# The female cephalothorax of *Stylops ovinae* Noskiewicz & Poluszyński, 1928 (Strepsiptera: Stylopidae)

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## Abstract

External and internal features of the female cephalothorax of the strepsipteran species *Stylops ovinae* (Stylopidae) are described in detail. Many derived features are closely related with the obligatory endoparasitism. A conspicuous characteristic is the secondary tagmosis with an anterior cephalothorax and a large, sack-shaped posterior body region. The cephalothorax comprises the head, thorax and anterior half of abdominal segment I and protrudes from the host's abdomen. It contains the mouth and birth opening, vestiges of cephalic appendages, and most internal organs including the large Nassonov's glands. The posterior body part contains mainly the reproductive organs. The constriction of abdominal segment I probably prevents the exposed anterior body from slipping back into host's body cavity. The narrow mouth opening is used for the uptake of the host's hemolymph by the secondary larval stage and is maintained as a non-functional structure by the adult females. The tentorium is absent. The mandibles are immobilized in the adult female. They are used for penetrating the host's body wall by the second larval instar. The brood canal formed by the larval and pupal exuvia is an autapomorphy of Stylopidia, the birth opening where the first instars are released from the female an autapomorphy of Stylopiformia, and the cephalothoracic invagination an autapomorphy of *Stylops*. A single pair of functional spiracles is laterally placed on abdominal segment I. An unusual circular hemolymph vessel is present in the pharyngeal region. The brain is shifted to the thorax. The suboesophageal complex and the thoracic and abdominal ganglia form a single ganglionic mass in the thoracic region. The musculature of the cephalothorax is extremely reduced. Nassonov's glands, which produce sex pheromones, are the largest internal structures in the cephalothorax. Despite of the loss of the digestive function, the digestive tract is not degenerated including a foregut with well-developed muscles.

## Key words

*Stylops*, Strepsiptera, female, cephalothorax, morphology.

## 1.    Introduction

With approximately 600 described species the highly specialized endoparasitic Strepsiptera are one of the smallest groups of holometabolous insects (POHL & BEUTEL 2005, 2008). The systematic position of the cosmopolitan order was one of the most obstinate enigmas in insect phylogenetics ("the Strepsiptera problem"; KRISTENSEN 1981). Only recently a sistergroup relationship with Coleoptera was unambiguously confirmed with analyses of entire genomes (NIEHUIS et al. 2012) and transcriptomes (PETERS et al. 2014).

The females of the basal extant strepsipteran family Mengenillidae (and probably Bahiaxenidae) are free-living, whereas those of Stylopidia remain in the body of the host (KINZELBACH 1971; POHL & BEUTEL 2005). Within the monophyletic Stylopidia, which comprise ca. 97% of all known strepsipteran species, Stylopidae and Xenidae

are the groups with the highest degree of specialization (Pohl & Beutel 2008).

One of many conspicuous autapomorphies of Strepsiptera is the extreme sexual dimorphism (Ulrich 1943). The short-lived adult winged males are characterized by an excellent flying capacity, enabling them to find the females within short time (Pix et al. 1993). Sexual pheromones produced by the female (e.g., Stylopsal) play a decisive role in this context (Cvačka et al. 2012; Tolasch et al. 2012). The adult females are always flightless but those of Mengenillidae possess thoracic legs and have retained a certain degree of motility. In contrast, the endoparasitic females of Stylopidia are legless and extremely simplified morphologically, with a large, unsclerotized sack-shaped posterior body (Kinzelbach 1971). Only their sclerotized cephalothorax protrudes from the host's abdomen (Lauterbach 1954).

The terminology for the developmental stage of the adult females is inconsistently used in the strepsipteran literature, especially for females of Stylopidia. They are described as paedomorphic, partially paedomorphic (Kinzelbach 1971), or more recently as neotenic (e.g. Kathirithamby 1989; Kathirithamby et al. 2015; Beani et al. 2005; Erezyilmaz et al. 2014). Kinzelbach (1971: 132) used the term paedomorphism (Pädomorphose) or paedomorphic (pädomorph) in the sense that an adult stage develops but retains larval features to a considerable degree. He mainly referred to Lauterbach (1954), who demonstrated that the female of *Stylops* is enclosed by three layers of cuticle, interpreted as larval, pupal and adult cuticle. Using transmission electron microscopy Beani et al. (2005) also found three cuticular layers enclosing the female of *Xenos vesparum* Rossius, 1793 (Xenidae). They interpreted the female as the fourth neotenic instar and the cuticular layers as second, third and fourth instar cuticle. Erezyilmaz et al. (2014) found increased levels of the pupal determinant *broad* in male fourth instar larvae of *X. vesparum* but not in the same instar of females. They suggested that neoteny in stylopidian females could be linked to the suppression of the pupal determination. However, this interpretation remains ambiguous. The authors point out that only five female larvae were examined and that higher levels of *broad* expression might be revealed with a larger sample size.

In any case, like in other stylopidian species females of *S. ovinae* permanently remain within two exuviae of preceding stages and form a functional unit with these layers of cuticle. This structural and functional complex is commonly referred to as *the female* (e.g., Kinzelbach 1971), which is actually an oversimplification. The external features of this unit are always characters of the secondary larvae, whereas internal structures are those of the true female.

External and internal structures of male adults and larvae are relatively well known (Silvestri 1941a,b, 1943; Rohnstein 1953; Baumert 1958; Borchert 1963; Kinzelbach 1971, 1978; Pohl 2000, 2002; Pohl & Beutel 2004, 2005, 2008; Beutel et al. 2005; Osswald et al. 2010; see also Pohl & Beutel 2013). In contrast to this, the cephal-

ic and thoracic anatomy of females is almost completely unknown. Only the head and thorax of the free-living females of *Eoxenos laboulbenei* De Peyerimhoff, 1919 (Mengenillidae) was described in two unpublished diploma theses (Müller 2009; Marquart 2011). The available studies on females of Stylopidia cover the external morphology and selected internal structures (Lauterbach 1954; Borchert 1970; Kinzelbach 1971), the mating behaviour (Hrabar et al. 2014; Peinert et al. 2016), and also physiological aspects (Solulu et al. 1998; Cvačka et al. 2012). Detailed anatomical studies of females using modern techniques were missing so far. Consequently, the main purpose of the present study was to document the morphology of the cephalothorax of females of a species of Stylopidia. External and internal structures of *Stylops ovinae* were examined and documented using scanning electron microscopy, photomicrography, microtome section series, and computer based 3D reconstructions. The results are compared to conditions in free-living females of the mengenillid species *Eoxenos laboulbenei*.

## 2. Material and methods

**Material.** A total of 13 females of *Stylops ovinae* Noskiewicz & Poluszyński, 1928 were obtained from the host *Andrena vaga* Panzer, 1799 (Hymenoptera, Apidae), a solitary bee breeding in larger aggregations. The stylopized bees were dug out from sandy soil at the 10.ii.2014 at the edge of the Niedringhaussee in the vicinity of Osnabrück (52°20′06″N 07°54′48″E). That *A. vaga* was still hibernating by that time ensured that the females of *S. ovinae* were not fertilized yet.

After they were removed from their habitat specimens of *A. vaga* were transferred to glass jars half filled with sand and closed with a screw-cap (0.5 l). During transportation the jars were cooled with crushed ice and kept dark in order to prevent premature hatching of males and mating. Until fixation the females of *Stylops* were kept dark in the fridge at +4°C.

**Fixation.** Females of *Stylops* were extracted from the gaster of the host bees, fixed in Dubosq-Brasil over night, and then dehydrated in an ascending ethanol series (70, 80, 90, 96, 100%) for embedding (n = 2). Specimens used for measurements, photomicrography, and SEM were directly fixed in 70% ethanol (n = 11). Females used for photomicrography, and SEM were then dehydrated as described above.

**Measurements.** The total length of eight females was measured under a Zeiss Stemi SV11 with a calibrated ocular micrometer.

**Scanning electron microscopy (SEM).** Females were transferred to 100% acetone and dried at the critical point using liquid $CO_2$ in an Emitech K 850 (Sample

preparation division, Quorum Technologies Ltd., Ashford, England). After this they were glued to the tip of minute insect pins with super glue. A specimen holder developed by Pohl (2010) was used. The specimen was sputter coated with gold with an Emitech K 500 (Sample preparation division, Quorum Technologies Ltd., Ashford, England). The SEM micrographs were taken with an ESEM XL30 (Philips, Amsterdam, Netherlands) equipped with Scandium FIVE (Olympus, Münster, Germany). To increase the depth of field several images were taken of some aspects and processed with Helicon Focus Version 4.2.7 (HeliconSoft, Kharkov, Ukraine).

**Photomicrography.** For the documentation of the original coloration of the cephalothorax of *S. ovinae* a specimen dried at the critical point was glued to the tip of an minute insect pin and pictures were taken with a Nikon D 90 and a 63 mm Zeiss Luminar macro lense, plus an adjustable extension bellows. The specimens were illuminated using two flashlights fitted with a transparent cylinder for even and soft light. Zerene Stacker (Zerene Systems LLC, Richland, USA) was used to process a stack of partially focused images.

**Microtome sectioning.** Two females were embedded in Araldit-CY 212 (Agar Scientific, Stansted/Essex, England). Semithin sections (1 µm) were cut with a diamond knife mounted on a Microtom HM 360 (Microm, Walldorf, Germany) (cross and longitudinal section). The sections were stained with toluidine blue and pyronine G (Waldeck GmbH and Co. KG/Division Chroma, Münster, Germany). For the 3D-Modelling, pictures of every second section were taken with an Axioplan (Carl Zeiss AG, Oberkochen, Germany) (magnification 5×) equipped with a camera (PixeLink Capture Oem) and software AnalySis (Soft Imaging Systems, Münster, Germany). Individual sections were documented with an Olympus dot.Slide microscope (BX51, software version 3.4, Olympus, Tokyo, Japan).

