in the spermatophore (Fig. 7B, C) is limited to a small space in the posterior region of the main body. The volume of this area was calculated based on µCT images as only 3.5% of the total volume of the entire jelly-like structure (space occupied by sperm: 1.0×10^5 µm^3, spermatophore: 2.9×10^6 µm^3). The volume of the spermatheca of the partner was calculated as ca. 8.4×10^4 µm^3.

Figure legends

Appendix **Figure 9.** Anatomy of the male genitalia and its musculature of *Z. caudelli.* A: line drawing of male copulatory organ in lateral view. B: schematic drawing of A. Origins and insertions of muscles 3-5 are shown. Hairs are regularly arranged on a corresponding area of the blue line. Morphological inner areas were painted by light grey. C-D: muscles related to copulatory organ in lateral (C) and ventral view, basal plate and muscles 1-2 removed (D). Each muscle or pair of muscles was numbered in the text. Same numbers in B, C, and D refer to homologous muscles.

Appendix **Figure 10.** Spermatophore morphology of *Z. caudelli*. A-B: spermatophore in male genital cavity (stage two) in ventral (A) and lateral view (B), elongated filaments severed. C-D: spermatophore in female vagina but still enclosed by male dorsal membrane (stage three) in ventral view.



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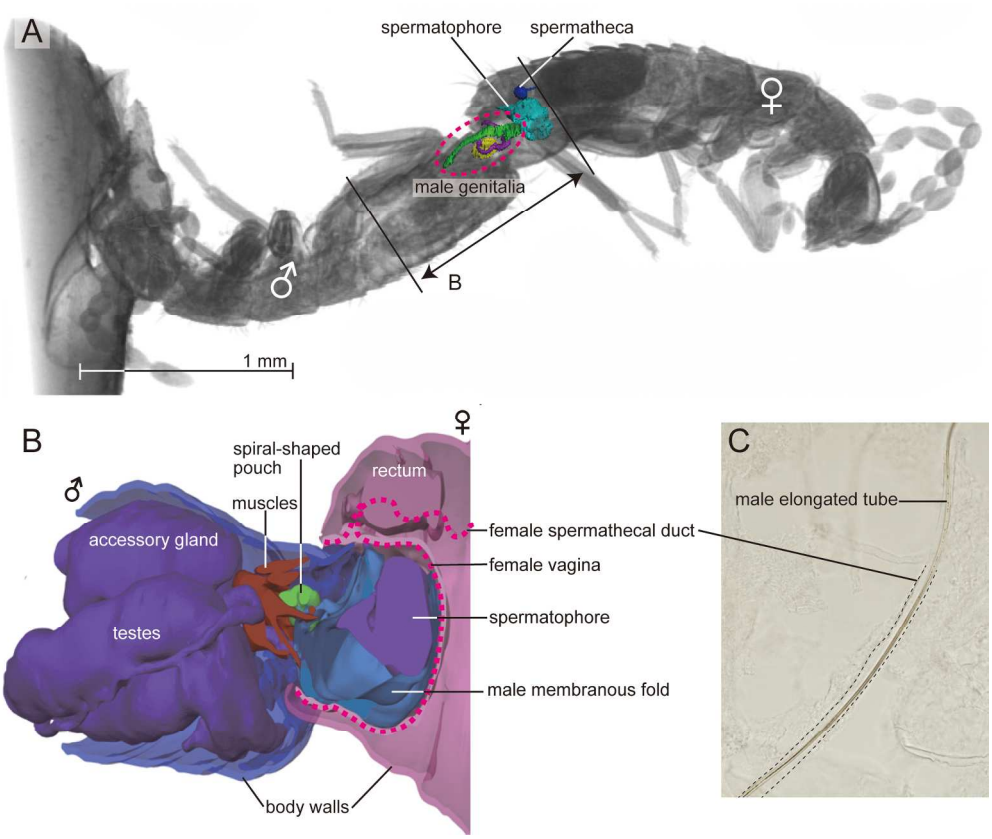

