

# Serial block-face scanning electron microscopy sheds new light on the head anatomy of an extremely miniaturized insect larva (Strepsiptera)

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Accepted 21.iv.2016.

Published online at [www.senckenberg.de/arthropod-systematics](http://www.senckenberg.de/arthropod-systematics) on 21.ix.2016.

Editor in charge: Christian Schmidt

## Abstract

Serial cross sections (80 nm) and an image stack (353 sections) of the head of the extremely miniaturized first instar larvae of *Stylops ovinae* (Stylopidae) were obtained with serial block-face scanning electron microscopy. This approach made it possible for the first time to reconstruct the head anatomy of a strepsipteran larva precisely, partly to cell level. The cephalic anatomy is described in detail, based on a 3D-reconstruction. It was possible to show the proportions of the exo- and endocuticle and the epidermis. Endoskeletal structures are the pharyngeal skeleton and the anterior tentorial arms, which form an articulation with the mandibles. The dorsoventrally flattened mandibles are the only functional mouthparts and used for penetrating the host's cuticle. The maxillae are fused with the head capsule to form the ventral plate, and the strongly modified and reduced labium functions as a closure of the ventral head opening. The nerves ending at the anterior head margin were reconstructed. The innervation of setae, olfactory pits and the vestigial maxillary palps are also documented for the first time. Five fat body cells are located in the head. Antennae, hypopharynx and labrum are not present as defined structures. A structure resembling a labrum is a secondary formation. Only nine cephalic muscles are present. Muscles of the antennae, hypopharynx, labrum and maxillae are missing. Some of the muscles originate in the thorax. Homology issues, possible phylogenetic implications, functional aspects of head structures, and also possible correlations of structural features with miniaturization and parasitism are discussed.

## Key words

First instar larvae, *Stylops*, Strepsiptera, head, anatomy, miniaturization, parasitism, phylogeny.

## 1. Introduction

Despite intensive investigations (e.g., KINZELBACH 1971; WHITING et al. 1997; BEUTEL & GORB 2001; WHEELER et al. 2001; KUKALOVÁ-PECK & LAWRENCE 2004; BEUTEL & GORB 2006) see also KRISTENSEN (1981, 1991), BEUTEL & POHL (2006) the highly specialized endoparasitic Strepsiptera remained a systematic enigma until very recently. Analyses of single copy nuclear genes (WIEGMANN et al. 2009), an extensive morphological data set (BEUTEL et al. 2011) and transcriptomes and entire genomes (NIEHUIS et al. 2012; MISOF et al. 2014) finally converged on a

reliable solution, a sistergroup relationship with Coleoptera.

Few studies were dedicated to the morphology of the first instar strepsipteran larva, which is the topic of the present study (e.g., ROHNSTEIN 1953; BORCHERT 1963; POHL 2000; OSSWALD et al. 2010). The first section series was produced by HOFFMANN (1913, 1914) in the framework of an embryological investigation of *Xenos bohlsi* Hoffmann, 1914 (Xenidae). However, this material allowed only a superficial character documentation (POHL 2000).

COOPER (1938) sectioned first instar larvae of *Corioxenos antestiae* Blair, 1936 (Corioxenidae) and BAUMERT (1958) first instar larvae of *Elenchus tenuicornis* (Kirby, 1815) (Elenchidae). SILVESTRI (1941a,b) investigated the larval anatomy of *Halictophagus tettigometrae* Silvestri, 1934 (Halictophagidae) and *Eoxenos laboulbenei* Peyerimhoff, 1919 (Mengenillidae). ROHNSTEIN (1953) examined the tracheal system of the first instar larvae of *Stylops ovinae* Noskiewicz & Poluszyński, 1928 (Stylopidae) making the tracheae visible by treating the specimens with glycerin gelatin. A comparative study of *S. ovinae* and other first instar larvae of the genus from different hosts was carried out by BORCHERT (1963), who also presented a reconstruction of the head anatomy. By using chloralphenol he improved the visibility of cuticular elements.

POHL'S (2000) comprehensive study of first instar larvae was mainly focused on external features, mostly using scanning electron microscopy, whereas OSSWALD et al. (2010) treated the thorax and its muscle system based on microtome sections and presented the first detailed 3D-reconstruction. The internal structures of the extremely small head were addressed by POHL (2000). However, mainly due to the extreme degree of miniaturization and the strong sclerotization of the cephalic skeleton, a detailed reconstruction was not possible with the techniques available by that time. It turned out as impossible to obtain microtome sections of good quality of the larval head.

The serial-block face scanning electron microscopy (SBFSEM) was developed by DENK & HORSTMANN (2004) for 3D-reconstructions of nervous tissue with ultrastructural resolution. The value of this technique in other areas of biology was recognized early. It was applied in insect morphology by HÖRNSCHEMEYER et al. (2012) and appeared promising for a new detailed investigation of the cephalic anatomy of first instar larvae of Strepsiptera. The species *S. ovinae* was chosen for this study as it is relatively easy to obtain and some data were already available (ROHNSTEIN 1953; BORCHERT 1963; SCHNEIDERIT 1986; POHL 2000).

One purpose of the present study was to evaluate the capacity of the new technique to obtain data for an unusually challenging object, i.e. an extremely small, strongly sclerotized and wedge-shaped head. The main aim was the detailed documentation of the cephalic anatomy, with the focus on the musculature, the nervous system, the endoskeleton, and the mouthparts.

## 2. Material and methods

**Material.** First instar larvae of *S. ovinae* were examined, a species parasitizing the bee *Andrena vaga* Panzer, 1799 (Hymenoptera, Apidae) (Fig. 1). Specimens prepared for SBFSEM were obtained from stylopized individuals of *A. vaga* collected on 17.iv.2004 in the surroundings of Prisannewitz, Mecklenburg-Vorpommern, Germany by

H. Pohl. The hosts were kept in glass jars (ca. 0.5 l) with moist sand and covered with gauze until the first instar larvae hatched. Temperatures were ca. 10–15°C during the night and ca. 20°C during 14 h of daylight. The bees were provided with honey dissolved in water. Females of *S. ovinae* were extracted from the abdomen of the bees and the first instar larvae from the brood canal. For scanning electron microscopy (SEM) first instar larvae obtained from stylopized *A. vaga* from the surroundings of Bad Freienwalde, Brandenburg, Germany (16.iii.2012 leg. H. Pohl) were used. They were kept like the other specimens and the first instar larvae were obtained in the same manner.

**Scanning electron microscopy.** First instar larvae were fixed with Dubosq-Brasil (150 ml 80% ethanol + 1 g picric acid, 60 ml formaldehyde + 15 ml acetic acid) and then transferred to 70% ethanol. After dehydration using an ascending series of ethanol (80, 90, 96, 100%) specimens were dried at the critical point in a microporous capsule (Plano GmbH, Wetzlar, Germany) with an EMITECH K850 (Sample preparation division, Quorum Technologies Ltd., Ashford, England). A fine hair glued to a Pasteur pipette was then used to mount the samples on a specimen holder with conductive tape (Plano GmbH, Wetzlar, Deutschland). After sputter coating with gold (EMITECH K500, Sample preparation division, Quorum Technologies Ltd., Ashford, England) images were taken with a Philips ESEM XL 30 (Philips, Amsterdam, Netherlands) equipped with Scandium Five-Software (Olympus, Münster, Germany). To improve the depth of field in some cases image stacks were produced and processed with Helicon Focus 4.2.7 software (HeliconSoft, Kharkov, Ukraine).

**Serial block-face scanning electron microscopy.** To facilitate infiltration, specimens were cut transversally into two halves under a stereomicroscope using a razor blade in a drop of KARNOVSKY fixative. Samples were fixed for 24 h at 4°C in in KARNOVSKY (1965) primary fixative modified after MÜLLER et al. (2009). They were postfixed for 1 h with 1% osmium tetroxide in 0.1 M, pH 7.3 phosphate buffer at 4°C. After rinsing with distilled water and dehydration via an ascending series of ethanol the larvae were transferred to a mixture of 50% Araldite and acetone, kept in this substrate overnight in an open jar, and then transferred to pure Araldite CY 212 (Agar Scientific, Stansted/Essex, England) poured in silicon rubber forms. Prior to sectioning the Araldite block containing the specimen was sputter-coated with gold (Balzers SCD 030 Sputter coater) to reduce image distortions from charging effects. Serial sectioning (80 nm) was carried out with a FEI Quanta 250 FEG® scanning electron microscope equipped with a Gatan 3View® ultramicrotome in its chamber (Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Göttingen). Images were acquired at low vacuum with 40 Pa chamber pressure, an acceleration voltage of 2 kV, spot size 4 and at 1939 × magnification with 2048 × 2048 (resolution



**Fig. 1.** *Stylops ovinae*, first instar larva, head blue, SEM-micrograph, lateral view.

0.035 × 0.035 μm per pixel) and 3072 × 3072 (resolution 0.023 × 0.023 μm per pixel) pixels and a dwell time of the electron beam of 2 × 4 μs per pixel. Images were constructed from backscattered electrons detected with the Gatan BE-detector delivered with the 3View setup. Images of sections were saved as tiff-files from the Gatan 3View control software. Prior to 3D-reconstruction images were converted to 8 bit grey scale and smoothed with a Gaussian blur filter (radius 0.7) to reduce noise.

**3D-Reconstruction.** The 3D models are based on the SEM micrographs obtained with SBFSEM. AMIRA 5.3.1 (Visage Imaging GmbH, Berlin, Deutschland) was used for segmentation and VGStudio MAX 2.0.5 (Volume Graphics, Heidelberg, Deutschland) for 3D visualization.

**Processing of images.** Figure plates were produced using Adobe Photoshop CS5 and Adobe Illustrator CS5.

**Terminology and nomenclature.** The terminology of POHL (2000) is used for the setae, the nomenclature of WIPFLER et al. (2011) for the cephalic muscles.

### 3. Results

#### 3.1. Head capsule

The head is light brown and very strongly sclerotized, without any exposed membranous regions. It appears flat and broad in frontal view (Fig. 2D) and wedge-shaped in lateral view, with a sharp anterior edge (Fig. 3A). Posterodorsally it is slightly retracted into the prothorax and as wide as the anterior pronotal edge (Fig. 2C). The lateral walls of the head capsule are convex. The dorsal side appears trapezoid with the anterior margin rounded and curved towards the ventral side (Fig. 2C,D). The ventral surface is formed by a flat and undivided ventral plate

(“Ventralplatte”; BORCHERT 1963) (Fig. 2A,B). The lateral margins are more strongly rounded than the anterior edge, which bears a small lip-shaped structure (**li** in Fig. 2A,C,D). The caudal basal margin is nearly straight with the exception of the strongly rounded lateral edges. Only one cephalic suture is visible on the anterodorsal region (Figs. 2C,D, 3A), but a notch is present at the anterior margin of the head (**fpc** in Fig. 2A,D). The apical part of this incision appears like a channel connected to the posterior opening of the head, which is recognizable on SEM images like a more or less rounded or ellipsoid concavity shaped like an inverted U (Fig. 2A). In most cases it is more or less completely covered by a small plate formed by the labium (**lb** in Fig. 3B). On some SEM images the opening is visible as a broad fissure anteriorly (**vpc** in Fig. 2B). It is very clearly exposed in the 3D-reconstruction of a specimen with retracted labium (Fig. 7B). The anterior part of the ventral plate is formed by the medially fused maxillae and the labium. The cuticle is folded inwards at the region where the lateral margin of the head and the ventral surface are connected, thus forming a fissure distinctly separating the ventral plate from the adjacent parts of the head (Figs. 2A,D, 3A). This also results in the formation of the caudolateral part of the ventrally curved lateral edges of the head capsule. All cephalic elements are very closely connected with scarcely recognizable borders between them. Two small and semicircular flattened areas are recognizable at the end of the anterior 1/3 of the ventral plate (Fig. 2A,D). A distinct fissure is visible at their base and directly caudad a short thorn-shaped seta inserted in a concavity (**ms** in Figs. 2A, 3A). Posteromedially the ventral plate reaches slightly beyond the anteroventral margin of the prothorax. In the anterior cephalic region the cranial edge of the plate forms the lower edge of the fissure-shaped frontal opening of the preoral cavity, which is dorsally limited by the anterior edge of the dorsal head capsule (**fpc** in Fig. 2A,B). The adjacent dorsoventrally flattened anterior preoral cavity contains the mandibles. Posteriorly the preoral cavity (buccal cavity) is almost round in cross section and has a reinforced cuticular wall. The preoral cavity connects

the frontal opening with the ventral opening of the head (Fig. 6A). In its structure its cuticle resembles that of the pharyngeal skeleton or the external exocuticle. The ventrocaudal margin of the buccal cavity is connected with the labium by a slightly chitinized semimembranous area which appears folded in in the 3D-reconstruction. The endoskeleton is distinctly reduced. The mandibular base and the anterior part of the tentorial bar are enclosed by a chitinous meshwork. The internal part of the stemmata is supported by a ridge. It is about as wide as the stemmata and originates close to the ventral invagination of the lateral walls of the head capsule (**ri** in Fig. 10A,B, 11).

