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Two intromittent organs in *Zorotypus caudelli* (Insecta, Zoraptera): the paradoxical coexistence of an extremely long tube and a large spermatophore

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Very unusual genitalia of the species *Zorotypus caudelli* are described. It contains the unique configuration of two different intromittent organs, one of them strongly elongated. Hyper elongated genitalia are known 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 do species with elongated genitalia cope with these requirements? To investigate this, we studied key features enabling storage, insertion, and withdrawal of the elongated genitalia. The co-existence of an elongated narrow tube and a bulky spermatophore is a highly unusual and apparently paradoxical condition. However, we demonstrate that the tube is not involved in sperm transmission, whereas the large spermatophore is transferred to females by a membranous fold of the genitalia. The movement of the spermatophore is caused by haemolymph pressure, which likely also promotes the insertion of both intromittent organs. A comparison with the genital anatomy and reproductive mode in related groups suggests that the elongated tube and its accommodating pouch is a *de novo* structure, and that the ancestral sperm transport via spermatophore is a preadaptive condition for the acquisition of this unusual structure. *Biological Journal of the Linnean Society*, 2014, **112**, 40–54.

ADDITIONAL KEYWORDS: copulation – insertion – novelty – penis – preadaptation – sexual selection – withdrawal.

INTRODUCTION

The structure of animal genitalia is often very complex, despite their apparently simple function, which is the transportation of sperm (Eberhard, 1985). The 'hyper elongation' of the genitalia is a conspicuous novelty observed in males of different groups. This condition occurs only sporadically in the animal kingdom, although it has apparently evolved independently in a considerable number of insect groups (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 (Tadler, 1999; Gschwentner & Tadler, 2000; Rodriguez, Windsor & Eberhard, 2004; Kamimura, 2005; van

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Lieshout & Elgar, 2011). The behavioural ecological issue solely attracts most of the attention, although anatomical specializations allowing for the evolution of elongated genitalia also merit investigation. Genitalia in repose have to be stored within a limited space of the abdomen in pterygote insects (Snodgrass, 1935), even in species with genitalia as long as the entire body (Matsumura & Yoshizawa, 2012). Males have to accommodate this and move it during copulation without tangling, injury, and breakage. These requirements appear to be problematic, and lacking anatomical specializations and/or mechanisms for accommodation and efficient manipulation of elongated genitalia could represent an impeding factor for the evolution of such genitalia (Briceño *et al*., 2011; Eberhard, 2005; Gack & Peschke, 2005; Matsumura & Yoshizawa, 2010, 2012). This raises the question: how were apparent constraints circumvented several times in insects to allow for the evolution of hyper elongated genitalia?

To answer this question, it is necessary to understand key features enabling insects to store, insert, and withdraw an hyper elongated (part of) genitalia without functional or structural damage (e.g. tangling or breakage) of what are often fine structures. However, there is a dearth of knowledge about the mechanisms of movement of elongated genitalia, with scattered and partly complete information available for only a few taxa (Briceño *et al*., 2011; Eberhard, 2005; Gack & Peschke, 2005; Matsumura & Yoshizawa, 2010). For example, Eberhard (2005) showed how the specifically modified anatomy of male medflies enables them to insert the elongated genitalia quickly and completely (Fig. 1A). A possible insertion mechanism in another fruit fly was discussed by Briceño *et al*. (2011), suggesting a stiffening and straightening of the genitalia by rhythmic cycling of the inflation and deflation of its membranous region. In a very distantly-related insect, a leaf beetle, Matsumura & Yoshizawa (2010) suggested a specialized structure of the genitalia and mechanisms, allowing for efficient insertion and withdrawal simply as a result of increased haemolymph pressure and muscle contraction (Fig. 1B). These previous studies further 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 genitalia just by increased haemolymph pressure and to withdraw it by contractions of muscles. Notably, insertion through haemolymph pressure and withdrawal by muscle contraction are also characteristic of other insects without elongated genitalia (Verma & Kumar, 1972; Dallai, Del Bene & Lupetti, 1997).

Even though morphological variability within the specific structures of hyper elongated genitalia is high, our literature survey (mainly covering taxonomic works) suggests that a spiral-shaped structure is widespread in insects (Fig. 1). Of these, the zorapteran species *Zorotypus caudelli* Karny, 1927 appeared to be ideal for use in the present study for several reasons. It was successfully reared in the laboratory (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 apparently widespread spiral-type genitalia using the zorapteran species. In the very small order Zoraptera (39 extant species), males of at least some species (e.g. *Zorotypus hubbardi*, *Zorotypus impolitus*) are known to transfer sperm packed in spermatophores, which are large in relation to their body size (*Z. hubbardi*: 2 mm × 25 μm; *Z. impolitus*: 100 μm × 100 μm; Dallai *et al*., 2012a, 2013, 2014a). Because this appears to be in conflict with the presence of an elongated tube in the study species *Z. caudelli* (New, 2000), we investigated the morphology and dynamics of male and female genitalia with a focus on sperm transmission by fixation of copulating pairs. Comparing the genital structures, mechanical explanation, and reproductive strategies of *Z. caudelli* with those found in other zorapterans and potentially related groups (polyneopteran orders), we discuss the background that may have enabled *Z. caudelli* to acquire its hyper elongated genitalia and associated mechanisms facilitating the evolution of similar configurations in other groups of insects.

MATERIAL AND METHODS

Specimens

We used specimens of *Z. caudelli* from the rearing stock of Mashimo *et al*. (2011) in addition to their rearing methods. The initial population was collected in Malaysia. To obtain copulating pairs, we placed two males and two females into a plastic case (3.5 × 3.5 × 1.0 cm) and observed them at intervals of approximately 30 min. The copulation of this species starts quite abruptly, probably with a very short pre-copulatory behaviour (Dallai *et al*., 2013). The copulation 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 10 min. This method instantly stopped the movement of animals. Afterwards, all couples were preserved in 70% ethanol in a container for anatomical investigation. The exact stage of the copulating pairs was unknown prior to the anatomical

