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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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1 1 **Two separate intromittent organs in *Zorotypus caudelli* (Insecta, Zoraptera): a seemingly paradox coexistence of**
2 2 **an extremely long and narrow tube and a large spermatophore**

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16
17 17 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

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

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

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

87

88 MATERIALS AND METHODS

89 SPECIMENS

90 In the very small order Zoraptera (39 extant species), males of species related to *Zorotypus caudelli* (e.g., *Z. hubbardi*, *Z.*
91 *impolitus*) are known to transfer sperm packed in spermatophores which are large in relation to the body size (Dallai et al.,
92 2012a, 2013, RD pers. obs.). As this is seemingly in conflict with the presence of a thin and elongated tube in the
93 studying species *Z. caudelli* (New, 2000) we investigated the morphology and dynamics of the male and female genital
94 apparatus with a focus on the sperm transmission by fixation of copulating pairs

95 We used specimens of *Z. caudelli* from the rearing stock of Mashimo et al. (2011) and followed the methods
96 described by these authors. The initial population was collected in Malaysia. To obtain copulating pairs, we placed two
97 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
98 copulation of this species starts quite abruptly, probably with a very short pre-copulatory behaviour (Dallai et al., 2013).
99 The copula is completed within 11.5 - 23.2 min (Dallai et al., 2013). When we found a copulating pair, alcohol cooled in
100 a normal household freezer was poured into the case, which was then kept in a freezer for more than ten minutes.
101 Afterwards, all couples were preserved in 70% ethanol in a container for anatomical investigation. The exact stage of the
102 copula was unknown prior to the anatomical investigation. Following the methods outlined above, we obtained 32 fixed
103 pairs in copula, and additionally we used single males and females preserved in 70% ethanol for anatomical study.

104
105 ANATOMY

106 In addition to manual dissection we used histological sectioning, micro-computed tomography (μ CT), computer-based
107 3D reconstruction, and confocal laser scanning microscopy (CLSM). Transverse and longitudinal semithin sections were
108 made of two copulae and of one single male of *Z. caudelli*. All samples were embedded in araldite CY 212® (Agar
109 ScientiWc, Stansted/Essex, England) and cut at 1 μ m using a microtome HM 360 (Microm, Walldorf, Germany)
110 equipped with a diamond knife. Sections were stained with toluidine blue and pyronin G (Waldeck GmbH and Co. KG/
111 Division Chroma, Münster, Germany). Pictures were taken of every second or every third section using a microscope
112 (Zeiss Axioplan, Germany) equipped with a camera (Pixelink Capture OEM). The images were aligned using Amira
113 4.1.2 software (Visage Imaging, Berlin, Germany). Based on the aligned image stacks (cross sections of a single male:
114 518 images; cross sections of a pair in copula: 534; longitudinal sections of a pair in copula: 458) we evaluated the
115 arrangement of internal structures and manually traced each element to reconstruct three dimensional images. For
116 smoothing and coloring them we used MAYA7 (Alias Wavefront, Toronto/Ontario, Canada). As histological sections are

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5 117 never completely free of deformations, we also used μ CT (Skyscan1172) to obtain a perfectly aligned three dimensional
6 118 image. The resolution is lower compared to the sections but this non-invasive technique is largely artifact free (e.g.
7
8 119 Friedrich & Beutel, 2008). To examine the genital fitting using μ CT, an alcohol preserved copula of *Z. caudelli* was
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10 120 dried at the critical point (Emitech K850 critical point dryer, Ingenieurbüro Peter Liebscher, Wetzlar, Germany) and
11
12 121 scanned. Then we traced selected structures using Amira software to highlight genital structures.

13
14 122 We used scanning electron microscopy (SEM, Philips XL 30 ESEM) to observe surface structures of the male
15
16 123 genitalia. We gradually dehydrated specimens preserved in 70% ethanol with ethanol-hexamethyldisilazane series and
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18 124 dried them in a draft chamber. The samples were mounted on the tip of a fine needle and fixed on the rotatable specimen
19
20 125 holder developed by Pohl (2010).

21 126 The material composition of the male genitalia was analyzed applying CLSM without staining. The preparation and
22
23 127 visualization methods described by Michels & Gorb (2012) were used, with the only difference that instead of a
24
25 128 longpass emission filter transmitting light with wavelengths ≥ 560 nm we applied wavelengths of ≥ 640 nm to detect the
26
27 129 autofluorescence excited by 639 nm. However, the filter differences did not have any influence on the results.

28
29 130 We describe the genitalic anatomy briefly in the results section. A detailed description of the complex genital
30
31 131 morphology is provided in an appendix. As Zoraptera are arguably the most enigmatic order in insect systematics (e.g.
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33 132 Beutel & Weide, 2005), our anatomical data on genital morphology, which are presented here for the first time, may turn
34
35 133 out as useful in a phylogenetic context in the future.

36 134 In addition to the morphological investigation, to assess the movement of the elongated tube during copulation, we
37
38 135 measured the length of the elongated tube and the spermatheca based on photographs of slide mounted specimens using
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40 136 a curvimeter (Koizumi COMCURVE-9 Junior, Japan). We followed the methods described by Matsumura & Yoshizawa
41
42 137 (2010). Twenty three of 32 pairs fixed in copula were disconnected after fixation during a transportation process from
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44 138 Hokkaido University to Friedrich-Schiller-Universität Jena. They were used for an indirect assessment of the rates of the
45
46 139 length of the elongated tube inserted into the female genital tract. The length of the part of the elongated tube remaining
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48 140 in the spiral-shaped pouch (of the male) was measured, and also the complete length of the elongated tube of the single
49
50 141 males. Using the average length of the elongated tube of the single males ($n=18$) we calculated the length of the part
51
52 142 inserted, before the couple was disconnected. The part of the elongated-tube remaining in the male abdomen ($n=19$) is
53
54 143 significantly shorter than that of the single males (Wilcoxon signed rank test, $Z=3.80$, $P<0.001$, range: 220-1895 μ m),
55
56 144 which is indirect evidence that a part must have been inserted in the female genital tract. At least in the connected pairs
57
58 145 ($n=9$), we observed that some males had inserted the elongated tube into the female spermatheca ($n=5$, Fig. 2C).

