

# The labral gland in termite soldiers

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## **Abstract**

The evolutionary success of termites is driven largely by a complex communication system operated by a rich set of exocrine glands. As many as 20 different exocrine organs are known in termites. While some of these organs are relatively well-known, only anecdotal observations exist for others. One of the exocrine organs that has received negligible attention so far is the labral gland. In this study, we examined the structure and ultrastructure of the labrum in soldiers of 28 termite species. We confirm that the labral gland is present in all termite species, and made up of two secretory regions located on the ventral side of the labrum and the dorso-apical part of the hypopharynx. The labrum of Neoisoptera is endowed with a hyaline tip, which was secondarily lost in Nasutitermitinae, *Microcerotermes*, and species with snapping soldiers. The epithelium of the gland generally consists of class 1 secretory cells, with an addition of class 3 secretory cells in some species. A common feature of the secretory cells is the abundance of smooth endoplasmic reticulum, an organelle known to produce lipidic and often volatile secretions. Our observations suggest that the labral gland is involved in communication rather than defence as previously suggested. Our study is the first to provide a comprehensive picture of the structure of the labral gland in soldiers across all termite taxa.

## Introduction

Termites are an important resource for a range of animals (Redford & Dorea 1984), and they compete for resources with other wood- and soil-feeding taxa (Šobotník et al. 2010a). Termites protect themselves through passive and active defence mechanisms, including a cryptic lifestyle, the construction of defensive structures (Korb 2011), and investments into a caste of special defenders: the soldiers (Haverty 1977). While the primary weapon of termite soldiers is generally their powerful mandibles, glands that produce defensive compounds are of comparable importance (Prestwich 1984, Šobotník et al. 2010a).

Termites use intricate communication systems which complexity is reflected in the development of 20 different signal-producing exocrine organs (Billen & Šobotník 2015). Four glands are found in most termite species: the frontal gland, the sternal gland, the labial gland, and the mandibular gland. The presence of other exocrine organs is restricted to specific termite lineages, or to certain castes. The function of these lineage/caste-specific glands is not fully understood, apart from the defensive function of the crystal glands in *Neocapritermes taracua* workers (Šobotník et al. 2012, 2014, Bourguignon et al. 2016). The labral gland is one of these poorly known exocrine glands, which is only known from the soldier caste of three termite species (Deligne et al. 1981, Quennedey 1984, Šobotník et al. 2010b, Costa-Leonardo & Haifig 2014), and from some imagoes (Křížková et al. 2014).

The labral gland was first discovered on the ventral side of the labrum in *Macrotermes bellicosus* (Deligne et al. 1981) and was later found also on the dorsal side of the hypopharynx in other Macrotermitinae species (Quennedey 1984). The presence of labral glands in other taxa is thought to be indicated by a hyaline tip, located on the tip of the labrum (Deligne et al. 1981). The labral gland of *M. bellicosus* is composed of class 1 secretory cells only (according to the classification by Noirot & Quennedey, 1974), while additional class 3 secretory cells have been found in the labral glands of *Glossotermes oculatus* and *Cornitermes cumulans* soldiers (Šobotník et al. 2010b, Costa-Leonardo & Haifig 2014). The function of the labral gland was not studied in any termite species, and the literature suggests that it produces toxic secretions that impregnate the mandibular edges (Deligne et al. 1981, Quennedey 1984). In this paper, we provide the first comprehensive description of the structure of the labral gland in the soldiers of 28 species, representatives of the termite tree of life.

## Material and Methods

### *Direct observations*

Living termites were observed and photographed using Canon EOS 6D and Canon EOS 5D SR, combined with lenses Canon EF 100mm f/2.8L Macro IS USM and Canon MP-E 65mm f/2.8, and equipped with

the Canon Macro Twin Lite MT-24EX flash. The photos were used to compare the shape of the labrum and the presence of a hyaline tip in termite soldiers.

#### *Optical microscopy and transmission electron microscopy*

Soldier labral glands were studied using three different fixatives: fixative with phosphate buffer (0.2M, pH 7.2 buffer: formaldehyde 10%: glutaraldehyde 8% = 2: 1: 1), cacodylate buffer (0.2M, pH 7.3 buffer: glutaraldehyde 8%: distilled water = 2: 1: 1), and standard Bouin's solution (for detail see Table S1). For electron microscopy (EM), soldier heads were cut off and the mandibles removed in order to facilitate sectioning. The mandibles were left intact in the minor soldiers of *Rhinotermitinae* and *Nasutitermitinae*. Samples were postfixed using 2% osmium tetroxide, and embedded into Spurr resin. The samples were cut into 0.5 µm sections using a Reichert Ultracut ultramicrotome and stained with Azure II for analysis with optical microscopy.

