

1 **Are males just passive? Coupling mechanism of the Brazilian cave insects with**
2
3 **inverted genitalia**

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56 19 **Abstract**

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59 20 Species of the Brazilian cave barklouse genus *Neotrogla* (Psocodea: “Psocoptera”:

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1 21 Trogiomorpha: Prionoglarididae: Sensitibillini) are known to have a “female penis
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3 22 (gynosome)” that functions as an intromittent organ inserted into the membranous
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6 23 pouches in the simple male genital chamber during copulation to receive semen.
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9 24 However, the functions of other male and female genital structures and the copulatory
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11 25 processes of *Neotroglia* were completely unknown to date. Based on μ CT observation
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14 26 of the male and female postabdomen and connected muscles both before and in copula,
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17 27 we clarified the functions of the male and female genital structures. In addition, based
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20 28 on the analyses of the established 3D models, we concluded that precise and rigid
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23 29 contact of multiple genital structures, and step-by-step releases of each holding
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26 30 mechanism achieved by the cooperation of both sexes are involved in the copulatory
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29 31 processes. The coevolution between the male and female genital structures in *Neotroglia*
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32 32 may provide a new example for the evolution of tolerance traits.

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35 **Keywords**

36 genitalia, copulatory processes, sexual selection, muscles

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1 43 **Introduction**

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3 44 The Brazilian cave insect genus *Neotrogla* (Psocodea: Trogiomorpha: Prionoglarididae:
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5 45 Sensitibillini) has been receiving much attention since it was discovered that the
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7 46 females have a penis-like intromittent structure called gynosome (Lienhard *et al.*, 2010).
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9 47 The gynosome, generally known as a female penis, is inserted into a vagina-like male
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11 48 structure during copulation to obtain semen (Yoshizawa *et al.*, 2014). The female penis
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13 49 has also been found in the African genus *Afrotrogla* (Lienhard, 2007). Both *Neotrogla*
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15 50 and *Afrotrogla* belong to the small tribe Sensitibillini, containing three genera and
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17 51 eleven named species only, and the well-developed female penis is considered to have
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19 52 evolved independently in these two genera (Yoshizawa *et al.*, 2018b; Cheng *et al.*, 2023).
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21 53 In recent years, several in-depth studies have been conducted on the factors affecting
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23 54 the formation of female intromittent structures, such as the oligotrophic environment,
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25 55 male nuptial gifts, female multiple sperm storage, female-above copulating position,
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27 56 elongated spermathecal duct, and the absence of male penetrative genitalia (Yoshizawa
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29 57 *et al.*, 2014, 2018ab, 2019; Kamimura *et al.*, 2021).

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31 58 All these studies focused on the intromittent female penis, but the morphology and
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33 59 function of other genital structures in these sex-reversed insects are still poorly
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35 60 understood. Referring to muscle homology, Cheng *et al.* (2023) determined that the
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37 61 female penis evolved from the spermapore plate, and its unique intromittent function is
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39 62 brought by two pairs of novel muscles formed only within the Sensitibillini. Are there
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41 63 novel structures and muscles in the other genital structures? What functions do other
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43 64 genital structures have during copulation? Do females copulate coercively, and are
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1 65 males just passive for mating? What types of sexual selection are strongly reflected in
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3 66 the copulatory processes? All these questions need to be answered under the overall
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6 67 grasp of the structures of male and female genitalia and the copulatory processes of
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9 68 these cave insects with inverted genitalia.

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11 69 In this study, by using the synchrotron μ CT technique, we present 3D models of
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14 70 the whole male and female genital structures and their associated muscles of *Neotrogl*
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17 71 *curvata*. By comparing the morphological changes of the genital structures and
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20 72 associated muscles before and during copulation, we clarify the functions of these
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23 73 structures and estimate the complete copulatory processes. We also analysed the
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26 74 homology of the muscles between *Neotrogl*a species and the other non-genital-reversed
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28 75 Psocodea and traced the origin of each structure and muscle of *Neotrogl*a.

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32 33 77 **Materials and Methods**

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36 78 A copulating pair and a noncopulating male and female of the coupling-role reversed
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39 79 cave psocid *Neotrogl*a *curvata* Lienhard & Ferreira, 2013 (Trogiomorpha) were
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42 80 examined. A noncopulating male and female of *N. brasiliensis* Lienhard, 2010
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45 81 (Lienhard et al., 2010) and noncopulating male specimens of *Trichadenotecnum*
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48 82 *pseudomedium* Yoshizawa, 2001 (Psocomorpha: Cheng & Yoshizawa, 2019), the latter
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51 83 of which has normal genital structures, were also examined for comparison. All samples
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54 84 were subjected to μ CT examination, and voucher specimens were stored in the
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57 85 Hokkaido University Insect Collection. Samples were fixed either with hot water, FAA
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60 86 solution (formalin:alcohol:acetic acid = 6:16:1), or 80% ethanol and then preserved in
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1 87 80% ethanol. Dehydration was conducted in ascending order with 80–100% ethanol
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3 88 before drying them at the critical point (EM CPD300, Leica, Wetzlar, Germany) to
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6 89 remove water without serious organ shrinkage. Samples were then scanned by
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9 90 synchrotron μ CT at the BL47XU (Uesugi et al., 2012) beamline of the Super Photon
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11 91 ring-8 GeV (SPring-8; Hyogo, Japan) using a stable beam energy of 8 keV in
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14 92 absorption-contrast mode. The tomography system consists of a full-field X-ray
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17 93 microscope with Fresnel zone plate optics (Uesugi et al., 2017). We used semiautomatic
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20 94 segmentation algorithms based on grey-value differences in ITK-SNAP software
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23 95 (Yushkevich et al., 2006) to obtain 3D representations of the terminalia of all three
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26 96 species.

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30 98 **Results**

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33 99 We first describe the muscles associated with male and female terminalia of *Neotrogla*
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36 100 *curvata*. We grouped the muscles according to their origin as follows: muscles of the
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39 101 epiproct [ep]; paraproct [pa]; subgenital plate [sg]; dorsal valve [do]; external valve
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42 102 [ex]; spermapore plate/gynosome or the membrane surrounding it [sp/gy]; hypandrium
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45 103 [hy]; and phallosome [ph].

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49 104 Abbreviations: O – origin; I – insertion; and F – assumed function based on
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52 105 morphological conditions.