146

147 TERMINOLOGY

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

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

157

158 RESULTS

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

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

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

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

178 MUSCULATURE OF MALE GENITALIA (Fig. 3D)

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

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

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

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

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

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

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

203 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
 204 the spermathecal duct tangled up with hairs on the spiral-shaped pouch membrane (Fig. 7E, F, compare with an image of
 205 sperm in a spermatophore in Fig. 7B, C and a spermatheca shown in Fig. 7D). The sperm of this male are still packed in
 206 an intact spermatophore, but free sperm, which have likely been deposited in a previous copulation, were observed in the
 207 spermatheca of the female.

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

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

220 DISCUSSION

221 Elongated tube-like genitalia are almost automatically considered as sperm transporting organs. In contrast to such an
 222 intuitive assessment, the current study clearly shows that males of *Z. caudelli* transport their sperm packed in a large
 223 spermatophore, and that the elongated tube is not directly involved in this process. This is evident as the ejaculatory duct
 224 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
 225 that the males insert the membranous fold into the vagina, but the elongated tube into the spermathecal duct (Figs. 5, 7G).
 226 This means that two intromittent organs are inserted during copulation in this species and have different functions. Such
 227 a highly unusual condition is known in sea slug species which have a branched penis and transfer sperm and prostate
 228 secretion separately into female genital tract and body cavity (Anthes & Michiels, 2007a, b). The odd penis shown here
 229 is the second example in animals as far as we know.

231 THE POSSIBLE FUNCTION OF THE ELONGATED ORGAN

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

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

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

258

259 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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We deeply thank Katharina Schneeberg, Hans Pohl, Frank Hünefeld, Rommy Peterson, Janin Naumann, and Shin-ichi Akimoto for their technical supports, Yoshitaka Kamimura for valuable comments and information on a *Z. caudelli*'s behaviour and articles, Akeo Iwasaki for bibliographic information, and Nobuyuki Matsumoto for stimulating discussions. This study was supported by JSPS Postdoctoral Fellowships for Research Abroad to YoM, the VolkswagenStiftung (to RGB), the MIUR (PRIN 2008/FL2237 to RD), and a Grant-in-Aid from the JSPS (Scientific Research C: 21570089 to RM).

REFERENCES

Alexander RD, Otte D. 1967. The Evolution of genitalia and mating behaviour in Crickets (Gryllidae). *Miscellaneous Publications Museum of Zoology University of Michigan* **133**: 1–62.

346 Anthes N, Michiels NK. 2007a. Precopulatory stabbing, hypodermic injections and unilateral copulations in a
347 hermaphroditic sea slug. *Biology Letters* **3**: 121–124.

348 Anthes N, Michiels NK. 2007b. Reproductive morphology, mating behavior, and spawning ecology of cephalaspid sea
349 slugs (Aglajidae and Gastropteridae). *Invertebrate Biology* **126** (4): 335–365.

350 Aspöck U, Aspöck H. 2008. Phylogenetic relevance of the genital sclerites of Neuropterida (Insecta: Holometabola).
351 *Systematic Entomology* **33** (1): 97–127.

352 Beutel RG, Weide D. 2005. Cephalic anatomy of *Zorotypus hubbardi* (Hexapoda: Zoraptera): new evidence for a
353 relationship with Acercaria. *Zoomorphology* **124** (3): 121–136.

354 Bolivar y Pieltain C. 1940. Estudio de un Nuevo Zoráptero de México. *Anales de la Escuela Nacional de Ciencias*
355 *Biológicas* **1** (3/4): 515–523.

356 Bragg PE. 1991. Spermatophores in Phasmida. *Entomologist* **110** (2): 76–80.

357 Briceño RD, Orozco D, Quintero JL, Hanson P, Hernández MDR. 2011. Copulatory behaviour and the process of
358 intromission in *Anastrepha ludens* (Diptera: Tephritidae). *Revista de Biología Tropical* **59** (1): 291–297.

359 Chapman RF. 1998. *The Insects: Structure and Function*. Cambridge: Cambridge University Press.

360 Choe JC. 1995. Courtship feeding and repeated mating in *Zorotypus barberi* (Insecta: Zoraptera). *Animal Behaviour* **49**
361 (6): 1511–1520.

362 Dallai R, Gottardo M, Mercati M, Machida R, Mashimo Y, Matsumura Y, Beutel RG. 2013. Divergent mating
363 patterns and a unique mode of external sperm transfer in Zoraptera: an enigmatic group of pterygote insects.
364 *Naturwissenschaften* **100** (6): 581–594.

365 Dallai R, Mercati D, Gottardo M, Machida R, Mashimo Y, Beutel RG. 2011. The male reproductive system of
366 *Zorotypus caudelli* Karny (Zoraptera): sperm structure and spermiogenesis. *Arthropod Structure & Development* **40**
367 (6): 531–547.

368 Dallai R, Mercati D, Gottardo M, Dossey AT, Machida R, Mashimo Y, Beutel RG. 2012a. The male and female
369 reproductive systems of *Zorotypus hubbardi* Caudell, 1918 (Zoraptera). *Arthropod Structure & Development* **41** (4):
370 337–359.

371 Dallai R, Mercati D, Gottardo M, Machida R, Mashimo Y, Beutel RG. 2012b. The fine structure of the female
372 reproductive system of *Zorotypus caudelli* Karny (Zoraptera). *Arthropod Structure & Development* **41** (1): 51–63.