#### *Histology*

The samples were dehydrated using ethanol series, transferred to xylene and embedded in paraffin. Polymerization was carried out in oven at 56-58°C for 2 hours. The samples were cut into 5-10 µm thick sections using microtome Bamed pfm Rotary 3004 M, placed on a slide coated with egg-white/glycerol and stained with Mallory's trichrome stain, and made clear with xylen. For additional details see Table S1.

#### *EM*

We dissected the heads of freshly freeze-killed soldiers, and removed the mandibles, maxillae and labium. The heads were thereafter dehydrated using acetone series. The samples were dried using a critical point method and glued onto an aluminium holder using a thermoplastic adhesive. The samples were then sputter-coated with gold and observed using Jeol 6380 LV scanning EM. The mouthparts of three species (*Embiratermes neotenicus*, *Coptotermes formosanus* and *Sphaerotermes sphaerotherax*) were cleaned through argon plasma etching in a sputter coater machine (Bal-Tec SCD 050).

Ultrastructural features were studied in selected samples (see Table S1) using a Jeol 1011 transmission EM, as described in Šobotník et al. (2003).

#### *Evolution of the hyaline tip*

We reconstructed the presence of hyaline tip using previously published phylogenetic trees (Bourguignon et al. 2015, 2017). We carried ancestral state reconstruction with Mesquite (Maddison & Maddison 2010), on the presence/absence of the hyaline tip, using the Mk1 likelihood model and parsimony analyses.

## Results

The labral gland is a constituent part of the labrum (Fig. 1A, B). The labrum is dorsally sclerotized, and membranous on the ventral side, with lower sclerotization towards the tip, often with transparent inflated apical part called “hyaline tip”. The hyaline tip appears as a transparent extensible protrusion of the labrum occurring in many taxa of Rhinotermitidae and Termitidae (Fig. 1C). The presence of the hyaline tip is variable, depending on developmental stage and species. The hyaline tip was lost in several lineages, including the snapping soldiers and all Nasutitermitinae (Figs. 1C, S1).

### *Scanning EM*

The ventral facies of the labrum were flexible and appeared wrinkled (Fig. 2A), the dorsal facies were more rigid with sclerotized cuticle. The ventral side of the labrum generally carried a few tens of sensillae (Fig. 2B), likely acting as contact chemoreceptors (based on combined SEM and TEM evidence, see below), with possible mechanosensitive function (based on striking similarity to campaniform sensillae). While the dorsal side of the labrum was usually smooth, the ventral facies of the labrum usually showed borders between the underlying epidermal cells, which appeared as irregular angular structures between 4 and 6  $\mu\text{m}$ . These borders were well-delimited in certain parts of the ventral surface of the labrum, often appearing as ridges or spines extending beyond the cell border. These features were especially developed in *Neotermes cubanus*, *Glossotermes oculatus*, *Neocapritermes taracua*, *Spinitermes* sp., and *Labiotermes labralis*. The same pattern was also observed along the midline of the labrum in *Prorhinotermes simplex*, the basal half of the labrum in *Coptotermes formosanus* (Fig. 2A) and *Sphaerotermes sphaerotherax*, and the basal part of the labrum in *Embiratermes neotenicus*. In all specimens, the apical and ventro-lateral part of the labrum possessed numerous pores typically about 110 nm in diameter (Fig. 2C).

### *Optical microscopy*

The labral gland appeared as a thickened epithelium located on the ventral side of the labrum, with possible extension to the dorsal side at the labrum apex. An independent portion of secretory

epithelium appeared also on the dorso-apical part of the hypopharynx (Figs. 3A and 3B). Labral gland secretions were shown to accumulate in the space between the secretory epithelium and the overlaying cuticle with no reservoir.

The labral gland secretory epithelium varied in thickness among species, most commonly ranging between 20 and 30  $\mu\text{m}$ . The thinnest epithelium was found in *Nasutitermes lujae* (2  $\mu\text{m}$ ) and the thickest epithelium was found in the large soldier of *Psammotermes hybostoma* (147  $\mu\text{m}$ ) (Table S1). The hypopharyngeal thickness varied between 4 to 30  $\mu\text{m}$ . The ultrastructural features were nearly identical between the labral and hypopharyngeal regions of the labral gland in all species. The shape and overall size of the labral gland were diverse and not proportional to the size of the labrum. While some labral glands covered the entire labrum, others covered less than half of the labral ventral area. Within the four studied species with soldier sub-castes, the thickness of the labral gland increased with the size of the soldier morph (Table S1).

#### *Transmission EM*

The transmission EM revealed that the labral and hypopharyngeal epithelium were made up of secretory cells. The ultrastructural features of the secretory cells in the labral and hypopharyngeal regions of the labral gland were almost identical, and are thus described together.