**3D-Modelling.** The 3D models are based on photographs of serial sections. Amira 5.3 (Visage Imaging GmbH, Berlin, Germany) was used for the alignment and segmentation. An image stack series was obtained for each segmented structure and surface objects were generated. For final post processing in Maya 2013 (Alias Wavefront, Toronto/ Ontario, Canada) all surface files (.iv) were converted into object files (.obj) using Transform2 64 bit software (Heiko Stark, Jena, Germany; URL: http://starkrats.de). Additionally the smoothing function implemented in Maya 2013 was applied.

**Processing of images.** Images were further processed with Adobe Photoshop® CS5 (Adobe System Incorporated, San Jose, USA) and Adobe Illustrator® CS5 (Adobe Systems Incorporated, San Jose, USA).

**Terminology.** For the female anatomy we use the terminology of Lauterbach (1954) except for the following

structures: pocket of the brood canal (Brutkanaltasche) is replaced by cephalothoracic invagination, and closure membrane (Verschlussmembran) is replaced by brood canal membrane.

# 3. Morphological results

## 3.1. Habitus

In the following, the exuvia of the cephalothorax of the single secondary larval stage and the adult female are described separately.

The female completely lacks legs and its external structures are strongly simplified (Fig. 1). The ventral side is oriented towards the host. The total length is between 5.6 and 7.2 mm (mean 6.7 mm, n = 8). The anterior part is formed by the dorsoventrally flattened cephalothorax, which also includes the anterior half of the first abdominal segment (Figs. 1, 2). Abdominal segment I is tapering towards its middle region where it is connected with the main part of the abdomen, located within the host's body and comprising ca. 80% of the body length of the female. The cephalothorax is strongly sclerotized, whereas the remaining sack-shaped and whitish body is largely membranous (Fig. 1). Only the cuticle in the region of the brood canal has a light ochre coloration (Fig. 1A).

The cuticle of the dorsal part of the cephalothorax, which is oriented towards the host's tergites, is of a uniform ochre coloration. The cuticle of the cranial part of the ventral side and of the margins is brownish (Fig. 1). The region of the brood canal is distinctly less pigmented and extends to the first abdominal segment (Fig. 1B). Segment I including its pair of spiracles differs distinctly from the metathorax in its coloration. Its cuticle is dark brown (Fig. 1B,C). The lateral region posterior to the spiracle has a less dark brownish coloration (Fig. 1C).

## 3.2. Cephalothorax of the secondary larval stage

The cephalothorax is more complex in its external features than the sack-shaped posterior body. It appears roughly triangular or parabolic in dorsal and ventral view (Fig. 2A,B). It is slightly longer than wide. In cross section it has a flattened elliptic shape (Fig. 7).

**Head capsule.** The prognathous cephalic region is about ¼ as long as the entire cephalothorax (Fig. 2A). Its orientation is horizontal and it is distinctly flattened dorsoventrally (Fig. 2D). The region in front of the mandibles is ventrally arched. The posterior part is almost completely fused with the prothorax, but still separated from it by an indistinct transverse furrow dorsally ($sb_{hp}$ in Fig. 2B), and by the birth opening in the prosternal region (**bo** in Figs. 1B,C, 2A, 3A). Cephalic sutures and eyes are absent.
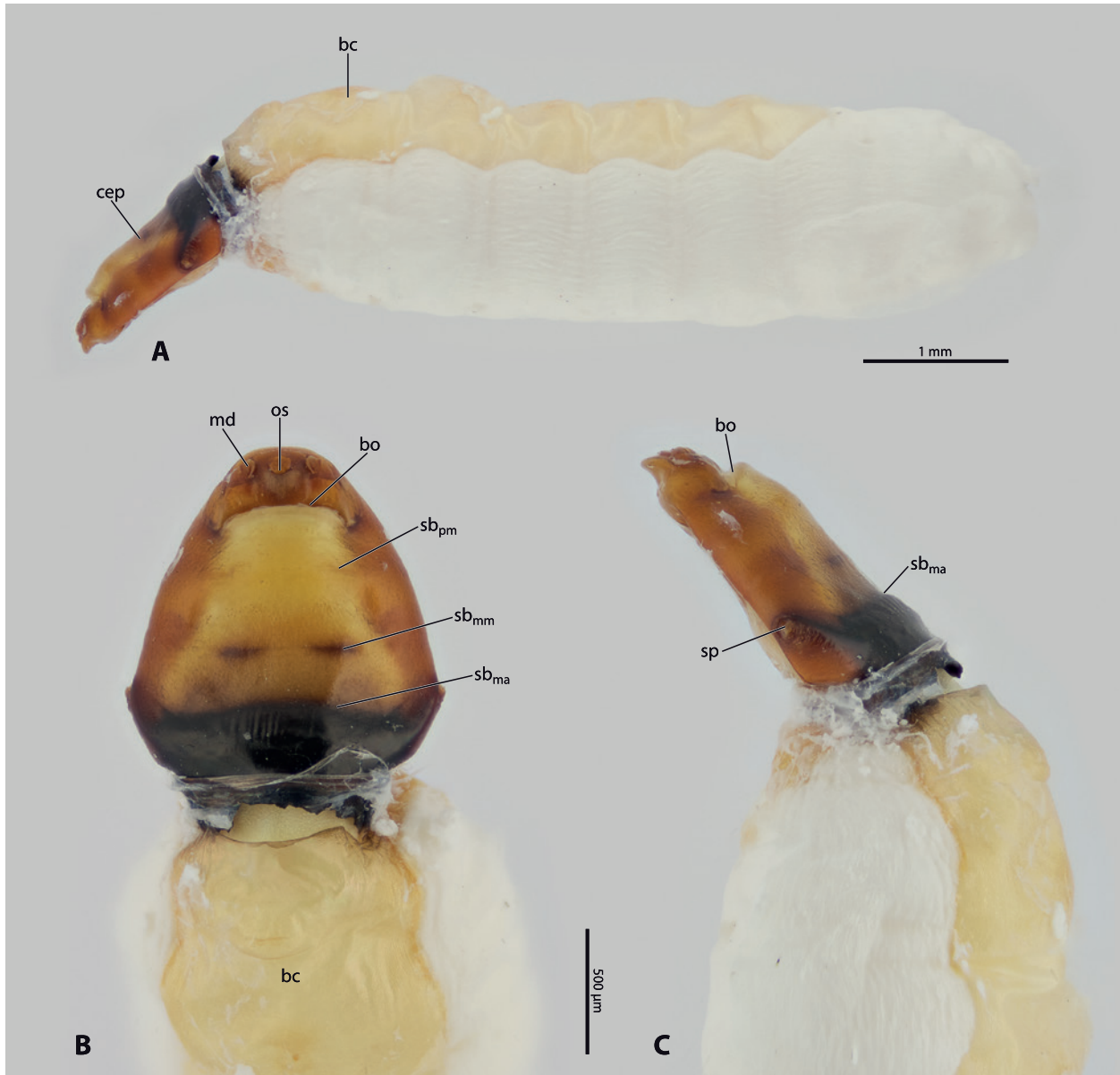
**Fig. 1.** *Stylops ovinae*, female, habitus, photomicrographs. **A**: lateral view, anterior is toward the left and ventral side (physiological dorsal side) facing upwards; **B**: ventral view of cephalothorax and anterior abdomen; **C**: lateral view of cephalothorax and anterior abdomen. — *Abbreviations*: bc – brood canal, bo – birth opening, cep – cephalothorax, md – mandible, os – mouth opening, sb$_{ma}$ – segmental border between metathorax and abdominal segment I, sb$_{mm}$ – segmental border between meso- and metathorax, sb$_{pm}$ – segmental border between pro- and mesothorax, sp – spiracle.

**Antenna.** The antenna is absent.

**Labrum.** A distinct labrum is not developed, but an indistinctly delimited semicircular structure, possibly of labral origin, is present anterior to the mouth opening (**ses** in Figs. 2A, 3A, 4A).

**Mandible.** The mandibles are anteriorly directed. They converge obliquely with their distal parts (Figs. 3A, 4). The tips are rounded (Figs. 3A,B, 4). A single tooth is present distally (**mdt** in Figs. 3B, 4B) and a knob on the ventral side (**mdk** in Figs. 3B, 4B,C). The cuticle is smooth in the region between both structures, but it is wrinkled between the knob and the ventral mandibular joint. The axis of movement is oblique and externally directed. However, the mandibles are immobilized, since their base is fused with the head capsule. The dorsal mandibular joint is absent. The ventral joint is formed by a strongly developed condyle of the mandible articulating with the anterior cephalic part of the cephalothorax (**co** in Figs. 3B, 4B,C).

**Maxilla.** Absent.

**Labium and hypopharynx.** The labium is fused with the wall of the anteroventral cephalic region. It is slightly indented medially (**lb** in Figs. 3A, 4A). The hypopharynx is absent.

