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**Evolution of cephalic glands in termites**

**Evoluce hlavových žláz termitů**

Dizertační práce

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"Prohlašuji, že jsem disertační práci na téma *Evolution of cephalic glands in termites* vypracovala samostatně s použitím uvedené literatury a na základě konzultací a doporučení školitele. Souhlasím se zveřejněním disertační práce dle zákona č. 111/1998 Sb. o vysokých školách v platném znění, a to bez ohledu na výsledek její obhajoby."

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## Abstract

Eusocial animals rely on communication, their cooperation allowed them being among the most competitive organisms on the planet. Termites are in fact eusocial cockroaches and the general consensus treats them as Blattodea epifamily Termitoidae. They are fully adapted for wood and soil digestion, and host rich symbiotic microbial communities in their hind gut. It makes them a group responsible for enormous biomass turnover, and important players influencing the climate on Earth. Their exocrine glands are source of various semiochemicals. 23 exocrine glands were described so far in termites. 18 are exclusive for them, 5 glands were common for termites and cockroaches. This thesis deals with two novel exocrine glands unique for termites, and one gland ancestral to all termites and their sister group Cryptocercidae. Optical and electron microscopy techniques were employed together with ancestral state reconstruction and behavioural tests to learn gland's function and evolution of traits related to their occurrence. The two novel glands are **nasus gland** and **clypeal gland**. The latter is present in three most derived termite families, however the older lineages of termites must be also inspected by future studies. The nasus gland is only found in one genus of Nasutitermitinae (Termitidae or "higher" termites), but similar structures were found in other unrelated species within the subfamily. The **labral gland** has previously been described only in soldiers of several termite species, however it is the organ common to all castes and species of termites and to *Cryptocercus punctulatus*.



## Abstrakt

Eusociální živočichové jsou závislí na komunikaci mezi jedinci. Jejich spolupráce vyústila v jejich vysokou početnost a přizpůsobivost. Termiti jsou nyní definováni jako eusociální švábi, jelikož nedávno ztratili status řádu a stali se epičeledí Termitoidea v rámci Blattodea. Jsou plně adaptováni na trávení potravy jako je dřevo či hlína, a to díky bohatým mikrobiálním společenstvům obývajících jejich zadní střevo. Tato vlastnost je činí zodpovědnými za rozklad biomasy v globálním měřítku, čímž ovlivňují i klimatické podmínky na Zemi. Jejich exokrinní žlázy jsou zdrojem mnoha semiochemikálií. Doposud bylo popsáno 23 exokrinních žláz termitů, z čehož 18 je unikátních pro termity a 5 je společných i švábům. Tato práce se zabývá dvěma novými žlázami nalezenými výlučně u termitů a jednou žlázou, která je společná i sesterské skupině švábů, Cryptocercidae. Byly využity techniky optické a elektronové mikroskopie spolu s rekonstrukcí ancestrálního stavu znaků u jednotlivých linií termitů a s behaviorálními testy. Cílem bylo získat informace nejen o struktuře a ultrastruktuře žláz, ale i o jejich funkci a evoluci s ní spřažených morfologických adaptací. Mezi nově popsané žlázy patří nasální žláza a klypeální žláza. **Nasální žláza** je společná jednomu rodu Nasutitermitinae (Termitidae neboli vyšší termiti), zároveň však byly podobné struktury pozorovány i u jiných nepříbuzných druhů v rámci podčeledi. **Klypeální žláza** se vyskytuje u 3 nejodvozenějších čeledí termitů, přičemž bazální skupiny budou subjektem následujících prací. **Labrální žláza** byla původně popsána pouze u vojáků několika druhů, přestože je orgánem, který je společný všem kastám i druhům termitů i švábům *Cryptocercus punctulatus*.

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## Aims of the thesis

- 1) Understanding termite biology and complexity of termite glands through background research.
- 2) Description of the newly-discovered nasus gland in *Angularitermes soldiers*.
- 3) Description of the clypeal gland, a new exocrine gland of termite imagoes.
- 4) Evolution of the labral gland in termite soldiers.

# 1 Background

## 1.1 Introduction

Insects (Class: Insecta) represent the most abundant and speciose living group. The still-growing number of described species has recently reached 1 000 000 (Stork, 2018), forming thus over 50 % of all described Eukaryota. Insects are similarly important in terms of abundance (Bar-On et al., 2018). The colossal biomass and biodiversity of insects implies its key role in ecology of terrestrial biomes on the planet. Insects act as grazers, decomposers or pollinators; they are food source for birds and other animals, they regulate their own population through predation and parasitism. The first insects evolved approximately 400 million years ago, in Silurian era, and the oldest current orders are e. g. Ephemeroptera, Odonata or Orthoptera with the last common ancestors living roughly 300 million years ago. Nevertheless, many of recent insect groups appeared in the fossil record later on, mostly in cretaceous era, approx. 150 to 100 million years ago, and insects evolved plethora of extreme adaptations derived from the common body plan since then (Grimaldi & Engel, 2005).