The labral gland was predominantly made up of columnar class 1 secretory cells (according to the classification by Noirot & Quennedey 1974) that were characterized by the abundance of smooth endoplasmic reticulum (ER), vesicles of different electron-densities, abundant mitochondria, numerous microtubules oriented apico-basally, glycogen granules, myelin figures and sparse rough ER mainly located around the nucleus (Figs. 4A, 4B, 4C). The secretory cells could easily be differentiated from the non-modified cells (Fig. S3A) as the latter are thinner and lack the characters mentioned above. Electron lucent vesicles were also relatively common within the cells, although they were rarely observed to be released (then including the membrane) at the cell apex, while electron-dense granules were rare. The secretory cell cytoplasm often contained lipid-like droplets (around 1–2  $\mu\text{m}$  in diameter; Figs. S3B, S3C) that were located freely in the cytoplasm and particularly abundant in major soldiers of *Dolichorhinotermes longilabius*. The droplets in *D. longilabius* had a foamy appearance and turned into lucent vesicles that were occasionally excreted at the secretory cell apex. Junctions between neighbouring class 1 cells were formed by apical zonula adherents followed by septate junctions, while the basal parts of the membranes were devoid of any junctions. Basal invaginations were well developed throughout the gland, on average about 5  $\mu\text{m}$  deep (up to 20  $\mu\text{m}$  in *Labiotermes labralis*) (Fig. 4A) and showed frequent pinocytotic activity (Fig. S3D). The nucleus of the class 1 cells was basally

located and elliptic or slightly irregular in shape. The largest dimension of the nucleus was 5  $\mu\text{m}$  (rarely up to 10  $\mu\text{m}$ ) and predominantly filled with dispersed chromatin with few aggregates. Microvilli were well-developed, about 1.5  $\mu\text{m}$  in length (rarely up to 3-4  $\mu\text{m}$ ), approximately 100 nm thick, and always revealed a central ductule of about 40 nm in diameter (Figs. 4A, S3C, S3E). The basal invaginations and microvilli of the hypopharyngeal region of the labral gland were always shorter than that of the labral region. Microvilli were in some cases longer in the central part of the gland compared with the gland margins.

The cuticle was in general made up of three layers including endocuticle of helicoid structure, exocuticle showing no discernible layers, and a thin epicuticle (see Table S1). The labral gland secretions were stored in the space between the secretory epithelium, the overlaying cuticle and inside the porous cuticle. There was no invaginated reservoir in any of the studied species. The cuticle showed numerous adaptations for release of the secretion, which were more pronounced towards the labral tip (Figs. 2C, 4D, S4A, S4B). The cuticular modifications included an increase in the number and width of the pore canals, which widened towards the cuticle base (Fig. 4D), and the occurrence of epicuticular pores allowing for the secretion to be evacuated from the body.

Secretory cells were innervated by free axons frequently observed at the base of the secretory epithelium (Fig. 4C). The singular axons without envelope cells often occurred among the basal invaginations, and sometimes contained typical electron-dense grains of neurosecretions. A different kind of neural tissue was represented by groups of sensillae located along the central line of the labrum, each comprising between 2 to 5 sensory neurons (represented by distal dendrites) and corresponding envelope cells (Figs. 4D, S4C).

Apart from the common organelles, large microtubule bundles running through secretory cells were found in *Mastotermes darwiniensis*, *Hodotermopsis sjoestedti* and *Embiratermes neotenicus* (Fig. S3D). Additionally, tracheae going through class 1 cells were found in *M. darwiniensis* and *H. sjoestedti* (Fig. S3E). Major soldiers of *Dolichorhinotermes longilabius* possessed particularly large amounts of lipid droplets, with electron dense granules that dissolved into lucent vesicles. In all studied Nasutitermitinae the labral gland was relatively underdeveloped, although, the cells retained the general labral gland characteristics.

Class 3 secretory cells, when present, commonly occurred on the dorsal side of the labrum and were generally separated from the secretory epithelium by non-modified epidermal cells. However, the class 3 cells were in few occasions mixed with class 1 cells (Fig. 4E) in *Glossotermes oculatus*, *Termes hospes*, and in the minor soldiers of *Dolichorhinotermes longilabius*. In *Mastotermes darwiniensis*, on the other hand, the class 3 secretory cells were located adjacent to the class 1 secretory cells.

Class 3 cells were neither touching the apex nor the basement membrane of the gland. Their cytoplasm predominantly contained vesicles of moderate electron density (Fig. 4E), but also contained rough ER and free ribosomes, Golgi apparatus, mitochondria, microtubules and rare electron dense granules. The cells were equipped with porous receiving canals continuous with a conducting canal of approximately 0.4  $\mu\text{m}$  in diameter. The conducting canal was made of inner (approximately 40 nm thick) and outer (approximately 6 nm thick) epicuticles (Fig. 4E).