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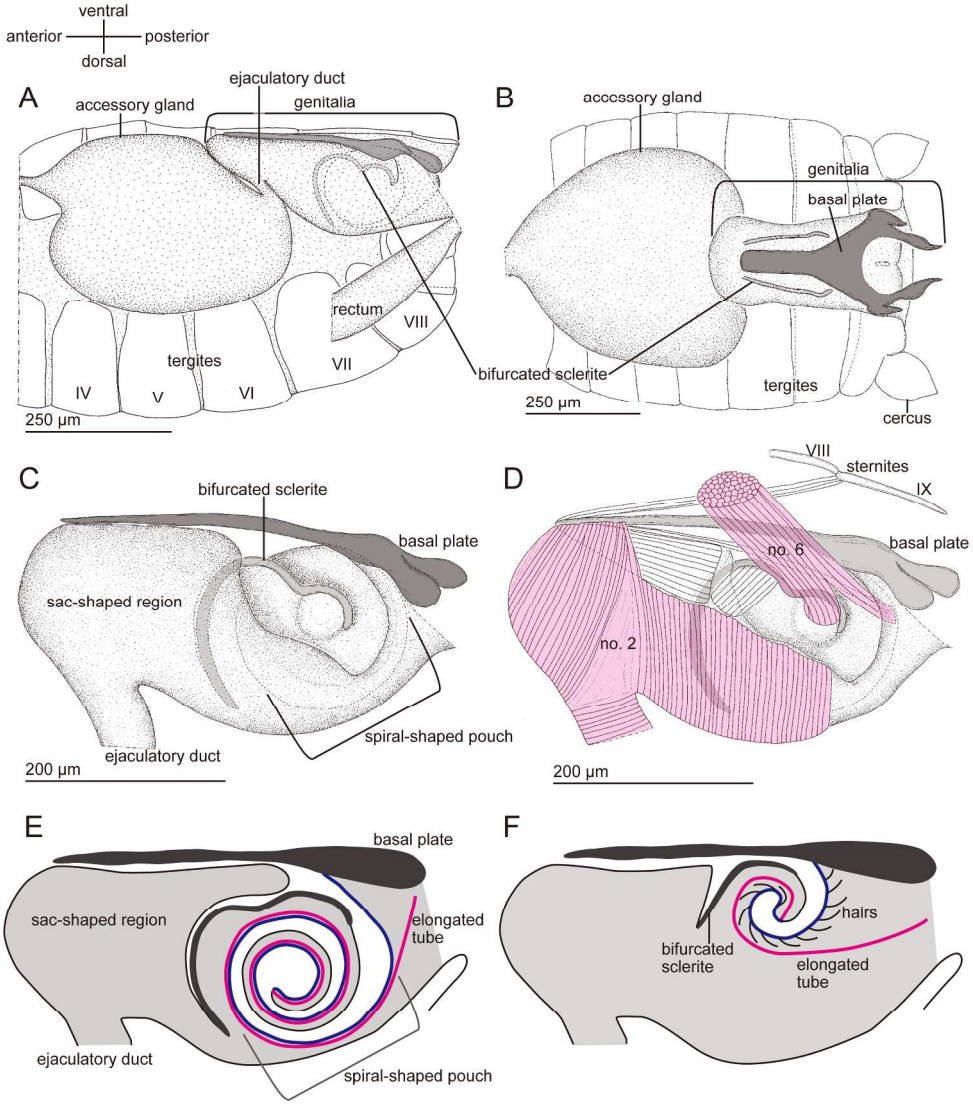

