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Author(s)	Matoba, Tatsuki; Kamimura, Yoshitaka; Yoshizawa, Kazunori
Citation	Organisms diversity & evolution, 23, 871-879 https://doi.org/10.1007/s13127-023-00621-y
Issue Date	2023-10-18
Doc URL	https://hdl.handle.net/2115/93199
Rights	This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature' s AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: http://dx.doi.org/10.1007/s13127-023-00621-y
Type	journal article
File Information	2023_ODE.pdf



Is the copulation of silver-washed fritillary inverted? Function of the female genital projection (Lepidoptera: Nymphalidae: Argynnini)

Tatsuki Matoba 1, Yoshitaka Kamimura 2, Kazunori Yoshizawa 1*

1 Systematic Entomology, School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

2 Department of Biology, Keio University, Yokohama 223-8521, Japan

Correspondence: K. Yoshizawa, psocid@agr.hokudai.ac.jp

Abstract

Females of the silver-washed fritillary butterfly, *Argynnis paphia* (Linnaeus), and its relatives are known to have a unique genital projection called the cornucopia. Previous observations showed that the cornucopia partly penetrates the male genitalia during copulation, but its detailed structures and functions are unknown to date. Our observations using light microscopy, confocal laser scanning microscopy, scanning electron microscopy, and microcomputed tomography suggested that penetration by the female cornucopia occurs passively, and it is hooked and pulled by the male towards the male genitalia using sharp teeth on the uncus. This hooking and pulling causes wounds on the dorsal region of the cornucopia. Artificial amputation of a cornucopia confirmed that it is indispensable for spermatophore transfer from males and functions to break off the male phallic spines (cornuti). The broken spines may be used by females to help the digestion of spermatophores in the female's corpus bursae. Females with larger body sizes had more broken spines in the corpus bursae, indicating that secure holding of a large female during copulation is challenging for males.

Keywords: *Argynnis paphia* • Copulatory wounds • Functional morphology • Inverted copulation

Introduction

Genitalia tend to evolve much faster than other external structures in animals and are considered to be some of the most diverse structures. Thus, genitalia, especially male ones, have been widely studied in evolutionary contexts (Eberhard, 1985). In contrast, female genitalia have been generally regarded as less diverse and less informative than male genitalia. One of the reasons is that, in contrast to well sclerotized insect male genitalia, female genitalia are largely membranes and are difficult to observe and evaluate. However, in recent years, it has become increasingly apparent that female genitalia are as highly diverse as male genitalia (e.g., Simonsen, 2006a, 2008; Sloan & Simmons, 2019). The evolution of female genitalia attracts special attention to understanding the mechanisms of sexual selection underlying co-evolution between male and female genitalia.

One of the most significant examples of highly diversified female genitalia is known from species showing inverted copulation (i.e., females possess extensible intromittent organs for collecting sperm). For example, a highly species-specific female intromittent organ (or gynosome) is known from a group of cave-dwelling barklice (Insecta: Psocodea: Prionoglarididae: Sensitibillini), whereas their male genitalia are largely membranous and thus are less variable among species (Lienhard, 2007; Lienhard et al., 2010; Yoshizawa et al., 2014, 2019). Inverted copulation is an extremely rare phenomenon, but a few possible examples are also known from Lepidoptera and Coleoptera insects (Jolivet, 2008). One such example has been reported for the females of the silver-washed fritillary butterfly, *Argynnis paphia* (Linnaeus) (Bayard, 1944).

The silver-washed fritillary is a species of nymphalid butterfly widely distributed throughout the Palearctic region. The females of this species possess a large, bizarre-shaped, bellows-like projection called "clitoris", "horn of plenty", or "cornucopia (the latter term is adopted hereafter)" (Fig. 1: cor). Bayard (1944) observed that the cornucopia of *A. paphia* penetrates male genitalia during copulation, and Jolivet (2008) expressed this phenomenon as "female erection". Bayard (1944) and Jolivet (2008) speculated that the cornucopia functions to pump sperm from the male, but the detailed anatomy of cornucopia and its movement during copulation are largely unknown.

In this study, we examined the cornucopia and associated structures of *A. paphia* using light microscopy, confocal laser scanning microscopy, scanning electron microscopy (SEM), and synchrotron microcomputer tomography (μ CT) techniques. By examining virgin and ex-virgin females, copulating pairs, and females from which the cornucopia was experimentally amputated, we estimated the function of the cornucopia. We also discuss the sperm-pumping and erectile functions of the cornucopia proposed by Bayard (1944) and Jolivet (2008).

Materials and Methods

The terminology of the lepidopteran genital structures followed Klots (1970), and an overview of male and female genitalia and their terminology is shown in Figs 1–2.

Specimens were collected from several sites in Hokkaido Prefecture, Japan, and were preserved at -20°C . The abdomen was removed from male and female specimens and soaked in 10% KOH solution at 40°C for 24 hours to clear tissue. Then, these specimens were dissected and observed in 75% ethanol under a SZX12 stereomicroscope (Olympus Corp, Tokyo, Japan). A confocal laser scanning microscope (CLSM; Leica TCS SP5, Wetzlar, Germany) was also used to examine detailed structures. For CLSM observations, the specimens were mounted with glycerol. We used an excitation wavelength of 405 nm and an emission wavelength of 420–480 nm (blue) to detect resilin and an excitation wavelength of 488 nm and emission wavelengths of 510–580 nm (green: muscles and membranous part) and 580–680 nm (red: sclerotized part), following Michels & Gorb (2011).

To examine the coupling of the genitalia during copulation, larvae were collected at Mashike, Hokkaido Prefecture, and were reared to obtain virgin males and females. At 5–6 days after emergence, a virgin female and a virgin male were placed into a net cage (20 cm diameter, 110 cm height). Fifteen minutes after the initiation of copulation, a mating pair was sprayed with freezing insecticide spray (Earth Corporation, Tokyo, Japan), placed into cold 75% ethanol for fixation, and then stored at -20°C for 3 weeks. A total of four pairs were fixed. For microscopy observations, coupling samples were soaked in BABB (benzyl alcohol/benzyl benzoate = 1:2) at 40°C for one month to make them transparent following Kamimura & Mitsumoto (2011). After this treatment, we observed the samples in BABB under a stereomicroscope. When necessary, a portion of the male valva was removed to observe the internal part.

In addition to the light microscopy examinations, a mating pair was also subjected to synchrotron microcomputed tomography (μCT) scanning. Samples were dehydrated in an increasing series of 80–100% ethanol before critical-point drying (EM CPD300, Leica, Wetzlar, Germany) to remove water without serious organ shrinkage. The sample was then scanned using synchrotron μCT at the BL20XU beamline (Uesugi et al., 2012) of the Super Photon ring-8 GeV facility (SPring-8; Hyogo, Japan) using a stable beam energy of 15 keV in absorption-contrast mode. The tomography system consisted 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 structures. As a complement to this analysis, five females (collected from Okaya, Nagano Prefecture, Japan) were examined for the orifice of the ductus seminalis using a JSM-6510 scanning electron microscope (JEOL Ltd., Tokyo, Japan; acceleration voltage, 10 kV). The samples were prepared basically following the protocols of Kamimura and Mitsumoto (2011), but the amputated

cornucopia were outstretched on a piece of rubber sheet during fixation in ethanol–butanol.

