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2 Report

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4 **Female penis, male vagina, and their**

5 **correlated evolution in a cave insect**

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17 Running head: Reversed intromittent organs

18

19 Key words: sexual selection, sexual conflict, sex-role reversal, genital evolution

20 **Highlights**

- 21 ► Females of the cave insect genus *Neotrogla* have an elaborate penis-like organ
- 22 ► The female penis acts as an intromittent organ and anchors the female to the male
- 23 ► Correlated evolution is detected between the female penis and male genitalia

24 **Summary**

25 Sex-specific elaborations are common in animals and have attracted the attention of many
26 biologists, including Darwin [1]. It is accepted that sexual selection promotes the evolution of
27 sex-specific elaborations. Due to the faster replenishment rate of gametes, males generally have
28 higher potential reproductive and optimal mating rates than females. Therefore, sexual selection
29 acts strongly on males [2], leading to the rapid evolution and diversification of male genitalia [3].
30 Male genitalia are sometimes used as devices for coercive holding of females as a result of
31 sexual conflict over mating [4, 5]. In contrast, female genitalia are usually simple. Here we
32 report the reversal of intromittent organs in the insect genus *Neotroglia* (Psocodea:
33 Prionoglarididae) from Brazilian caves. Females have a highly elaborate, penis-like structure, the
34 gynosome, while males lack an intromittent organ. The gynosome has species-specific
35 elaborations, such as numerous spines that fit species-specific pouches in the simple male genital
36 chamber. During prolonged copulation (ca. 40–70 hours), a large and potentially nutritious
37 ejaculate is transferred from the male via the gynosome. The correlated genital evolution in
38 *Neotroglia* is probably driven by reversed sexual selection with females competing for seminal
39 gifts. Nothing similar is known among sex-role reversed animals.

40 **Results and Discussion**

41 The genus *Neotroglia* (Figure 1A) contains four named species (adult body length 2.7–3.7 mm)
42 [6, 7]. Its most striking feature is the presence of a large penis-like structure in the female,
43 termed a gynosome (Figures 1–3 and S1–3). We show here that the gynosome is erectile, basally
44 membranous and apically sclerotized. Its sclerotized part consists of a proximal rod-like
45 extension and a penis-like distal prominence. The latter encloses a duct leading to the sperm
46 storage organ (spermatheca), and is interpreted as a novel structure differentiated from the
47 opening region of the spermathecal duct (Figure 1: light blue) [6]. In contrast, the male genitalia
48 (phallosome) consist of a simple, thin arc lacking an intromittent organ (Figures 1G and 3E). In
49 related insects, the spermathecal duct has a simple opening and the phallosome is well developed
50 (Figure 1).

51 We observed coupling in all *Neotroglia* species and found that the gynosome acts as an
52 intromittent organ to receive voluminous spermatophores from the male. As in most related taxa,
53 including those having well developed male genitalia (Figure 1C) [8], the male is positioned
54 under the female during copulation (Figure 1A). The apical sclerotized part of the gynosome,
55 bearing the opening of the spermathecal duct, deeply penetrates the male (Figures 2–3 and S2–
56 3), and its tip fits the opening of the seminal duct (Figures 2D and 3C). The membranous part
57 inflates within the male genital chamber, and numerous spines on the membrane internally
58 anchor the female to the male (Figures 2B, E, 3A, D and S2). In this position, the male sternum
59 is gripped between the female paraprocts and inflated gynosome (Figure 2B–C). Only the
60 connection of the abdominal tips holds pairs fixed in copula together. Furthermore, pulling apart
61 coupled specimens (*N. curvata*: $N=1$) led to separation of the male abdomen from the thorax

62 without breaking the genital coupling, showing that the female can hold the male tightly using
63 the gynosome and paraprocts.

64 The gynosomal structures are species-specific. The distal sclerotized part is strongly curved
65 in *N. curvata* (Figure 2A–D), but is straight or only slightly curved in other species (Figures 3
66 and S2). The membranous region of *N. curvata* has a smooth dorsal lobe (yellow) and five areas
67 bearing sclerotized spines: a dorsal (red), a pair of lateral (green) and a pair of ventrolateral
68 (purple) spiny areas (Figure 2). In *N. aurora* and *N. brasiliensis*, the dorsal and lateral spiny
69 areas are present (Figure S2), but the dorsal lobe and ventrolateral spiny areas are absent. The
70 gynosome of *N. truncata* lacks all elaborations, but its membranous part is densely covered by
71 tiny bristled spines (Figure 3).

72 Male genitalia are simple, but also species-specific, corresponding to the gynosomal
73 structures (Table S1). In *N. curvata*, the seminal duct is strongly curved as is the gynosomal
74 apical sclerite (Figure 2B–D), whereas these are straight or only slightly curved in the others
75 (Figure 3 and S2). The male genital chamber of *N. aurora*, *N. brasiliensis* and *N. curvata* has
76 lateral pouches corresponding to the lateral spiny areas of the gynosome (Figures 1G, 2, S1B and
77 S2: green arrowheads), while ventrolateral spiny areas and corresponding male pouches are only
78 present in *N. curvata* (Figures 2B, E and S1B: purple arrowheads). During copulation, the spiny
79 areas fit into the corresponding pouches and anchor the female (Figures 2 and S2). The
80 gynosome of *N. truncata* lacks strong spines (Figure 3A, D), and the female of this species
81 anchors itself using the entire surface of the bristled gynosomal membrane (Figure 3A, D). The
82 male genital chamber of this species lacks any pouches (Figure 3E: empty green arrowheads).

83 Spiny genitalia are present in many male animals [3–5, 9]. These may be used as

84 stimulatory devices [10, 11] or may result from sexual conflict [12, 13], in addition to an
85 anchoring function to grasp and hold mates. Species-specific membranous pouches in female
86 genitalia are reported in some insects to accommodate the male genitalia [5, 9], and thereby
87 reduce the cost of mating imposed by the corresponding male genital spines. The relative
88 function and pattern of elaboration of male and female genitalia in *Neotroglia* are completely
89 reversed relative to that generally observed.

90 In certain astigmatan mites [14] and scirtid beetles [15], male genitalia are reduced and
91 females possess an elongated intromittent tube or an eversible genital duct, respectively.
92 Although these organs are used to obtain sperm or spermatophores, no anchoring mechanism has
93 been observed. Female seahorses have an intromittent ovipositor to deposit eggs in the male
94 brood pouch [16], but this is not a penis. The evolution of a female penis is likely to be strongly
95 constrained because, in internally fertilizing animals, the ancestral condition is exclusively that
96 of an inseminating male requiring an intromittent organ and a receiving female, so that integrated
97 modifications in male and female structures and behaviors are required [17]. Therefore very few
98 animals have reversed intromittent copulatory organs.

99 It is known that reversed sexual selection has sometimes caused the evolution of masculine
100 characteristics or of secondary genitalia in females. For example, female ground weta
101 (Orthoptera: Anostostomatidae: *Hemiandrus pallitarsis*), benefit from seminal gifts and compete
102 intensely for them [18], using an elbow-shaped structure located in the middle of the abdomen to
103 obtain them from males. An anchoring intromittent organ, such as the gynosome, might be even
104 more effective at taking up seminal gifts from males. Nevertheless, reversed intromittent organs
105 were previously unknown among animals with reversed sexual selection.

106 Insects related to *Neotroglia* suggest the potential of nuptial gifts favoring the evolution and
107 diversification of the gynosome. In *Lepinotus patruelis* (Trogiidae), the direction of sexual
108 selection is reversed (without reversal of the intromittent organ). Males of this species transmit
109 specialized spermatophores (seminal capsules formed during copulation within the female's
110 spermatheca), which are produced by the enlarged seminal duct [19]. Males are choosier about
111 mates than females, indicating the costs of spermatophore production, while females compete for
112 the nutritious seminal gift [19, 20]. In *Neotroglia*, similarly shaped spermatophores (Figures 3F
113 and S3C–D) and an enlarged seminal duct producing voluminous spermatophore material
114 (Figures 2B and S3A–B) are present, suggesting that *Neotroglia* males also donate a nutritious
115 seminal gift to females. All known *Neotroglia* species inhabit extremely dry oligotrophic caves
116 feeding on bat guano and bat carcasses, which are relatively scarce resources [6, 7]. Under such
117 circumstances, nutritious seminal gifts cause a strong selection pressure for increased female
118 mating rate [21]. During their life, *Neotroglia* females may acquire several spermatophores (up to
119 11 have been observed in *N. brasiliensis*) (Figures 3F and S3C) [6]; they are evidently
120 polyandrous. We also observed that females consumed the contents of the spermatophores after
121 their first mating before producing mature eggs ($N=5$: Figure S3D), suggesting that the contents
122 of the spermatophores are probably used for nutrition as well as fertilization.