### 3.2. Cuticle

The endocuticle is thicker than the exocuticle in most areas (Figs. 4–6). The apical part of the anterior region is mainly formed by endocuticle (**en** in Fig. 6). Endocuticle is apparently missing in the posterior half of the dorsal side of the head capsule. Instead the epidermis is very thick in this region (Fig. 4G,H). Exceptions are the areas of origin of muscles where the epidermis is probably missing or too thin to distinguish with SBFSEM. The muscles are apparently directly attached to the exocuticle (Figs. 5, 6).

### 3.3. Epidermis

Epidermis cells, nerves, fat body cells and nuclei of muscles are hardly clearly distinguished in the sections, especially in the posterior region of the head above the pharynx. The epidermis is apparently missing in the lateral region of the anterior 1/3 of the head (Fig. 4C–F). Posterior to this area it becomes visible as a flat layer covering the internal surface of the posterior head capsule except for muscle attachment areas (see 2.). Dorsally its thickness increases. At the cervical region it is 4 × as thick as the thin anterior layer.

### 3.4. Tentorium

Paired angled anterior tentorial arms are present laterad the buccal cavity, and the pharyngeal skeleton (“laterale Chitinbalken”; ROHNSTEIN 1953) (**te** in Figs. 4, 5). The anterior part is angled median and flattened. The slightly longer posterior section is approximately parallel to the pharyngeal skeleton. The mandibles articulate on the anterior part (Fig. 4E), whereas the posterior part serves as attachment area for the muscle of the pharyngeal skeleton (m3) (Figs. 4H, 5A,B). In cross sections the posterior part is round almost along its entire length (Figs. 4, 5). In the specimen examined it bifurcates on one side, whereas it is undivided and on the other (Fig. 4A). The tentorial bars are largely embedded in the endocuticle. A connection to the exocuticle is only recognizable in the anteriormost region.

### 3.5. Labrum

A labrum is absent but a narrow lip-like structure is formed at the anterior margin of the dorsal wall of the head capsule (**li** in Fig. 2). It overtops the anterior edge of the ventral head surface when the apical opening of the head is closed. In dorsal and frontal view this structure appears medially interrupted. A bulge is present posterior to it on both sides (Fig. 2A,C). Posteriorly it is delimited by a medially interrupted suture (Fig. 2CD).

**Musculature:** absent.

### 3.6. Antenna

The antenna is completely reduced. Neither external nor internal vestiges are recognizable.

**Musculature:** absent.

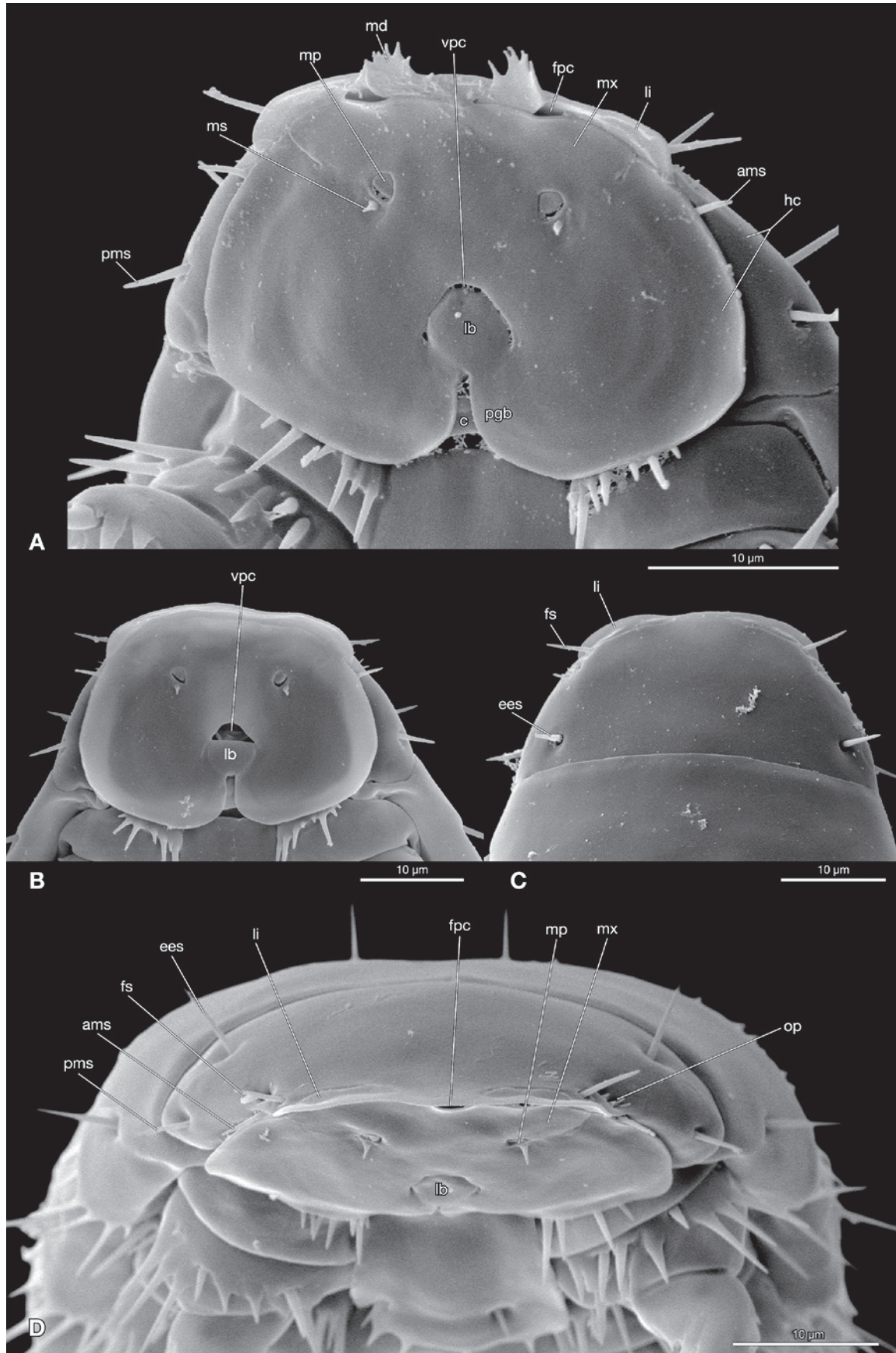
### 3.7. Mandibles

The mandibles, the only functional mouthparts, are inserted in the preoral cavity and articulate with the basal part of the tentorial bars. The basal part is broad and concave. Its ventral joint is enclosed by the tentorial bar in a tongue-and-groove manner (Fig. 4E). The secondary mandibular joint is absent. The elongated distal mandibular part is strongly flattened. Both mandibles are curved ventrad along their mesal edges. The apical part appears obliquely truncated. Its external edge bears 6–12 teeth (**in** in Fig. 3), whereas the mesal edge is rounded. Several rounded teeth are present along the margin of a large mesal extension close to the mandibular base (**ml** in Fig. 3C). Distad this molar part the mandible is abruptly narrowed but again widens towards the apex. In their resting position the distal parts of mandibles intersect in the median line. The apical part can protrude far through the frontal opening of the preoral cavity (Fig. 3B,C) but in the resting position they are completely enclosed in the preoral cavity (Fig. 2B–D). The apparent torsion of the mandibles on SEM micrographs (Fig. 3C) is an artifact.

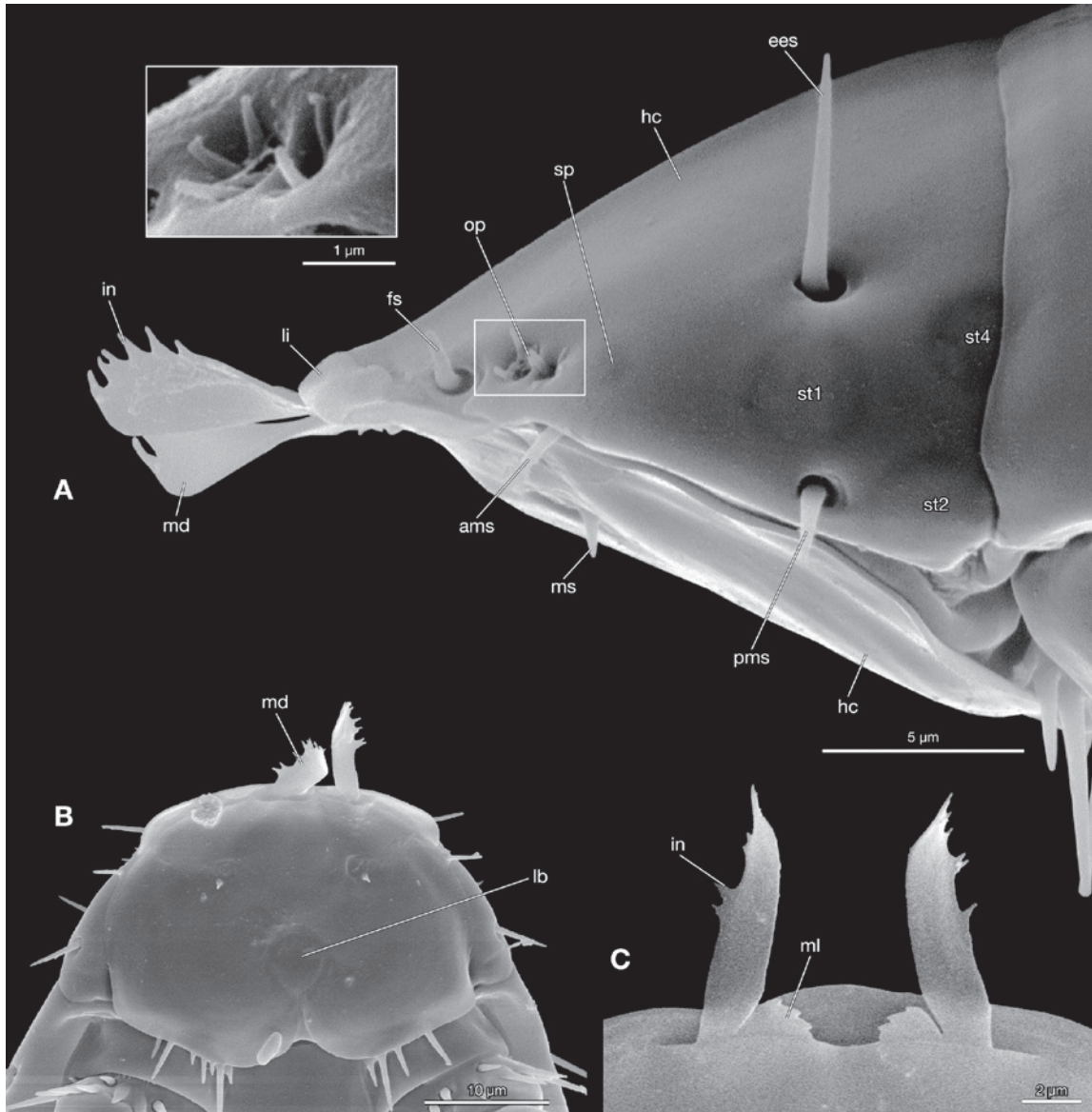
**Musculature:** Both mandibular muscles are large and about equally sized (Fig. 8C,D). Muscle m1 originates at the posterodorsal margin of the head capsule (Figs. 4, 5) and inserts posterodorsally on the mandibular base with a tendon. Muscle m2 originates posterolaterally on the head capsule and inserts on the dorsal region of the mandibular base. The nuclei of both muscles are contained in a ventromesal lobe-like extension (**rn** in Fig. 8D), which reaches into the prothorax posteriorly (Fig. 8C,D).

