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Morphology and Ontology

When mayflies have an erection: functional morphology of the genitalia in *Ecdyonurus* (Ephemeroptera: Heptageniidae)

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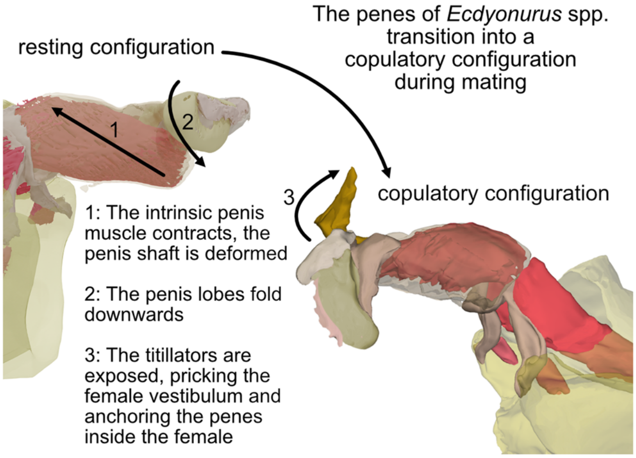
The aerial copulation of the mayfly *Ecdyonurus venosus* (Fabricius, 1775) and *Ecdyonurus submontanus* Landa, 1969 is described based on SEM, dissections, histological serial sections, and μ-CT. The abdomina of a pair in copula and a resting male specimen are reconstructed, with emphasis on sclerites, musculature and associated muscles of the genitalia and the male gonopods. The resulting models reveal a change in penis configuration while the penis is located inside the female vestibulum. This change is facilitated by a pair of intrinsic penis muscles. Its action leads to a depression of the penis shaft sclerite, which is associated with an upward rotation of the penis lobes and the eversion of a pair of spike-like titillators. Once exposed inside the female vestibulum, the penes are slightly moved dorsally. This movement is facilitated by a pair of extrinsic penis muscles. Thereby, the everted titillators are pricking into the female intersegmental membrane between sternites VII and VIII. In this way, the titillators act as an anchoring device, fixing the penes within the female vestibulum and enlarging the latter during copulation. The evolutionary implications of these findings are discussed, and a new nomenclature for the copulatory organs is proposed.

Keywords: abdomen, function, gonopod, musculature, sex

Graphical abstract



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Introduction

Mayflies (Ephemeroptera) constitute one of the basal branches of winged insects (Staniczek 2000, Misof et al. 2014). While the aquatic nymphal stages last for at least several months, the adults are short-lived with a life span of only a few hours to a few days. During this time, mating and oviposition are accomplished. Mayflies usually perform an aerial mating: Males swarm above the water, and females fly into the swarm (Wesenberg-Lund 1943). The males then approach the females from below and grasp the forewing bases of the female with their elongated forelegs. Additionally, males bend their abdomen dorsad and grab the females with an abdominal pair of gonopods from ventrally (Brinck 1957).

Apart from their highly derived mode of areal mating, mayflies also exhibit numerous, putatively plesiomorphic characters, like the so-called subimago, a penultimate winged life stage that undergoes another moulting to become the imago. The subimaginal moulting is generally regarded as a vestigial adult moulting, as adult moults still occur in all primarily apterygote insect orders (Maiorana 1979).

Other putatively plesiomorphic characters within Pterygota concern the reproductive system: Unlike in the vast majority of all other insects, the primarily paired phallic lobes, or mesomeres, do not entirely fuse during ontogenesis, so mayflies usually exhibit a pair of well-separated penis lobes, each with a separate genital duct and genital pore. Likewise, the female genital ducts in most species also remain entirely separated, and 2 genital openings are present (Grimm 1985).

While the inner anatomy and reproductive organs of both sexes of several mayfly species have been extensively studied (eg Palmén 1884, Needham et al. 1935, Snodgrass 1935, Qadri 1940, Levy 1948, Brinck 1957, Grandi 1960a, 1960b, 1962, 1964; Grimm 1977, 1985; Soldán 1981, Harker 1986, Takemon 1990, Gaino and Rebora 1995), the internal interactions of male and female genitalia during copulation have not yet been described in detail. This may be due to the fact that mayflies usually copulate in flight and are very sensitive to disturbance, so couples tend to separate when they are captured. To our knowledge, there are only a few photographs in scientific literature that document mating mayfly couples (Brink 1957: *Parameletus chelifer*; Bauernfeind and Soldán 2012: *Epeorus assimilis*; Voigt et al. 2017: *Siphlonurus armatus*).

Kimmins (1942) described differences in the male genitalia of various *Ecdyonurus* species (Heptageniidae). Harker (1986), however, assumed that some of these differences in penis shape are not species-specific, but may reflect configurational penis changes during different stages of mating. She observed such different penis configurations in *E. venosus* and assumed that the penis lobes rotate during mating. However, the exact mechanism and its significance remained unclear.

Subsequently, Takemon (1990) investigated the mating in *Epeorus ikanonis*, which mate on the ground, and are thus easier to observe and access than species with aerial mating. By dissecting couples at different times during mating, he was able to prove that the penis indeed changes its shape by the time it is introduced into the female vestibulum. Accordingly, he distinguished between a solitary rest form ("sole form") and a "copula form" of the penis that is adopted during mating. However, Takemon (1990) did not investigate the underlying functional mechanisms responsible for these configurational changes.

Grimm (1985) in a comparative work studied abdominal musculature and reproductive organs throughout Ephemeroptera. As Levy (1948) and Grandi (1960b, 1964) before him, he also documented a pair of intrinsic and 2 pairs of extrinsic penis muscles in most of the investigated species (Grimm 1985). Likewise, the gonobasis, which together with the gonostylus forms the gonopod, is generally equipped with intrinsic muscles.

Grimm (1985) assumed that the intrinsic penis muscles play an important role in sperm transfer. He was not aware of configurational changes that may occur in the penes of mayflies during copulation, so he did not associate any of the described muscles with these spatial changes.

The aim of the present work is to clarify the function of genital interactions during copulation in the mayfly genus *Ecdyonurus* and to unravel the mechanisms that lead to the change in the penis configuration during mating. Therefore, we study males and females of *Ecdyonurus venosus* (F., 1775) by manual dissections, maceration, SEM, histological serial sections, and mating couples by synchrotron X-ray microtomography (SR-µCT). For comparison, an additional copula of *Ecdyonurus submontanus* Landa, 1969 is investigated using SR-µCT.

Materials and Methods

Material

Single male and female adult specimens of *Ecdyonurus venosus* (F., 1775) as well as couples of *E. venosus* and *E. submontanus* Landa, 1969 in copula, all housed in the Ephemeroptera Collection of the State Museum of Natural History Stuttgart, Germany (SMNS), were used for this study. The material of *E. venosus* in copula was almost entirely collected during several field trips to the river Wutach in the southern Black Forest, Germany (see also Frey and Staniczek 2010). Additionally, further material from the SMNS collection was used. For the collections in the nature reserve Wutachschlucht/Wutachflühen, a respective permit of the Regional Council of Freiburg had been obtained. The couple of *E. submontanus* was collected during a field trip to the Goldersbach close to Tübingen-Bebenhausen, Germany.

E. venosus:

SMNS_EPH_002686_A, ♂I: Deutschland, BW, Steibis, Weissach, 4 September 1972, leg. P. Malzacher.

SMNS_EPH_004631_A, ♀I: Deutschland, BW, Umg. Wutach bei Mündung Gauchach, GPS 8,43917E; 47,85378N; 518 m, 01 July 2006, leg. A. Staniczek & M. Pallmann.

SMNS_EPH_004672_A, ♀I: Deutschland, BW, Umg. Münchingen, Wutachschlucht, Schurhammerhütte, Wutach, GPS 8,39637E; 47,84295N; 623 m, 23 August 2007, leg. B. Frey.

SMNS_EPH_005017_A: Deutschland, BW, Umg. Göschweiler, Wutachschlucht, Schattenmühle, GPS 8,31687E; 47,84386 N; 651 m, 3 September 2008, leg. B. Frey.

SMNS_EPH_005053_A, ♂I: Deutschland, BW, Umg. Achdorf, Wutachflühe bei ehemaliger Moggerenmühle, Wutach, GPS 8,51064E; 47,81683N; 531 m, 11 September 2008, leg. B. Frey.

SMNS_EPH_005033_S, ♂/♀I in copula: Deutschland, BW, Münchingen, Wutachschlucht, Schurhammerhütte, Wutach, GPS 8,39637E; 47,84295N; 623 m, 25 August 2008, leg. B. Frey.

SMNS_EPH_005033_A, ♂/♀I in copula: Deutschland, BW, Münchingen, Wutachschlucht, Schurhammerhütte, Wutach, GPS 8,39637E; 47,84295N; 623 m, 25 August 2008, leg. B. Frey.

SMNS_EPH_005974_A, ♂+♀I: Deutschland, BW, Umg. Dobel, Eyachmühle, Eyach, GPS 8,527671E; 48,784116 N; 487 m, 28 June 2013, leg. A. Staniczek

E. submontanus:

SMNS_EPH_012513_B, ♂/♀I in copula: Deutschland, BW, Tübingen-Bebenhausen, Goldersbach, GPS 9,05265E; 48,55875N; 360 m, 25 May 2024, leg. B. Stocker & S. Belschner.

Collection and Fixation

Using an insect net with a handle length of 2.5 m, copulating pairs were netted and immediately fixed in 80% ethanol. Alternatively, some pairs were immobilized in the net using a commercial freeze spray prior to fixation. In about 50% of cases, the animals separated despite freezing. Those couples, which did not separate, were immediately transferred into AFAA fixative (a mixture of 80% ethanol (90 parts), 37% formaldehyde (5 parts), and acetic acid (5 parts)). The material was then transferred to 100% ethanol after 24 h.

Manual Dissection and Maceration

For fuchsin staining, specimens were cut along the sagittal plane while submerged in 75% ethanol using a commercial razor blade (Wilkinson Sword) and dissected using sharpened insect needles and Dumont forceps (Biology E). Stained specimens were studied using a Leica M205 C stereo microscope. In order to observe inner chitinous structures of the genital segments, individual specimens were macerated for 24 h in potassium hydroxide at room temperature (see Mulisch and Welsch 2015). The macerates obtained were stained for one minute using 0.12% aqueous Chlorazol Black E solution and then transferred to 50% ethanol. Photographic documentation was carried out with a Leica DMC5400 digital camera on a Leica Z16 APO Macroscope, using Leica Application Suite Version 3.1.8 and Helicon Focus Pro to obtain stacked photographs with an extended depth of field. All photographs were subsequently sharpened and adjusted for contrast and tonality in Adobe Photoshop Version 26. Figures were arranged and labelled using Affinity Designer 2.

Scanning Electron Microscopy

Genital segments of males and females were prepared by dehydration through a stepwise immersion in ethanol, critical point drying (Leica EM CPD300), and mounting on SEM stubs. The mounted material was coated with a 5 nm Au/Pd layer (Leica EM ACE200) and subsequently scanned using a Zeiss EVO LS 15 scanning electron microscope at 10 kV. Resulting photographs were sharpened and adjusted for contrast and tonality in Adobe Photoshop Version 26.

For 1 female of *E. venosus*, the subgenital plate was removed to reveal the vestibulum. It was dried in an ascending ethanol series (75%, 80%, 90%, and twice at 100%) followed by critical point drying (Leica EM CPD300). Objects were gold-coated for 5 min from various angles to improve electrical properties (Quorum Emitech K550X). Images were taken using a Scanning Electron Microscope (ZEISS EVO LS 10), by using the secondary electron detector. An acceleration voltage of 10 kV was used.

Histological Serial Sections

To obtain serial sections, genital segments of single male and female specimens were dehydrated by stepwise immersion in ethanol. Subsequently, samples were embedded for 24 h each in a 1:1 mixture of isopropyl alcohol and paraffin, in pure paraffin (both steps at 50 °C), and twice in Paraplast Plus at 60 °C. Sections of 5 µm thickness were cut using a Leitz 1516 rotary microtome. Sections were stained with Delafield’s hematoxylin, counterstained with aqueous eosin, and embedded in Euparal. Photographs were taken using a Keyence VHX-500FD microscope. Photographs were subsequently adjusted for contrast and tonality in Adobe Photoshop CC, Version 26.

Synchrotron Microtomography and 3D-Modeling

Synchrotron X-ray microtomography (SR-µCT) of two critically point-dried copulating couples of *E. venosus* was performed at the TOPO-TOMO beamline of the KIT Light Source. The polychromatic X-ray beam was produced by a 1.5 T bending magnet and filtered to achieve an average energy of approximately 20 keV. The detector system consisted of a LAG scintillator (50 µm), a PCO 4000 CCD digital camera (4,006 × 2,672 pixels), a tube lens, and a 5× objective, resulting in an effective pixel size of 1 µm. During each scan, 3,000 projections were acquired over an angular range of 180°. Projection data were processed using the ANKAphase phase-retrieval software (Weitkamp et al. 2011), and tomographic reconstruction was performed using the PyHST algorithm (http://ufo.kit.edu/pyhst/).

Two stacks of tomographic slice images of *E. venosus* in copula were generated, one of which was selected for subsequent 3D modeling. The scan used consisted of a total of 1,336 single-image planes.

From the acquired tomographic image series, a 3D segmentation model was reconstructed using Amira 6.01. Important structures were pre-segmented in the segmentation editor by manually labelling every fifth slice. Semi-automated segmentation was done using Biomedisa (Lösel et al. 2020). Segmentation results were imported in Amira 6.01, and all individual parts were converted into polygon meshes by employing the “SurfaceGen” tool. The meshes were exported as OBJ files and reassembled in CINEMA 4D R19 to smooth the reconstruction.