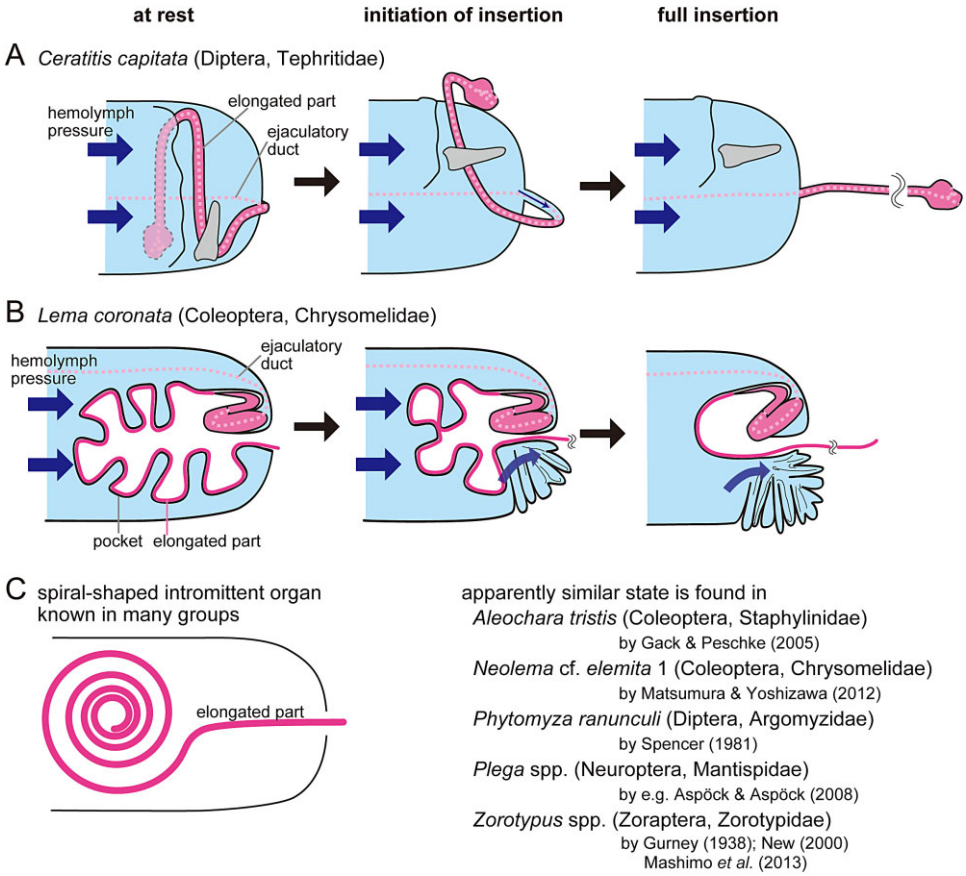

Figure 1. Schematic drawings of structures and the insertion mechanism of elongated genitalia. The focus is on areas that are actually inserted into the female genitalia. A, male genitalia of the medfly *Ceratits capitata*. The phallus is elongated (pink), and an ejaculatory duct passes through it (Marchini *et al*., 2001); haemolymph pressure (blue regions and arrows), 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, a part of the male genitalia in the leaf beetle *Lema coronata*; a specialized pocket is present in their internal sac; the elongated tube, flagellum (pink), is stored within it in the resting state; haemolymph 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 structure; this type is widespread in pterygote insects, although morphological details and mechanisms of insertion and withdrawal remain largely unknown.

investigation. 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 three-dimensional reconstruction, and confocal laser scanning microscopy (CLSM). Transverse and longitudinal semithin sections were made from two copulating pairs and one single male of *Z. caudelli*. All samples were embedded in araldite CY 212 (Agar Scientific) and cut at 1 μm using a microtome HM 360 (Microm) equipped with a diamond knife. Sections were stained with toluidine blue and pyronin G (WaldeckGmbH and Co. KG). Images were taken of every second or every third section using a light microscope (Zeiss Axioplan) equipped with a camera (PixeLink Capture OEM). The images were aligned using AMIRA, version 4.1.2 (Visage Imaging). Based on the aligned image stacks (cross sections of a single male, 518 images; cross sections of a pair in copula, 534 images; longitudinal sections of a pair in copula, 458 images), we evaluated the arrangement of internal structures and manually traced each element to reconstruct three-dimensional images. For smoothing and colouring, we used MAYA7 (Alias Wavefront). Because 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 artefact free (Friedrich & Beutel, 2008). To examine the genital fitting using μCT, an alcohol preserved copulating pair of *Z. caudelli* was dried at the critical point (Emitech K850 critical point dryer; Ingenieurbüro Peter Liebscher) and scanned. We then traced selected structures using AMIRA to highlight genital morphology.

We used scanning electron microscopy (Philips XL 30 ESEM) to observe surface structures of the male genitalia. We gradually dehydrated specimens preserved in 70% ethanol with a 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. Accordingly, the confocal laser scanning microscope and the preparation and visualization methods described by Michels & Gorb (2012) were used, with the only difference being that, instead of a long pass emission filter transmitting light with wavelengths ≥ 560 nm, we applied a long pass emission filter transmitting light with wavelengths of ≥ 640 nm to detect the autofluorescence excited by the laser light with a wavelength of 639 nm. However, the filter differences did not have any influence on the results.

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 (COMCURVE-9 Junior; Koizumi Sokki Mfg Co. Ltd) and in accordance with the methods described by Matsumura & Yoshizawa (2010).

Terminology

We adopted the terminology used by Gurney (1938), who studied the genitalia of different zorapteran species. However, the genitalia vary widely among species (Gurney, 1938), greatly impeding homologization of the parts. Consequently, we also used general terms for some of the muscles. The musculature related to the male genitalia of *Z. hubbardi* was described in an earlier study (Hünefeld, 2007), although a reliable homologization among zorapteran species is unfeasible with the information presently available.

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

RESULTS

We describe the genital anatomy only briefly here. A detailed description will be provided elsewhere because Zoraptera are arguably the most enigmatic order in insect systematics (Beutel & Weide, 2005).

The reproductive organs

The male reproductive organs are composed of the paired testes, paired ducts connecting them with the accessory gland, and the genitalia. The entire male reproductive organs fill out a large part of the male's abdominal lumen, whereas the genitalia occupy only a relatively small posterior portion of abdomen. A short ejaculatory duct connects the accessory gland and the genitalia (Fig. 2B).