Termites, formerly Isoptera (iso – the same, pteron - wing), are now classified as epifamily Termitoidae (sensu Lo et al., 2007; Eggleton et al., 2007), as they are an inner group of cockroaches (Blattodea) (Lo et al., 2000, Inward et al. 2007a). Termites are prognathous insects. They possess two pairs of wings, they are mostly blind, compound eyes are found in alate individuals. Albeit small in taxonomical sense (currently comprising of approximately 3 000 species; Krishna et al., 2013), they largely dominate warm regions and comprise biomass comparable to mankind or ants, if not higher (Bignell, 2016; Bar-On et al., 2018). Their abundance is truly tremendous as about 5 000 of individuals per m<sup>2</sup> live in central Africa (Eggleton et al., 1996). Termites are the most important decomposers of dead plant matter (Davies et al., 2003), playing a crucial role in nutrient recycling in the tropics and subtropics. They can convert 50 to 100 % of

crude plant biomass to frass (Bignell & Eggleton, 2000; Bignell, 2016), and are thus labelled “ecosystem engineers” (Jouquet et al., 2006). Termite actions have remarkable impact on their environment, and they are suspected from ceasing the coal layers deposition after they expanded to all continents (Engel et al., 2009). Similarly, they are responsible for a great deal of CO<sub>2</sub> and methane production due to the digestion processes (Sugimoto et al., 2000). Other vital effects of termite activities is soil aeration, changing topography of a locality via moving immense amounts of organic material (counted by tons of material per hectare and year in the tropics), participation on soils formation and increasing its heterogeneity important for many other groups (Jouquet et al., 2006; Eggleton, 2011; Evans et al., 2011; Bignell, 2016). Due to their enormous abundance, termites represent an important source of food for a number of less or more specialised predators (Deligne et al., 1981; Redford & Dorea, 1984; Bourguignon et al., 2017). Although the importance of termites is indisputable, their taxonomy is still not satisfactorily resolved, and dozens of new genera were described in last few years (Scheffrahn et al., 2018). On the other hand, the higher classification suffers from pre-molecular phylogenies and obsolete  $\beta$ -taxonomical opinions, and several important taxa remain paraphyletic or even polyphyletic (see Bourguignon et al., 2015a; 2017), and the higher classification will have to change considerably in the future.

My thesis focuses on exocrine glands of termites, of which I conducted morphological, anatomical and functional research on predominantly cephalic glands. Social and eusocial insect possess numerous exocrine glands since communication is the key factor for information transmission and the colony coherence. Social insects always develop more exocrine organs compared to their non-social relatives (Billen & Šobotník, 2015), and the same is true also for termites having 23 glands described so far, while other cockroaches possess only 17 of them (Křížková et al., 2014). While some glands are common to all termites, others are in their taxonomic distribution restricted to few or even one

species only. My work led to description of several new cephalic glands that are specific to Neoisoptera imagoes (Křížková et al., 2014) or *Angularitermes* (Termitidae: Nasutitermitinae) soldiers (Šobotník et al., 2015), but I also participated on the evolutionary study of the labral gland present in all termites together with their sister group, *Cryptocercus* wood roach (Palma-Onetto et al., 2018; 2019).

## 1.2 Phylogeny and classification of termites

Termites, formerly ranked as the order Isoptera, are now classified as epifamily Termitoidae within the order Blattodea with the closest living relative, the woodroach *Cryptocercus* (Lo et al., 2000; Inward et al., 2007a; Xiao et al., 2012; Bourguignon et al., 2015a). Blattodea, together with Mantodea, form superorder Dictyoptera that belongs into the cohort Polyneoptera, along with grasshoppers, katydids, earwings, web-spinners, stick insects, stone flies and some other groups. In 2007 (a), Inward et al. came with a hypothesis that termites are supposed to be a family within the order Blattodea, since Cryptocercidae is a family and sister taxons should have the same rank. However during that year, a series of replies in scientific journals provided termites with an epifamily status in order to help the classification undergo minimal changes (Lo et al., 2007). This rank was later confirmed by an independent study (Xiao et al., 2012). Common ancestor of termites and *Cryptocercus* lived roughly 170 million years ago during the Jurassic era, well-before the breakup of Pangea (Bourguignon et al., 2015a; Evangelista et al., 2019).