Additionally, SR-µCT of the abdomen of a male *E. venosus* specimen and a copulating pair of *E. submontanus* fixed in 100% ethanol was conducted at the IMAGE beamline (Cecilia et al. 2025) of the KIT Light Source. The polychromatic X-ray beam generated by a superconducting wiggler was filtered with 10 mm pyrolytic graphite sheets, yielding a spectrum peaking at approximately 16.5 keV. The imaging setup comprised a fast indirect detector system with a LSO scintillator (25 µm), visible-light optics, a white-beam microscope (Optique Peter, Lentilly, France; see Douissard et al. 2012), and a 12-bit pco.dimax high-speed camera (Excelitas PCO GmbH, Kelheim, Germany). A 5× magnification yielded an effective pixel size of 2.44 µm. For each scan, 3,000 equiangularly spaced radiographic projections were recorded over 180°. Automated data acquisition was controlled via the Concert framework (Vogelgesang et al. 2016). Tomographic reconstruction was performed with tofu (Faragó et al. 2022), including ring-artefact removal, 8-bit conversion, and blending of phase- and absorption-based 3D reconstructions (Faragó et al. 2024). The segmentation took place by first labelling every 20th slice in Amira 3D 2022.1 and selecting slices in other, orthogonal orientations. The image was then automatically segmented using Biomedisa (Lösel et al. 2020) all axis segmentation. The resulting segmentation was then cleaned and corrected in Amira, exported to 3D Slicer (5.6.2) via the Bio-Formats plugin in ImageJ 1.54f. The segmentation was then converted into a polygon mesh and exported as .obj files for further processing in Blender 4.3.2. Here, holes were closed using the shrink-wrap modifier, and the meshes were decimated and remeshed. The images were rendered using the Cycles engine, final adjustments were made using Photoshop version 26.

Terminology

The terminology of muscles was established according to their respective origins, directions, and attachments within the observed abdominal segments. For convenience and better readability in the descriptions and figures, the muscles were also consecutively numbered. Numbering of abdominal segments is given in Roman numerals. An overview of the musculature is compiled in Appendix 1.

Designation of Spatial Relationships

During copulation, the tip of the male abdomen bends forward, producing an inverted orientation: the abdominal sternites (S) are above and the tergites (T) below. For clarity, all directional terms refer to the original anatomical orientation of the animal, not to its position after flexion.

Results

The abdomen in *Ecdyonurus venosus* has a circular cross section and consists of 10 visible segments (Fig. 1). The 10 segments are differentiated into pregenital, genital, and postgenital segments. Each of the segments I to IX is divided into a larger tergite and a smaller sternite, which are connected ventrolaterally by a thin intrasegmental membrane. Additionally, the segments are connected to each other by a thin intersegmental membrane (ism). Segment X is represented by its tergite as well as paired paraprocts (ppt), and an epiproct (ept) surrounding the anus (an) in both sexes (Fig. 2A and B). Long, paired cerci (ce) originate lateral to the epiproct, while dorsal to the epiproct, the unpaired medial terminal filament (tf), or paracercus, is heavily reduced and only represented by a short, unsegmented, membranous tip.

External Morphology of the Male Genital Segments

In the male, sternite IX is caudally extended to give rise to a pair of genital appendages (Figs 2A and 3A). These paired gonopods are each composed of a basal gonobasis (gb) and an apical gonostylus (gst). The gonobases are medially fused to form a plate which has been termed in mayfly literature as subgenital plate (Needham et al. 1935, Domínguez et al. 2006), basal plate or coxopodite (Snodgrass 1935), styliger plate (Brinck 1957), or styliger (Studemann et al. 1992, Bauernfeind and Humpesch 2001). Here, we refer to the medially fused gonobases as gonobasal plate (gbp). The caudal end of the gonobasal plate is convex. The gonobasal plate covers the basal part of the penes ventrally. Each gonostylus is secondarily divided into 4 parts. Parts of secondarily divided appendages are usually indicated by the ending "–mere" (like tarsomeres, palpomeres, or antennomeres, etc.), so we refer to these secondarily subdivided parts not as segments, but as gonostylomeres (gsm): Gonostylomere 1 is broader than the remaining ones, short, and rounded. In its lateral half it is adjoined by gonostylomere 2, which is the longest one. A shorter third and fourth gonostylomere follow apically. The gonostyli are bent inwards, thus giving the genital claspers the appearance of a pair of forceps. They are densely covered with microtrichia (mtr) along their entire length (Fig. 3A and B). Especially on gonostylomeres 3 and 4, these microtrichia are dorsally, ventrally, and laterally densely packed and scale-like (Fig. 3B). Additionally, hair-like setae (se) are scattered laterally along the full length of the gonostyli (Fig. 3A to C). On their entire medial sides, the gonostyli are equipped with modified, papillose setae (pse) (Fig. 3C and D). On gonostylomeres 1-3, they are evenly distributed (Fig. 3C), at the tip of gonostylomere 4 they are medially densely clustered (Fig. 3B). Most of these papillae are pear-shaped, with a thinner basal end (Fig. 3D). In some SEM images they appear apically slightly dented, others are apically stretched to a short, thin thread. They are all surrounded by a basal ring (br), as it is typical for setae (Fig. 3D).

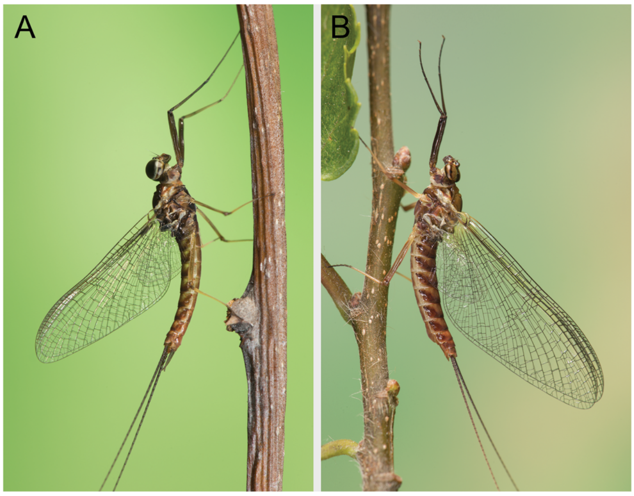

Fig. 1. Male (A) and female (B) imago of *Ecdyonurus venosus* in lateral view.

Penis Morphology

The male genitalia originate at the caudal end of abdominal segment IX between the gonobasal plate and abdominal segment X (Fig. 3A). They are bilaterally symmetrical and consist of a penis shaft (ps) and a pair of apical penis lobes (pl, Figs 3A and 4), reflecting their paired origin during ontogenesis. Consequently, the male genitalia of mayflies are often referred to as paired penes, as each penis lobe bears a separate gonopore. Each penis lobe also features several isolated sclerites (see below). Medial to the penis lobes, the titillators (ti) are located. These are heavily sclerotized stylets, which are basally fused and part of a larger titillator sclerite (tis, Fig. 5B, D, and F). The basal arms of the titillator sclerite (tia) are connected to the medioanterior ends of the apical, lateral, and basal sclerites (Fig. 5E). The penis shaft forms the basal part of the penes. The penis shaft sclerite is a cylinder that is dorsally contiguous

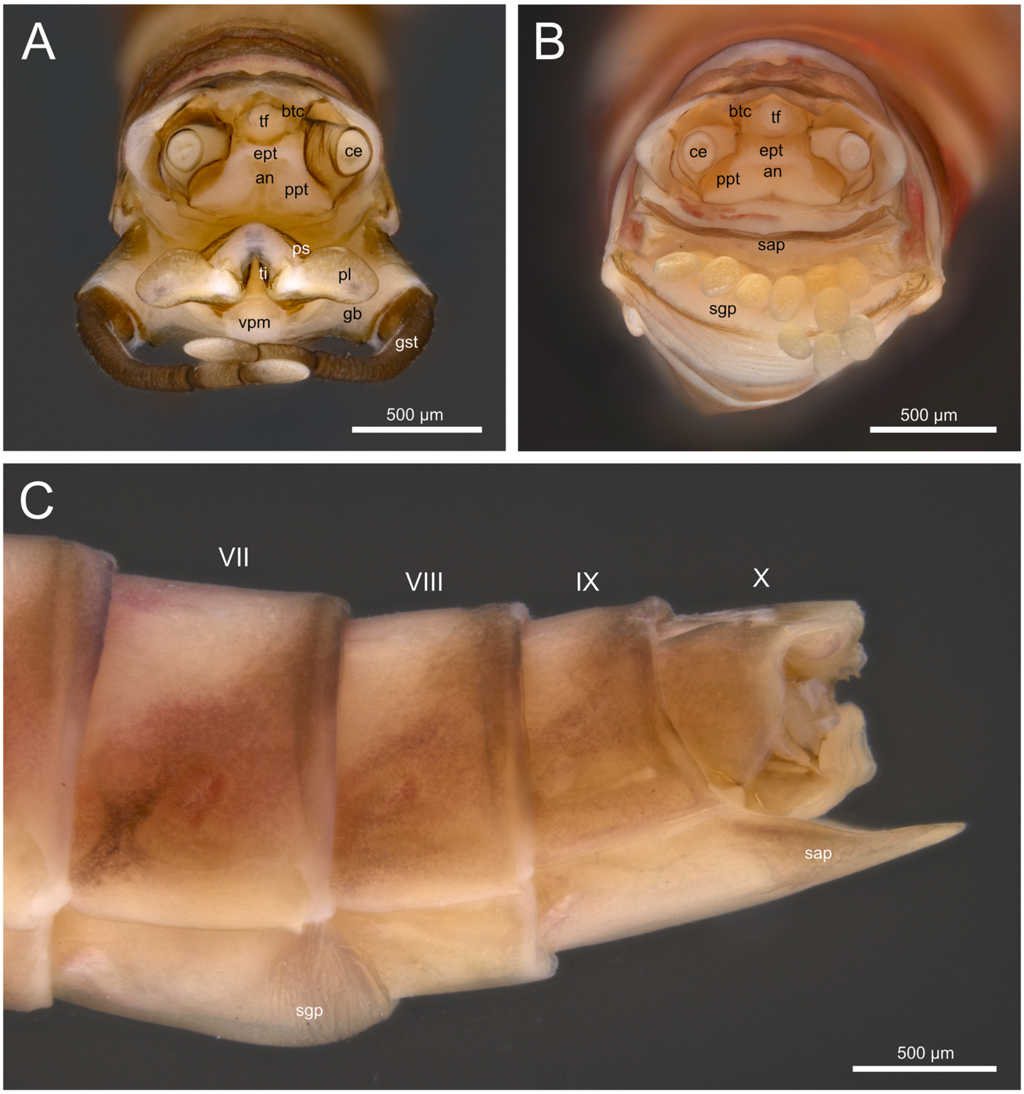

Fig. 2. Imaginal abdomen of *E. venosus*. (A) Male in caudal view. (B) Female in caudal view. (C) Female in lateral view.

in its basal half, with an apical, V-shaped cleft bridged by an interlobal membrane (ilm, Fig. 4A). It also leaves a longitudinal medial cleft on the ventral side that is partially connected by a ventral penis membrane (vpm). Basally, the penis shaft is thickened on its dorsal side to form a transverse penis rod (pr), which supports and stiffens the penis. Medially, there is a membranous triangular gap between penis shaft and penis rod (Fig. 5A). The penis rod is laterally extended into 2 curved penis arms (pa), which are directed dorsolaterally to articulate with the caudal processes of tergum IX (Fig. 13E and F).

Each of the shoe-shaped penis lobes is equipped with 3 sclerites: The basal sclerite (bs) encompasses the penis lobes basolaterally (Figs 4 and 5). The rather broad lateral sclerite (ls) is located distal to the basal sclerite (Fig. 4A). The titillators are dorsally covered by the interlobal membrane (Fig. 4A,C,E). The acute tips of the titillators point caudad. Medially, each penis lobe is equipped with an apical sclerite (as). The latter medially possesses several short, apically directed spines (Fig. 4C and D). At the caudal end of each penis lobe, the elongate gonopores are located, which are covered by membranous, serrated lobe flaps (lf, Fig. 4E). Ventrobasally the penis shaft is connected to the gonobasal plate by a ventral penis membrane (vpm), which may medially form a lappet and laterally a hump (Figs 2A and 3A).

Campaniform sensilla (cs) are found on the penis shaft sclerite, the lateral sclerite, and the apical sclerite. Trichoid sensilla (ts) are found on the apical sclerites only (Fig. 4E, inlay). The sensilla appear to be similar to those found in *Rhithrogena semicolorata* Curtis, 1834 by Gaino et al. (2009).