373 Eberhard WG. 1985. *Sexual selection and animal genitalia*. Cambridge: Harvard University Press.

- 374 **Eberhard WG. 1996.** *Female control: sexual selection by cryptic female choice*. Princeton, New Jersey: Princeton
375 University Press.
- 376 **Eberhard WG. 2005.** Threading a needle with reinforced thread: intromission in *Ceratitis capitata* (Diptera,
377 Tephritidae). *The Canadian Entomologist* **137** (2): 174–181.
- 378 **Eberhard WG, Pereira F. 1995.** The process of intromission in the Mediterranean fruit fly, *Ceratitis capitata* (Diptera:
379 Tephritidae). *Psyche* **102** (3–4): 99–120.
- 380 **Friedrich F, Beutel RG. 2008.** Micro-computer tomography and a renaissance of insect morphology. *Proceedings of*
381 *SPIE* 7048: 1–6.
- 382 **Gack C, Peshke K. 2005.** ‘Shouldering’ exaggerated genitalia: a unique behavioural adaptation for the retraction of the
383 elongate intromittant organ by the male rove beetle (*Aleochara tristis* Gravenhorst). *Biological Journal of the*
384 *Linnean Society* **84** (2): 307–312.
- 385 **Gschwentner R, Tadler A. 2000.** Functional anatomy of the spermatheca and its duct in the seed bug *Lygaeus simulans*
386 (Heteroptera: Lygaeidae). *European Journal of Entomology* **97** (3): 305–312.
- 387 **Gurney AB. 1938.** A synopsis of the order Zoraptera, with notes on the biology of *Zorotypus hubbardi* Caudell.
388 *Proceedings of the Entomological Society of Washington* **40** (3): 57–87.
- 389 **Holwell GI. 2007.** Spermatophore feeding and mating behaviour in praying mantids (Mantodea: Liturgusidae). *Journal*
390 *of Zoology* **271** (3): 255–260.
- 391 **Hünefeld F. 2007.** The genital morphology of *Zorotypus hubbardi* Caudell, 1918 (Insecta: Zoraptera: Zorotypidae).
392 *Zoomorphology* **126** (3): 135–151.
- 393 **Kamimura Y. 2000.** Possible removal of rival sperm by the elongated genitalia of the earwig, *Euborellia plebeja*.
394 *Zoological Science* **17** (5): 667–672.
- 395 **Kamimura Y. 2005.** Last-male paternity of *Euborellia plebeja*, an earwig with elongated genitalia and sperm removal
396 behavior. *Journal of Ethology* **23** (1): 35–41.
- 397 **van Lieshout E, Elgar MA. 2011.** Longer exaggerated male genitalia confer defensive sperm-competitive benefits in an
398 earwig. *Evolutionary Ecology* **25** (2): 351–362.
- 399 **Marchini D, del Bene G, Falso LF, Dallai R. 2001.** Structural organization of the copulatory site in the medfly
400 *Ceratitis capitata* (Diptera: Tephritidae) and observations on sperm transfer and storage. *Arthropod Structure &*
401 *Development* **30** (1): 39–54.

402 **Mashimo Y, Machida R, Dallai R, Gottardo M, Mercati D, Beutel RG. 2011.** Egg structure of *Zorotypus caudelli*
403 Kary (Insecta, Zoraptera, Zorotypidae). *Tissue and Cell* **43 (4)**: 230–237.

404 **Mashimo Y, Yoshizawa K, Engel MS, Ghani AB, Dallai R, Beutel RG, Machida R. in press.** *Zorotypus* in
405 Peninsular Malaysia (Zoraptera: Zorotypidae), with the description of three new species. *Zootaxa*.

406 **Matsumura Y, Yoshizawa K. 2010.** Insertion and withdrawal of extremely elongated genitalia: a simple mechanism
407 with a highly modified morphology in the leaf beetle, *Lema coronata*. *Biological Journal of the Linnean Society* **99**
408 **(3)**: 512–520.

409 **Matsumura Y, Yoshizawa K. 2012.** Homology of the internal sac components in the leaf beetle subfamily Criocerinae
410 and evolutionary novelties related to the extremely elongated flagellum. *Journal of Morphology* **273 (5)**: 507–518.

411 **Michels J, Gorb SN. 2012.** Detailed three-dimensional visualization of resilin in the exoskeleton of arthropods using
412 confocal laser scanning microscopy. *Journal of Microscopy* **245 (1)**: 1–16.

413 **Naomi S. in press.** The morphology of the endophallic flagellum in the family Staphylinidae (Insecta: Coleoptera).

414 **New TR. 1978.** Notes on Neotropical Zoraptera, with descriptions of two new species. *Systematic Entomology* **3 (4)**:
415 361–370.

416 **New TR. 2000.** Zoraptera (Insecta) in east Malaysia: notes on *Zorotypus caudelli* Kary. *Oriental Insects* **34 (1)**: 77–82.

417 **Pohl H. 2010.** A scanning electron microscopy specimen holder for viewing different angles of a single specimen.
418 *Microscopy Research and Technique* **73 (12)**: 1073–1076.

419 **Proctor HC. 1998.** Indirect sperm transfer in arthropods: behavioral and evolutionary trends. *Annual Review of*
420 *Entomology* **43**: 153–74.

421 **Rodriguez V. 1994.** Function of the spermathecal muscle in *Chelymorpha alternans* Boheman (Coleoptera:
422 Chrysomelidae: Cassidinae). *Physiological Entomology* **19 (3)**: 198–202.

423 **Rodriguez V. 1995.** Copulatory courtship in *Chelymorpha alternans* Boheman (Coleoptera: Chrysomelidae:
424 Cassidinae). *The Coleopterists Bulletin* **49 (4)**: 327–331.

425 **Rodriguez V, Windsor D, Eberhard WG. 2004.** Tortoise beetle genitalia and demonstration of a selected advantage
426 for flagellum length in *Cherymorpha alternans* (Chrysomelidae, Cassidini, Stolinai). In: Jolivet P, Santiago-Blay JA,
427 Schmitt M, eds. *New Developments in the Biology of Chrysomelidae*. The Hague: SBP Academic Publisher, 739–
428 748.

429 **Ross ES. 1970.** Biosystematics of the Embioptera. *Annual Review of Entomology* **15**: 157–172.

430 **Snodgrass RE. 1935.** *Principles of Insect Morphology*. New York and London: McGraw-Hill Book Co.