### 3.8. Maxilla

The maxillae do not function as mouthparts but form the anterior region of the ventral plate (**mx** in Fig. 2A,D). The primarily paired elements are medially fused without



**Fig. 2.** *Stylops ovinae*, first instar larva, head, SEM-micrographs. **A:** slightly oblique ventral view; **B:** ventral view; **C:** dorsal view; **D:** frontal view. — **Abbreviations:** ams – anterior marginal seta, c – cervix, ees – external eye seta, fpc – frontal opening of preoral cavity, fs – frontal seta, hc – head capsule, lb – labium, li – lip-like structure, md – mandible, mp – maxillary palp, ms – maxillary seta, mx – maxilla, op – olfactory pit, pgb – postgenal bridge, pms – posterior marginal seta, vpc – ventral opening of preoral cavity.



**Fig. 3.** *Stylops ovinae*, first instar larva, head, mandibles protruded, SEM-micrographs. **A:** lateral view, inset olfactory pit enlarged; **B:** ventral view; **C:** mandibles, ventral view. — **Abbreviations:** ams – anterior marginal seta, ees – external eye seta, fs – frontal seta, hc – head capsule, in – incisivi, lb – labium, li – lip-like structure, md – mandible, ml – mola, ms – maxillary seta, op – olfactory pit, pms – posterior marginal seta, sp – sensillum placodeum, st1, st2, st4 – corneal lens of stemma 1, 2, 4.

a recognizable trace. Two anterolateral incisions extend from the anterolateral margin of the ventral plate towards the vestigial palps but do not reach them (Fig. 2A). The palps (see ventral plate) are inserted in shallow grooves and shifted into the head lumen to a considerable degree (**mp** in Fig. 2A,D).

**Musculature:** absent.

### 3.9. Labium

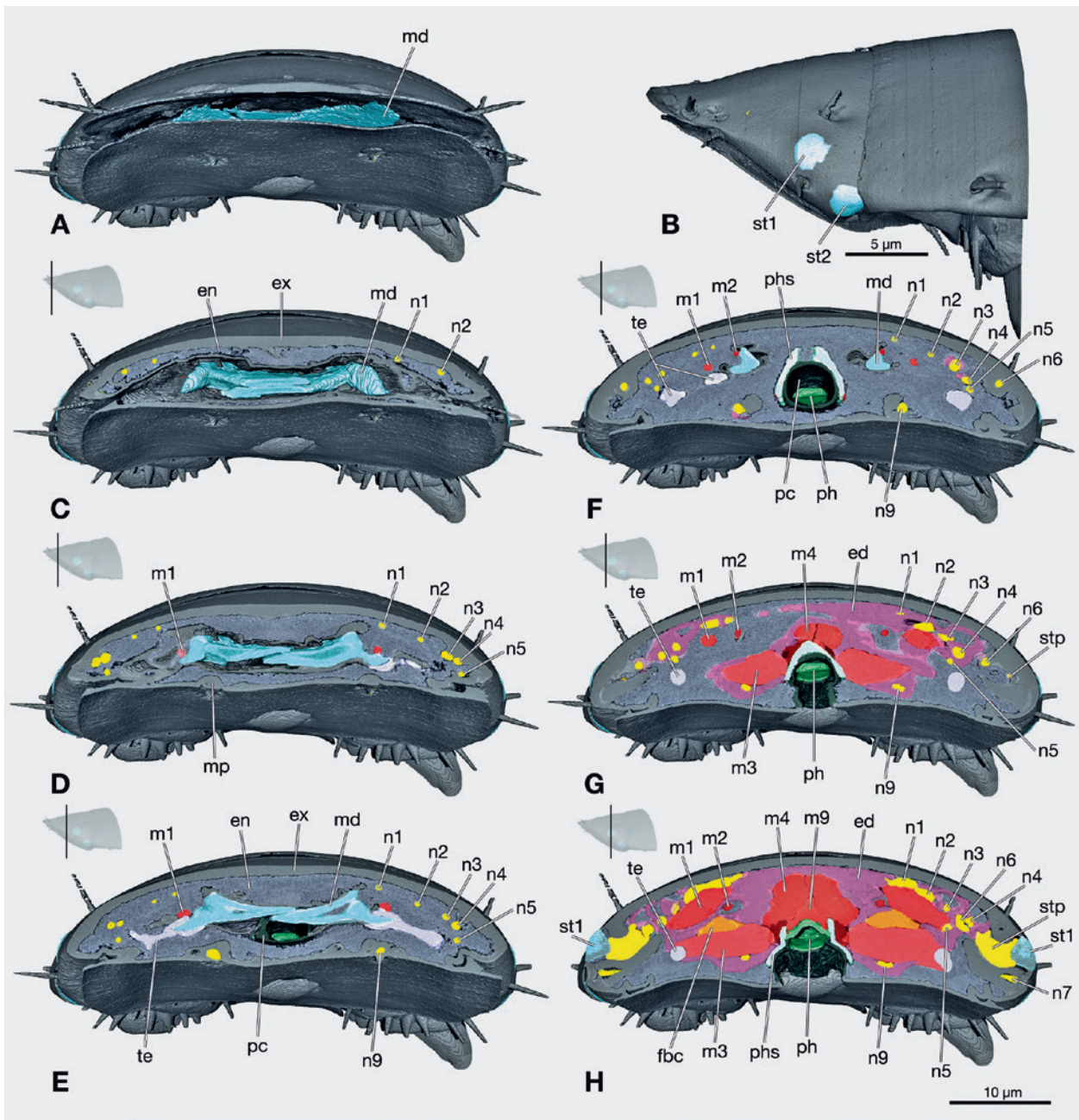
The labium, which is completely fused with the head capsule posteriorly, closes the ventral opening of the preoral cavity (**lb** in Fig. 2). Often, only a narrow fissure is visible. However, it is retracted on some SEM micrographs and in the specimen used for SBFSEM (Figs. 2B,

7B). The labium is completely undivided and palps and endite lobes are missing.

**Musculature:** A paired retractor (m7) inserts on the anterior region of the labium (Figs. 5, 6, 8E,F). It extends along the posteroventral part of the pharyngeal skeleton and originates in the thorax.

### 3.10. Pharyngeal skeleton

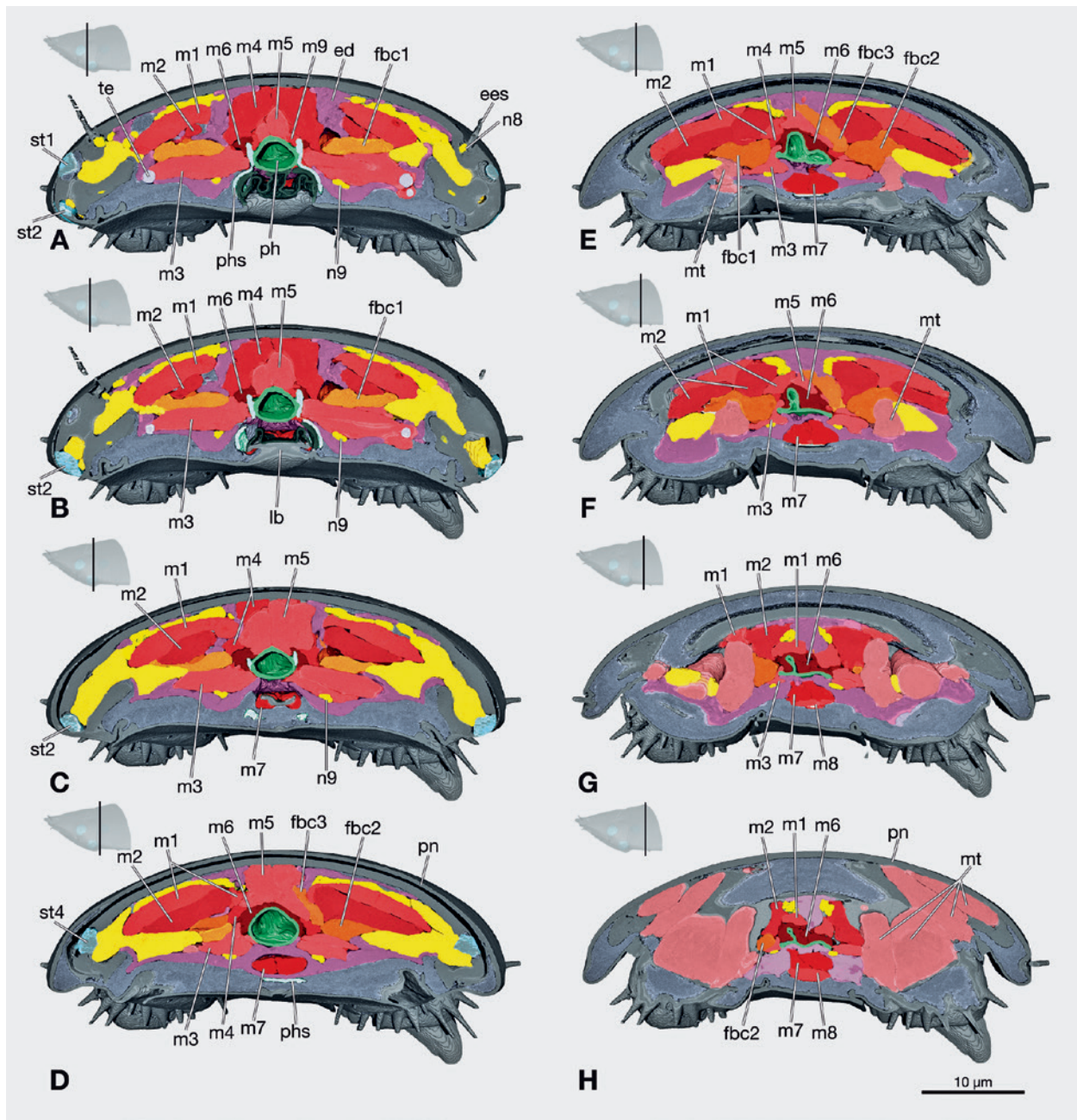
The buccal cavity and the anterior pharynx are enclosed by a complex chitinous structure, the pharyngeal skeleton (“Pharynxspange”; ROHNSTEIN 1953) (**phs** in Figs. 4–9). It lies above the buccal cavity and encloses it dorsally and laterally (Figs. 6, 7D, 8A,B). Its anterior section is tunnel-shaped and the posterior region is widened and



**Fig. 4.** *Stylops ovinae*, first instar larva, head, 3D-reconstructions. **A:** frontal view; **B:** lateral view; **C–H:** Cross-sections from the anterior head capsule to the level of the ventral opening of preoral cavity, position of sections indicated in thumbnail images. — **Abbreviations:** ed – epidermis, en – endocuticle, ex – exocuticle, fbc – fat body cell, m – muscle, md – mandible, mp – maxillary palp, n – nerve, ph – pharynx, phs – pharyngeal skeleton, pc – preoral cavity, st1 – corneal lens of stemma 1, st2 – corneal lens of stemma 2, stp – proximal part of stemmata, te – tentorium.

bulbous (Fig. 8A,B). Two dorsocaudally directed wing-shaped extensions are present at the anatomical mouth region, laterally and directly adjacent with the pharyngeal walls (**phsw** in Fig. 6E). Posterior to the labial region and below the pharynx the lateral walls of the pharyngeal skeleton are medially fused and form a semi-circular structure (Fig. 8B). Posteriorly, the pharyngeal skeleton is extended caudally, thus forming a broad plate-like structure and gradually narrows posteriorly (**phs** in Fig. 5D). It reaches into the prothorax posteriorly, where its terminal region is enclosed by a muscle (Fig. 5F,G).