The male genitalia are composed of three main regions: a basal plate (hardly sclerotized), a spiral-shaped pouch (hereafter referred to as a pouch) (membranous), and a sac region (membranous) (Fig. 2C). Around the pouch region, two other sclerotized elements are present (Fig. 2D, E): a bifurcated sclerite placed on the pouch and an elongated tube inside of this structure. The pouch is rolled up in layers and accommodates the elongated tube, apparently being formed as a deep invagination of the genital ventral membrane (Fig. 2D, E, F). The elongated tube can only be released when the pouch is uncoiled. Relatively long hairs are arranged on the pouch and come into contact with the elongated tube (Fig. 2D, E, on the blue line).

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 (Fig. 3). This indicates that these structures consist mainly of sclerotized chitinous material.

The female reproductive organs that contact with the male reproductive organs are simple and are composed of the vagina and spermatheca (sperm receptacle organ). The spermatheca opens on the posterior dorsal region of the vagina, and its distal end is swollen, forming a spherical capsule for sperm storage.

The length of the elongated tube and spermatheca is summarized in Table 1.

Musculature of male genitalia

Five pairs of muscles and one unpaired muscle are directly connected to the male genitalia. Two of them

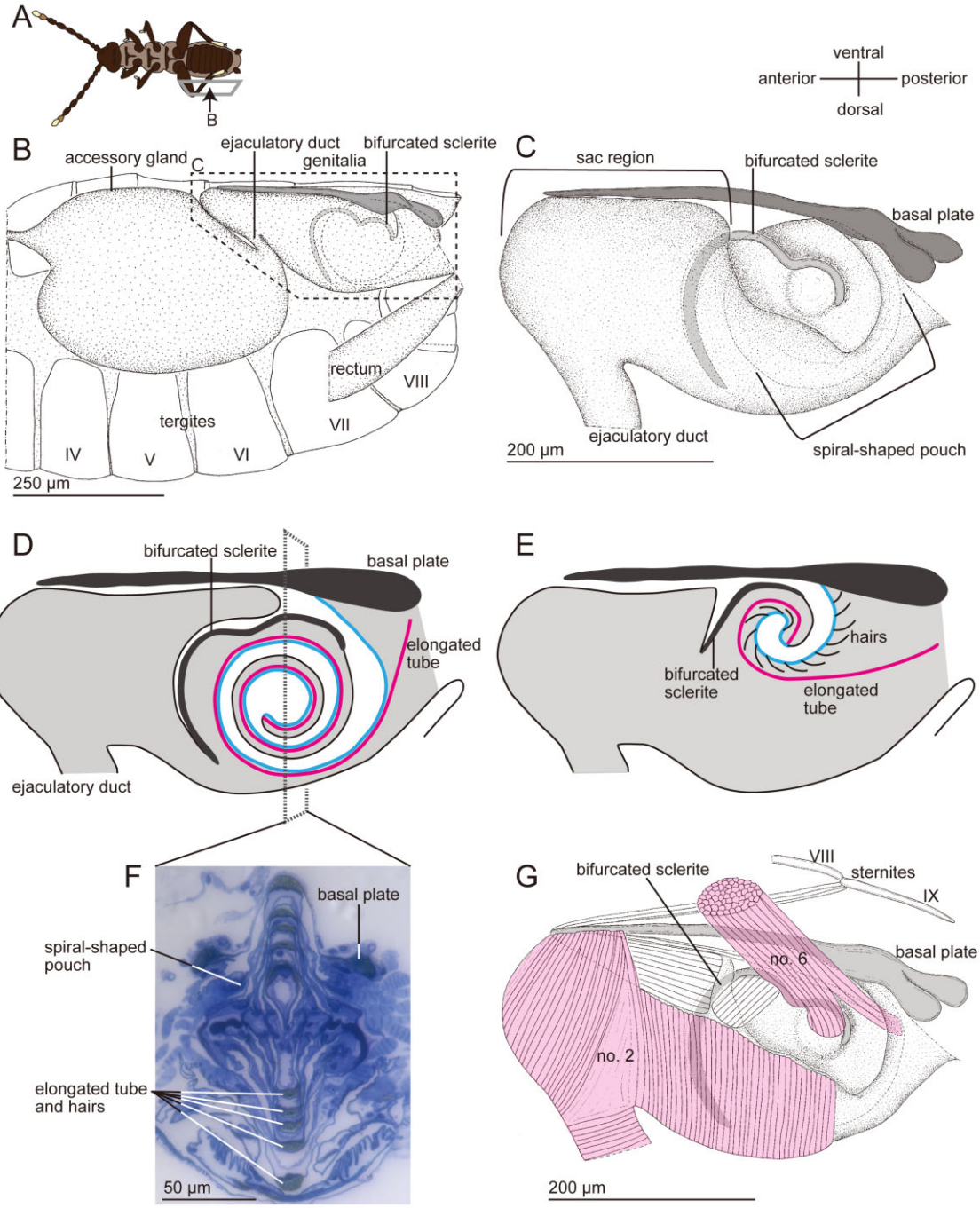

Figure 2. Male reproductive organs of *Zorotypus caudelli*. All figure parts except for (A) and (F) were drawn with the same direction of the indicator above C. A, schematic view of male *Z. caudelli* in ventral view. B, male post abdomen, lateral view (testes removed). C, habitus of genitalia in lateral view. D, schematic drawing of C. E, pouch accommodating elongated tube simplified. F, cross section of pouch around its central region. G, muscles related to genitalia, lateral view. Morphologically outer spaces indicated by light grey in D, E; roman numerals refer to segments.

play a major role in the mechanisms effecting movements of the male genitalia, as discussed below.

The first muscle is unpaired and transverse; it is broad and encloses the sac region and the ejaculatory duct (Fig. 2G, number 2); the sac 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 (eighth sternite) and the pouch (Fig. 2G, number 6).