- 431 **Spencer KA. 1981.** *A revisionary study of the leaf mining flies (Agromyzidae) of California*. Berkeley: Division of
432 Agricultural Sciences, University of California, Special Publication.
- 433 **Suzuki K. 1988.** Comparative morphology of the internal reproductive system of Chrysomelidae (Coleoptera). In:
434 Jolivet P, Petitpierre E, Hsiao TH, eds. *Biology of Chrysomelidae*. Dordrecht, Boston and London: Kluwer Academic
435 Publishers, 317–355.
- 436 **Tadler A. 1999.** Selection of a conspicuous male genitalic trait in the seedbug *Lygaeus simulans*. *Proceedings of the*
437 *Royal Society of London B: Biological Sciences* **266**: 1773–1777.
- 438 **Thornhill R. 1983.** Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *The American*
439 *Naturalist* **122** (6): 765–788.
- 440 **Verma KK, Kumar D. 1972.** The aedeagus, its musculature, and ‘retournement’ in *Aspidomorpha miliaris* F.
441 (Coleoptera, Phytophaga, Chrysomelidae). *Journal of Natural History* **6**: 699–719.
- 442 **Villavaso EJ. 1975.** Function of the spermathecal muscle in the boll weevil, *Anthonomus grandis*. *Journal of Insect*
443 *Physiology* **21** (6): 1275–1278.
- 444 **Yoshizawa K. 2011.** Monophyletic Polyneoptera recovered by wing base structure. *Systematic Entomology* **36** (3): 377–
445 394.
- 446
- 447 Figure legends
- 448 **Figure 1.** Schematic drawings of structures and the insertion mechanism of elongated intromittent organs. The focus is
449 on areas which are actually inserted into the female genital cavity. A: male genitalia of the medfly *Ceratits capitata*.
450 The phallus is elongated (pink), and an ejaculatory duct passes through it (Marchini et al., 2001); hemolymph
451 pressure (blue regions and arrows), which is probably produced by an ejaculatory apodeme and sperm sac, pushes
452 the elongated part; a folded site of the elongated part gradually moves distally, and then almost the entire elongated
453 part is inserted (Eberhard & Pereira, 1995; Eberhard, 2005); grey regions show a kind of stopper of the elongated
454 part, which is called phalloapodeme. B: the intromittent region of the male genitalia of the leaf beetle *Lema*
455 *coronata*; a specialised pocket is present in their internal sac; the elongated sperm transmitting tube, flagellum (pink),
456 is stored within it in the resting state; hemolymph pressure (blue regions and allows) that is probably generated by
457 contraction of abdominal segments everts the pocket membrane and the flagellum (Matsumura & Yoshizawa, 2010).
458 C: male intromittent organ with spiral-shaped elongated part; this type is widespread in pterygote insects, but
459 morphological details and mechanisms of insertion and withdrawal remain largely unknown.

Figure 2. Outline of the male reproductive system of *Z. caudelli*. A: μ CT 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 μ CT (n=1).

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

	N	mean $\pm$ s.e.	range
Male elongated tube	18	1795 $\pm$ 38 μ m	1470 - 2015 μ m
Female spermathecal duct	7	2274 $\pm$ 87 μ m	1810 - 2537 μ m

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 $\times$ 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.

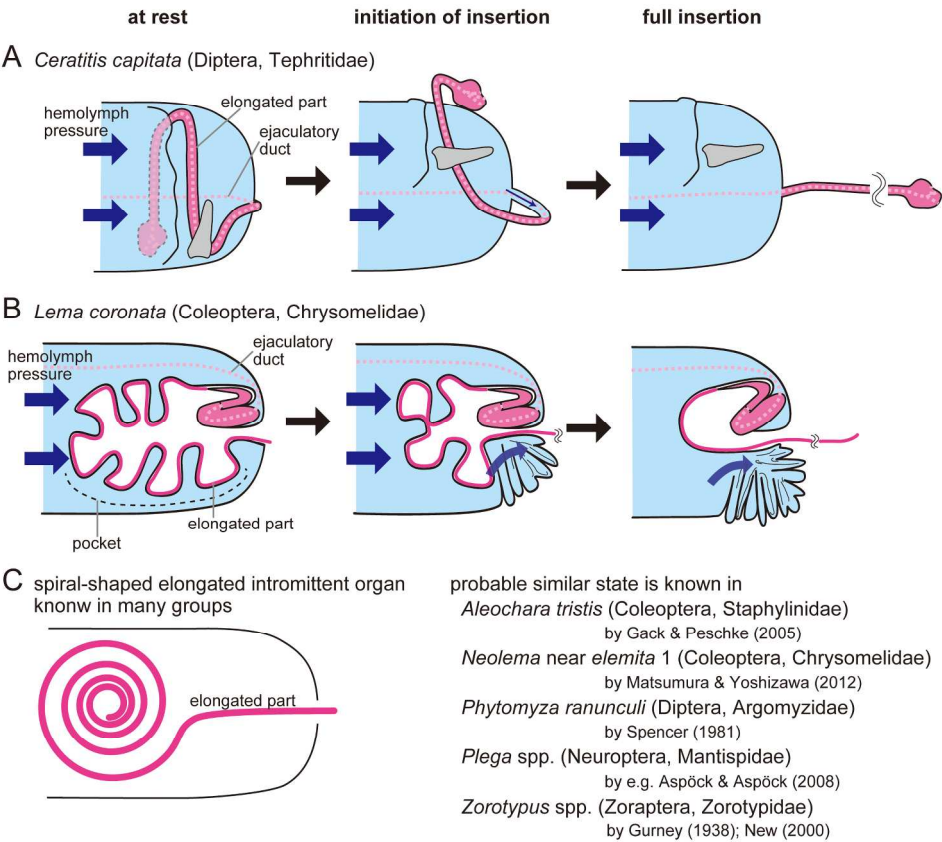
The shape has distinctly changed after the transfer to the female. The elongated filaments are tangled and clinging to the ventral surface of the main body, and the projection of the ventral surface directs in the opposite direction compared to the second stage (Fig. 10).

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: $1.0 \times 10^5 \mu\text{m}^3$, spermatophore: $2.9 \times 10^6 \mu\text{m}^3$). The volume of the spermatheca of the partner was calculated as ca. $8.4 \times 10^4 \mu\text{m}^3$.