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55 106 **epX01** (male/female) (Figs. 2C, D and 8A: = 01 of Cheng & Yoshizawa, 2019); O:
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58 107 mid-dorsal site of clunium (segment IX); I: posterior end of the epiproct; and F: closure
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1 108 and/or flipping of the epiproct.
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5 109 **paX01** (male/female) (Figs. 2C, D and 8A: = 02 of Cheng & Yoshizawa, 2019); O:
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7 110 anterolateral region of the clunium (segment X); I: anterodorsal end of the paraproct,
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9 111 very close to the posterolateral margin of the epiproct; and F: involved in opening the
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11 112 paraproct.
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17 113 **paX02** (male/female) (Figs. 2C, D and 8A: = 02 of Cheng & Yoshizawa, 2019); O:
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19 114 mediodorsal region of the clunium (segment X); I: anterodorsal end of the paraproct,
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21 115 very close to the anterolateral corner of the epiproct; and F: involved in opening the
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23 116 paraproct.
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29 117 **paX03** (male/female) (Figs. 2C, D and 8A: = 03 of Cheng & Yoshizawa, 2019); O:
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31 118 mediolateral region of the clunium (segment X); I: internal margin of the paraproct near
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33 119 the anal opening; and F: involved in retracting the paraproct.
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39 120 **paX04** (male/female) (Figs. 2C, D and 8A); O: mediolateral region of the clunium
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41 121 (segment IX); I: anterolateral margin of the paraproct; and F: involved in retracting the
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43 122 paraproct.
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49 123 **dosp01** (female) (Figs. 2E, F and 8A: = 05 of Cheng & Yoshizawa, 2019); O: base of
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51 124 the dorsal valve; I: on the membrane connected to the spermapore plate/gynosome, near
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53 125 the posterior tip of the spermapore plate; and F: involved in restoring the position of
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55 126 the gynosome (spermapore plate) and the dorsal valve.
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59 127 **doex01** (female) (Figs. 2E, F and 8A); O: base of the dorsal valve; I: base of the external
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1 128 valve; and F: involved in restoring the position of the dorsal valve and closure of the
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3 129 external valve.
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6 130 **exsp01** (female) (Figs. 2E, F and 8A: = 06 of Cheng & Yoshizawa, 2019); O: base of
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8 131 the external valve; I: on the membrane connected to the spermapore plate/gynosome,
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10 132 near the posterior tip of the spermapore plate; and F: involved in restoring the position
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12 133 of the gynosome (spermapore plate) and closure of the external valve.
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15 134 **exIX02** (female) (Figs. 2E, F and 8A: = 08 of Cheng & Yoshizawa, 2019); O:
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17 135 anterolateral margin of the clunium; I: base of the external valve, near the middle of the
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19 136 junction with the clunium; and F: involved in opening the external valve.
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22 137 **exVIII01** (female) (Figs. 2E, F and 8A); O: medioventral region of sternum VIII; I:
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24 138 base of the external valve; and F: involved in closure of the external valve.
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27 139 **spIX01** (female) (Figs. 2G, H and 8A); O: mediolateral region of the clunium; I: on the
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29 140 spermapore membrane; and F: involved in restoring the gynosomal spiny membrane
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31 141 (*Neotroglia*) or the position of the spermapore plate (other Psocodea).
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34 142 **spIX02** (female) (Figs. 2G, H and 8A); O: mediolateral region of the clunium; I: on
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36 143 the spermapore membrane; and F: involved in restoring the gynosomal spiny
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38 144 membrane (*Neotroglia*) or the position of the spermapore plate (other Psocodea).
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41 145 **gyIX01** (female) (Figs. 2G, H and 8A); O: gynosome membrane very close to dorsal
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43 146 valves (=ventral membrane of segment IX); I: anterior end of the basal shaft; and F:
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45 147 protractors of the gynosome.
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48 148 **gy-01** (female) (Figs. 2G, H and 8A); O: an internal organ (specific insertion site not
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50 149 detected); I: anterior end of the basal shaft; and F: retractors of the gynosome.
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1 150 **papa01** (male) (Figs. 5C, D and 8B); O: internal margin of the paraproct near the anal
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3 151 opening; I: anterolateral margin of the paraproct; and F: involved in opening the anus.

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6 152 **pahy01** (male) (Figs. 3F, 5C, D and 8B: = 04 of Cheng & Yoshizawa, 2019); O:
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8 153 anteroventral end of the paraproct; I: anterolateral region of the hypandrium (segment
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10 154 IX); and F: involved in indirectly opening the hypandrium and retracting the male
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12 155 paraproct (holding the female dorsal valve during copulation: see below).

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17 156 **hyVIII01** (male) (Figs. 3F, 5E, F and 8B: = 05 of Cheng & Yoshizawa, 2019); O:
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19 157 posterolateral region of segment VIII; I: mediolateral margin of the hypandrium; and F:
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21 158 involved in restoring the hypandrium.

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25 159 **phIX01** (male) (Figs. 3F-I, 5G, H and 8B: = 07 of Cheng & Yoshizawa, 2019); O: U-
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27 160 shaped end of the sclerite of the phallosome; I: anterolateral region of the hypandrium
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29 161 (segment IX); and F: involved in opening the hypandrium/protrusion of the phallosome.

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33 162 **phIX02** (male) (Figs. 3F-I, 5G, H and 8B: = 08 of Cheng & Yoshizawa, 2019); O:
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35 163 middle of the sclerite of the phallosome; I: anterolateral region of the hypandrium
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37 164 (segment IX), phIX01, phIX02 and the sclerite of the phallosome present a triangle in
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39 165 position; and F: involved in opening the hypandrium/retraction of the phallosome.

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43 166 **phVIII01** (male) (Figs. 3F-I, 5G, H and 8B); O: posterolateral region of segment VIII;
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45 167 I: posterior of the sclerite of the phallosome; and F: involved in protrusion of the
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47 168 phallosome (holding the female dorsal valve during copulation: see below).

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54 55 56 57 170 **Skeletal and muscle structures of female terminalia**

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59 171 Among the female terminal structures of *Neotroglia*, the morphology of the clunium and
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1 172 the epiproct is not much different from those of the other females of Psocodea. In
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3 173 contrast, the subgenital plate (posterior extension of the 8th sternum) is completely
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6 174 reduced in *Neotroglia*. A distinct difference was also detected in the paraproct between
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9 175 *Neotroglia* and other psocodeans, i.e., two concavities are present on the ventrolateral
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12 176 surface of the paraproct in *Neotroglia* (Fig. 1B). One group of muscles (epX01; Fig. 2C)
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14 177 is associated with the epiproct, and four groups of muscles (paX01-04; Fig. 2C) with
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17 178 the paraproct, all of which originate from the clunium. All of these muscles are
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20 179 homologous to those associated with the epiproct or paraproct of other psocodeans
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23 180 (Cheng & Yoshizawa, 2022).

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25 181 The bases of the external valve of the gonapophyses are connected by a transverse
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28 182 sclerite (Fig. 3B). The dorsal valve of the gonapophyses is located posteromedially to
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31 183 the transverse sclerite of the external valves and forms an independent structure (Fig.
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34 184 1A). The dorsal valve is not truly a paired structure like the external valves. It is usually
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37 185 paired in Psocodea, but forms a single lobe-like structure with a posterior and lateral
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39 186 marginal ridge shaped like a chair with a central depression (Fig. 3A). Two groups of
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42 187 muscles (dosp01 and doex01; Fig. 2E) are connected to the dorsal valve. They are
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45 188 homologous with those associated with the dorsal valves of other psocodeans (Cheng
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47
48 189 & Yoshizawa, 2022).

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50 190 The external valves form a pair of projections, together forming a crab claw-like
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53 191 structure, basally connected by a transverse sclerite. The transverse sclerite is laterally
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56 192 articulated with the clunium and anteriorly connected with sternum VIII (Fig. 1A, C).
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59 193 The external valves of both *Neotroglia* species show a similar morphology (Fig. 3C).
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1 194 There are four groups of muscles (doex01, exsp01, exIX02, exVIII01; Fig. 2E)
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3 195 connected to the external valve, and three groups of those muscles are homologous with
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6 196 those associated with the external valves of other psocodeans (Fig. 2E and 8A; Cheng
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9 197 & Yoshizawa, 2022). The exVIII01 muscle is unique to *Neotroglia*, and is observed in
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12 198 both species (Fig. 3C).

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14 199 The gynosome is composed of the apical sclerite, spiny membrane and basal shaft
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17 200 (Cheng et al., 2023). The gynosome is entirely placed anteriorly to the gonapophyses
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20 201 in the noncopulating state (Fig. 2A). During copulation, all parts other than the basal
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23 202 shaft protrude from the opening between the paraproct and gonapophyses (Fig. 1C, D).

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25 203 There are six groups of muscles (dosp01, exsp01, spIX01, spIX02, gyIX01, gy-01; Fig.
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28 204 2 E, G) connected to the gynosome, and four groups of those muscles are homologous
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31 205 with those associated with the spermapore plates of other psocodeans (Cheng &
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34 206 Yoshizawa, 2022). The gyIX01 (=M5 of Cheng et al., 2023) and gy-01 (= M6 of Cheng
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36
37 207 et al., 2023) muscles are unique to *Sensitibillini*.

