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Defensive glands in Stylotermitidae (Blattodea, Isoptera)

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ABSTRACT

The large abundance of termites is partially achieved by their defensive abilities. Stylotermitidae represented by a single extant genus, *Stylotermes*, is a member of a termite group Neoisoptera that encompasses 83% of termite species and 94% of termite genera and is characterized by the presence of the frontal gland. Within Neoisoptera, Stylotermitidae represents a species-poor sister lineage of all other groups. We studied the structure of the frontal, labral and labial glands in soldiers and workers of *Stylotermes faveolus*, and the composition of the frontal gland secretion in *S. faveolus* and *Stylotermes halumicus*. We show that the frontal gland is a small active secretory organ in soldiers and workers. It produces a cocktail of monoterpenes in soldiers, and some of these monoterpenes and unidentified proteins in workers. The labral and labial glands are developed similarly to other termite species and contribute to defensive activities (labral in both castes, labial in soldiers) or to the production of digestive enzymes (labial in workers). Our results support the importance of the frontal gland in the evolution of Neoisoptera. Toxic, irritating and detectable monoterpenes play defensive and pheromonal functions and are likely critical novelties contributing to the ecological success of these termites.

1. Introduction

Termites (Isoptera) are eusocial insects of prime ecological importance, contributing significantly to tropical invertebrate faunas (Bar-on et al., 2018; Tůma et al., 2020). Indeed, termites are among the main decomposers of dead plant tissues on a global scale and are broadly recognized as key ecosystem engineers (Jouquet et al., 2011; Eggleton, 2011; Bignell, 2016). There are about 3000 described species of termites, out of which about 10% are of economic importance, with many acting as serious invading pests of timber and agriculture (Evans et al., 2013; Krishna et al., 2013). Termites are classified into ten families, i.e., Mastotermitidae, Hodotermopsidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae; the last four of which comprise the crown group Neoisoptera, characterized by the presence of a frontal

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gland and the associated fontanelle (Krishna et al., 2013). The family Stylotermitidae occupies a unique position among the Neoisoptera as it is the extant sister group to all other Neoisoptera (Engel et al., 2009, 2016; Wu et al., 2018). It is for this reason that understanding of stylotermitid biology and anatomy is crucial to study the evolution of the Neoisoptera including the most diverse family, Termitidae. It is from among the stylotermitids that insights into the earliest origins of key specializations, such as the frontal gland and its defensive capabilities in the well-studied Neoisoptera, are to be discovered. Yet, while there is a solid body of information about many aspects of termites' lives, there are no data on fundamental features of the colony life, caste development, symbiotic associations, etc., for *Stylotermes*, the sole living genus of the family. *Stylotermes* comprises 45 described species distributed in Southeast Asia (Liang et al., 2017; Wu et al., 2018) and exhibits an intermingling of termite plesiomorphic and apomorphic morphological characters, and also unique features such as feeding on living trees or having trimerous tarsi (Engel et al., 2009; Liang et al., 2017; Wu et al., 2018). The genus *Stylotermes* is among some of the most poorly studied termites as its species are difficult to collect and rear.

Because termites are the source of food of many animals, their defense mechanisms are of prime importance against predation. Among all social organisms, it was in the Isoptera that a truly altruistic caste first evolved – the soldiers (Krishna et al., 2013; Engel et al., 2016). Termite soldiers are usually characterized by elongate biting mandibles (secondarily short in several groups), enlarged robust heads, generally higher body sclerotization, and development of novel defensive glands. Other castes also reveal certain defensive skills including the use of exocrine secretions; however, soldiers are more efficient at defensive actions (Deligne et al., 1981; Prestwich, 1984). Soldiers can, according to mandibular shape, be classified as *crushing*, when robust mandibles with rich dentition are used to bite; *reaping*, when slender, saber-shaped mandibles inflict deeper wounds; or *piercing*, when hooked mandibles are used to attach soldier onto an enemy and in order to prolong the application of defensive secretion release on the wound. In many groups, the long mandibles lost their biting function, and work as springs (i.e., in symmetric or asymmetric snapping soldiers). In other groups, the soldier mandibles have secondarily evolved as a shortened form, such as in phragmotic soldiers who use their massive and modified heads to plug the entrance holes to the colony, or in soldiers whose heads have evolved to smear or squirt defensive compounds from the frontal gland via a nozzle-like head, as in nasutoids (Rhinotermitidae) or nasutes (Termitidae: Nasutitermitinae) (Deligne et al., 1981; Prestwich, 1984). Apart from the active defenses performed primarily by soldiers, termites also largely benefit from their hidden way of life, making them inaccessible to generalized predators, and mostly competing with other detritivores.

Termites in general release numerous semiochemicals, similar to other advanced eusocial insects (Billen and Morgan, 1989; Bordereau and Pasteels, 2011; Šobotník et al., 2010a). The blends of signals originate from various glands and provide fundamental information regarding foraging, attraction to food sources, defense, mate location, and the status of their colony (Billen and Morgan, 1989; Costa-Leonardo et al., 2009). Out of 23 exocrine glands hitherto described in termites (Aumont et al., 2023), only nine occur across termite groups and castes, and these are: frontal (Noirot, 1969; Deligne et al., 1981; Šobotník et al., 2004, 2010b, 2010c, 2010d), oral (Synek et al., 2019), labral (Palma-Onetto et al., 2018, 2019), mandibular (Brossut, 1973; Šobotník and Hubert, 2003), labial (Šobotník and Weyda, 2003), lateral thoracic (Gonçalves et al., 2010), tarsal (Costa-Leonardo et al., 2015), sternal and tergal glands (Ampion and Quennedey, 1981). One third of these glands is known to be involved in defensive activities — the labral, frontal, and labial glands.

The labral gland is found in two secretory regions, located ventrally on the labrum and apico-dorsally on the hypopharynx, and occurs in workers, soldiers, and imagoes (Deligne et al., 1981; Quennedey, 1984; Šobotník et al., 2010c; Costa-Leonardo and Haifig, 2010; Křížková et al., 2014; Palma-Onetto et al., 2018, 2019). Although the function of the labral gland secretion has not been identified, behavioral observations suggest it serves as a warning signal and leads to avoidance by other colony mates (Palma-Onetto et al., 2018). The frontal gland, probably the most studied organ in termites, occurs in presoldiers, soldiers, imagoes, and some workers of Neoisoptera, and its presence has been confirmed in Rhinotermitidae, Serritermitidae, and Termitidae (Deligne et al., 1981; Quennedey, 1984; Šobotník et al., 2004, 2010b, 2010d). Most of the sources refer to the frontal gland as a saccular invagination at the anterior of the frons, often filling a significant portion of the soldier body. Indeed, the proportion of the frontal gland secretion may exceed a third of the live body weight of soldiers (Waller and La Fage, 1987). At the same time, the frontal gland also occurs in many imagoes as a saccular organ or epidermal thickening, and in certain workers also as an epidermal thickening (Šobotník et al., 2010b; Kutalová et al., 2013). The frontal gland synthesizes, stores, and discharges copious amounts of a defensive secretion, playing a role as a contact poison, irritant or repellent, adhesive glue or incapacitating agent, anti-healing, or alarm pheromone (Eisner et al., 1976; Kriston et al., 1977; Prestwich et al., 1977; Clément et al., 1988; Deligne et al., 1981; Prestwich, 1984; Šobotník et al., 2008a, 2010a). The labial gland is a paired organ located along the gut in the thorax and anterior abdomen in all termite species, castes, and developmental stages (Noirot, 1969; Billen et al., 1989; Šobotník and Weyda, 2003). The labial gland comprises numerous clumps of secretory cells, the acini, reservoir (also called water sac), and ducts connecting all components to the base of the labium (Šobotník and Weyda, 2003; Šobotník et al., 2010c). Labial gland secretions serve for food digestion in workers, feeding the dependent castes, and also for cement during building activities, or as a food-marking pheromone (Noirot, 1969; Grassé, 1982; Reinhard et al., 2002; Tokuda et al., 2002; Sillam-Dussès et al., 2012). The secretion is defensive in all termite soldiers but becomes a dominant source of contact poisons only in *Mastotermes darwiniensis* and in several Macrotermitinae (Moore, 1968; Maschwitz et al., 1972; Plasman et al., 1999; Sillam-Dussès et al., 2012).