**Mouth opening.** The fissure-shaped functional mouth opening lies between the mandibles (**os** in Figs. 2A, 3A, 4A).

**Salivarium.** A salivarium is not present.
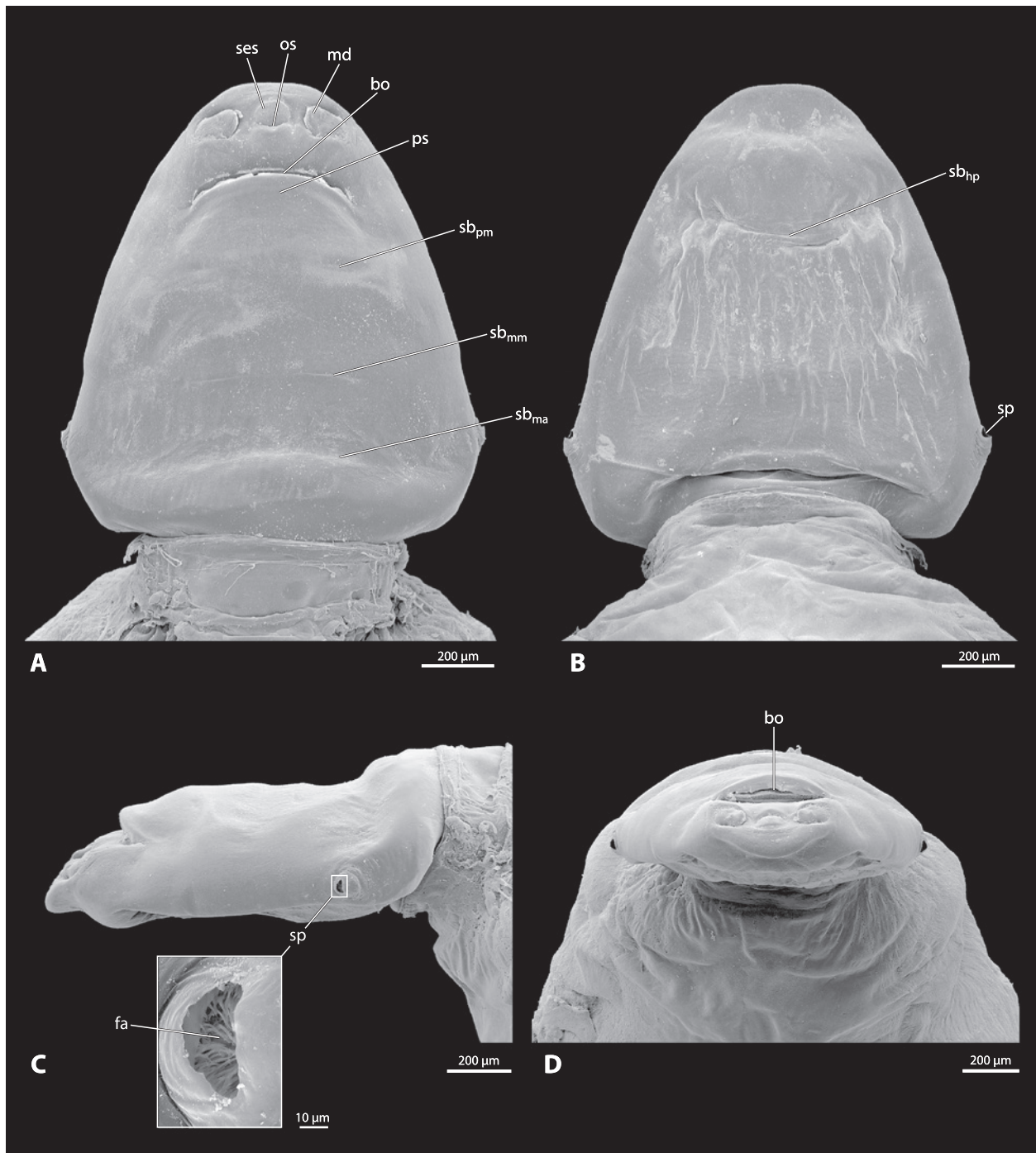
**Fig. 2.** *Stylops ovinae*, female, cephalothorax, SEM-micrographs. **A**: ventral view; **B**: dorsal view; **C**: lateral view with details of the spiracle (box), ventral side (physiological dorsal side) facing upwards; **D**: frontal view, ventral side (physiological dorsal side) facing upwards. — ***Abbreviations***: bo – birth opening, fa – filter apparatus, md – mandible, os – mouth opening, ps – prosternum, $sb_{hp}$ – segmental border between head and prothorax, $sb_{ma}$ – segmental border between metathorax and abdominal segment I, $sb_{mm}$ – segmental border between meso- and metathorax, $sb_{pm}$ – segmental border between pro- and mesothorax, ses – semicircular structure, sp – spiracle.

**Birth opening.** The narrow transverse birth opening posterior to the labium indicates the border between the head and the prosternum (**bo** in Figs. 1B,C, 2A,D, 3A, 4A). Its lateral margins are more intensively pigmented as the rest of the structure and the cuticle is thickened and invaginated (Figs. 1B,C, 7B,C). The larval cuticle (brood canal membrane) is very thin in this area (ruptured in the SEM image; bcm in Figs. 4A, 7B).

**Thorax and abdominal segment I.** Pro-, meso-, metathorax, and abdominal segment I are completely fused and widen successively towards the anterior border of abdominal segment I. The cephalothorax is broadest at the level of the spiracles. Thereafter, the abdominal segment I is strongly constricted. The thoracic tergites, pleurites and sternites are also completely fused. Anlagen of thoracic legs are absent. Segmental limits are not recog-
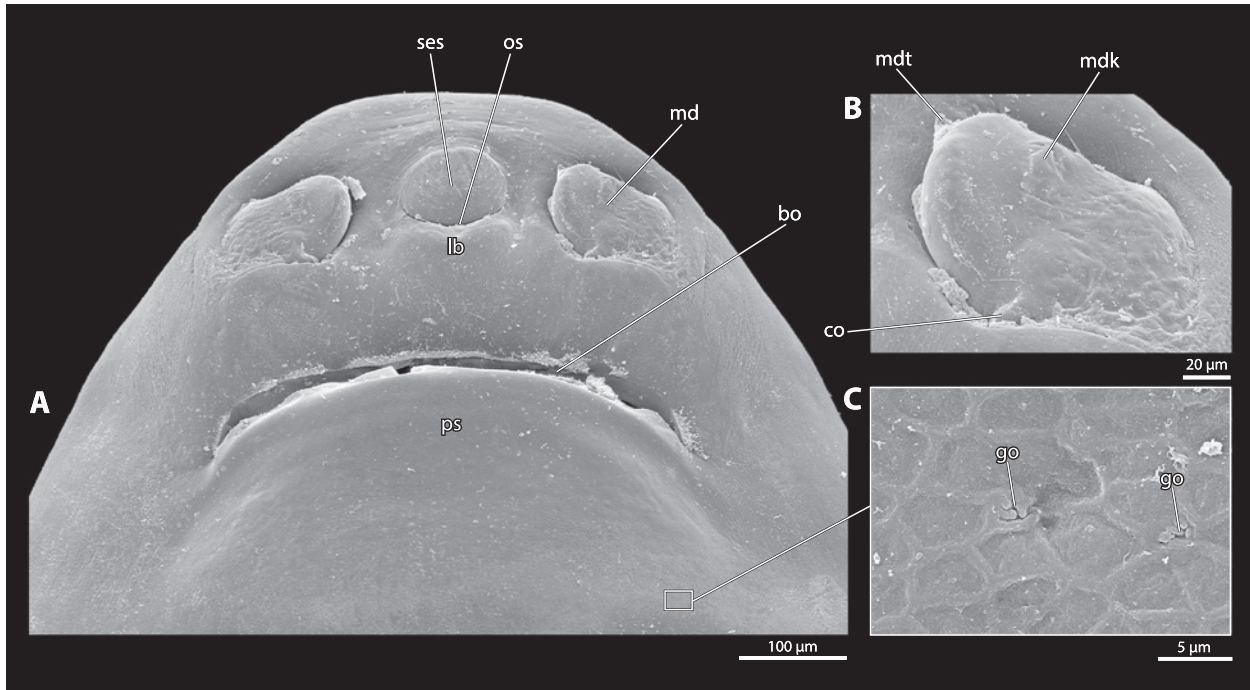
**Fig. 3.** *Stylops ovinae*, female, cephalic region, ventral view, SEM-micrographs. **A**: cephalic region; **B**: left mandible; **C**: surface structure of the cuticle with external openings of Nassonov's glands. — **Abbreviations**: bo – birth opening, co – condylus, go – opening of the Nassonov's gland, lb – labium, md – mandible, mdk – mandible knob, mdt – mandible tooth, os – mouth opening, ps – prosternum, ses – semicircular structure.

nizable on the dorsal side but a distinct fold is present on abdominal segment I (Fig. 2B). Ventrally indistinct segmental borders are visible in the region of the brood canal between the pro- and mesothorax ($sb_{pm}$ in Fig. 2A) and the meso- and metathorax ($sb_{mm}$ in Fig. 2A). Ventrally abdominal segment I is delimited from the metathorax by a distinct convexity and the dark brown coloration (Figs. 1B,C, 2A,C). Numerous openings of the Nassonov's glands are visible on the dorsal surface of the prothorax and the ventral surface of the pro-, meso-, and metathorax (**go** in Fig. 3C).

**Spiracles.** The paired spiracles are placed on approximately hemispherical elevations of the cuticle of the first abdominal segment of the secondary larva (**sp** in Figs. 1C, 3B,C). The spiracular opening is anteriorly oriented and semicircular, with an irregularly structured margin (Fig. 2C). The opening is about twice as long as wide. In the atrium microtrichia form a filter apparatus (**fa** in Fig. 2C).

### 3.3. Cephalothorax of adult female

The female cephalothorax below the pupal and larval cuticle is very similar to that of secondary larva in terms of general shape and proportions (see 3.2.).

**Head capsule.** The head capsule of the adult is very similar to that of the secondary larval stage in its external features (see above). The head is separated from the prothorax by an indistinct segmental border in the dorsal region ($sb_{hp}$ in Fig. 5B), the cephalothoracic invagination in the prosternal region (**in** in Fig. 5A, 6), and by an indistinct furrow in the pleural region ($sb_{hp}$ in Figs. 5A, 6). A Y-shaped cuticular fold is present on the ventral side (**cf** in Fig. 5B).