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40 41 42 209 **Skeletal and muscle structures of male terminalia**

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44 210 Among the male terminal structures, no obvious specificity was detected for the
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46
47 211 clunium and epiproct. On the ventral side of each paraproct, a ridge is present, which
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50 212 is triangular in the lateral view (Fig. 4A, C). Hook-like protrusions are also observed
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53 213 on the paraproct of some other species of Psocodea but are generally located at the
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56 214 posterior end of the paraproct (Yoshizawa, 2005). One group of muscles is associated
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59 215 with the epiproct (epX01; Fig. 5C), and six groups of muscles with the paraproct

1 216 (paX01-04, papa01, pahy01; Fig. 5C). Among the latter, five groups of muscles
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3 217 originate from the clunium, and one group of muscles originates from and inserts within
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6 218 the paraproct (Fig. 5C). Since the epiproct, paraproct and clunium are present in both
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9 219 sexes and have similar functions, five groups of muscles (epX01, paX01-04) can be
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11 220 homologized between sexes (Fig. 5C and 8B; Cheng & Yoshizawa, 2022). The
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14 221 following two groups of muscles are unique to males.
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17 222 The hypandrium represents sternum IX and articulates laterally with the clunium.
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20 223 The hypandrium has no obvious protrusions in *Neotroglia* (Fig. 4A, C). One group of
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23 224 muscles is connected to the hypandrium (hyVIII01; Figs. 5E), which is homologous to
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26 225 that observed in *Trichadenotecnum* (Fig. 3D).
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29 226 The phallosome consists of two parts: a membranous pouch and a reversed U-
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31 227 shaped thin sclerite surrounding the posterodorsal margin of the entrance of the
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33
34 228 membranous pouch (Figs. 3F, G, 4E and 5A), which is only observed in *Neotroglia*
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37 229 species. The phallosome closely fits into the concavity formed by the hypandrium in a
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39
40 230 noncopulating state (Fig. 4E). During copulation, the sclerotized part of the phallosome
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43 231 is raised upwards, and its apex rests on the protrusions of the paraproctal ridges (Fig.
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45
46 232 4F). There are three groups of muscles (phIX01-02, phVIII01; Figs. 3F-I and 5G, H)
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49 233 connected to the phallosome. Among them, phIX01 and phIX02 were also observed in
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52 234 *Trichadenotecnum* (Fig. 3E).
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54 235 **Male–female genital interaction**

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56 236 In the copulating state, the male paraproct and epiproct are partly retracted inwardly.
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59 237 The ventral paraproctal ridges, which are separated in the noncopulated condition, are
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1 238 closely associated during copulation, together forming a single projection (Fig. 4A-D).
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3 239 The sclerite of the phallosome protrudes upwards, and its apex fits with the anterior
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6 240 margin of the male paraproctal projection (Fig. 4E, F). During copulation, these two
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9 241 male structures securely sandwich the female dorsal valve from both sides (Fig. 6B:
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12 242 blue arrow and Fig. 7: black dotted arrows).

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14 243 The male genital cavity formed by the hypandrium and phallosome is opened and
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17 244 exposed in the copulating condition (Fig. 4B, D). During copulation, the gynosome,
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20 245 with the exception of the basal shaft, extends into the male's genital cavity. The apical
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23 246 sclerite of the gynosome deeply penetrates the membranous pouch of the phallosome
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26 247 (Fig. 6B: red arrow and Fig. 7). The spiny membrane of the gynosome expands to form
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29 248 an internal anchor within the male genital cavity. The female epiproct and paraproct are
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32 249 slightly retracted inwards. The concave ventral surface of the female paraproct and the
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35 250 swollen gynosomal spiny area together anchor the male by sandwiching the
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38 251 hypandrium externally (paraproct) and internally (gynosomal spines) (Fig. 6B: white
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41 252 arrow and Fig. 7: red dotted arrows). The female external valves are opened and grasp
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44 253 the lateral sides of the hypandrium (Figs 1F, 6A and 7).

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47 255 **Discussion**

50 256 **Copulation process and holding mechanisms**

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53 257 The present observation revealed that the following holding/locking systems are at
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56 258 work during copulation to stabilize the coupling condition: (1) the female dorsal valve
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59 259 is tightly sandwiched by the male paraproctal ridges and the tip of the phallosomal

1 260 sclerite; (2) the gynosomal spiny membrane anchors male internally by its inflation
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3 261 within the male genital cavity; (3) the female paraproctal ventral concavities support
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6 262 the ventral surface of the male hypandrium, and the hypandrium is sandwiched by the
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9 263 female paraproct (ventrally) and the gynosomal spines (dorsally); and (4) the female
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12 264 external valves grasp the lateral side of the male abdominal tip.

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14 **265 Fixation of the female dorsal valve by the male**

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17 266 During copulation, the female dorsal valve is almost fully inserted into the male and is
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20 267 tightly held by the male's structures (the paraproctal ridges and the tip of the
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23 268 phallosome sclerite; Figs 6B, red arrow and 7). In the copulating state, dosp01
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26 269 connected to the female dorsal valve is in a relaxed state (Fig. 2F). Therefore, it is
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29 270 assumed that this muscle is related to the re-storage of the dorsal valve at the end of
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32 271 copulation. No muscle related to the protrusion of the dorsal valve were found.

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34 272 Contractions of the male epiproctal and paraproctal muscles (epX01, paX01,
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36 273 paX02, paX03, and pahy01) guide the male epiproct and paraproct to partly retract
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39 274 inwardly (Figs 4C, D and 5D). The paired male paraproctal ridges are tightly associated
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42 275 during copulation, together forming a single process, due to the contraction of paX03
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45 276 (Figs 4B, D and 5D). In the meantime, the contraction of the phVIII01 muscle pulls the
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48 277 phallosome upwards towards the paraproct (Figs 3I and 5H). The phIX01 muscle may
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51 278 also have the function of protruding the phallosome. The merged male paraproctal
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53
54 279 ridges buckle the ventral ridge of the female dorsal valve, and the other side of the
55
56
57 280 female dorsal valve is supported by the tip of the phallosomal sclerite (Figs 6B, blue
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59 281 arrow and 7, black dotted arrows). Furthermore, the contraction of the pahy01 muscle
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1 282 provides strong power to securely make the paraproct hooking and pulling the female
2
3 283 dorsal valve inward. The phIX01 and phIX02 muscles may also produce additional
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5
6 284 power to move the apex of the phallosome inwards (i.e., pull the female dorsal valve
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8
9 285 inwards, Fig. 1E). This clearly shows that the male *Neotrogla* actively holds the female
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11
12 286 during copulation.

13
14 287 Similar movement of the phallosome to hold and fix the female genital structure
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16
17 288 is also known in *Trichadenotecnum* (Psocomorpha: Psocidae: Cheng & Yoshizawa,
18
19
20 289 2019). However, in the case of *Trichadenotecnum*, the male holds the subgenital plate,
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22
23 290 not the dorsal valve. In addition, the phIX01 and phIX02 muscles function to restore
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25
26 291 the phallosome, and the phVIII01 muscle is absent in *Trichadenotecnum*. Therefore,
27
28
29 292 the female holding mechanism is apparently non-homologous between *Neotrogla* and
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31 293 *Trichadenotecnum*.

32
33
34 294 At the end of the copulation, the paX01 and 02 muscles of the males are likely
35
36
37 295 contracted to move the paraproct outwardly, and phIX01 is contracted to restore the
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39
40 296 phallosome. Both movements (active movements by the male) can function to release
41
42
43 297 the grasp on the female dorsal valve.

44 298 **Penetration of the gynosome and anchoring of the male**

45
46
47 299 To open the male genital cavity is very likely a prerequisite for the insertion of the
48
49
50 300 gynosome by a female. This is probably caused by two factors: 1) retraction of the male
51
52
53 301 paraprocts (see above), which are located above the genital chamber under
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55
56 302 noncopulating conditions, thus blocking the male genital opening, and 2) backwards
57
58
59 303 movement of the posterior margin of the hypandrium. There is no muscle that causes
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1 304 direct backwards movement of the posterior margin of the hypandrium. However, as
2
3 305 seen from Fig. 4B, the male terminalia are strongly compressed laterally during
4
5
6 306 copulation, which is very likely to cause the indirect backwards movement of the
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8
9 307 posterior margin of the hypandrium (such as the mouth of a spring-loaded coin purse).
10
11 308 From the reconstructed 3D model of the copulating condition, two factors can be
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13
14 309 assumed to be associated with the lateral compression of the male terminalia: 1)
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16
17 310 contraction of the pahy01, phIX01 and 02 muscles causes the inward movement of the
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19
20 311 lateral side of the hypandrium (i.e., male active movement); 2) grasping by the female
21
22
23 312 external valves causes compression force of the male terminalia (i.e., passive movement
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25
26 313 for the male: see below).

27
28 314 The phIX01 and phIX02 muscles share the same origin on the hypandrium,
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30
31 315 together forming a triangular configuration with the sclerite of the phallosome (Fig. 3H,
32
33
34 316 I). This state allows the hypandrium to move more effortlessly because the forces
35
36
37 317 generated by the two muscles are in the same plane. If the insertion site of these two
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39
40 318 muscles on the hypandrium is not consistent, it is difficult to ensure that all the forces
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43 319 are on the same plane, which will easily lead to conflicting forces. A similar
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46 320 arrangement of the muscles has also been observed in the female external valves
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49 321 (exIV01 and exIV02) of other psocodeans, such as *Lepinotus* and Amphientomidae,
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51
52 322 which is also considered to cause effective movements of the valve (Cheng &
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54
55 323 Yoshizawa, 2022).