External Morphology of Female Genital Segments

In the female, sternite VII is caudally extended to form a subgenital plate (sgp), which ventrally also covers the anterior part of the subsequent sternite VIII (Figs 2C and 6A). The subgenital plate appears to be weakly sclerotized with numerous transversal folds (Figs 2C and 6A). Sternite VIII is laterally dented, sternite IX is caudally extended to form a broad, sclerotised subanal plate (sap), which ventrally covers the entire segment X (Figs 2C and 6A). Sternum VII (as the preceding segments) is densely covered with microtrichia (Fig. 6B and C) except where longer, hair-like setae (Fig. 6B and C) are present, which are conspicuous because of the circular blank area without microtrichia surrounding their cuticular insertions. The subgenital plate is also covered with numerous

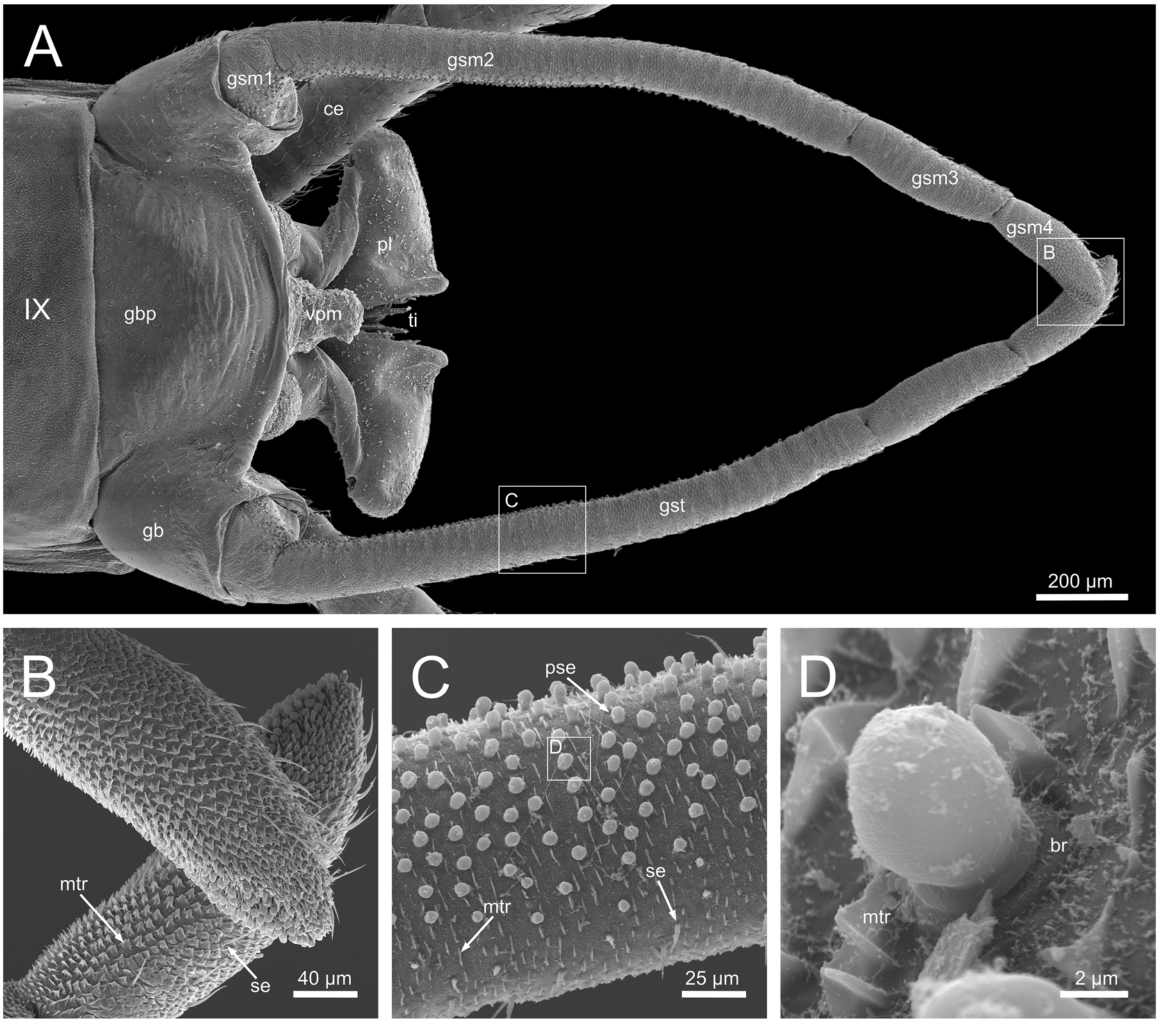

Fig. 3. Male genitalia of *E. venosus*, SEM. (A) Penes and gonopods in ventral view. (B) Tips of gonostyli. (C) Detail of gonostylomere 2 with numerous pear-shaped setae medially. (D) Detailed view of pear-shaped seta.

microtrichia and occasionally equipped with setae (Fig. 6C). The cuticular microsculpture of sterna VIII and IX, as well as that of the subanal plate, is less pronounced (Fig. 6D).

Internal Organization of the Abdomen

Beneath the cuticle, various muscle groups surround the vegetative organs, including the aorta (ao), mesenteron (mes), tracheae (tr), ventral nerve cord (vn), and the reproductive organs (Fig. 7). The different muscle groups allow for the movement of tergites against sternites and of entire segments against each other. Not only the musculature of the genital segments but also the muscles of the pregenital segments differ significantly between both sexes. The pregenital segments include the segments I-VI in the female, in the male, the segments I-VIII. Unlike all other preceding abdominal segments, in segment VII, there are 2 ganglia (ga) present. The respective ganglion VII is situated medioventrally at the anterior end of segment VII. It is directly followed by the darkly pigmented synganglion VIII, formed by the fusion of abdominal ganglia VIII, IX, and X.

Male Inner Reproductive Organs

The paired testes are atrophied and located laterally in abdominal segment VIII. They lead into the likewise paired, tube-like vasa deferentia (vd), which in segment IX widen into bipartite vesiculae seminales (vsa, vsp, Fig. 8). The anterior vesiculae are unchambered, while the posterior vesiculae are subdivided into numerous chambers. The transition between the anterior and the posterior vesiculae is narrowed. Along their entire length, the vasa deferentia and vesiculae seminales are equipped with a thin layer of longitudinal muscles (Fig. 7A). In segment X, each posterior vesicula seminalis leads into an unmusculated ductus ejaculatorius (de, Figs 16, 17). Both ductus ejaculatorii remain separated along their entire length and open separately into the gonopores located at the apices of the penis lobes.

Female Inner Reproductive Organs

The paired ovaries (ov) are located dorsally in the anterior abdomen and close to the tergites filling the entire dorsal third of the anterior segments (Fig. 7C). In the caudal half of segment VI, they taper into the tubular oviducts (ovd), which obliquely lead ventrad towards the caudal end of segment VII (Fig. 15). Dorsal to the subgenital plate, the intersegmental membrane between sternite VII and VIII is invaginated to form a largely membranous cavity, the so-called vestibulum (ves), into which the penis is inserted during mating. The cavity is dorsally delimited by the anteriorly deeply notched sternite VIII. The notch encompasses the dorsal portion of the intersegmental membrane

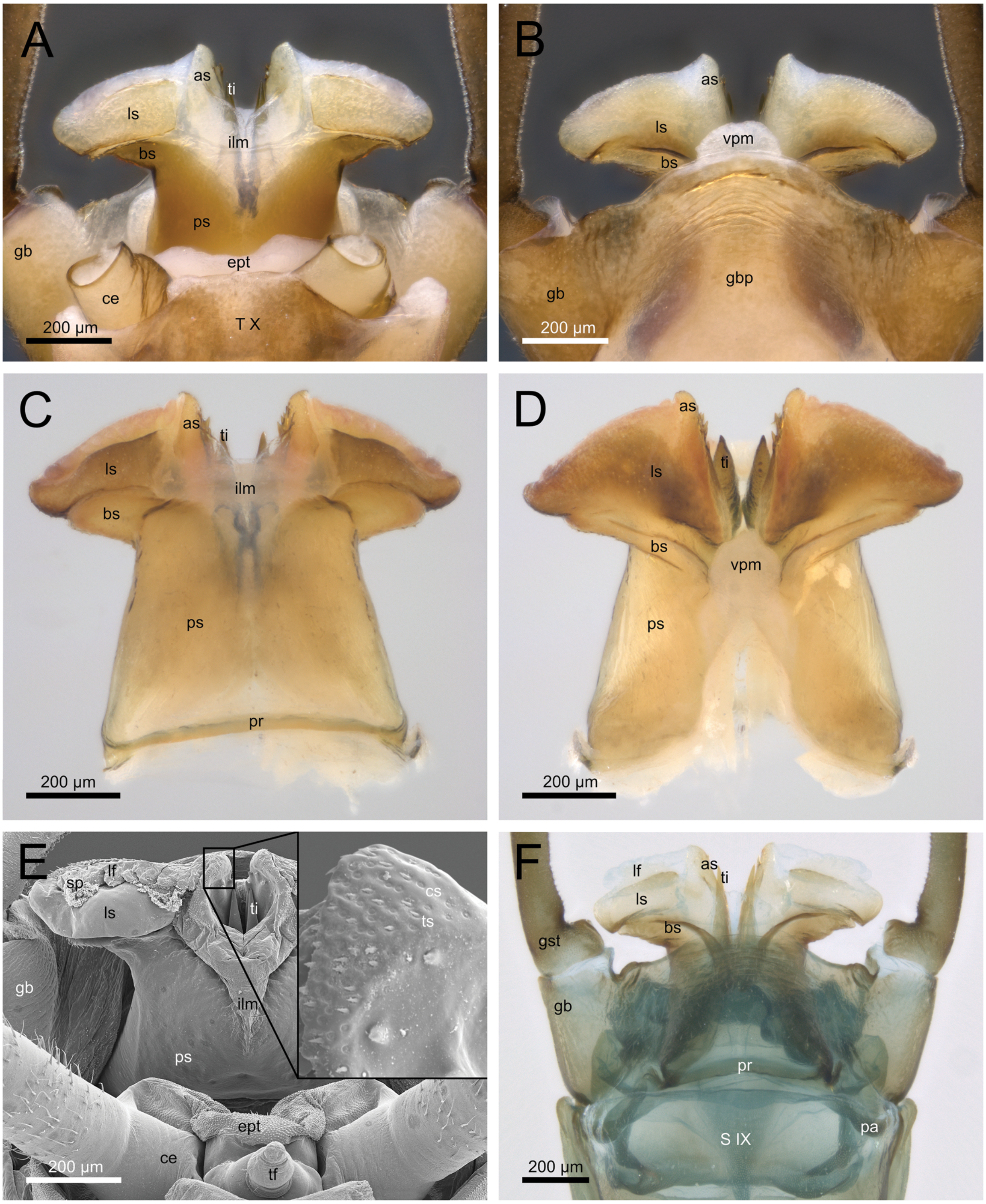

Fig. 4. Penes of *E. venosus* in resting position. (A, C, and E) in dorsal view, (B, D, and F) in ventral view. (A and B) in situ, (C and D) excised, (E) SEM with inlay of surface details of apical sclerite, (F) macerate stained in Chlorazol Black.

VII. Inside the vestibulum, the paired female gonoducts open into ovipores laterally at the posterior end of the enlarged intersegmental membrane VII, near the anterior margin of sternite VIII. Each ovipore is covered by a valve (va, Fig. 9). The valve is a lateral, lobe-like outgrowth of the intersegmental membrane. The latter is hypertrophied medially and presents multiple folds (htm, Fig. 9). The opening of the vestibulum itself is covered by the weakly sclerotised subgenital plate.

Muscles of the Male Pregenital Segments II -VIII (Fig. 10, Appendix 1)

The male pregenital segments II to VIII are each equipped with 11 muscles, except for segment VIII, which has 12 (Fig. 10):

The *inferior sternal longitudinal muscle* (1) is a minute and short muscle with a flattened cross section. It originates in the medioposterior area of the sternite and inserts at the anteromedial margin of the consecutive sternite. It supports the

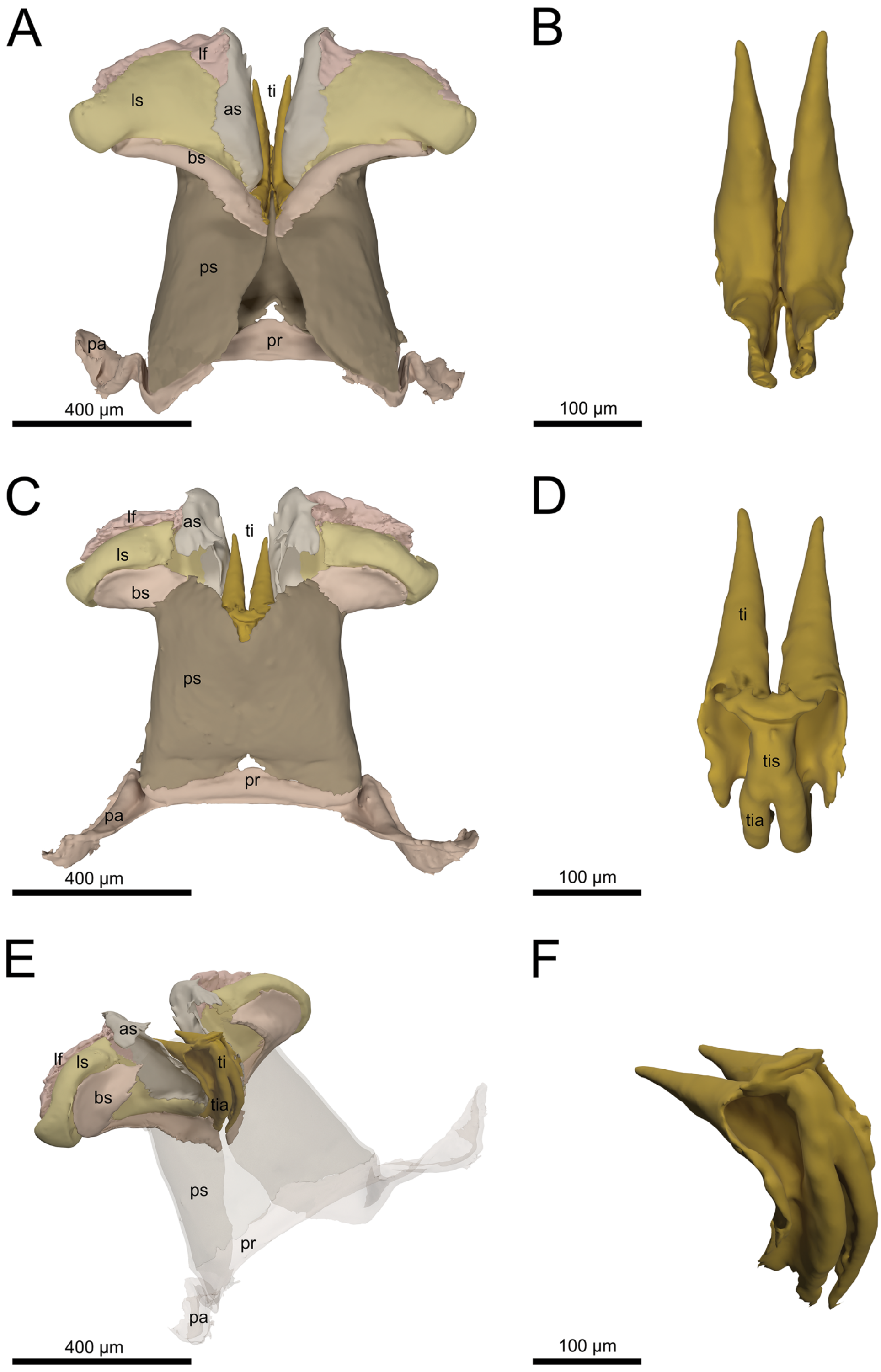

Fig. 5. Penes of *E. venosus* in resting position, reconstructed µCT model. (A, C, and E) Penes and (B, D, and F) titillators in (A and B) ventral, (C and D) dorsal, and (E and F) anterolateral view.

muscles (2) and (3) in the ventral flexion and shortening of the abdomen.

