Are males just passive? Coupling mechanism of the Brazilian cave insects with inverted genitalia

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Abstract

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

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

Keywords

genitalia, copulatory processes, sexual selection, muscles

Introduction

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

All these studies focused on the intromittent female penis, but the morphology and function of other genital structures in these sex-reversed insects are still poorly understood. Referring to muscle homology, Cheng et al. (2023) determined that the female penis evolved from the spermapore plate, and its unique intromittent function is brought by two pairs of novel muscles formed only within the Sensitibillini. Are there novel structures and muscles in the other genital structures? What functions do other genital structures have during copulation? Do females copulate coercively, and are

males just passive for mating? What types of sexual selection are strongly reflected in the copulatory processes? All these questions need to be answered under the overall grasp of the structures of male and female genitalia and the copulatory processes of these cave insects with inverted genitalia.

In this study, by using the synchrotron μCT technique, we present 3D models of the whole male and female genital structures and their associated muscles of *Neotrogla curvata*. By comparing the morphological changes of the genital structures and associated muscles before and during copulation, we clarify the functions of these structures and estimate the complete copulatory processes. We also analysed the homology of the muscles between *Neotrogla* species and the other non-genital-reversed Psocodea and traced the origin of each structure and muscle of *Neotrogla*.

Materials and Methods

A copulating pair and a noncopulating male and female of the coupling-role reversed cave psocid *Neotrogla curvata* Lienhard & Ferreira, 2013 (Trogiomorpha) were examined. A noncopulating male and female of *N. brasiliensis* Lienhard, 2010 (Lienhard et al., 2010) and noncopulating male specimens of *Trichadenotecnum pseudomedium* Yoshizawa, 2001 (Psocomorpha: Cheng & Yoshizawa, 2019), the latter of which has normal genital structures, were also examined for comparison. All samples were subjected to μCT examination, and voucher specimens were stored in the Hokkaido University Insect Collection. Samples were fixed either with hot water, FAA solution (formalin:alcohol:acetic acid = 6:16:1), or 80% ethanol and then preserved in

80% ethanol. Dehydration was conducted in ascending order with 80–100% ethanol before drying them at the critical point (EM CPD300, Leica, Wetzlar, Germany) to remove water without serious organ shrinkage. Samples were then scanned by synchrotron μCT at the BL47XU (Uesugi et al., 2012) beamline of the Super Photon ring-8 GeV (SPring-8; Hyogo, Japan) using a stable beam energy of 8 keV in absorption-contrast mode. The tomography system consists of a full-field X-ray microscope with Fresnel zone plate optics (Uesugi et al., 2017). We used semiautomatic segmentation algorithms based on grey-value differences in ITK-SNAP software (Yushkevich et al., 2006) to obtain 3D representations of the terminalia of all three species.

Results

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

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

epX01 (male/female) (Figs. 2C, D and 8A: = 01 of Cheng & Yoshizawa, 2019); O: mid-dorsal site of clunium (segment IX); I: posterior end of the epiproct; and F: closure

and/or flipping of the epiproct.

paX01 (male/female) (Figs. 2C, D and 8A: = 02 of Cheng & Yoshizawa, 2019); O: anterolateral region of the clunium (segment X); I: anterodorsal end of the paraproct, very close to the posterolateral margin of the epiproct; and F: involved in opening the paraproct.

paX02 (male/female) (Figs. 2C, D and 8A: = 02 of Cheng & Yoshizawa, 2019); O: mediodorsal region of the clunium (segment X); I: anterodorsal end of the paraproct, very close to the anterolateral corner of the epiproct; and F: involved in opening the paraproct.

paX03 (male/female) (Figs. 2C, D and 8A: = 03 of Cheng & Yoshizawa, 2019); O: mediolateral region of the clunium (segment X); I: internal margin of the paraproct near the anal opening; and F: involved in retracting the paraproct.

paX04 (male/female) (Figs. 2C, D and 8A); O: mediolateral region of the clunium (segment IX); I: anterolateral margin of the paraproct; and F: involved in retracting the paraproct.

dosp01 (female) (Figs. 2E, F and 8A: = 05 of Cheng & Yoshizawa, 2019); O: base of the dorsal valve; I: on the membrane connected to the spermapore plate/gynosome, near the posterior tip of the spermapore plate; and F: involved in restoring the position of the gynosome (spermapore plate) and the dorsal valve.

doex01 (female) (Figs. 2E, F and 8A); O: base of the dorsal valve; I: base of the external

valve; and F: involved in restoring the position of the dorsal valve and closure of the external valve.

exsp01 (female) (Figs. 2E, F and 8A: = 06 of Cheng & Yoshizawa, 2019); O: base of the external valve; I: on the membrane connected to the spermapore plate/gynosome, near the posterior tip of the spermapore plate; and F: involved in restoring the position of the gynosome (spermapore plate) and closure of the external valve.

exIX02 (female) (Figs. 2E, F and 8A: = 08 of Cheng & Yoshizawa, 2019); O: anterolateral margin of the clunium; I: base of the external valve, near the middle of the junction with the clunium; and F: involved in opening the external valve.

exVIII01 (female) (Figs. 2E, F and 8A); O: medioventral region of sternum VIII; I: base of the external valve; and F: involved in closure of the external valve.

spIX01 (female) (Figs. 2G, H and 8A); O: mediolateral region of the clunium; I: on the spermapore membrane; and F: involved in restoring the gynosomal spiny membrane (*Neotrogla*) or the position of the spermapore plate (other Psocodea).

spIX02 (female) (Figs. 2G, H and 8A); O: mediolateral region of the clunium; I: on the spermapore membrane; and F: involved in restoring the gynosomal spiny membrane (*Neotrogla*) or the position of the spermapore plate (other Psocodea).

gyIX01 (female) (Figs. 2G, H and 8A); O: gynosome membrane very close to dorsal valves (=ventral membrane of segment IX); I: anterior end of the basal shaft; and F: protractors of the gynosome.

gy-01 (female) (Figs. 2G, H and 8A); O: an internal organ (specific insertion site not detected); I: anterior end of the basal shaft; and F: retractors of the gynosome.

papa01 (male) (Figs. 5C, D and 8B); O: internal margin of the paraproct near the anal opening; I: anterolateral margin of the paraproct; and F: involved in opening the anus.

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

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

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

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

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

Skeletal and muscle structures of female terminalia

Among the female terminal structures of *Neotrogla*, the morphology of the clunium and

the epiproct is not much different from those of the other females of Psocodea. In contrast, the subgenital plate (posterior extension of the 8th sternum) is completely reduced in *Neotrogla*. A distinct difference was also detected in the paraproct between *Neotrogla* and other psocodeans, i.e., two concavities are present on the ventrolateral surface of the paraproct in *Neotrogla* (Fig. 1B). One group of muscles (epX01; Fig. 2C) is associated with the epiproct, and four groups of muscles (paX01-04; Fig. 2C) with the paraproct, all of which originate from the clunium. All of these muscles are homologous to those associated with the epiproct or paraproct of other psocodeans (Cheng & Yoshizawa, 2022).