56 324 Regarding the muscles attached to the gynosome, gy-01 relaxes and gyIX01
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58
59 325 contracts in the copulating condition, and the status of these two muscles vice versa in
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1 326 the non-copulating condition. Therefore, the contraction of gyIX01 apparently has a
2
3 327 function to extend the gynosome, and gy-01 works to restore the stuck gynosome to the
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5
6 328 resting position (Fig. 2G, H). Two stout muscles, spIX01 and spIX02, relax during
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8
9 329 copulation, thus restoring the gynosome membrane to its original shape and position
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11 330 (Fig. 2G, H). The apical sclerite of the gynosome deeply penetrates the membranous
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13
14 331 pouch of the phallosome during copulation, from which the semen is transported to the
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16
17 332 female (Yoshizawa et al., 2014). The spiny membrane of the gynosome also enters the
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19
20 333 male genital cavity (Figs. 1C, D, 6B and 7). Because no muscle is involved in inflating
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22
23 334 the spiny membrane, the anchoring function of this specialized membrane is likely
24
25
26 335 achieved by increased body pressure. See also the next section for the additional
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28
29 336 holding function of the gynosomal spines.

30
31 337 At the end of copulation, reduction of the internal pressure can cause the deflation
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33
34 338 of the spiny membrane, and the contraction of the spIX01 and 02 muscles acts to restore
35
36
37 339 the gynosomal spiny membrane. The gynosome is retracted by the contraction of the
38
39
40 340 gy-01 muscle.

41 42 341 **Ventral fixation of the hypandrium**

43
44 342 The female paX01 and paX02 muscles are contracted during copulation, causing the
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46
47 343 paraproct to be slightly indented in the direction of the female epiproct (Fig. 2D). The
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49
50 344 female paX03 muscle retracts during copulation, directing the protrusions on the ventral
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52
53 345 surface of the female paraproct inward (Figs. 1D and 2D). By this movement, the
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56 346 ventrolateral paraproctal concavities form a continuous single circular concavity, which
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58
59 347 fits perfectly to the curve of the ventral margin of the hypandrium (Fig. 6B, white
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1 348 arrow).

2
3 349 The jointed female paraproctal cavities support the ventral surface of the male
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5
6 350 hypandrium externally during copulation, which sandwich the hypandrium together
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8
9 351 with the gynosomal spiny anchor within the male genital chamber (Figs. 6B, white
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11
12 352 arrow and 7). Therefore, it is apparent that the female actively holds the male during
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14
15 353 copulation by using the female paraproct and gynosome. Such close contact between
16
17 354 the female paraproct and the male hypandrium is not observed in *Trichadenotecnum*
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19
20 355 (Cheng & Yoshizawa, 2019).

21
22
23 356 At the end of copulation, paX01 and 02 are probably contracted to release the
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25
26 357 ventral paraproctal support of the male hypandrium.

27 28 358 **Grasping the male abdomen by the female external valves**

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30
31 359 The exIX02 muscle is contracted during copulation and thus is associated with the
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33
34 360 active opening of the female external valve at the beginning of copulation (Fig. 1C, F).

35
36 361 The female external valves grasp the lateral surface of the male hypandrium with
37
38
39 362 the contraction of the stout doex01 and exVIII01 muscles (Figs. 2E, F, 6A and 7). As
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41
42 363 also mentioned above, this probably comprises two functions: opening the male genital
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44
45 364 cavity at the beginning of copulation and holding the male during copulation, both of
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47
48 365 which are active movements of the female.

49
50 366 At the end of copulation, exIX02 is probably contracted to open the external valves
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52
53 367 to release the grasp on the male.

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56 368 The functions of the muscles related to the external valve (opening/closing the
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58
59 369 external valves) are also common in *Trichadenotecnum*. However, the external valve

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1 370 of *Trichadenotecnum* does not have a function to grasp the male, and its closure muscles
2
3 371 hold the female subgenital plate, possibly to resist coercive mating by males (Cheng &
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6 372 Yoshizawa, 2019).
7

8
9 **373 Sexual selection**

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11 374 In animals with conventional sex roles, females usually gain fewer fitness benefits from
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13
14 375 multiple matings, and thus, males more actively seek mating opportunities (Trivers,
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16
17 376 1972). Accordingly, male animals sometimes develop elaborate genital structures for
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20 377 coercive copulation with unwilling mates (Arnqvist & Rowe, 2002; Lange et al., 2013).
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22
23 378 As counteradaptations, two types of traits are known to occur in female genitals:
24
25 379 “resistance” traits that decrease the male fitness component (e.g., efficiency of mate
26
27
28 380 holding) and “tolerance” traits that mitigate the costs of mating without affecting male
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31 381 mating success (Reinhardt et al., 2014). In *Neotrogla*, males give nutritious seminal
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33
34 382 gifts to females during copulation, which last up to 72 hours, and females mate multiply,
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37 383 as is evident from the occurrence of multiple seminal capsules in the spermatheca
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39 384 (Yoshizawa et al., 2014, 2018a). In addition, female *Neotrogla* possess twin
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41
42 385 insemination slots with switching valves to receive double seminal gifts at the same
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45 386 time (Yoshizawa et al., 2018a; Kamimura et al., 2021). Therefore, it has been assumed
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47
48 387 in previous studies that female *Neotrogla* gain more net benefits from multiple
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51 388 copulations than males and that females actively control copulatory processes by using
52
53 389 the unique intromittent organ (coupling-role reversal: Yoshizawa et al., 2014, 2019).

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56 390 Our present study confirmed this view by showing that the multiple female organs
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59 391 actively (and probably partly coercively) hold the male mate: this is achieved by
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1 392 anchoring within the male genital chamber using the gynosomal spines and external
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3 393 grasping using the female paraproct and the external valves. However, at the same time,
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6 394 the 3D models also revealed that autonomous movements of male genital muscles are
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9 395 necessary for the initiation of the copulatory processes and that male *Neotrogla* actively
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11
12 396 hold the female mate during copulation.

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14 397 The male paraproctal ridges and the tip of the phallosomal sclerite are tightly
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17 398 associated even in the non-copulating condition in both *N. curvata* and *N. brasiliensis*
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20 399 (Figs. 4A, C and 6C, D) and, without active upwards movement of the paraproct
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23 400 (contraction of the paX01 and 02 muscles) of the male and downwards movement of
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26 401 the phallosome (contraction of the phIX02 muscle), the female cannot insert the dorsal
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28 402 valve into the genital cavity. To open the genital cavity is the most important
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30
31 403 prerequisite for gynosomal insertion, and although its opening may partly be achieved
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34 404 by the female's active movements (i.e., grasping of the terminalia using the external
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37 405 valve), some male muscles (pahy01, phIX01 and phIX02) seem to be related to the
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39 406 autonomous opening of the genital cavity. These male structures may also act to resist
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41
42 407 coercive copulation by females.

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45 408 In addition, the male securely fixes the female dorsal valve during copulation using
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48 409 the male paraproctal ridges and the phallosomal sclerite. The well-developed male
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51 410 muscles related to this fixation function, including some uniquely developed in
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53 411 *Neotrogla*, strongly suggest that the secure holding of the mate during copulation is
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55
56 412 also crucial for the male.

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59 413 For some animals with conventional sex roles, mismatching in genital coupling is
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1 414 known to result in leakage of seminal fluid, which can cause the female and male bodies
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3 415 to stick together and thus reduce the rate of sperm transfer (Kamimura & Mistumoto,
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5
6 416 2012; Tanaka et al., 2018; Polak & McEvey, 2022), or increasing the wounding
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9 417 probability of the female genitals (Sota & Kubota, 1998; Kamimura, 2012; Masly &
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11
12 418 Kamimura, 2014). In many of those cases, the females develop specific structures (such
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14
15 419 as membranous pockets) that accommodate the wound-inflicting structures of the male
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18 420 genitalia and thus mitigate the copulatory costs (Sota & Kubota, 1998; Kamimura,
19
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21 421 2012). Interestingly, male *Neotroglia* possess species-specific pockets on the walls of
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23
24 422 the genital chamber to receive gynosomal spines, although no copulatory wounds have
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26
27 423 been detected there (Yoshizawa et al., 2014). Given that the copulatory mechanism of
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30 424 *Neotroglia* is complicated, seminal fluid passed to the female is voluminous and
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32
33 425 possibly reactive (Yoshizawa et al., 2014). Considering that the females' anchoring
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35
36 426 power is very strong (an artificial trial to separate a coupled specimen led to separation
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38
39 427 of the male abdomen from the thorax without breaking the genital coupling: Yoshizawa
40
41
42 428 et al., 2014), a precisely connected genital complex and step-by-step releases of each
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44
45 429 holding mechanism achieved by the cooperation of both sexes are probably beneficial
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47
48 430 for both males and females. Such concordance of the interests between the sexes may
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50
51 431 have prompted the evolution of "tolerance" traits in male *Neotroglia*, as in a completely
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54 432 sex-role reversed mating scenario.