The *median sternal longitudinal muscle* (2) has a flattened oval cross section. It originates in the lateromedial area of the sternites and inserts at the anteromedial margin of the consecutive sternite. Its function is the ventral and lateral flexion as well as the shortening of the abdomen.

The *superior sternal longitudinal muscle* (3) has a flattened oval cross section. It originates lateromedially in the anterior area of the sternites and inserts at the anterolateral margin of the consecutive posterior ventral sclerite. Its function is the ventral and lateral flexion as well as the shortening of the abdomen.

The *anterior dorsoventral muscle* (4) has a flattened cross section. It spans dorsoventrally from the lateral regions of the

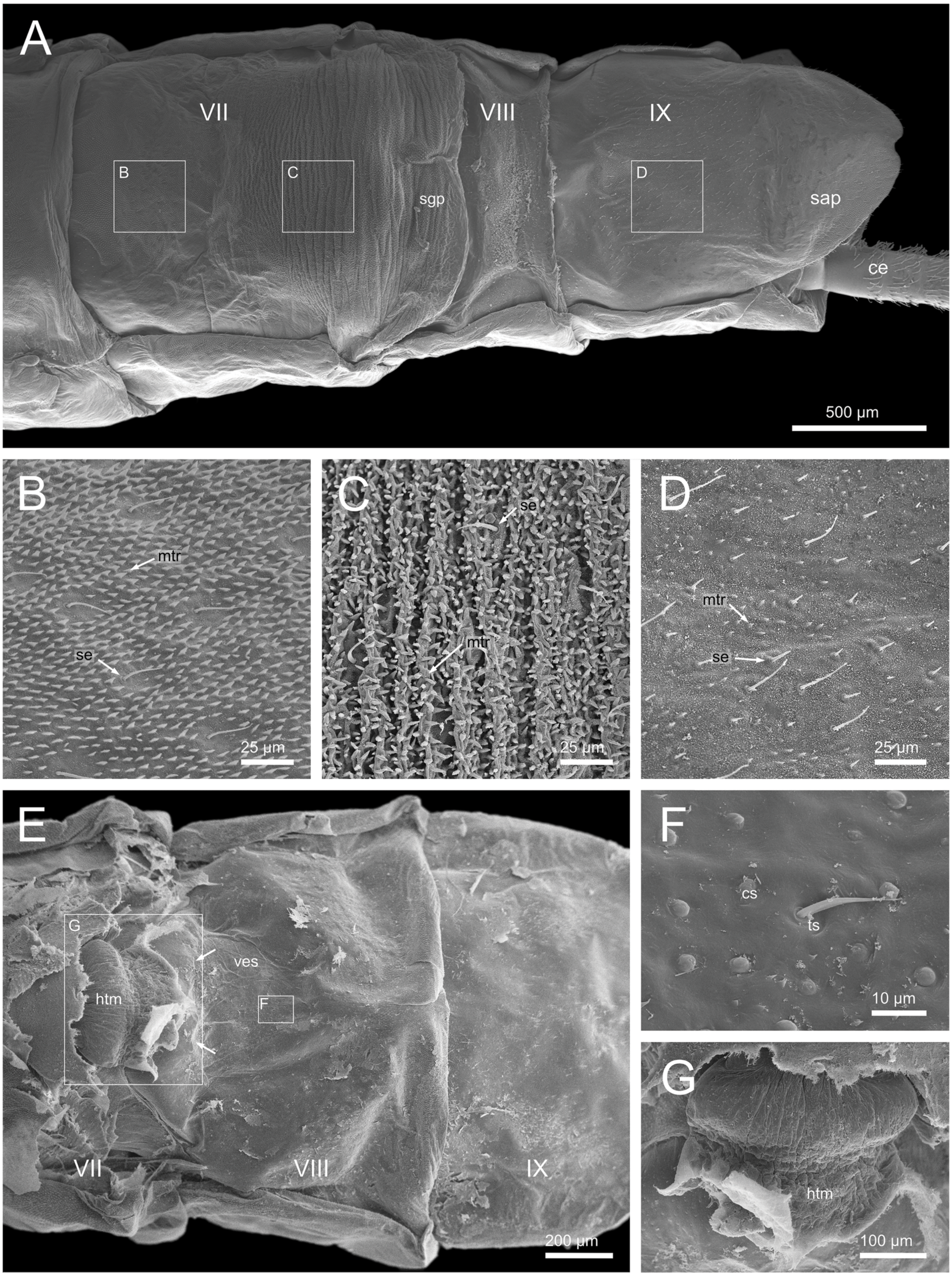

Fig. 6. *E. venosus*, end of female abdomen in ventral view, SEM. (A) Total view, (B to D) detailed views of cuticular microsculpture of (B) sternite VII, (C) subgenital plate, (D) subanal plate, (E) total view with subgenital plate removed, (F) sensilla of sternite VIII, (G) hypertrophied membrane. Arrows in (E) mark the approximate position of the pricking titillator apices during mating.

corresponding tergite to the respective sternite and extends along the anterior quarter of each sternite. Its ventral attachment lies closer to the lateral margin of the sternite than its dorsal attachment does to the lateral margin of the tergite. Its function is the interlocking of the lateral margins of the sternite and tergite. Its action may also indirectly lead to the closure of the corresponding abdominal stigma, as the stigmata of mayflies are lacking direct occlusor muscles.

The *posterior dorsoventral muscle* (5) has a flattened cross section. It spans dorsoventrally from the lateral regions of the corresponding tergite to the respective sternite and extends to the posterolateral area of the sclerites. It is similar to (4) in function.

The *tergosternal laterolongitudinal muscle* (6) only occurs in segment VIII. It has a flattened oval cross section, originates lateroventrally on tergite VIII and inserts at anterolateral

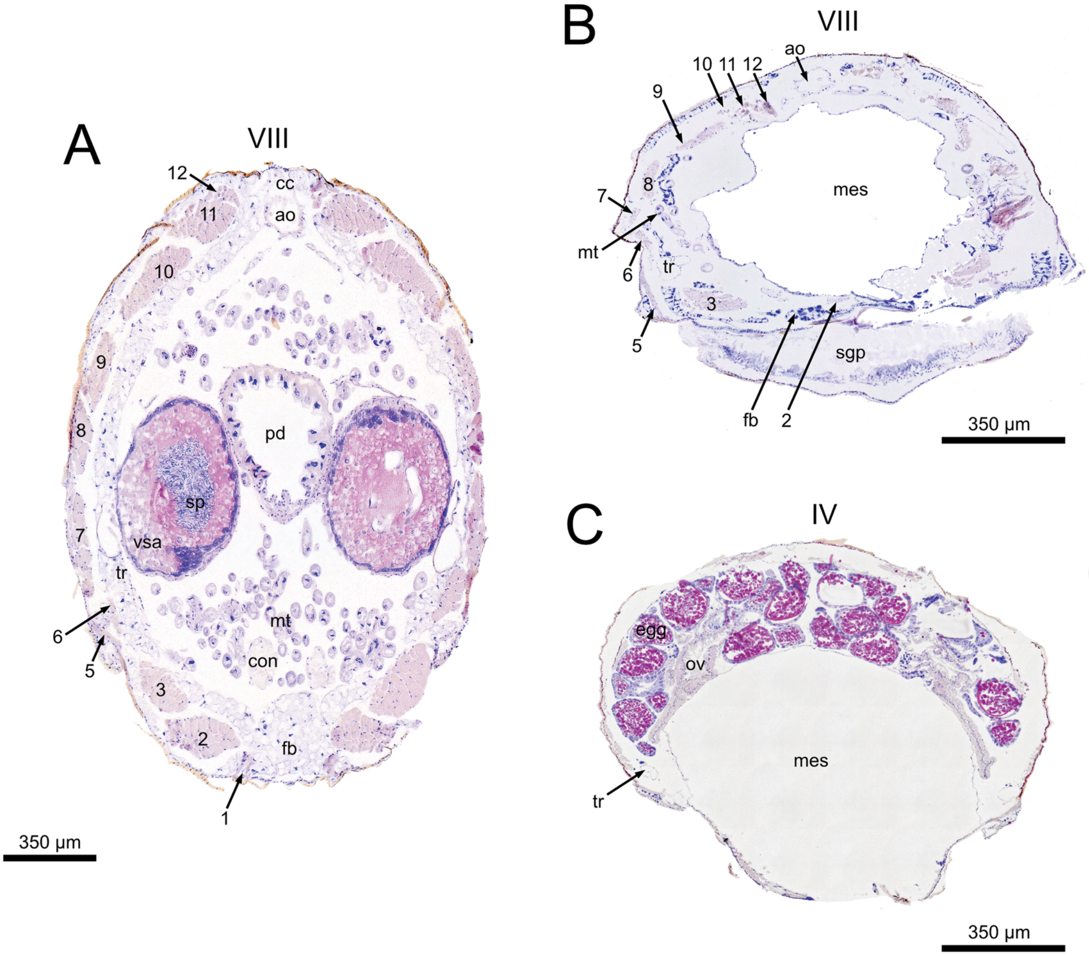

Fig. 7. *E. venosus*, histological crosscuts of male and female abdomina, stained in Delafield's Hematoxylin/Eosin. (A) male segment VIII, (B) female segment VIII, (C) female segment IV.

margin of sternite IX. Its function is the lateral flexion and the shortening of the abdomen.

The *inferior tergal laterolongitudinal muscle* (7) has a flattened oval cross section. It originates close to the dorsal plane in the lateral region of the tergites and inserts ventrally at the anterolateral margin of the consecutive tergite. Its function is the lateral flexion as well as the shortening of the abdomen.

The *median tergal laterolongitudinal muscle* (8) has a flattened oval cross section. It originates close to the dorsal plane in the lateral region of the tergite and inserts at the anterior margin of the consecutive tergite close to the dorsal plane. Its function is the lateral flexion as well as the shortening of the abdomen.

The *superior tergal laterolongitudinal muscle* (9) has a flattened cross section. It originates close to the dorsal plane in the lateral region of the tergite and inserts at the anterior margin of the consecutive tergite slightly dorsal to the dorsal plane. Its function is the lateral flexion as well as the shortening of the abdomen.

The *inferior tergal dorsolongitudinal muscle* (10) has a flattened cross section. It originates lateromedially in the anterior area of the tergites and inserts at anterolateral margin of the consecutive posterior tergite. Its function is the dorsal and lateral flexion as well as the shortening of the abdomen.

The *median tergal dorsolongitudinal muscle* (11) has a flattened cross section. It originates directly medioposterior of (10) and has a similar form and function.

The *superior tergal dorsolongitudinal muscle* (12) has an oval to triangular cross section. It is distinctly shorter and thinner than (10) and (11) and originates medioposterior of the former. It inserts at the medial margin of the consecutive tergite and supports (10) and (11) in their function.

Muscles of the Female Pregenital Segments II-VI (Fig. 11A, Appendix 1)

The female pregenital segments II to VI each are equipped with fewer muscles than their male counterparts:

The *superior sternal longitudinal muscle* (3) has a flattened oval cross section. It originates mediolaterally close to the anterior margin of the sternites and inserts at the anterolateral margin of the consecutive posterior sternite. Its function is the ventral and lateral flexion, as well as the shortening of the abdomen.

The *posterior dorsoventral muscle* (5, II to VII) is similar in shape and function to its counterpart described for the male, but extends from the anterior quarter to the posterior third of each sternite.

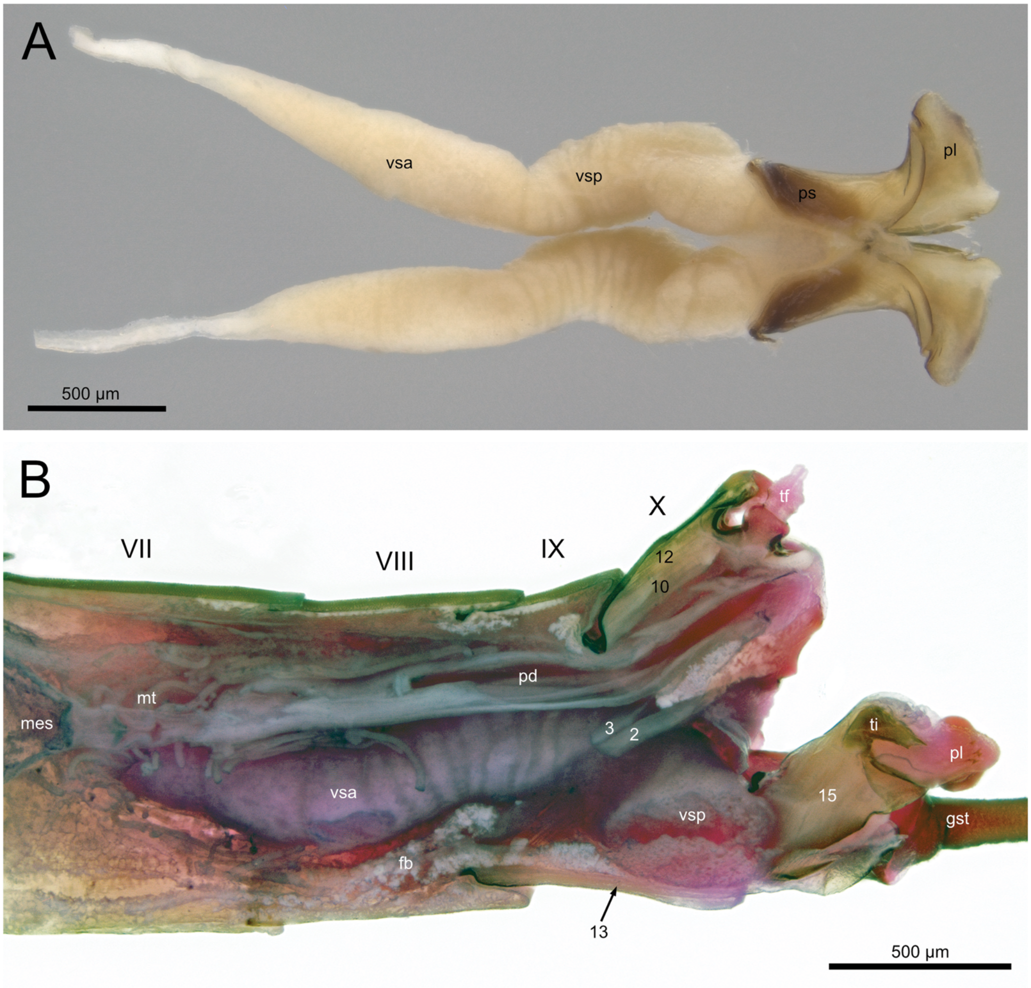

Fig. 8. Male *E. venosus*. (A) Excised vasa deferentia and penes in ventral view, (B) right half of end of abdomen in situ, stained in fuchsin.