123 This interpretation may explain the following unique characteristics of the female internal
124 genitalia and of the coupling behavior of *Neotroglia*. The spermathecal duct of *Neotroglia* is
125 divided by a spermathecal plate, such that the female can simultaneously maintain two filled
126 spermatophores (Figures 3F and S3C), something unknown in related taxa [19, 22]. The duration
127 of copulation in *N. curvata* is exceptionally long (52.5 ± 11.2 hours: mean \pm SD, range 41-73

128 hours, $N = 12$: Table S2) in comparison with related taxa: approximately 40 minutes in
129 *Prionoglaris stygia* [8], approximately 2 hours in *Trogium pulsatorium* [22] and a maximum of 4
130 hours in the genus *Lepinotus* [8, 19]. In *Neotroglia*, females have structures that can coercively
131 hold males. The very long copulation, as well as polyandry, is probably controlled by females to
132 obtain more seminal gifts from males._

133 Sexual conflict over the donation of a nutritious seminal gift is thus the most likely factor
134 favoring the evolution of the gynosome. This organ may have a pre-mating function grasping
135 reluctant mates or a post-mating function holding mates to ensure prolonged copulation,
136 although these functions are not mutually exclusive. Because other paternal investments, such as
137 parental care, are not observed in *Neotroglia*, the correlated evolution of morphologically and
138 functionally novel genital organs has probably been driven by reversed sexual selection on
139 copulatory function. Sex-role reversed animals are valuable for testing the generality of theories
140 of sexual selection [23]. Because sex-role reversed females usually cannot coercively hold males,
141 they may be constrained in terms of evolving adaptations that relate to sexual conflict. The
142 female genital anchoring mechanism of *Neotroglia*, correlating morphologically with specialized
143 reduced male genitalia is unique, and nothing similar is known among other sex-role reversed
144 animals. However, in addition to anchoring, the gynosomal spines may have other functions,
145 such as genital stimulation or inflicting harm. The evidence for reversed sexual selection and
146 sexual conflict provided here is mostly circumstantial, but further controlled studies of the
147 mating system of *Neotroglia* species, together with an exploration of their phylogeny, would
148 provide an extremely rare opportunity to test the generality and relative importance of some
149 hypotheses about sexual selection [1–5, 9–13]. *Neotroglia* also offers a significant opportunity to

150 study evolutionary novelty, an area of central interest in contemporary evolutionary biology [17,
151 24].

152

153 **Experimental Procedures**

154 See Supplemental Information for details.

155

156 **Sampling**

157 *Neotroglia* specimens in copula were killed with hot water (ca. 80 °C) and fixed with 80% ethanol
158 in caves. We observed three pairs of *N. aurora*, four pairs of *N. brasiliensis*, eleven pairs of *N.*
159 *curvata*, and six pairs of *N. truncata*.

160

161 **Morphological Observations**

162 We used BABB (1:2 benzyl alcohol: benzyl benzoate) to make muscles and sclerites transparent
163 for examination [25]. This method does not dissolve soft tissues, and specimens in copula can be
164 observed *in situ* (Figures 2D and 3C). However, BABB could not make fat bodies transparent,
165 and we used 1% KOH to dissolve soft internal tissues after embedding abdomens in 1% agarose.
166 Observations were performed in glycerol (unmounted) or after slide mounting (dissected female
167 and male genitalia). An Olympus SZX16 stereo microscope and a Zeiss Axiophot compound
168 light microscope were used for examination. Photographs were taken with an Olympus E-330 or
169 OM-D E-M5 digital camera attached to the microscopes. Partially focused photographs were
170 combined using Helicon Focus (Helicon Soft Ltd.) to obtain images with high field depth.

171

172 **Behavioral Observations**

173 A total of 12 couplings of *N. curvata* were observed (Table S2). Specimens were kept in
174 Styrofoam boxes during observation. Adults were placed together, and when a couple formed it
175 was placed in a separate vial for observation. Copulations were observed at 30-minute intervals.
176 After copulation, some pairs were kept for observation until they died ($N = 2$), sometimes in the
177 presence of their F1 nymphs.

178

179 **Supplemental Information**

180 Supplemental Information includes three figures, two tables and the Supplemental Experimental
181 Procedures and can be found with this article online at http://dx.doi.org/****

182

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189

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247

248 **Figure legends**

249 Figure 1. Male and female genitalia of Prionoglarididae.

250 (A) *Neotroglia curvata* in copula.

251 (B–C) *Prionoglaris dactyloides*, spermathecal duct opening (light blue) and phallosome.

252 (D–E) *Speleketor irwini*, same as in (B–C).

253 (F–G) *Neotroglia aurora*, gynosome (parts highlighted as in Figures 2–3) and phallosome. Green

254 arrowhead in G indicates the left lateral pouch in the male genital chamber.

255 Scale = 0.1 mm.

256

257 Figure 2. Terminal structures of *Neotroglia curvata*. The following parts of the gynosome are

258 highlighted: distal sclerite (light blue), basal rod (orange), membranous region with dorsal lobe

259 (yellow) and lateral (green), dorsal (red) and ventrolateral (purple) spiny areas. Corresponding

260 male genital pouches are indicated by arrowheads of the same color.

261 (A) Erect gynosome, dorsolateral view.

262 (B–E) Terminal abdomens in copula, lateral (B–D) and ventral (E) views. Gynosome tip and

263 seminal duct opening are magnified in (D). In the schematic drawing (C), female structures,

264 except for the distal part of the gynosome, are indicated in orange, male structures in grey.

265 Scale = 0.1 mm.

266

267 Figure 3. Terminal structures of *Neotroglia truncata*. Color scheme as in Figure 2, plus pink color

268 indicating the basal gynosomal membrane.

269 (A–D) Terminal abdomens in copula, lateral (A–C) and ventral (D) views. Gynosome tip and

270 seminal duct opening are magnified in C.

271 (E) Slide-mounted male genitalia. Green open arrowheads indicate lack of membranous pouches.

272 See Figure 1G for comparison.

273 (F) Spermatheca fixed during copulation. Seven spermatophores are present, of which two

274 attached to the spermathecal plate are filled (indicated by white asterisks); the others are

275 separated from the plate and are empty (black asterisks).

276 Scale = 0.1 mm.

Figure 1

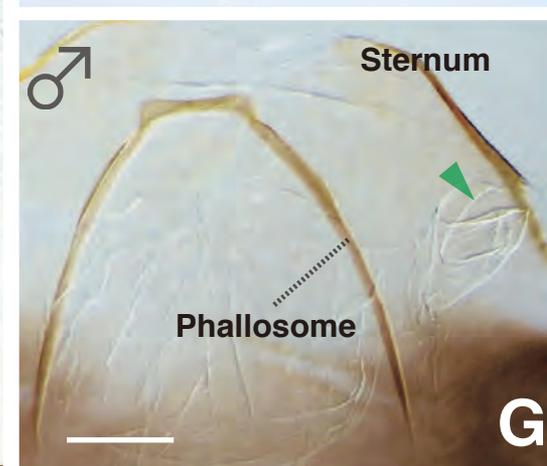
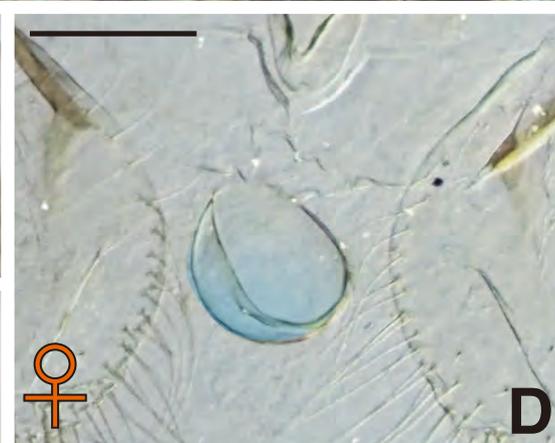
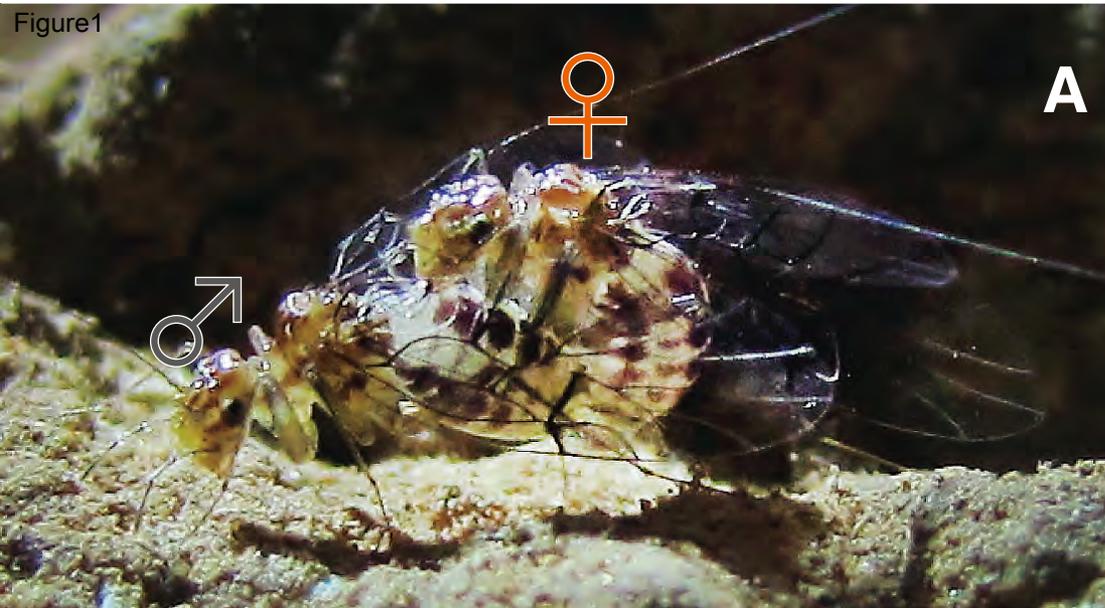