**Musculature:** The paired dilator m4 connects the anterior tunnel-shaped part with the median region of the dorsal wall of the head capsule (Figs. 6A,B, 9A). The nuclei are contained in lobe-like posterior appendages of the muscle which rest dorsolaterally on the pharynx (**m4** in Fig. 5D). The dilator m3 originates in the second half of the posterior part of the tentorial bar. It inserts on the entire surface of the wing-shaped extensions of the pharyngeal skeleton (Figs. 4, 5, 9). The nuclei of this muscle are shifted posteriorly into lobe-shaped extensions. The unpaired m9 is located at the median margin of the wing-



**Fig. 5.** *Stylops ovinae*, first instar larva, head, 3D-reconstructions. Cross-sections from the posterior region of the preoral cavity to the level of the prothorax, position of sections indicated in thumbnail images. — **Abbreviations:** ed – epidermis, ees – external eye seta, fbc – fat body cell, lb – labium, m – muscle, mt – thoracic muscles, n – nerve, ph – pharynx, phs – pharyngeal skeleton, pn – pronotum, st1, st2, st4 – corneal lens of stemma 1, 2, 4, te – tentorium.

shaped extensions of the pharyngeal skeleton and at the anterior margin of the pharynx. It attaches to the dorsal wall of the head capsule with a narrow offshoot enclosed by the paired m4, anterior to the origin of m4 (Figs. 4, 5, 6A,B, 9A,C). The retractor m8 inserts at the posterior plate-like end of the pharyngeal skeleton and completely encloses its posterior apex (Figs. 5G,H, 9B). It widens strongly posteriorly and extends far into the prothorax. The area of origin could not be identified.

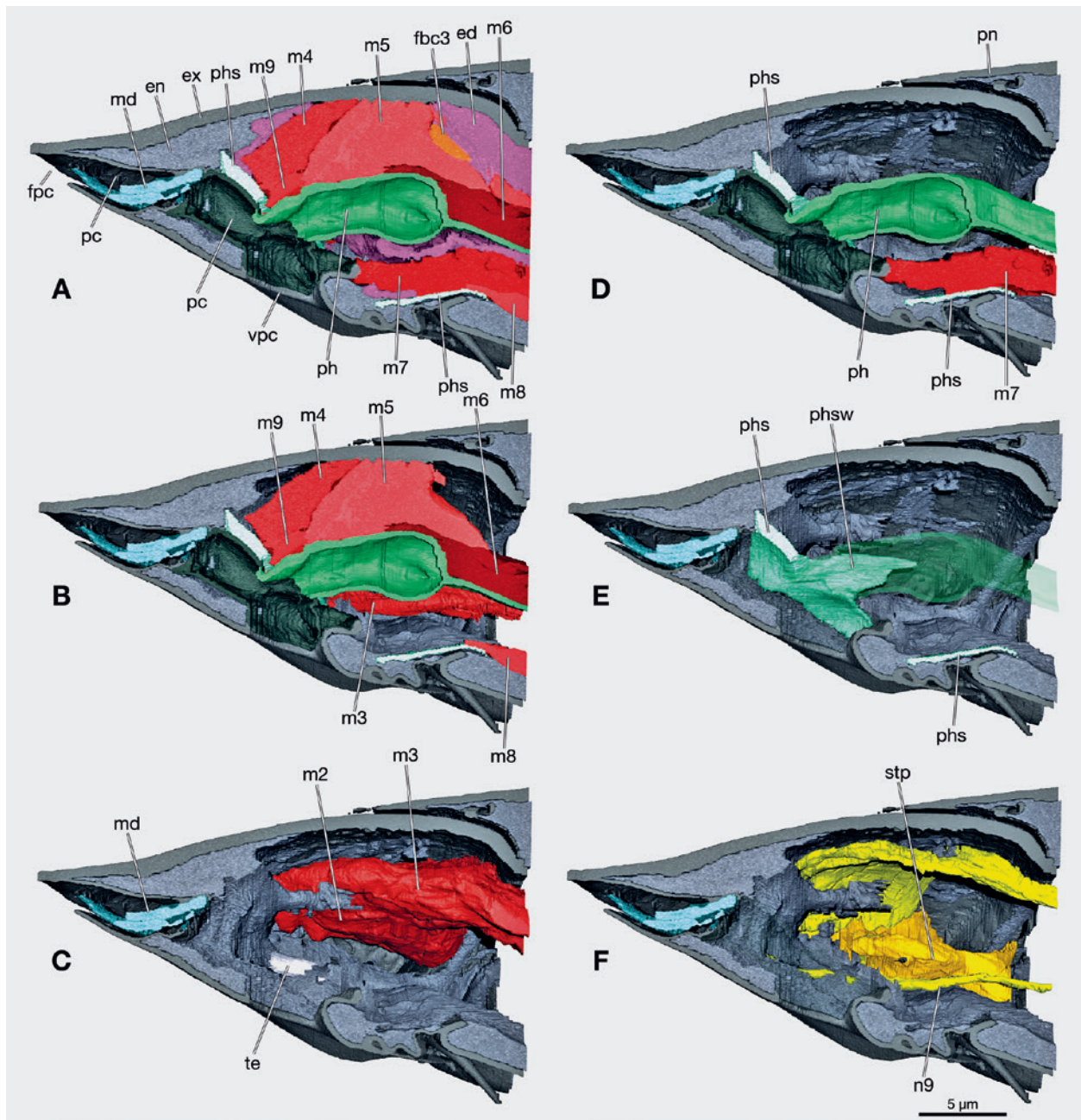
### 3.11. Epipharynx

The epipharynx is represented by the roof of the preoral cavity (Fig. 6A,B). Microtrichia or sensilla are not recognizable.

### 3.12. Hypopharynx

A hypopharynx is not present as a recognizable defined structure.





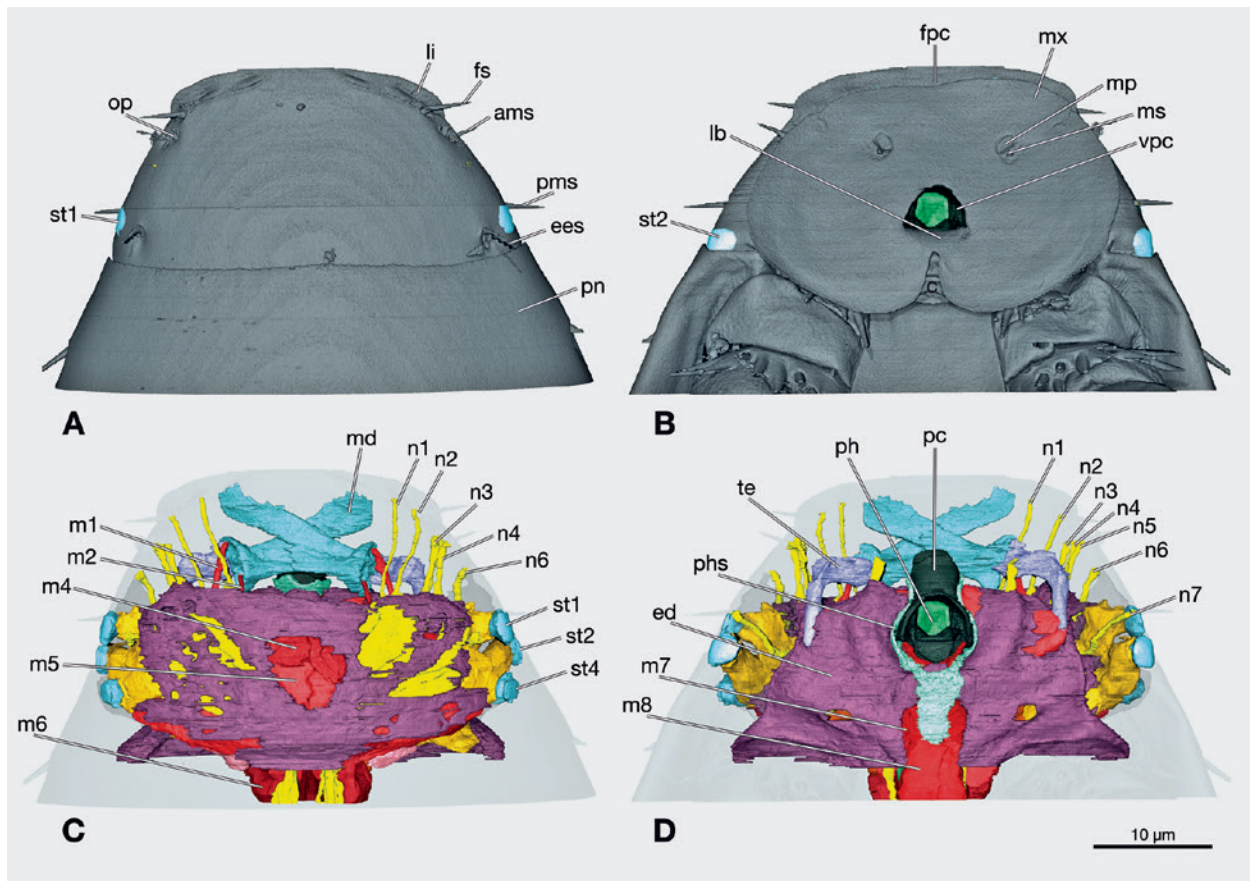
**Fig. 6.** *Stylops ovinae*, first instar larva, head, 3D-reconstructions, mediosagittal view. **A:** complete anatomy; **B:** pharyngeal musculature; **C:** mandibular musculature; **D:** labial musculature; **E:** pharyngeal skeleton; **F:** nervous system and stemmata. — **Abbreviations:** ed – epidermis, en – endocuticle, ex – exocuticle, fbc – fat body cell, fpc – frontal opening of preoral cavity, m – muscle, md – mandible, n – nerve, pc – preoral cavity, ph – pharynx, pbs – pharyngeal skeleton, pbsw – wing-shaped extension of pharyngeal skeleton, pn – pronotum, stp – proximal part of stemmata, te – tentorium, vpc – ventral opening of preoral cavity.

### 3.13. Pharynx

The pharynx is dorsally connected to the buccal cavity, directly posterior to the roof-like section of the pharyngeal skeleton. Its anterodorsal margin forms a lip-like projection extending into the buccal cavity. Its anterior section ascends slightly (**ph** in Figs. 5, 6A,B,D). This part is almost circular in cross section and largely filled with secretions. After reaching its highest point the pharynx is strongly flattened with the ventral and dorsal walls touch-

ing each other medially (Fig. 6A). The pharyngeal lumen is restricted to the lateral areas and obliterates completely in the posterior region, where the pharynx slightly descends towards the prothorax.