Phylogeny of termites went through major changes in last 20 years, along with the rapid development of molecular techniques (Miura et al., 1998; Donovan et al., 2000; Lo et al., 2000; Austin et al., 2004; Inward et al., 2007a; Legendre et al., 2008; Davis et al. 2009, Engel et al., 2009; Cameron et al., 2012; Bourguignon et al., 2015a; 2017). The phylogenetic hypothesis relevant for my thesis is adopted

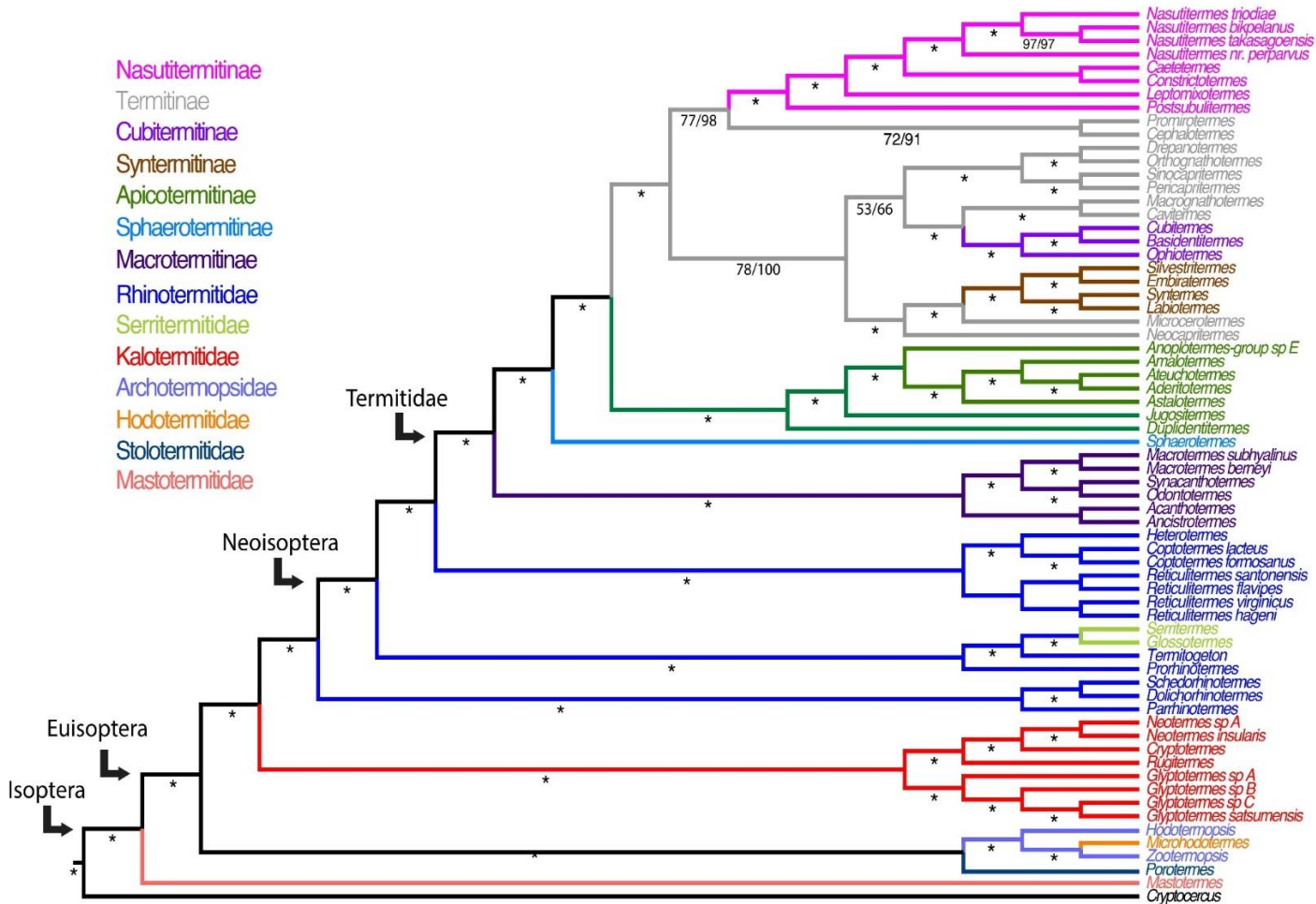


Figure 1: Phylogenetic tree with methodology from Bourguignon et al., 2015a, unused in the publication.