Figure 2

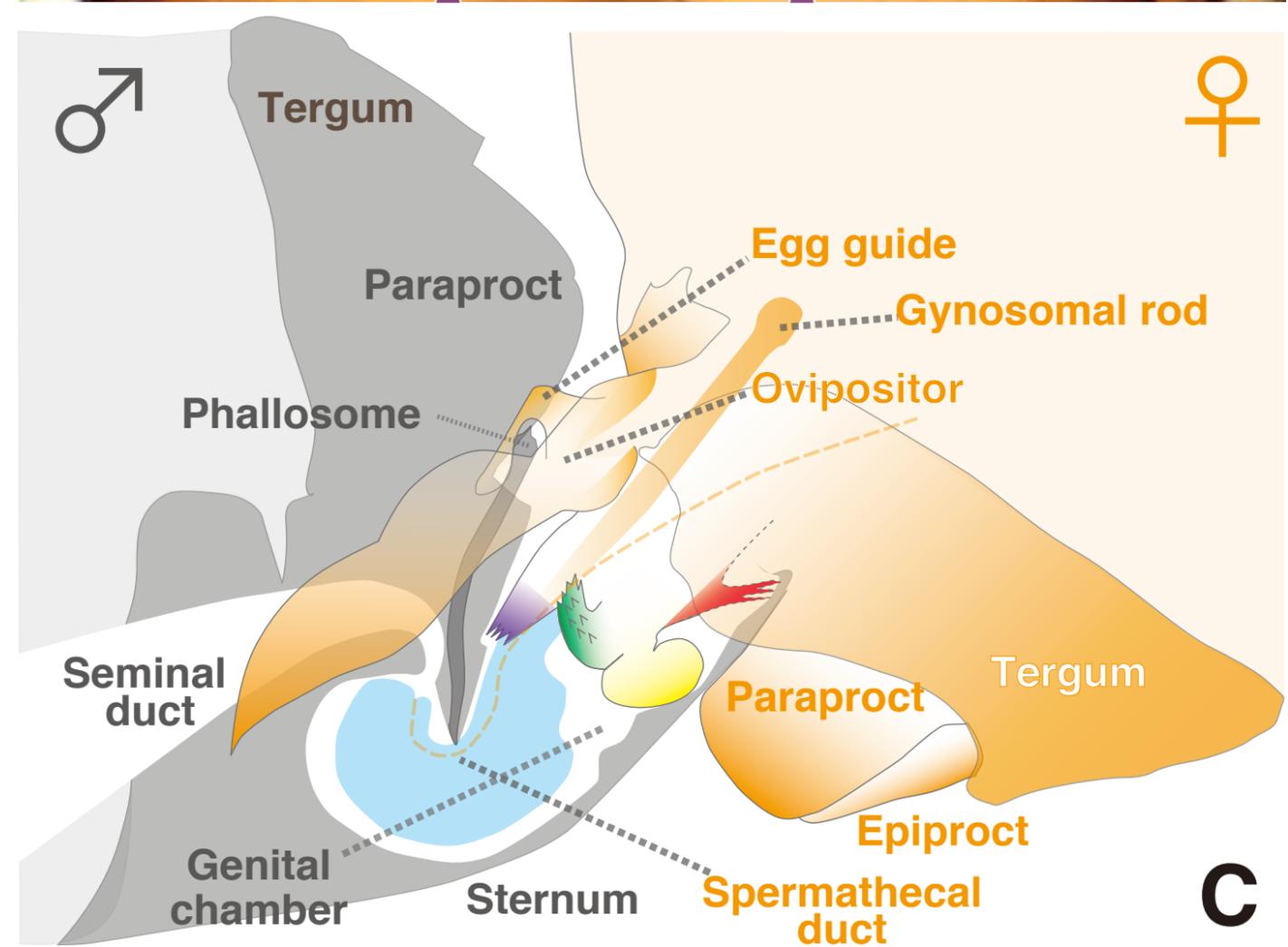
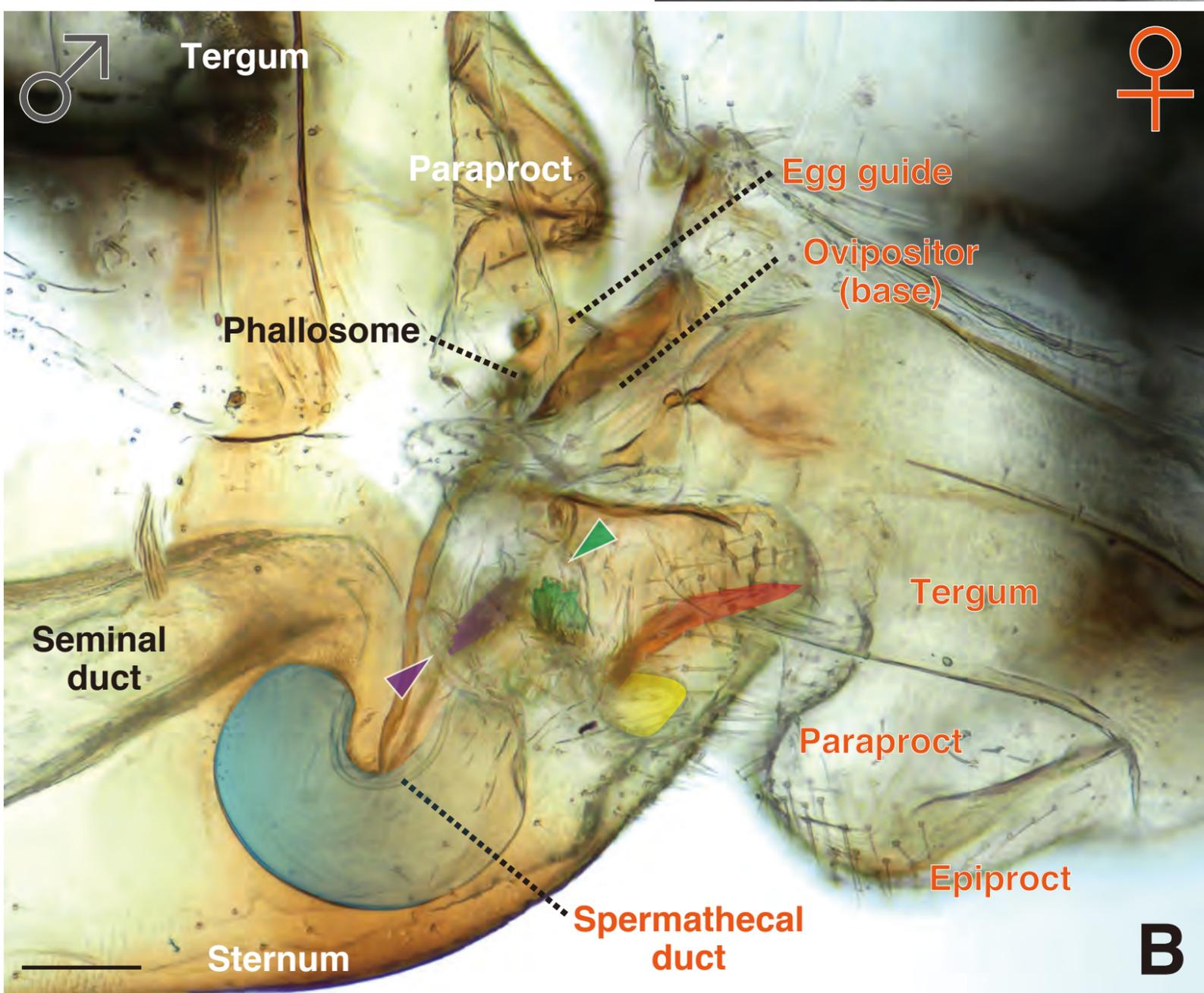
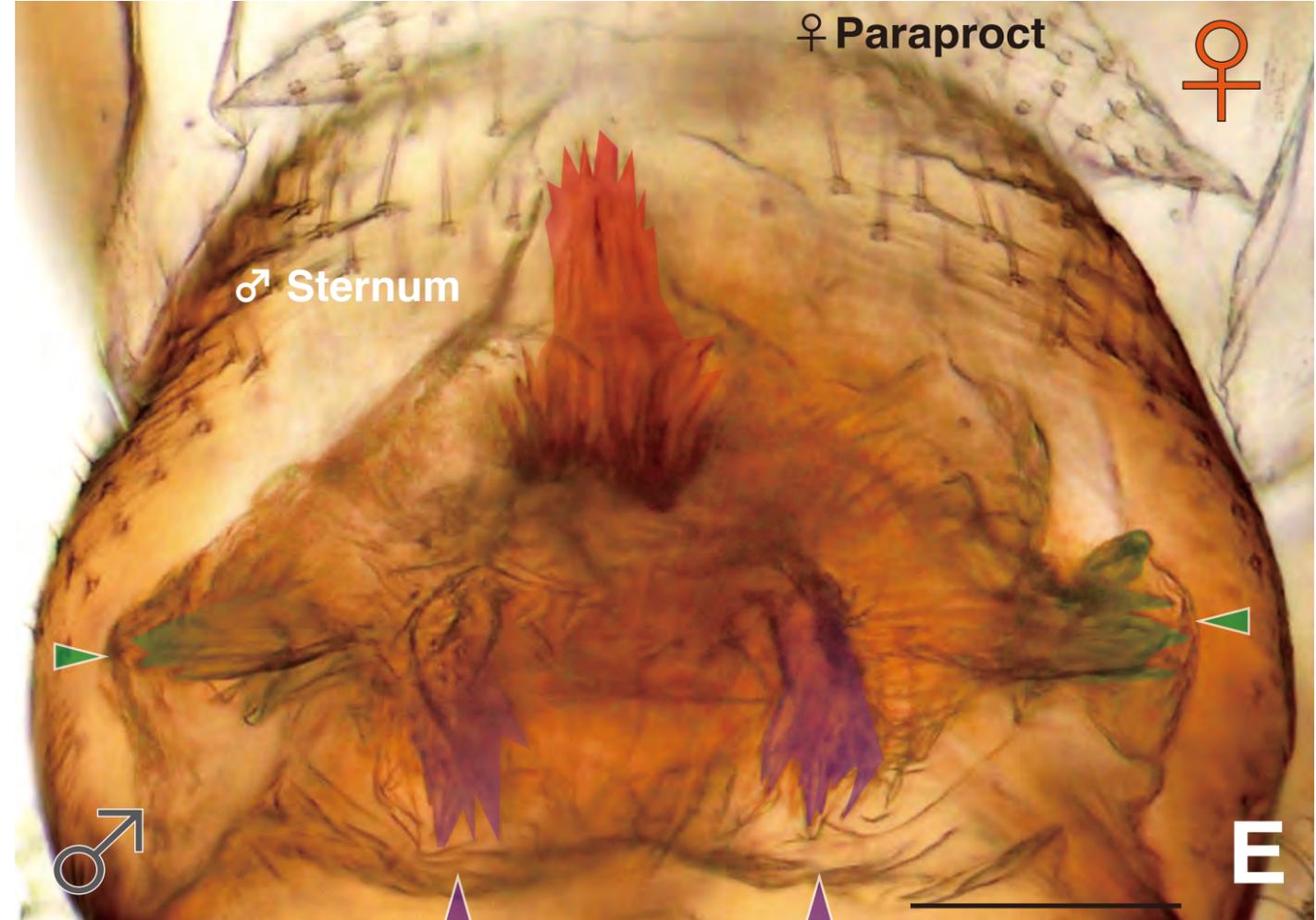