The bases of the external valve of the gonapophyses are connected by a transverse sclerite (Fig. 3B). The dorsal valve of the gonapophyses is located posteromedially to the transverse sclerite of the external valves and forms an independent structure (Fig. 1A). The dorsal valve is not truly a paired structure like the external valves. It is usually paired in Psocodea, but forms a single lobe-like structure with a posterior and lateral marginal ridge shaped like a chair with a central depression (Fig. 3A). Two groups of muscles (dosp01 and doex01; Fig. 2E) are connected to the dorsal valve. They are homologous with those associated with the dorsal valves of other psocodeans (Cheng & Yoshizawa, 2022).

The external valves form a pair of projections, together forming a crab claw-like structure, basally connected by a transverse sclerite. The transverse sclerite is laterally articulated with the clunium and anteriorly connected with sternum VIII (Fig. 1A, C). The external valves of both *Neotrogla* species show a similar morphology (Fig. 3C).

There are four groups of muscles (doex01, exsp01, exIX02, exVIII01; Fig. 2E) connected to the external valve, and three groups of those muscles are homologous with those associated with the external valves of other psocodeans (Fig. 2E and 8A; Cheng & Yoshizawa, 2022). The exVIII01 muscle is unique to *Neotrogla*, and is observed in both species (Fig. 3C).

The gynosome is composed of the apical sclerite, spiny membrane and basal shaft (Cheng et al., 2023). The gynosome is entirely placed anteriorly to the gonapophyses in the noncopulating state (Fig. 2A). During copulation, all parts other than the basal shaft protrude from the opening between the paraproct and gonapophyses (Fig. 1C, D). There are six groups of muscles (dosp01, exsp01, spIX01, spIX02, gyIX01, gy-01; Fig. 2 E, G) connected to the gynosome, and four groups of those muscles are homologous with those associated with the spermapore plates of other psocodeans (Cheng & Yoshizawa, 2022). The gyIX01 (=M5 of Cheng et al., 2023) and gy-01 (= M6 of Cheng et al., 2023) muscles are unique to Sensitibillini.

Skeletal and muscle structures of male terminalia

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

(paX01-04, papa01, pahy01; Fig. 5C). Among the latter, five groups of muscles originate from the clunium, and one group of muscles originates from and inserts within the paraproct (Fig. 5C). Since the epiproct, paraproct and clunium are present in both sexes and have similar functions, five groups of muscles (epX01, paX01-04) can be homologized between sexes (Fig. 5C and 8B; Cheng & Yoshizawa, 2022). The following two groups of muscles are unique to males.

The hypandrium represents sternum IX and articulates laterally with the clunium. The hypandrium has no obvious protrusions in *Neotrogla* (Fig. 4A, C). One group of muscles is connected to the hypandrium (hyVIII01; Figs. 5E), which is homologous to that observed in *Trichadenotecnum* (Fig. 3D).

The phallosome consists of two parts: a membranous pouch and a reversed U-shaped thin sclerite surrounding the posterodorsal margin of the entrance of the membranous pouch (Figs. 3F, G, 4E and 5A), which is only observed in *Neotrogla* species. The phallosome closely fits into the concavity formed by the hypandrium in a noncopulating state (Fig. 4E). During copulation, the sclerotized part of the phallosome is raised upwards, and its apex rests on the protrusions of the paraproctal ridges (Fig. 4F). There are three groups of muscles (phIX01-02, phVIII01; Figs. 3F-I and 5G, H) connected to the phallosome. Among them, phIX01 and phIX02 were also observed in *Trichadenotecnum* (Fig. 3E).

Male–female genital interaction

In the copulating state, the male paraproct and epiproct are partly retracted inwardly. The ventral paraproctal ridges, which are separated in the noncopulated condition, are

closely associated during copulation, together forming a single projection (Fig. 4A-D). The sclerite of the phallosome protrudes upwards, and its apex fits with the anterior margin of the male paraproctal projection (Fig. 4E, F). During copulation, these two male structures securely sandwich the female dorsal valve from both sides (Fig. 6B: blue arrow and Fig. 7: black dotted arrows).

The male genital cavity formed by the hypandrium and phallosome is opened and exposed in the copulating condition (Fig. 4B, D). During copulation, the gynosome, with the exception of the basal shaft, extends into the male's genital cavity. The apical sclerite of the gynosome deeply penetrates the membranous pouch of the phallosome (Fig. 6B: red arrow and Fig. 7). The spiny membrane of the gynosome expands to form an internal anchor within the male genital cavity. The female epiproct and paraproct are slightly retracted inwards. The concave ventral surface of the female paraproct and the swollen gynosomal spiny area together anchor the male by sandwiching the hypandrium externally (paraproct) and internally (gynosomal spines) (Fig. 6B: white arrow and Fig. 7: red dotted arrows). The female external valves are opened and grasp the lateral sides of the hypandrium (Figs 1F, 6A and 7).

Discussion

Copulation process and holding mechanisms

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

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

Fixation of the female dorsal valve by the male

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

Contractions of the male epiproctal and paraproctal muscles (epX01, paX01, paX02, paX03, and pahy01) guide the male epiproct and paraproct to partly retract inwardly (Figs 4C, D and 5D). The paired male paraproctal ridges are tightly associated during copulation, together forming a single process, due to the contraction of paX03 (Figs 4B, D and 5D). In the meantime, the contraction of the phVIII01 muscle pulls the phallosome upwards towards the paraproct (Figs 3I and 5H). The phIX01 muscle may also have the function of protruding the phallosome. The merged male paraproctal ridges buckle the ventral ridge of the female dorsal valve, and the other side of the female dorsal valve is supported by the tip of the phallosomal sclerite (Figs 6B, blue arrow and 7, black dotted arrows). Furthermore, the contraction of the pahy01 muscle

provides strong power to securely make the paraproct hooking and pulling the female dorsal valve inward. The phIX01 and phIX02 muscles may also produce additional power to move the apex of the phallosome inwards (i.e., pull the female dorsal valve inwards, Fig. 1E). This clearly shows that the male *Neotrogla* actively holds the female during copulation.

Similar movement of the phallosome to hold and fix the female genital structure is also known in *Trichadenotecnum* (Psocomorpha: Psocidae: Cheng & Yoshizawa, 2019). However, in the case of *Trichadenotecnum*, the male holds the subgenital plate, not the dorsal valve. In addition, the phIX01 and phIX02 muscles function to restore the phallosome, and the phVIII01 muscle is absent in *Trichadenotecnum*. Therefore, the female holding mechanism is apparently non-homologous between *Neotrogla* and *Trichadenotecnum*.

At the end of the copulation, the paX01 and 02 muscles of the males are likely contracted to move the paraproct outwardly, and phIX01 is contracted to restore the phallosome. Both movements (active movements by the male) can function to release the grasp on the female dorsal valve.