**Musculature:** The origin of m5 on the dorsal wall of the head capsule lies directly posterior to that of the similarly shaped m4 (Figs. 6A,B, 9A). However, unlike m4 this dilator is not inserted on the pharyngeal skeleton but between its wing-shaped extensions directly on the dorsal pharyngeal wall. Its nuclei are also distinctly shifted pos-



**Fig. 7.** *Stylops ovinae*, first instar larva, head, 3D-reconstructions. **A:** dorsal view; **B:** ventral view; **C:** complete anatomy, cuticle transparent, dorsal view; **D:** complete anatomy, cuticle transparent, ventral view. — **Abbreviations:** ams – anterior marginal seta, ed – epidermis, ees – external eye seta, fpc – frontal opening of preoral cavity, fs – frontal seta, lb – labium, li – lip-like structure, m – muscle, md – mandible, mp – maxillary palp, ms – maxillary seta, mx – maxilla, n – nerve, op – olfactory pit, pc – preoral cavity, ph – pharynx, phs – pharyngeal skeleton, pms – posterior marginal seta, pn – pronotum, st1, st2, st4 – corneal lens of stemma 1, 2, 4, te – tentorium, vpc – ventral opening of preoral cavity.

teriorly. Another paired muscle, m6, rests on the dorsal side of the pharynx along its entire length (Figs. 6A,B, 9A,C). It inserts anteriorly on the lateral dorsal margin of the pharyngeal skeleton. This muscle encloses the point of insertion of m5 and reaches into the prothorax with its proximal part.

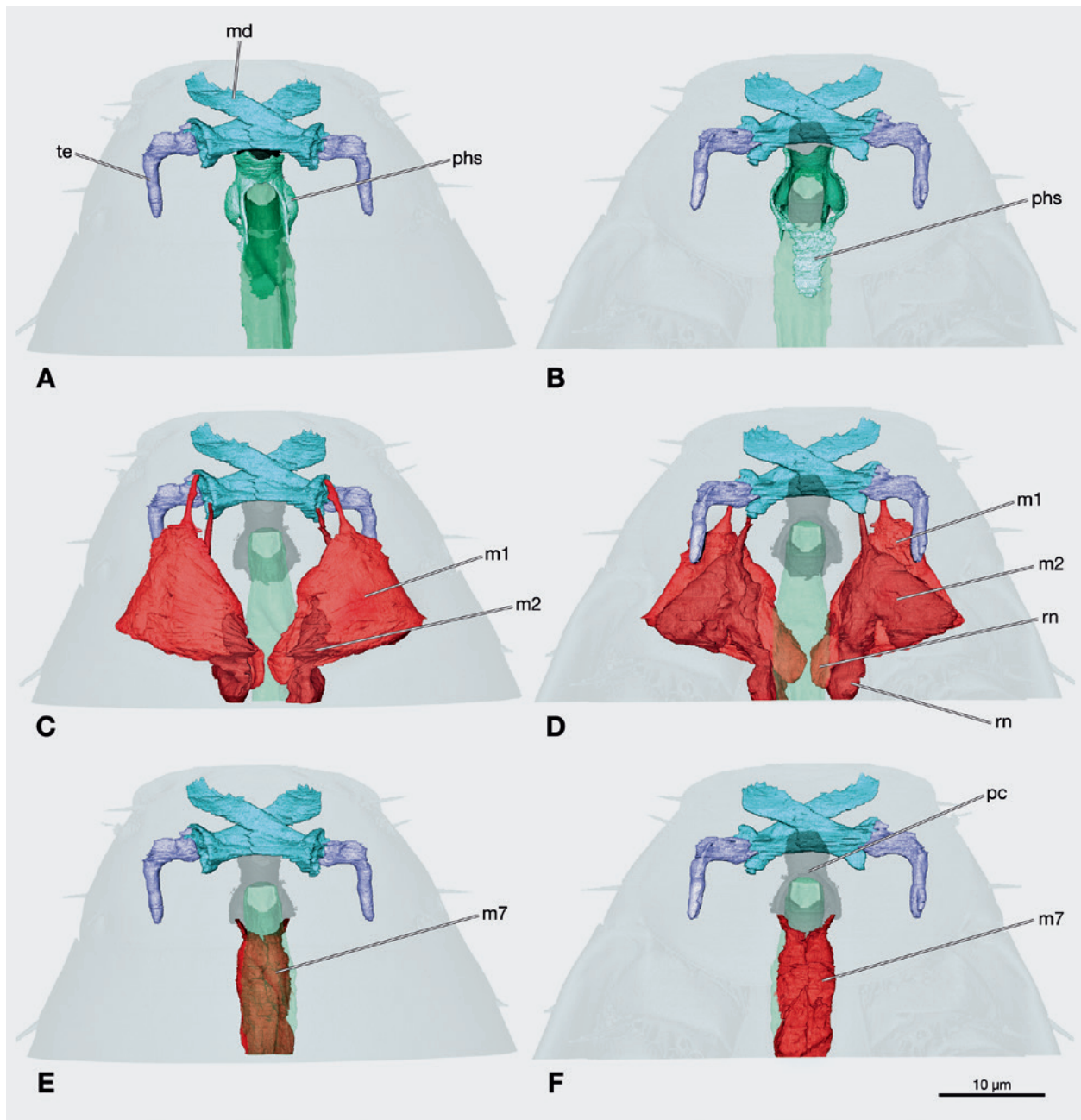
### 3.13. Nervous system

The brain and the suboesophageal ganglion are shifted to the middle region of the postcephalic body. Mainly nerves associated with sensory organs enter the head. All except one extend along the dorsal wall of the head capsule as a compact bundle dorsolaterad the pharynx (Fig. 10A,B). Approximately at the level of the caudal margin of the plate-like structure of the pharyngeal skeleton the bundle bends laterad and the nerves disperse in a fan-like manner. The peripheral network of nerves lies directly below the dorsal wall of the head capsule and is largely embedded in the epidermis, which impedes the identification of individual nerves (Figs. 4, 5).

**Table 1.** Nerves and effectors of the head of first instar larva of *Stylops ovinae* (optical nerves omitted).

Nerves	Effectors
n1	lip-like structure
n2	anterior frontal seta
n3	anterior olfactory pit
n4	posterior olfactory pit
n5	anterior marginal seta
n6	sensillum placodeum
n7	posterior marginal seta
n8	external eye seta
n9	maxillary seta

The optical nerves of the stemmata are the rearmost nerves of the fan-shaped complex, followed by the nerve (n8) of the external eye seta. However, individual nerves of the stemmata are hardly recognizable (**on** in Fig. 10). All other nerves except the anteriormost n1 and n2 then turn towards their sensory organs along the external body

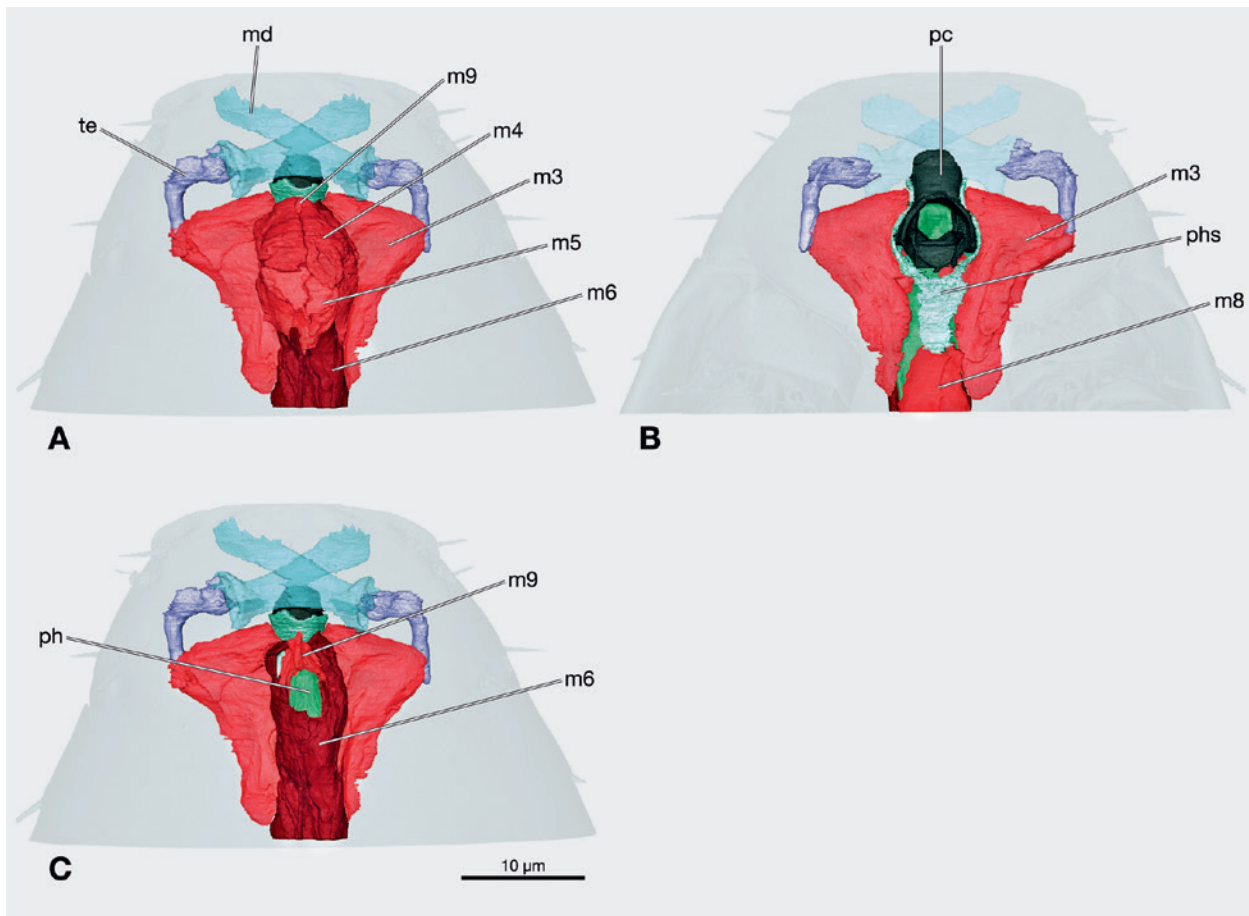


**Fig. 8.** *Stylops ovinae*, first instar larva, head, endoskeleton and musculature of mandibles and labium, 3D-reconstructions, cuticle transparent. **A:** endoskeleton, dorsal view; **B:** endoskeleton, ventral view; **C:** mandibular musculature, dorsal view; **D:** mandibular musculature, ventral view; **E:** labial musculature, dorsal view; **F:** labial musculature, ventral view. — **Abbreviations:** m – muscle, md – mandible, pc – preoral cavity, phs – pharyngeal skeleton, rn – region containing the nuclei, te – tentorium.

wall (Table 1). Nerve n6 innervates the sensillum placodeum located caudally of the olfactory pit. Nerves n3 and n4 innervate the olfactory pit. Very close to them n5 runs to the anterior marginal seta. The two nerves n1 and n2 innervate the lip-like structure and the frontal seta, respectively. A part of n7, which innervates the posterior marginal seta, lies within the mass of nerve and pigment cells of the stemmata. It emerges from it dorsad the posterior end of the tentorial arms and forms a loop before it reaches the base of the seta. In contrast to the other nerves n9 runs laterally below the pharynx after emerging from the prothorax (Figs. 4, 5). It innervates the maxillary seta.