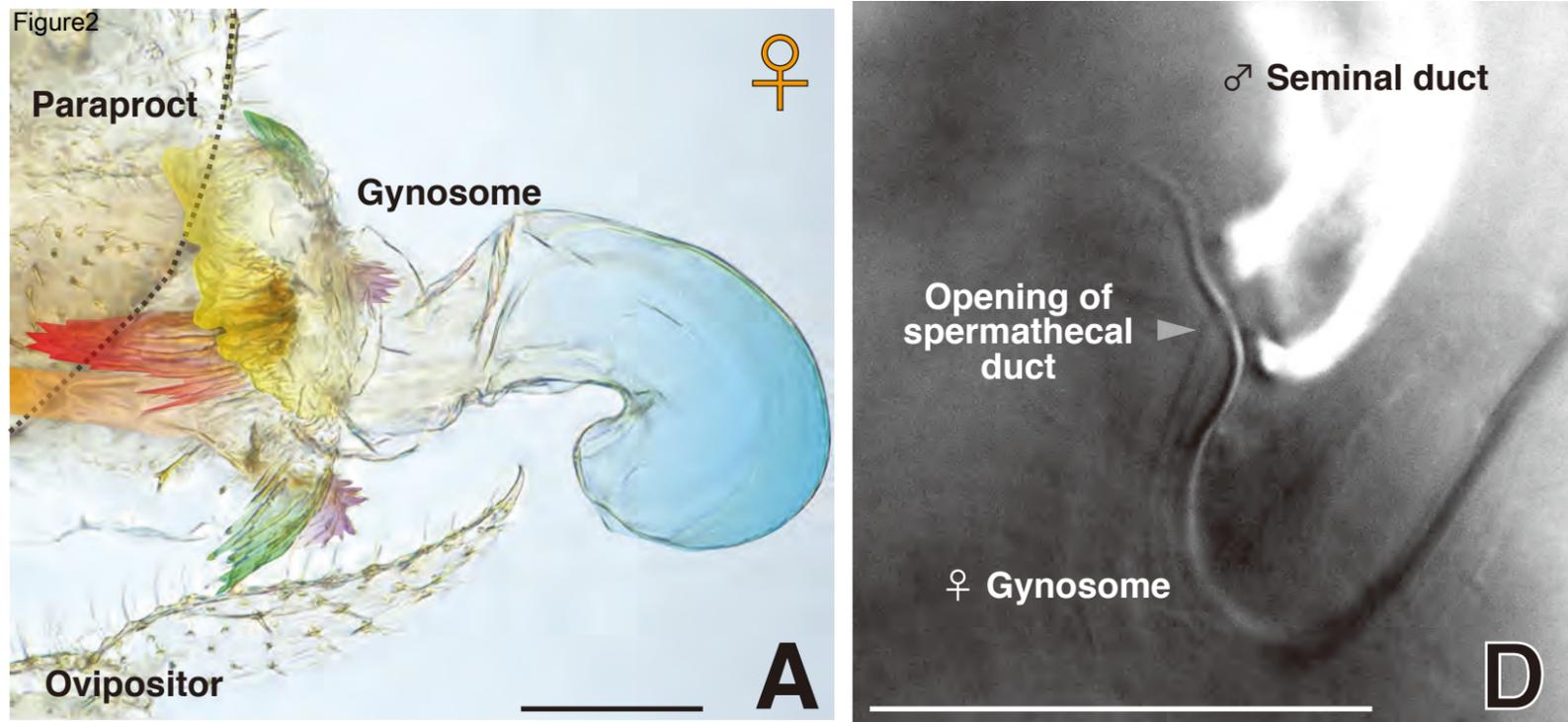
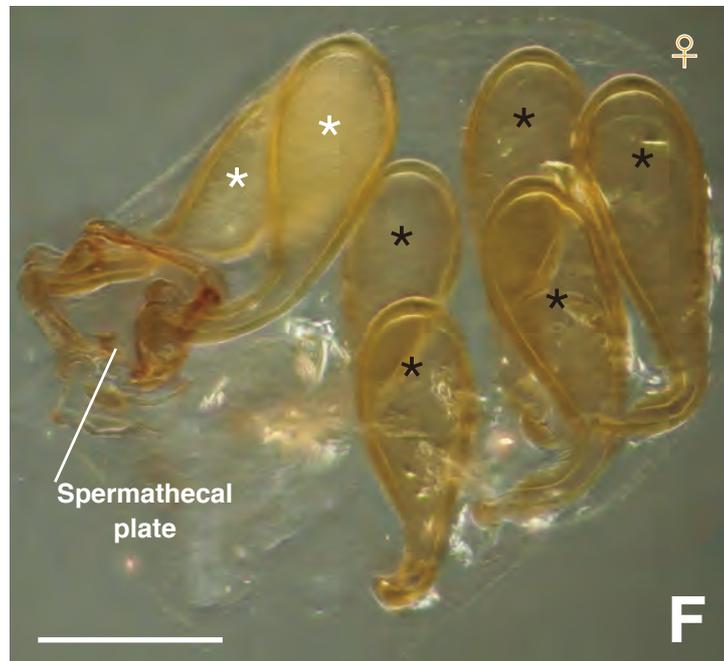
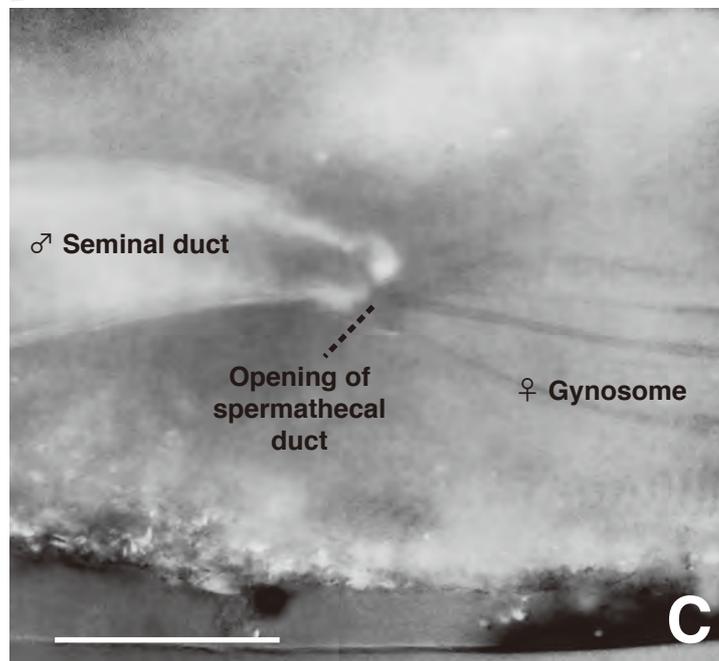
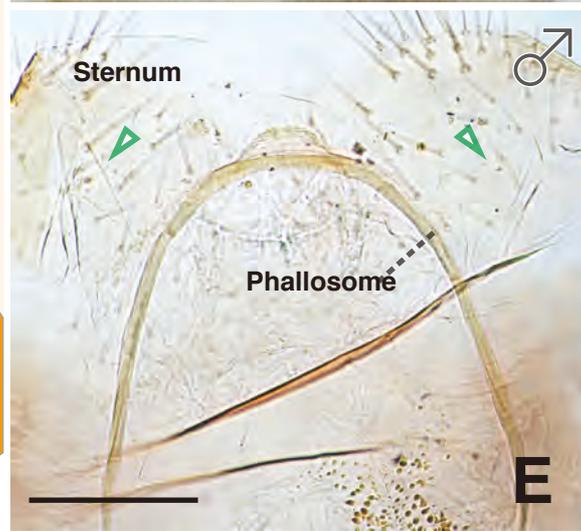