While the secretion of the labral gland has not yet been characterized, the labial glands are known to contain a variety of polar and water-carried products, such as quinones, macrocyclic lactones, ancistrodial, ancistrofuran, cavidial, α- and β-cyclogeraniolen, and larger-molecule components (proteins, polysaccharides) responsible for its congealing properties (Moore, 1968; Maschwitz et al., 1972; Plasman et al., 1999; Sillam-Dussès et al., 2012). A much higher diversity of compound classes was discovered from frontal gland secretions, with hydrocarbons, alcohols, mono-, sesqui-, diterpenes, aldehydes, ketones, fatty acids, macrocyclic lactones, heterocyclic, aromatic compounds, nitro-compounds, and ceramids being more common (Howse, 1984; Prestwich, 1984; Šobotník et al., 2010a). In imagoes the frontal gland secretion has only been studied in *Prorhinotermes*, in which the secretion is similar to soldiers albeit with many more sesquiterpenes present (Piskorski et al., 2009).

The anatomy, physiology, and chemistry of defensive glands have been studied in various termite taxa (Costa-Leonardo et al., 2023), but no data are available for *Stylotermes*, a genus crucial to understanding the origin of adaptations key to the unprecedented success of the Neoisoptera. The frontal gland is the most important autapomorphy of Neoisoptera, and, as noted above, *Stylotermes* occupies a significant position as the extant sister group to all other living Neoisoptera (Engel et al., 2009, 2016; Wu et al., 2018). Historically, stylotermitids have been difficult to locate and study because their habitat is inside living tree trunks at higher elevations in tropical Asia, the result being that many biological details for the family are virtually unknown.

In this work, we bring the first observation of the caste-specific development of defensive glands and the composition of the frontal gland secretion in Stylotermitidae corroborated by results from two *Stylotermes* species, and provide a discussion on the importance of these findings for the evolution of termites, among the main cellulose decomposers on Earth.

2. Material and methods

A colony of *Stylotermes faveolus* (Chatterjee and Thakur, 1964) was found at the Hill Agricultural Research & Extension Centre, Bajaura, Kullu, Himachal Pradesh, India (N 31°50′11″, E 77°10′23″, 1050 m amsl) in the trunk of a living tree of *Alnus nitida* (Spach) Endl. (Betulaceae), and a fragment of it was brought to the lab. Living workers and soldiers of *S. faveolus* were extracted from the wood and immediately placed into a droplet of fixative (0.2 M PBS at pH 7.2: 8% glutaraldehyde: 10% paraformaldehyde = 2:1:1), and the individual tagmata (head, thorax, abdomen) isolated with a razor blade. All appendages were removed, as well as the mandibles. After 48 h at +4 °C, the samples were postfixed with 2% OsO_4 solution in PBS, dehydrated through an acetone series, and embedded into Spurr resin (standard mixture). The polymerized samples were sectioned using a Reichert-Jung ultramicrotome. For optical microscopy, semithin sections (0.5 μm thick) were cut to the water surface with a glass knife and placed on a slide in 10% acetone. The dried specimens were stained with toluidine blue and imaged under an Olympus BX63 light microscope (Olympus, Japan) equipped with an Olympus DP74 CMOS camera (Olympus, Japan). For transmission electron microscopy, ultrathin sections were cut on the diamond knife to the water surface. After stretching by chloroform the sections were placed on the cooper nets and contrasted by uranyl acetate and lead citrate and coated by carbon. The samples were imaged under the transmission electron microscope Jeol 1011 (Jeol, Akishima, Japan).

In addition, *Stylotermes halumicus* individuals were collected at Dakeshan Forest Recreation Area, Zhuolan Township, Miaoli County, Taiwan (N 24°19′45.5″ E 120°53′45.6″, 650 m amsl) in the tree hole of a living *Trema orientalis* (L.). Individuals were collected from the same colony 3 times within 1 month. After each collection, they were placed in moistened tissue paper for safe transport. Upon returning to the laboratory, they were immediately processed with the subsequent extraction work.

Living soldiers of both *Stylotermes* species were for analytical purposes treated by two distinct ways, and we prepared either samples for the secretion composition (quality) or quantity of its constituent parts. To collect pure frontal gland secretion, the ice-immobilized soldiers were gently squeezed with forceps and the secretion droplet appearing from the fontanelle was collected using a thin-tipped capillary tube, which was then extracted with p.a. *n*-hexane. To learn the quantity of secretion components per average soldier, we prepared two samples, each containing five crushed soldier heads of *S. faveolus*, and extracted them twice with the same solvent for 12 h at 4 °C; workers were treated the same way as controls. For *S. halumicus*, we prepared nine samples, each containing one crushed soldier head, but workers were not studied. The samples were analyzed by using a comprehensive two-dimensional gas chromatographic-mass spectrometric system employing time-of-flight mass analyzer (Pegasus 4D, LECO, USA), in the same setup and conditions described in our recent paper on identification of *Stylotermes* trail-following pheromone (Thakur et al., 2023). Only difference was that samples were evaporated to last drop and then redissolved into 50 μl of hexane containing 5 μg/ml of 1-bromododecane as an internal standard. Identification was performed using mass spectral similarity of deconvoluted mass spectrum and spectra from NIST mass library (NIST, 2019). For confirmation, comparison of measured retention indexes was used for all compounds. The identity of compounds for which standards were available was confirmed also with the comparison of the retention time and these compounds were quantified using external calibration curve and internal standard.

Synchrotron X-ray microtomography (SR-μCT) scans of one soldier and one worker of *S. halumicus* preserved in 80% ethanol were performed at the IMAGE beamline of the Imaging Cluster at KIT Light Source. The beam produced by the superconducting wiggler was filtered by 2 mm pyrolytic graphite and monochromatized at 18 keV by a Double Multilayer Monochromator (DMM). The specimens were scanned at 5× magnification and an effective voxel size of 2.44 μm. We employed a fast indirect detector system consisting of a scintillator, visible light optics, a white beam microscope (Optique Peter) (Douissard et al., 2012) and a 12-bit pco.dimax high-speed camera (Excelitas PCO GmbH) with 2016 × 2016 pixels of 11 μm physical size. For each scan, we took 200 dark field images, 200 flat field images, and 3000 equiangularly spaced radiographic projections in a range of 180° with a frame rate of 50 fps. The control system concert (Vogelgesang et al., 2016) served for automated data acquisition. Data processing including dark and flat field correction and phase retrieval was performed by the UFO framework (Vogelgesang et al., 2012). The final 3D tomographic reconstruction was done with Tofu (Faragó et al., 2022) and yielded phase and absorption contrast data sets. These were blended and converted into 8-bit volumes.

The selected organs were segmented in Amira-Avizo software (Thermo Fisher Scientific) using manual visual selection of the tissues on a subset of parallel slices followed by interpolation. The data were visualized using the "volume rendering" module and the volumes were calculated using "material statistics" module in Amira.

3. Results

The labral, frontal, and labial glands are present in both castes of *S. faveolus* and *S. halumicus*. Our data (see Table 1 and Fig. 1) shows the labral gland occurring in two isolated regions in the labrum ventral, and dorsal part of hypopharynx, respectively. The frontal gland appears as a small organ located behind the brain in both castes, saccular in soldiers and epidermal thickening in workers. Labial glands are located mostly in thorax, and consist of clumps of secretory cells, acini, and thin-walled reservoirs (called also water sacs in workers). Surprisingly, the left water sac of the worker is collapsed and squeezed among the acini, while the right one pushes all acini to the left side.

3.1. The labral gland

The labrum is an oval-shaped structure above the mandibles, much longer in soldiers, without a visible hyaline tip. The labral gland occurs on the ventral part of the labrum and apico-dorsal part of hypopharynx (Fig. 2A, B, C). The secretory epithelium is slightly thicker in soldiers, usually 20–30 μm, while it is only from 15 to 20 μm in workers. In both castes, the epithelium is made of a single cell type, secretory cells class I (according to Noirot and Quennedey 1974). The gland is covered by a highly modified cuticle, with loose layers of endocuticle (1–3 μm thick), exocuticle (about 1.5 μm

Table 1
Volumes of the head capsules and the defensive glands of one soldier and one worker in *Stylotermes halumicus* by using computed tomography scans.