Penetration of the gynosome and anchoring of the male

To open the male genital cavity is very likely a prerequisite for the insertion of the gynosome by a female. This is probably caused by two factors: 1) retraction of the male paraprocts (see above), which are located above the genital chamber under noncopulating conditions, thus blocking the male genital opening, and 2) backwards movement of the posterior margin of the hypandrium. There is no muscle that causes

direct backwards movement of the posterior margin of the hypandrium. However, as seen from Fig. 4B, the male terminalia are strongly compressed laterally during copulation, which is very likely to cause the indirect backwards movement of the posterior margin of the hypandrium (such as the mouth of a spring-loaded coin purse). From the reconstructed 3D model of the copulating condition, two factors can be assumed to be associated with the lateral compression of the male terminalia: 1) contraction of the pahy01, phIX01 and 02 muscles causes the inward movement of the lateral side of the hypandrium (i.e., male active movement); 2) grasping by the female external valves causes compression force of the male terminalia (i.e., passive movement for the male: see below).

The phIX01 and phIX02 muscles share the same origin on the hypandrium, together forming a triangular configuration with the sclerite of the phallosome (Fig. 3H, I). This state allows the hypandrium to move more effortlessly because the forces generated by the two muscles are in the same plane. If the insertion site of these two muscles on the hypandrium is not consistent, it is difficult to ensure that all the forces are on the same plane, which will easily lead to conflicting forces. A similar arrangement of the muscles has also been observed in the female external valves (exIV01 and exIV02) of other psocodeans, such as *Lepinotus* and Amphientomidae, which is also considered to cause effective movements of the valve (Cheng & Yoshizawa, 2022).

Regarding the muscles attached to the gynosome, gy-01 relaxes and gyIX01 contracts in the copulating condition, and the status of these two muscles vice versa in

the non-copulating condition. Therefore, the contraction of gyIX01 apparently has a function to extend the gynosome, and gy-01 works to restore the stuck gynosome to the resting position (Fig. 2G, H). Two stout muscles, spIX01 and spIX02, relax during copulation, thus restoring the gynosome membrane to its original shape and position (Fig. 2G, H). The apical sclerite of the gynosome deeply penetrates the membranous pouch of the phallosome during copulation, from which the semen is transported to the female (Yoshizawa et al., 2014). The spiny membrane of the gynosome also enters the male genital cavity (Figs. 1C, D, 6B and 7). Because no muscle is involved in inflating the spiny membrane, the anchoring function of this specialized membrane is likely achieved by increased body pressure. See also the next section for the additional holding function of the gynosomal spines.

At the end of copulation, reduction of the internal pressure can cause the deflation of the spiny membrane, and the contraction of the spIX01 and 02 muscles acts to restore the gynosomal spiny membrane. The gynosome is retracted by the contraction of the gy-01 muscle.

Ventral fixation of the hypandrium

The female paX01 and paX02 muscles are contracted during copulation, causing the paraproct to be slightly indented in the direction of the female epiproct (Fig. 2D). The female paX03 muscle retracts during copulation, directing the protrusions on the ventral surface of the female paraproct inward (Figs. 1D and 2D). By this movement, the ventrolateral paraproctal concavities form a continuous single circular concavity, which fits perfectly to the curve of the ventral margin of the hypandrium (Fig. 6B, white

arrow).

The jointed female paraproctal cavities support the ventral surface of the male hypandrium externally during copulation, which sandwich the hypandrium together with the gynosomal spiny anchor within the male genital chamber (Figs. 6B, white arrow and 7). Therefore, it is apparent that the female actively holds the male during copulation by using the female paraproct and gynosome. Such close contact between the female paraproct and the male hypandrium is not observed in *Trichadenotecnum* (Cheng & Yoshizawa, 2019).

At the end of copulation, paX01 and 02 are probably contracted to release the ventral paraproctal support of the male hypandrium.

Grasping the male abdomen by the female external valves

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

The female external valves grasp the lateral surface of the male hypandrium with the contraction of the stout doex01 and exVIII01 muscles (Figs. 2E, F, 6A and 7). As also mentioned above, this probably comprises two functions: opening the male genital cavity at the beginning of copulation and holding the male during copulation, both of which are active movements of the female.

At the end of copulation, exIX02 is probably contracted to open the external valves to release the grasp on the male.

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

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

Sexual selection

In animals with conventional sex roles, females usually gain fewer fitness benefits from multiple matings, and thus, males more actively seek mating opportunities (Trivers, 1972). Accordingly, male animals sometimes develop elaborate genital structures for coercive copulation with unwilling mates (Arnqvist & Rowe, 2002; Lange et al., 2013). As counteradaptations, two types of traits are known to occur in female genitals: "resistance" traits that decrease the male fitness component (e.g., efficiency of mate holding) and "tolerance" traits that mitigate the costs of mating without affecting male mating success (Reinhardt et al., 2014). In *Neotrogla*, males give nutritious seminal gifts to females during copulation, which last up to 72 hours, and females mate multiply, as is evident from the occurrence of multiple seminal capsules in the spermatheca (Yoshizawa et al., 2014, 2018a). In addition, female *Neotrogla* possess twin insemination slots with switching valves to receive double seminal gifts at the same time (Yoshizawa et al., 2018a; Kamimura et al., 2021). Therefore, it has been assumed in previous studies that female *Neotrogla* gain more net benefits from multiple copulations than males and that females actively control copulatory processes by using the unique intromittent organ (coupling-role reversal: Yoshizawa et al., 2014, 2019).

Our present study confirmed this view by showing that the multiple female organs actively (and probably partly coercively) hold the male mate: this is achieved by

anchoring within the male genital chamber using the gynosomal spines and external grasping using the female paraproct and the external valves. However, at the same time, the 3D models also revealed that autonomous movements of male genital muscles are necessary for the initiation of the copulatory processes and that male *Neotrogla* actively hold the female mate during copulation.

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

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

For some animals with conventional sex roles, mismatching in genital coupling is

known to result in leakage of seminal fluid, which can cause the female and male bodies to stick together and thus reduce the rate of sperm transfer (Kamimura & Mistumoto, 2012; Tanaka et al., 2018; Polak & McEvey, 2022), or increasing the wounding probability of the female genitals (Sota & Kubota, 1998; Kamimura, 2012; Masly & Kamimura, 2014). In many of those cases, the females develop specific structures (such as membranous pockets) that accommodate the wound-inflicting structures of the male genitalia and thus mitigate the copulatory costs (Sota & Kubota, 1998; Kamimura, 2012). Interestingly, male *Neotrogla* possess species-specific pockets on the walls of the genital chamber to receive gynosomal spines, although no copulatory wounds have been detected there (Yoshizawa et al., 2014). Given that the copulatory mechanism of *Neotrogla* is complicated, seminal fluid passed to the female is voluminous and possibly reactive (Yoshizawa et al., 2014). Considering that the females' anchoring power is very strong (an artificial trial to separate a coupled specimen led to separation of the male abdomen from the thorax without breaking the genital coupling: Yoshizawa et al., 2014), a precisely connected genital complex and step-by-step releases of each holding mechanism achieved by the cooperation of both sexes are probably beneficial for both males and females. Such concordance of the interests between the sexes may have prompted the evolution of "tolerance" traits in male *Neotrogla*, as in a completely sex-role reversed mating scenario.