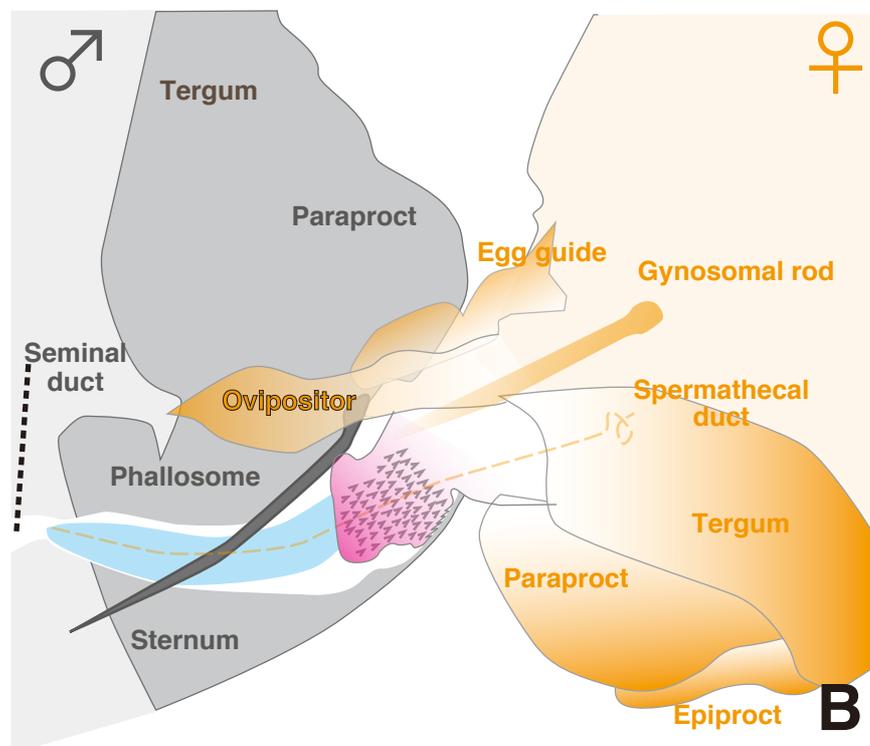
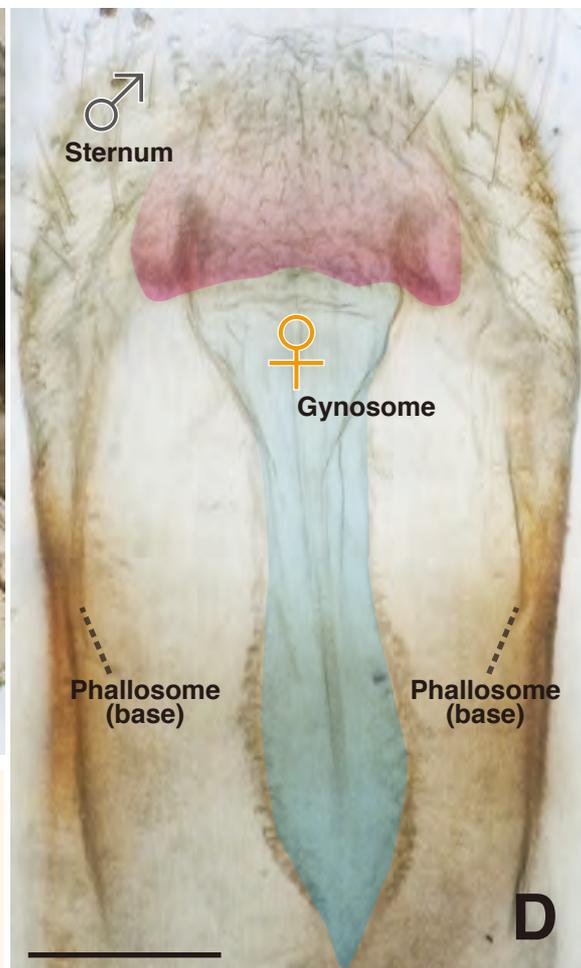
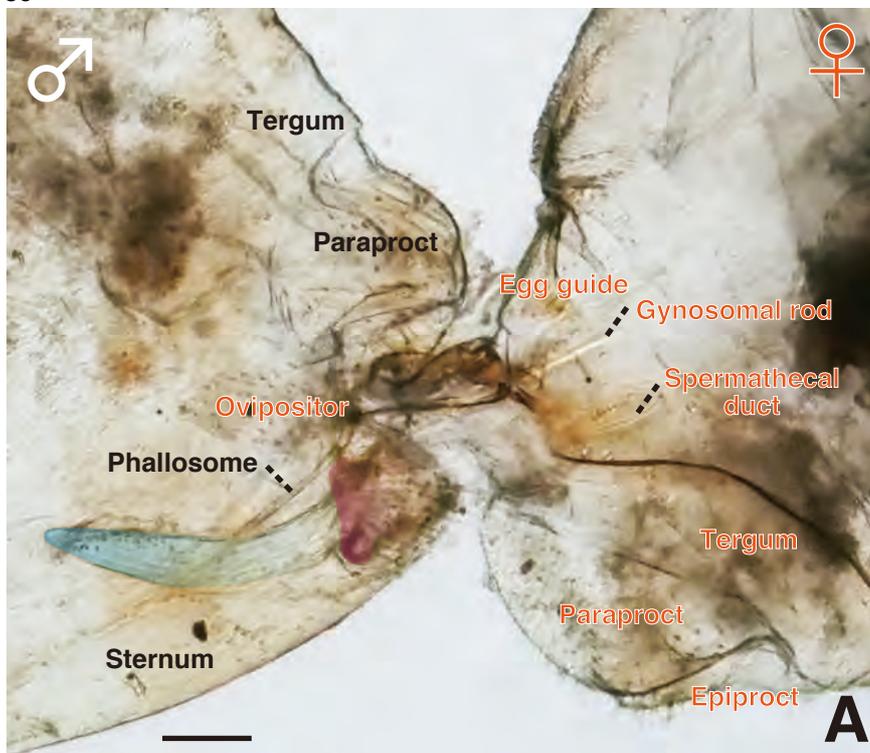