Caste	Body part	Relative volume	Volume (mm^3)	Relative to head capsule volume (%)
Soldier	Head capsule	24531927410	2.8475	100.00
	Acini of the labial glands	107703861	0.0125	0.44
	Dorsal part of the labral gland	62880153	0.0073	0.26
	Ventral part of the labral gland	4648123	0.0005	0.02
	Reservoirs of the labial glands	689894184	0.0801	2.81
	Frontal gland	232374421	0.0270	0.95
Worker	Head capsule	7806686355	0.9062	100.00
	Acini of the labial glands	1011977057	0.1175	12.96
	Dorsal part of the labral gland	37014893	0.0043	0.47
	Ventral part of the labral gland	7461633	0.0009	0.10
	Reservoirs of the labial glands	880973629	0.1023	11.28
	Frontal gland	3246189	0.0004	0.04

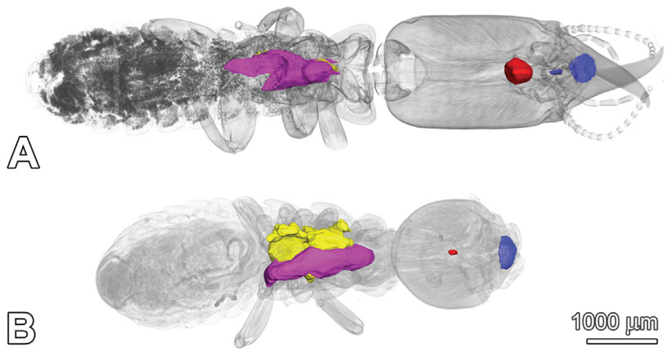

Fig. 1. µCT of one soldier (A) and one worker (B) of *Stylotermes halumicus*. The position and the reconstructed volume of the labral (in blue), frontal (in red), and labial (made of acini in yellow and reservoirs in purple) glands are shown on individuals from dorsal views.

thick) with enlarged pore canals, and epicuticle (15 nm for outer epicuticle and 30 for the inner epicuticle) with numerous pores. The secretory cells are columnar, with well-developed apical microvilli (1.2–1.5 µm long, about 90 nm thick) facing to the cuticle. Microvilli reveal a central ductule about 30 nm thick (Fig. 2D, E, F). The basal parts of the secretory cells reveal well-developed basal invaginations, reaching up to 5 µm deep in the secretory cells, and a high pinocytotic activity. The basement membrane is mostly made of a single, rarely more, layer about 100 nm thick. Nuclei occupy the central portion of the secretory cells and are slightly irregular and filled predominantly with dispersed chromatin with, apart from the nucleolus, a few larger condensates. The main secretory organelle is the abundant smooth endoplasmic reticulum (ER), mixed with small patches of rough ER. The secretory inclusions comprise small (0.2–1 µm in diameter) electron-lucent vesicles occurring predominantly in the apical part of the secretory cells and are sometimes excluded at the microvilli bases. Large droplets (2–5 µm in diameter) located freely in the cytoplasm are frequently observed in the middle parts of the secretory cells. Mitochondria are moderately abundant, and 400–800 nm long. The neighboring secretory cells are connected by an apical zonula adherens followed by a long septate junction. The gland is developed similarly in both castes, and although the gland is much larger in soldier, the volume relative to the head capsule volume is smaller due to larger head size in the soldier caste (Table 1). Workers also reveal smaller secretory cells with less abundant secretory organelles, such as smooth and rough ER. Labral gland cells in workers also contain higher amounts of glycogen rosettes and electron-dense granules (Fig. 2E, F).

3.2. *The frontal gland*

This gland is present in both workers and soldiers of *S. faveolus*, but its development is quite different between them, in soldiers being much larger in absolute and relative numbers (Table 1). The gland occurs as an ovoid saccular organ in soldiers, about 1 mm long and 0.5 mm wide, located in the middle part of the head capsule and opening at the fontanelle on the frons. The reservoir wall is up to 75 µm thick including the thick cuticle lining the reservoir. The fronto-tentorial muscle is attached to the anteroventral part of the frontal gland epithelium (Fig. 3A, B, C). In workers the frontal gland is located at the junction of the Y-suture as an epidermal thickening, about 150 µm in diameter and up to 80 µm thick, and the fronto-tentorial muscle is attached at the ental tip of the secretory epithelium (Fig. 4A, B). The glandular cuticle in soldiers is 4–6 µm thick, composed of a loose system of cuticular filaments, leaving much space for the secretion. The most apical layer is more compact, made of tightly packed cuticular elements. The epicuticle is only about 7 nm thick and made of outer and inner epicuticles of similar thickness. The secretory cells are columnar, 20–50 µm long but invariably only about 3 µm thick medially. There is a broad gap between the secretory cells and the cuticular lining, varying in width between 5 and 10 µm, filled with an electron-dense secretion originating from the secretory cells. The apical plasma membrane is differentiated into long (2–3 µm long, about 100 nm thick) and dense microvilli. The basal cell parts reveal moderately abundant basal invaginations reaching some 2–3 µm deep into the cells. The basement membrane is always thick, strengthened by collagen fibers, 100–200 nm thick laterally, and up to 500 nm thick close to the muscle attachment. The nuclei are placed basally, and are rounded to ovoid, slightly irregular, up to 6 µm in the longest dimension, and made predominantly of dispersed chromatin. Both smooth and rough endoplasmic reticulum (ER) occur in the secretory cells, the smooth ER is mostly at the cell apices, while the rough ER is found more around the nucleus. The mitochondria are large (1–3 µm long, about 250 nm thick) and abundant throughout the secretory cells. The cytoplasm contains large amounts of lipid-like droplets (0.25–5 µm in diameter), located freely in the cytoplasm, with sizes gradually decreasing towards the cell apex. The small droplets are excluded at the microvilli bases, along with rather rare electron-dense granules. Neighboring cells are connected by an apical zonula adherens followed by a long septate junction.

The frontal gland in workers is present as an epidermal thickening of triangular cross-section, up to 80 µm thick at the point of the fronto-tentorial muscle attachment (Fig. 4A). While the head capsule cuticle is about 25 µm thick, the cuticle overlying the frontal gland decreases in thickness to about a third. At the apex, the epicuticle is made of outer and inner layers, about 7 and 20 nm