The *inferior tergal laterolongitudinal muscle* (7) has a flattened cross section. It originates closely below the dorsal plane in the anterolateral region of the tergite and inserts at the anterolateral margin of the consecutive tergite. Its function is the lateral flexion as well as the shortening of the abdomen.

The *median tergal laterolongitudinal muscle* (8) has a flattened cross section. It originates close to the dorsal plane in the lateral region of the tergite and inserts at the anterior margin of the consecutive tergite close to the dorsal plane. Its function is the lateral flexion as well as the shortening of the abdomen.

The *superior tergal laterolongitudinal muscle* (9) has a flattened cross section. It originates close to the dorsal plane in the lateral region of the tergite and inserts at the anterior margin of the consecutive tergite dorsal of the dorsal plane. Its function is the lateral flexion as well as the shortening of the abdomen.

The *inferior tergal dorsolongitudinal muscle* (10) has a flattened cross section. It originates lateromedially in the anterior area of the tergites and inserts at anterolateral margin of the consecutive posterior tergite. Its function is the dorsal and lateral flexion as well as the shortening of the abdomen.

Male Genital Segment IX (Figs 10 and 13, Appendix 1)

The musculature of segment IX is largely similar to that of the pregenital segments. Notable differences concern the *median sternal longitudinal muscle* (2) and the *superior sternal longitudinal muscle* (3), which lay on top of each other and follow almost the same course. Muscle (3) originates lateromedially at the very anterior margin of sternite IX, and takes an oblique course mediocaudad towards the medial anterior margin of the paraproct. The origin of muscle (2) is slightly lateroposterior of the origin of (3), its course is similar, and it inserts slightly anterior to (2) at the paraproct. They have a rectangular cross section. Their function is the ventral flexion of segment X and the shortening of the abdomen, possibly also the indirect ventral movement of the cerci via the paraprocts and the basitricaudale (btc), for the latter see also Fig. 2A and B and Kluge (2004). Medial to the origins of (2) and (3), the *gonobasal plate muscle* (13) originates along the medioanterior margin of sternite IX. It inserts at the medioanterior margin of the gonobasal plate and has a flattened cross section. Its function is the ventral movement of the gonobasal plate. Here and in the following segment, the *inferior sternal longitudinal muscle* (1) is absent.

The penes originate dorsal to the gonobasal plate as a mediocaudal protuberance of the ventral intersegmental membrane between segments IX and X, extending posteriorly beyond segment X.

The *ventral extrinsic penis muscle* (14) originates laterally in sternum IX and inserts at the ventroanterior margin of the penis arm. It has a flattened cross section and a triangular shape. Its function is the lateral and ventral movement of the penis.

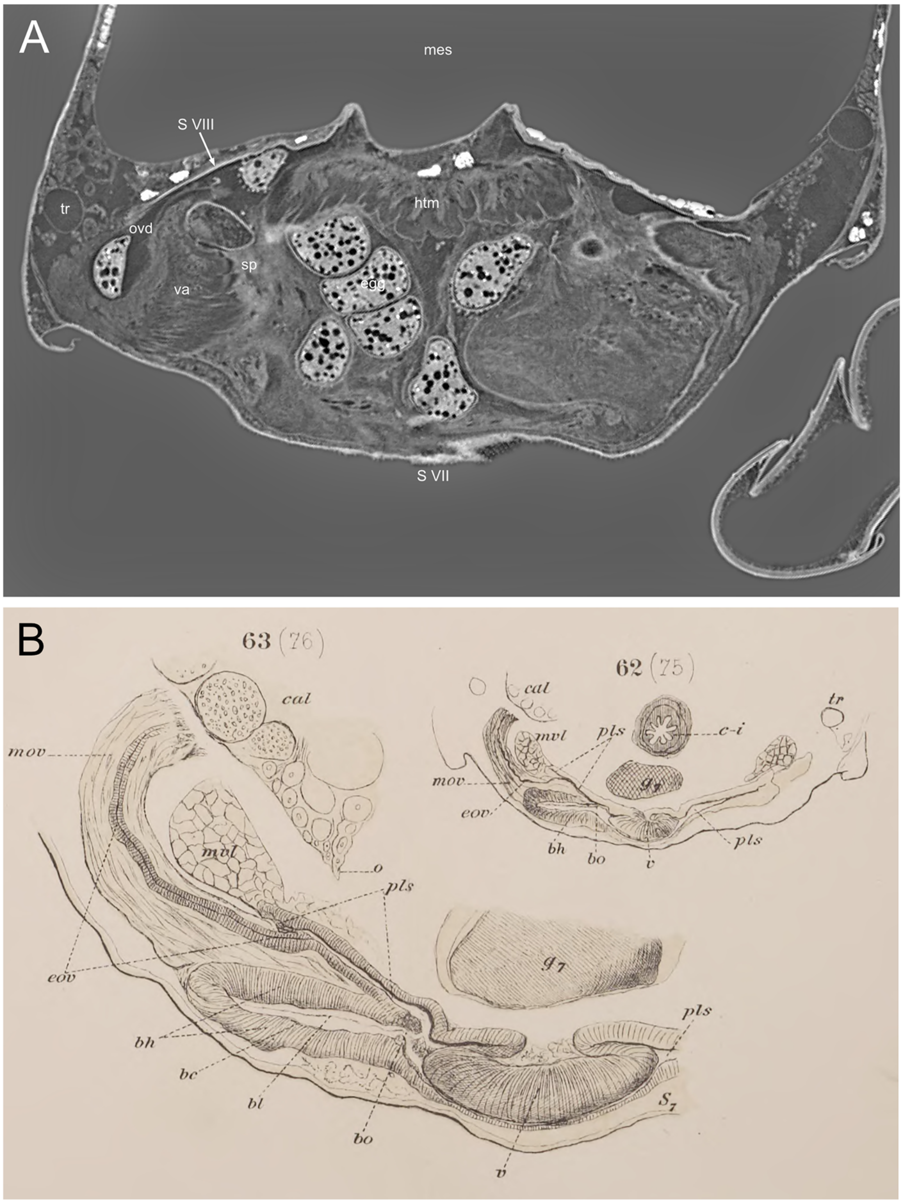

Fig. 9. Cross section of female vestibulum. (A) µCT of copulating pair of *E. submontanus*, (B) female *E. venosus*, as depicted by Palmén (1884: Figs 62 and 63) for an approximately similar section. Literal translation of abbreviations in Palmén (1884: pp. 102-103): *bc*: chitinous skin of bursa copulatrix (*b*); *bl*: larval skin of *b* (author's interpretation from unclear legend); *bh*: hypodermis of *b*; *bo*: mouth of *b*; *cal*: calyx; *c-i*; close connection between colon and intestine; *eov*: epithel layer of oviduct; *g*: abdominal ganglion; *mvl*: the laterally situated longitudinal ventral muscle (3); *mov*: muscle layer of oviduct; *o*: ova; *pls*: plica intersegmentalis; *v*: a median torus in pls VII-VIII of the female.

The *intrinsic penis muscle* (15) has a rectangular cross section. It originates both at the ventral posterior processes of the basal penis shaft sclerite and the ventral anterior area of the penis shaft, inserting at the dorsal medial area of the penis shaft. Its function is the dorsal flexion and creasing of the penis shaft sclerite, resulting in the rotation of the penis lobes, as well as the ventral movement of the titillators.

The *intrinsic gonobasal muscle* (16) has an apically oval and basally crescent-shaped cross section (Fig. 13). It originates at the lateroanterior margin of the gonobasis and inserts mediobasally at the basal gonostylomere. Its function is the mediodorsal movement of the gonostylus, which results in the clasping movement of the forceps.

The *dorsal extrinsic penis muscle* (17) has a rectangular cross section. It originates at the ventrocaudal processes of tergite IX and inserts at the anterolateral processes of the penis arm. Its function is the dorsal movement of the penis.

Female Genital Segment VII (Fig. 11, Appendix 1)

The female genital segment contains similar musculature to the respective pregenital segments. The *superior sternal longitudinal muscle* (3) is smaller than in the pregenital segments.

Male Postgenital Segment X (Figs 10 and 12, Appendix 1)

Some muscles of previous segments also occur in segment X, albeit in a different configuration:

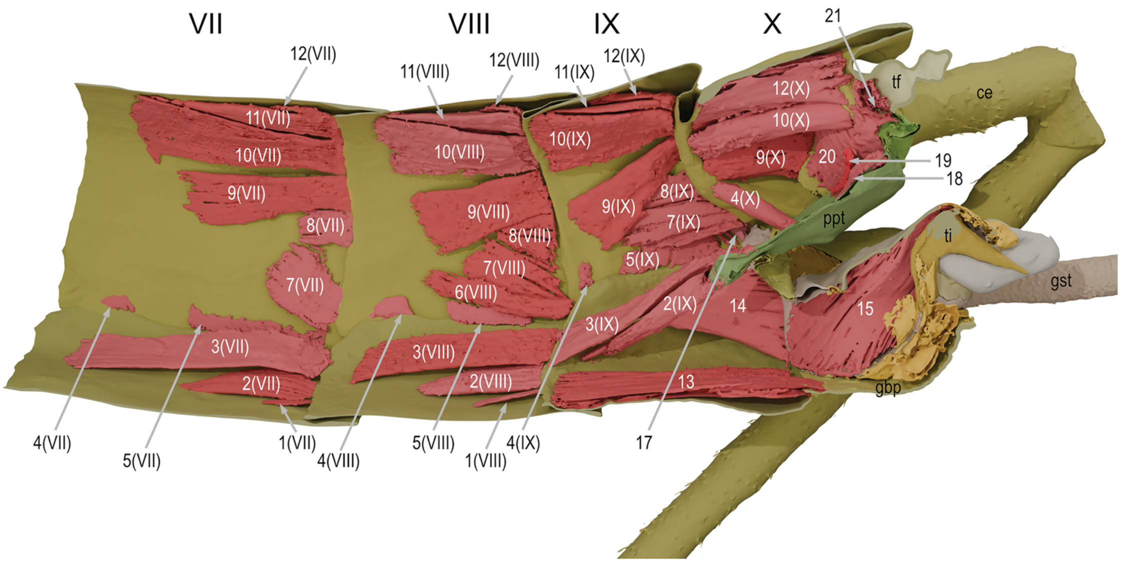

Fig. 10. Male *E. venosus*, reconstructed µCT model, end of abdomen, sagittal cut, right half, showing musculature.

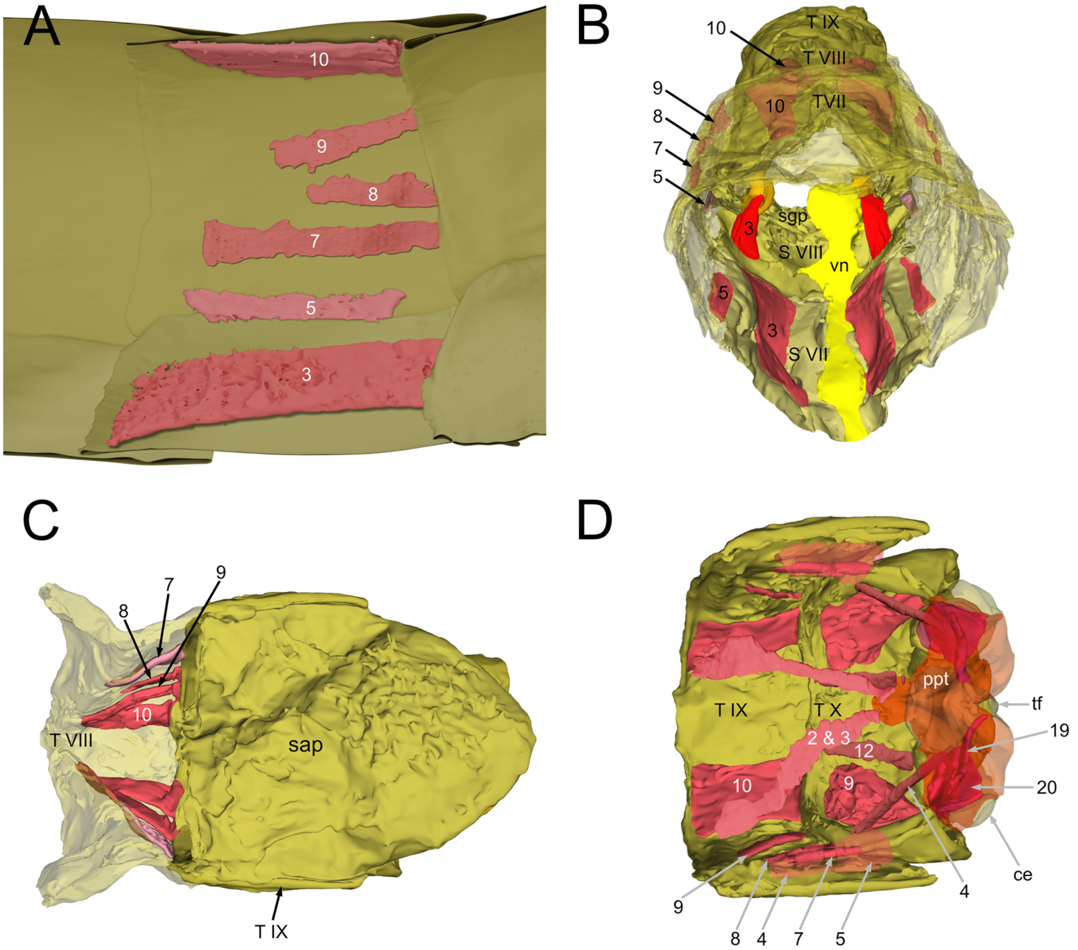

Fig. 11. Female *E. venosus*, 3D model of the abdominal musculature, (A) segment VI lateral view of sagittal cut, (B) frontal view of last abdominal segments (VII to IX), (C) ventral view of abdominal segment VIII with its sternite removed, its musculature, and the subanal plate, (D) ventral view of horizontal section of segments IX and X as well as their musculature.