Figure 3



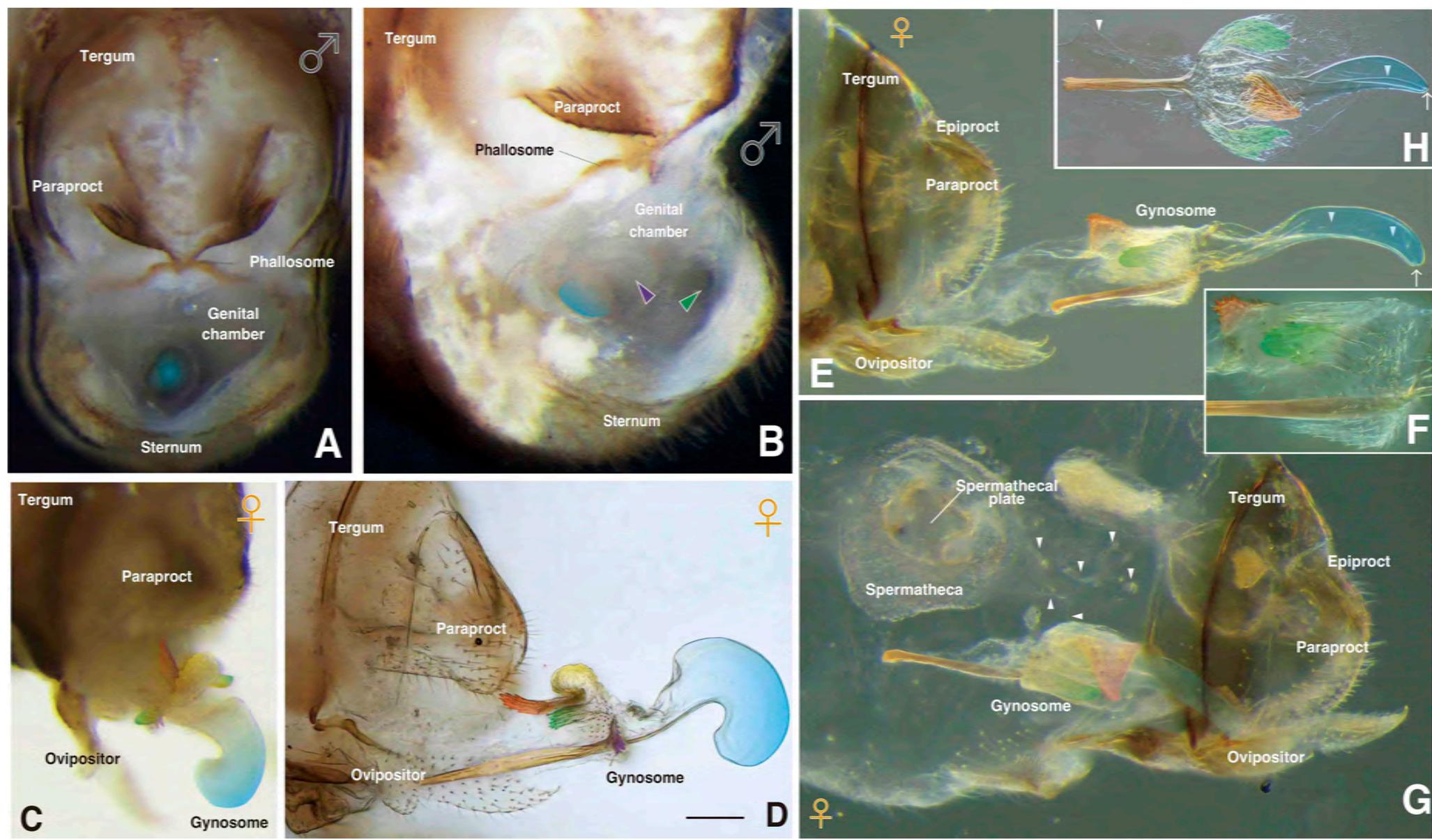


Figure S1 (related to Figure 1). Structures of male and female genitalia of *Neotrogla*. (A–D) *Neotrogla curvata*. Specimens in Figure 2A and Figure S1A–D were fixed in the copulating position and later separated; the specimen in Figure S1C–D is the same as that in Figure 2A. (A) Male terminal abdomen, posterodorsal view, showing the genital chamber and the hole (highlighted by light blue) into which the sclerotized distal part of the gynosome is inserted. (B) Same specimen as (A), postero-dorsolateral view. Arrowheads indicate the right-hand membranous pouches corresponding to the right-hand spiny areas indicated in Figure 2A–C and E. The hole in which the distal sclerite is inserted is highlighted in light blue. (C) Female terminal abdomen and erect gynosome before KOH treatment, postero-dorsolateral view. (D) Same as (C), after KOH treatment, lateral view. The gynosome was extended artificially to show its detailed structure. (E–F) *N. aurora*. (E) Female terminal abdomen and artificially erect gynosome, lateral view. An arrow indicates the opening of the spermathecal duct enclosed in the distal part of the gynosome (indicated by arrowheads). (F) Same as (E). The membranous part of the gynosome is magnified. (G) Terminal abdomen of a virgin female (spermatheca empty) with non-erect gynosome, in lateral view. Spermathecal duct indicated by arrowheads. (H) Non-erected gynosome of *N. brasiliensis* (slide-mounted holotype), dorsal view. Gynosome parts highlighted according to the color scheme used in Figure 2. Scale = 0.1 mm.