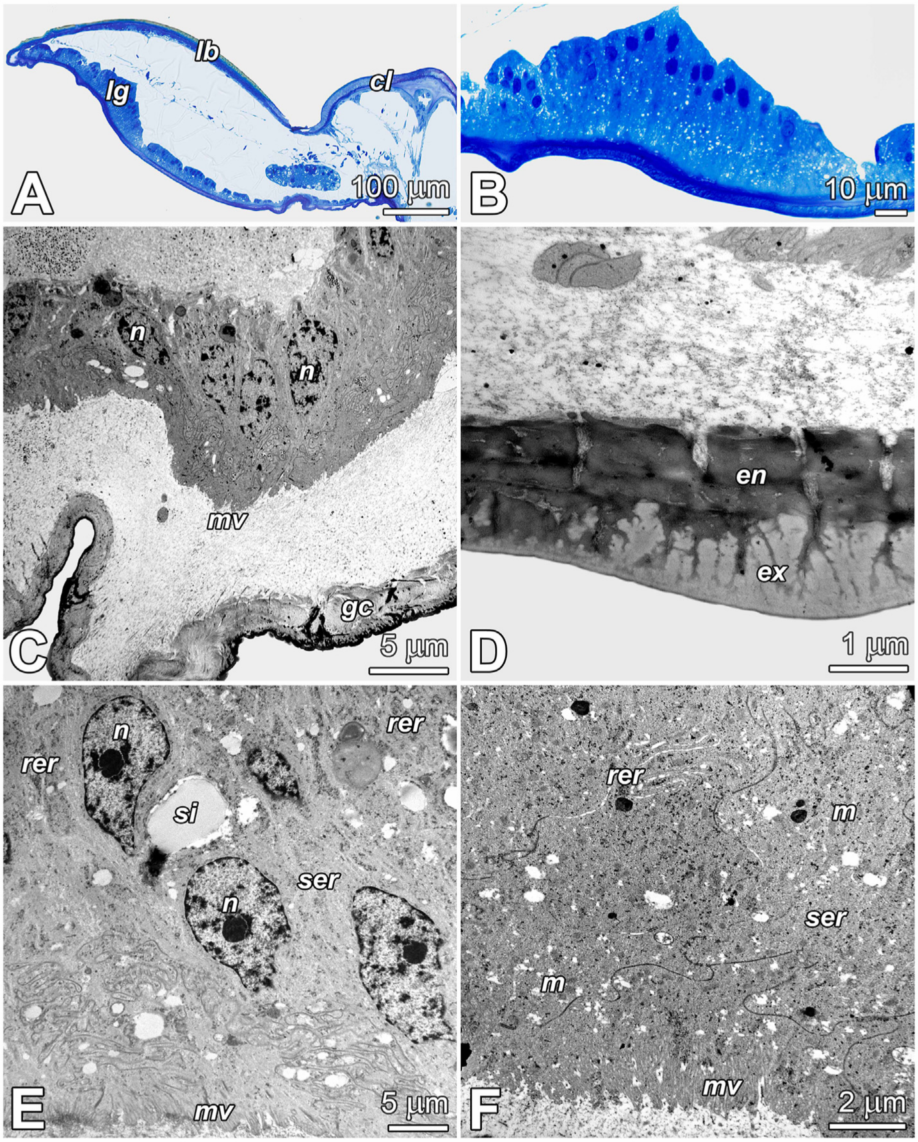

Fig. 2. Labral gland in soldier (A-B, E-F) and worker (C–D) in *Stylotermes faveolus*. **A.** Sagittal section of labrum in a soldier. **B.** Detail of Fig. 2A. **C.** Labral gland in a worker. **D.** Detail of secretory cuticle in a worker. **E.** Apical part of a secretory cell in a soldier. **F.** Detail of the apex of the secretory epithelium in a soldier. **Abbreviations**: *cl*, clypeus; *en*, endocuticle; *ex*, exocuticle; *gc*, glandular cuticle; *lb*, labrum; *lg*, labral gland; *m*, mitochondria; *mv*, microvilli; *n*, nucleus; *rer*, rough endoplasmic reticulum; *ser*, smooth endoplasmic reticulum; *si*, secretory inclusion.

thick, respectively. Both the exocuticle and the endocuticle, of similar thicknesses, are perforated by numerous enlarged pore canals. The secretory cells are columnar, 20–70 µm long and about 2 µm wide apically and up to 7 µm wide basally. The apical plasma membrane forms dense microvilli about 1 µm long, however, sometimes pieces of apical cytoplasm, devoid of microvilli, are found to detach from the secretory cells and burst open in the space below the modified secretory cuticle (Fig. 4B, C). The secretory cells are differentiated into three distinct regions: apical, central, and basal (Fig. 4C, D, F). The apical region comprises 2–5 µm of the cell apex and contains just lucent cytoplasm with numerous microtubules, rare rough ER, and groups of small (50–100 nm in diameter) electron-dense secretory vesicles. The central part comprises 15–50 µm of the mid-section of a given cell and contains an elongated ovoid nucleus up to 10 µm in the longest dimension, filled predominantly with dispersed chromatin. The neighboring secretory cells are connected by an apical zonula adherens and a septate junction, which disappears along the gradual change of cytoplasm content in the cells' central parts. The central part's cytoplasm is rich in electron-dense granules, which seem to start as homogeneous granules, rounded and slightly irregular, 0.8–2.5 µm in the largest dimension, of moderate electron-density, in which darker granulate material appears and gradually changes into a heterogeneous structure (Fig. 4E). Mitochondria (1.5–2 µm long, 250–400 nm thick) are highly abundant here, and tubular rough ER is also fairly common. The basal part is characterized by the presence of many deep invaginations of the basal membrane, which may eventually reach to the nucleus but more often are 5–10 µm deep. The cytoplasm contains huge numbers of mitochondria, and smaller amounts of rough ER, while other organelles and inclusions are missing. The basal lamina is thick and reinforces the collagen fibers, and its thickness increases from 500 nm up to 3 µm close to the fronto-tentorial muscle attachment (Fig. 4D, F).

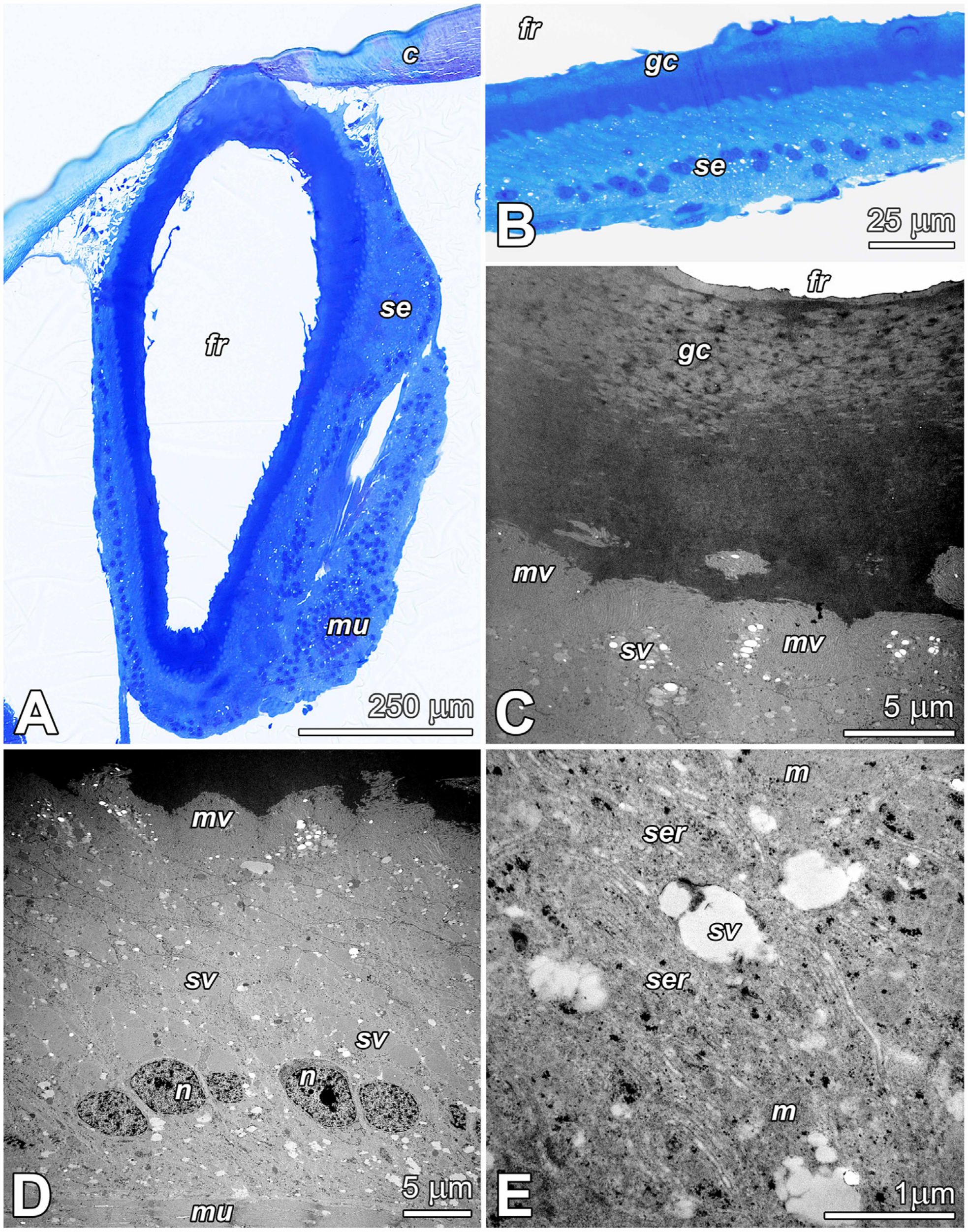

Fig. 3. Frontal gland in soldiers in *Stylotermes faveolus*. **A.** Parasagittal section of the frontal gland. **B.** Detail of the frontal gland secretory epithelium. **C.** Secretory cuticle and epithelium apex. **D.** Cross section of secretory epithelium. **E.** Detail of secretory organelles. **Abbreviations**: *c*, cuticle; *fr*, frontal gland reservoir; *gc*, glandular cuticle; *m*, mitochondria; *mu*, muscle; *mv*, microvilli; *n*, nucleus; *se*, secretory epithelium; *ser*, smooth endoplasmic reticulum; *sv*, secretory vesicle.