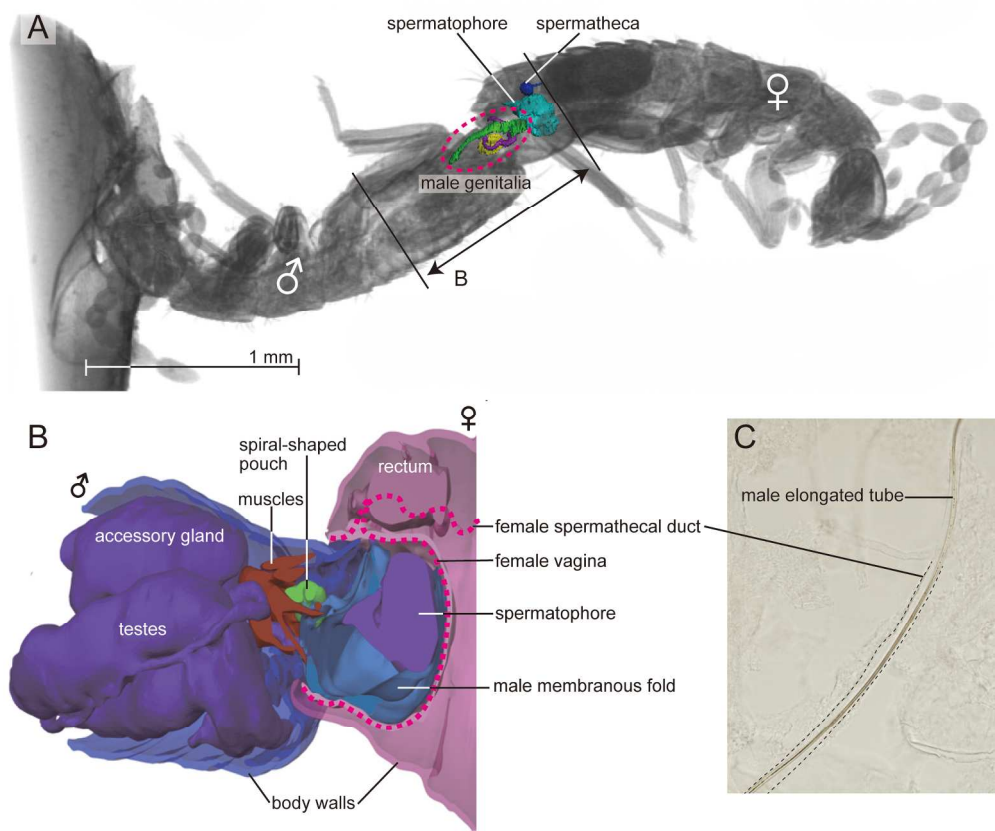
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Appendix **Figure 9**. Anatomy of the male genitalia and its musculature of *Z. caudelli*. A: line drawing of male copulatory organ in lateral view. B: schematic drawing of A. Origins and insertions of muscles 3-5 are shown. Hairs are regularly arranged 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.