The *anterior dorsoventral muscle* (4) has an oval cross section. It spans dorsoventrally from the anterolateral region of the tergite to the lateromedial anterior margin of the paraproct. Its ventral attachment lies closer to the lateral margin of the paraproct than its dorsal attachment does to the lateral margin of the tergite. Its function is the compression and flattening of segment X.

The *superior tergal laterolongitudinal muscle* (9) has a kidney-shaped cross section and a triangular shape. It

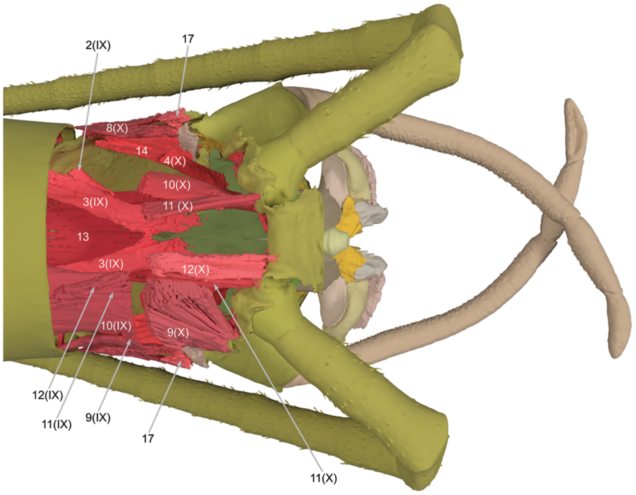

Fig. 12. Dorsal view of male *E. venosus*, segments IX and X with removed tergites; additionally, on the right half, the muscles 9, 10(IX), 11(IX) and 12 are removed.

originates close to the dorsal plane at the anterior margin of the tergite and inserts at the lateral margin of the basitricaudale. Its function is the lateral movement of the cercus. It is the largest muscle in segment X.

The *inferior tergal dorsolongitudinal muscle* (10) has a flattened oval cross section. It originates lateromedially in the anterior area of the tergite and inserts at the mediodorsal region of the basitricaudale. Its function is the medial movement of the cercus.

The *median tergal dorsolongitudinal muscle* (11) has a flattened oval cross section. It originates directly dorsal to (10) and inserts at the mediodorsal margin of the basitricaudale. It has a similar form and function as (10).

The *superior tergal dorsolongitudinal muscle* (12) has a triangular cross section. It originates and inserts directly medial to (11) and has a similar function as (10) and (11).

The *ventral anus muscle* (18) and the *dorsal anus muscle* (19) have a flattened rectangular cross section. They originate at the lateral margin of the paraproct and insert at the posterior end of the hindgut, with (19) originating and inserting lateral to (18). Their function is the dilation of the hindgut.

The *cercal dorsoventral muscle* (20) and the *paracercal dorsoventral muscle* (21) span dorsoventrally across the bases of the cerci and the terminal filament, respectively. Their function is the compression and compartmentalisation of these appendages; in doing so, they support the function of the circulatory system (see Gereben-Krenn and Pass 2000).

The Female Postgenital Segments VIII-X (Fig. 11B-D, Appendix 1)

Segment VIII is mostly similar in musculature to the male pregenital segment VIII, except for the absence of the *inferior sternal longitudinal muscle* (1).

In segment IX, the ventral longitudinal musculature differs from that of the male in the absence of the *gonobasal plate muscle* (13) and the *median sternal longitudinal muscle* (2).

The lateral musculature is similar to that of the corresponding male segment except for the lack of the *tergosternal laterolongitudinal muscle* (6) and the *inferior tergal laterolongitudinal muscle* (7).

The female possesses 2 tergal longitudinal muscles: The *inferior tergal dorsolongitudinal muscle* (10) and the *median tergal dorsolongitudinal muscle* (11) with similar form and function as their male counterparts.

The musculature of segment X is identical to that found in the male, except for the lack of a distinct (10) and (11).

The Mating Position

The aerial mating in *E. venosus* is typical for mayflies: Male imagines form aerial swarms above the water surface or nearby, which the females join for copulation. According to Fischer (1992), *E. venosus* is a species whose flight pattern is primarily characterized by horizontal forward movement along a linear, one-dimensional path. When a male detects a female entering the swarm, it is approached from below. The male grasps the female with its elongated front legs during flight, encompassing the wing base from below. Simultaneously, the male bends its abdomen dorsally and clasps the female abdominal segment VIII with its gonostyli from ventrally (Fig. 14). The gonobases are bent ventrally, additionally the gonostyli are moved mediodorsally by the contraction of the intrinsic gonobasal muscle (16). The penes are thereby inserted into the female vestibulum, which is located dorsally of the subgenital plate. The male cerci are bent ventrally between their second and third segment. As the cerci are not equipped with intrinsic muscles, this is either an artifact of the fixation, the result of external forces, or due to a change in haemolymph pressure.

Configurational Change of the Penes During Copula

As revealed by µ-CT analysis, the penes undergo a conformational change within the female vestibulum (Fig. 13): Compared to the resting position, the shape of the penis shaft is heavily altered due to the contraction of the complex intrinsic penis muscle. The dorsomedial part of the penis shaft is pulled ventrally and basally and thereby indented, while its ventromedial part is pulled dorsally. This results in a flattening of the penis shaft and the widening of its ventral gap. The flattening of the formerly convex dorsal surface of the penis shaft results in a concave V-shaped crease of the latter. As the penis lobes are connected to the penis shaft, they become erected by rotating 90° dorsally along the longitudinal axis. Additionally, the change in conformation of the penis shaft also affects the shape and position of the titillator sclerite. Due to the rotation of the penis lobe sclerites, to which the titillator sclerite is connected via its basal arms, the titillators and the basal titillator arms are spread, so the titillator sclerite takes on an X-shaped appearance. Thereby, the titillators are tilted ventrally and become exposed.

Position of the Penes Inside the Vestibulum

The penes are positioned medially at the anterior end of the vestibulum (Figs 15, 16A, and 17A). In this region, the intersegmental membrane is strongly hypertrophied (htm, va, see Fig. 17A), presenting multiple folds. The penis lobes are pressed closely against this hypertrophied membrane, with sperm being directed towards its folds. The close contact of the sperm with the hypertrophied membrane can be directly observed (Figs 16 and 17). Simultaneously, the titillators are pricking, not piercing, the dorsal membrane of the vestibulum directly at the transition between sclerite VII and the hypertrophied region (Figs 6E, 16B, and 18). Thereby the roof of the vestibulum is

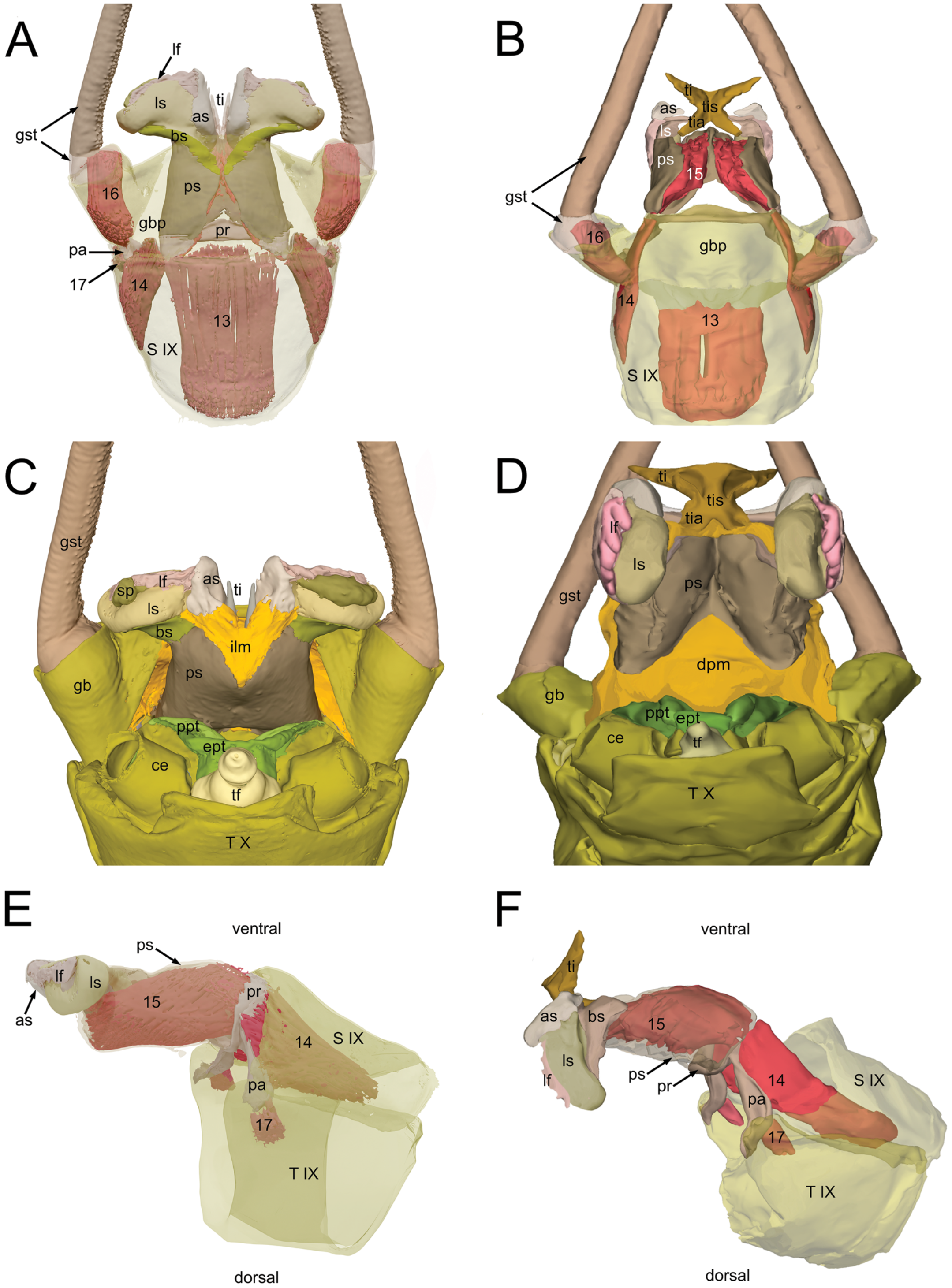

Fig. 13. Male *E. venosus*, reconstructed µCT models of penes, surrounding muscles, and sclerites in (A, and B) ventral view, (C, and D) dorsal view, (E and F) lateral view. (A, C, and E) Resting position (B, D, and F) copulatory position.

expanded, much like the canopy of a tent by its supporting poles. In consequence, a voluminous cavity is formed in which the insemination takes place.

Discussion

Musculature of the Abdomen

The abdominal musculature of several Ephemeroptera species has been described previously, most notably by Birket-Smith (1971) for *Povilla adusta* Navás, 1912 (Polymitarcyidae), Grimm (1985) for a wide selection of species, among them *Ecdyonurus torrentis* Kimmins, 1942, and Grandi (1962) for several species. The genital musculature has additionally been described by Grandi (1960b, 1964), among others for *Ecdyonurus helveticus* Eaton, 1887. Boudinot (2018) compiled a summary of the genital musculature described by Birket-Smith (1971) and the larval musculature described by Brinck (1957).

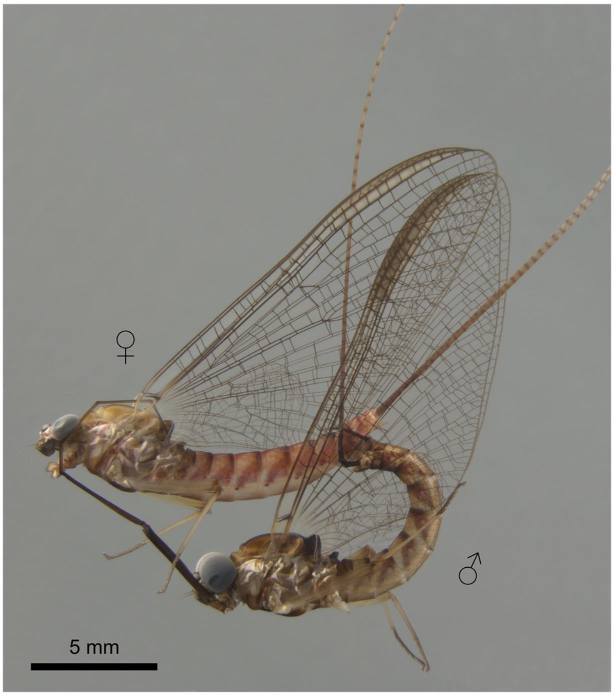

Fig. 14. Copulating pair of *E. venosus*, fixed in ETOH, lateral view.