**Antenna.** Absent.

**Labrum.** Not recognizable as a defined structure.

**Mandible.** The mandibles of adults are characterized by a broad base and a tapering distal part (Fig. 6). The cuticle is covered with microtrichia. A tooth as it is found in the secondary larva is absent. The mandibular joint is not clearly recognizable.

**Maxilla.** The maxillae are strongly simplified lobe-like structures (**mx** in Fig. 6). Maxillary palps are absent. The cuticle is covered with microtrichia.

**Labium and hypopharynx.** Like in the secondary larvae the labium is largely fused with the exoskeleton of the cephalic region. However, a division in a postmentum (**pom** in Fig. 6) with slightly convex anterior border and a smaller prementum (**prm** in Fig. 6) is recognizable. Palps are absent. The labium is located between the external mouth opening and the cephalothoracic invagination. The hypopharynx is absent.

**Mouth opening.** The external mouth opening of adults is also transverse. It is enclosed by cuticular folds and is very narrow and slightly curved (**os** in Fig. 6).

**Salivarium.** A salivarium is not developed.

**Cephalothoracic invagination.** The cephalothoracic invagination is a transverse, fissure-shaped invagination of the adult cuticle, in front of the birth opening (Fig. 5A,D, 6, 7B–E). Its ventral surface is covered with microtrichia
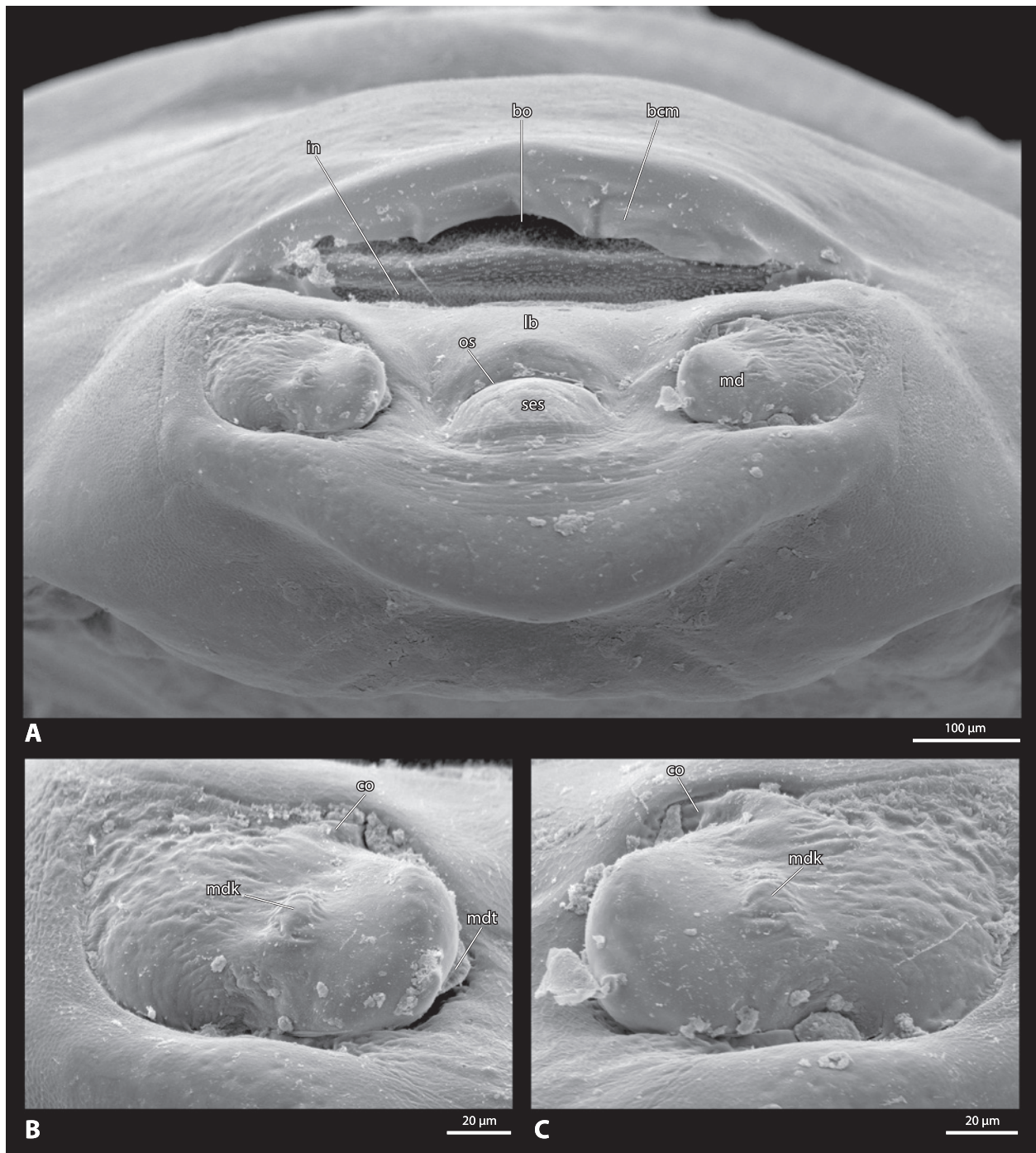
**Fig. 4.** *Stylops ovinae*, female, cephalic region, frontal view, SEM-micrographs, ventral side (physiological dorsal side) facing upwards. **A**: cephalic region, overview; **B**: right mandible; **C**: left mandible. — *Abbreviations*: bcm – brood canal membrane, bo – birth opening, co – condylus, in – cephalothoracic invagination, lb – labium, md – mandible, mdk – mandible knob, mdt – mandible tooth, os – mouth opening, ses – semicircular structure.

(Figs. 6, 8D). The invagination extends into the body lumen from the ventral side of the cephalic region to the region of the prothorax (Fig. 10). The cuticle of the ventral side of the invagination is greatly thickened and the epidermis is multi-layered (**cta** in Fig. 8D).

**Thorax and abdominal segment I.** The three thoracic segments are largely fused like in the secondary larva (Fig. 5). Ventrally the segmental borders are recognizable as transverse furrows (Fig. 5A). The border between the cephalic region and the prothorax is marked by the cephalothoracic invagination. Tergites, pleurites and sternites are largely fused with scarcely recognizable borders. The dorsal side lacks segmental borders except for the hind margin of the cephalic region ($sb_{hp}$ in Fig. 5B). The border between the thorax and abdominal segment I is distinct (Fig. 5A), indicated by the different surface structure, fine wrinkles on the adult thoracic cuticle in the region of the brood canal (Fig. 6), and microtrichia
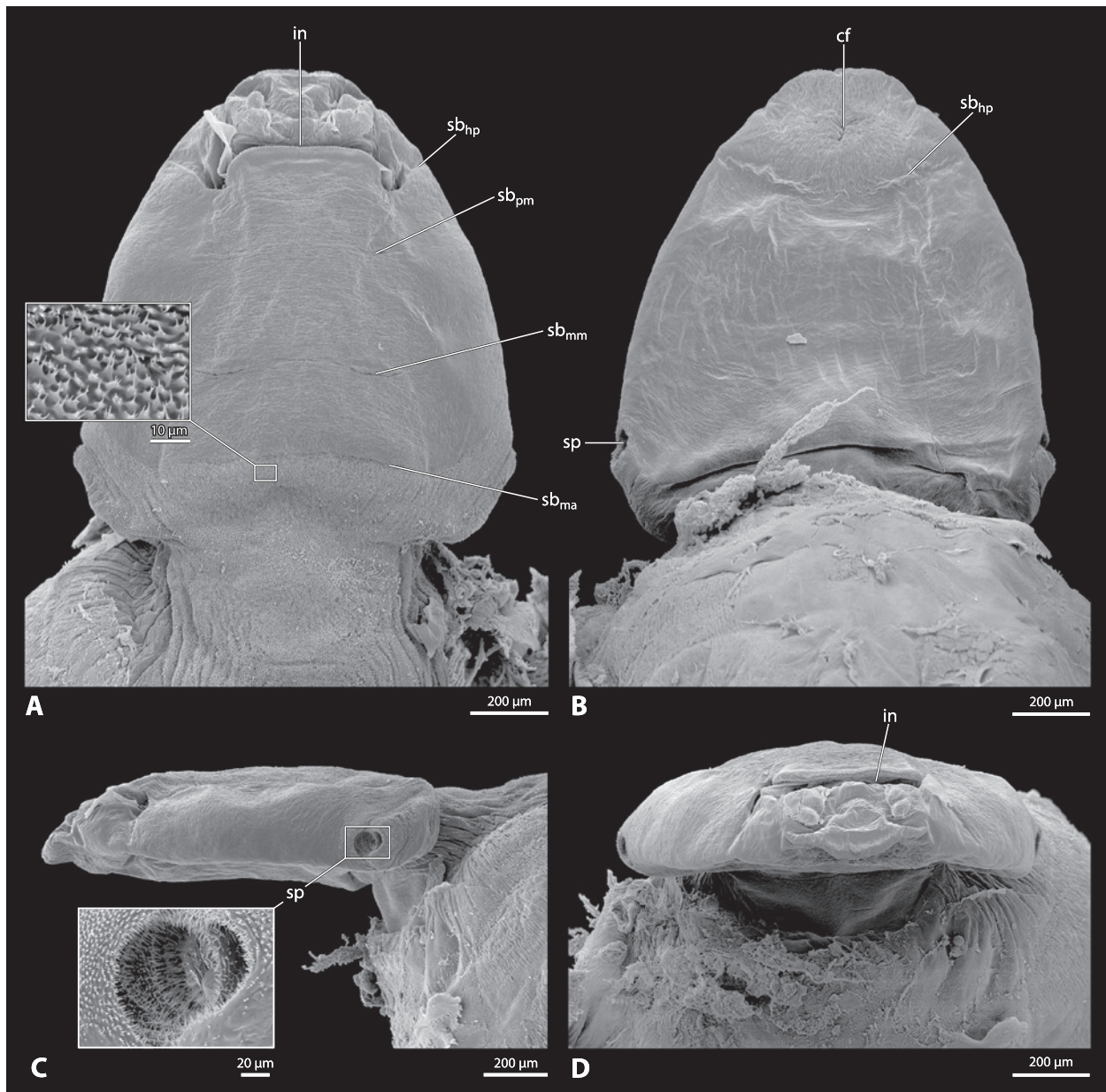
**Fig. 5.** *Stylops ovinae*, female, larval- and pupal cuticle removed, cephalothorax, SEM-micrographs. **A**: ventral view with details of the cuticular structure of I abdominal segment (box); **B**: dorsal view; **C**: lateral view with details of the spiracle (box), ventral side (physiological dorsal side) facing upwards; **D**: frontal view, ventral side (physiological dorsal side) facing upwards. — ***Abbreviations***: cf – cuticular fold, in – cephalothoracic invagination, sb$_{hp}$ – segmental border between head and prothorax, sb$_{ma}$ – segmental border between metathorax and abdominal segment I, sb$_{mm}$ – segmental border between meso- and metathorax, sb$_{pm}$ – segmental border between pro- and mesothorax, sp – spiracle.