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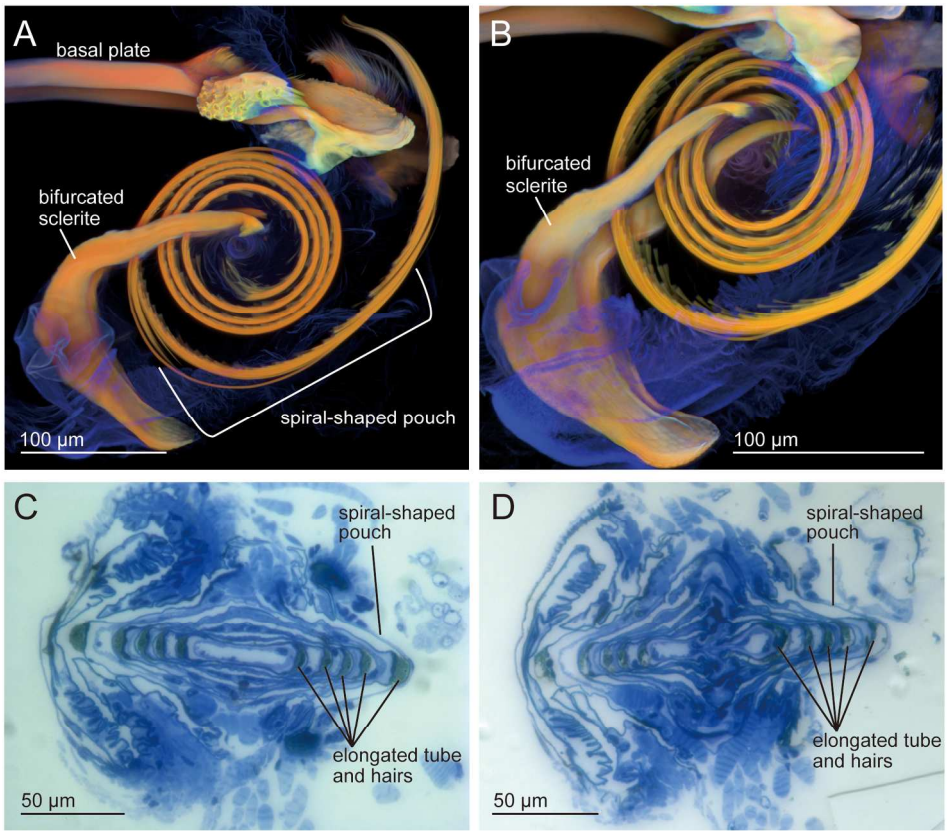

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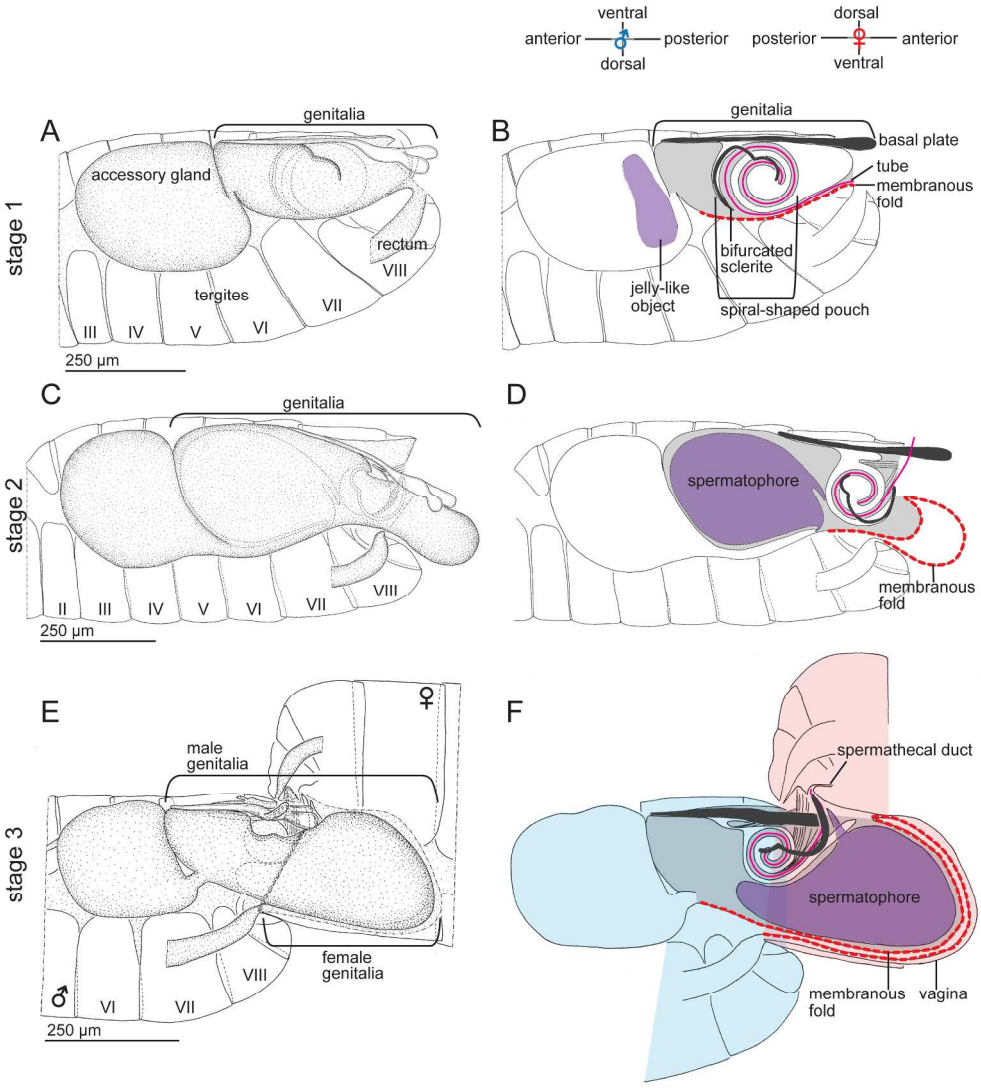