Conclusion

Inverted genital couplings of *Neotrogla* provide an extremely rare opportunity to test

hypotheses on the evolution of genital structures (House & Simmons, 2006; Simmons, 2014; Michels et al., 2015) under the reversed direction of sexual selection. We found evidence of the complicated coexistence of genital structures, suggesting male resistance, female coercion, and cooperation between the sexes. The absence of harmful structures that inflict wounds on the opposite sex in both males and females is notable. The coevolution between the male and female genital structures in *Neotrogla* may provide a new example for the evolution of tolerance traits, a newly proposed idea concerning genital evolution (Michels et al., 2015).

For further understanding of the sexual selection and evolution of novelties, detailed information of the courtship behaviour of *Neotrogla* and its relatives is key but is very poorly understood to date. Accumulation of basic behavioural information is highly desired for a more accurate and detailed understanding of the evolution of the gynosome in *Neotrogla*.

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Declarations

Conflict of Interest The authors declare no conflict of interests

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Figure Legends

Fig. 1. 3D reconstruction of the female terminalia of *Neotrogla curvata*; (for E-G left: noncopulating state; right: copulating state): (A) ventral view, noncopulating state; (B) posterior view, noncopulating state; (C) ventral view, copulating state; (D) posterior view, copulating state; (E) lateral view (highlighting dorsal valve); (F) lateral view (highlighting the external valve); (G) lateral view (highlighting the gynosome). Abbreviations: ep = epiproct; pa = paraproct; cl = clunium; st = sternum; gy = gynosome; do = dorsal valve; ex = external valve.

Fig. 2. 3D reconstruction of the female terminalia of *Neotrogla curvata*, internal view; noncopulating state (A, C, E and G); copulating state (B, D, F, and H); highlighting different structures and associated muscles (C-H): (1) epX01; (2) paX01; (3) paX02; (4) paX03; (5) paX04; (6) dosp01; (7) doex01; (8) exsp01; (9) exIX02; (10) exVIII01; (11) spIX01; (12) spIX02; (13) gygy01; (14) gy-01. See Fig. 1 for abbreviations.

Fig. 3. 3D reconstruction of the terminalia in the noncopulating state (A-H) and copulating state (I), highlighting different structures and associated muscles: female *Neotrogla curvata* (A,B); female *N. brasiliensis* (C); male *Trichadenotecnum pseudomedium* (D,E); male *N. brasiliensis* (F,G); male *N. curvata* (H,I). (A-C) Ventral view; (D-F) internal view; (G-I) lateral view. Abbreviations: ep = epiproct; pa = paraproct; hy = hypandrium; cl = clunium; ph = phallosome.

Fig. 4. 3D reconstruction of the male terminalia of *Neotrogla curvata*, noncopulating

state (A, C and E); copulating state (B, D and F): (A, B) posterior view; (C, D) lateral view; (E, F) lateral view (highlighting the phallosome). Abbreviations: sg = segment VIII; m= membrane; for others, see Fig. 3.

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

Fig. 6. *Neotrogla curvata*, 3D reconstruction of terminalia of the copulating pair, lateral view (A,B); *N. brasiliensis*, 3D reconstruction of terminalia of the male in the noncopulating state (C,D). (B) highlighting the gynosome, phallosome, paraproct (m) and epiproct (m); (C) posterior view; (D) lateral view, highlighting the phallosome and paraproct (m). See Figs. 1 and 3 for abbreviations.

Fig. 7. Schematic drawing of the male and female genitalia of *Neotrogla curvata* in copulated condition. Gray indicates male structures, and orange indicates female ones. Black arrows indicate holding system by male, and red arrows indicate that by female.

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

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

Figure 1

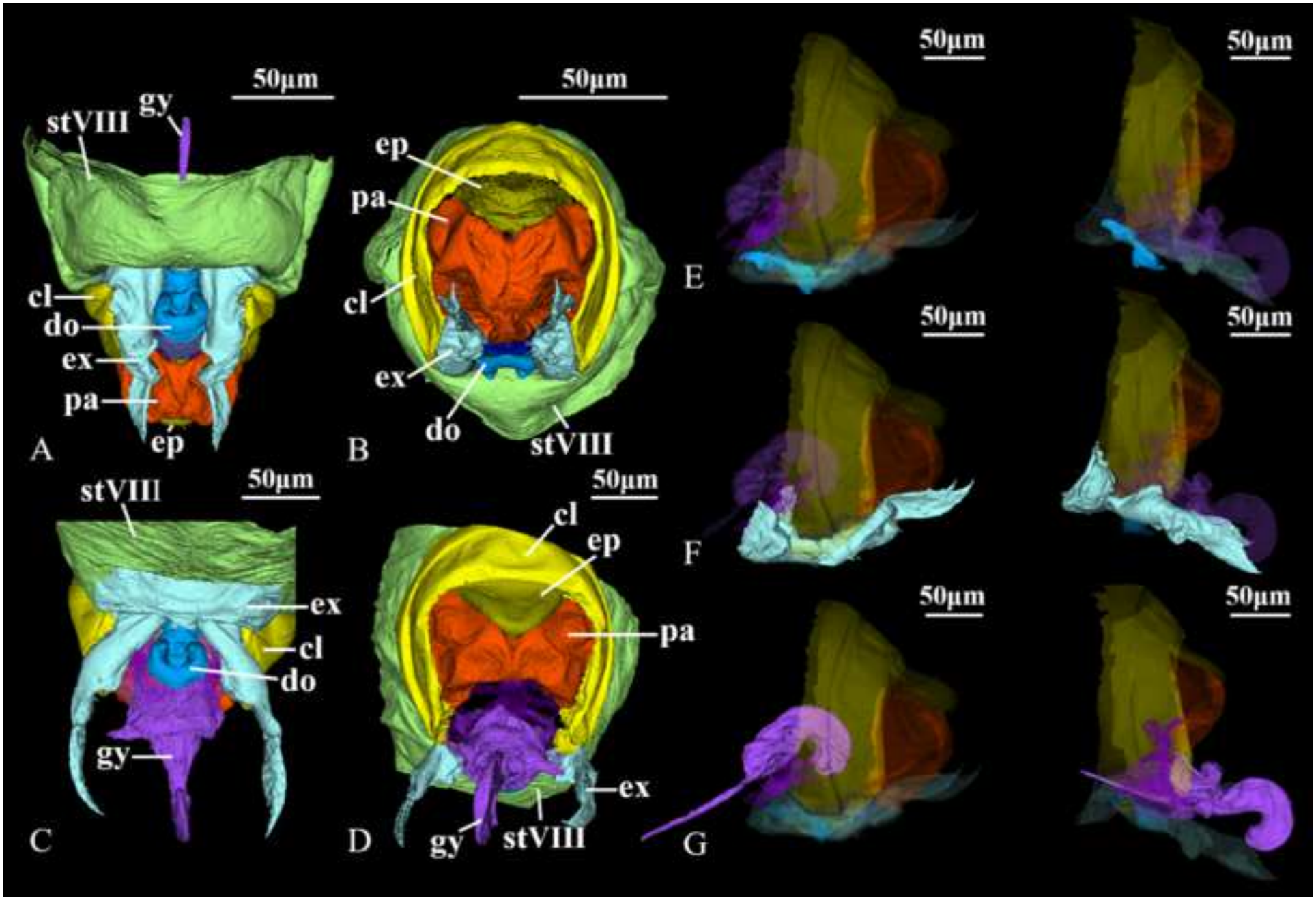
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Figure 2