## Discussion

The labral gland is an integral part of the labrum, which is a thin lid-like structure that covers the dorsal side of the pre-oral cavity. The labral gland belongs to the basic body plan of termites. However, its presence has rarely been investigated. Here we report on its presence and cytological features in soldiers of 28 species across the termite phylogeny. The presence of the labral gland in all observed species was unexpected as the gland has only been reported in soldiers of 3 termite species previously (Deligne et al. 1981, Quennedey 1984, Šobotník et al. 2010b, Costa-Leonardo & Hafig 2014). The labral gland was originally recognized as an exocrine organ (Deligne et al. 1981). Quennedey (1984) described the hypopharyngeal part of the labral gland and suggested that the occurrence of the hyaline tip proves the presence of the labral gland in termite soldiers. It was only recently, and following Šobotník et al.'s (2010b) study on the defensive glands in *Glossotermes oculatus*, and Costa-Leonardo & Hafig's (2014) study on the labral gland in *Cornitermes cumulans*, that additional data on the labral gland appeared. In addition to the presence of the labral gland in termite soldiers, it was also recently observed in some imagoes (Křížková et al. 2014) and certain workers (unpublished). These random observations suggest that labral gland might be present in all termite castes, pointing out its importance during termite evolution.

Labral gland is split into two secretory regions located in the ventral part of labrum and dorso-apical part of hypopharynx, respectively. Although the secretory epithelium is always thicker in the labral part, the ultrastructure of secretory cells present in these two secretory regions is virtually identical. We therefore expect that both secretory regions play the same role, and should be thus treated as a single gland. The nomenclatural change from “labral gland” into “cibarial gland” proposed by Quennedey (1984), based on the gland development in two regions, is therefore redundant and the original name, well-accepted by the scientific community, should prevail.

The hyaline tip is a traditional morphological character. The dorsal side of the labrum is always sclerotized, while the ventral part is always formed by lucent membranous cuticle. However, species may differ in level of sclerotization of the dorsal side, especially at the labrum apex. While some



soldiers reveal unchanged level of labrum sclerotization (hyaline tip absent), the level of sclerotization often decreases towards the labrum apex in others (hyaline tip present). All basal taxa primarily lack the hyaline tip, which evolved in a common ancestor of Rhinotermitidae and Termitidae, and was subsequently lost at least four times independently. Once in Nasutitermitinae, in which the entire labrum is greatly reduced in size, twice independently in lineages with snapping soldiers, *Pericapritermes* and *Neocapritermes* + *Planicapritermes*, and once in *Microcerotermes*. While the hyaline tip has been shown to disappear in some lineages, the labral gland was found in all studied termite families in this study. This infers that the evolution of the snapping mandibles did not see a loss of the labral gland and suggests that the evolution of mandibles has not necessarily been accompanied by a reduction or loss of chemical adaptation (Kyjaková et al. 2015).

The cytological features of the labral gland revealed many similarities among all studied species. Additionally, the four species with polymorphic soldiers that we studied, showed that the labral gland volume increased with subcaste size, what was particularly pronounced in *Psammotermes hybostoma*.

The common features shared by labral and hypopharyngeal parts of the labral glands include: (i) the abundance of smooth endoplasmic reticulum, (ii) the presence of apical microvilli with a central ductule, (iii) well-developed basal invaginations ensuring the intake of precursors from the haemolymph, and (iv) cuticular modifications in the tip of the labral gland allowing gland secretions to reach the exterior (see also Deligne et al. 1981, Quennedey 1984, Šobotník et al. 2010b, Costa-Leonardo & Hafig 2014). These ultrastructural features are a conservative account of the characteristics of the two secretory regions in the studied species, which suggest that the labral gland has the same function among all species. The labral gland secretion is stored between the secretory epithelium and the overlaying cuticle, as well as within the cuticle itself. Labral secretions from the glandular cells are under neural control, supposedly from the brain, as singular axons were often detected at the base of the secretory epithelium.

The function of the labral gland is most likely not defensive due to the absence of a reservoir, a feature characteristic of defensive glands (Chapman 2013). Additionally, the labral gland is present in soldiers of all species, irrespective of their defensive strategies, including species having soldiers with nasus, snapping mandibles, or performing body rupture. The composition of the labral gland secretion remains unknown despite our repeated attempts to identify labral gland-specific compounds. This may be due to the small size of the labral gland and the unknown nature of its secretion. Nevertheless, the high abundance of a smooth ER suggests that the secretion may have a lipidic and volatile nature and could be used in communication (Percy-Cunningham & MacDonald 1987, Nakajima 1997, Tillman et al. 1999, Alberts et al. 2002).

The presence of specialized receptors at the ventral side of the labrum is likely to aid the dosage of labral secretions. As all observed receptors contained several dendrites, the chemosensory function is likely to be present in all species while the mechanoreceptive function remains hypothetical. The idea that the labral receptors respond to mechanical pressure has a functional parallel in the sternal gland, whose secretion releases are controlled by groups of campaniform sensillae (Stuart & Satir 1968, Quennedey et al. 2008).