To clarify the function of the female cornucopia and the male cornuti (spines borne on the phallus: see below), two additional examinations were conducted. First, the cornucopia was removed from a laboratory-reared virgin female using a scalpel, and the treated female and a virgin male were introduced into the same net cage until copulated (N=3: of which two were derived from Mashike and one from Okaya). Three to five days after copulation, the females were dissected to examine the presence of spermatophores, broken spines (or deciduous cornuti hereafter) within the corpus bursae, and copulatory wounds on their body. Second, we counted the number of deciduous cornuti remaining in wild-caught mated females (Mashike population) and examined possible factors related to it, including mating frequency, female body size, and cornucopia size. To evaluate female body size, forewing length (from the base of the wing to the end of the R4 vein: Supplementary Figure) was measured to the nearest 0.1 mm. For individuals, both the right and left wings could be measured, and the mean value was used as an index of body size. Cornucopia size was determined as the sum of the distances between cornucopia ridges under resting conditions (Supplementary Figure). Mating frequency was estimated by the number of spermatophores within the female corpus bursae and by copulatory wound lines on the female cornucopia (see below for detail). The effects of these factors on the number of deciduous cornuti were evaluated by multiple linear regression models (generalized linear models [GLMs] with a Gaussian error structure and an “identity” link function). Two different approaches, *viz.*, backwards variable selection by likelihood ratio tests (Crawley, 2012) and model selection based on AIC (Akaike's information criterion) (Burnham & Anderson 2002), were adopted. All measurements were conducted using Fiji (version 2.3.0), and statistical analyses were carried out using R software version 4.2.1 (R Core Team 2022), implemented with the “MuMIn” package version 1.47.1 for multi-model inference (Bartoń 2022).

Results

Cornucopia during copulation

The female cornucopia of *Argynnis paphia* (Fig. 1) is well developed and bellows-like in shape, protruding from the dorsal surface at the entrance of the bursa copulatrix. In the resting condition, it is strongly bent medially and then directed posteriorly. The dorsal surface (in the copulated condition: Fig. 3) and the grooves of the bellows structure of the cornucopia are mostly membranous, whereas the ventral ridges are well sclerotized (Fig. 1). The area around the cornucopia, including the sinus vaginalis and antrum, is membranous and wrinkled. Muscles were observed inside the cornucopia (Fig. 3C). The ductus seminalis opens at the bottom of a ventral groove of the cornucopia near its base (Figs 1B, 3CD). The corpus bursae lacks signum (spines frequently observed in the lepidopteran corpus bursae).

1 The male uncus (Fig. 2) is long, bearing several dorsal teeth. The ampulla of the valva is large
2 and covered with dense, strong denticles. The dorsal side of the phallus, which functions as a tube
3 for spermatophore transfer, includes areas bearing dense strong spines, termed cornuti.

4 During copulation, the dorsal teeth of the male uncus penetrate the dorsal membranous region
5 of the female cornucopia (Fig. 3B). The cornucopia is pulled into the male body by inwards
6 movement of the uncus (Figs 3–4). The male phallus is inserted into the female ostium bursae, but
7 its apex does not reach the antrum (Fig. 4), the entrance of the ductus bursae. The spines on the
8 dorsal part of the phallus (i.e., cornuti) are tightly associated with the ventral sclerotized ridges of
9 the female cornucopia (Fig. 3B). The denticulated ampulla of the valva touch abdominal segment
10 VIII near the base of the cornucopia (Fig. 4).

18 *Copulatory wounds and broken cornuti*

20 Copulatory wounds were almost always observed on the cornucopia of mated females. The
21 repaired copulatory wound patches were usually arranged in straight lines (Fig. 5A), corresponding
22 to the teeth of the male uncus (Figs 2B, 3B). A total of 71 wild-caught females were dissected to
23 investigate the patterns of copulatory wounds and deciduous cornuti. We removed the following 13
24 individuals from the subsequent analysis: number of cornuti uncountable by problems during
25 dissections (N = 2), with too large and uncountable wounds on the cornucopia (N = 3), with
26 copulatory wounds but no spermatophore (N = 3), and with copulatory wounds arranged in two
27 lines but only one spermatophore (N = 5). Accordingly, in eight cases, females possessed more
28 copulatory wound lines than spermatophore envelopes. In the other 58 females (87.9% out of 66
29 females), the two estimates of mating frequency were identical, with a mean of 1.1 (min–max: 1–3).

30 Deciduous cornuti were present in the bursa copulatrix, a digestive organ of male-derived
31 spermatophores, of all mated females. The number of deciduous cornuti was 13.1 ± 9.3 (mean $\pm$
32 SD) in these females (N = 58): 11.8 ± 9.0 in once-mated (N = 49), 20.9 ± 9.8 in twice-mated (N =
33 8), and 11 in thrice-mated (N = 1) females. The CLSM observation of the male phallus surface
34 showed that cornuti of various regions were broken at various lengths (Fig. 6).

35 The size of the cornucopia could not be measured in eight females, which were removed from
36 the GLM analysis. The size of the cornucopia was not significantly correlated with forewing length
37 ($r = 0.17$, $P = 0.23$, $N = 50$). Our GLM analysis revealed that twice-mated females had significantly
38 more deciduous cornuti than once-mated females (Table 1; Fig. 7a). Female body size (forewing
39 length) was also positively correlated with the number of deciduous cornuti (Table 1; Fig. 7a). The
40 size of the cornucopia was also selected as an explanatory factor based on AICs, but its effect was
41 marginally non-significant (Table 1; Fig. 7b).

42 In the bursa copulatrix, deciduous cornuti were always present in the corpus bursae but not in

the ductus bursae. Several broken spines were also observed between the phallus and the cornucopia during copulation (Fig. 3B). A portion of the deciduous cornuti in the corpus bursae penetrated the spermatophore. When the spermatophore was not broken, the deciduous cornuti were found around it. Wounds were also detected in the corpus bursae in 3 out of 71 females.

Neither spermatophores nor deciduous cornuti were detected in the treated females from which the cornucopia was amputated (N = 3). The treated females had wounds on sternum VIII where copulatory wounds were not observed in untreated females (Fig. 5B). In addition, fragments of repaired wound patches detached from where the cornucopia had been amputated were present in the corpus bursae.

Discussion

Function of the female cornucopia

The copulatory mechanisms of *A. paphia* were estimated based on detailed observation of copulating pairs. For males, the uncus likely has the functions of hooking, pulling, and holding the female cornucopia during copulation (Figs 3–4). Although the tip of the uncus is not associated with the cornucopia in the μ CT image (Fig. 3), it hooks the tip of the cornucopia in another pair (Fig. 4). The presence of the largest wound at the tip of the cornucopia (Fig. 5, where the dorsal spines of the uncus are not associated in all pairs: Figs. 3–4) and the relative positions of the spines on the uncus suggest that the tip of the uncus hooks at this position to pull the cornucopia into the male body. Judging from the arrangements of the muscles in the cornucopia (Fig. 3C, white dotted arrows), these muscles probably function to only straighten the bent cornucopia (Fig. 1). However, all these muscles do not have the function of extending the cornucopia into the male body during copulation. Active erection of the cornucopia by body fluid pressure may be possible. However, inwards movement of the male uncus during copulation (Fig. 2) and tight fixation of the apical part of the cornucopia by the uncus (Fig. 3B) suggest that the penetration of the cornucopia into the male genitalia likely occurs passively by the movement of the male uncus. Hannemann (1954a) reported a large bundle of depressor muscles for the uncus of *A. paphia*, which was also confirmed in the present μ CT observation (not reconstructed in Fig. 3). Therefore, active erection of the cornucopia by females, as suggested by Bayard (1944) and Jolivet (2008), is less likely, at least for *A. paphia*. Passive shallow penetration of a portion of the female genitalia into the male body has been reported from some barklice (Klier, 1956; Cheng & Yoshizawa, 2019), but this is apparently a different phenomenon from the reversed copulatory function observed in Brazilian cave-dwelling barklice (Yoshizawa et al., 2014, 2019).