Our description of the abdominal musculature of *Ecdyonurus* fits well with Grandi's (1960b, 1964) descriptions of the genital musculature, and generally also with Grimm's (1985) findings for this genus. Grimm (1985), however, reported the inferior sternal longitudinal muscle (1) as missing from segment VII onwards for *Ecdyonurus*, while we clearly identified its presence (see Fig. 10). Also, we identified a superior tergal dorsolongitudinal muscle (12) not described by Grimm (1985). Furthermore, we noticed discrepancies regarding the homology of his muscles 39 and 40 across his work. The description of his muscle 39 (fig. 20, p. 19) corresponds to our dorsal and ventral anus muscles (18, 19, see Fig. 10), while in his drawing of *Rhithrogena semicolorata* (Fig. 26, p. 24), which was also used for the description of *E. torrentis*, it clearly corresponds to our anterior dorsoventral muscle (4). This is also the case in his drawing of *E. torrentis*, which was used for the description of the internal genitalia (Fig. 34, p. 32). At the same time, in Fig. 25, his muscle 40 clearly corresponds to our anus muscles (18, 19). He appears to have mixed up his muscles 39 and 40. Apart from these inconsistencies, we found Grimm's descriptions of the muscles in *E. torrentis* to largely match our findings in *Ecdyonurus*. Also, Boudinot (2018) in his compilation did not consider the substantial findings of Grimm (1985) and Grandi (1960b, 1964), so his considerations neither reflect the variations in the genital muscles of Ephemeroptera nor do they reflect their ground plan. Grimm provided a hypothetical ground plan of the abdominal muscles in adult Ephemeroptera. It would exceed the scope of this work to discuss this in detail; therefore, we are going to address this issue in a separate contribution.

The differences in abdominal musculature between males and females may be explained by their differences in behavior, necessitating higher abdominal mobility in the males opposed to the required volume for the storage of eggs in females. The stronger and more complex musculature of the male abdomen should be of importance for the abdominal flexion during mating (dorsal muscles) and for manoeuvring during the mating flight and approaching the females (musculature of X and ventral muscles).

An overview of the described abdominal musculature in *E. venosus*, which generally also applies to *E. submontanus*, is provided in Appendix 1. It also summarizes the assumed function of the muscles and provides the corresponding muscles described by Grimm (1985) for *E. torrentis*.

Origin and Terminology of the Male Gonopods

In the male, sternite IX caudally gives rise to a pair of genital appendages (Figs 2A and 3A), which facilitate the grabbing of the female during mating. There is general consensus that these genital appendages are derived from abdominal leglets, which still occur on many abdominal segments in primarily wingless Hexapoda (Snodgrass 1935, Matsuda 1976, Kristensen 1991). In morphological literature, the abdominal appendages of the genital segment are therefore generally referred to as gonopods. The paired gonopods in basal hexapods each consist of a proximal gonobasis and a distal gonostylus (Willmann 1998). Different authors refer to the gonobasis as coxopodite (Snodgrass 1935), gonocoxopodite (Weidner 1982), gonocoxite (Grimm 1985), or gonocoxa (Boudinot 2018). In Archaeognatha and Zygentoma, the gonobases are still medially separated, and the gonostyli are generally not subdivided (Delany 1959, 1961, Bitsch 1974, Birket-Smith 1974). Each gonobasis is equipped with intrinsic and extrinsic musculature (Bitsch 1974). In most taxa of mayflies, the gonobases are medially fused to form a gonobasal plate and the gonostyli are secondarily subdivided. This, however, does not represent a true segmentation (see also Willmann 1998), as the stylomeres are not equipped with intrinsic muscles. The gonostyli are medially equipped with numerous specialized, pear-shaped sensilla (Fig. 3C and D), which have mechanosensory functions during copulation (Gaino and Rebora 2002).

In taxonomic mayfly literature, the terminology for male genital appendages has often been inconsistent. In species descriptions or determination keys, gonostyli are commonly referred to as forceps or claspers. Most native English-speaking authors use forceps as the plural form for a pair of gonostyli and also refer to a single gonostylus as a forceps (see also Torre-Bueno 1962). The Latinized plural form forcipes rather refers to multiple pairs of gonostyli (Gordh and Headrick 2001, Webster's Third New International Dictionary 1981), and this term is indeed also used by some authors (eg Malzacher 2022).

Likewise, the fused gonobases, which are situated posterior to the ninth sternite and ventral to the penis, have been referred to as the styliger plate (Needham et al. 1935), subgenital plate (Edmunds et al. 1976; Landa 1969), or simply styliger (Brinck 1957, Grandi 1960a, Illies 1968, Verrier and Brinck 1970, Matsuda 1976, Kluge 2004, Bauernfeind and Soldán 2012). Kluge (2004) refers to the lateral portions of the fused gonobases, which contain the intrinsic gonobasal muscle, as the pedestal of gonostylus. In Baetidae, where the gonobases are separate, these have been termed unistyligers by Kluge and Novikova (2011).

However, considering the combined morphological and molecular phylogeny of the Ephemeroptera presented by Ogden et al. (2009), the fusion of the gonobases appears to be a character that has evolved within Ephemeroptera, as the 2

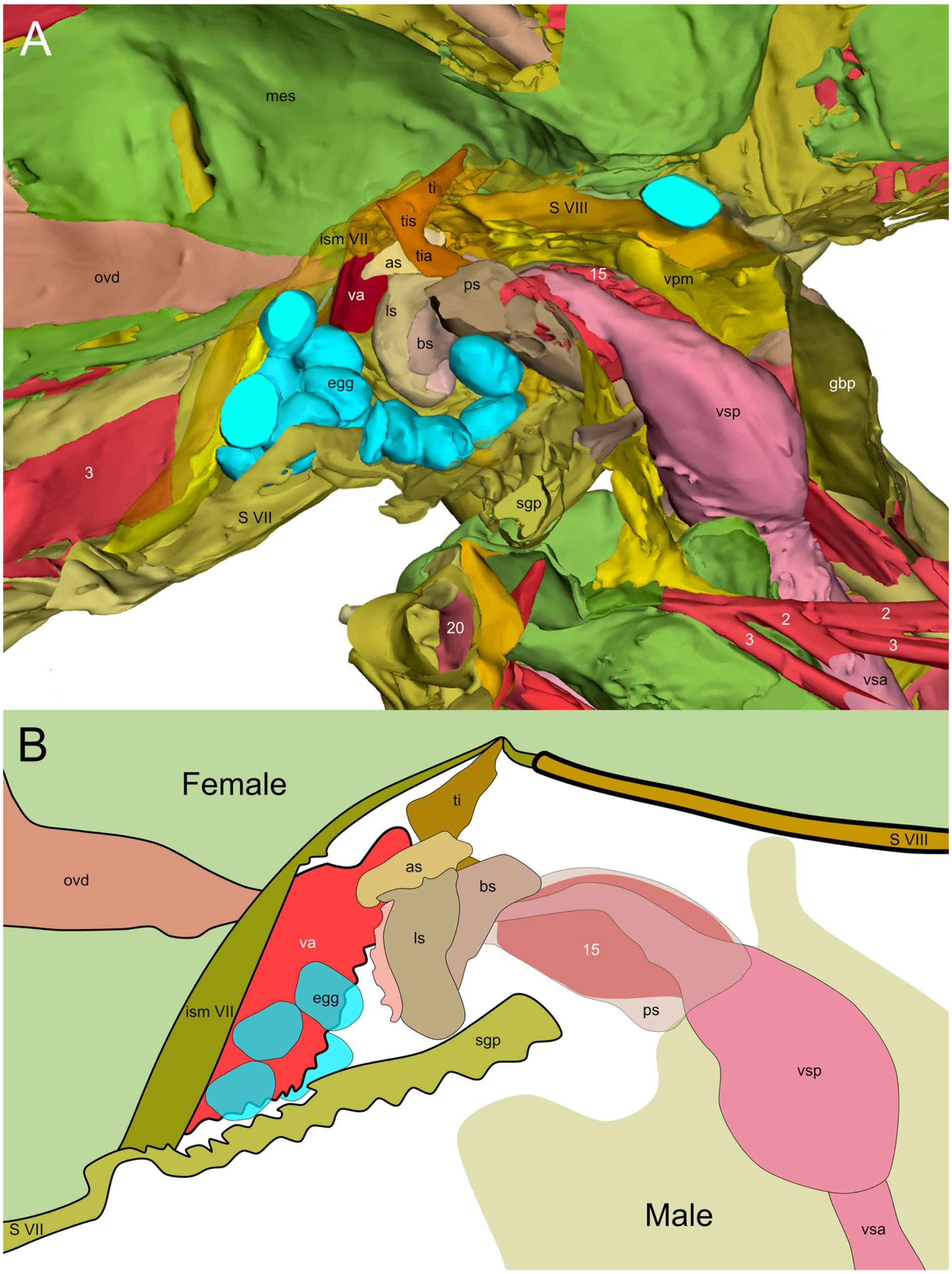

Fig. 15. Copulating pair of *E. venosus*. (A) Reconstructed µCT model of genitalia and surrounding muscles and sclerites, lateral view of sagittal cut (right half). (B) Simplified schematic drawing of principal components.

most basal clades, Siphluriscidae and Baetidae, still possess separate gonobases (for Baetidae, see Kluge 2004; for Siphluriscidae, see Ulmer 1920). To avoid misunderstandings, we define the term gonobasis as synonymous with the term styliger and the term gonobasal plate as synonymous with the term styliger plate. We also define the individual gonostylus to be synonymous with the term clasper and the functional unit of both gonostyli to be synonymous with the term forceps. The plural term forcipes should therefore only be used when discussing multiple pairs of gonostyli. As the subdivision of the gonostyli is secondary and does not reflect a true segmentation, these subdivisions of the gonostyli should accordingly be termed as gonostylomeres, since secondarily divided appendages are usually indicated by the ending "–mere" (like tarsomeres, palpomeres, or antennomeres, etc.).

Female External Genitalia

The female subgenital plate externally is equipped with a large amount of conspicuous microtrichia (see Fig. 6). In our investigated copulae, we could not identify any interaction of the male abdomen or genitalia with this region. Since the egg deposition in Heptageniidae usually takes place by dipping the end of the abdomen on the water surface (Encalada and Peckarsky 2006) with a dorsally flexed abdominal tip (own observation), the microstructure of the subgenital plate may be the only

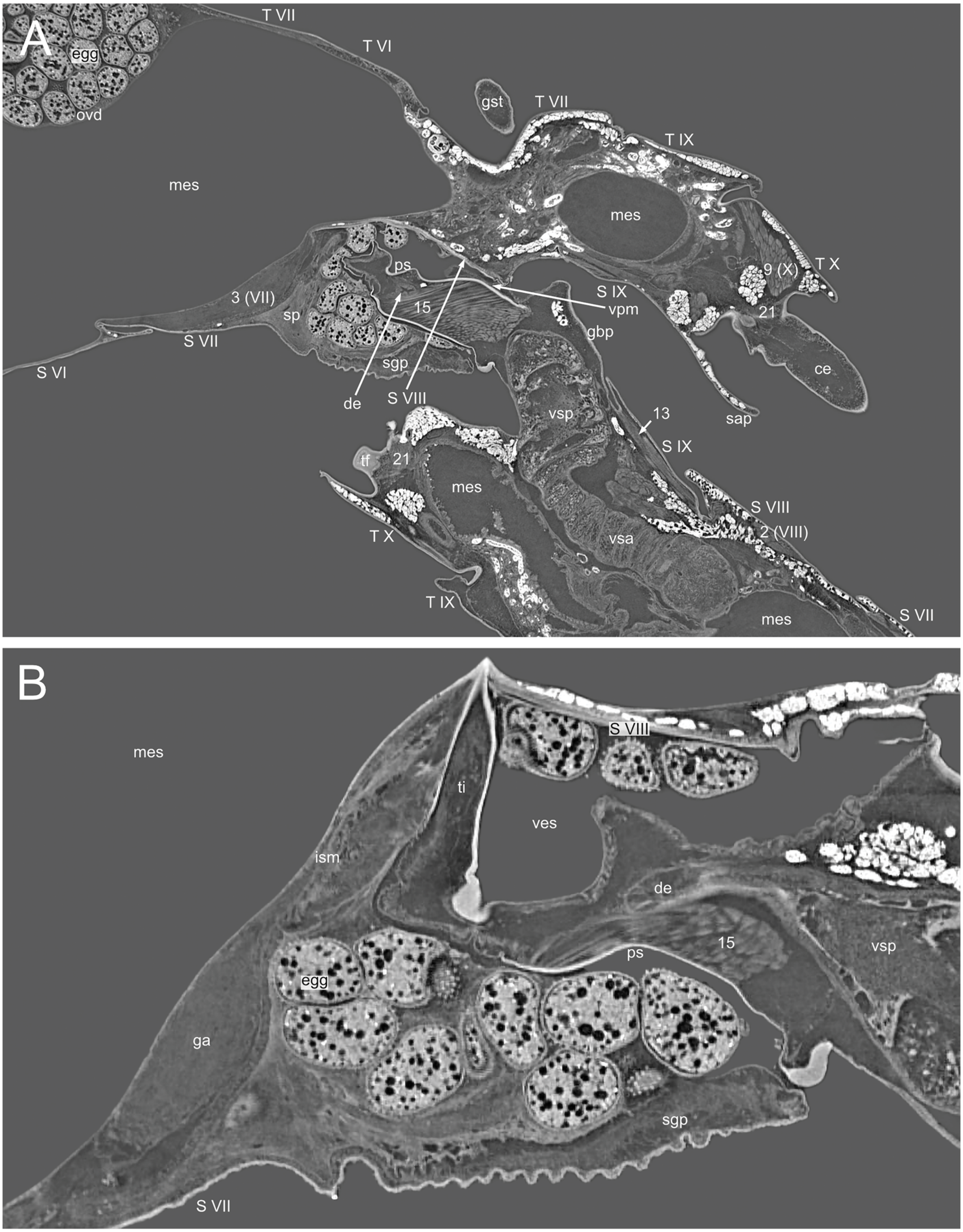

Fig. 16. Copulating pair of *E. submontanus*, µCT of genitalia, parasagittal section. (A) Overview of the posterior abdomen, (B) laterally offset parasagittal section with titillator pricking the vestibulum.

contact area with the water surface and could be hydrophobic, as is often observed in nature for similarly structured surfaces.