from Bourguignon et al. (2015a & 2017; Fig. 1). Within termites, 2 big groups are recognized: Euisoptera and Neoisoptera. The former are characterized by absence of ovipositor and lack of anal lobe in the wings and less than 5 tarsomeres in at least one pair of legs. The latter share presence of frontal gland and specific wing venation (Eggleton, 2011). “Higher” termites (Termitidae) are usually differentiated from the “lower” termites (all other families), which is a paraphylum, therefore not a valid taxonomical rank. The basalmost family Mastotermitidae is a sister group of Euisoptera. The next branch is made of Stolotermitidae and Archotermopsidae, within which the family Hodotermitidae



is nested. Next branch is speciose family Kalotermitidae, a sister group to Neoisoptera. Stylotermitidae represent the basal group of Neoisoptera, and the



Figure 2: *Mastotermes darwiniensis*, JŠ, pers. archive

next branch is one of independent taxa of „Rhinotermitidae“, currently subfamily Rhinotermitinae. Following monophylum is formed by genera *Prorhinotermes*, *Psammodermes* and *Termitogeton*, together with family Serritermitidae. The last group of „Rhinotermitidae“ is monophylum formed by genera *Reticulitermes*, *Coptotermes* and *Heterotermes*, being together a sister group of the crown family Termitidae.

### 1.2.1 Mastotermitidae

Mastotermitidae have probably evolved roughly 150 million years ago in upper Jurassic (Bourguignon et al., 2015a). Although it was formerly distributed worldwide, this lineage is represented by a single living species *Mastotermes darwiniensis* (Fig. 2). Original distribution of *Mastotermes* comprises the north of Australia, however it was introduced by man to south Papua (Thorne et al., 2000;

Evans et al., 2013). *Mastotermes darwiniensis* is an example of mosaic evolution, as it reveals combination of plesiomorphic and apomorphic traits. The former comprise presence of anal lobe of hind wing pair, 5-segmented tarsi, eggs laid in ootheca by female ovipositor, *Blattabacterium* as a symbiont in fat body, and highest number of sternal and tergal glands among termites (Noirot, 1969; Ampion & Quennedey, 1981; Watson & Gay 1991; Grimaldi & Engel, 2008). The advanced traits include life in large populous colonies, complicated structure of nests and food galleries, bifurcated ontogeny, alarm pheromone communication by labial gland secretion (Moore, 1968; Gay & Calaby 1970; Noirot & Pasteels, 1987; Delattre et al., 2015). A special case is the sperm cells that reveal approximately 100 flagella, a character unique among all animals (Baceti & Dallai, 1978).

Mastotermitidae are wood feeders foraging through complicated system of underground galleries on hard sound woody items. In the natural environments, the colonies are relatively small reaching roughly tens of thousands of individuals, while in the man-made habitats the colonies can grow much bigger and become serious threat to the timber (Krishna et al., 2013; JŠ, pers. comm.).

1.2.2 Termopsidae, Stolotermitidae, Archotermopsidae and Hodotermitidae Families, forming the most primitive, yet monophyletic clade of Euisoptera, have the most recent common ancestor dated back approximately 130 million years ago (Bourguignon et al., 2015a). Termopsidae is an extinct family from the Palearctic region, and the recent families Archotermopsidae and Stolotermitidae were split off it later on (Inward et al., 2007b, Engel et al., 2009). Stolotermitidae is the most basal group of this clade, and, according to modern phylogenies, Archotermopsidae are paraphyletic since Hodotermitidae are their inner group (Bourguignon et al., 2015a; Inward et al., 2007b).

Archotermopsidae (*Archotermopsis*, *Hodotermopsis*, *Zootermopsis*; 6 species) and Stolotermitidae (*Stolotermes*, *Porotermes*; 10 species) reveal primitive traits such as rich wing venation, long cerci and genital anatomy (Weesner, 1969). Sternal gland is always located on the 4<sup>th</sup> sternite (Quennedey et al., 2008). All species feed on a damp wood, where they also reproduce (Pearce & Waite, 1994; Eggleton, 2000). Stolotermitidae are relics of Gondwanan distribution, while Archotermopsidae occupy relict distribution in Neotropic, Nearctic and Oriental region.

The ontogeny of Archotermopsidae is linear, and so they have no true workers, but pseudergates (Roisin, 2000; Krishna et al., 2013; see Chapter 1.6). Their colonies are formed by a small number of individuals, for example only around 40 in *Archotermopsis wroughtoni* (Roonwal et al., 1984). Their reproduction is not strictly limited to royal couple, since they can reproduce also as neotenic reproductives or even fertile soldiers (Shellman-Reeve, 1997).