The large teeth on the dorsal side of the uncus are a characteristic of *A. paphia* (Dos Passos & Grey, 1945; Simonsen, 2006a). Our present study revealed that the teeth penetrate and cause

1 copulatory wounds on female the cornucopia (Fig. 3B). Copulatory wounds were also seen on both
2 sides of female segment VIII and its neighbours (Fig. 1). These wounds appear to be caused by the
3 male valva (Fig. 2). Some females had wounds on the sclerotized ridges of the ventral cornucopia
4 (Fig. 1). According to the observation of mating pairs, the cornuti on the phallus are tightly
5 associated with these sclerotized ridges (Fig. 3). This suggests that cornuti can also cause wounds
6 on the ventral side of the cornucopia.
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9 The CLSM observation showed that the dorsal membranous side of the cornucopia contains
10 resilin (blue in Fig. 6B). Resilin is a rubber-like protein often found in insect cuticles. In bed bugs
11 (Hemiptera: Cimicidae), the resilin protein in the cuticle of the female spermaledge functions to
12 reduce copulatory damage by sealing the puncture immediately after male stabbing for traumatic
13 insemination (Michels et al., 2015). In conclusion, the cornucopia likely functions as a receiving
14 organ of the uncus during copulation, similar to the spermaledge of bed bugs, and evolved as a
15 counterpart structure.
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17 In the cornucopia-amputated females, spermatophores and deciduous cornuti were not
18 detected in the corpus bursae. Instead, a copulatory wound was observed at an abnormal position
19 after mating (Fig. 5B). These results strongly suggest that the cornucopia is a necessary structure
20 for proper copulation. In contrast, detached pieces of repairing wounds were observed in the corpus
21 bursae of the treated female. This clearly shows that females can transfer an object from the
22 entrance of the antrum to the corpus bursae without using the cornucopia, possibly due to several
23 sets of muscles dilating the corpus bursae (Hannemann, 1954b; Arnold & Fischer, 1977).
24 Therefore, the lack of the spermatophores in the treated and copulated females was probably caused
25 by the male not ejaculating sperm properly, not because the female failed to transfer the formed
26 spermatophore into the corpus bursae due to the absence of the cornucopia.
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28 *Function of male cornuti*

29 This study is the first to examine the patterns of deciduous cornuti in mated females in
30 lepidopterans lacking signum (spines developed within the corpus bursae). Deciduous cornuti are
31 not common in Lepidoptera but are known in several groups, especially in Tortricidae (Cordero &
32 Miller, 2012; Anzaldo et al., 2014). There are several hypotheses about the function of deciduous
33 cornuti (Cordero, 2010).
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35 According to Camacho-Garcia et al. (2018), the deciduous cornuti in a tortricid moth,
36 *Amorbia cuneana*, probably has the function of reducing the chance of female remating. In this
37 species, cornuti are designed to break off easily during copulation, and most of the detached cornuti
38 occur at the ductus bursae near the ostium (i.e., at the entrance of the female genital tract). They can
39 block the insertion of the phallus and/or eversion of the endophallus of subsequent males. However,
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in *A. paphia*, cornuti are not designed to break off easily without external force (Fig. 6A). Moreover, in the mated females, deciduous cornuti were not found at the entrance of the genital tract (ductus bursae) but occurred in the corpus bursae. Judging from the phallus size and the observations of mating pairs, it is unlikely that the male intromittent organ reaches the corpus bursae (see also Bayard, 1944; Jolivet, 2008). Therefore, the hypothesis that deciduous cornuti prevent female remating by blocking the insertion of the phallus of other males cannot be applied to *A. paphia*. It is known in fritillary butterflies that the secretion from the valval pocket glands possibly has an antiaphrodisiac effect, preventing subsequent mating (Ockenfels et al., 1998; Simonsen, 2006b). This effect is possibly related to the nearly monandrous mating behaviour observed in this species (average number of matings = 1.1).

Copulatory wounds were present on the corpus bursae in three out of 71 females, which indicates that deciduous cornuti could sometimes penetrate female internal genitalia during or after copulation. However, such wounds were not found in most females, so it is probably a rather rare phenomenon and is not a fundamental function for deciduous cornuti to damage the female genital tract to stimulate them.

Females of lepidopteran species do not remate while a spermatophore remains in their corpus bursae (Wedell, 2005). Therefore, lepidopteran males are considered to reduce the possibility of females subsequently remating by interfering with the contraction of the corpus bursae or signum, thus delaying the breakup of the spermatophore. However, the signum is absent in *A. paphia*. The broken cornuti within the corpus bursae frequently penetrated the spermatophore. Therefore, the broken cornuti may have the function of speeding up the breakup of spermatophores, not delaying their destruction.

The following observations suggest that the breaking of the male cornuti is actively caused by *A. paphia* females using the cornucopia. First, there are many muscles in the cornucopia, and females are likely able to actively move its bellows-like structure (Fig. 3C). The male cornuti were found to tightly contact the sclerotized cornucopia ridges during copulation (Fig. 3AB). Our cornucopia removal experiment demonstrated that mated females with amputated cornucopia did not have deciduous cornuti within the corpus bursae (N = 3). In addition, although marginally non-significant, females with larger cornucopias tended to receive more deciduous cornuti (Table 1; Fig. 7B). The cornucopia is a bellows-like, extendable structure, so it might be variable in length according to its condition at fixation, which may hinder determining the actual relationship between the cornucopia size and number of deciduous cornuti. These results suggest that females may actively break male cornuti by using the cornucopia. Second, larger females (= with longer forewing length) possessed more deciduous cornuti in the corpus bursae (Table 1; Fig. 7A). Our field and laboratory observations showed that males fly while holding a female during copulation. It

can be assumed that larger females impose a larger impact on their movement while mating. In this situation, the friction between the phallus and the ridges of the cornucopia could cause more cornuti to break off. Our preliminary observations also showed that in the species without a developed cornucopia but with cornuti on the phallus (*Fabriciana adippe* and *Speyeria aglaja*), deciduous cornuti were not found in the corpus bursae after copulation (Matoba et al., unpublished data). Thus, the cornucopia ridges could function in breaking cornuti in *A. paphia*, and females seem to actively participate in damaging male genitalia.

The active participation of females in breaking cornuti suggests that deciduous cornuti have some beneficial function for females. As mentioned above, when the spermatophores were broken, the deciduous cornuti in the corpus bursae were found to penetrate them. This suggests that deciduous cornuti are used by females to break the spermatophore after copulation. Faster destruction of a spermatophore may accelerate female access to the resources contained in it and acquisition of another spermatophore. In contrast, they may be disadvantageous for males because faster breakup of spermatophores will make it possible for females to remate with other males within a shorter period. Therefore, cornuti of *A. paphia* might have non-deciduous functions for males, such as rigid holding of the mate during mating.

Our present observation also indicate that the cornuti are not designed to break-off as they lack breaking point in the individual spines (Fig. 6). In addition, the proportion of the broken spines seems to be small (Figs 3, 6). These observations suggest that the break-off of cornuti occurs accidentally and that broken cornuti within the female corpus bursae do not have a special function. The spermatophore envelope could be broken by the contraction of the muscles surrounding the corpus bursae, without the assistance of a signum or broken cornuti (Cordero, 2005; Sánchez & Cordero, 2014). In fact, our observations of *Fabriciana adippe* and *Speyeria aglaja*, which lack signa and broken cornuti in the corpus bursae (Matoba et al., unpublished data), suggest that these species are able to break the spermatophore envelope without the presence of signa or cornuti. To answer this question, further studies, including experimental treatments, must be performed in a wide variety of fritillary butterflies.