3.3. The labial glands

The paired labial glands are made of clumps of secretory cells (acini) connected by excretory ducts to the thin-walled reservoirs and together open at the base of the labium. The glands are developed similarly in both castes in *S. faveolus*, but the volume of secretory cells is about 10-times larger in workers compared to soldiers while the reservoirs are roughly of the same volume in both castes (Table 1). The acini are located in the pro- and mesothorax, along the esophagus and the crop, and the reservoirs are placed posteriorly, reaching to the first abdominal segment (Fig. 5A). Acini consist of principal and parietal cells. The parietal cells (5–10 μm in the longest dimension) are scattered throughout the acini. They reveal long and dense apical microvilli (about 2 μm long and 90 nm thick), nuclei made of dispersed chromatin, extraordinarily numerous mitochondria (up to 1.5 μm long and 200 nm thick) filling much of the cell volume, and relatively rare rough ER and electron-dense granules. The basal invaginations are well-developed, sometimes reaching almost to the apical microvilli. The principal cells are large (15–30 μm in the largest dimension) and have a limited contact with the excretory duct, and the apex is formed by spaced microvilli about 1.5 μm long. The basally placed nuclei are slightly irregular, ovoid, 6–8 μm in the longest dimension, filled predominantly with dispersed chromatin. Mitochondria are rather rare and small (0.6–1.2 μm long and about 250 nm thick) in the principal cells. The cytoplasm includes predominantly rough ER and the Golgi apparatus, and secretory vesicles. The ratio between vesicles to rough ER volume varies among cells, with type A

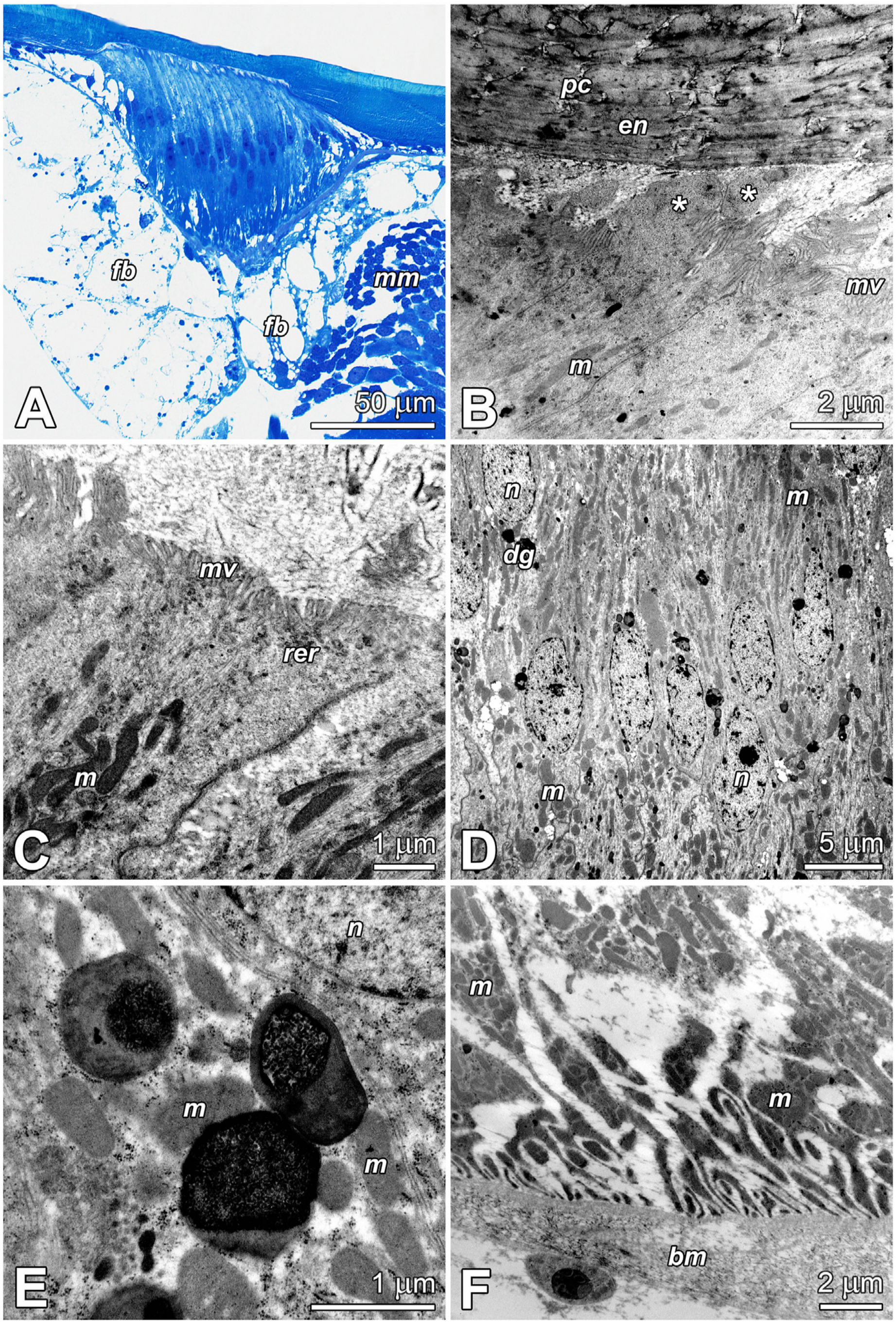

Fig. 4. Frontal gland in workers in *Stylotermes faveolus*. **A.** Sagittal section of the frontal gland. **B.** Apex of secretory cells showing the detachment of the apical cytoplasm (marked by asterisks). **C.** Apical region of the secretory cells. **D.** Central region of the secretory cells. **E.** Detail of the different stages of transformation of electron-dense granules into heterogeneous material. **F.** Apical region of a secretory cell close to the fronto-tentorial muscle attachment showing a rich system of basal invaginations. **Abbreviations**: *bm*, basement membrane; *dg*, electron-dense granule; *en*, endocuticle; *fb*, fat body; *m*, mitochondria; *mm*, mandibular muscles; *mv*, microvilli; *pc*, pore canal; *n*, nucleus; *rer*, rough endoplasmic reticulum.

containing only electron-lucent vesicles with granular material, or type B with only homogeneous electron-dense material. In soldiers, type A is more common than type B (3:1 ratio), and the number of secretory vesicles is exceptionally high in some cells while rather low in others, upon expense of rough ER and the Golgi apparatus. The type-B cells contain rather stable moderate amounts of secretory vesicles. The opposite is true for workers, in which type-A cells are less common and contain moderate amounts of secretion, while the type-B cells are more common and have a highly variable ratio between secretory vesicles and rough ER (Fig. 5B, C, D, E). The intra-acinar ducts are made of simple flat cells almost devoid of organelles, apart from a few mitochondria, small amounts of rough ER, and a nucleus made of predominantly condensed chromatin. Outside of the acinus, duct cells abruptly change into thick epithelium made of cubic cells, with dense apical microvilli, populous mitochondria, and nuclei made of dispersed chromatin. The cuticular intima consists of epicuticular layers (outer epicuticle about 10 nm and inner about 5 nm thick) inside of the acini, while