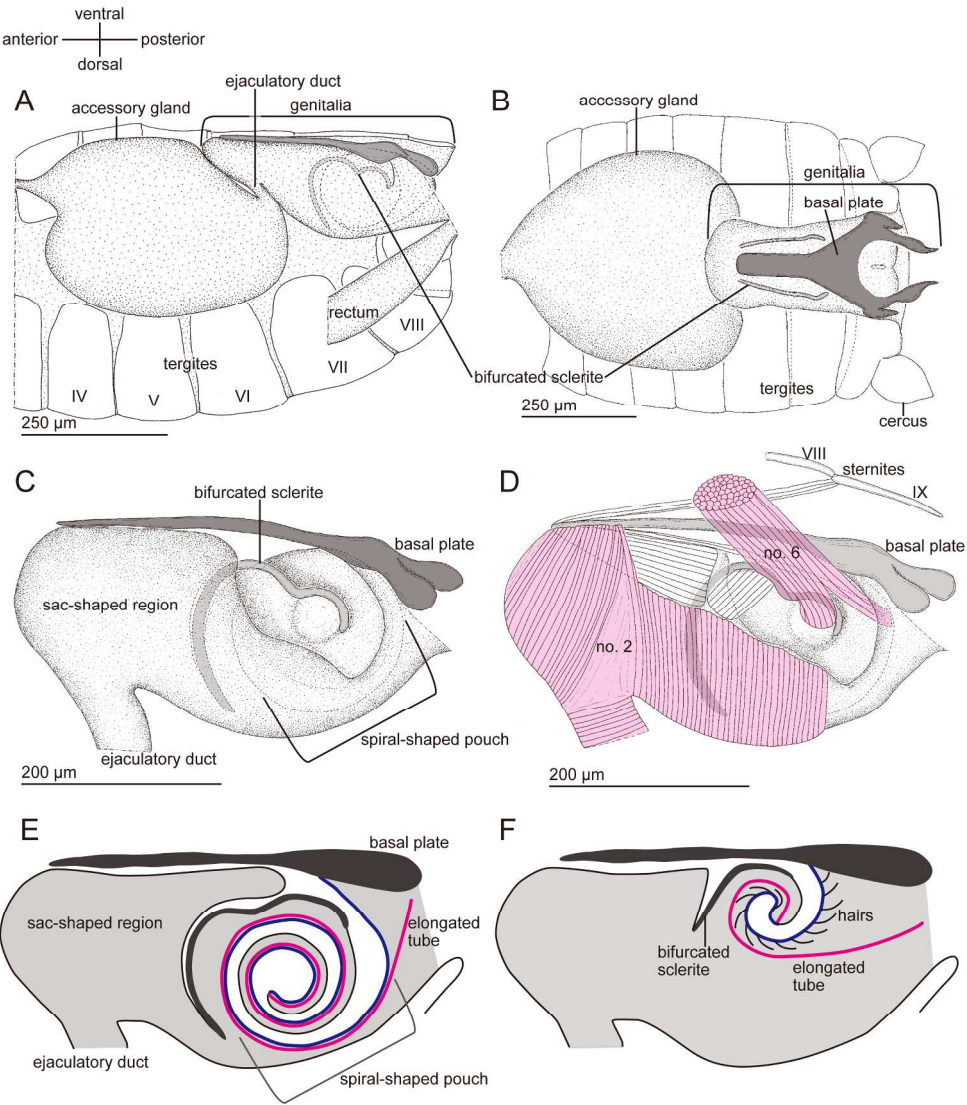


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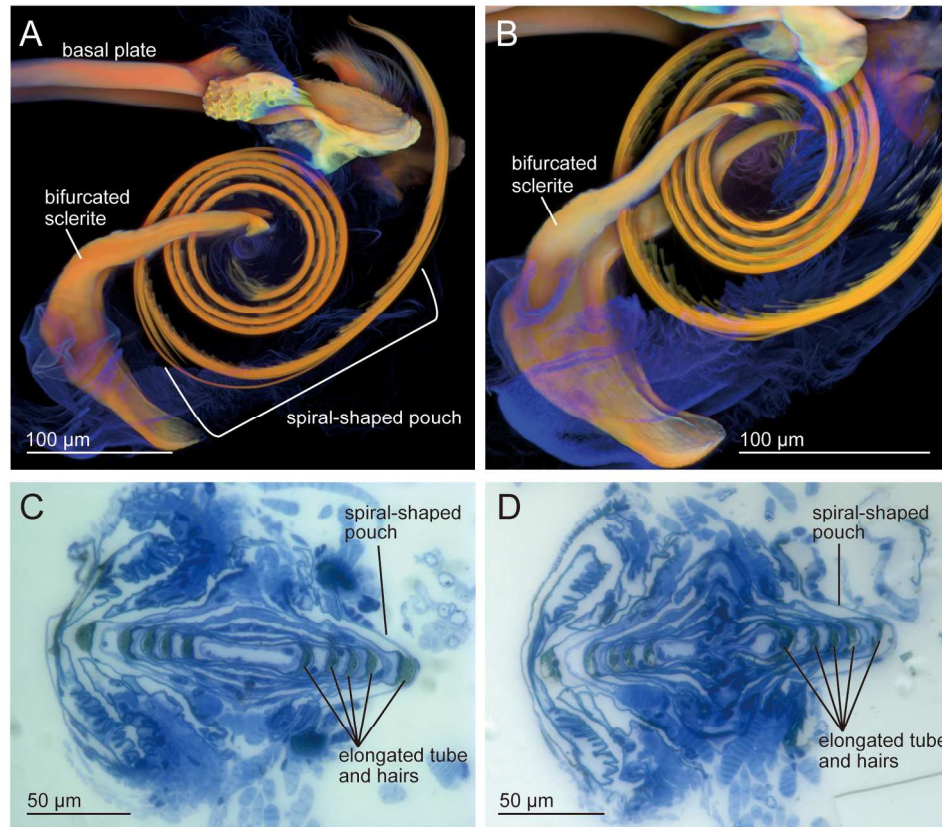