Concluding summary

Our present examinations suggest that the female cornucopia may have evolved as a receptive device for the uncus during copulation, as suggested by copulatory wounds occurring on the cornucopia. Moreover, females might actively break male cornuti using the cornucopia during copulation. The active penetration by females hypothesis, suggested by Bayard (1944) and Jolivet (2008), is less likely, at least for the cornucopia of *A. paphia*. Therefore, this case should not be regarded as an example of inverted copulation.

Acknowledgments

We thank Toyohi Saigusa and Takashi Adachi- Yamada for valuable advice about rearing and collection of the butterflies, and Akihisa Takeuchi and Masayuki Uesugi for their support at SPring-8. The research at SPring-8 was approved through project number 2202A1201 to KY. We also thank Thomas J. Simonsen and an anonymous reviewer for their constructive comments. This study was partly supported by JSPS grant numbers 15H04409 and 19H03278 to KY and 15K07133 to YK. This study was conducted to fulfill the master's degree of TM, who thanks Shin-ichi Akimoto and Masahiro Ohara for their continuous guidance.

Author contribution

YK and KY conceived the project. TM designed the experiments, collected specimens, analyzed morphology, and performed experiments. KY performed μ CT analysis. YK performed SEM imaging and statistical analyses. All authors wrote and approved the final manuscript.

Statements and Declarations

All authors approved the final version of the manuscript. The authors declare no competing interests.

Statements of Data Availability

The statistical data generated or analyzed during this study are included in the online supplementary files. Scanned μ CT images (zip file) are available from Figshare at DOI:10.6084/m9.figshare.21992420.

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Figure captions

Fig. 1. Photograph (A: ostium bursae removed) and schematic drawing (B) of female genitalia (lateral view) of *Argynnis paphia*. Head comes to left. Red arrowheads indicate copulatory wounds caused by the male cornuti (C), uncus (U), and valva (V). Scale = 1.0 mm. Abbreviations: antr, antrum; bu.co, bursa copulatrix; cor, cornucopia; crp.bu, corpus bursae; du.bu, ductus bursae; du.sml, ductus seminalis; ost.bu, ostium bursae; ovar, ovaria; sin.v, sinus vaginalis; sp, spermatheca; SVIII, 8th sternum.

Fig. 2. Male genital structures of *Argynnis paphia*. Head comes to right (except for D). A. Schematic drawing (front valva removed), lateral view. B. Uncus, lateral view. C. Valva, internal view. D. Phallus, dorsal view (head comes to bottom). The arrow indicates the movement of the uncus during copulation. Scale = 1.0 mm. Abbreviations: amp, ampulla; cot, cornuti; ph, phallus; un, uncus; val, valva.

Fig. 3. A. 3D reconstruction of the cornucopia and its related structures during copulation. B. Ditto, with cornucopia transparent. Blue arrowheads indicate broken cornuti; black arrowheads indicate the spines of the uncus penetrating the cornucopia. C. Internal structures of the cornucopia (orange: ductus seminalis; orange arrowhead: opening of ductus seminalis; yellow: muscles). White broken arrows indicate the direction of muscle fibres. Muscles are only reconstructed posterior to the ductus seminalis opening. D. SEM image of the opening of the ductus seminalis (scale = 0.1 mm).

Fig. 4. Photograph (A) and schematic drawing (B) of male and female genitalia in copulation. See Figures 1–2 for abbreviations.

Fig. 5. A. Copulatory wounds (indicated by red arrowheads) observed on the dorsal membranous region of the female cornucopia. Two rows of wound lines (indicated by arrowheads in different directions) were detected in this individual. B. Copulatory wound occurred at an abnormal position in the female without cornucopia. "S" indicates surgically removed cornucopia (ventral view). Head comes to bottom.

Fig. 6. CLSM images of the male cornuti (A) and section of the female cornucopia (B). White arrowheads in A indicate broken cornuti. In B, v and d indicate the ventral and dorsal sides in the copulatory condition. Red indicates strongly sclerotized portions, and blue indicates

resilin-rich portions.

Fig. 7. Relationship between forewing length (A) or cornucopia size (B) and the number of deciduous cornuti in once- (circle) and twice- (triangle) mated female *Argynnis paphia*.

Supplementary Figure. Measurement methods for forewing (left) and cornucopia (right).

Supplementary Data. Dataset used for Table 1 and Fig. 7 (analysis of the number of deciduous cornuti and mating frequency).

Tables

Table 1. Comparison of multiple regression models for explaining the numbers of deciduous cornuti in the corpus bursae of female *Argynnis paphia*. In the column titled "Log-likelihood ratio test", *P* values (compared with the full model) are given with the χ^2 statistic in parentheses. The smallest AIC value is in bold and italicized.

Models	Log-likelihood ratio test (vs. Full model)	AIC
Full model: No. cornuti ~ Forewing length + Cornucopia size + No. copulations	-	<i>359.8</i>
Without "Forewing length": No. cornuti ~ Cornucopia size + No. copulations	0.0012 ($\chi^2_1 = 726.2$)	368.1
Without "Cornucopia size": No. cornuti ~ Forewing length + No. copulations	0.106 ($\chi^2_1 = 181.3$)	360.6
Without "No. copulations": No. cornuti ~ Forewing length + Cornucopia size	0.0086 ($\chi^2_1 = 480.2$)	364.8