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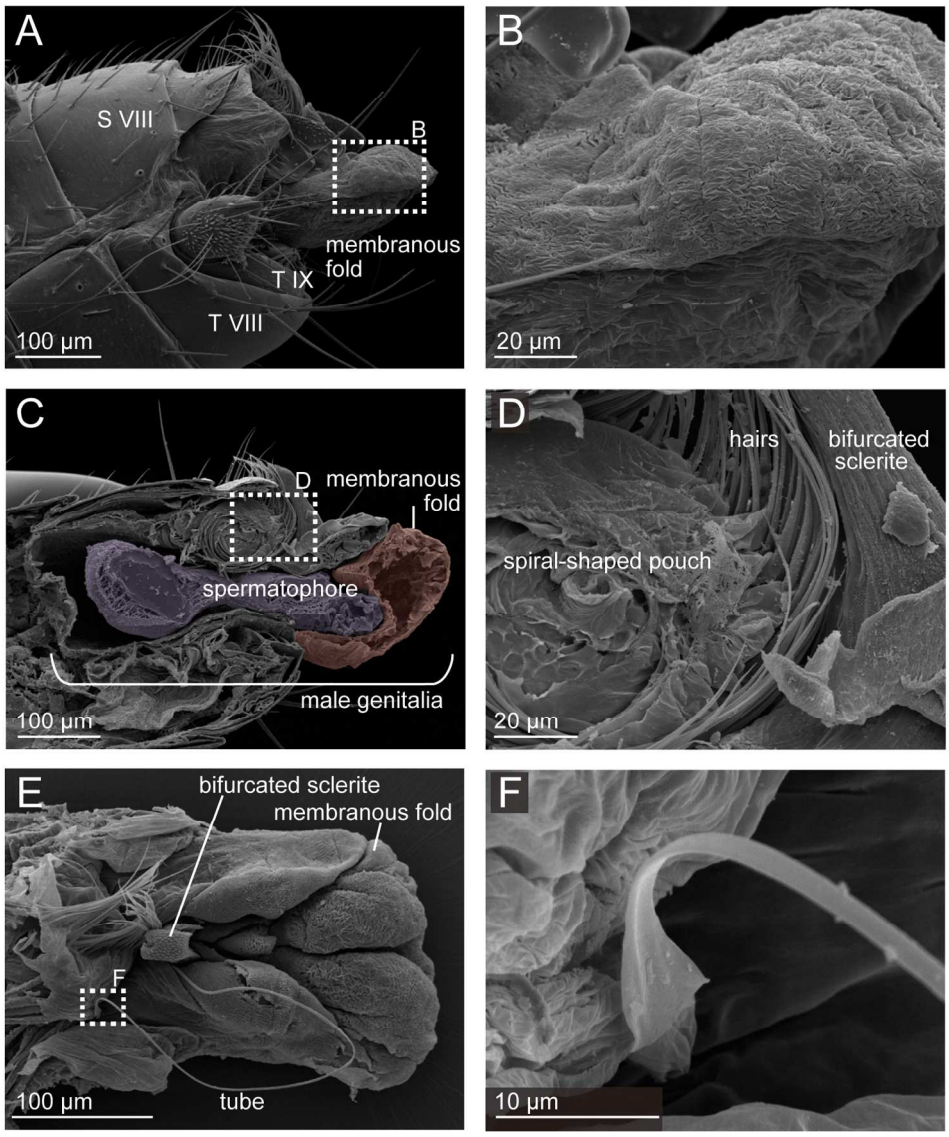