Hodotermitidae live in Palearctic region, they comprise of 3 genera (*Anacanthotermes*, *Hodotermes* and *Microhotermes*) and 21 species in total. They are inner group of Archotermopsidae with *Zootermopsis* as their sister taxon (Bourguignon et al., 2015a). Their external morphology is similar to the previous families (Krishna, 1970), and they also share the same position of the sternal gland on the 4<sup>th</sup> abdominal sternite (Quennedey et al., 2008). Among the “lower” termites, they are the only grass-feeders specialized on dry habitats. They forage in open space and mostly in the night, therefore all castes retain compound eyes (Heidecker & Leuthold, 1984). The way-of-life is derived and different from all “lower” termites in Hodotermitidae. They build underground nests of soft fragile material, which is inhabited by several tens of thousands of individuals. The ontogeny is bifurcated (see Chapter 1.6), the workers are of both sexes, while soldiers are all males (Watson, 1973; Watson & Sewell, 1981; Grassé, 1986; Noirot & Pasteels, 1987).



Figure 3: *Epicalotermes kempae*, JŠ, pers. archive

### 1.2.3 Kalotermitidae

Kalotermitidae are a monophyletic group (Bourguignon et al., 2015a; Fig. 3). They currently comprise of 21 genera and 456 species which is making them the second most diverse family of termites. Their most recent common ancestor appeared roughly 80 million years ago. They are spread worldwide, however they rather live on the edge of tropical region, on top of hills or on dry patches in the tropics. It is given by their low competitiveness. They inhabit dry branches in tree corolla, even though they can also colonize dry wood lying on the ground. In lowland tropical rainforests, they specialize only on wood that is too hard for other termite species (JŠ, pers. comm.). Since their colonies are small and the ontogeny is linear (Roisin, 2000), they are very good in dispersal. That is the reason they have the broadest area of distribution of all termite families (Pearce & Waite, 1994; Eggleton, 2000; Krishna, 1961). Kalotermitid soldiers often show phragmotic strategy in defence (see Chapter 1.8).

#### 1.2.4 Stylotermitidae

Family Stylotermitidae has only one recent genus and 45 species (Krishna, 2013). They are nested at the base of the group Neoisoptera (Wu et al., 2018). They all inhabit Southeast Asia, nevertheless they were rarely collected and are thus poorly understood. They share some of the traits with Neoisoptera, mainly presence of the frontal gland and wing venation (Wu et al., 2018). They are specialized on hard living trees in the tropics as the only termites on the planet. Their ecology and caste system needs more attention of researchers as it is unknown.

#### 1.2.5 Serritermitidae

Serritermitidae is a very small family of Neoisoptera. According to Bourguignon et al. (2015a), they are inner group of „Rhinotermitidae“ and they originated about 75 million years ago. They are based in Neotropic region where both living genera are found, comprising of 3 species only. They have unique linear ontogeny combined with specialization according to sex. Only males are soldiers and workers (Bourguignon et al., 2009; Barbosa & Constantino, 2017). *Serritermes serrifer* is inquiline of *Cornitermes cummulans* in Brazil (Araujo, 1970; Shellman-Reeve, 1997), being actually the only inquiline among “lower” termites. *Serritermes* has specific shape of mandibles in soldiers and their frontal gland is capable of autothysis (see Chapter 1.8) (Costa-Leonardo & Kitayama, 1991). The genus *Glossotermes* is native to Brazil and French Guyana, its food is decaying wood (Jš, pers. comm.).

#### 1.2.6 „Rhinotermitidae“

„Rhinotermitidae“ is not a monophyletic group (Bourguignon et al., 2015a). They are created of three independent monophyla (Bourguignon et al. 2015a; 2017).

Altogether they comprise of 12 genera and 315 species (Krishna, 2013). The first monophylum is current subfamily Rhinotermitinae and it is represented by the genera *Acorhinotermes* (1 species), *Dolichorhinotermes* (7 species), *Macrorhinotermes* (1 species), *Parrhinotermes* (13 species), *Rhinotermes* (5 species) and *Schedorhinotermes* (34 species). In the future, this subfamily should be elevated to family rank (Bourguignon et al., 2015a; JŠ, pers. comm.). The second monophylum is formed by *Prorhinotermes* (11 species; Fig. 4),



Figure 4: *Prorhinotermes simplex*, JŠ, pers. archive

*Psammotermes* (6 species), *Termitogeton* (2 species) and by the family Serritermitidae. This group will probably get a new family name, Psammotermitidae, according the firstly described genus (Bourguignon et al., 2015a; JŠ, pers. comm.). The last monophyletic group formally still within „Rhinotermitidae“ is formed by *Reticulitermes* (138 species), *Coptotermes* (67 species) and *Heterotermes* (30 species). This group will be nominated Heterotermitidae (Bourguignon et al., 2015a; JŠ, pers. comm.).