Fig. 1

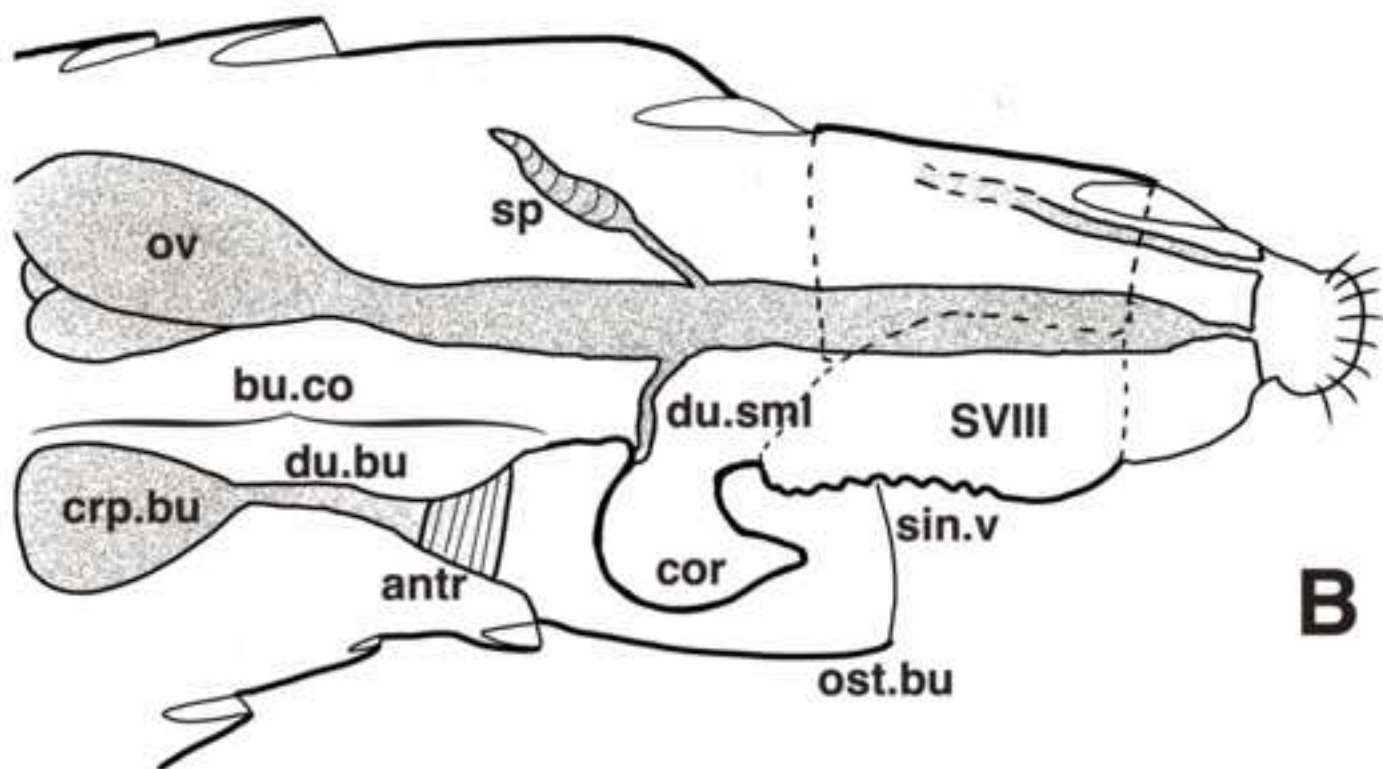
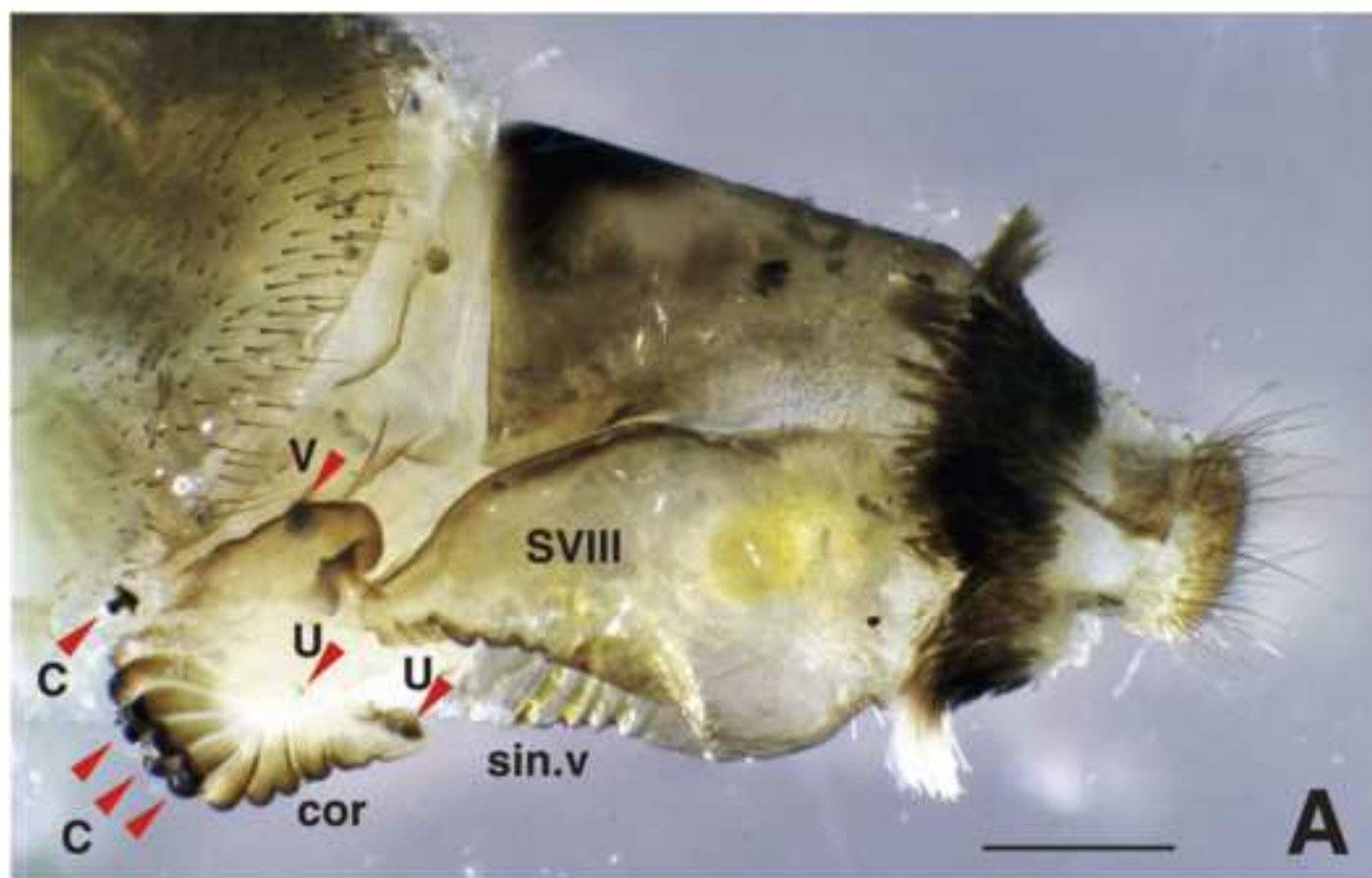