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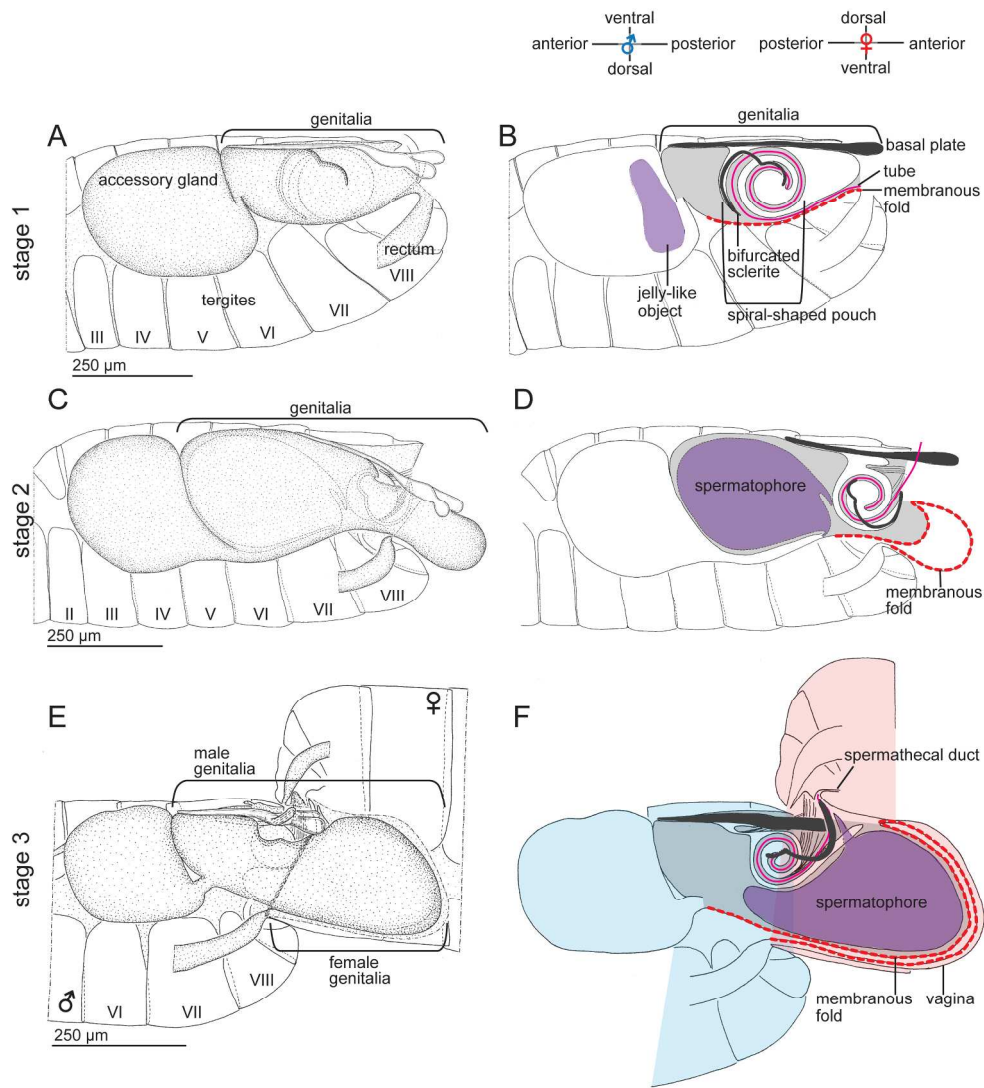
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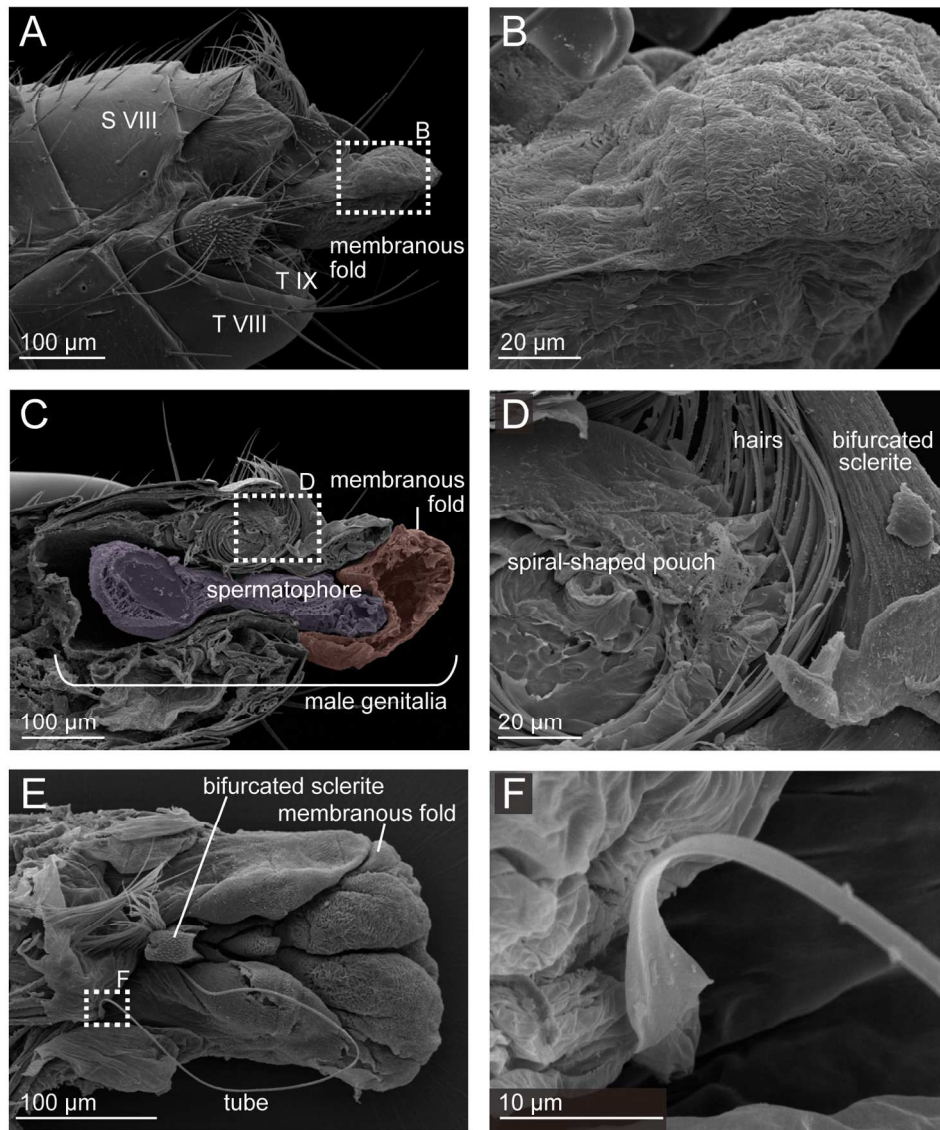
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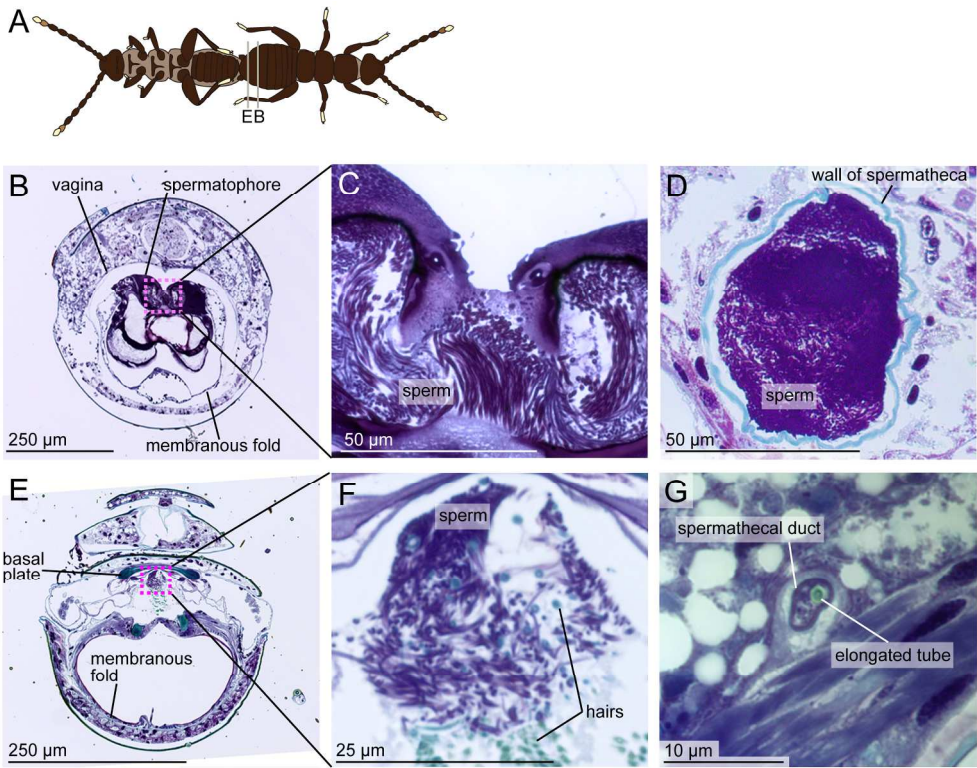