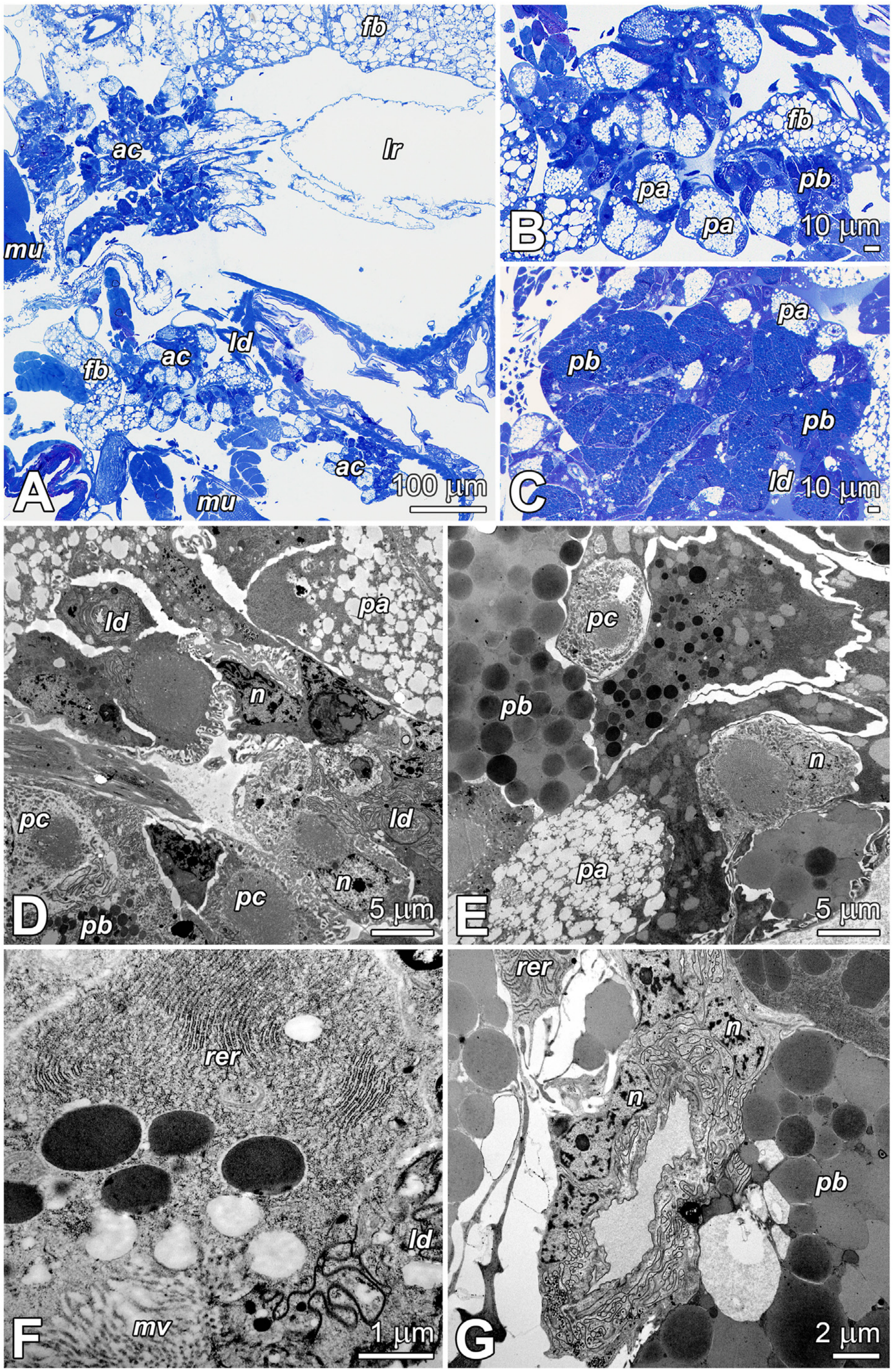

Fig. 5. Labial glands in workers and soldiers in *Stylotermes faveolus*. **A.** Oblique section of worker thorax/abdomen boundary. **B.** Part of the labial gland acini in soldier showing prevalence of type-A principal cells. **C.** Part of the labial gland acini in worker showing prevalence of type-B principal cells. **D.** Acini in a soldier showing prevalence of type-A principal cells. **E.** Acini in a worker showing prevalence of type-B principal cells. **F.** Detail of rough endoplasmic reticulum and secretory vesicles. **G.** The intra-acinar duct in worker. **Abbreviations**: ac, acini; *fb*, fat body; *mu*, muscle; *ld*, labial gland duct; *lr*, labial gland reservoir; *mv*, microvilli; *n*, nucleus; *pa*, type-A principal cells; *pb*, type-B principal cells; *pc*, parietal cell; *rer*, rough endoplasmic reticulum.

changes into ribbed epicuticle (outer about 10 nm and inner about 20 nm thick) along with the change of the duct cells ultrastructure. The thicker ducts are supported with a taenidium, which starts as a thin strand (100 nm in diameter) getting much thicker (up to 500 nm) with increasing duct diameter. The water sac reveals wrinkled intima made of 15 nm thick epicuticle, and simple epithelial cells with no marked organelles (Fig. 5F, G).

3.4. Composition of the frontal gland secretion

Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry shows 11 monoterpenes produced by the frontal gland of soldiers in both species, *S. faveolus* and *S. halumicus* (see Table 2 and Figs. 6 and 7). Seven of these compounds are quantified (Table 2). In soldiers, the overall quantity of

Table 2
Identification and quantification of terpenes from the frontal gland of the soldiers in *Stylotermes faveolus* (n = 2) and *S. halumicus* (n = 9). β-pinene and β-myrcene were coeluting together in *S. halumicus* so the sum of both is reported for this species. NQ = Not quantified because standards were not available.

Compound	Similarity (%)	RI measured	RI Nist	RI difference	Amount (ng±SD) per soldier	
					Stylotermes faveolus (n = 2)	*Stylotermes halumicus* (n = 9)
α-pinene	93.2	937	935	2	110 ± 19	12. ± 14
Camphene	93.0	952	953	−1	8.4 ± 0.9	4.5 ± 5.5
β-pinene	94.3	981	978	3	137 ± 13	–
β-myrcene	93.0	1005	1001	4	67 ± 7.4	–
β-pinene + β-myrcene	–	–	–	–	203 ± 5.8	52 ± 53
α-phellandrene	75.4	1007	1005	2	NQ	NQ
α-terpinene	86.9	1018	1018	0	80 ± 37	≤0.5
Limonene	93.2	1033	1030	3	223 ± 82	26 ± 27
β-phellandrene	76.6	1033	1031	2	NQ	NQ
cis-ocimene	81.8	1044	1040	4	NQ	NQ
γ-terpinene	84.9	1063	1054	9	6.6 ± 1.2	≤0.5
Terpinolene	91.1	1096	1089	7	NQ	NQ

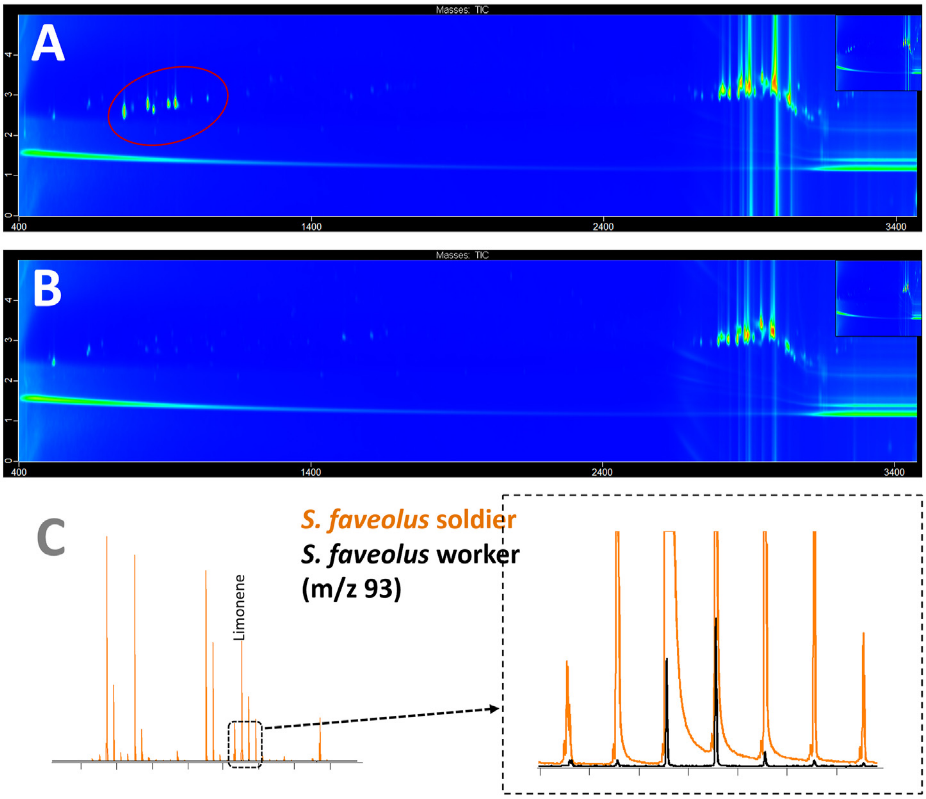

Fig. 6. Two-dimensional GC–MS analysis of an extract of 5 frontal gland equivalents of *Stylotermes faveolus* soldier (A), worker (B) and both (C). The spots in the circle of the sub-figure A correspond to the terpenes identified in Fig. 7. The quantity of terpenes differs between soldiers and workers. As an example, the specific mass for the terpene *m*/*z* 93 is highlighted in sub-figure C to illustrate the difference in limonene content between soldier (in orange) and worker (in black).

the frontal gland secretion reaches over 700 ng in *S. faveolus*, while we observed only less than 150 ng in *S. halumicus*. α and β-pinene and α-terpinene are present below the limit of quantification (0.5 ng/gland), in *S. faveolus* workers. Only one terpene, limonene, is below the limit of quantification, presenting only traces (1–2 ng/gland) in soldiers of the same species (Fig. 6).