Fig. 2

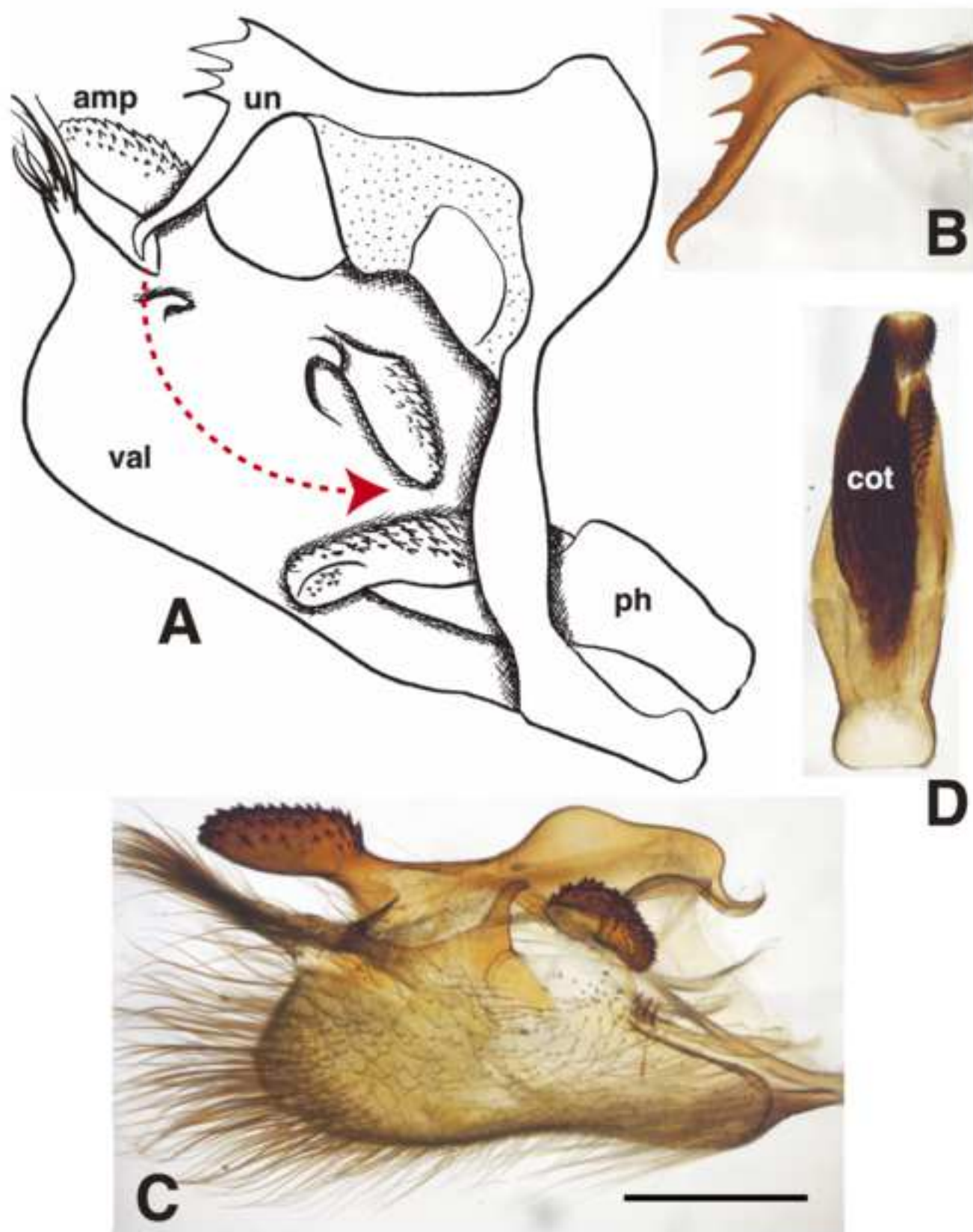


Fig. 3

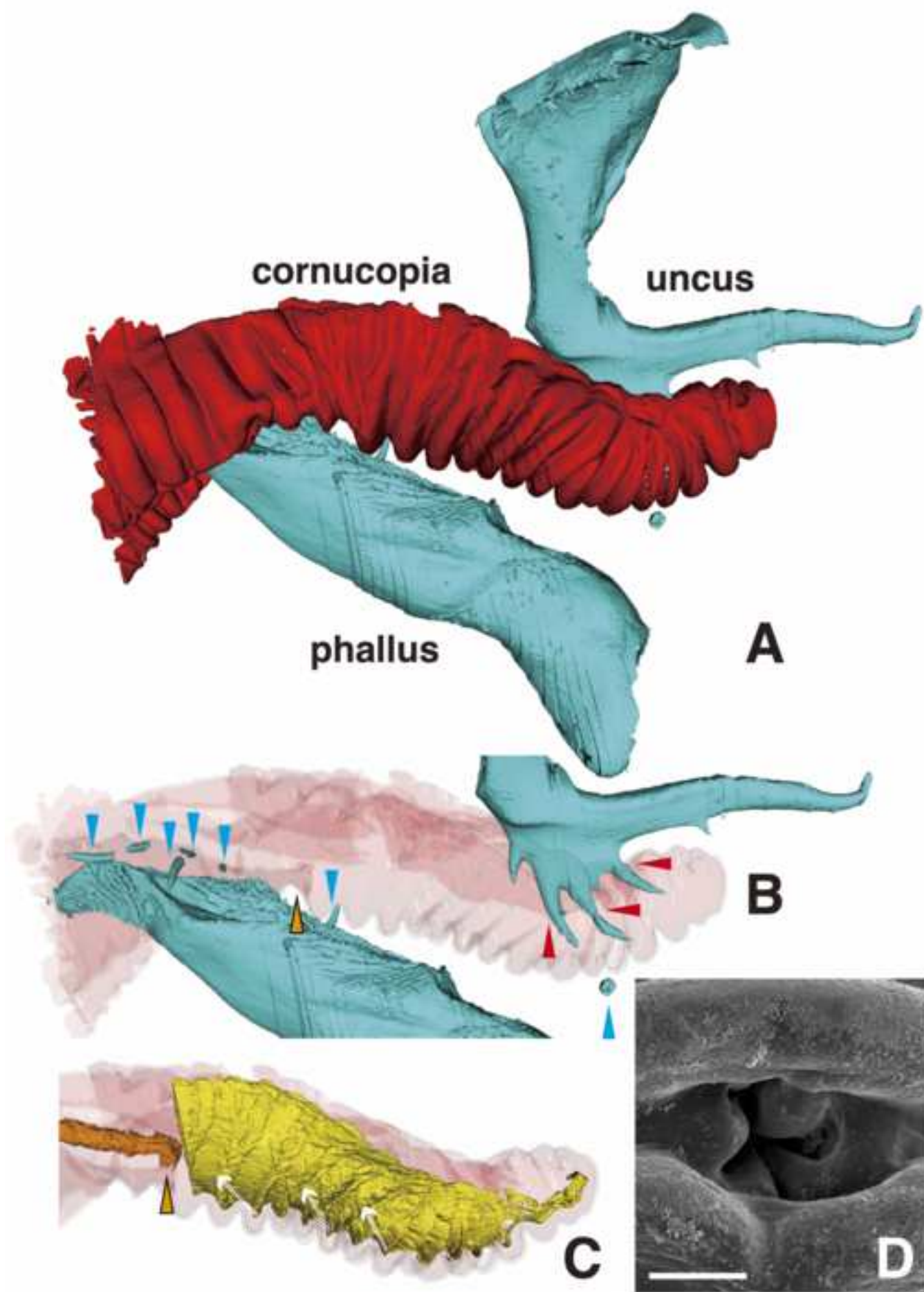


Fig. 4