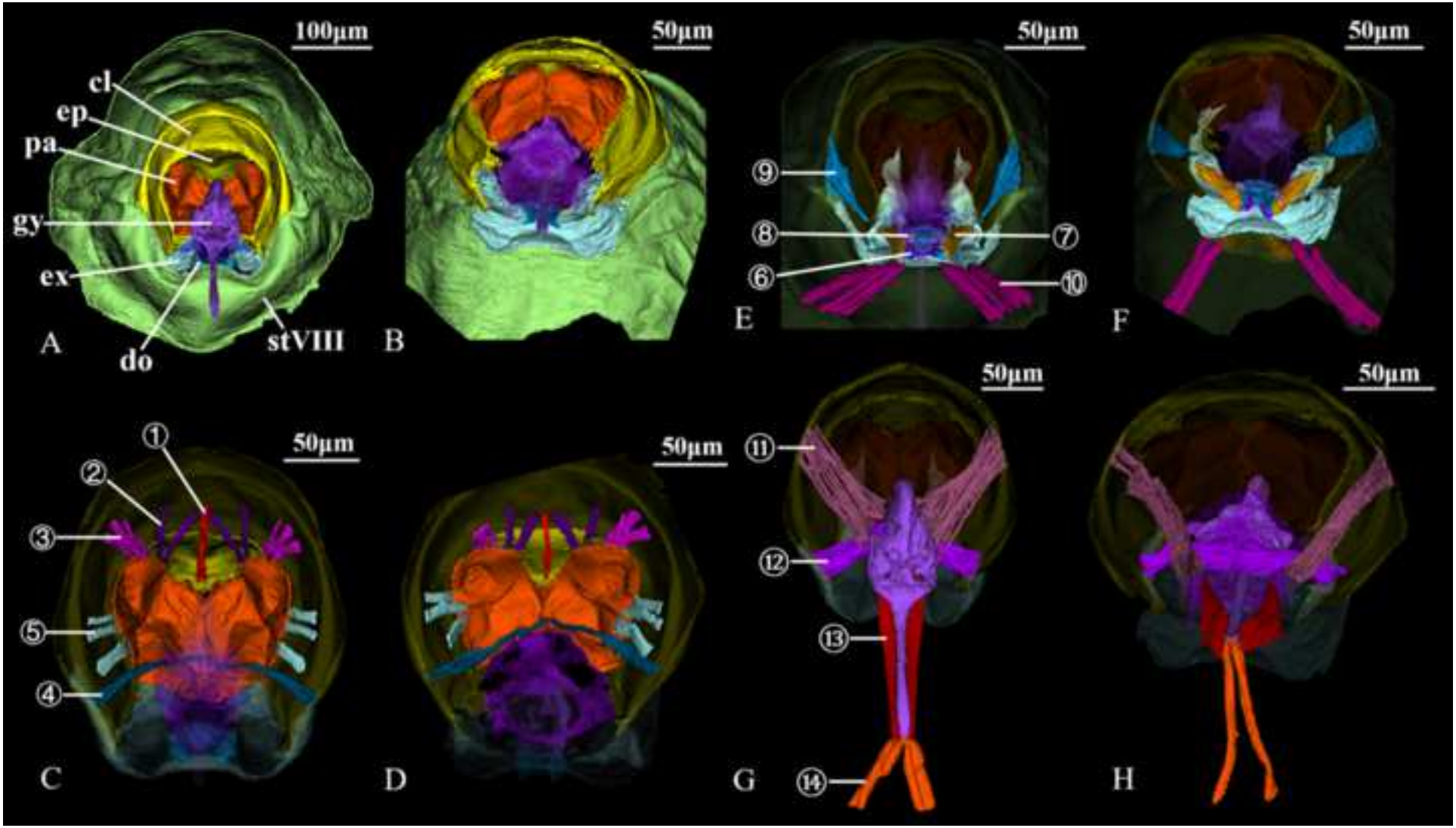
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Figure 3

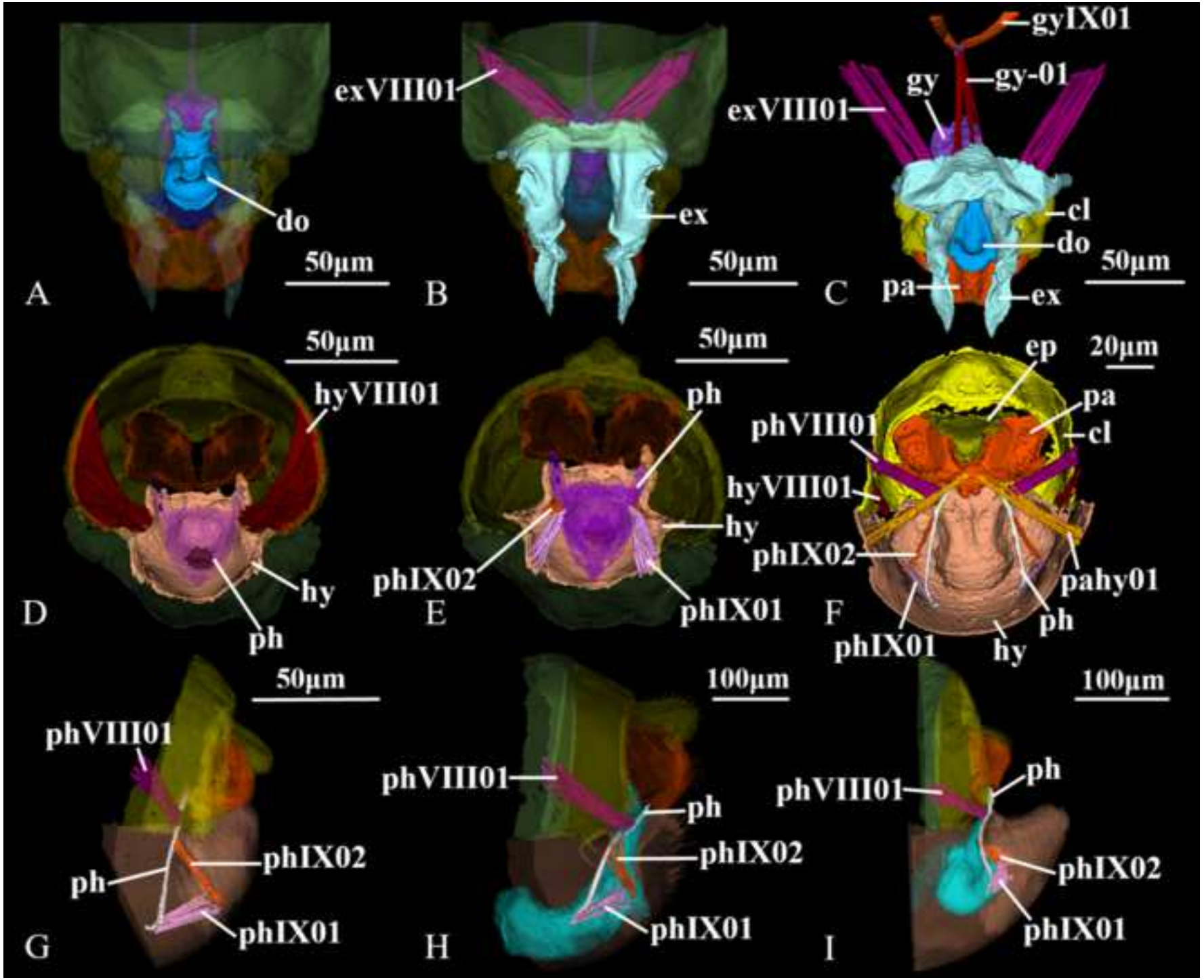
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Figure 4