on the ventral and lateral abdominal regions (Fig. 5A). The vestiture of microtrichia is also enclosing the surface of the brood canal in the abdomen. The dorsal prothoracic surface and the ventral surface of the pro-, meso-, and metathorax are characterized by numerous openings of the Nassonov's glands (**go** in Fig. 6). The microtrichia of the first and the following abdominal segments are arranged in a comb-like manner. Several microtrichia are often combined on ventrally directed plates (Fig. 5A, box).

**Spiracle.** The hemispherical spiracular elevation is absent after the larval cuticle is removed. A large round opening is recognizable in front of a smaller semi-circu-

lar opening (Fig. 5C). The cuticle is covered by microtrichia. The external microtrichia are shorter and thinner than those of the spiracular filter apparatus.

## 3.4. Internal morphology

**Adult cuticle, pupal and last larval exuvia.** The adult cuticle is largely unsclerotized and fits closely with the outer cuticular layers except in the region of the brood canal where a wider gap is present (Fig. 7). The pupal exuvia is very thin and also present in the cephalothoracic invagination (**exp** in Fig. 6, 8C). The cuticle of the
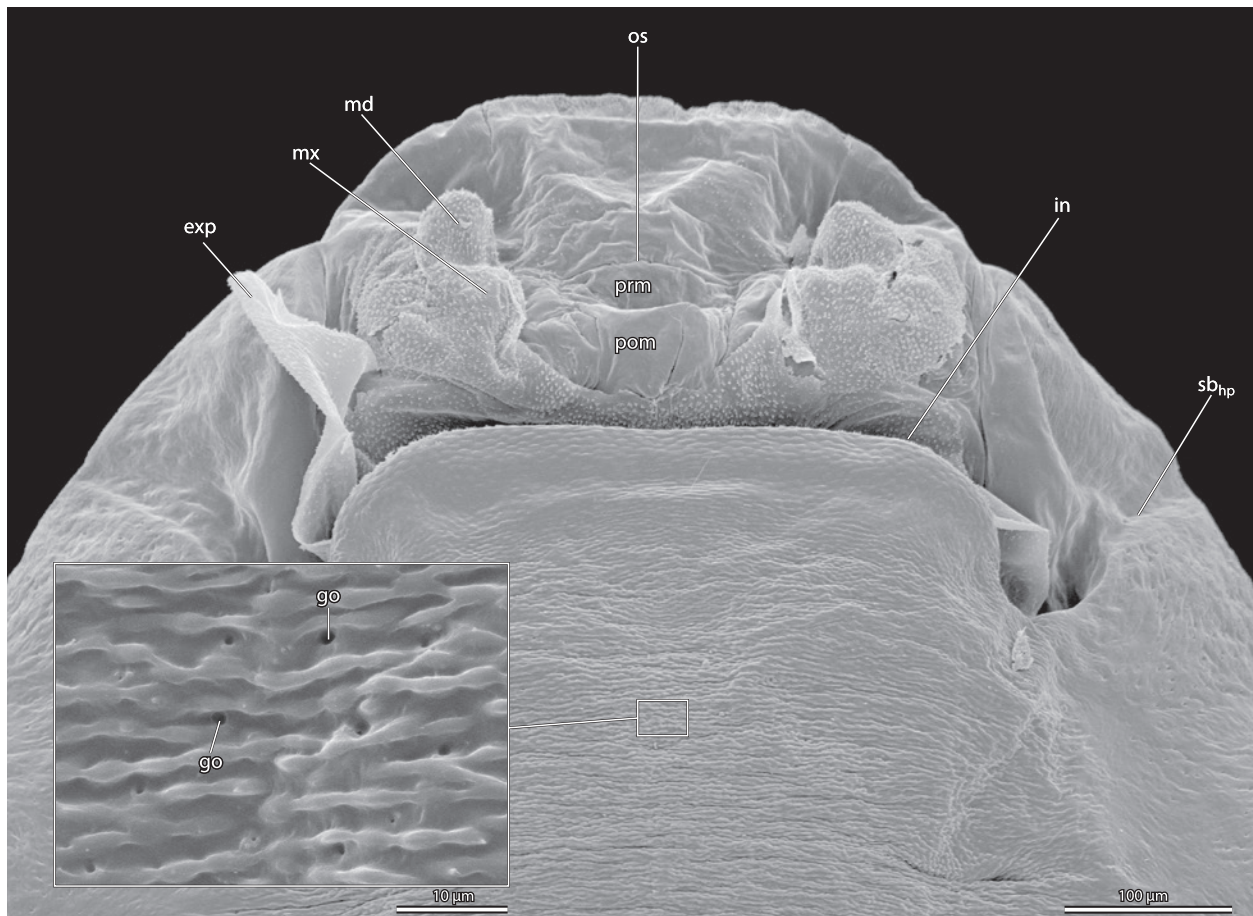
**Fig. 6.** *Stylops ovinae*, female, overview of cephalic region, larval- and pupal cuticle removed with details of prothoracic surface structure (box), ventral view, SEM-micrograph. — *Abbreviations*: exp – pupal exuvia, go – opening of Nassonov's gland, in – cephalothoracic invagination, md – mandible, mx – maxilla, os – mouth opening, pom – postmentum, prm – prementum, sb$_{hp}$ – segmental border between head and prothorax.

secondary larva is strongly sclerotized and the dorsal and ventral areas have the same thickness (ca. 15 μm) in the cephalic region (Fig. 7A). The thickness of the dorsal region is declining gradually from the prothorax to the first abdominal segment, (Fig. 7). The last larval exuvia is absent in the cephalothoracic invagination.

**Tracheal system.** The main tracheal stems extend posteriorly close to the external adult cuticle from the spiracles of abdominal segment I towards the posterior body region, with a curve before entering the abdomen (**tra$_m$** in Fig. 9C,D). Two side branches arise in the anterior abdominal segments and enter the cephalothorax (**tra$_s$** in Fig. 9C). An anastomosis connecting the two secondary tracheae is present in the region of abdominal segment I (**ana** in Fig. 9C). Anterior to this the secondary branches divide into many smaller tracheae, which extend far into the cephalothorax.

**Dorsal vessel.** The main part of the circulatory system is located in the posterior body region. It is spiral-shaped in the constricted region of abdominal segment I and adjacent to the dorsal body wall in the thoracic segments (**dv** in Figs. 7J,K). The dorsal vessel is nearly straight in the cephalothorax. It bifurcates in the anterior prothorax and forms a ring above the functional mouth opening, which

encloses the pharyngeal dilators (Fig. 7C,D, **dvr** in Fig. 9A,C). A short anteriorly directed vessel originates from the ring.