4. Discussion

Termites primarily rely upon passive defenses, i.e., strategies that render them undetectable to non-specialized predators (Šobotník et al., 2010a). Additionally, they are exceptional architects and builders, spending much of their lives enclosed in their highly modified habitat within a constructed nest, wood, or soil galleries (Grassé, 1984). They are sensitive to disturbance, and their behavior abruptly changes after exposure to light or air currents (Sillam-Dussès et al., 2023). While larvae and other developing stages can only retreat when they are discovered by a predator, workers, soldiers, and alates often participate in defensive activities. Several derived groups of termites have secondarily lost the soldier caste, and in such lineages the workers have a greater degree of aggressiveness and sometimes also well-developed defensive glands, such as the frontal gland in many genera of Apicotermitinae (Šobotník et al., 2010b) or dehiscent glands in *Ruptitermes*, unrelated *Apolemotermes*, and several other African genera (Poiani and Costa-Leonardo, 2016; JŠ, personal observation). However, the

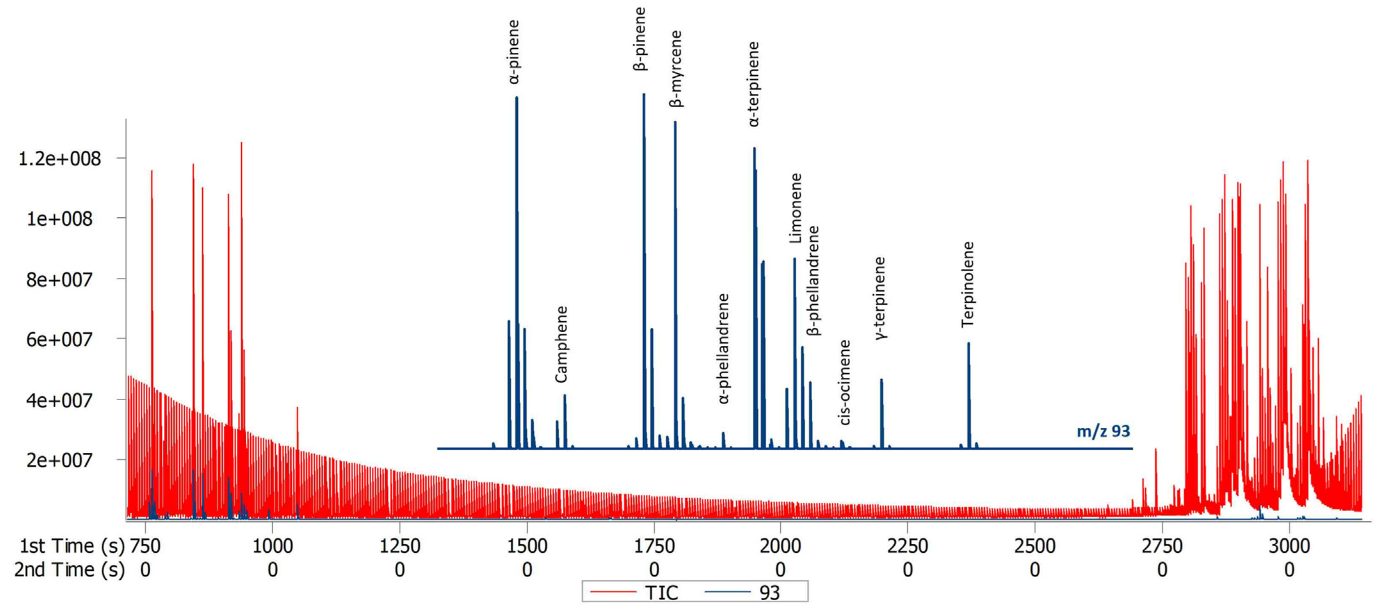

Fig. 7. Chemical identification of the terpenes detected in the frontal gland of soldiers in *Stylotermes faveolus*. Ion chromatogram (in red), extracted characteristic mass of terpene compounds *m*/*z* 93 (in blue).

soldiers, when they occur, are the primary defenders, and their mandibles are usually (but not always) long and robust. The soldier mandibles have a rich dentition in Mastotermitidae, Hodotermopsidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, and many Kalotermitidae, while many Neoisoptera, including Stylotermitidae, have narrow, saber-shaped mandibles. In *Stylotermes*, the soldiers guard the openings of their nest and use their mandibles to bite any predators attempting to enter. Because colonies live inside living and hard trunks used both for nesting and foraging, the defensive strategy of the soldiers is quite efficient even though individuals of this caste are quite rare (8% in *S. faveolus* and only 2% in *S. halumicus*, unpublished). Moreover, the transition from crushing to reaping/slashing mandibles in the evolution of termites was accompanied by much higher amplitude of the mandibular movements (see Deligne et al., 1981), which can thus inflict much deeper wounds with a smaller power. The diminished volume of mandibular muscles along with the transition from crushing to reaping mandibles allowed for the evolution of the frontal gland, which originates as an epithelial invagination in the region of the most anterior portion of the frons (Grassé, 1982; Bordereau et al., 1997). The presence of the frontal gland in soldiers is the defining feature of Neoisoptera, and *Stylotermes* is no exception, although the frontal gland is much smaller than all remaining non-termitid Neoisoptera.

The frontal gland was mostly studied in soldiers, where it usually forms a large sac often reaching deep into the abdomen, especially in Rhinotermitidae and Serritermitidae, while in Termitidae it is more commonly confined to the head only (Deligne et al., 1981; Quennedey 1984; Šobotník et al., 2010a, 2010c). In *Stylotermes* soldiers, however, it forms a rather small (about 1% of the total head capsule volume), thick-walled reservoir, similar to imagoes of Rhinotermitidae (especially *Prorhinotermes*), Serritermitidae (especially *Glossotermes*), and Macrotermitinae and Foraminitermitinae among termitids (Šobotník et al., 2005, 2010d; Kutalová et al., 2013). Irrespective of the frontal gland size, there are three distinct modes of the secretion discharge in Neoisoptera: (*i*) hydrostatic power, i.e., increase of inner body pressure by contraction of intersegmental muscles, (*ii*) pinching, i.e., the frontal gland reservoir is squeezed by an anterior branch of the mandibular adductor (musculus craniomandibularis internus), and (*iii*) piston, when the whole reservoir is placed between anterior and posterior branches of the mandibular adductor (Kaji et al., 2016). The same mechanisms are expected to occur also in neoisopteran imagoes, in which the extent of the frontal gland is largely variable, from absent to a huge sac reaching deep into the abdomen (Šobotník et al., 2010d; Kutalová et al., 2013). However, the increase of pressure on the reservoir does not alone allow the secretion to flow out, and an additional fronto-tentorial muscle is needed to open the fontanelle. The contraction of this muscle pulls the anterior part of the secretory epithelium further from the fontanelle, which is otherwise kept blocked (Šobotník et al., 2010d; Kutalová et al., 2013), allowing the soldiers and imagoes to control the secretion release. At the same time this muscle has three ental attachments - to the median tentorium, to the mouth, and to the hypopharyngeal base (Wipfler et al., 2016, 2022) - and its contraction surely affects each of these structures, and this may explain repeated mandible openings accompanying the release of the frontal gland secretion (Sieber and Leuthold, 1981; Šobotník et al., 2008b).