### 3.14. Stemmata

The large stemmata are conspicuous due to the voluminous assemblage of subcuticular pigment. They occupy a large part of the lateral region of the dorsal part of the head capsule. A large mass of pigment cells and nervous tissue is present ventrolaterad the fan-shaped complex of nerves (Fig. 10A,B). It is ventrally supported by the cuticular ridge of the lateral wall of the head capsule (**ri** in Fig. 10A,B, 11C). The individual stemmata are distinctly recognizable within this complex. The anterior stemma (POHL 2000: 1<sup>st</sup> stemma) is larger than the two



**Fig. 9.** *Stylops ovinae*, first instar larva, head, musculature of pharynx and pharyngeal skeleton, 3D-reconstructions, cuticle transparent. **A:** dorsal view; **B:** ventral view; **C:** dorsal view, m4 and m5 omitted. — **Abbreviations:** m – muscle, md – mandible, pc – preoral cavity, ph – pharynx, phs – pharyngeal skeleton, te – tentorium.

others (**st1** in Fig. 3A). It is enclosed by the nerves of the external eye seta and the posterior marginal seta. It proceeds ventrad approximately in a 45° angle. The posterior two stemmata are covered by the anterior margin of the pronotum in most specimens due to the retraction of the posterior head region. The dorsal stemma (POHL 2000: 4<sup>th</sup> stemma) is directly adjacent to the fan-shaped nerve complex laterally (**st4** in Fig. 3A). Its longitudinal axis is at a right angle to the sagittal plane of the head. The 3<sup>rd</sup> stemma (POHL 2000: 2<sup>nd</sup> stemma) is slightly inclined ventrad (**st2** in Fig. 3A). All three stemmata are equipped with a cornea lens. The external surface is only slightly convex and scarcely distinguishable from the adjacent cuticular surface (Fig. 3A).

### 3.15. Setae

Five pairs of setae are present. The frontal seta is inserted between the lip-like structure and the olfactory pit (**fs** in Fig. 3A). The external eye seta (**eess** in Fig. 3A) is inserted above the first stemma, and the posterior marginal seta below it (**pms** in Fig. 3A). The external ocular seta is longer than the others. The anterior marginal seta (**ams** in Fig. 3A) is inserted in the lateral fissure between the dor-

sal head capsule and the margin of the ventral plate, very close to the olfactory pit. The shortest pair is inserted directly posterior the maxillary palps (**ms** in Figs. 2A, 3A).

### 3.16. Olfactory pit

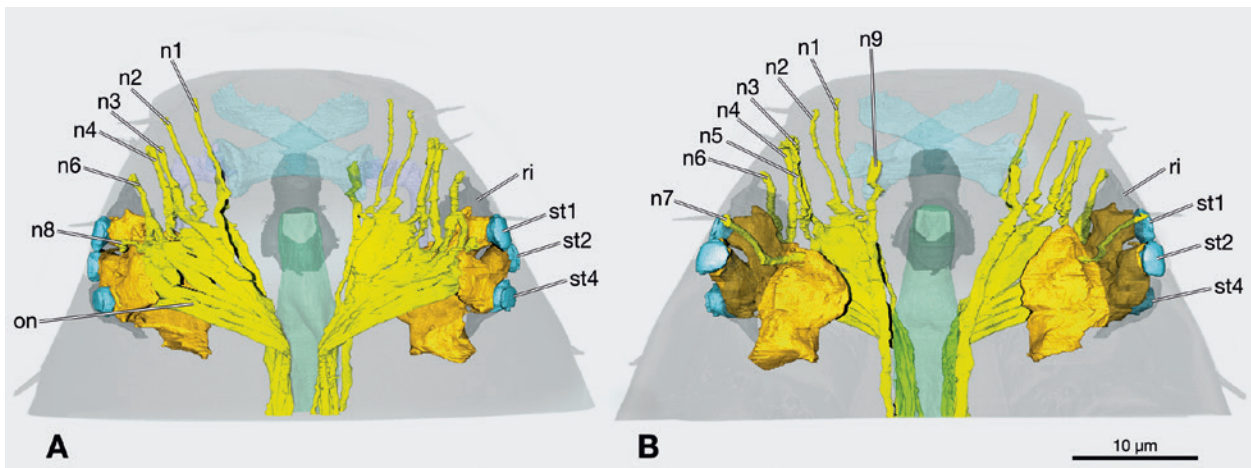
The olfactory pit between the anterior frontal seta and the anterior marginal setae is composed of two grooves on both sides each containing three sensilla (**op** in Figs. 2D, 3A). Each groove is connected with two nerves.

### 3.17. Tracheal system

Tracheae could not be identified in the head and are apparently absent. A transverse tracheal branch is present in the anterior prothorax.

### 3.18. Fat body cells

Five fat body cells are present in the posterior region of the head lateral between the pharynx and the stemmata (Figs. 4, 5). On both sides two are placed above each



**Fig. 10.** *Stylops ovinae*, first instar larva, head, 3D-reconstructions, cuticle transparent. **A:** nervous system and stemmata, dorsal view; **B:** nervous system and stemmata, ventral view. — **Abbreviations:** n – nerve, on – optical nerve, ri – ridge, st1, st2, st4 – corneal lens of stemma 1, 2, 4.

other. The upper ones are smaller and slightly shifted posterad. These cells are enclosed by m3 ventrally, and dorsally and anteriorly by the mandibular muscles. The unpaired fat body cell is enclosed by epidermal cells and lies above the paired cells on the left body half.

## 4. Discussion

### 4.1. Objects and techniques

The investigation of the anatomy of strepsipteran first instar larvae is greatly impeded by the extremely small size (average body length ca. 230 µm; POHL 2002) and the strong sclerotization (SIEBOLD 1843; NASSONOV 1910; POHL 2000). Additionally, the penetration of fixative is impeded by the specific condition of the body surface, the cuticle, and the body openings and the resulting extreme resistance of the larvae against chemicals. First instar larvae of *S. nevinsoni* Perkins, 1918 (named *S. melittae* ex *Andrena fulva*, following the species concept proposed by KINZELBACH 1978) survived 12 hours in 2.5% glutaraldehyde (POHL 2000). Observations of internal features using light microscopy are possible to a certain degree due to the transparency of the cuticle. However, most structures remained obscure due to lacking suitable staining techniques (BORCHERT 1963). Exceptions are some cuticular structures, the pharyngeal skeleton, the tentorium, and some muscles (ROHNSTEIN 1953; BORCHERT 1963; POHL 2000). However, the spatial arrangement of the visible internal structures remained largely unclear.

Several earlier attempts to reconstruct the cephalic anatomy based on light microscopy or traditional microtome sections were only partly successful (HOFFMANN 1913, 1914; COOPER 1938; SILVESTRI 1941a,b; ROHNSTEIN 1953; BAUMERT 1958; BORCHERT 1963). POHL (2000) examined *M. chobauti* and *S. nevinsoni* based on transverse

and longitudinal sections. Fixation and penetration problems were avoided by cutting the larvae in half. Nevertheless, unlike in the study of OSSWALD et al. (2010) on the distinctly larger thorax, a complete reconstruction of internal structures was not possible.

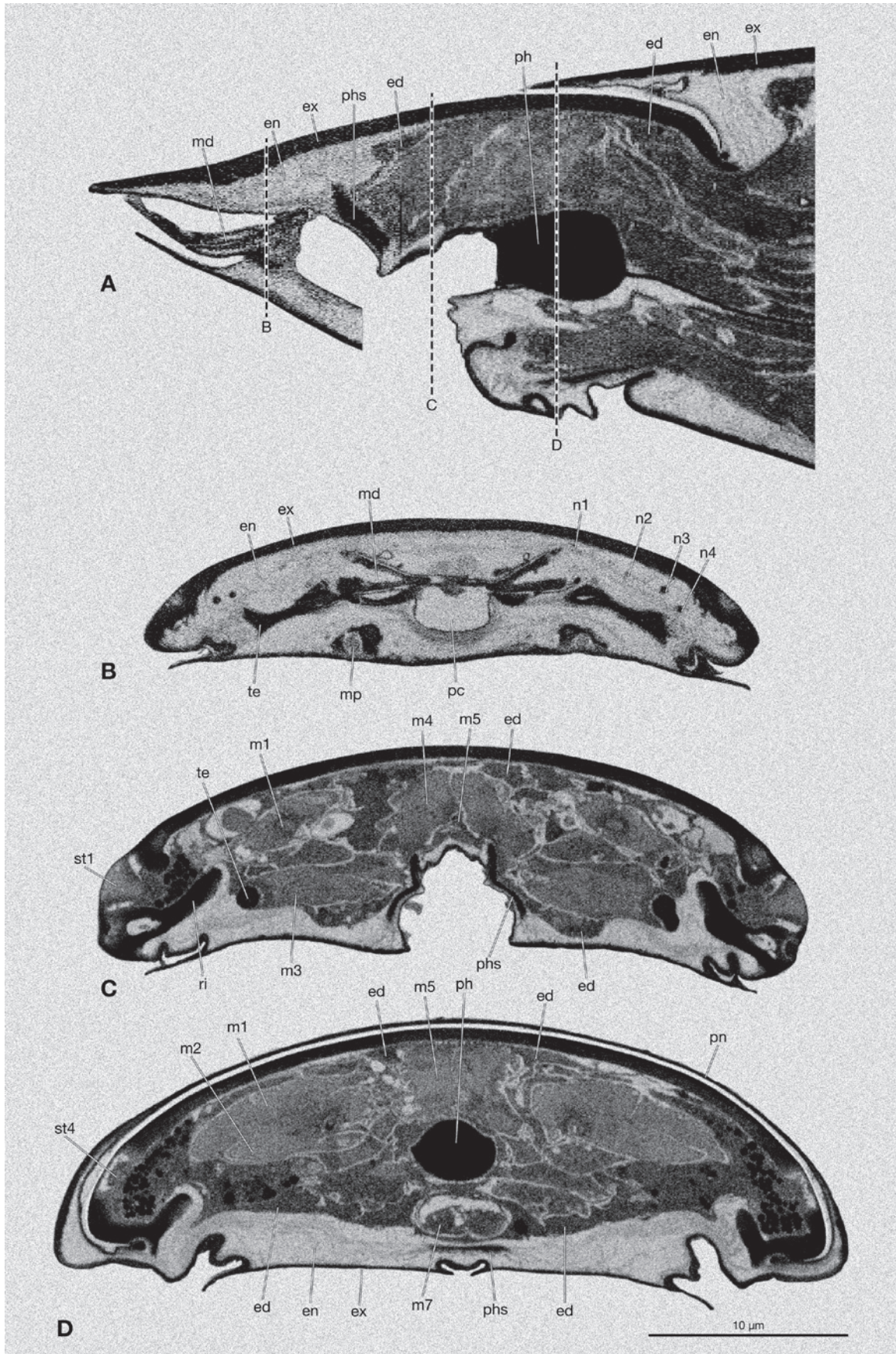
The SBFSEM technique made it possible for the first time to reconstruct the cephalic anatomy of a strepsipteran first instar larva. Using a fixation with Karnovsky followed by contrasting with osmium tetroxide and SBFSEM a complete series of micrographs of sections of the head could be obtained (Fig. 11). With this data set it was possible to reconstruct the head anatomy, partly on the cellular level. The results surpassed previous attempts by far, with all endoskeletal elements, the cephalic foregut, and the entire cephalic musculature and nervous system clearly documented.

### 4.2. Homology of head structures

#### 4.2.1. Head capsule and preoral cavity

Our observations of the cephalic surface structures largely confirm earlier observations (NASSONOV 1910; BORCHERT 1963; SCHNEIDERREIT 1986; POHL 2000). The structure superficially resembling a labrum is a neof ormation (POHL & BEUTEL 2005). In contrast to HOFFMANN (1913) who postulated the presence of a true labrum in *Xenos*, new embryological investigations clearly suggest that this structure is absent (FRAULOB et al. 2015). Consequently, N1 is not the labral nerve, but connected to the apical margin of the head capsule. The absence of the labrum implies that the medially interrupted anterodorsal suture is not homologous with the transverse clypeo-labral suture (POHL 2000).