Class 3 cells occur frequently on the dorsal side of the labrum as well as on the sclerotized body cuticle (Šobotník et al. 2004, 2005). Class 3 cells may also occur adjacent to the labral gland secretory epithelium but should not be considered as part of the labral gland until the two cell classes are combined, as seen in *G. oculatus* (Šobotník et al. 2010b), the minor soldiers of *D. longilabius* (presented here), *C. cumulans* (Costa-Leonardo & Hafig 2014) and *T. hospes* (presented here). The class 3 cells were never observed in the hypopharyngeal part of the labral gland in none of above-mentioned species. The ultrastructure of the class 3 secretory cells is uniform in termites, irrespective of their caste (Costa-Leonardo & Shield 1990, Šobotník et al. 2004) and position in the gland, such as mandibular (Lambinet 1959, Cassier et al. 1977), sternal (Noirot & Quennedey 1974, Quennedey et al. 2008), tergal (Ampion & Quennedey 1981, Šobotník et al. 2005), and epidermal (Šobotník et al. 2003). The secretory cells are always rich in rough ER and Golgi apparatus, and contain variable amounts of moderately electron-lucent vesicles released to the extracellular reservoir ("end apparatus"), into which the cuticular canal is inserted. This ultrastructure suggests that rough ER produces proteinaceous water-soluble secretions that are configured in the Golgi apparatus (Hand & Oliver 1984) before being released on the surface of the body cuticle. These secretions may appear as the uppermost layer of the epicuticles protecting the lower layers from abrasion (Chapman 2013).

### *Conclusion and further hypotheses*

The labral gland has previously been suggested to be a synapomorphy of Neoisoptera (Šobotník et al. 2010a). The presence of the labral gland in termite soldiers of all studied species suggests that the labral gland evolved with the soldier caste where it has remained an important organ. Moreover, the labral gland has long been thought to primarily have a defensive function. Gland secretion was thought to be secreted on the mandibles and deposited into the wound following bite (Deligne et al. 1981, Quennedey 1984, Šobotník et al. 2010b, Costa-Leonardo & Hafig 2014). However, preliminary observations based on the morphology, structure and ultrastructure of the labral gland suggest that the gland secretion has a communicative function.

The presence of labral gland in soldiers of all termite species suggests that it has a fundamental role in colony survival and success. Our data suggest that the function of the labral gland may be related to communication. This hypothesis is supported by personal observations of soldiers wiping their labrum against the floor after encountering an enemy. Better understanding of the function of the labral gland in termites is called for in order to enhance understanding of termite defence mechanisms and communication behaviour.