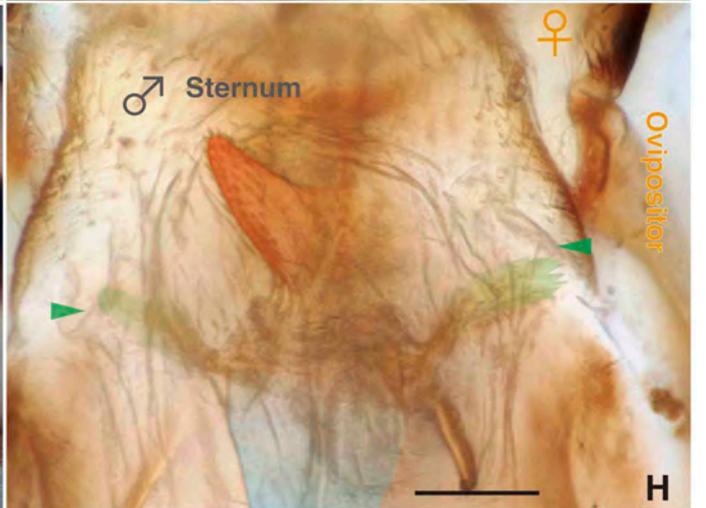
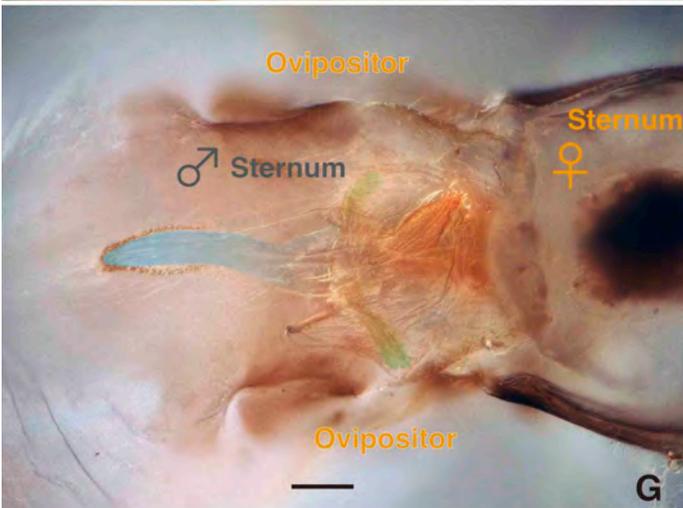
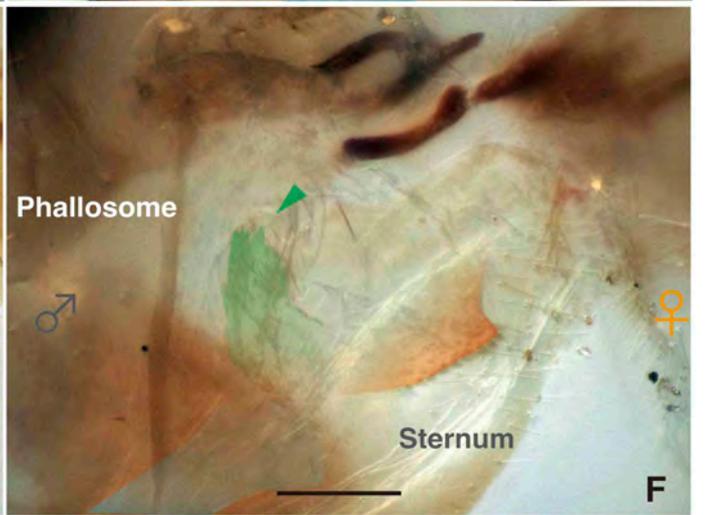
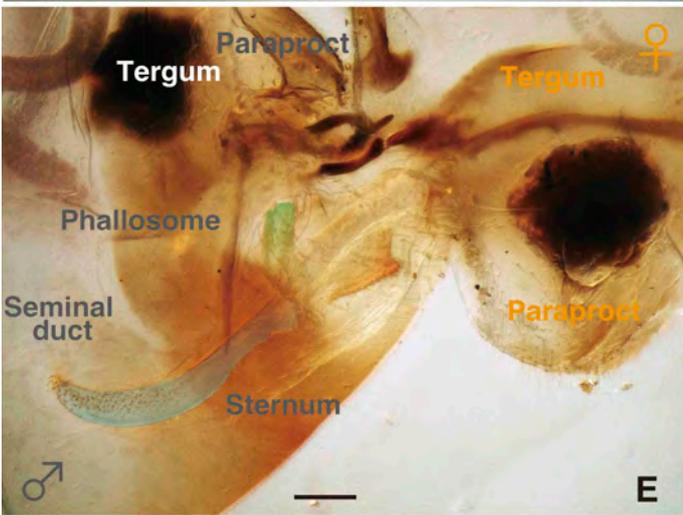
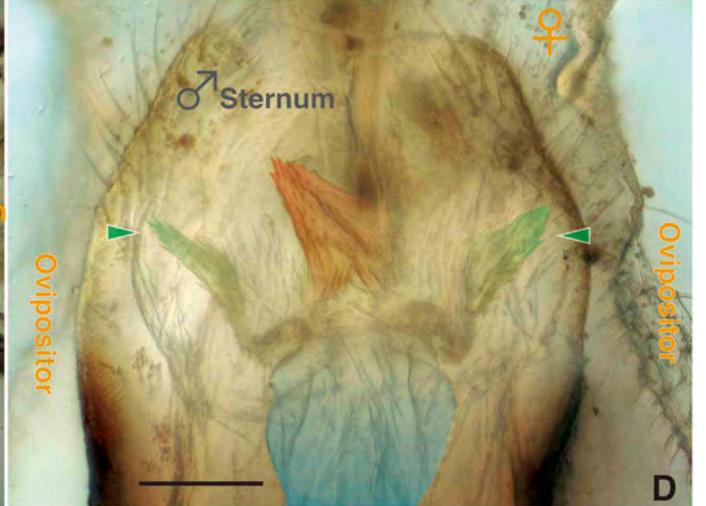
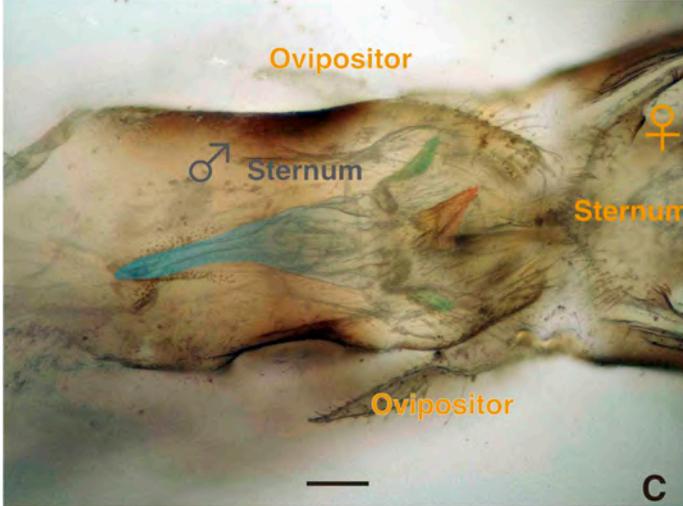
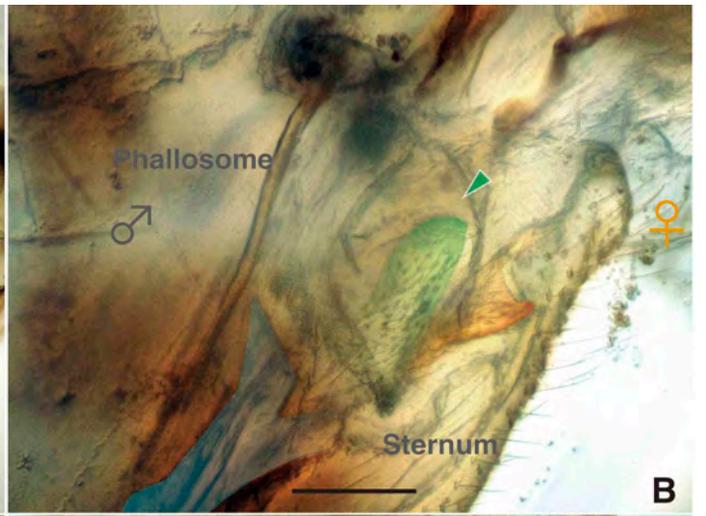
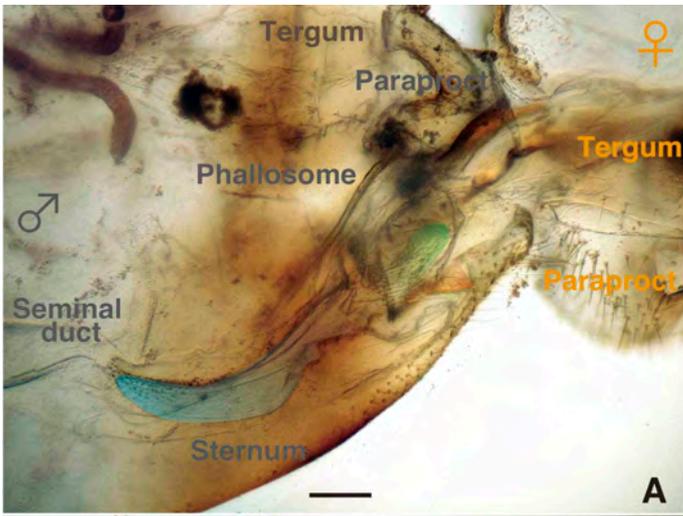


Figure S2 (related to Figure 2). *Neotroglia brasiliensis* (A–D) and *N. aurora* (E–H) in copula. (A–B, E–F) Lateral view. (C–D, G–H) Ventral view. Gynosomal structures are highlighted by the same colors as used in Figures 1–3. Green arrowheads indicate the lateral pouches of the male genital chamber. Scale = 0.1 mm.

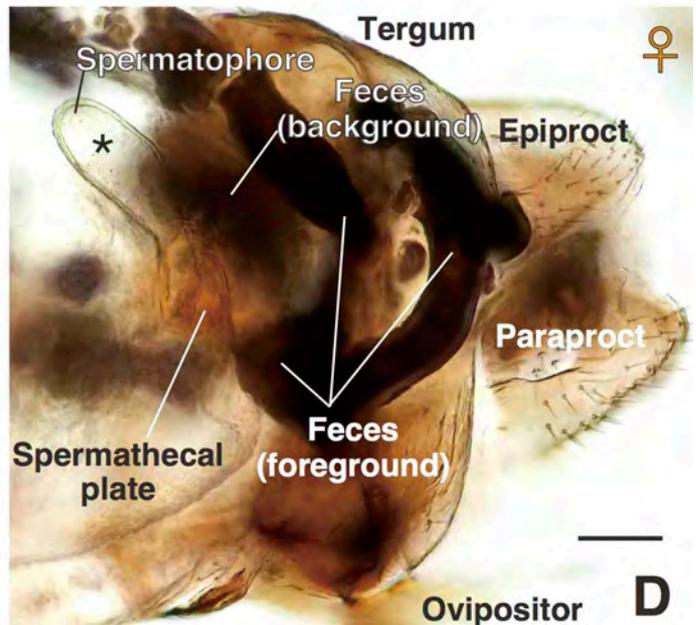
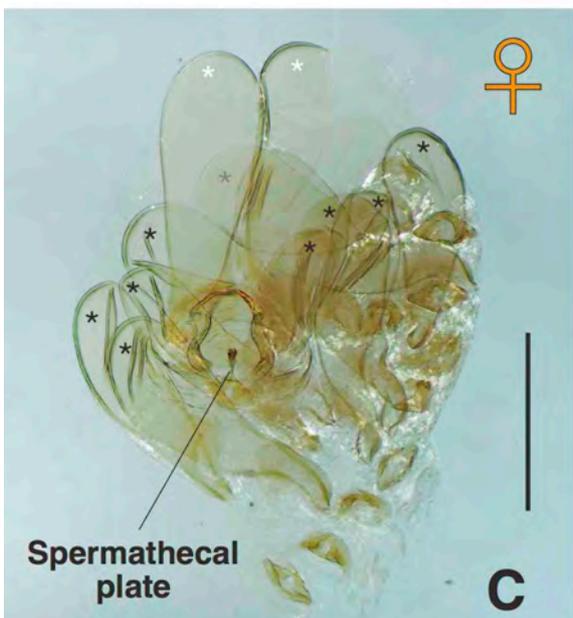
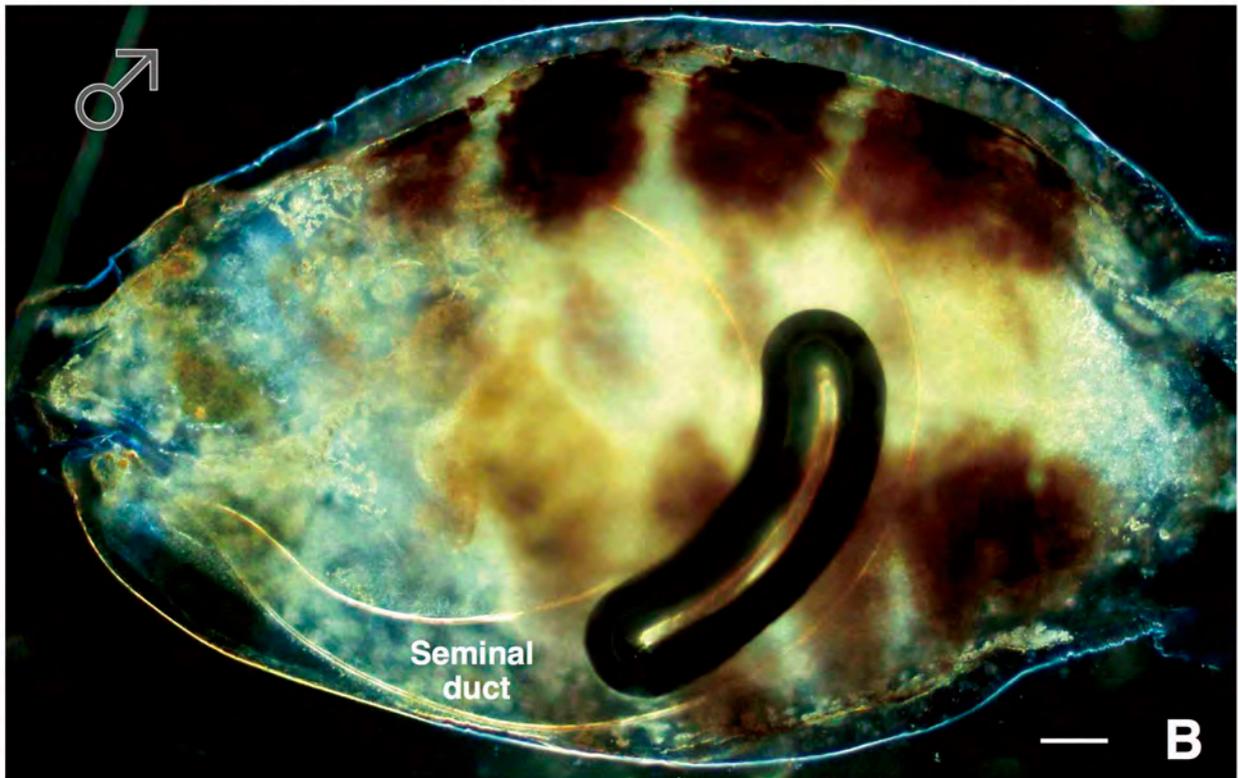