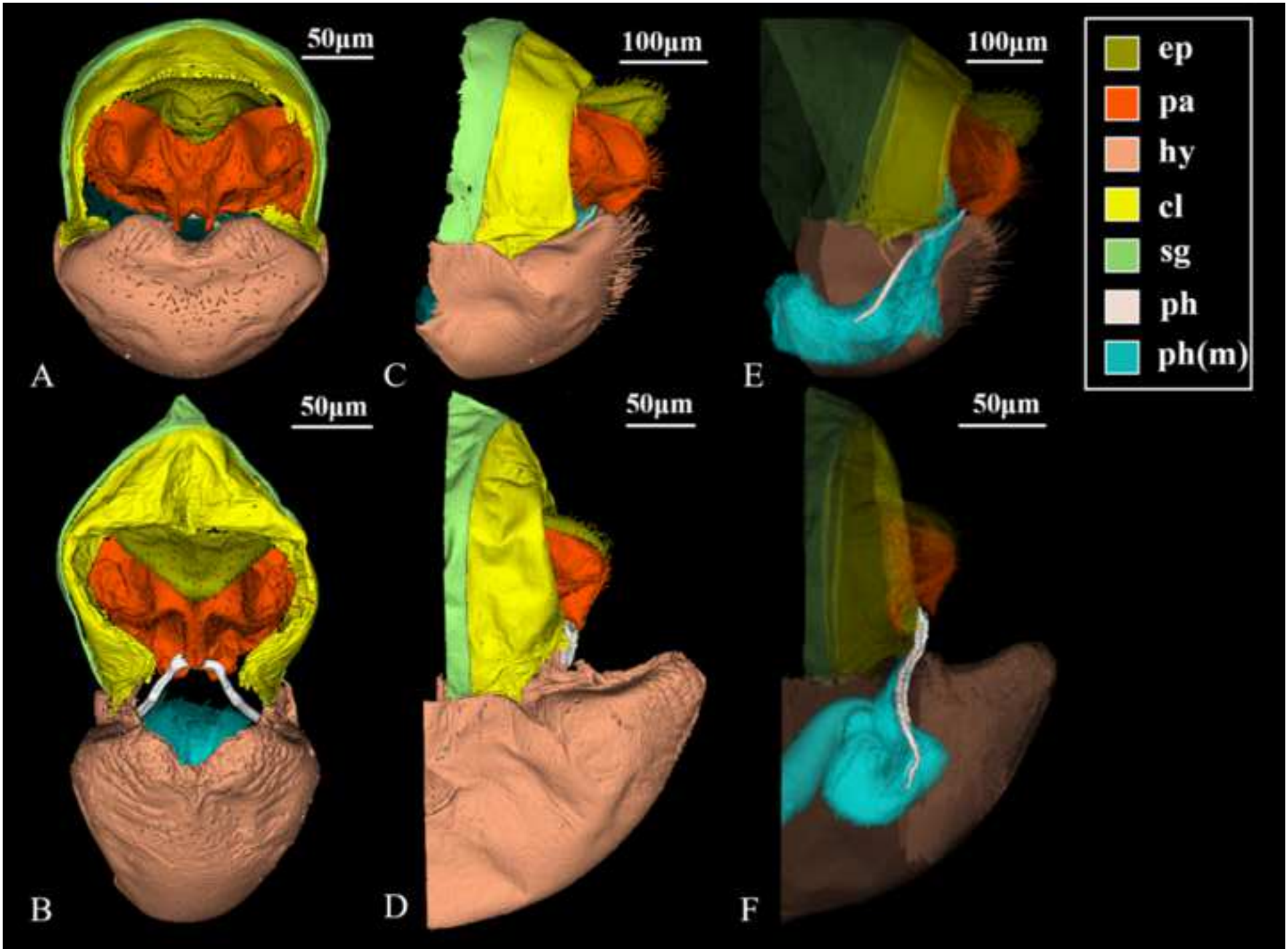
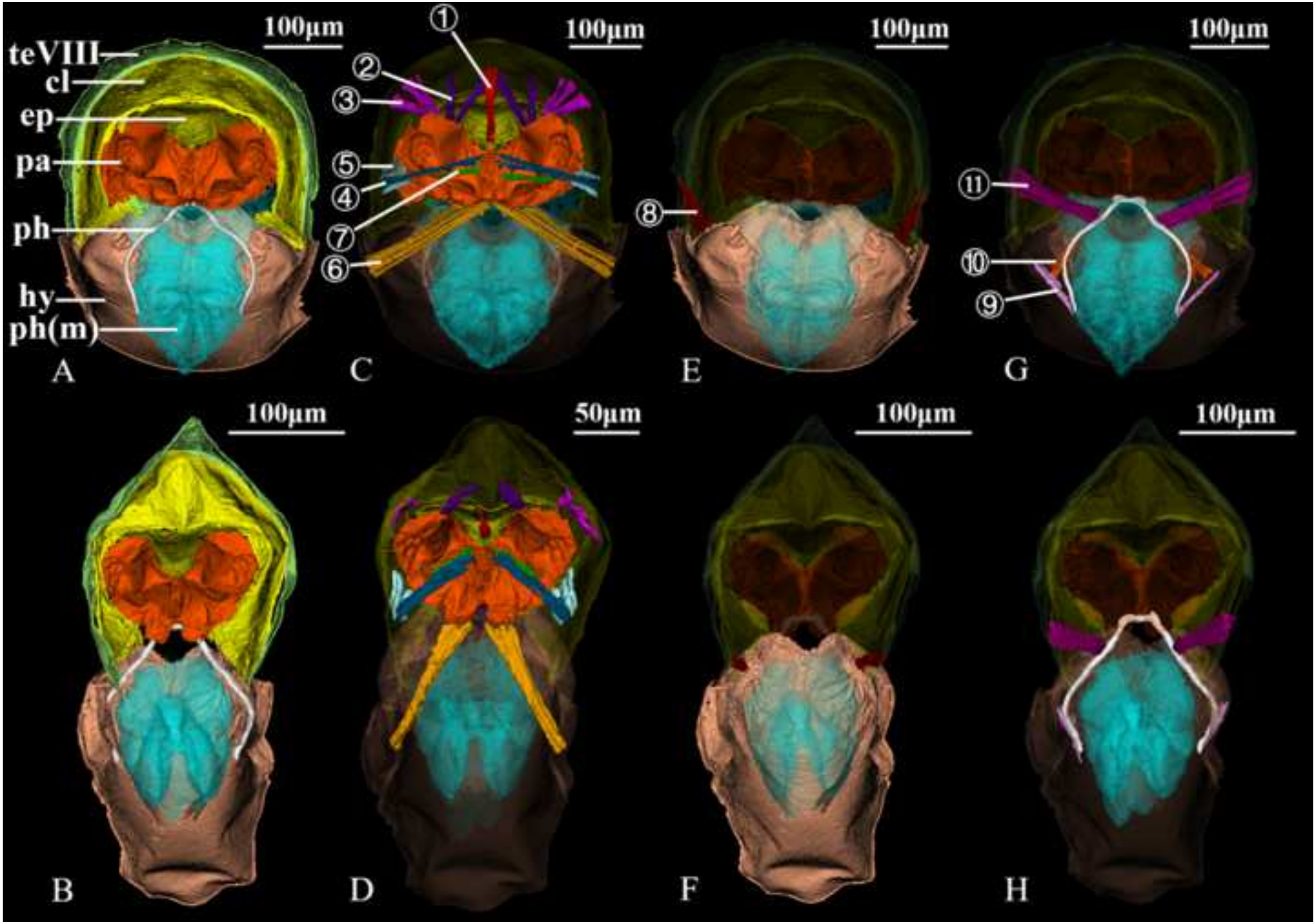