They are distributed worldwide, however the highest diversity is reached in Southeast Asia (Pearce & Waite, 1994; Eggleton, 2000). Future Psammotermitidae have linear ontogeny, while all other species show branched ontogeny (Roisin, 1988; Bourguignon et al., 2009; Barbosa & Constantino, 2017). Often, the workers and soldiers are polymorphic due to sexual or instar specification (Roisin, 2000). Soldiers are usually mandibulate, however in Rhinotermitinae, they are nasutoid, their labrum is prolonged to enable application of frontal gland products, which are mostly contact poisons (Weesner, 1969; Prestwich, 1984a).

### 1.2.7 Termitidae

The family Termitidae is also known as “higher” termites thanks to its position in the crown of the phylogenetic tree. It contains 238 genera and 2 072 species (Krishna, 2013) which is great proportion of the biodiversity of termites (approximately 80 % of species; Bourguignon et al., 2015a). They evolved around 50 millions years ago after the breakup of Pangea and Gondwana (Bourguignon et al., 2015a). Within the family, the system of subfamilies is not completed yet. The monophyla that have been denominated and positioned correctly in the phylogenetic diagram are certainly Macrotermitinae, Foraminitermitinae, Sphaerotermitinae and Apicotermitinae (Bourguignon et al., 2017). Macrotermitinae are the most basal and the following subfamilies are in order of appearance in the cladogram. Further, Nasutitermitinae, Syntermitinae and Cubitermitinae form monophyletic clades within the group of Termitinae. The family will have to go through revision and it will be divided into several new subfamilies.

The morphology and physiology shared by Termitidae is wing venation, where the costal, subcostal and radial region merge (Weesner, 1969), and absence of flagellate protozoa in their hind gut (Noirot & Noirot-Timothee, 1969; Grassé,

1986). Ontogeny is always branched, rigid and very uniform, the workers are different from the larvae and nymphs. The caste system and sexual polymorphism is the most advanced in this family (Noirot & Pasteels, 1987; Roisin, 2000). Neotenic reproductives are rare, scarcely found only in Termitinae and Syntermitinae during asexual queen succession (see Chapter 1.6; Fougeyrollas et al., 2015). The distribution of Termitidae is circumtropical, they do not live in temperate regions often (Pearce & Waite, 1994; Eggleton, 2000). There is great diversification in mandible types in soldiers (see Chapter 1.8) and in feeding strategies. The Termitids are capable of digesting plant and fungal material in any decomposition stage, including degraded detritus or fungal



*Figure 5: Macrotermes sp., incipient colony, JŠ, pers. archive*

gardens. Among ecological adaptations found in this group, there are inquilines, soldierless termites living mostly underground, as well as fungus and bacteria growing species. Macrotermitinae (Fig. 5) are the fungus growing termites, they primarily feed on dead wood, leaf litter or grass. They bring this food to the fungal gardens and secondarily feed on nodules (asexual spores) of the genus *Termitomyces* (Basidiomycota: Agaricales). Subfamily Sphaerotermitinae consists



of only one species, *Sphaeroterme sphaerothorax*, feeding on dead wood and creating underground spherical nests in central Africa. They have bacterial gardens instead of the fungal ones. Foraminitermitinae have three genera, all of them are soil-feeders (Krishna, 2013; Eggleton et al., 1995). In Apicotermitinae, two groups are differentiated; *Apicotermes*-group and *Anoplotermes*-group,



Figure 6: *Fulleritermes tenebricus*, soldiers with nasus, JŠ, pers. archive

both of them are soldierless and soil-feeding. Termitinae are very diversified, soil-feeders as well as wood-feeders are in this group. Cubitermitinae are feeding on soil only. Syntermitinae and Nasutitermitinae (Fig. 6) can be both, soil-feeding or wood-feeding.