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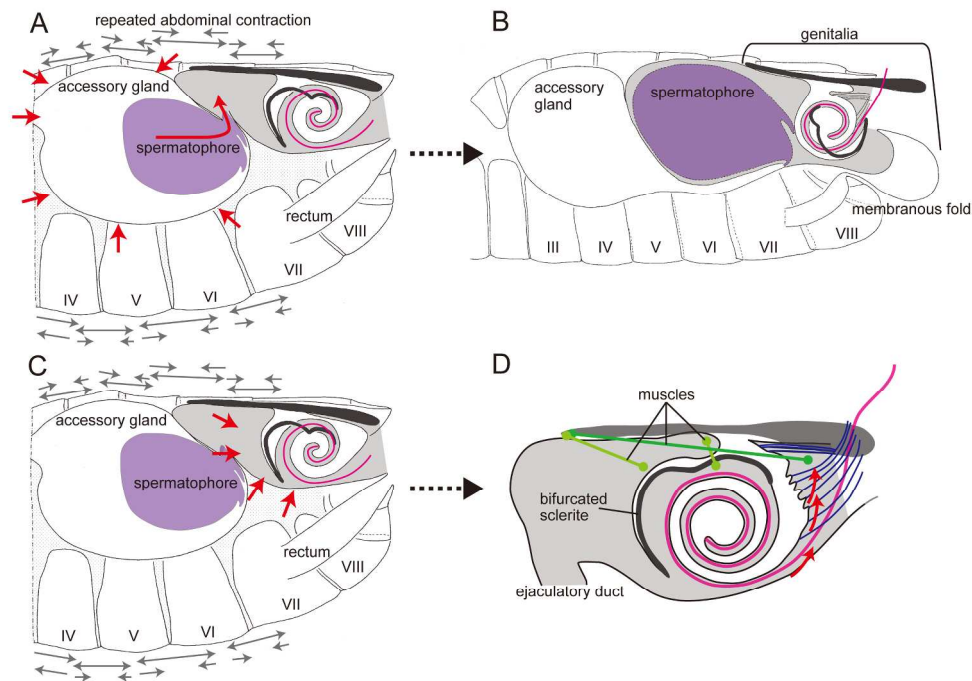
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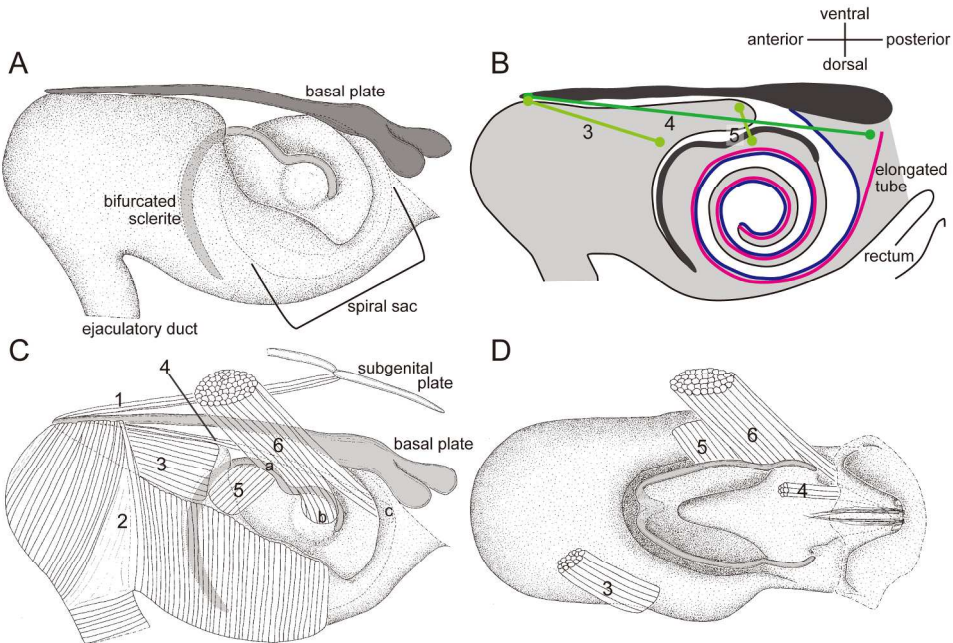
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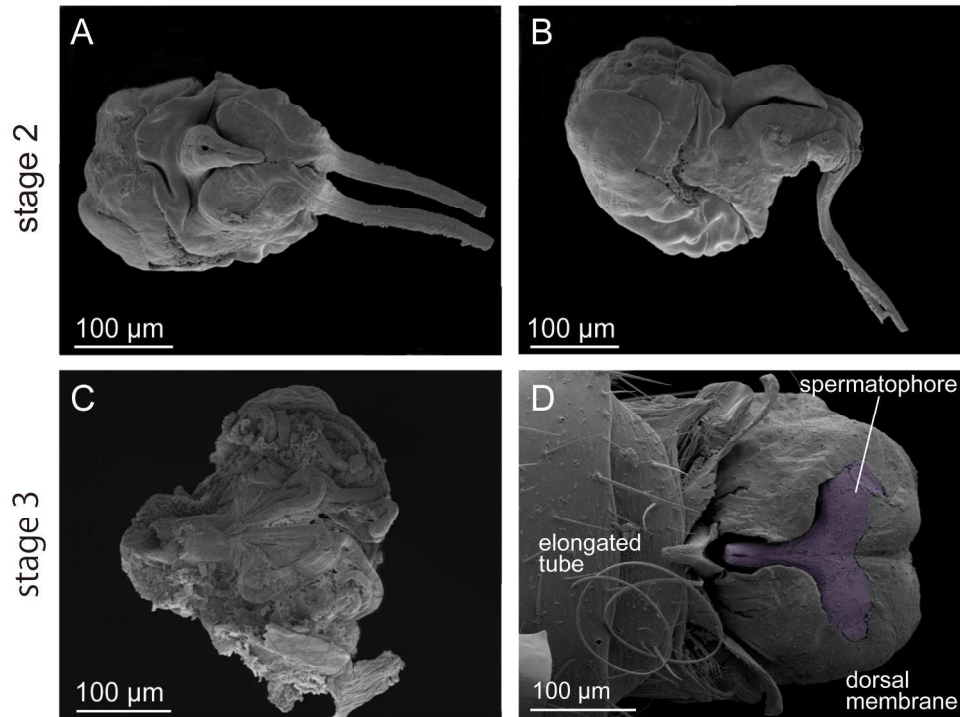
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