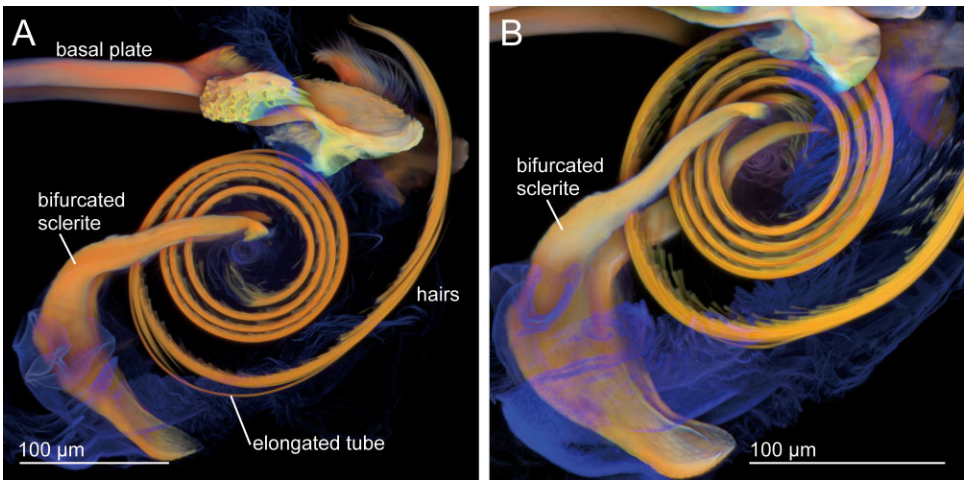

Figure 3. Pouch of *Zorotypus caudelli*. A, B, confocal laser scanning micrographs (maximum intensity projections) showing the autofluorescence composition of the copulatory organ in lateral view.

Table 1. Length of the elongated tube and spermatheca

	N	Mean ± SE.	Range
Male elongated tube	18	1795 ± 38 µm	1470–2015 µm
Female spermatheca	7	2274 ± 87 µm	1810–2537 µm

Validity of measurement data

Thirty-two pairs could be fixed in copula. Twenty-three of them were disconnected after fixation during transport from Japan to Germany. These were used for an indirect assessment of the rates of the length change of the elongated tube inserted into the female. The length of part of the elongated tube remaining in the pouch of disconnected males was measured along with the complete length of the elongated tube of the single males. Using the mean length of the elongated tube of the single males ($N = 18$), we calculated the length of the extruded part in disconnected couples. The part of the tube remaining in the pouch ($N = 19$) is significantly shorter than that of single males (Wilcoxon signed-rank test, $Z = 3.41$, $P < 0.001$, range 464–1895 µm). Based on this indirect evidence, we assumed that the disconnected males also had their tube inserted into the spermatheca before separation, and we used the estimated data (length of the inserted part) for the subsequent analysis. At least in some of the connected pairs ($N = 5$ out of 9), we directly observed that a part of the tube was inserted into the spermatheca.

Genital fitting during copulation

Observations of the reproductive organs of fixed pairs at different stages of copulation ($N = 30$) suggest four stages after males and females connect (stages 1–4 in the following overview of a pair: Fig. 4A). Even though the exact timing of the initiation of the copulation 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. Throughout copulation, the male genitalia are in contact with the vagina and spermatheca. The exit of the elongated tube is placed in front of the entrance of the spermatheca, and it is inserted in the spermatheca during copulation (Fig. 6G).

In stage 1, the posterior part of the basal plate is inserted into the vagina, and a part of the elongated tube is inserted into the spermatheca (Fig. 4B, C, Table 2; $N = 9$). An incompletely formed spermatophore is present in the accessory gland (Fig. 4C).

In stage 2, the membranous fold of the male genitalia is everted posteriorly and inserted into the vagina (Fig. 4D, E, Table 2; $N = 13$). The membranous folds are everted to different degrees in different individuals. The lumen of the membranous fold contains haemolymph, although this space is relatively narrow and the entire surface is covered with wrinkles (Fig. 5A, B). A spermatophore is located in the male genitalia at this stage (Fig. 4D, E).

At stage 3, we found the spermatophore including sperm (Fig. 6B, C) within the vagina. The transfer was not completely accomplished at this time because it was still enclosed by the membranous fold (Fig. 4F, G, Table 2; $N = 5$). The spermatophore (longitudinal axis, approximately 260 µm) is relatively large compared to the genital tract (e.g. the ejaculatory duct: 114 µm at rest; the genital opening on the posterior tip of the abdomen: approximately 136 × 296 µm). Histological sections of a mating pair of *Z. caudelli* at this stage