55 433

56 434 **Conclusion**

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58 435 Inverted genital couplings of *Neotroglia* provide an extremely rare opportunity to test
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1 436 hypotheses on the evolution of genital structures (House & Simmons, 2006; Simmons,
2
3 437 2014; Michels et al., 2015) under the reversed direction of sexual selection. We found
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5
6 438 evidence of the complicated coexistence of genital structures, suggesting male
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9 439 resistance, female coercion, and cooperation between the sexes. The absence of harmful
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11
12 440 structures that inflict wounds on the opposite sex in both males and females is notable.
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14
15 441 The coevolution between the male and female genital structures in *Neotroglia* may
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17
18 442 provide a new example for the evolution of tolerance traits, a newly proposed idea
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21 443 concerning genital evolution (Michels et al., 2015).

22
23 444 For further understanding of the sexual selection and evolution of novelties,
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26 445 detailed information of the courtship behaviour of *Neotroglia* and its relatives is key but
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29 446 is very poorly understood to date. Accumulation of basic behavioural information is
30
31
32 447 highly desired for a more accurate and detailed understanding of the evolution of the
33
34
35 448 gynosome in *Neotroglia*.

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37 449

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5 458 **Declarations**

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8 459 **Conflict of Interest** The authors declare no conflict of interests

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14 461 **References**

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1 549 **Figure Legends**

2
3 550 **Fig. 1.** 3D reconstruction of the female terminalia of *Neotroglia curvata*; (for E-G left:
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6 551 noncopulating state; right: copulating state): (A) ventral view, noncopulating state; (B)
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8 552 posterior view, noncopulating state; (C) ventral view, copulating state; (D) posterior
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10 553 view, copulating state; (E) lateral view (highlighting dorsal valve); (F) lateral view
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12 554 (highlighting the external valve); (G) lateral view (highlighting the gynosome).
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17 555 Abbreviations: ep = epiproct; pa = paraproct; cl = clunium; st = sternum; gy =
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19 556 gynosome; do = dorsal valve; ex = external valve.
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22 557 **Fig. 2.** 3D reconstruction of the female terminalia of *Neotroglia curvata*, internal view;
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24 558 noncopulating state (A, C, E and G); copulating state (B, D, F, and H); highlighting
25
26 559 different structures and associated muscles (C-H): (1) epX01; (2) paX01; (3) paX02;
27
28 560 (4) paX03; (5) paX04; (6) dosp01; (7) doex01; (8) exsp01; (9) exIX02; (10) exVIII01;
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30 561 (11) spIX01; (12) spIX02; (13) gygy01; (14) gy-01. See Fig. 1 for abbreviations.
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40 563 **Fig. 3.** 3D reconstruction of the terminalia in the noncopulating state (A-H) and
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42 564 copulating state (I), highlighting different structures and associated muscles: female
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44 565 *Neotroglia curvata* (A,B); female *N. brasiliensis* (C); male *Trichadenotecnum*
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46 566 *pseudomedium* (D,E); male *N. brasiliensis* (F,G); male *N. curvata* (H,I). (A-C) Ventral
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48 567 view; (D-F) internal view; (G-I) lateral view. Abbreviations: ep = epiproct; pa =
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50 568 paraproct; hy = hypandrium; cl = clunium; ph = phallosome.
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59 570 **Fig. 4.** 3D reconstruction of the male terminalia of *Neotroglia curvata*, noncopulating
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1 571 state (A, C and E); copulating state (B, D and F): (A, B) posterior view; (C, D) lateral
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3 572 view; (E, F) lateral view (highlighting the phallosome). Abbreviations: sg = segment
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6 573 VIII; m= membrane; for others, see Fig. 3.

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9 574 **Fig. 5.** 3D reconstruction of the male terminalia of *Neotroglia curvata*, internal view;
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11 575 noncopulating state (A, C, E and G); copulating state (B, D, F and H), highlighting
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13 576 different structures and associated muscles (D-H): (1) epX01; (2) paX01; (3) paX02;
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15 577 (4) paX03; (5) paX04; (6) pahy01; (7) papa01; (8) hyVIII01; (9) phIX01; (10) phIX02;
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17 578 (11) phVIII01. Abbreviations: te = tergum; m= membrane; for others, see Fig. 3.

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20 579 **Fig. 6.** *Neotroglia curvata*, 3D reconstruction of terminalia of the copulating pair, lateral
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22 580 view (A,B); *N. brasiliensis*, 3D reconstruction of terminalia of the male in the
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24 581 noncopulating state (C,D). (B) highlighting the gynosome, phallosome, paraproct (m)
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26 582 and epiproct (m); (C) posterior view; (D) lateral view, highlighting the phallosome and
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28 583 paraproct (m). See Figs. 1 and 3 for abbreviations.

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31 584 **Fig. 7.** Schematic drawing of the male and female genitalia of *Neotroglia curvata*
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33 585 in copulated condition. Gray indicates male structures, and orange indicates female
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35 586 ones. Black arrows indicate holding system by male, and red arrows indicate that by
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37 587 female.

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40 588 **Fig.8.** *Neotroglia curvata* in an uncopulated state, putative groundplan configuration of
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42 589 the terminalia (internal view), schematic (based on the 3D reconstruction of the *N.*
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44 590 *curvata*). (a) female; (b) male. Lines indicate muscles. Except for muscle paX04, all
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46 591 dotted lines indicate the muscles attached to the gynosome membrane. (1) epX01; (2)
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48 592 paX01; (3) paX02; (4) paX03; (5) paX04; (6) dosp01; (7) doex01; (8) exsp01; (9)

1 593 exIX02; (10) exVIII01; (11) spIX01; (12) spIX02; (13) gygy01; (14) gy-01; (15)
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3 594 pahy01; (16) papa01; (17) hyVIII01; (18) phIX01; (19) phIX02; (20) phVIII01. See
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6 595 Figs. 1 and 3 for abbreviations.
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Figure 1