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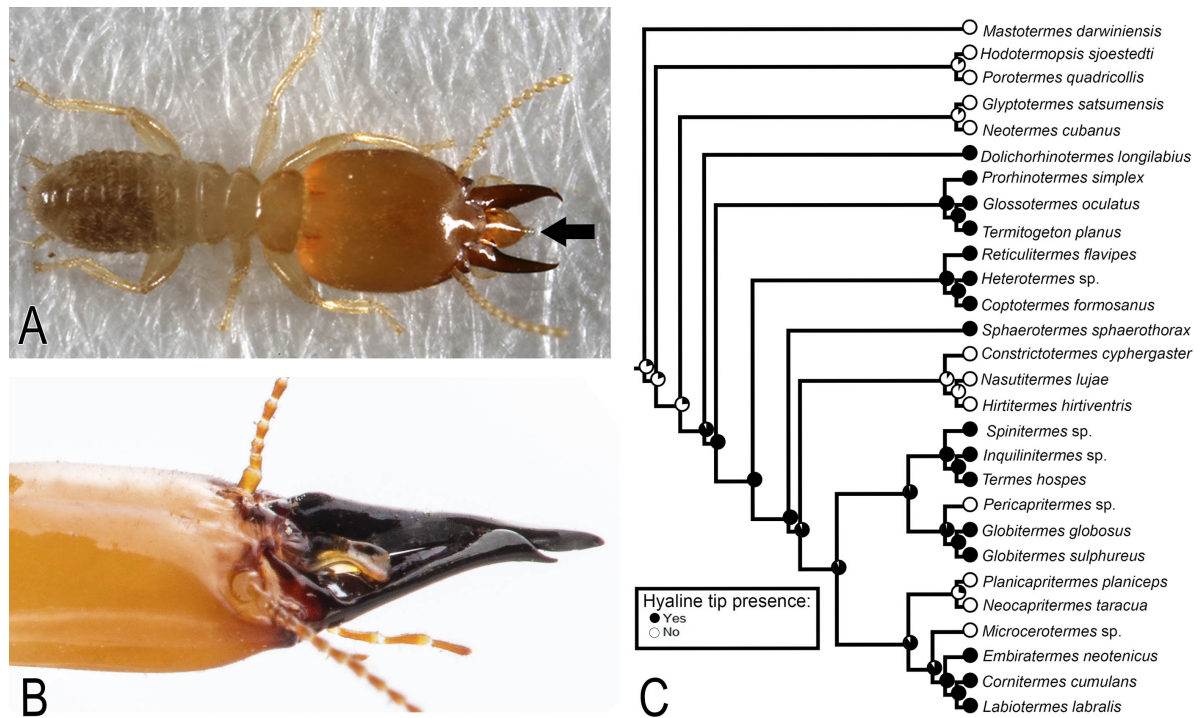
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