The presence of a preoral cavity was already postulated by NASSONOV (1910) and its formation was described by HOFFMANN (1913). This structural complex comprises the anterior flat cavity containing the mandibles and the



**Fig. 11.** *Stylops ovinae*, first instar larva, head, serial-block face scanning electron microscopic images. **A:** virtual mediosagittal section (Amira) with section planes; **B–D:** cross-sections (raw images). — **Abbreviations:** ed – epidermis, en – endocuticle, ex – exocuticle, m – muscle, md – mandible, mp – maxillary palp, n – nerve, pc – preoral cavity, ph – pharynx, phs – pharyngeal skeleton, pn – pronotum, ri – ridge, st1, st4 – corneal lens of stemma 1, 4, te – tentorium.

posteriorly adjacent buccal cavity connecting both cephalic openings with the anatomical mouth POHL (2000). The position and extension of the buccal cavity clearly indicate that the ventral opening of the head and the mouth opening are connected, in contrast to BORCHERT (1963). The roof of the preoral cavity is formed by the posterior epipharynx. It can be assumed that the anterior epipharynx was reduced along with the true labrum.

Enclosed by endocuticle an exocuticular structure extends towards the tentorium far into the lumen of the head capsule (**ri** in Figs. 10, 11). It mechanically supports the stemmata on their ventral side but does not serve as muscle attachment area. It is almost certainly not a vestige of the tentorial bridge or the posterior tentorial arms, but very likely a neof ormation that evolved within Strepsiptera.

#### 4.2.2. Ventral plate

Profound transformations take place during the embryogenesis in connection with the formation of the ventral plate. This structure is formed by different elements: the maxillae which fuse with each other medially and posterior with ventral parts of the head capsule, which extend mesad and also fuse in the midline posterior to the labium (HOFFMANN 1913, 1914; FRAULOB et al. 2015). The cardines and stipites of both maxillae fuse medially without a trace of a separating line. They form the ventral, plate-like anterior closure of the preoral cavity, reaching the ventral opening posteriorly (POHL 2000: “Maxillarbrücke”). In contrast to *S. ovinae* a median separating line is recognizable in some other strepsipteran species (POHL 2000). Only two lateral incisions indicate the position of the maxillae within the ventral plate of *Stylops*.

As the anterior part of the ventral plate is formed by the maxillae, the circular flat convexity is equivalent with a greatly reduced maxillary palp. Compared to other species of Strepsiptera it is greatly reduced. In strepsipterans outside of Stylopidae the palp is represented by a cone- or bolt-shaped socket mostly with a strongly developed seta (POHL 2000). The short seta inserted posterior to the maxillary palp is certainly not homologous with the seta emerging from the tip of palp in other strepsipterans (POHL 2000).

Two minute, paired chitinous columns (“Chitinsäulchen”) are described in the literature, located between the anterior region of the pharyngeal skeleton and the tentorial arms, with an origin on the ventral head capsule (ROHNSTEIN 1953; BORCHERT 1963; POHL 2000). BORCHERT (1963) assumed a connection with the mouthparts, with a possible function of the columns as attachment sites of small muscle fibres. Our observations support an interpretation as internalized parts of the maxillary palps as already suggested by POHL (2000).

The labium closes the ventral opening of the preoral cavity. During the embryogenesis its size is greatly reduced in contrast to the maxillae (FRAULOB et al. 2015). The labium functions as a retractable lid closing the ventral opening of the preoral cavity. The paired muscle

m7 retracts the labium into a cavity between the ventral plate and pharynx. In this process the ventral opening is exposed and the buccal cavity becomes accessible as a functional mouth. As the labium is connected with the ventrocaudal margin of the buccal cavity, this space is narrowed ventrally and closes the opening when the retractor is relaxed and the labium assumes its resting position. SCHNEIDERREIT (1986) assumed that the U-shaped fissure in the middle of the ventral plate is the opening of a salivary duct. However, neither ducts nor glands are present within the head or in the postcephalic body.

Labial palps are described for other genera of Strepsiptera but missing in *Stylops* (SCHNEIDERREIT 1986; POHL 2000). A fusion of the hypopharynx with the labium was postulated by POHL (2000), a condition also characteristic for larvae of Coleoptera. Whether the strepsipteran hypopharynx is also involved in the formation of the ventral plate is uncertain, as no discernible trace of this structure is recognizable in the embryonic development of *S. ovinae* (FRAULOB et al. 2015). However, in other species (*Elenchus tenuicornis* and *Eoxenos laboulbenei*) sclerites at the anterior labial margin suggest the presence of hypopharyngeal elements (POHL 2000). They were interpreted as processes of the hypopharyngeal lingual sclerites (POHL 2000) and are completely missing in *S. ovinae*.

#### 4.2.3. Endoskeleton

Two endoskeletal structures can be identified: the remnants of the tentorium and the pharyngeal skeleton. SIEBOLD (1843) referred to the lateral chitinous structures as hook-shaped corneous ridges (“hakenförmige Hornleisten”), whereas the more frequently used term lateral chitinous rods (“laterale Chitinbalken”) was introduced by ROHNSTEIN (1953). BORCHERT (1963) assumed a connection with the mouthparts. POHL (2000) showed that a rigid connection with the external exocuticle is present and interpreted the structure as a strongly reduced tentorium, with the rostral and caudal arms corresponding with the anterior and posterior tentorial arms, respectively. Our results suggest that these structures are homologous with the anterior arms only and that the other tentorial elements are reduced (see also MATSUDA 1965). The arms are also present in other strepsipteran species (POHL 2000).

The presence of a condylar socket at the rostral part of the tentorial arms was already described by BORCHERT (1963). This concavity articulates with the mandibular base and forms a guiding device which contributes to the restriction of mandibular movements to the horizontal plane (POHL 2000). A ball-and-socket articulation which is present in unmodified mandibles is missing.

The pharyngeal skeleton was already described in detail by ROHNSTEIN (1953) and BORCHERT (1963) based on light microscopical observations. BORCHERT (1963) suggested that the structure is formed as a sclerotization of parts of the pharyngeal wall and this interpretation was followed by POHL (2000). In contrast, SILVESTRI (1941a,b) and MATSUDA (1965) homologized the pharyngeal skel-

eton with tentorial elements. The plate-like structure formed by the two converging ventral branches serves as an extensive attachment site for a retractor muscle (m8). Apparently this element and the entire pharyngeal skeleton play an important role in the process of closing the access to the functional mouth. Contraction of the muscles shifts the anterior roof-shaped part backwards and ventrad, thus also lowering the mouth opening towards the ventral head opening. When the muscle relaxes, the roof-shaped part is lifted and thus opens the connection between the apical head opening and the mouth. These processes are likely coordinated with the contraction and relaxation of m7, resulting in the closure or opening of the direct connection between the mouth opening and the ventral opening. The membrane between the buccal cavity and labium is presumably involved in this process. The pharyngeal skeleton also functions as an internal support structure, like the partially reduced tentorium, and as attachment site for most cephalic muscles (m3, m4, m8, m9).

#### 4.2.4. Functional mouthparts

Only the mandibles of *S. ovinae* function as active mouthparts. SIEBOLD (1843) and NASSONOV (1910) already described structures functioning as feeding devices (“Fresswerkzeuge”), but the shape and number was uncertain for a long time. BORCHERT (1963) described two pairs of mouthparts, an anterior pair of larger, knife-shaped elements, and a posterior pair of chisel-shaped structures with a thickened base. It is likely that he interpreted the molar part as an independent pair of mouthparts. As both elements always move together ROHNSTEIN (1953) correctly pointed out that only one pair is present.

The orientation of the mandibles is clearly prognathous, as already suggested by POHL (2000). Their enclosure in the preoral cavity results in a specific type of endognathous condition (FRAULOB et al. 2015). The precise function of the molar cutting edge is unclear. The mandibles move only in a horizontal plane as already observed by BORCHERT (1963) and POHL (2000). The degrees of freedom at the mandibular bases are reduced by the articulation with the tentorium and by the fissure-shaped frontal opening of the preoral cavity (see above).

#### 4.2.5. Cephalic nervous system

The main cephalic elements of the nervous system, i.e. the brain and suboesophageal ganglion, are shifted to the middle region of the postcephalic body. The entire central nervous system is extremely concentrated, forming a single complex (ROHNSTEIN 1953; POHL 2000; BEUTEL et al. 2005). Using SBFSEM the individual nerves entering the head could be documented for the first time, even though the reconstruction of single nerve cells and their precise pathways was only possible in some cases. The borders between nerve cells and epidermal cells are often indistinct. Except for the two branches innervating the setae inserted posterior the maxillary palps, all nerves proceed dorsally directly below the wall of the head cap-

sule, densely packed and with a fan-shaped arrangement. From this complex the individual branches extend to their respective effectors.

A large proportion of the posterior cephalic region is occupied by the proximal cells of the stemmata. This area is distinctly separated from the adjacent tissues, but individual cells cannot be distinguished. The connection between nerves and effectors can be easily recognized. It is surprising that no nerves associated with the pharynx and its muscles could be identified and that the frontal ganglion is absent. It is possible that the lobe-shaped muscular extensions reaching into the prothorax are innervated. However, this interpretation is unconfirmed. An alternative explanation would be that the applied technique or the fixation is insufficient for detecting the nerves in the dense tissues in the neck region.

A 3D-reconstruction of the brain of the first instar larva of *M. chobauti* was presented in an earlier study (BEUTEL et al. 2005). A thick and elongated branch, which also contains pigment grana innervating the stemmata was the only identified nerve extending into the head. Interestingly the optic nerves of *S. ovinae* do not contain pigment grana.

#### 4.2.6. Sense organs and sensory structures

The homologization of setae (e.g., SILVESTRI 1943; SCHNEIDERREIT 1986; POHL 2000) is greatly impeded by the absence of delimited head regions. Even comparisons within Strepsiptera beyond the family level are problematic (POHL 2000). In our study the innervation of setae could be documented for the first time. However, corresponding data for other strepsipteran larvae are still missing.

The olfactory pits are probably an olfactory sense organ but not homologous with the antennal fields of more basal strepsipterans. SCHNEIDERREIT (1986) assumed that it facilitates the orientation in the host’s nest. The porous membranous structure revealed by intensive crystal-violet staining (POHL 2000) is typical for olfactory sense organs (SLIFER 1960). That this structure is derived from two sensilla as discussed by POHL (2000) is supported by the innervation by n3 and n4.

The three stemmata of the first instar larvae of *S. ovinae* were already described by BORCHERT (1963), SCHNEIDERREIT (1986), and POHL (2000). POHL (2000) accepted a terminology suggested by SCHNEIDERREIT (1986) for his homologization among strepsipteran groups and postulated a groundplan condition of six. The largest one corresponds with stemma 4 in the groundplan. A small circular structure (ca. 1 µm) was described by BORCHERT (1963), close to the external ocular seta of *S. ovinae*. He discussed its possible homology with a fourth partly reduced stemma. However, this structure could not be identified in the present study.

#### 4.2.7. Fat body

The fat body in the head of *S. ovinae* consists only of few cells. However, large numbers are only rarely found in



**Table 2.** Terminology and homology of head muscles of the first instar larva of *Stylops ovinae*. The musculature is homologized with muscles described for insect heads (v. KÉLER 1963; WIPFLER et al. 2011). ? = uncertain homology.