### 1.3 Ecology of termites

Termites live in tropical and subtropical parts of the world, reaching at most to south Canada, France, central Chile or New Zealand. Maximum of diversity and abundance is concentrated around the equator and drops fast beyond 10°

latitudes; in southern hemisphere, the diversity drops slower compared to the northern hemisphere (Eggleton, 2000). Termites represent important share of tropical and subtropical biomass (Bignell, 2016; Bar-On et al., 2018). They digest dead plant matter and thus influence soil topography and chemical properties, as well as the plant growth rates (Jouquet et al., 2006; Fox-Dobbs et al., 2010, Evans et al., 2011). They are capable of digesting cellulose, hemicelluloses and lignin, which is not common among animals. Due to their enormous impact on the land biomes, termites are called ecosystem engineers (Jouquet et al., 2006). While they digest any plant material that they are able to chew, each termite species specializes on only one stage of degradation of plant matter, starting from living trees (Stylotermitidae) or grass (Hodotermitidae), over dry grass (Hodotermitidae, many Termitidae), dry dead wood (Kalotermitidae), wet dead wood (Archotermopsidae) ending with topmost soil layers rich in humus (e.g. Termitidae: Foraminitermitinae, many Termitinae, Syntermitinae, and Nasutitermitinae) and bare soil (e.g. Termitidae: Apicotermitinae, Cubitermitinae). Among minor strategies belong feeding on microepiphytes (Termitidae: Nasutitermitinae: *Constrictotermes*, *Hospitalitermes* and related genera), leaf litter (several Termitidae genera) or commensalism of inquiline species that live in nests of other termites (eg. Serritermitidae: *Serritermes*, many Termitidae: Termitinae). Around 60 % of the termites are soil-feeders, 30 % are wood-feeders and around 10 % reveal the minor feeding strategies (Krishna et al. 2013). All “lower” termites (with exception of *Serritermes*, see above) feed on freshly dead plant materials, whilst Termitidae are able to digest the food in all states of decomposition and comprising also most of the inquiline species (Abe & Matsumoto, 1979). Donovan et al. (2001) established 4 ecological groups according to workers’ mandibles and gut morphology. **Group I** termites with relatively simple guts feed on dead wood or dead grass. **Group II** termites with more complex guts, feeding on wood, leaf litter of microepiphytes. **Group III** termites feed on decayed wood, humus or other materials where plant cell

structure is still visible. **Group IV** termites feed on humified soil with no cellular structure. In terms of biochemistry, the division among digestion types is even simpler (Bourguignon et al., 2011). Groups I+II comprise of wood feeders and groups III+IV include soil feeders *sensu lato*.

Several wood-feeding termites are important pests of timber (Su & Sheffrahn, 2000). These pests (183 reported species) belong mostly to families “Rhinotermitidae”, Kalotermitidae and Termitidae. “Rhinotermitidae” (especially genera *Coptotermes* and *Reticulitermes*) and Termitidae (*Odontotermes*, *Microcerotermes*) infest wood in contact with the ground, while Kalotermitidae (mainly *Cryptotermes*, *Incisitermes* and *Kalotermites*) are specialized on dry hard wood in height (Su & Sheffrahn, 2000). The field crop pests are mainly “higher” termites, “lower” termites ranked as pests are Hodotermitidae (altogether around 170 species). All other termites viewed as pests focus on woody plants. They can come from all the families since the roots of the trees are easily accessible for most of termite species (Lefèvre, 2011). Anti-termite treatments and protecting crops is extremely costly, in 2000 1,5 billion USD was spent on this subject only in the USA (Su & Sheffrahn, 2000).

#### 1.4 Termite digestion and intestinal symbionts

Digestive system of termites is adapted for processing lignocellulose matrix (formed by cellulose, hemicelluloses and lignin) of the plant tissues (Sugimoto et al., 2000). **Cellulose** is the most common polysaccharide and even the most common organic matter on the planet. Roughly 40 % of any plant material is formed by cellulose present in cell wall (Eriksson et al., 1990; Leschine, 1995). This linear biopolymer consists of  $\beta$ - (1-4) bonds that link together 500 to 14 000 D-glucose units (Sjostrom, 1993; Leschine, 1995). **Hemicelluloses** are also polysaccharides with up to 3 000 units, which are mostly D-pentose and various five- and six-carbon sugars. The structure is branched heteropolymer with

random sequence of sugars. Hemicelluloses occur in nearly all plant cell walls (Scheller & Uvlskov, 2010). **Lignin** forms roughly 30 % of cell walls in wood and bark where it serves as a matrix supporting the cellulose fibers. It is organic heteropolymer made predominantly from phenylpropanoid units (p-hydroxyphenyl, guaiacyl, syringyl, sinapyl and others) - aromatic cores with hydroxylated side chains linked together by ether or C-C bonds (Freudenberg & Neish, 1968; Sjoström, 1993).