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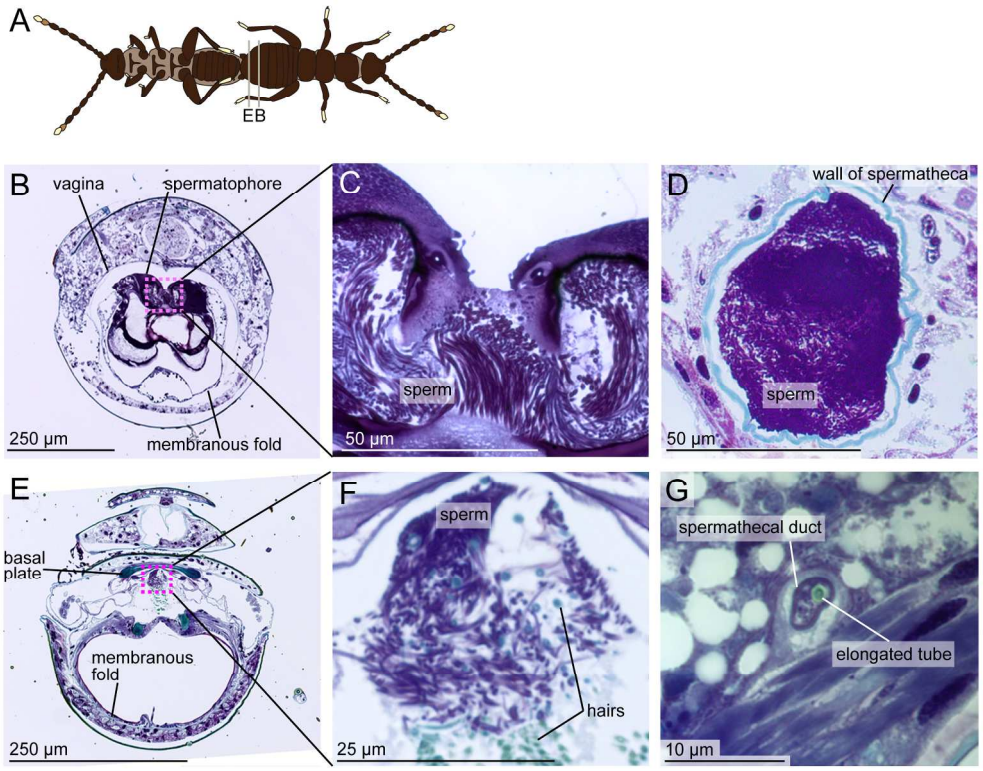

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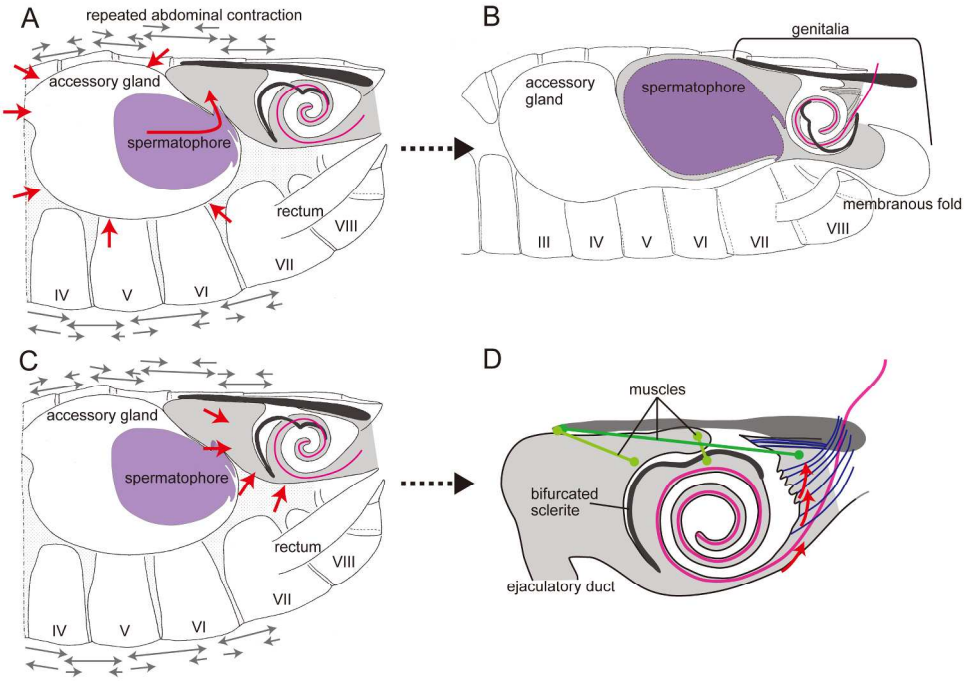