**Nervous system.** The cephalic region contains only peripherical elements of the nervous systems. The brain is located in the anterior mesothorax (Fig. 7F, 9C,D, 10) and the suboesophageal ganglion in the metathorax and anterior abdominal segment I (Fig. 7H,I, 9D, 10). Both are connected by circumpharyngeal connectives enclosing the midgut (Figs. 7G, 9D, 10C). Two large anteriorly directed nerve branches arise from the brain and divide shortly after their origin. This complex also contains nerves of the posterior and anterior pharyngeal dilators and of longitudinal and ring muscles of the pharynx (Fig. 9C,D). Additionally, a distinct nervus connectivus connects the brain with the frontal ganglion (Figs. 9C, 10C). Optic lobes and antennal nerves are absent. Six distinct nerve branches originate from a compact complex formed by the suboesophageal ganglion and the fused thoracic ganglia. Four pairs of nerves extend posteriorly and two pairs towards the anterior body region (Fig. 9D). The posterior branches extend in the vicinity of the curve of the main tracheal stems. The anterior nerves extend ventrolaterally and end in the vicinity of tracheal side
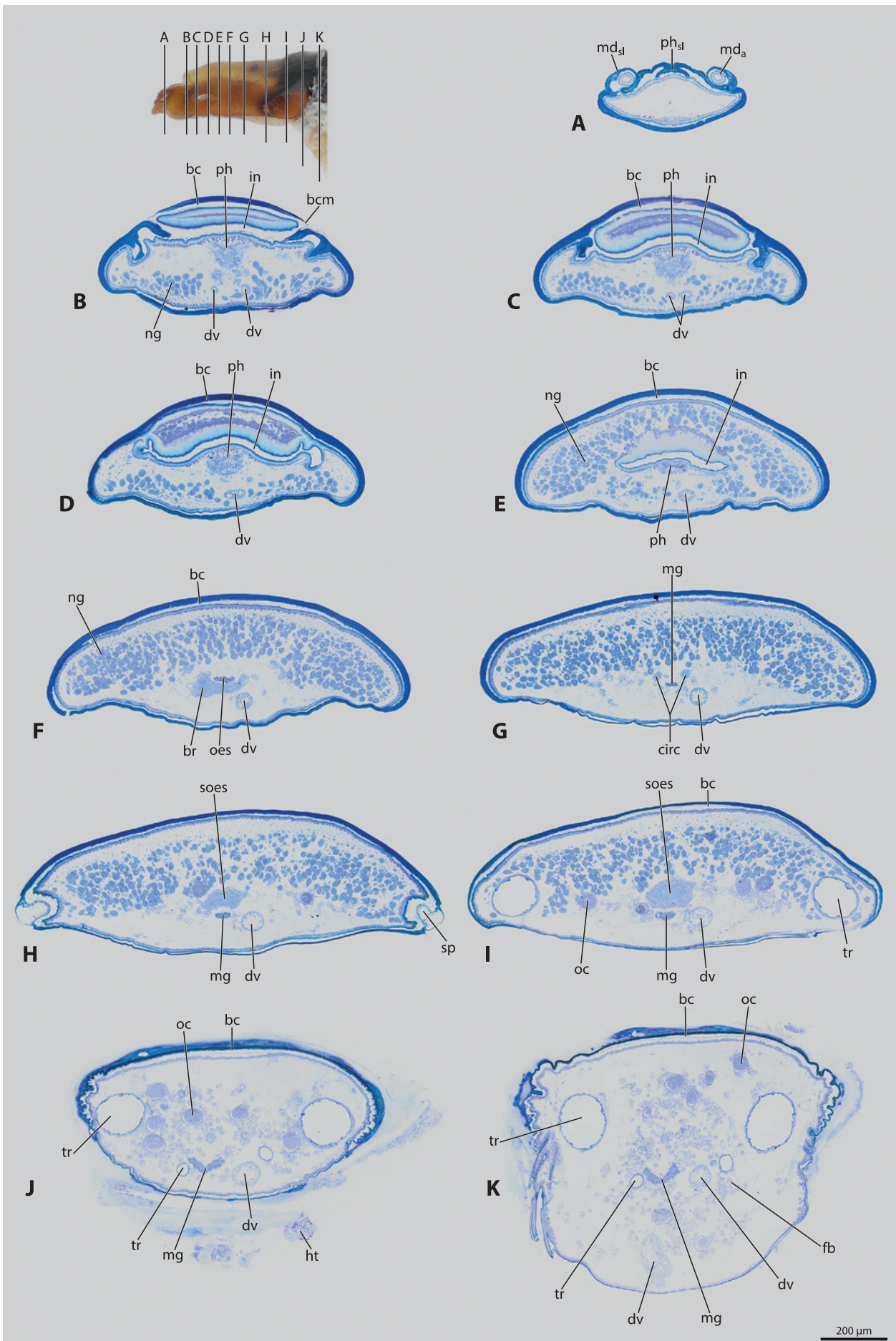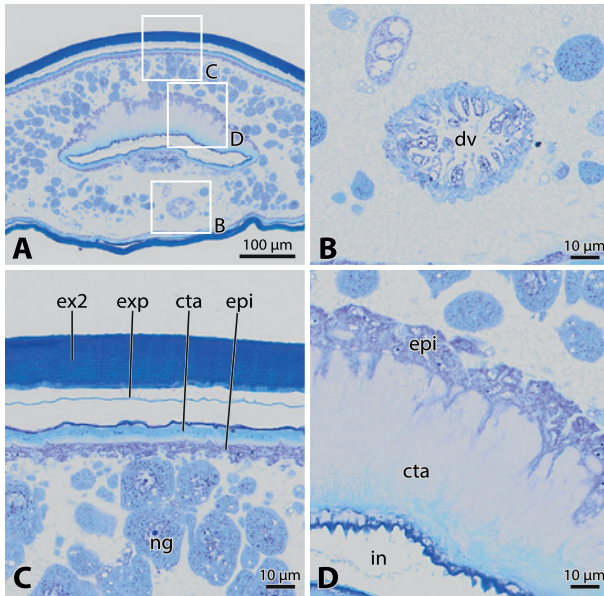
**Fig. 8.** *Stylops ovinae*, female cephalothorax, cross section in the region of the posterior cephalothoracic invagination, ventral side (physiological dorsal side) facing upwards. **A**: overview; **B**: dorsal vessel; **C**: ventral body wall; **D**: cephalothoracic invagination. — *Abbreviations*: cta – adult cuticle, dv – dorsal vessel, epi – epidermis, ex2 – exuvia of the second larval stage, exp – pupal exuvia, in – cephalothoracic invagination, ng – Nassonov's gland.

branches (Fig. 9C,D). The anteriormost pair extends towards the cephalic region and end laterad to the brain (Fig. 9C,D).

**Musculature.** The musculature of the cephalothorax is poorly developed (Table 1). An unusually large abductor and the adductor are attached to the mandibles (Fig. 9C,D). Another distinctly developed group is associated with the pharynx, the anterior and posterior dilators (Fig. 10B). A ring- and longitudinal muscle layer extend from the functional mouth opening to the pharynx (Fig. 10B).

**Nassonov's glands.** The complex Nassonov's gland is composed of numerous small units and by far the most voluminous structure in the cephalothorax (**ng** in Figs. 7B–I, 9A,B, 10). Each single lobe is composed of a globular terminal part with coarsely granular cytoplasm (Fig. 8C) and a duct opening on the cuticle of the adult female (Fig. 6, box). The glands fill out a large part of the lumen of the cephalothorax.

**Digestive tract.** The digestive tract of *S. ovinae* comprises the functional mouth opening, pharynx (anterior foregut), oesophagus and midgut (Figs. 7B–K, 9C,D). The entire system has lost its digestive function, which is underlined by the closed mouth opening (Fig. 6). Salivary glands are absent. The mouth opening is followed

by the pharynx, which is posteriorly continuous with the oesophagus and then the midgut. The digestive tract rests ventrally on the cephalothoracic invagination and extends along it in the dorsal region of the cephalothorax adjacent to the dorsal vessel (Figs. 7, 9A,C,D). In cross sections the midgut appears like a diaphragm without the typical pattern of high cylindrical cells.

## 4. Discussion

The following comparison with free-living females of Mengenillidae is based on descriptions of the head (Marquart 2010) and thorax (Müller 2009) of *Eoxenos laboulbenei*. Many derived features of females of *S. ovinae* are apparently correlated with the obligatory endoparasitism, among them the conspicuous secondary tagmosis with an anterior part protruding from the host's abdomen and a sack-shaped posterior body region (Lauterbach 1954). The former bears the mouth and birth opening, the vestiges of cephalic appendages and most internal organs including the very large Nassonov's glands. The much larger posterior part contains the reproductive organs, and the embryonic development takes place in its lumen.

### 4.1. Secondary larval stage

According to Kathirithamby et al. (2015) the cephalothorax of stylopidian females contains only the head and prothorax. This is in contrast to earlier findings that this secondary tagma comprises the head, pro-, meso-, metathorax and the anterior part of the first abdominal segment (e.g. Lauterbach 1954; Kinzelbach 1971; Pohl & Beutel 2005, 2008). The interpretation of Kathirithamby et al. (2015) is clearly refuted by the present contribution. Kinzelbach (1971) interpreted the flattening of the cephalothorax as an adaptation to mechanical strain caused by the host's cuticle. However, this appears unlikely as the male puparium is not flattened (Pohl & Beutel 2005). The distinct constriction in the middle region of abdominal segment I of *S. ovinae* marks the penetration point of the host's body wall and probably prevents the exposed anterior body part from slipping back into the host's body cavity. The narrow mouth opening is used for the uptake of the host's hemolymph by the secondary stylopidian larvae and is maintained as a non-functional structure by the adult females (Lauterbach 1954).

The birth opening is an autapomorphy of Stylopiformia (Pohl & Beutel 2005, 2008) and the area where

← **Fig. 7.** *Stylops ovinae*, female cephalothorax, cross sections, position of sections indicated in photomicrograph, ventral side (physiological dorsal side) facing upwards. — *Abbreviations*: bc – brood canal, bcm – brood canal membrane, br – brain, circ – circumpharyngeal connectives, dv – dorsal vessel, fb – fatbody cells, ht – host tissue, in – cephalothoracic invagination, md$_a$ – adult mandible, md$_{sl}$ – mandible of second larva, mg – midgut, ng – Nassonov's gland, oc – egg, oes – oesophagus, ph – pharynx, ph$_{sl}$ – pharynx of second larva, soes – suboesophageal complex, sp – spiracle, tr – trachea.

**Table 1.** Terminology and homology of adult head muscles of the female of *Stylops ovinae*. The musculature is homologized with muscles described for females of Stylopidia (KINZELBACH 1971) and general nomenclatures of insect head muscles (v. KÉLER 1963; WIPFLER et al. 2011).

| Name | Abbrevation | Origin | Insertion | v. KÉLER 1963 | WIPFLER et al. 2011 | KINZELBACH 1971 |
|------|-------------|--------|-----------|---------------|---------------------|------------------|
| M. craniomandibularis internus | m11 | concavity of the adult cephalic region at the level of the birth opening | mandibular base | 11 | 0md1 | 3′ |
| M. craniomandibularis externus | m12 | concavity of the adult cephalic region, close to M11 | mandibular base, close to m11 | 12 | 0md2 | 4′ |
| M. clypeopalatalis | m43 | anterodorsal region of the cephalic region, within the ring-shaped structure formed by the dorsal vessel | pharynx anterior to anatomical mouth opening | 43 | 0ci1 | 6′ |
| M. tentoriobuccalis anterior | m48 | anterodorsal region of the cephalic region, within the ring-shaped structure formed by the dorsal vessel | pharynx posterior to the anatomical mouth opening | 48 | 0bu5 | 7′ |
| M. anularis stomodaei | m68 | prothoracic region, end of the brood canal at the pharynx | ventral side of the cephalic region, level of the functional mouth opening | 68 | 0st1 | 8′ |
| M. longitudinalis stomodaei | m69 | prothoracic region, end of the brood canal at the pharynx | ventral side of the cephalic region, level of the functional mouth opening | 69 | 0st2 | 9′ |

the primary larvae are released. This function is correlated with the rupture of the membranous cuticle of this region (KINZELBACH 1971). The birth opening and the brood canal are formed by the larval cuticle and are removed when this layer of integument is artificially detached, whereas the cephalothoracic invagination is left in its position as it is formed by the pupa and adult.