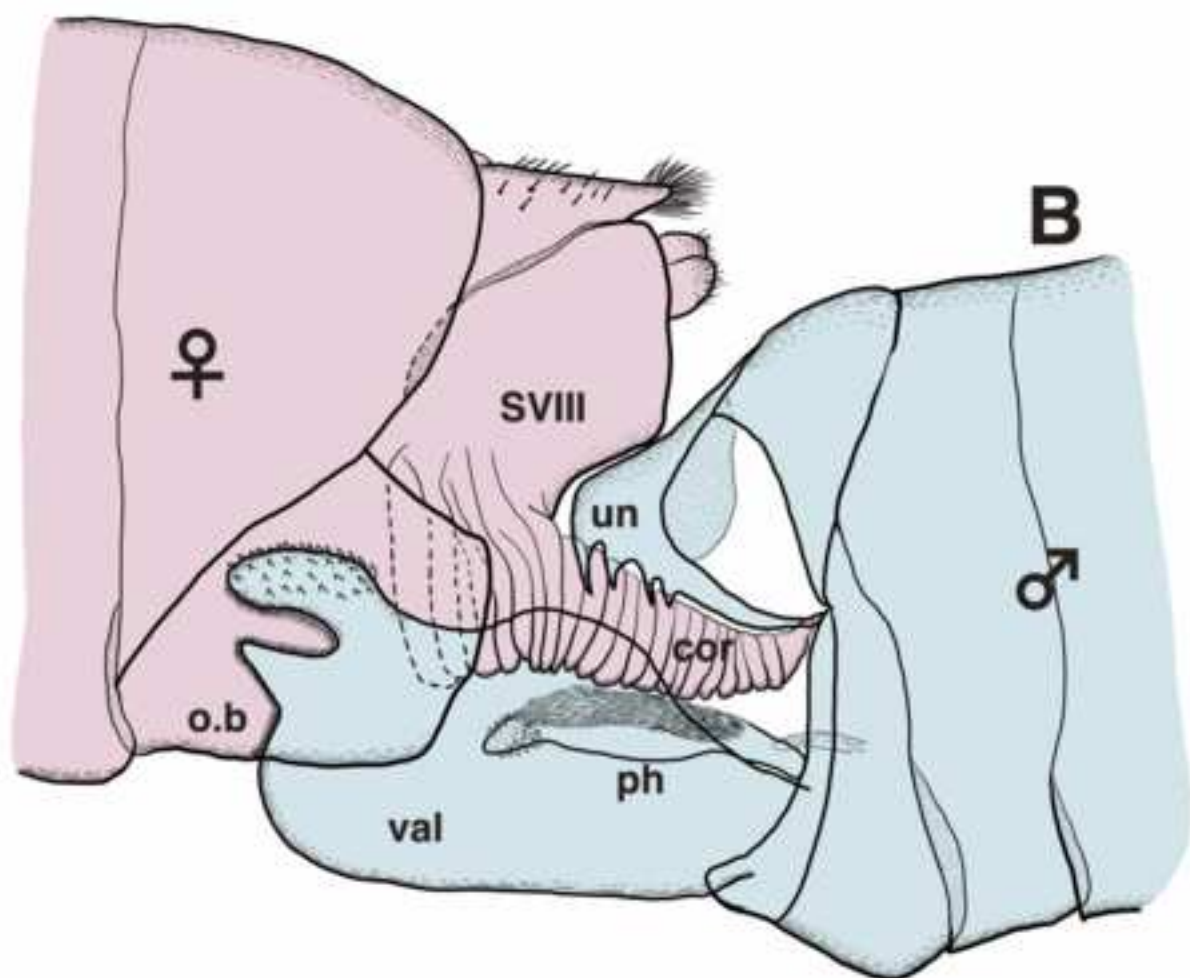
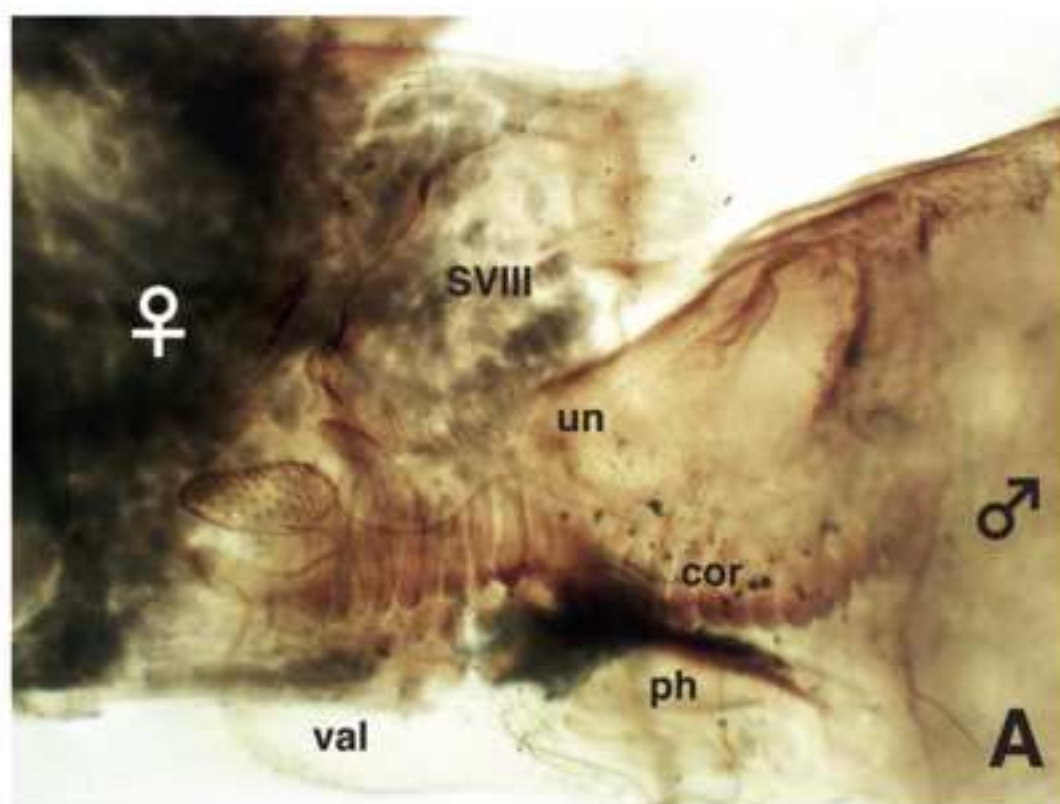
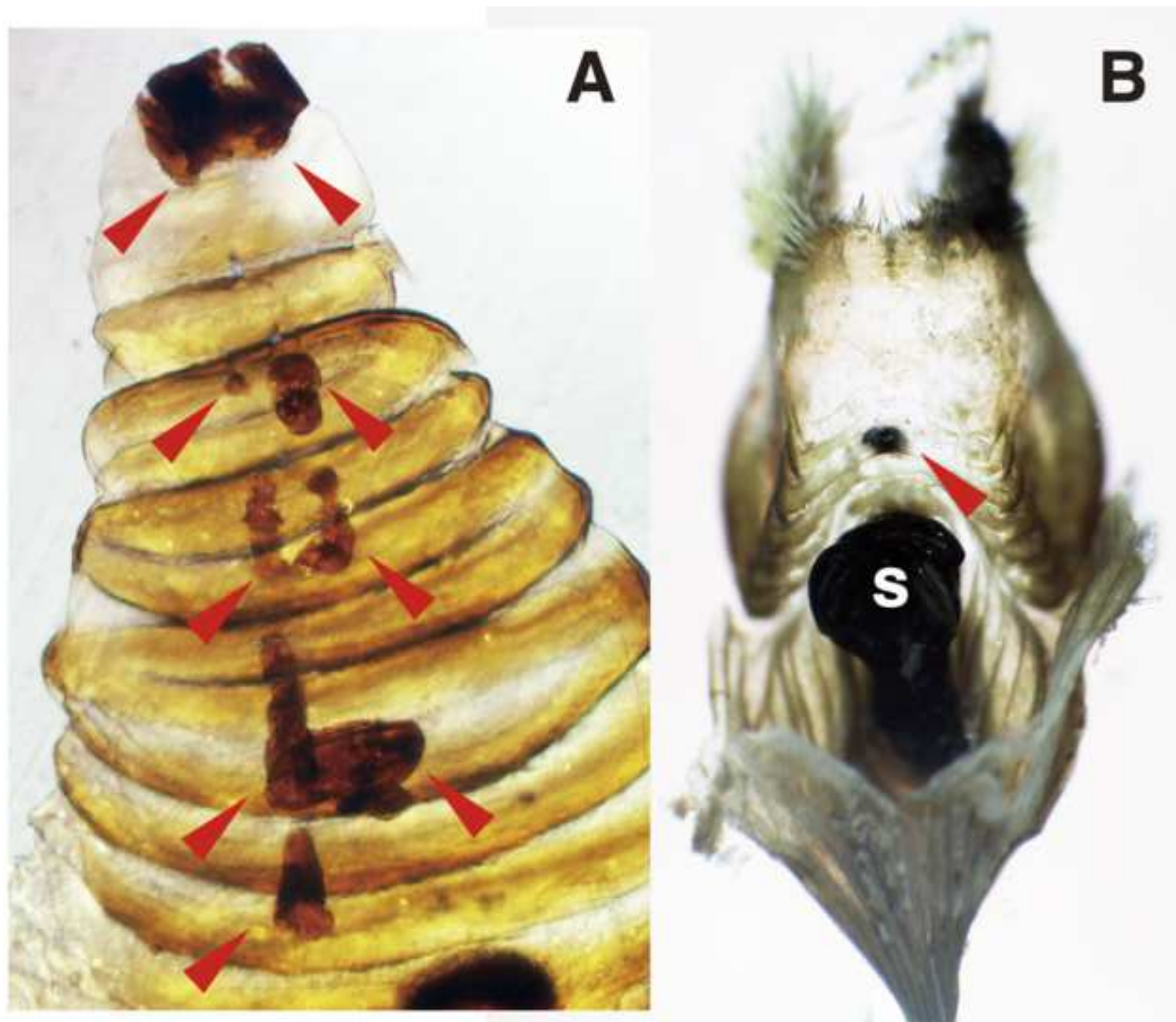