The frontal gland secretory epithelium is in *S. faveolus* made of class I secretory cells (*sensu* Noirot and Quennedey 1974) similarly to all other termites studied so far with the exception of *Coptotermes*, in which class I and III secretory cells form the epidermal lining (Quennedey 1984; Bordereau et al., 1997; Šobotník et al., 2005, 2010c). The observed secretory organelles in *Stylotermes* occur frequently in the exocrine organs of termites (Costa-Leonardo et al., 2023). The most important secretory organelle, smooth ER, is known to produce non-polar and/or volatile compounds including monoterpenes in many insect species (Noirot and Quennedey, 1974; Percy-Cunningham et al., 1987; Tillman et al., 1999), which is congruent with the observed secretion of the frontal gland in *Stylotermes* (see below). Moreover, the lipid-like droplets observed in the cytoplasm of the secretory cells in *Stylotermes* could represent the final secretion, as the monoterpenes do not dissolve in water and are thus expected to be located freely in the cytoplasm. The frontal gland secretion in *S. faveolus* soldiers contains a series of monoterpenes, α- and β-pinene, camphene, β-myrcene, α-terpinene, limonene, and terpinolene, known to play many important biological functions, especially in plant defenses, and insect defense and communication (Blum, 1982). The frontal gland of soldiers in *S. halumicus* contains the same compounds but the concentration is lower. It is even more true for the frontal gland of workers in *S. faveolus*, the compounds being below the detection limit with the exception of a single compound (limonene). The quantity of compounds found in *S. halumicus* soldiers is quite low and represents only about 0.45% of the overall frontal gland volume (recalculated from density of α-pinene 858 kg/m^3 and frontal gland volume in Table 1). The secretion of frontal gland usually reaches units or tens of μgs per soldier in Neoisoptera (for review see Šobotník et al., 2010a), but in most of Rhinotermitidae and Serritermitidae, the frontal gland is much larger and reaches deep into the abdomen (Deligne et al., 1981; Šobotník et al., 2004, 2010c; Costa-Leonardo et al., 2023). Monoterpenes also occur in many other termites, especially in Nasutitermitinae (Azevedo et al., 2006; Goh et al., 1990; Chuah et al., 1991; Valterová et al., 1993), but also in *Reticulitermes* (Bagnères et al., 1990; Nelson et al., 2008), *Amitermes* (Moore, 1968; Braekman et al., 1993), and *Cyrilliotermes* (Baker et al., 1981). The monoterpenes are generally considered toxic, and α-terpinene and limonene cause fast pathological changes in vertebrate models (for a review see Wojtunik-Kulesza, 2022). Perhaps more importantly, all monoterpenes occurring in *Stylotermes* are also known from the frontal gland secretions of Nasutitermitinae, in which they are used as irritating compounds against invertebrates, and they induce long-term scratching and cleansing behavior, as well as an avoidance of the source of release (Prestwich, 1984; Šobotník et al., 2010a). The same compounds may also acquire a pheromonal function, such as α- and β-pinene, limonene, and terpinolene, which are alarm pheromones in *Reticulitermes* spp. and in some Nasutitermitinae (Vrkoč et al., 1978; Roisin et al., 1990; Lindström et al., 1990; Reinhard et al., 2003; Šobotník et al., 2010a; Cristaldo et al., 2015; Delattre et al., 2019).

The epidermal thickening occurring in workers of *S. faveolus* can be homologized with the frontal gland in soldiers of other neoisopterans, due to its position as evidenced by attachment of the same fronto-tentorial muscle and its secretory function, although it is different from the one in soldiers. The frontal gland is often present in neoisopteran workers (JŠ, personal observation); however, it has hitherto been studied in detail only in soldierless Apicotermitinae, in which it is similarly sized with the exception of *Tonsuritermes* having a much larger frontal gland (Šobotník et al., 2010b; Constantini et al., 2018). The differences between *Aparatermes* and *Stylotermes* consist in the absence of specialized envelope cells in *Stylotermes*. Moreover, there are numerous adipocytes close to the frontal gland in *Stylotermes* workers (see Fig. 4), which may support the biochemical machinery of the secretory cells and reveal various degrees of specialization to this function. *Aparatermes* workers also showed much higher amounts of biocrystals, while much lower amounts of granular secretion and many more mitochondria per secretory cell were observed in *Stylotermes*.

As the secretory organelles comprise the rough ER and the secretion is proteinaceous, we do not expect the gland to be involved in direct defense or communication, but rather in producing antimicrobial peptides or in playing another protective function. Our observation confirms that the presence of the frontal gland in all older individuals is indeed an important autapomorphy of Neoisoptera, and this gland is directly involved in the defensive behavior of soldiers and imagoes, while the function of the proteinaceous secretion produced by workers remains unknown but is likely also somewhat defensive.

Likewise, the structure of the labral and labial glands corresponds to the general scheme observed in other termite species. All aspects of the labral gland development in workers and soldiers of *Stylotermes* are commonly seen in other termites (Palma-Onetto et al., 2018, 2019). The gland in *Stylotermes* is made of class I secretory cells only, similar to most termites with the exception of *Mastotermes*, *Dolichorhinotermes*, *Glossotermes*, and *Termes*, which also include class III cells. The main secretory organelle is smooth ER, which is compatible with the production of volatile signals of unknown composition. Due to the lack of knowledge on the mode of secretion, the secretion release is observed most easily in soldiers and sometimes also in workers after encountering an alien insect, when they wipe their mouths against the substrate (Palma-Onetto et al., 2019).

Labial glands belong to the body plan of all insects and have an acinous form in all Polyneoptera (Chapman, 2013). The development in *Stylotermes* is similar to other termites, and the differences between workers and soldiers closely resemble the situation observed in *Prorhinotermes* (Šobotník and Weyda, 2003). Labial glands are known to play several roles among termites – production of digestive enzymes, food for dependent castes, and cement for building activities in workers, while in soldiers they produce predominantly defensive compounds released from the mouth (Noirot, 1969; Grassé, 1982; Sillam-Dussès et al., 2012). At the same time, the reservoirs are used to carry water in workers (Grube and Rudolph 1999), while the soldiers store copious amounts of defensive secretion in the reservoirs, especially in *Mastotermes* and Macrotermitinae (Moore, 1968; Maschwitz et al., 1972; Plasman et al., 1999). In *Stylotermes,* the type-A secretory cells seem, similar to *Prorhinotermes*, to produce the defensive secretion while type-B cells secrete digestive enzymes, and this view is supported by the dominance of type-A cells in soldiers and type-B cells in workers (Šobotník and Weyda, 2003).

Termites are one of the few groups able to decompose abundant dead plant matter, from which they produce a remarkable biomass, especially in tropical areas. Living in wood, as is the case in several basal Neoisoptera including *Stylotermes*, is by itself a powerful defensive strategy, as the already existing tunnels can be sealed easily or defended by soldiers prepared to kill whatever intruder is capable of squeezing into the narrow passages. During aggressive encounters, the mandibular bites are always accompanied by the release of defensive secretions originating primarily from the labial glands, and secondarily also from the frontal gland in Neoisoptera, both having complementary functions due to their non-polar and polar natures, respectively. Although the labral gland is not involved directly in defensive actions in workers and soldiers in Neoisoptera, it is likely used to mark territories during periods of danger, where aliens are expected, and defensive measures should be taken (Palma-Onetto et al., 2018, 2019). The frontal gland, as the primary defensive organ in derived termites, produces in *Stylotermes* a series of monoterpenes known from other termite species. Given the ubiquity of terpenoid compounds in the frontal gland secretions of Stylotermitidae, Rhinotermitidae, and Termitidae, we can conclude that they comprise the principal cocktail components of the ancestral neoisopteran, necessary for the unprecedented ecological success of these derived termite lineages. While termites first diverged from their roach relatives in the Late Jurassic, the diversification of Isoptera was seemingly slow (Engel et al., 2009). Termites were successful and important specializations were critical to their initial success and persistence, such as the presence of a soldier caste, but simply lacked the ecological heft and ubiquity of their modern counterparts (Engel et al., 2009, 2016; Chouvenc et al., 2021). The extant families that derive from among this initial diversity are few in species and often confined to comparatively relic distributions (Krishna et al., 2013). In the Late Cretaceous we find the earliest Neoisoptera and during the Cenozoic this lineage diversified dramatically, particularly in the Termitidae, producing the considerable breadth of species that today dominate tropical ecosystems throughout the world. The frontal gland, as the key trait characterizing the lineage, seems to have played a critical role in the evolutionary success of termites as we understand them today. This gland, along with its unique cocktail of defensive components, provided the initial impetus that would, in conjunction with subsequent evolutionary novelties in diet, gut microbiomes, and soldier morphology, give us the world's greatest ecosystem engineers. Whether this was strictly the fortuitous accumulation of critical adaptations driving increased speciation or a complex interplay between speciation and a decline in natural extinction (i.e., more species persisting following speciation as a result of these specializations rather than strictly an increase in the rate of species generation), remains the next challenge in deciphering the remarkable evolution of termites.

CRediT authorship contribution statement

Himanshu Thakur: Writing – original draft, Investigation, Formal analysis, Data curation. **Surbhi Agarwal:** Formal analysis, Data curation. **Aleš Buček:** Formal analysis, Data curation. **Jaromír Hradecký:** Formal analysis, Data curation. **Hana Sehadová:** Formal analysis, Data curation. **Vartika Mathur:** Supervision, Resources, Funding acquisition. **Ulugbek Togaev:** Data curation. **Thomas van de Kamp:** Formal analysis, Data curation. **Elias Hamann:** Formal analysis, Data curation. **Ren-Han Liu:** Formal analysis, Data curation. **Kuldeep S. Verma:** Supervision, Resources. **Hou-Feng Li:** Supervision, Formal analysis. **David Sillam-Dussès:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation. **Michael S. Engel:** Writing – review & editing. **Jan Šobotník:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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