The absence of the antennae, labrum and maxillae is part of the overall morphological simplification and apparently correlated with endoparasitism. The mandibles as the only movable appendages (KINZELBACH 1971) have the sole function of penetrating the host's body wall. In contrast to normal insect mandibles they are moved outwards (LAUTERBACH 1954) and perforate the intersegmental membrane between the tergites of *A. vaga*. The plesiomorphic condition is maintained in Mengenillidae, with mandibles working inwards with very large flexor muscles (KINZELBACH 1971).

## 4.2.  Adults

The results of earlier authors (e.g., LAUTERBACH 1954; KINZELBACH 1971; POHL & BEUTEL 2005) and our investigations show that the exuviae of the secondary larva, pupa and the adult cuticle form a functional unit. As all three layers are very tightly packed the cephalothorax has a very similar shape in these stages (Figs. 2, 5).

In the following the adult morphology of *S. ovinae* and *E. laboulbenei* is compared. This is impeded by the fact that Mengenillidae do not form a complex with several cuticular layers and are not parasitic (MARQUART 2010). Some of the females of Mengenillidae remain in the puparia whereas a certain proportion of them hatch from this structure (POHL & BEUTEL 2005, 2008).

The mouth opening of adult females of *S. ovinae* is fissure-shaped, non-functional and lacks any specific structural features. In contrast, the free-living females of Mengenillidae, which do not consume food like those of Stylopidia, possess a functional mouth opening with a mouthfield sclerite, similar to that of males (MARQUART 2010). In *S. ovinae* labrum and antennae are absent, whereas these structures are preserved in females of Mengenillidae.

The mandibles of *S. ovinae* lack teeth (see section 3.3.) and are non-functional (KINZELBACH 1971). In contrast, the mandibles of Mengenillidae females are used to hatch from the puparium (H. Pohl unpubl. observations). The cephalothoracic invagination lacks a comparable counterpart in Mengenillidae and is an autapomorphy of *Stylops*. This paragenital organ (PEINERT et al. 2016) is lined with pupal cuticle and its ventral side is characterized by a multilayered epidermis (LAUTERBACH 1954; PEINERT et al. 2016). Moreover, the thickness of the cuticle is strongly increased ventrally (PEINERT et al. 2016). The specific properties are probably correlated with the traumatic insemination, with the penis penetrating the exuvia of the second larval stage (brood canal membrane), the pupal exuvia and the adult cuticle. The acumen of the penis enters the body cavity of the females (PEINERT et al. 2016). The ventral thickening of the cuticle in the region of the cephalothoracic invagination probably prevents larger lacerations and also infections (LAUTERBACH 1954).

Like in all other known females of Stylopidia, only a single pair of functional spiracles is present, placed laterally on abdominal segment I. It is used for air uptake after the anterior end of the body has penetrated the host's body wall (KINZELBACH 1971; POHL & BEUTEL 2005). The loss of the abdominal spiracles II–VIII is ap-
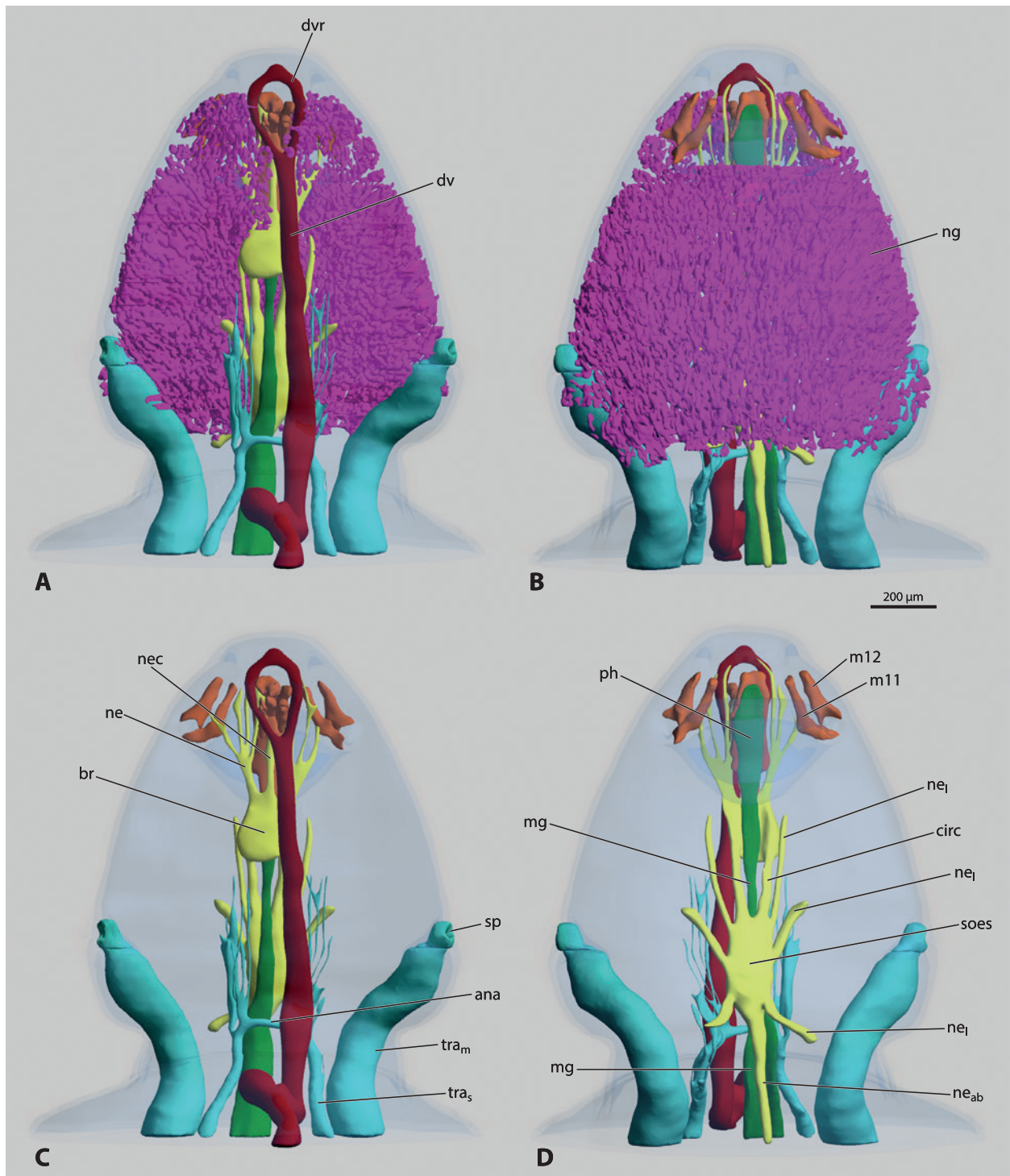
**Fig. 9.** *Stylops ovinae*, cephalothorax, 3d-reconstruction; **A** and **C** dorsal view, **B** and **D** ventral view. — ***Colours***: transparent light grey, exuvia of the second larval stage; transparent light blue, cuticle of the adult; pink, Nassonov's glands; blue-green, tracheal system; red, dorsal vessel; yellow, nervous system; green, digestive tract; orange, musculature. — ***Abbreviations***: ana – anastomosis, br – brain, circ – circumpharyngeal connectives, dv – dorsal vessel, dvr – ring of dorsal vessel, m11 – Musculus craniomandibularis internus, m12 – Musculus craniomandibularis externus, mg – midgut, ne – nerve, ne_{ab} – abdominal nerve, nec – nervus connectivus, ne_{l} – leg nerve, ng – Nassonov's glands, ph – pharynx, soes – suboesophageal complex, sp – spiracle, tra_{m} – main tracheal stem, tra_{s} – side branch of tracheal system.

parently correlated with endoparasitism (Pohl & Beutel 2005). Corioxenidae and Halictophagidae possess non-functional metathoracic spiracles (Pohl & Beutel 2005), whereas they are absent in Myrmecolacidae and Elenchidae. Functional metathoracic spiracles only occur in