Figure S3 (related to Figure 3). Internal genitalia of *Neotrogla*. (A) *N. curvata* in copula (same individuals as shown in Figure 2B–E). (B) Male abdomen of *N. truncata*, right lateral view, separated from female during copulation. (C) Spermatheca of *N. brasiliensis* (slide-mounted holotype) containing 11 spermatophores (two filled ones fixed on the spermathecal plate indicated by white asterisks and empty ones by black asterisks). (D) An empty spermatophore in the spermatheca of an ex-virgin female of *N. curvata* after first copulation but before egg laying. Scale = 0.1 mm.

Table S1 (related to Figures 2, 3 and S2). Correlated genital evolution in *Neotroglia*. The similarly numbered male and female structures contact each other during copulation.

	<i>N. curvata</i>	<i>N. aurora</i>	<i>N. brasiliensis</i>	<i>N. truncata</i>
Female 1: Sclerotized part of the gynosome	C	S	S	S
Male 1: Seminal duct opening	C	S	S	S
Female 2: Lateral spiny areas (highlighted in green in Figure 2A)	+	+	+	-
Male 2: Lateral membranous pouches (green arrowheads in Figures 1G and 2B, E)	+	+	+	-
Female 3: Ventrolateral spiny areas (highlighted in purple in Figure 2A)	+	-	-	-
Male 3: Anterior membranous pouches (purple arrowheads in Figure 2B, E)	+	-	-	-

C, strongly curved; S, nearly straight to slightly curved; +, present; -, absent.

Table S2 (related to Figure 1). Coupling durations of *Neotroglia curvata*. Data based on 12 observations in the laboratory. Mean \pm SD = 52.5 \pm 11.2 hours.

		Starting day	Starting time	Finishing day	Finishing time	Duration (hours)
Couple 1		05.xi.2012	16:30	07.xi.2012	11:30	43
Couple 2		06.xi.2012	08:30	08.xi.2012	11:30	51
Couple 3		19.xi.2012	16:00	21.xi.2012	23:00	55
Couple 4		19.xi.2012	20:00	21.xi.2012	13:30	41.5
Couple 5		24.xi.2012	12:00	26.xi.2012	12:30	48.5
Couple 6		27.xi.2012	17:00	29.xi.2012	10:00	41
Couple 7	Virgins	01.xii.2012	17:00	03.xii.2012	21:00	52
Couple 8	Virgins	02.xii.2012	14:00	04.xii.2012	09:30	43.5
Couple 9		20.v.2013	18:00	22.v.2013	16:00	46
Couple 10	Virgins	27.v.2013	12:30	30.v.2013	13:30	73
Couple 11	Virgins	03.vi.2013	12:30	06.vi.2013	10:30	70
Couple 12	Virgins	04.vi.2013	14:00	07.vi.2013	7:00	65

Supplemental Experimental Procedures

Taxon Sampling

Neotrogla specimens in copula were killed with hot water (ca. 80 °C) and fixed with 80% ethanol in caves in the São Félix do Coribe and Santa Maria da Vitória municipalities, Bahia State in October 2012 (*N. curvata*: 11 pairs), in caves in the Ouro-lândia municipality, Bahia State in January 2013 (*N. truncata*: 6 pairs), in caves in the Dianópolis municipality, Tocantins State in March 2013 (*N. aurora*: 3 pairs) and in caves in the Januária municipality, Minas Gerais State in March 2013 (*N. brasiliensis*: 4 pairs), all in Brazil. Additional specimens were observed as follows: 1 female and 1 male of *N. aurora* from Tocantins State, Arraias, cave Ponto 014, Brazil on 4–6.x.2010, collected by F. Pellegatti-Franco and colleagues and six pairs of *N. truncata* from Bahia State, Ouro-lândia, Toca dos Ossos, Brazil on 10.vi.2012, collected by RLF, originally in copula but separated at fixation. The examined specimens are preserved at Hokkaido University and the Geneva Natural History Museum.

Morphological Observations

Two methods were used to examine the coupling mechanisms of *Neotrogla* species. The first method used BABB (1:2 benzyl alcohol: benzyl benzoate) to make the muscles and sclerites transparent [S1]. This method does not dissolve any soft tissue, and specimens in copula can be observed *in situ* without any modifications (Figures 2D and 3C). The separated abdomens in copula were dehydrated with 100% ethanol and placed in BABB for a few days at room temperature, and observations were conducted in BABB. However, BABB does not make fat bodies transparent. Therefore, after the application of the BABB method, KOH treatment was used to dissolve the fat bodies. The

abdomens were removed from BABB and washed with 100% ethanol, 80% ethanol and distilled water. They were then embedded in 1% agarose, and the soft internal tissues of the embedded abdomens were cleared with 1% KOH for two days at room temperature. KOH-treated specimens embedded in agarose were rinsed with distilled water, and observations were conducted in glycerol. The KOH method dissolves muscles and other soft tissues and produces very slight modifications in certain membranous structures (e.g., the seminal duct); however, we confirmed that the genital structures were fixed by agarose and no recognizable modification occurred in the coupling condition. The specimen shown in Figure S1D was first embedded within agarose (Figure 2A) but later removed from the agarose, and the gynosome was positioned to more clearly observe its basal portion. To observe the genital structures in other specimens, the abdomens were cleared with 10% KOH at room temperature for one night. The cleared specimens were rinsed and stored in 80% ethanol. Observations were made in glycerol (unmounted) or after slide mounting (dissected female genitalia and male genitalia). An Olympus SZX16 stereo microscope and a Zeiss Axiophot compound light microscope were used for examination. Photographs were taken with an Olympus E-330 or OM-D E-M5 digital camera attached to the microscopes. Partially focused pictures were combined using Helicon Focus (Helicon Soft Ltd.) to obtain images with high depth of field.

Behavioral Observations

Several unpaired adults and nymphs of the *Neotroglia* species were collected from caves and reared in RLF's laboratory to observe mating behavior. A total of 12 couplings of *N. curvata* were observed (Table S2). The specimens were kept in Styrofoam boxes during observations. Adults were placed together, and when a couple formed, it was placed in a

separate vial for observation. When a nymph developed into an adult, it was placed together with other non-mating adults. Several virgin adults (raised in the laboratory) were placed together immediately after reaching adulthood. These individuals required several days to form mating pairs ($N = 2$). The copulations were observed at 30-minute intervals. Such observations began each time the presence of copulating adults was verified. After copulation, some pairs were kept for observation until they died, sometimes in the presence of their F1 nymphs.

Supplemental Reference

- S1. Kamimura, Y., and Mitsumoto, H. (2011). Comparative copulation anatomy of the *Drosophila melanogaster* species complex (Diptera: Drosophilidae). *Entomol. Sci.* *14*, 399–410.