Configurational Changes of the Penes: Mode and Consequences

During copulation, the male penes undergo a major change in configuration facilitated by the contraction of the intrinsic penis muscle (15). This change of configuration was previously observed by Harker (1986), whose descriptions match our findings. It was then further investigated by Takemon (1990), who also described two different configurations of the penes in *Epeorus ikanonis* Takahashi, 1924 (Heptageniidae) with titillators quite different from those of *Ecdyonurus*. However, the movement of the penis lobes was described to be quite similar to that of *Ecdyonurus* in our findings.

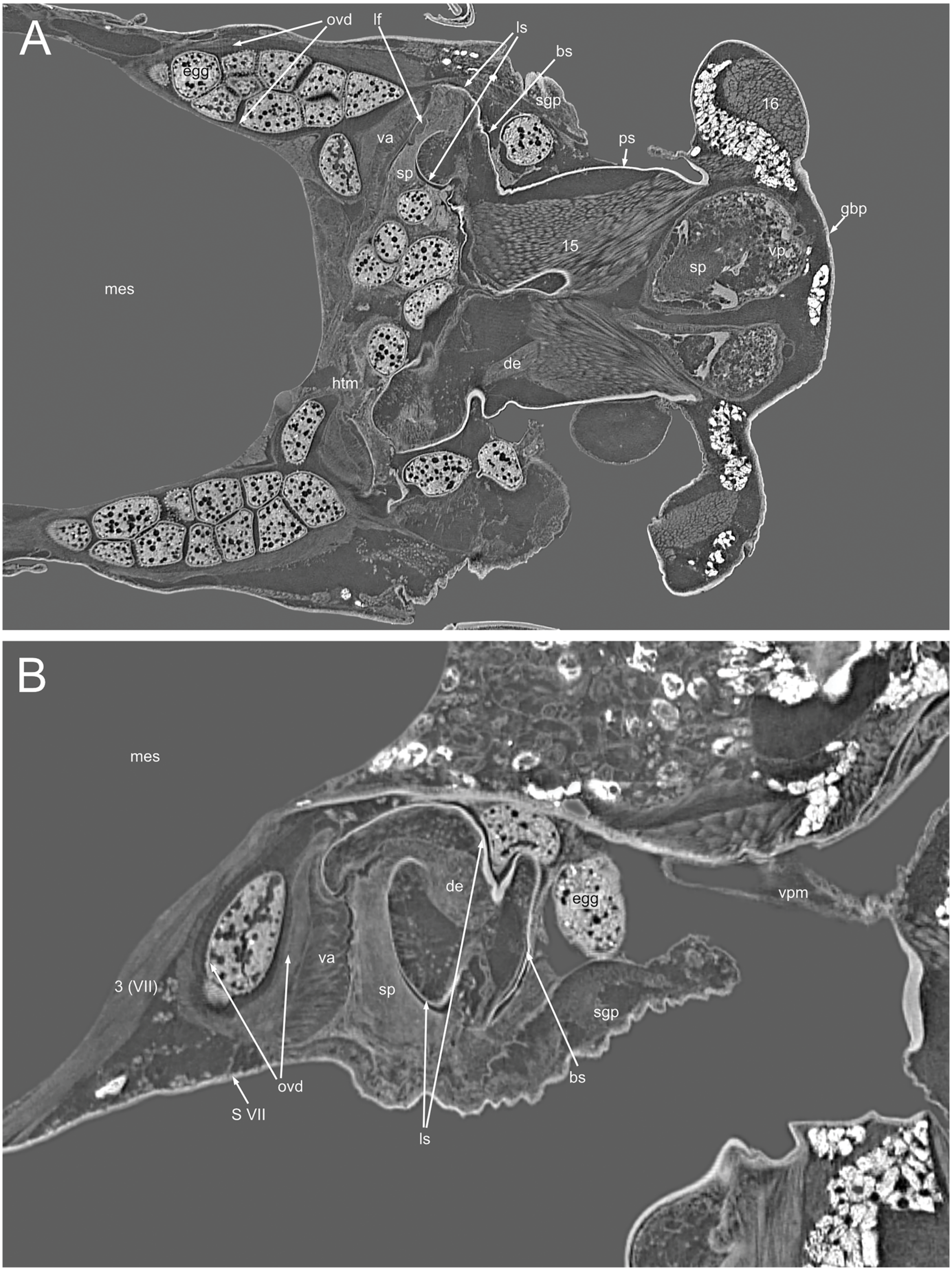

Fig. 17. Copulating pair of *E. submontanus*, µCT of genitalia. A) Horizontal section, B) sagittal section showing male and female gonopores.

Our morphological investigations clearly point to a change in configuration of the penis shaft sclerite facilitated by the contraction of the intrinsic penis muscles (15). Thereby, the dorsally convex penis shaft sclerite changes into a strongly concave form during mating. This in turn causes the rotation of the penis lobes and ultimately also the spreading of the basal arms of the titillator sclerite as well as the titillators, which become exposed (Fig. 13).

A possible explanation would be the elastic deformation of the penis shaft sclerite with continuous, full strength muscle

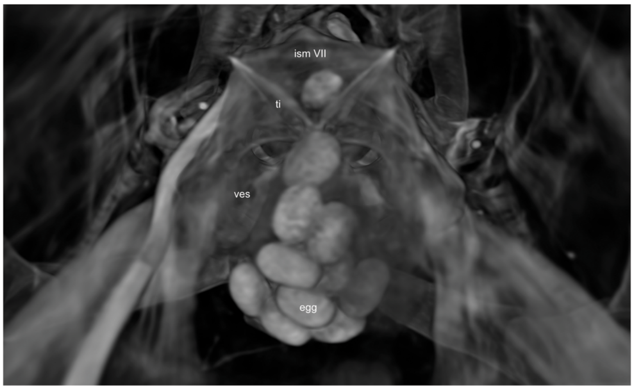

Fig. 18. Copulating pair of *E. venosus*, μCT volume rendering, frontal view with titillators pricking the roof of the vestibulum.

tension being applied to the penis shaft sclerite, which then returns to its resting position once this tension subsides. This would be the simplest scenario; however, the movement would be relatively slow and the energy cost high.

Another scenario would be that the penis shaft sclerite is in a bistable configuration, allowing the penis to remain in the mating or resting configuration without the need for muscular contraction to stabilize the respective configuration. Bistable mechanisms in insects have been suspected, for example, for dipteran flight (Gronenberg 1996, Brennan et al. 2003) and have even found applications in research of biomimetics (Liu et al. 2016). To the knowledge of the authors, no such mechanism has been described for an intromittent organ of insects. It would be of interest to further investigate the presence of such a mechanism in *E. venosus* by analyzing the distribution of resilin in the penis shaft sclerite and the titillator sclerite. Such a mechanism would allow for a rapid change of configuration due to the possibility of energy being stored before the movement takes place. However, there is no clear mechanism for resetting the penis-shaft sclerite from the copulatory to the resting configuration. In the case of a bistable condition, it may be possible that this is achieved via haemolymph pressure.

As a third alternative, the penis shaft sclerite may not possess true bistability, but rather a metastability where some amount of permanent muscle tension is needed to support the mating configuration, but significantly less than to achieve the transition toward it. This would then also eliminate the need for a reset mechanism opposing the intrinsic penis muscle. We consider this to be the most plausible scenario at present, but will address this issue in a further contribution.

Accommodation of the Penes Within the Vestibulum

The dorsal delimitation of the female vestibulum is provided laterally and posteriorly by sclerite VII. The spread out titillators insert at the transition between sclerite and intersegmental membrane. The width and position of this area may be species-specific and the lateral, sclerotized areas of the vestibulum may prevent successful copulation with males of species that may have differently sized titillators. Furthermore, the insertion of the titillator at the transition between membrane and sclerite may anchor the penes in a desired location for copulation and may prevent the penes from being anchored at a penetration depth that is too shallow for successful insemination. The penis lobes, and thereby the gonopores, are closely pressed against the folded and hypertrophied intersegmental membrane, specifically the valves covering the oviducts. This may facilitate the intrusion of sperm into said folds, which then, in turn, can act as a sperm storage for egg fertilization. We did not find any evidence for the intrusion of sperm or the penes into the oviducts. When examining the condition during copulation, this seems also generally unlikely, since the oviduct valve is pressed closed by the penis lobes. Given our observations, it is far more likely that the folds of the hypertrophied intersegmental membrane and the lumen of the vestibulum are filled with sperm, so that the eggs pass through this sperm-filled area and thereby are fertilized immediately before being deposited as described by Takemon (1990).

The structure described by Takemon (1990) as a sac-like receptaculum seminis in *Epeorus ikanonis* Takahashi 1924, we identified as being formed by the hypertrophied intersegmental membrane VII to VIII (Fig. 9). However, it is only present as a small, sac-like pouch in non-copulating females, since the vestibulum is dilated by the penes during copulation. As soon as the copulation ends, the pouch is formed to secure and store sperm adhering to the hypertrophied membrane. We could not identify a pouch dorsally of the receptaculum seminis as was described by Takemon (1990) for *E. ikanonis*. Also not present in *Ecdyonurus* are the paired depressions in the roof of the vestibulum as described by Gaino et al. (2009) for *Rhithrogena*, which were interpreted to accommodate the titillator during copula. Moreover, judging from the different shapes and positions of these titillators in *Rhithrogena*, we assume independent origins of these structures within Heptageniidae.

Palmén (1884) assumed that the titillators directly interact with the hypertrophied membrane and thereby induce a mechanical stimulus of importance for mating. However, we found no evidence for direct interaction of the titillators with the hypertrophied membrane. It furthermore appears to be implausible for the membrane to be responsible for this stimulus, as the hypertrophied membrane acts as a sperm storage and the titillators rather serve as an anchoring device. Instead, we identified numerous campaniform sensilla close to the area where the titillators interact with the roof of the vestibulum (Fig. 6F). Such proprioceptors are associated with the detection of cuticular deformations (Keil 1997) and may therefore signal a dilation of the vestibulum to the female. Furthermore, the anchoring of the titillators to the roof of the vestibulum may also lead to a more stable connection of males and females during copulation. This may also be a reason for the comparatively easy access to pairs in copula, as in other mayfly families, the pairs tend to separate quickly upon disturbance.

Variability of Genitalia Throughout the Ephemeroptera

While we describe 2 species of *Ecdyonurus* with rather similar genital morphology, even within the Heptageniidae vastly different genital morphologies can be found between different genera (for *Rhithrogena*, see Gaino et al. 2009; for *Epeorus*, see Takemon 1990). Other families show notable differences in the length of the female subgenital plate. It may be absent, for example, in Siphlonuridae (see Malzacher 1981) or in Baetidae (see Müller-Liebenau 1969). In contrast, it may be significantly extended to form an oviscapt in Leptophlebiidae: Hagenulini (see Domínguez et al. 2006). Likewise, the different positions and shapes of the titillators in different taxa even raise the question of their homology and possible functional differences across taxa (eg for Leptophlebiidae see Gaino and Rebora 1995). Additionally, the penes themselves differ vastly in shape throughout Ephemeroptera (Bauernfeind and Soldán 2012). Finally, there are different shapes and compositions of the gonopods observable (eg for some Polymitarcyidae, see McCafferty and Bloodgood 1989, for Caenidae see Malzacher 2022), which may be linked to differing mating positions (eg for Palingeniidae see Cornelius 1848) and mating habits (Brinck 1957). To further investigate the functional morphology, diversity and evolution of mayfly genitalia, it would be essential to compare the copulation of further genera within and outside of Heptageniidae.

Specimen Collection Statement

Insect Systematics and Diversity supports compliance with the Nagoya Protocol. The authors attest that all legal and regulatory requirements, including export and import collection permits, have been followed for the collection of specimens from source populations at any international, national, regional, or other geographic level for all relevant field specimens collected as part of this study.

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Author Contributions

Benedict F.C. Stocker (Formal analysis [equal], Investigation [equal], Methodology [equal], Resources [supporting], Visualization [equal], Writing—original draft [equal], Writing—review & editing [supporting]), Raffaele Gamba (Formal analysis [equal], Investigation [equal], Methodology [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [supporting]), Thomas van de Kamp (Data curation [lead], Formal analysis [supporting], Investigation [supporting], Project administration [supporting], Resources [equal], Validation [equal], Writing—original draft [supporting], Writing—review & editing [supporting]), Elias Hamann (Formal analysis [supporting], Investigation [supporting], Writing—review & editing [supporting]), Marcus Zuber (Formal analysis [supporting], Investigation [supporting], Writing—review & editing [supporting]), Patrik Vagovic (Formal analysis [supporting], Investigation [supporting], Writing—review & editing [supporting]), and Arnold H.H. Staniczek (Conceptualization [lead], Investigation [supporting], Methodology [supporting], Project administration [lead], Resources [equal], Supervision [lead], Validation [equal], Writing—original draft [supporting], Writing—review & editing [lead])

Supplementary Material

Supplementary data are available at *Insect Systematics and Diversity* online.

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Conflicts of Interest

The authors declare no competing interests.

Data Availability

The data underlying this article are available in the article itself and its online supplementary material. The SR-µCT volumes analysed in this study are accessible through the RADAR4KIT repository under https://dx.doi.org/10.35097/rbtuybw1s9qfu0ry.

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