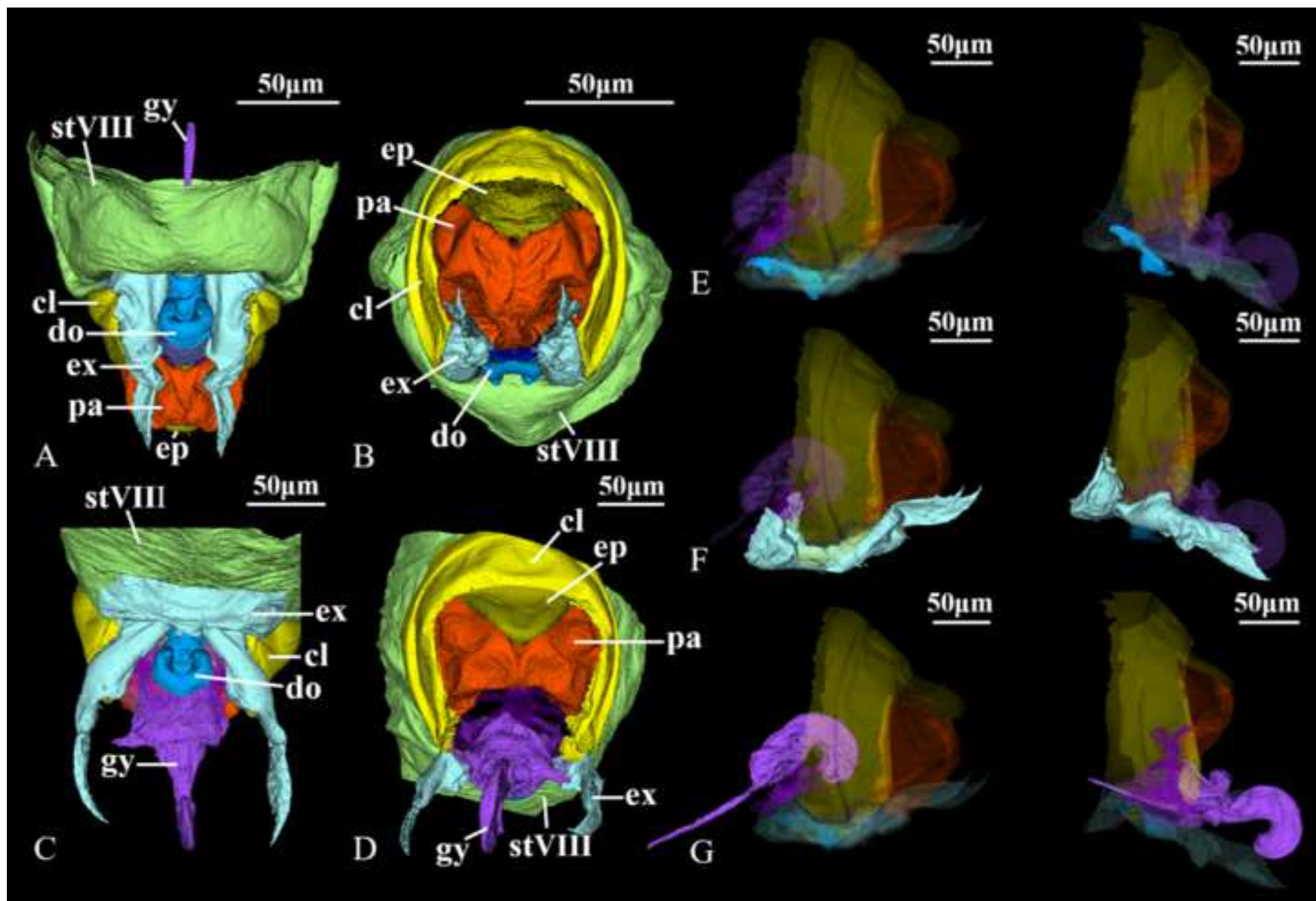


Figure 2

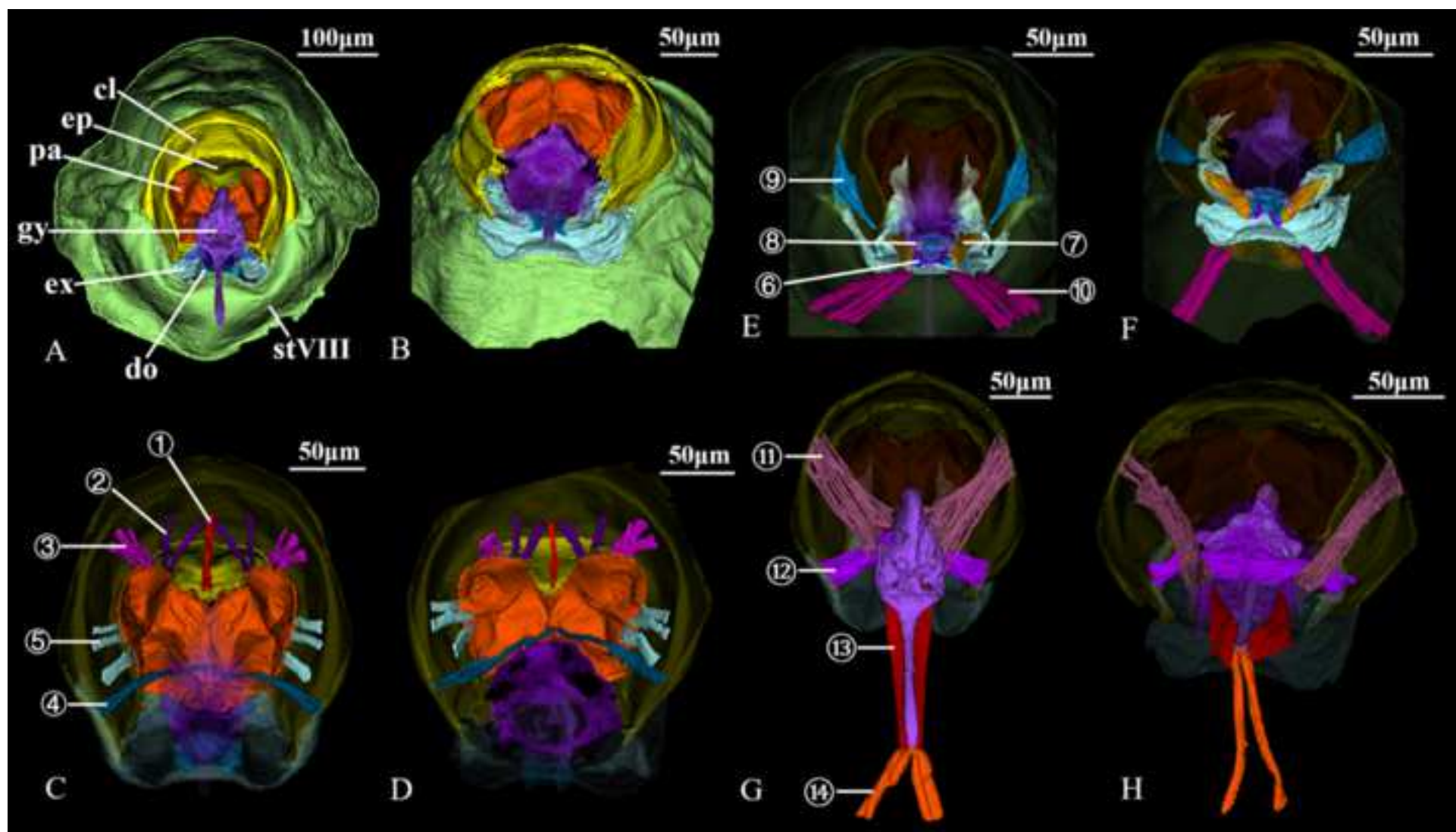


Figure 3