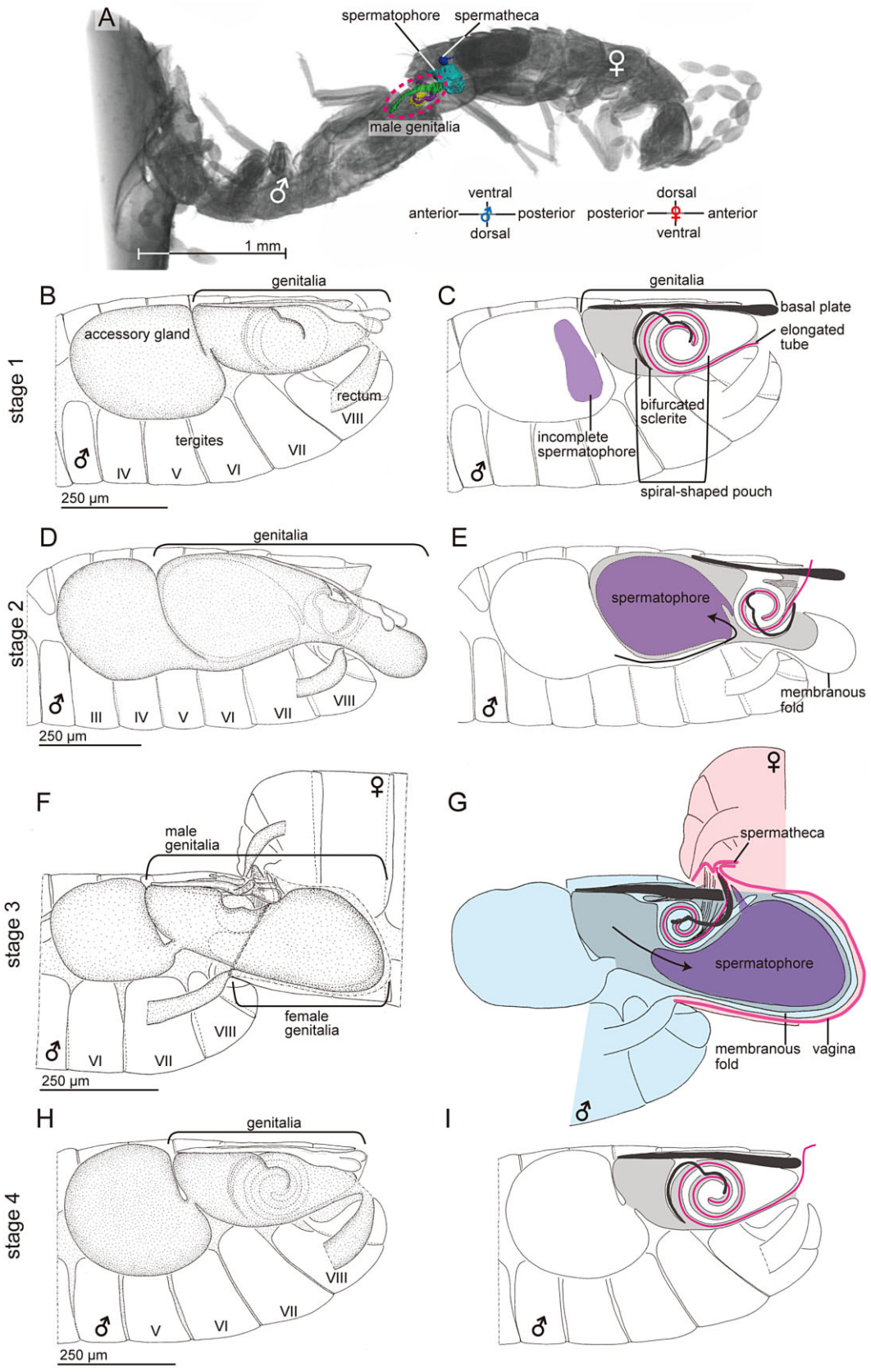

Figure 4. Serial movements during copulation of *Zorotypus caudelli*. A, micro-computed tomography scanned image of a copulating pair; some organs are highlighted to visualize their relative volume in comparison with the remaining body; coloured objects indicate parts of genitalia, such as the basal plate (green), the bifurcated sclerite (purple), and the pouch (yellow). Line drawings (B, D, F, H) and schematized illustrations (C, E, G, I), lateral view. B, C, male postabdomen, stage 1, female body removed. D, E, male postabdomen, stage 2, female body removed. F, G, couplings in stage 3. Arrows in (E) and (G) indicate spermatophore tracks from preceding stages. H, I, male postabdomen, stage 4, female body removed. Morphologically outer spaces indicated by light grey in (C), (E), (G), and (I); roman numerals (males) refer to segments.

Table 2. Summarized data for copulation stages

	Stages			
	1	2	3	4
Numbers of samples observed	9	13	5	2
Is the basal plate protruded?	Yes*	Yes	Yes	Yes
Is the membranous fold everted?	No	Yes	Yes	No
Where is a spermatophore found?	Accessory gland	Male genitalia	Female genitalia enclosed by male membranous fold	Probably vagina†
Ratio of insertion to the whole elongated tube length on average (%)	18.3 (N = 7‡)	22.0 (N = 10‡)	34.0 (N = 2§)	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

*Unclear in two cases.
†Because we preserved all couples in a container and many of them separated during transportation, we lost data about partnerships. However, as far as we could observe, some separated females had a jelly-like object, a spermatophore, in her vagina.
‡Some could not be used as slide specimens (N = 5).
§Some were used for histological sections (N = 2) or observations by micro-computed tomography (N = 1).

revealed free sperm in a posterior area of the opening of the spermatheca tangled up with hairs on the pouch (Fig. 6E, F, compare with an image of sperm in a spermatophore in Fig. 6C and the spermatheca shown in Fig. 6D). The sperm of this male are still packed in an intact spermatophore, although free sperm, which have likely been deposited in a previous copulation, were observed in the spermatheca.

At stage 4, we observed males with the elongated tube still partly inserted in the spermatheca but with the membranous fold retracted into the own body cavity (Fig. 4H, I, Table 2; N = 2). No spermatophore is present in these male specimens.

The elongated tube is slightly shorter than the spermatheca (Table 1). In most fixed specimens (N = 19 out of 21) where we measured the length of the elongated tube, we found this structure to be inserted into the spermatheca throughout the entire copulation. In some cases (N = 3), more than 70% of the elongated tube is inserted into the spermatheca during copulation (Table 2). The ratio of the inserted portion to the entire length of the elongated tube varied greatly among the couples within each stage (Table 2). When the elongated tube is inserted into the spermatheca, the membrane of the pouch is released from the spiral. At this stage, the morphological interior of this released membrane is not filled with haemolymph, and we observed that the membrane of the pouch is uncoiled and folded regularly and the hairs on the surface are tightly aligned around the bifurcated sclerite (Fig. 5 C, D). After we extracted the elongated tube from the pouch of anaesthetized males, it maintained a helical or at least sinuate shape (Fig. 5E) and the tip of the elongated tube widens (Fig. 5F).

DISCUSSION

Elongated parts of genitalia are almost automatically considered as sperm-transporting organs. By contrast to such an intuitive assessment, the present 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 because the ejaculatory duct is not connected to the base of the elongated tube but opens directly into the sac region of the male genitalia (Fig. 2B). In addition, the males insert the membra-