Abbrev.	Origin	Insertion	Function	v. KÉLER 1963	WIPFLER et al. 2011
m1	posterolateral head capsule	posteriorly on dorsal margin of the mandibular base	abductor	M. craniomandibularis externus posterior	Omd3
m2	Posterolaterally on head capsule	anteriorly on dorsal margin of the mandibular base	adductor	M. craniomandibularis internus	Omd1
m3	posterior end of tentorium	lateral wing of pharyngeal skeleton	pharyngeal dilator	M. tentoriobuccalis lateralis	Obu4
m4	dorsal head capsule	antero-dorsally on pharyngeal skeleton	pharyngeal dilator	M. frontobuccalis anterior	Obu2
m5	dorsal head capsule	anterior pharynx	pharyngeal dilator	M. frontobuccalis posterior	Obu3
m6	prothorax	anterodorsal pharyngeal skeleton and dorsal pharynx	longitudinal muscle (?), retractor of pharyngeal skeleton	M. longitudinalis stomodaei	Ost2
m7	prothorax	anterior labium	labial retractor	M. tentoriopraementalis	Ola5
m8	prothorax	postero-ventral apex of pharyngeal skeleton	retractor of pharyngeal skeleton	?	
m9	dorsal head capsule (?)	mesal side of wings of the pharyngeal skeleton	constrictor	?	

the head region of holometabolous larvae (e.g., BEUTEL 1993; BEUTEL & GE 2008). The borders between fat body cells and cells of the epidermis are indistinct. Larval fat body cells were only described for three other strepsipteran species, *X. vesparum* ROSSIIUS, 1793 (NASSONOV 1910), *H. tettigometrae* (SILVESTRI 1941b) and *M. chobauti* (POHL 2000). Missing observations may be partly due to inadequate fixation. The use of osmium tetroxide results in a stabilization of lipids in the fat body tissue (POHL 2000).

#### 4.2.8. Musculature

The homologization of the musculature is impeded by the strongly modified cephalic morphology, including reductions and other effects of miniaturization (see below) (Table 2). The origin of several muscles is shifted into the prothorax. Another unusual feature is that the nuclei of mandibular and pharyngeal muscles are placed in lobe-shaped extensions (m3, m4, m5).

**Mandibular muscles.** Based on the obvious function m1 can be easily identified as *M. craniomandibularis externus posterior* (Omd3) and m2 as *craniomandibularis internus* (Omd1). POHL (2000) assumed an origin of both of them on the tentorium. Our results show that this is not the case.

**Pharyngeal muscles.** ROHNSTEIN (1953) and BORCHERT (1963) interpreted the dorsal (m4, m5) and lateral muscles (m3) of the pharyngeal skeleton as pharyngeal dilators, which was later confirmed by POHL (2000). BORCHERT (1963) erroneously assumed that m3 moves the tentorial arms, which then act as levers moving the mandibles outwards. However, the preserved tentorial element is connected with the external cuticle and therefore immobilized. The insertion close to the anatomical mouth suggests that m3 may be homologous with *M. tentoriobuccalis lateralis* (Obu4), but this interpretation remains uncertain.

Muscle m4 is probably homologous to *M. frontobuccalis anterior* (Obu2). The adjacent m5 is similar and the

only muscle directly inserted on the pharynx and not on the pharyngeal skeleton. It is apparently *M. frontobuccalis posterior* (Obu3). The longitudinal muscle m6 is likely involved in the pharyngeal sucking apparatus and at the same time a dorsal retractor of the pharyngeal skeleton. Similarly m8 is likely a retractor of this structure. The origin of both muscles in the thorax is a feature apparently linked to miniaturization (see below). The homologization of m9 is particularly problematic. It probably functions as an antagonist of the dilators. The absence of ventral dilators is likely correlated with the reduction of the posterior tentorium.

**Labial muscle.** The labial muscles of *M. chobauti* originate on the caudal end of the tentorial arms according to POHL (2000). The only labial muscle identified in the present study is the retractor m7, which originates in the thorax. Its precise homologization is not possible due to the shifted origin.

#### 4.2.9. Effects of miniaturization

Effects of miniaturization were described in several recent studies on minute coleopteran larvae (BEUTEL & HAAS 1998: Myxophaga; GREBENNIKOV 2002, POLILOV & BEUTEL 2009: Ptiliidae; GE et al. 2012: Meloidae) and first instar larvae of Strepsiptera (BEUTEL et al. 2005). Miniaturized hexapods have one feature in common, the maximum use of the available space by the internal structures, resulting in a very dense arrangement and often in deformations and also losses (BEUTEL & HAAS 1998). The extremely small size of the first instar larvae (*S. ovinae*: ca. 200 µm) has enormous effects on the spatial arrangement within the head (length ca. 20 µm). Virtually no empty spaces are present between the single internal structures. The only exception is a cavity below the pharynx, which accommodates the retracted labium. Aside from several complete reductions (see 4.4.2.) some structures are shifted to the thorax. The compact complex formed by the brain, subesophageal ganglion and ven-

tral ganglionic chain is located in the meso- and metathorax and in the anterior abdomen (POHL 2000). The brain itself is larger than the entire head (BEUTEL et al. 2005). The nerves identified in the head innervate the olfactory pits, all setae, and stemmata, which are well developed despite of the extremely small size.

The dislocation of the cellular nuclei of several muscles (m1, m2, m3, m5) towards the posterior head region or even the prothorax is apparently also due to the extremely limited space. The origin of m6, m7 and m8 is shifted to the thorax. This is a very unusual feature even among strongly miniaturized insect larvae (e.g., GREBENNIKOV 2002; POLILOV & BEUTEL 2009).

#### 4.2.10. Reductions

Aside from shifts of structures various reductions occurred. Due to the absence of sutures and ridges defined cephalic regions like the clypeus, frons or subgena are missing. The antennae are missing in the first instar larvae. However, HOFFMANN (1913, 1914) and FRAULOB et al. (2015) identifiedanlagen which are completely reduced in the later stages of the embryonic development. The reduction contributes to a streamlined surface of the head and facilitates the penetration of the host's body wall (SCHNEIDERREIT 1986). The loss of the antennae as mechanoreceptors is compensated by innervated setae (SCHNEIDERREIT 1986). However, their number is also distinctly reduced in Strepsiptera, with a tendency to minimize the vestiture within the group. Only five paired setae are present of *S. ovinae*, whereas 12 or 11 pairs are preserved in the basal genera *Eoxenos* and *Mengenilla* (POHL & BEUTEL 2005).

The reduction of the tentorium is likely linked with the strongly increased stability of the cephalic exoskeleton and the far-reaching reduction of muscles normally originating on the tentorium. The musculature of the head is the character system most strongly affected by reductions. About 50 larval cephalic muscles are present in the groundplan of Holometabola (up to 90 in hemimetabolous insects) (WIPFLER et al. 2011). Only nine muscles (eight pairs and one unpaired) are preserved in the first instar larvae of *S. ovinae*, some of them with an origin in the thorax. Muscles of the transformed maxillae are absent and also labral, antennal and hypopharyngeal muscles. Only one muscle originates on the reduced tentorium (m3). Aside from the losses it is likely the muscles consist only of a single cell. In most cases only a single nucleus is present and placed in a lobe-shaped extension (m1, m2, m3, m4, m5). Further reductions occur in the tracheal system, which lacks anastomoses within the head. The fat body in the head comprises only five cells and glands are lacking.

#### 4.2.11. Features related with parasitism

Many features of the first larva are related to the essential function of attacking an insect host. Even though the larvae of *S. ovinae* likely attack the host in the egg stage,

most of the characteristics are maintained. Obvious modifications linked to penetrating a host's body wall are the reduction of prominent external structures such as antennae or elongated palps (SCHNEIDERREIT 1986; POHL 2000). Whether the fusion of the maxillae can be seen in this functional context is unclear.

A very unusual feature is that the entire anterior third of the head is exclusively formed by cuticle (Fig. 6). The solid anterior edge increases the penetrating capacity. Whereas the endocuticle of insects is usually several times thicker than the exocuticle, the ratio of most regions of the first instar larvae is about 1:2. On parts of the dorsal wall of the head capsule the endocuticle is completely absent (Fig. 6A,B). The strong sclerotization and the almost complete absence of exposed membranes and sutures likely also minimize the water loss, enabling the larvae to stay for a longer time exposed on flowers to wait for a suitable host (POHL 2000).

The specific condition of the ventral head opening and ventral plate of *Stylops* are probably linked with their specialized mode of attacking the host. First instar larvae of *Stylops* attack either soft bodied bee larvae or the eggs. The latter possibility appears more likely as the specialized ventral plate and ventral head opening facilitate the attachment to smooth surfaces with a sucking mechanism despite of the complete lack of glands, with the elongated and toothed mandibles functioning as the penetrating device. The direct infection of the eggs is only described for two species, *S. pacifica* Bohart, 1936 (LINSLEY & MAC-SWAIN 1957) and *Pseudoxenos hookeri* (Pierce, 1909) (Xenidae) (KROMBEIN 1967).

### 4.3. Phylogeny

#### 4.3.1. Intraordinal relationships

Strepsiptera are characterized by numerous autapomorphies (e.g., POHL & BEUTEL 2005, 2008), among them many features of the first instar larvae. This includes the sharp anterior edge of the prognathous head, the lack of a free labrum, the greatly or completely reduced antenna, and the pharyngeal skeleton. The postgenal bridge, the reduced maxillary palps, and the ventral plate are autapomorphies of Stylopidae (POHL & BEUTEL 2005). Another complex apomorphy is the partly reduced tentorium, with its specific articulations with the mandibles and pharyngeal skeleton. As this condition is only slightly differing in *M. chobauti* it belongs very likely to the groundplan of the order. Other derived groundplan features are the strongly flattened shape of the mandibles, the shift of the brain and suboesophageal ganglion to the middle body region, and the greatly reduced cephalic muscle system. A muscle between the reduced tentorium and the labium is present in the groundplan (POHL 2000) but missing in *S. ovinae*.

A flattened ventral plate combined with a closing mechanism of the ventral head opening is missing in other strepsipterans. The resulting sucking mechanism is a pos-

sible autapomorphy of *Stylops* (see above). It is an open question whether this specific variation of the parasitism has evolved once or several times within the group.

#### 4.3.2. The systematic position of Strepsiptera

The sistergroup relationship between Strepsiptera and Coleoptera is well established (WIEGMANN et al. 2009; BEUTEL et al. 2011; NIEHUIS et al. 2012; MISOF et al. 2014; PETERS et al. 2014; see also discussion in POHL & BEUTEL 2013). However, potential larval synapomorphies of both orders were rarely discussed so far (e.g., OSSWALD et al. 2010; BEUTEL et al. 2011).

As the monophyly of Coleoptera, Polyphaga and Cucujiformia is clearly confirmed (e.g., LAWRENCE et al. 2011; MCKENNA et al. 2015), similarities with first instar larvae of the specialized Meloidae and Ripiphoridae (e.g., GE et al. 2012) are clearly the result of parallel evolution. Like Strepsiptera both tenebrionoid families are characterized by parasitic habits and hypermetamorphosis, and very small and agile first instar larvae, which function as infectious stage.

A feature assigning Strepsiptera to the clade Aparaglossata (Holometabola excl. Hymenoptera) (PETERS et al. 2014) is the presence of typical stemmata. Prognathism of the larva is a potential apomorphy linking Strepsiptera with Neuropterida and Coleoptera. However, this is very uncertain as this condition also occurs in other groups (e.g., basal lepidopteran lineages, Diptera, Siphonaptera) and it is arguably not a groundplan feature of Coleoptera. Potential autapomorphies of Coleopterida (Strepsiptera + Coleoptera) are the fusion of the hypopharynx with the prelabium and the loss of the salivarium, salivary ducts, and the salivary glands.

## 5. Acknowledgements

We thank Benjamin Wipfler (Institut für Spezielle Zoologie und Evolutionsbiologie, FSU Jena) for his help with the three-dimensional reconstruction and Andy Sombke (Cytologie und Evolutionsbiologie, Zoologisches Institut und Museum, Universität Greifswald) for his help with the fixation of the first instar larvae. TH was supported by a Heisenberg grant of the DFG (German science foundation HO 2306/6-1, 6-2). He also wants to thank Rainer Willmann (Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Universität Göttingen, Germany) for supporting his work.

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