Figure 5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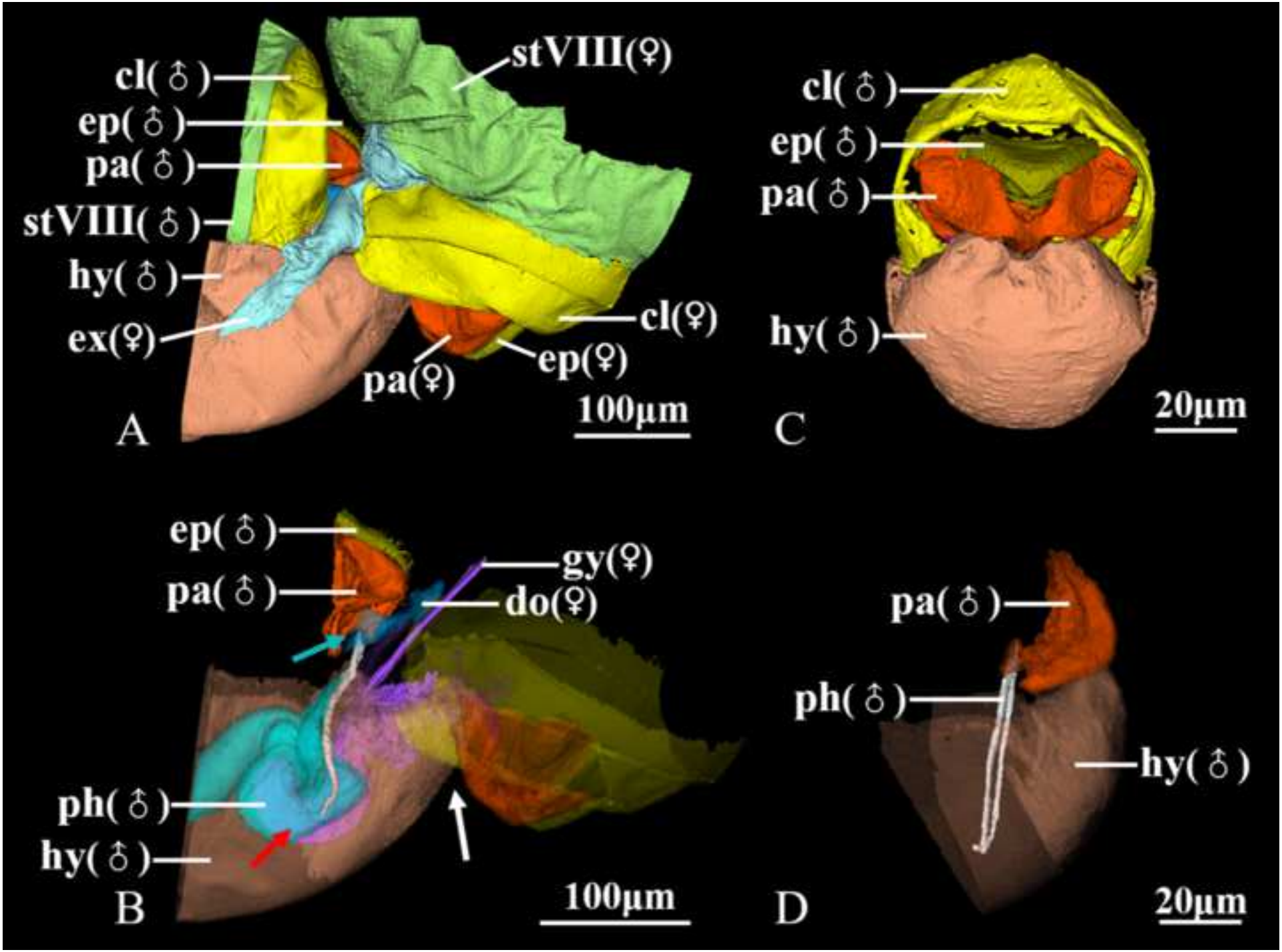
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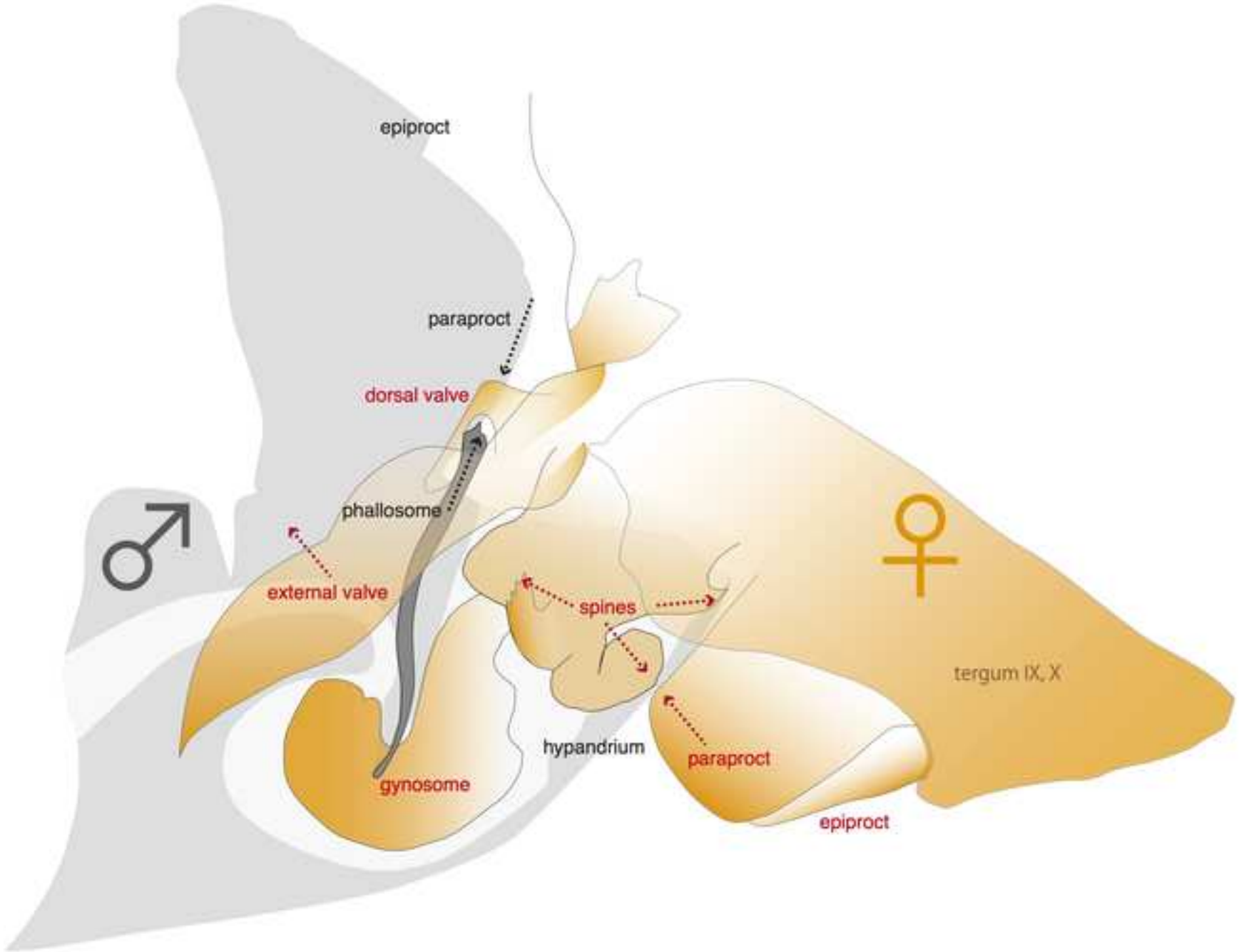