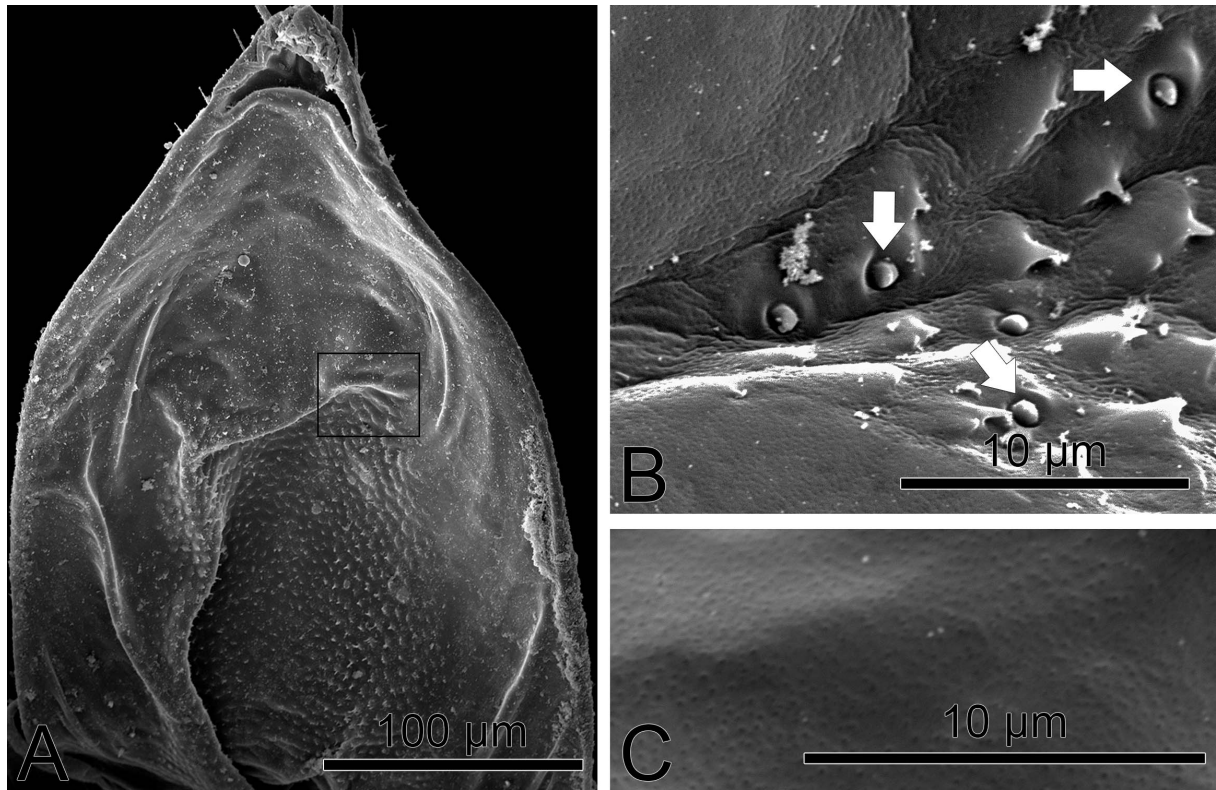
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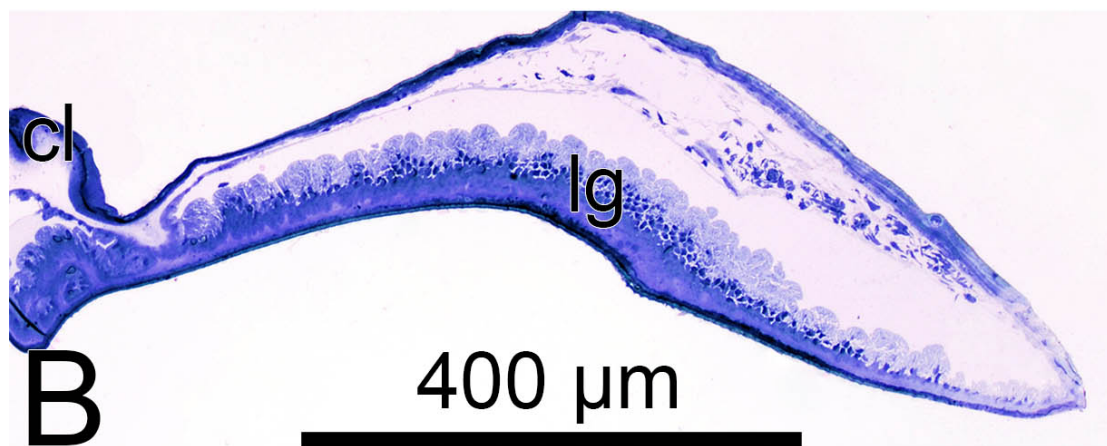
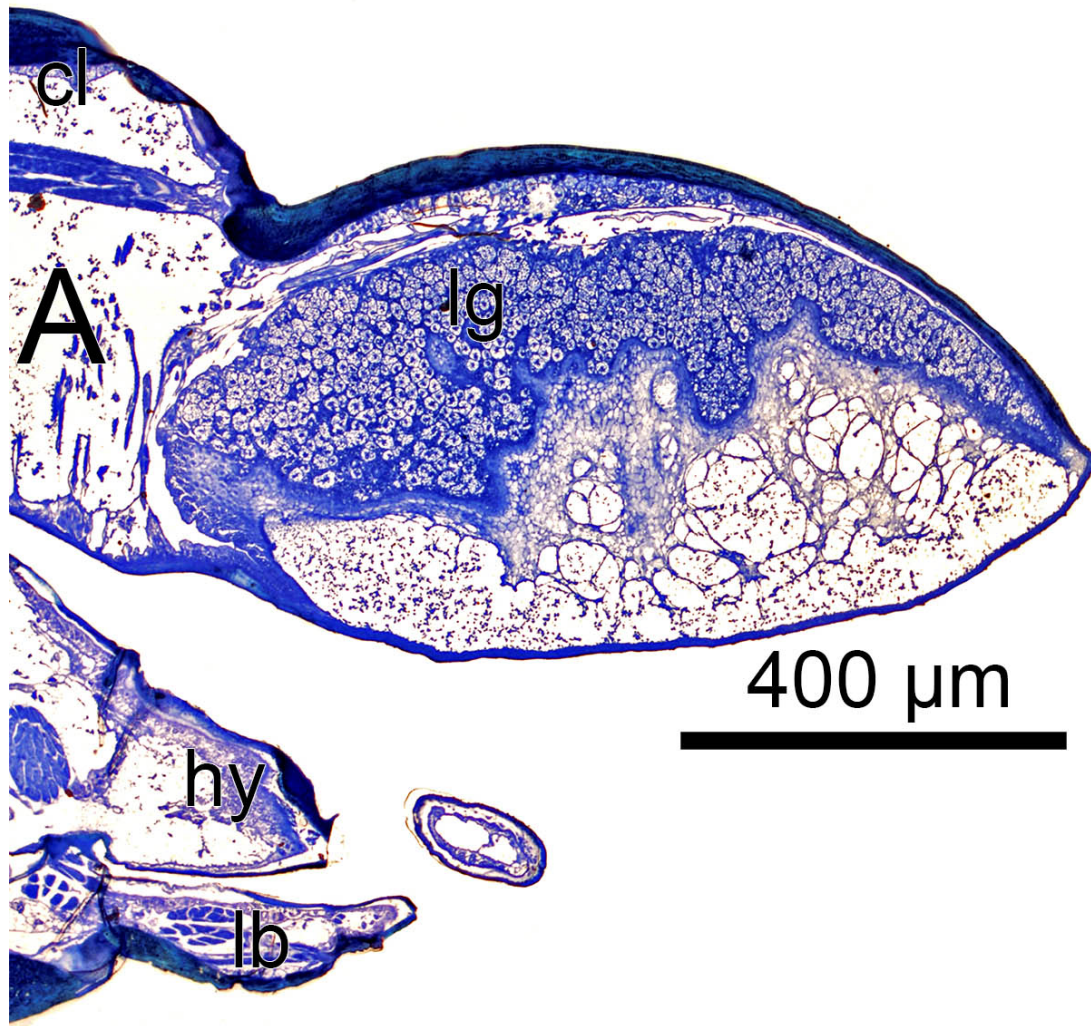