*Callipharixenos* (Callipharixenidae). According to Kinzelbach (1971), non-functional metathoracic spiracles are present in Xenidae and Stylopidae (Stylopidae *sensu* Kinzelbach). However, this could not be confirmed for *S. ovinae*. Females of Mengenillidae possess non-function-
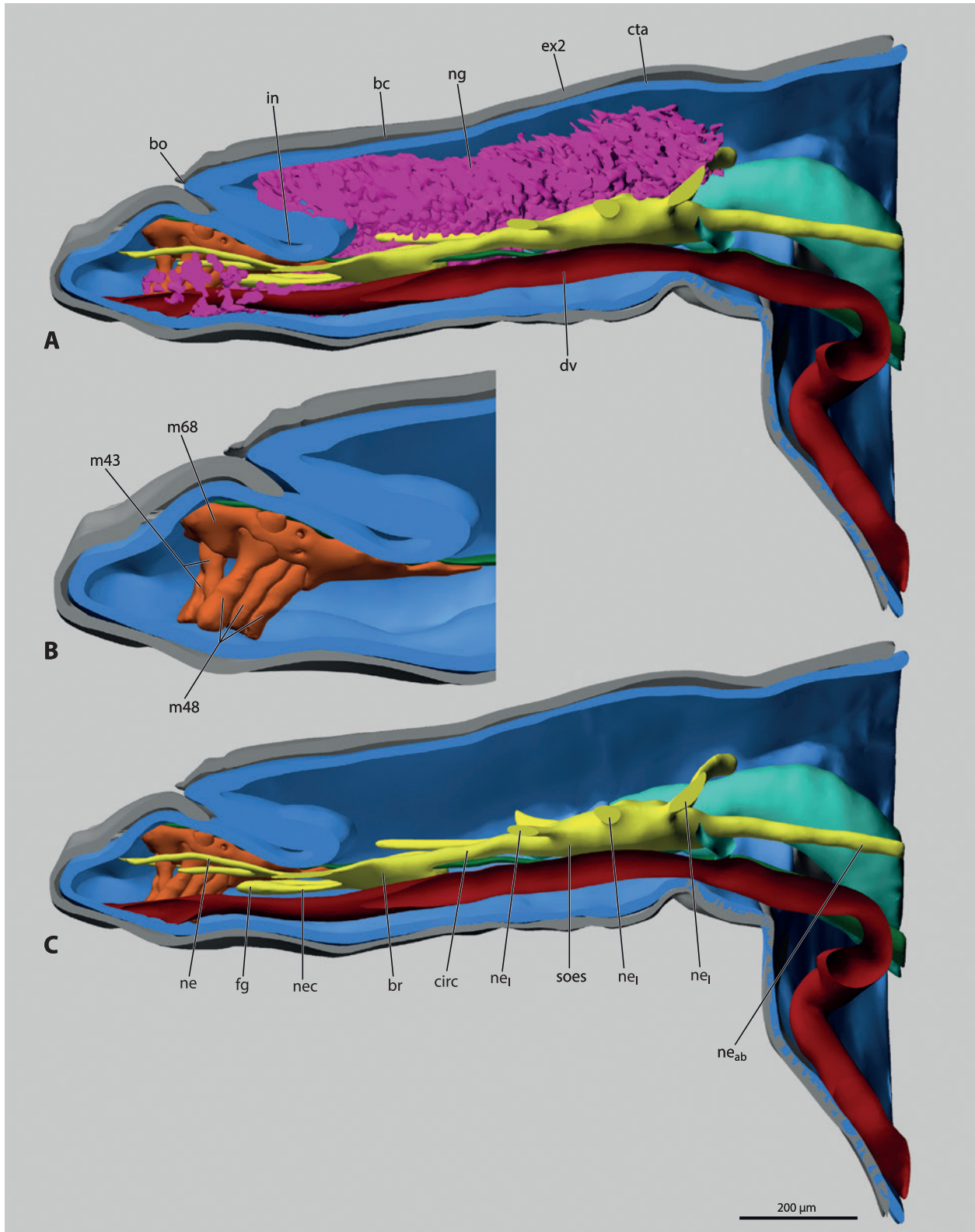
**Fig. 10.** *Stylops ovinae*, cephalothorax, 3d-reconstruction, sagittal view, anterior is toward the left and ventral side (physiological dorsal side) facing upwards. **A**: with complete interior structures; **B**: head and prothorax, Nassonov's glands, dorsal vessel and nervous system removed; **C**: Nassonov's glands removed. — ***Colours***: light grey, exuvia of the second larval stage; light blue, cuticle of the adult; pink, Nassonov's glands; blue-green, tracheal system; red, dorsal vessel; yellow, nervous system; green, digestive tract; orange, musculature. — ***Abbreviations***: bc – brood canal, bo – birth opening, br – brain, circ – circumpharyngeal connectives, cta – cuticle of the adult, dv – dorsal vessel, ex2 – exuvia of the last larval stage, fg – frontal ganglion, in – cephalothoracic invagination, m43 – musculus clypeopalatalis, m48 – musculus tentoriobuccalis anterior, m68 – musculus anularis stomodaei, ne – nerve, ne$_{ab}$ – abdominal nerve, nec – nervus connectivus, ne$_l$ – leg nerve, ng – Nassonov's glands, soes – suboesophageal ganglion.

al meso- and metathoracic spiracles and functional spiracles of abdominal segments I–VII (Kinzelbach 1971), which is likely part of the strepsipteran groundplan. The microtrichiae in the spiracular atrium of *S. ovinae* apparently prevent contamination of the tracheal system with small particles (Lauterbach 1954). The irregularly shaped margin of the spiracle is caused by the rupture of the closed spiracle of the last instar larva after protruding from the host's abdomen (Lauterbach 1954).

## 4.3.  Internal structures

In contrast to Kinzelbach (1971) vestiges of the tentorium are not present in *S. ovinae*. It is absent like in the basal Mengenillidae (Marquart 2010) and all males of Strepsiptera (Pohl & Beutel 2005). Apparently the cephalic endoskeleton was reduced very early in the evolution of the group.

The paired main stems of the tracheal system follow the outline of the cephalothorax as already described by Lauterbach (1954). The same author described their conjunction in abdominal segment VII and a second transverse anastomosis formed by secondary branches in abdominal segment I or in the metathorax (Fig. 9). Kinzelbach (1971) interpreted this condition of the tracheal system as a secondary simplification, resulting from paedomorphosis. He referred to a similar configuration in primary larvae of *S. ovinae* (Rohnstein 1953). Interestingly the tracheal system of free living females of *E. laboulbenei* is even more simplified. Only a single main stem extends along the digestive tract (Müller 2009).

The unusual circular structure of the dorsal vessel in the pharyngeal region is possibly correlated with the position and configuration of the dilators and may enhance the supply of this muscle complex. In contrast to our own observations Lauterbach (1954) did not describe a ring-shaped structure but a split into two branches close to the dilators of the pharynx. A ring-shaped structure is absent in *E. laboulbenei* (Marquart 2010).

In contrast to the free-living females of *E. laboulbenei* (Marquart 2010), where the brain lies within the head capsule, the brain of females of *S. ovinae* is completely shifted to the thorax. The nervous system is characterized by simplifications and fusions impeding the identification of individual segmental ganglia. In *E. laboulbenei* and *S. ovinae* the suboesophageal complex and the thoracic and abdominal ganglia form a single ganglionic mass in the thoracic region (Müller 2009). It is conceivable that a highly derived condition in the extremely miniaturized primary larvae is largely maintained throughout the postembryonic development. As a result of extreme size reduction the central nervous system of primary larvae is extremely concentrated in the middle body region, including the brain and suboesophageal ganglion (Pohl 2000; Beutel et al. 2005). The shift of the brain and different structural simplifications in Stylopidia are probably related to endoparasitism. This includes the absence of optic lobes, which are obviously consequences to the loss of eyes. In *E. laboulbenei* the nerves of the thoracic legs are the only nerves arising from the postcerebral complex and mandibular nerves were not identified (Müller 2009). We therefore interpret the nerves arising from the ganglionic complex of *S. ovinae* as leg nerves, preserved despite of the complete loss of the thoracic locomotor organs.

The musculature of the cephalothorax is extremely reduced. Only mandibular muscles, dilators, and a ring- and longitudinal muscle layer of the pharynx are maintained (Fig. 10B). Thoracic muscles are absent. Three muscles of the cephalothorax of *X. vesparum* were described by Kinzelbach (1971). In contrast, 13 muscle pairs are present in the head (Marquart 2010) and more than 50 muscle pairs in the thorax of free living females of *E. laboulbenei* (Müller 2009). The simplification in *S. ovinae* is certainly a result of the obligatory endoparasitism and the correlated immobility. Mainly muscles used by the secondary larvae for penetrating the host's body wall are maintained. This is in clear contrast to the mobile females of *E. laboulbenei* where legs and leg muscles are preserved (Müller 2009).

Nassonov's glands are by far the largest internal structures in the cephalothorax. They produce sex pheromones (Cvačka et al. 2012; Tolasch et al. 2012) attracting the males. The glands are restricted to the cephalothorax, the only freely exposed body part. In contrast to Lauterbach (1954), pores in the adult cuticle and in the exuvia of the secondary larva were identified with SEM. Kathirithamby et al. (2015) assumed that a closed brood canal membrane may impede the release of pheromones and that the brood canal membrane is ruptured during the super-extrusion of the female's cephalothorax in *X. peckii*, thus facilitating the release of pheromones through the birth opening. Our findings support Lauterbach (1954), who suggested that the pheromones penetrate the cuticle of the secondary larval stage.

Despite of the loss of the digestive function, the digestive tract is not degenerated. In agreement with Lauterbach (1954) a clear differentiation between pharynx and oesophagus could not be observed. A plug between the oesophagus and the midgut (Lauterbach 1954) was not recognizable in the available section series of *S. ovinae*. The lumen of the pharynx of *E. laboulbenei* is distinctly narrower than in *S. ovinae* (Marquart 2010). The musculature associated with the foregut is also more strongly developed in *E. laboulbenei*, with a well-developed pharyngeal layer of ring muscles, almost horizontally oriented pharyngeal dilators, and strongly developed epipharyngeal muscles (Marquart 2010). As all adults of extant strepsipteran species do not consume food (Kinzelbach 1971; Pohl & Beutel 2005) the presence of a distinctly developed musculature of the foregut is surprising. These structures which apparently have no function in the adults are probably vestiges, maintained from the larval stages, as it is also the case with the mandibles of females of *S. ovinae*.

# 5.    Acknowledgements

# 6.    References

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