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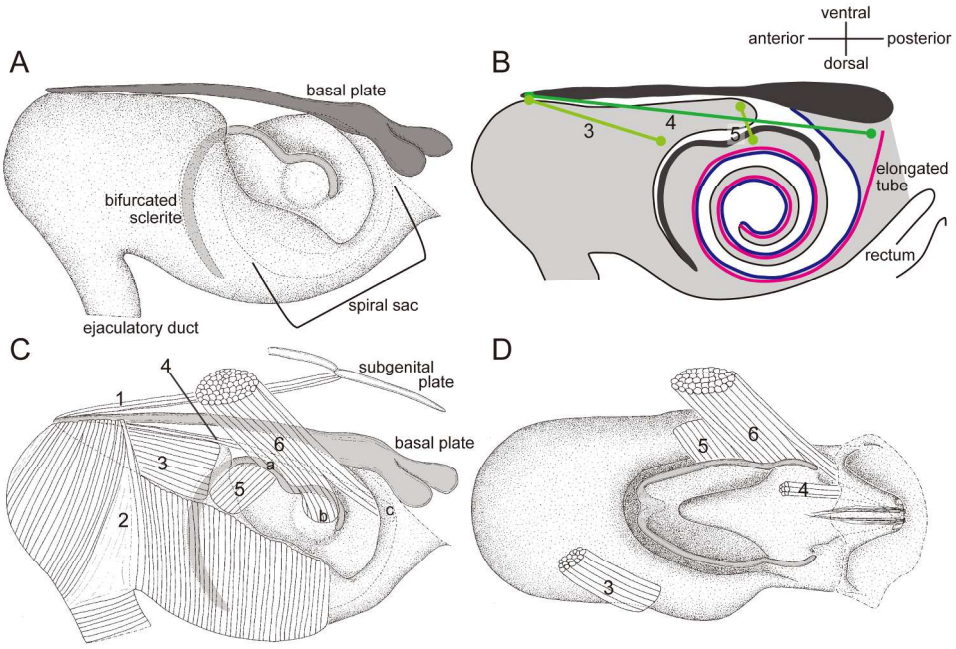

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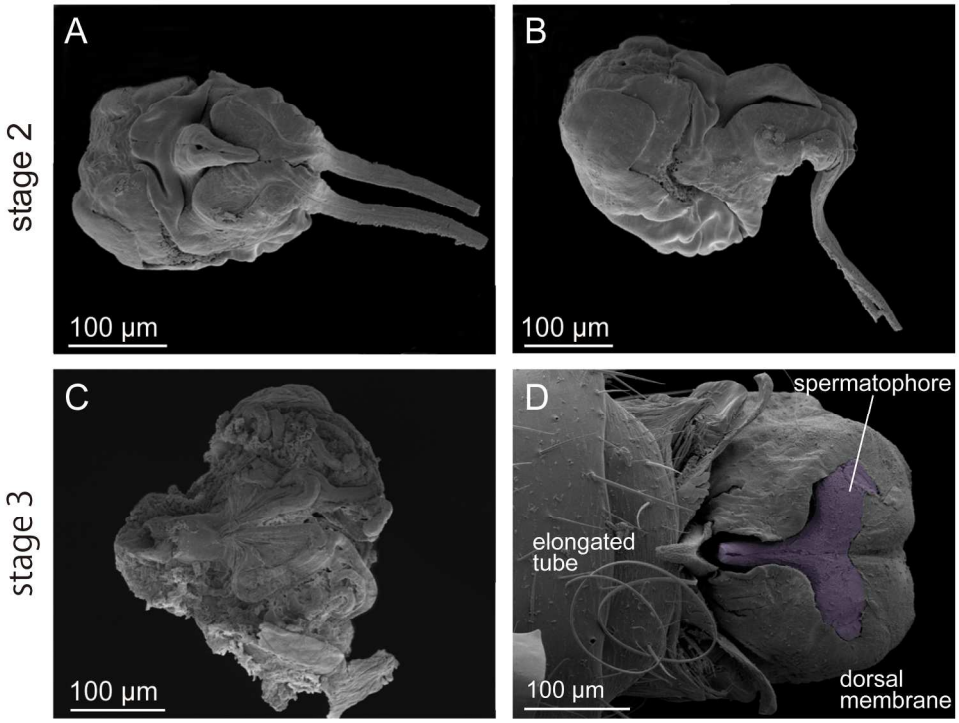

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