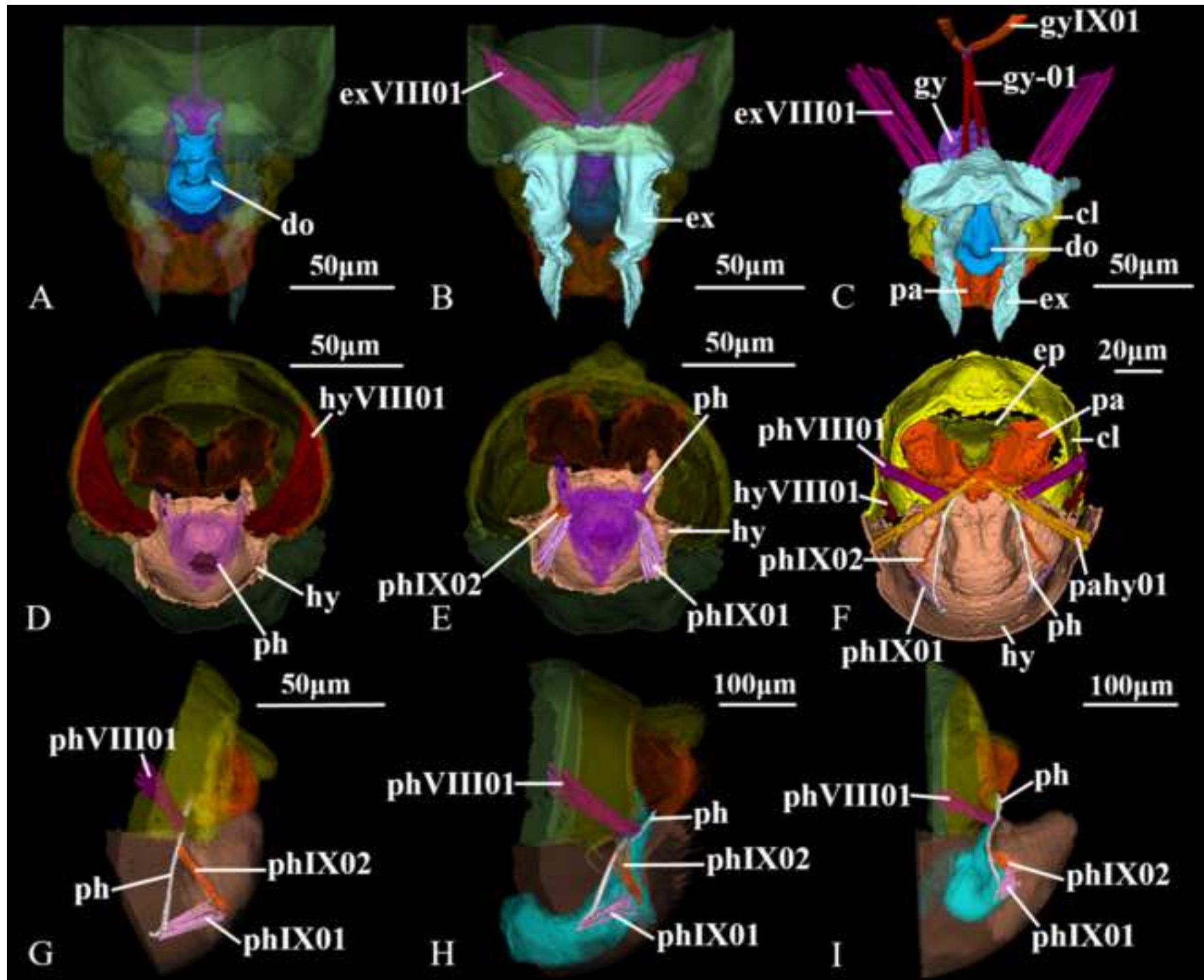


Figure 4

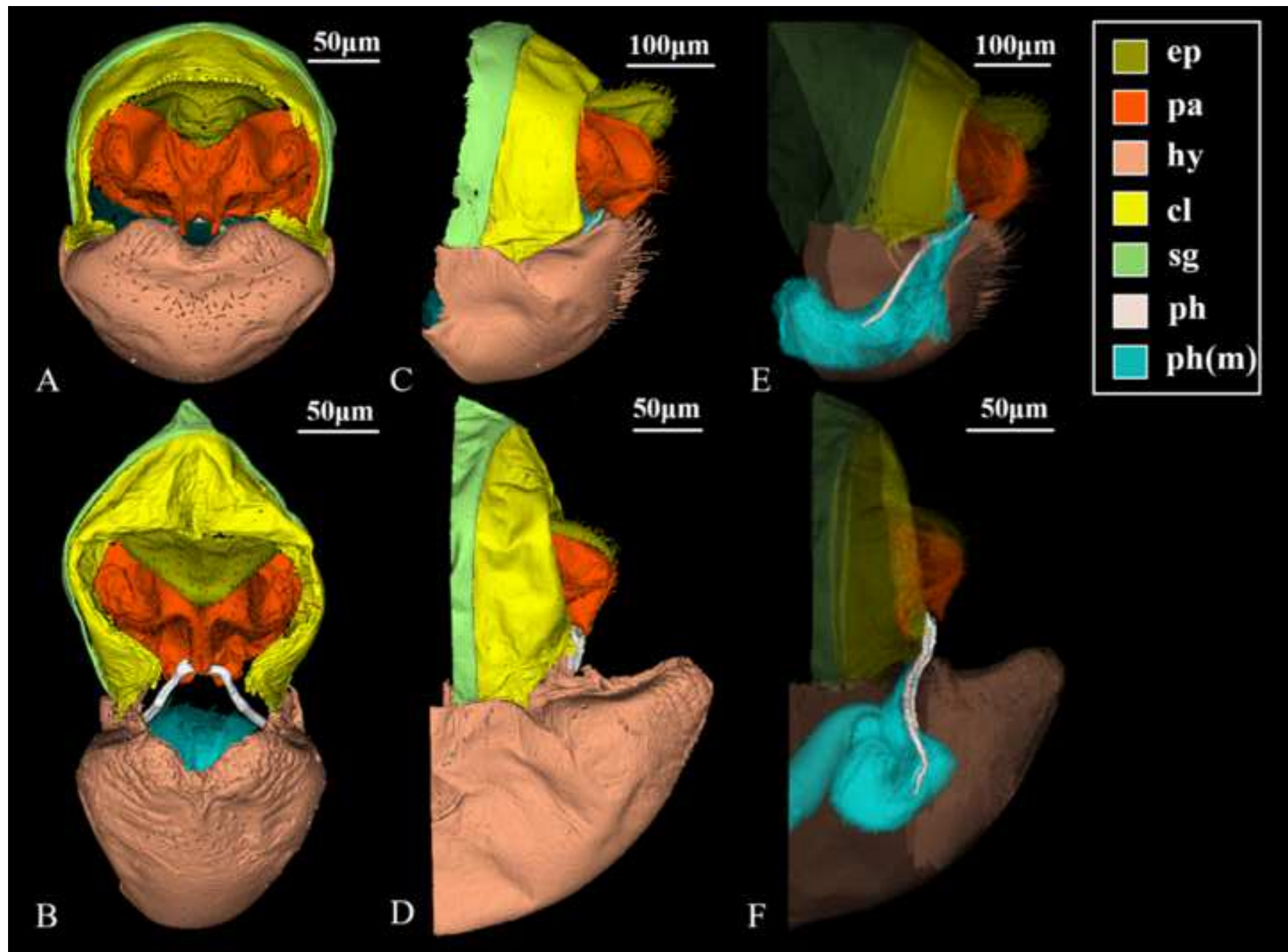


Figure 5

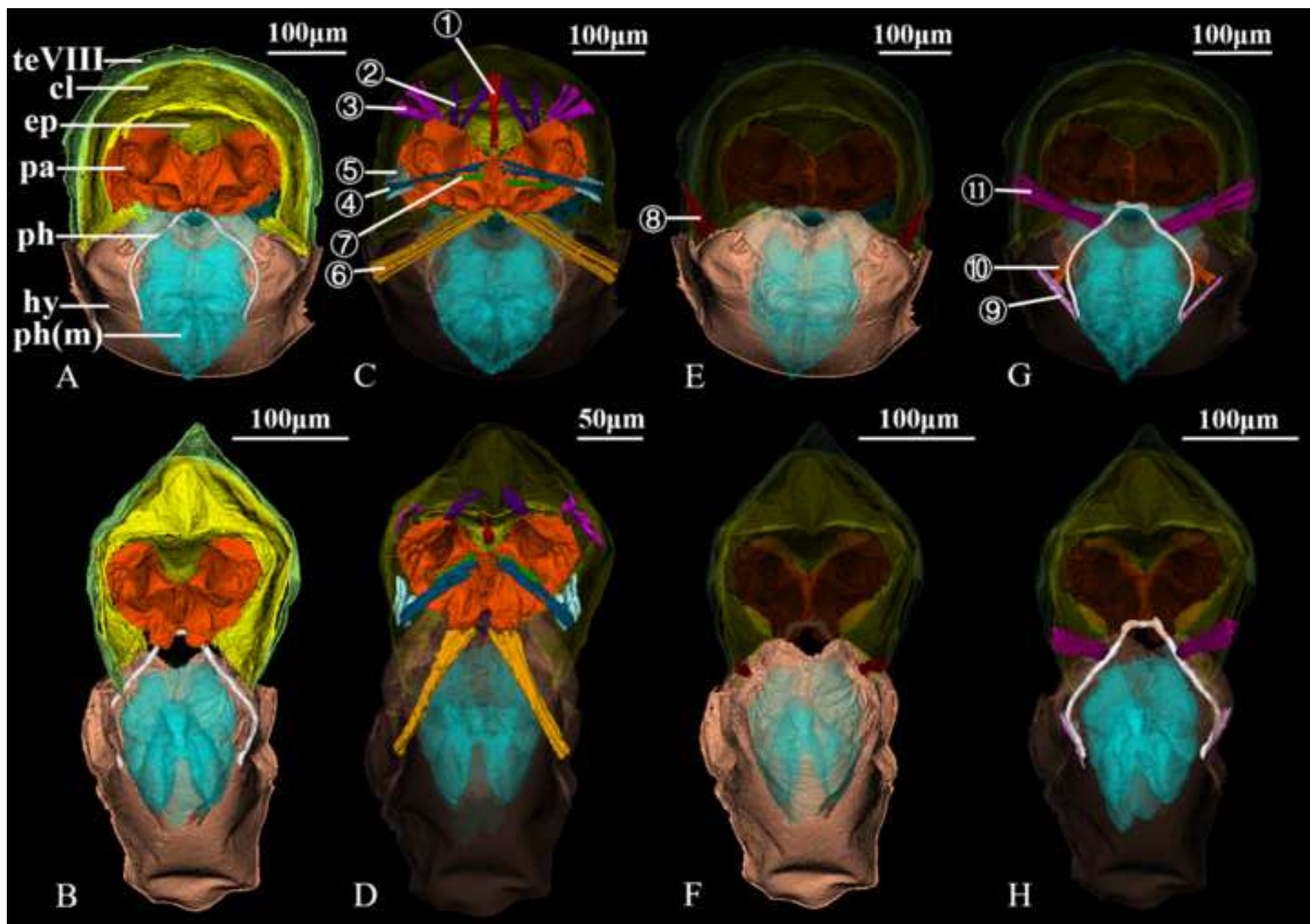