Fig. 5



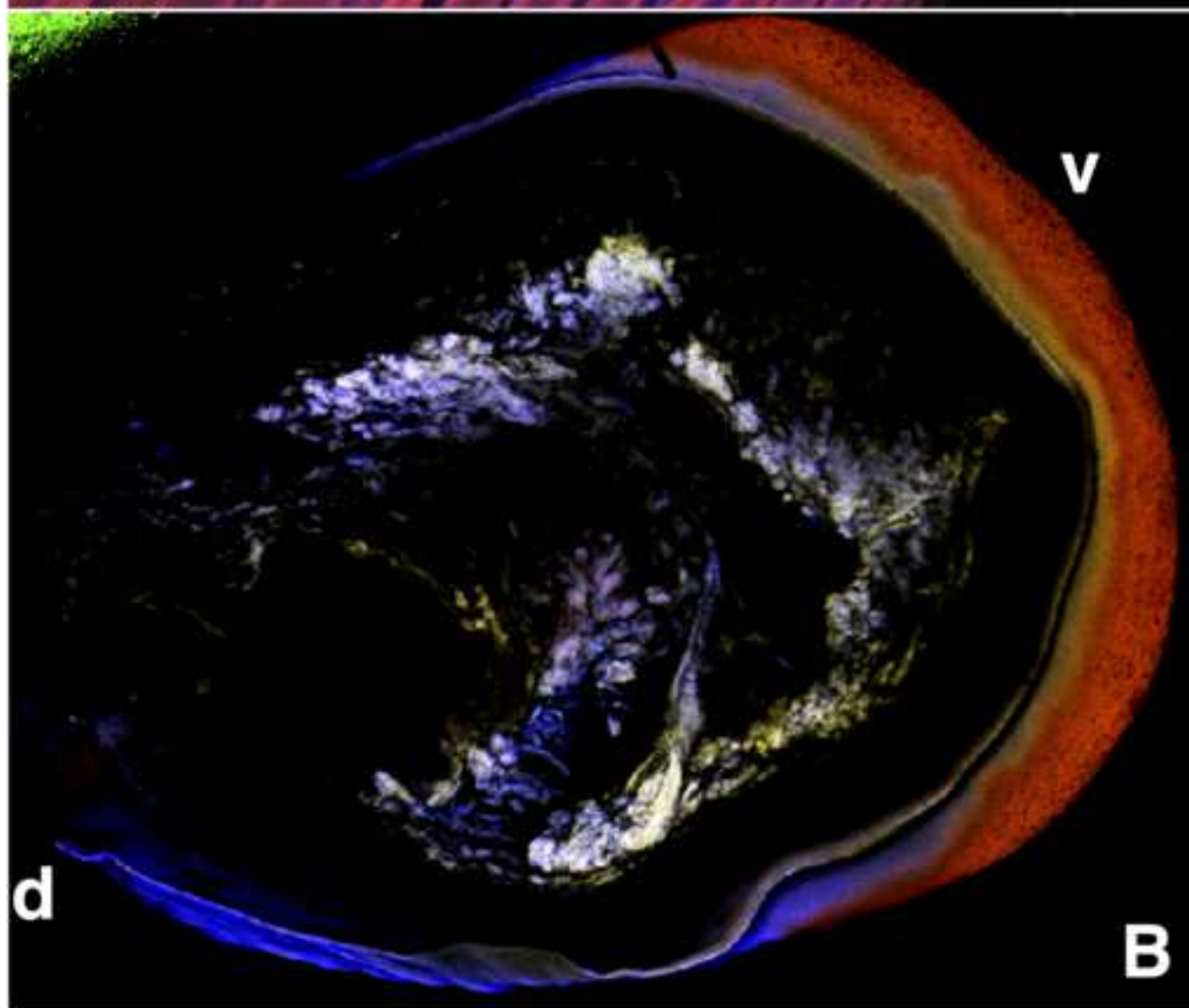
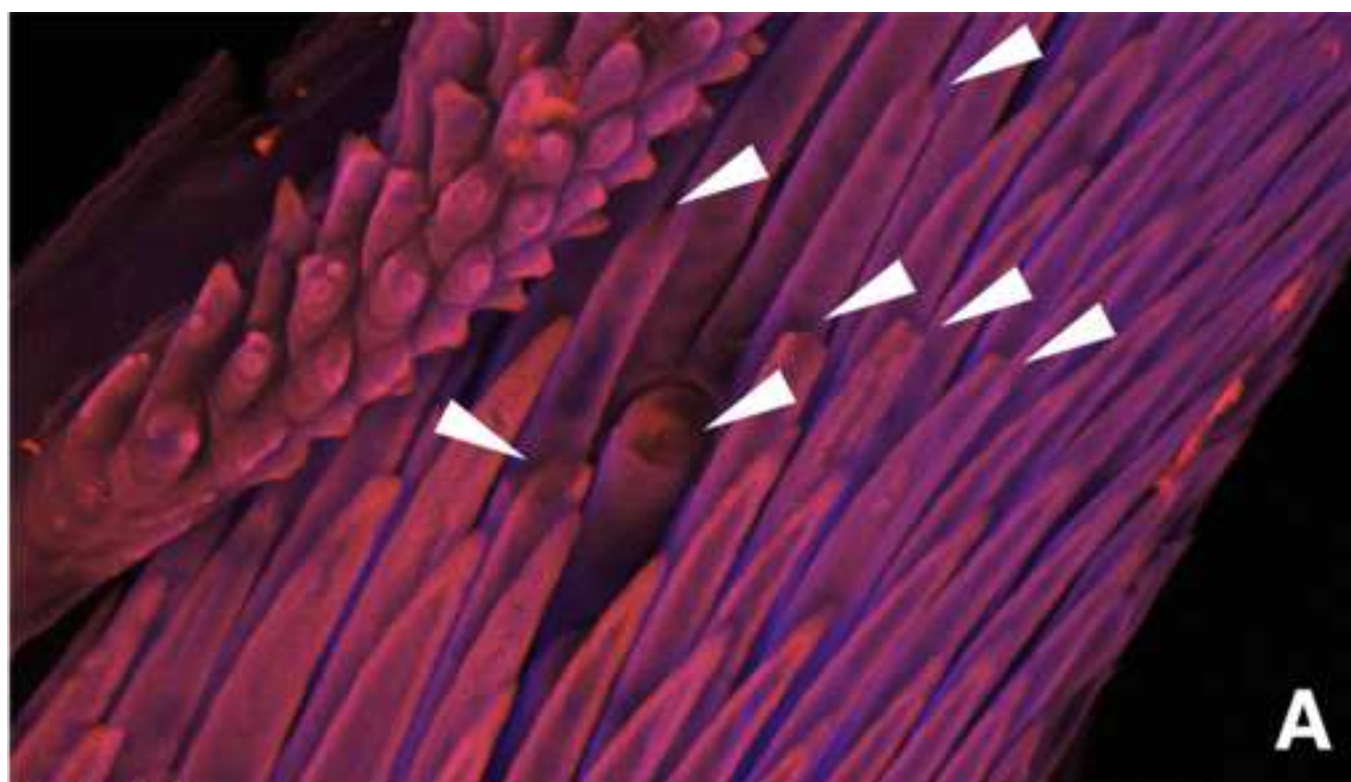


Fig. 7