**Figure 1.** (A) *S. sphaerotherax* soldier. Arrow marks the hyaline tip of the labrum. (B) Head of *N. taracua* soldier. (C) Phylogenetic tree showing the evolution of the hyaline tip in soldier caste. The presence or absence of the hyaline tip is marked with black or white circles, respectively.

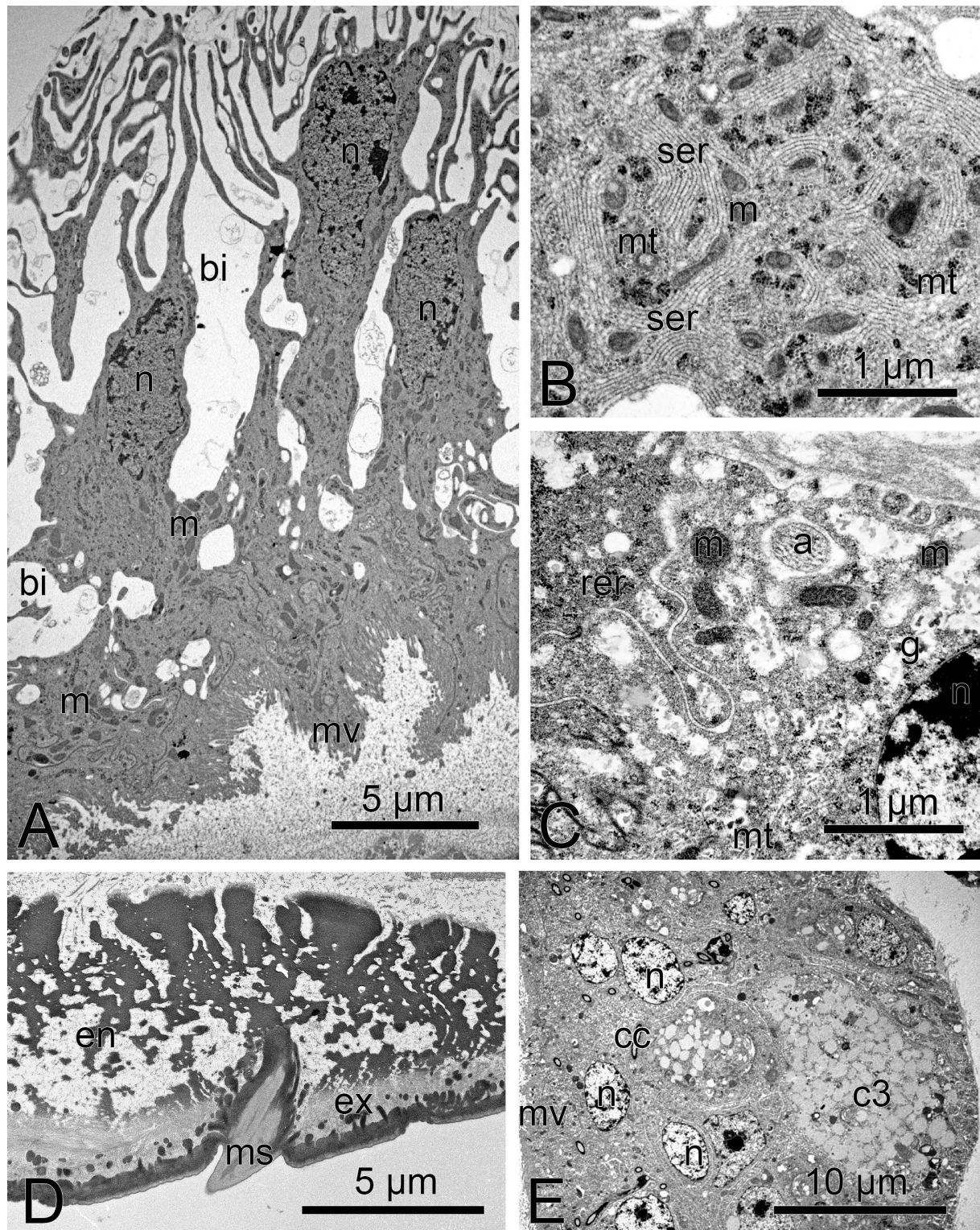


**Figure 2.** Labral gland development. (A) Micrograph of ventral side of labrum of *C. formosanus*, the small rectangle indicates the sector where was taken the micrograph B. (B) Micrograph of region with a group of sensillae (marked with white arrows) in *C. formosanus* labrum. (C) Large magnification micrograph of the apical region with epicuticular pores in *S. sphaerothorax* labrum.





**Figure 3.** Sagittal sections of the forehead of *P. hybostoma* medium soldier (A) and *N. taracua* soldier (B), showing the secretory epithelium in hypopharynx. Abbreviations: cl= clypeus, hy= hypopharynx, lb= labium, lg= labral gland.



**Figure 4.** Ultrastructure of the labral gland in soldiers. (A) Overall development of the labral gland in *L. labralis*. Note the development of the apical microvilli and basal invaginations. (B) Detailed labral gland secretory cell class 1 cytoplasm in *N. taracua* showing well developed smooth endoplasmic reticulum. (C) Detailed labral gland secretory cell class 1 cytoplasm in the large soldier of *D. longilabius* showing a free axon located at the base of the secretory epithelium. (D) Highly modified cuticle underlying the

labral gland in *E. neotenicus*. Note enlarged pore canals ensuring the secretion release and the margin of the sensillum. (E) Class 3 secretory cell in *G. oculatus*. Abbreviations: a = axon, c3 = class 3 secretory cell, cc= conducting canal, en= endocuticle, ex= exocuticle, g = glycogen, bi = basal invaginations, m = mitochondria, ms = margin of the sensillum, mt= microtubule, mv = microvilli, n = nucleus, rer = rough endoplasmic reticulum, ser = smooth endoplasmic reticulum.