Figure 7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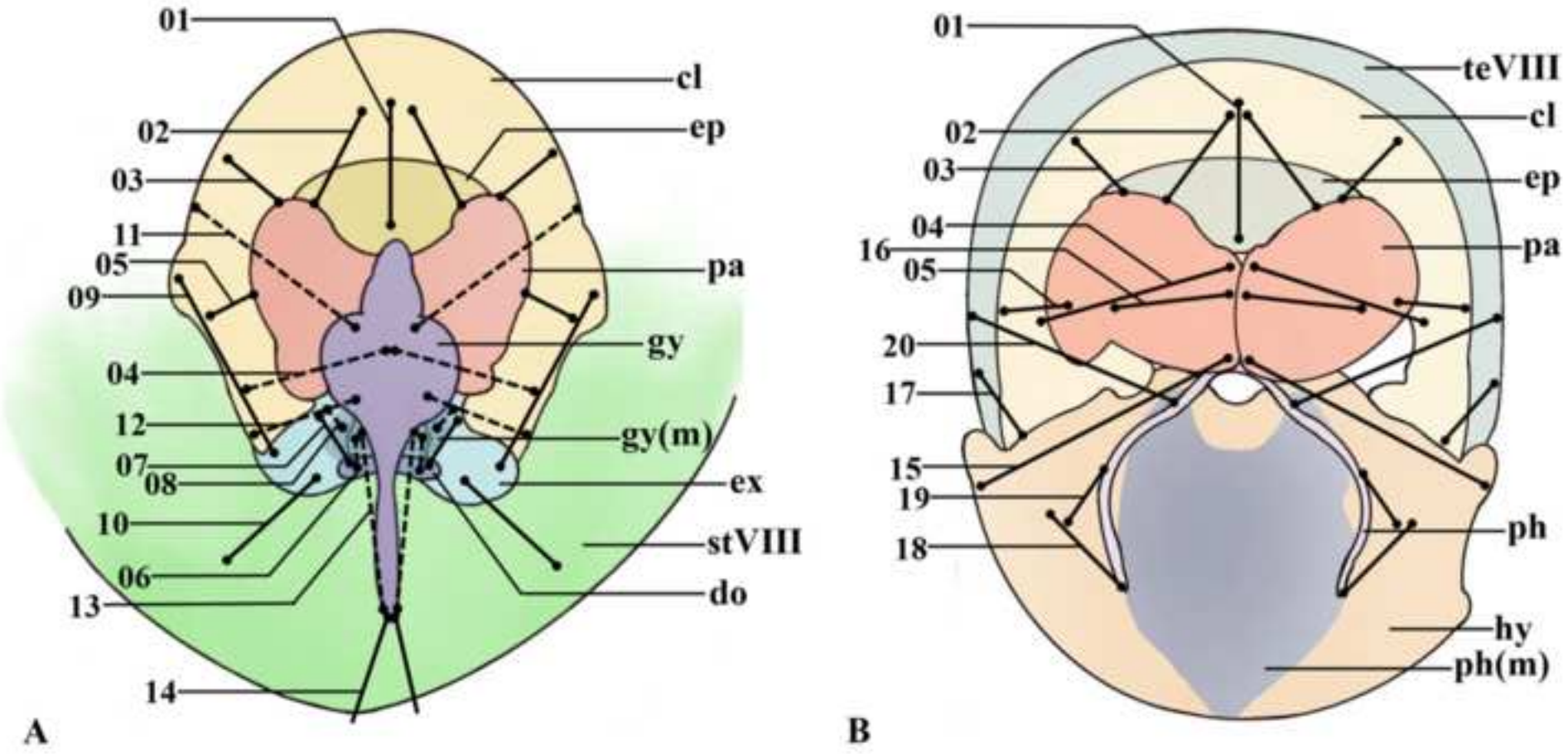
epiproct

Figure 8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