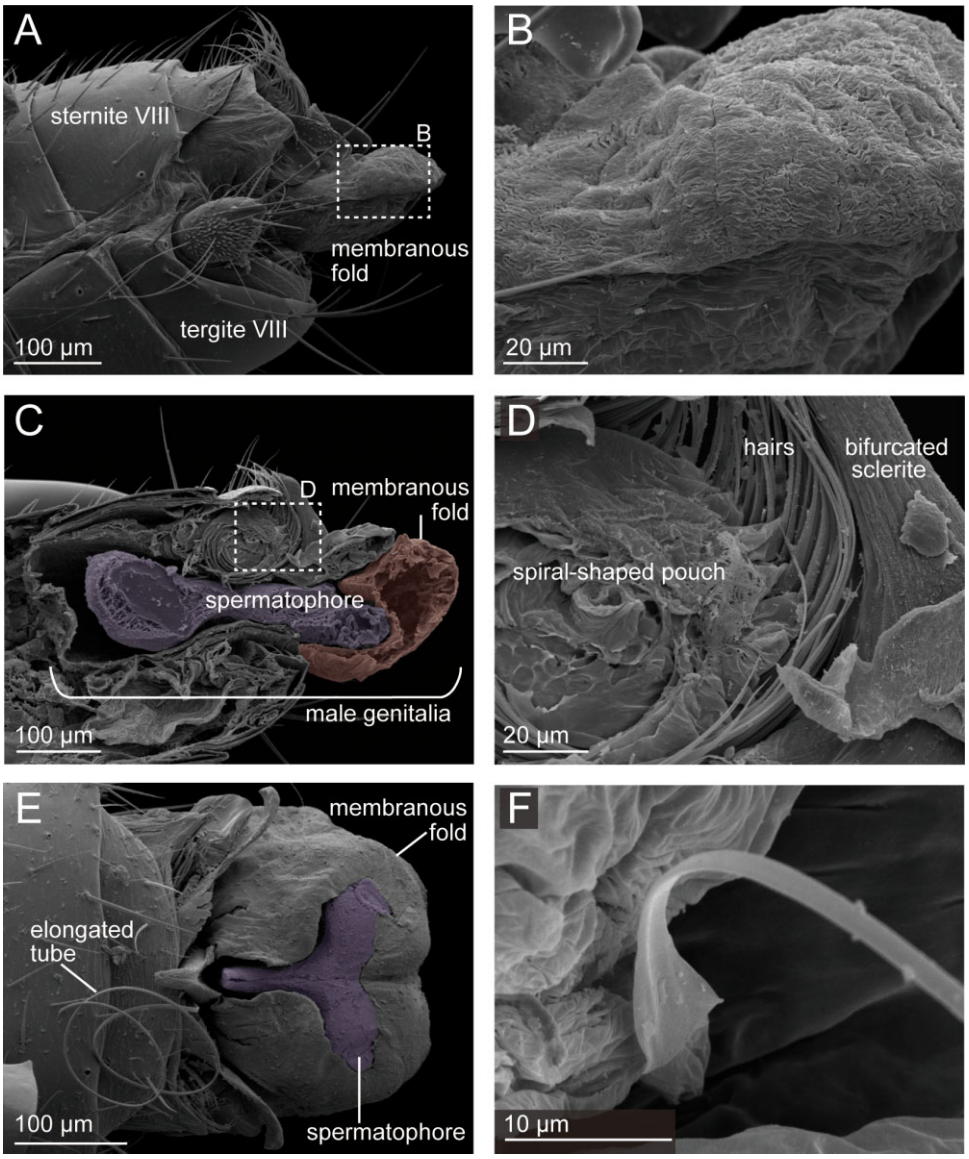

Figure 5. Scanning electron microscopy micrographs of male genitalia of *Zorotypus caudelli*. A, surface structure of the membranous fold in stage 2. B, enlargement of the square in (A). C, sagittal plane of postabdomen in stage 2. D, enlargement of the square in (C). E, membranous fold with a spermatophore and protruded elongated tube, ventral view. F, tip of elongated tube.

nous fold into the vagina and the elongated tube into the spermatheca (Figs. 4, 6G). This means that two intromittent organs with differing functions are inserted during copulation in this species. Such a highly unusual condition is known in some invertebrates (e.g. sea slug species) that have a branched genitalia and transfer sperm and prostate secretion separately into the female genital tract and body cavity (Anthes & Michiels, 2007a, b; Lange, Wermingshausen & Anthes, 2014). Similarly, nonsperm-transferring intromittent organs of the male genitalia are also known in snails (Koene & Chiba, 2006). The odd genitalia reported here is a further example of this rare trait in animals.

The possible function of the elongated tube

Different functions of elongated genitalia are known in different groups of insects. It is a guiding device involved in the transfer of spermatophores in staphylinid beetles (the elongated part of this group is relatively thick; S. Naomi, pers. comm.), it widens the spermatheca in a lygaeid bug (Gschwentner & Tadler, 2000), and it is used to remove rival sperm in an