Figure 6

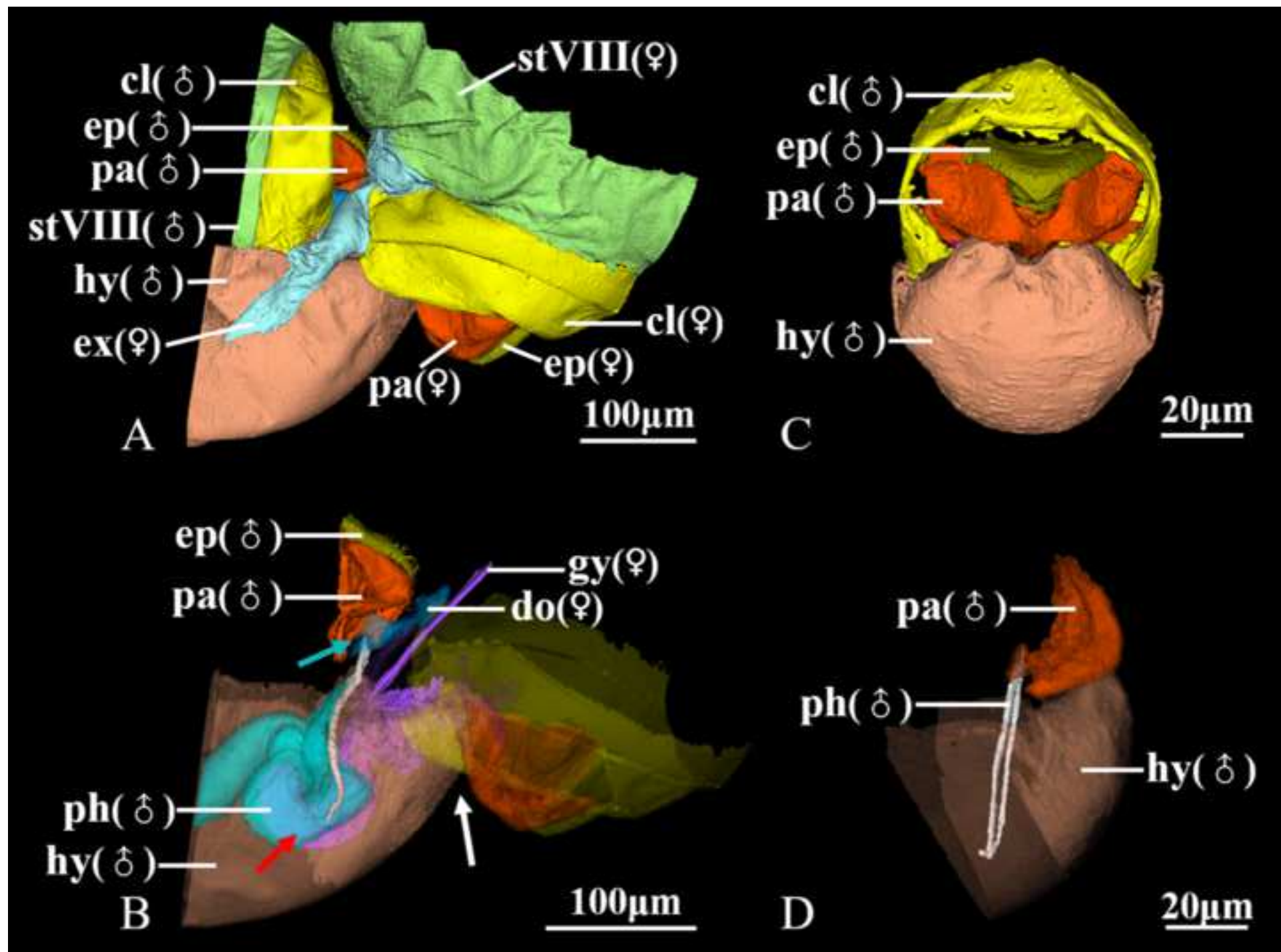


Figure 7

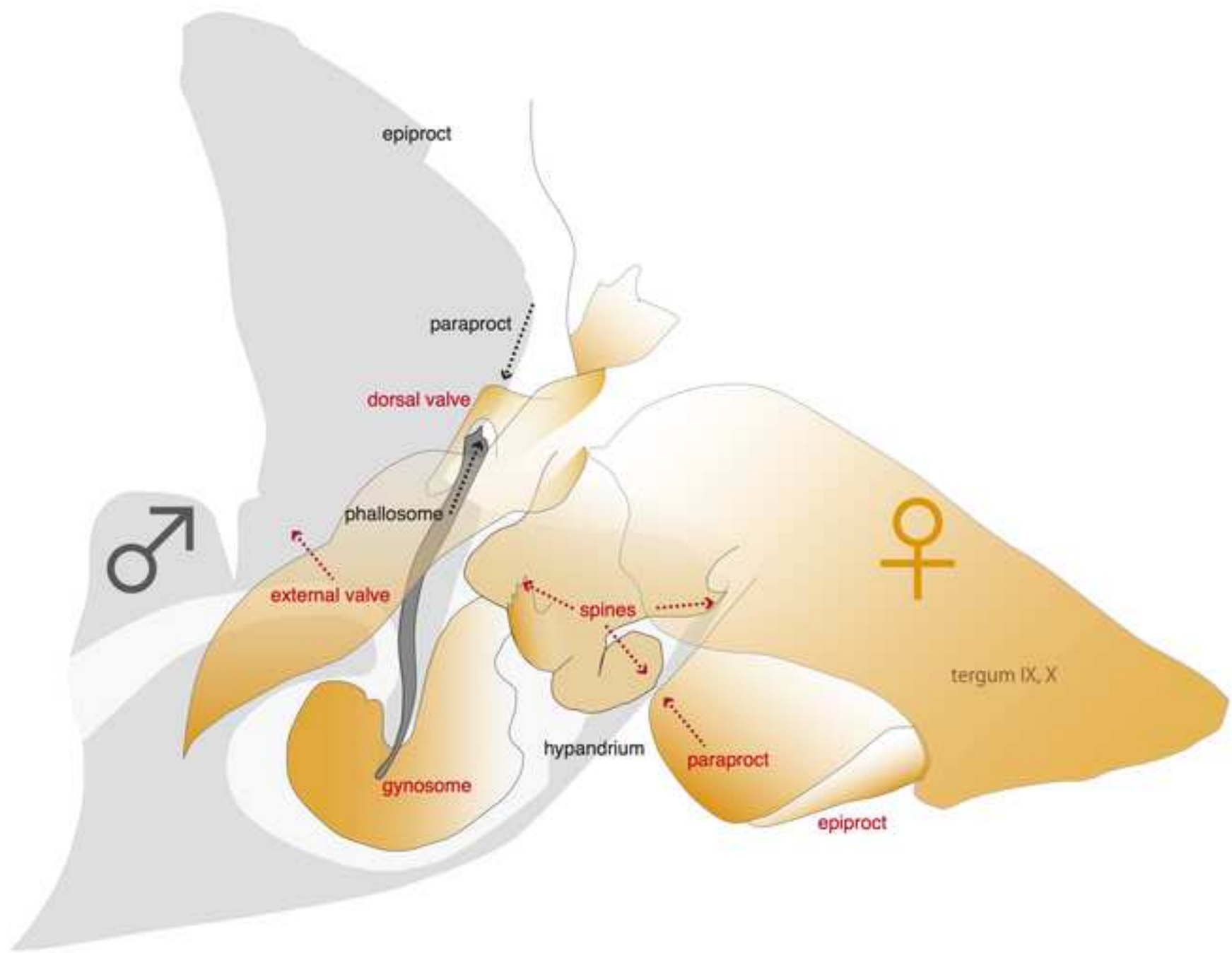


Figure 8