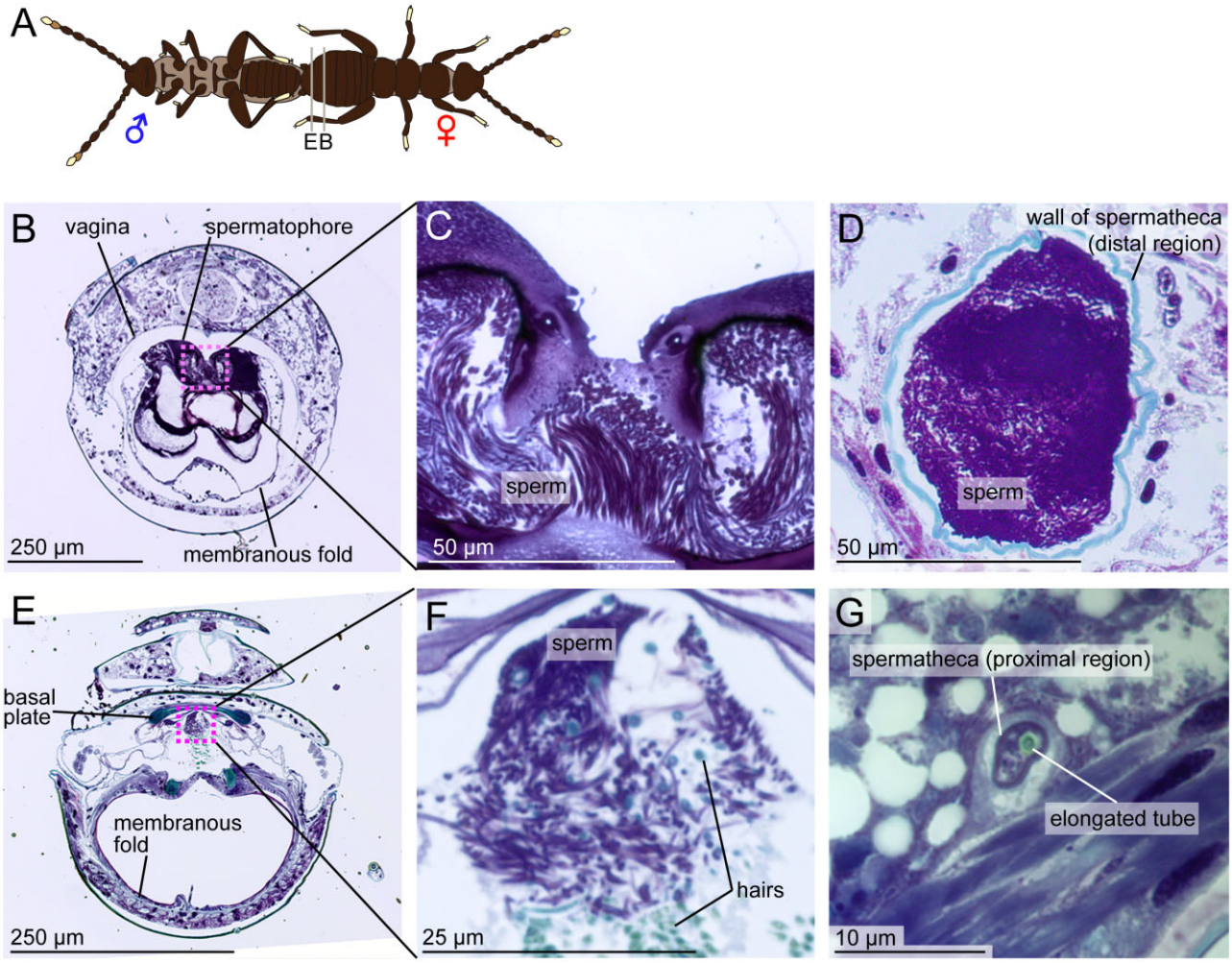

Figure 6. Histological cross sections of a copulating pair of *Zorotypus caudelli* (all sections from one pair in copula). A, positional diagram of sections shown below. B, C, female abdomen with spermatophore (B) and the sperm packing region enlarged (C). D, sperm in spermatheca. E, F, posterior region of male abdomen (E) with free sperm (F). G, male elongated tube in spermatheca.

anisolabidid earwig (Kamimura, 2000). In a chrysomelid beetle, it was identified as an indicator of cryptic female choice (Rodriguez, 1995; Rodriguez *et al*., 2004), which comprises a part of female choice occurring after the initiation of the copulation (Thornhill, 1983; Eberhard, 1996).

Because the elongated tube in *Z. caudelli* is definitely not involved in transferring sperm or a spermatophore, one can exclude a guiding function of sperm or a spermatophore as in staphylinid beetles. The second option, widening the spermatheca, is also highly unlikely. The spermatheca 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 elongated tube is definitely too small to widen it (diameter of spermatheca of approximately 20 µm, Dallai *et al*., 2012b) (Fig. 6G). An active choice in 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 upon the acceptance 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, given that *Z. caudelli* is polyandrous (Y. Kamimura, pers. comm.). It is conceivable that the widened distal tip of the male elongated tube (Fig. 5F)

interacts with the spermatozoa. The elongated tube is as long as the spermatheca on average (Table 1) and deeply inserted during copulation and the duct of the spermatheca is known to function as a sperm receptacle (Dallai *et al.*, 2012b). In addition, as a result of the relatively long sperm cells (770–800 μm) (Dallai *et al.*, 2011), the elongated tube can interfere with rival sperm in the spermathecal duct by wrapping the long spermatozoa around its distal part. We also found free sperm outside of the spermatheca in the vagina, possibly pulled back by the tip of the elongated tube (Fig. 6E, F). Only the lumen of the spermatheca is filled with secretion and is therefore suitable for sperm storage (Dallai *et al.*, 2012b). These observations suggest the 'removal of rival sperm' as a hypothesis that 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 small genitalia (relative to spermatophore size; Fig. 4). 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 haemolymph pressure on the genitalia (Fig. 7). Indeed, in the Malaysian species *Zorotypus magnicaudelli*, which has a spiral-like elongated tube in the genitalia (Mashimo *et al.*, 2013; Dallai *et al.*, 2014b) 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. 4D, E), contractions of the muscle surrounding the sac region of the genitalia (Fig. 2G, number 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 that could be involved in the insertion of the elongated tube (Table 2) and an extensor muscle inserted on the pouch and/or the elongated tube is missing. When the elongated tube is inserted into the spermatheca, the lumen of the uncoiled membrane of the pouch is not filled with haemolymph, and the membrane of the pouch is just folded (Fig. 5C, D). Repeated pressure pushing out the pouch, caused by repeated abdominal contractions for transmission of a spermatophore, would likely result in such a condition with the uncoiled pouch (Fig. 7D). At the same time, avoidance of rewinding movement of the elongated tube and uncoiled membrane is essential because our mechanical explanation predicts that pushing force is intermittent. The hairs arranged on the pouch membrane along the elongated tube (Fig. 6D) perhaps help to limit reverse movements of the membrane by interacting among hairs. Additionally, anatomical coupling would insure the insertion of the elongated tube into the spermatheca: the entrance of the spermatheca and the apex of the elongated tube face each other and the vagina is filled with the membranous fold (Fig. 4G).

By 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. 2G, number 6). The withdrawal of this structure is obviously achieved by contractions of this muscle. The withdrawal of the elongated tube is also, at least partly, caused by muscle contraction, in this case through the paired muscles connecting the centre of the pouch and the body wall (sternum VIII) (Fig. 2G, number 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 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 as a result of 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 pouch (Fig. 5E).

Evolutionary perspectives

Direct insemination with free sperm occurs in the zorapteran species *Zorotypus barberi* (Choe, 1995), although at least four of 39 described species form spermatophores (Dallai *et al.*, 2012a, 2013, 2014a, present study). Spermatophores evolved several times in arthropods (Proctor, 1998). In Polyneoptera, which include Zoraptera (Yoshizawa, 2011), spermatophore formation is a common feature (Blattodea: Chapman, 1998; Mantodea: Holwell, 2007; Orthoptera: Alexander & Otte, 1967; Embioptera: Ross, 1970; Phasmatodea:

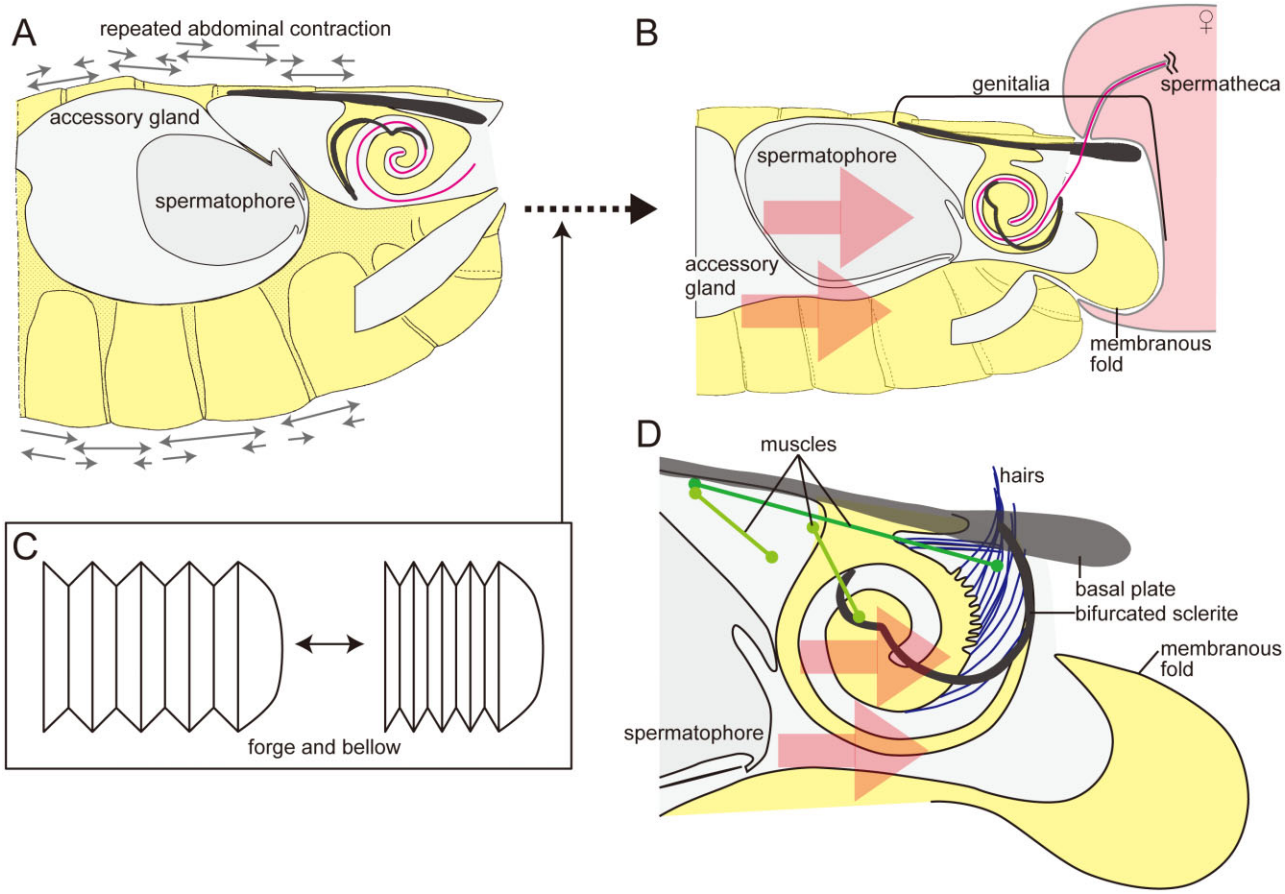

Figure 7. Schematic view of insertion mechanisms of spermatophore and elongated tube. A, B, male postabdomen. C, forge and bellow movement of male abdominal segments. D, male genitalia. Dotted areas in (A) indicate membrane (easily movable). Yellow regions indicate that it is filled with haemolymph. Arrows in (A) indicate possible movements provided by the abdominal segments. Red arrows in (B) indicate pressure acting on the accessory gland and subsequent movement of spermatophore, red arrows in (D) indicate possible pressure acting on the genitalia and subsequent movement of membrane of the pouch; muscles inserted on genitalia (D) function as stoppers for the movement of structures except for the pouch. Morphological outer regions are indicated by light grey. Roman numerals refer to segments.

Bragg, 1991). Considering the condition of sperm transfer in these potentially related groups, spermatophore formation is very likely a plesiomorphic state in Zoraptera (outgroup comparison). By contrast, the presence of the elongated tube accommodated in the pouch of *Z. caudelli* and probably in some other zorapteran species is a highly unusual apomorphic condition (Gurney, 1938; Bolivar y Pieltain, 1940; New, 1978; Mashimo *et al*., 2013). This polarity interpretation is suggested by the absence of the elongated tube in the majority of zorapteran species (missing in 14 out of 39 species, present in nine; males of nine species unknown; characters related to reproduction unknown in seven) and especially by the absence of this unusual structure in potentially related orders such as Embioptera (webspinners) and Phasmatodea (stick insects) (Yoshizawa, 2011). Considering the character state distribution in the ingroup (partly present in Zoraptera) and outgroup taxa (absent), implied complex morphological modifications, and the absence of potential precursors (with the possible exception of an elongated rod in *Z. barberi*; see below), it is likely that the combination of the pouch and the elongated tube is a *de novo* formation. This does not exclude that a moderately elongated tube without pouch was present in the zorapteran ground plan and was completely reduced in a number of the species. It is noteworthy that an elongated rod (= intromittent probe *sensu* Choe, 1995; see also Gurney, 1938) is present in *Z. barberi*. It is half as long as the entire body, inserted into the female spermatheca, and used as the transporting organ of free sperm (Choe, 1995). Its composition of several elements and its different function (Gurney, 1938; Choe, 1995) suggest that it is likely not homologous with the simple elongated tube in *Z. caudelli*. To address this issue and to clarify

the origin of the elongated tube itself, a broader morphological comparison and an intraordinal phylogeny are necessary.

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. haemolymph pressure caused by peristaltic movements of the abdomen). This suggests that spermatophore transfer is a preadaptation for the evolution of the pouch and elongated tube, although the coexistence of a hyper long tube and a large spermatophore initially appeared to be 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; present study) and Coleoptera (beetles; Matsumura & Yoshizawa, 2010) (i.e. increased haemolymph pressure and the contraction of muscles directly connected to the male genitalia). In the case of the beetle species, they transport sperm directly through the elongated part, and the haemolymph pressure is used for the protrusion of the male genitalia, as in other species without an elongated organ (Verma & Kumar, 1972; Matsumura & Yoshizawa, 2010). This explanation for the insertion process is identical with the mechanism suggested for a medfly (Diptera) (Eberhard, 2005). Although the species of Coleoptera, Diptera, and Zoraptera have elongated parts in their genitalia, the function and morphology of these modified elements are very different. Nevertheless, in the present study, we show how the acquisition of a unique and complicated feature is possible in conjunction with an already available ancestral mechanism for manipulating genitalia, and that this applies to groups widely separated phylogenetically.

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