All of those compounds have to be digested by a complex enzymatic apparatus. The enzymes are partially produced by termites, however, the plant tissue digestion is inevitably aided by microbiota inhabiting termite guts (Rouland et al., 1991; Watanabe et al., 1998; Lo et al., 2003; Brune & Ohkuma, 2011). Termite digestion is very efficient, and up to 90 % of polysaccharides from plant matter is digested during passage through the intestine (Breznak & Brune, 1994). Over  $10^{11}$  cells of microorganisms were found in one milliliter of intestinal fluid. “Lower” termites, as well as wood roach *Cryptocercus*, host apart of abundant bacteria and archaea also unique anaerobic flagellates that have not been found elsewhere in nature (Inoue et al., 2000). They mostly belong to the phylum Parabasalia or the order Oxymonadida (phylum Preaxostyla) (Cleveland et al., 1934; Inoue et al., 2000; Brune & Ohkuma, 2011). All of the “higher” termites are characterized by absence of flagellates in their digestive system (Noirot & Noirot-Timothee, 1969; Grassé, 1986), and depend on procaryotes only. The bacterial phylotypes are mostly unique to termites and have no close relatives among freely-living strains. Termite bacteria therefore represent novel candidate phyla. According to Bignell (2011), there are at least 7 functions ensured by termite symbionts. Most important is the **polysaccharide digestion** in wood feeders to provide energy to termites and to the symbionts themselves. They enable fermentation in the intestine lumen by **oxygen consumption**. **Reductive acetogenesis** is a means to consume hydrogen. **Nitrogen surplus recycling** and **nitrogen fixation** are important as well. **Lignin degradation**, mainly of the

aromatic cores side chains takes place in the gut as well. Last but not least, symbionts help with **humification of organic compounds** and providing environments with carbon in mineral form. Due to all these abilities, termites and their symbiotic microorganisms are a subject to research on **biofuels** production by lignocellulolytic processes. Bioethanol may be produced from sugars originating in polysaccharide decomposition (Scharf et al., 2011). Methane and hydrogen are also important products or side-products of termite digestion (Brune, 2014). Another consequence of microorganism importance in termite digestion is **anal trophallaxis** towards young larval stages to assure that they possess them.

The lignocellulose decomposition is a complex biochemical process taking place in termite guts being increasingly complicated along with the diet humification. The guts become compartmentalized with diverse ways of coiling, torsions and curvature in order to provide microbes with specific conditions, such as pH (Bignell, 2011). This effect was observed mainly in “higher” termites whose hind guts reveal immense variability (Noirot, 2001). In all termite lineages, the gut is diversified into three sections, namely foregut, midgut and hindgut. The foregut is adapted for mixing the chyme with products of labial glands. In “lower” termites, these products are innate cellulases (Sillam-Dussès et al., 2012). The midgut is a place where more innate enzymes digest the food, mainly in “higher” termites (Sillam-Dussès et al., 2012) and where nutrient uptake occurs. The junction between midgut and hindgut is called mixed segment. The hindgut is further divided into anterior part, enteric valve, pouch and posterior part. Pouch is the section where most of the microbes live, enabling digestion of the most intricate parts in lignocellulose matrix (Noirot & Noirot-Timothee, 1969; Brune, 2009). Hindgut often exceeds several times the length of the body of a worker and possesses unique arrangements (Bignell, 2011; Bourguignon et al., 2013).

## 1.5 Eusociality and caste patterns

Eusocial life-style is defined by three key characteristics: cooperative care for offspring, overlap of generations, and reproductive division of labour (Wilson, 1971). Most of eusocial organisms (although not all of them) reveal presence of **castes** – categories of individuals morphologically specialized for various tasks. Eusocial insects comprise termites, wasps, bees, ants, one beetle species (Curculionidae: *Austroplatypus incompertus*), and some gall-forming aphids and thrips. Eusociality is however present also in other taxa, such as crustaceans (Decapoda: *Synalpheus* spp.) and 2 mole rats (Rodentia: *Heterocephalus glaber*, *Cryptomys damarensis*; Jarvis et al., 1994; Duffy et al., 2000). The most popular explanation on the evolution of eusociality, so-called **kin selection theory** (Hamilton, 1964a; b), received significant support in experimental data on Hymenopterans, however, it barely fits at data from other taxa (Bourguignon et al., in preparation).

Termites is the oldest eusocial group of recent animals on the planet, however, there probably were some older eusocial groups that got extinct (Thorne et al., 2000; Bordy et al., 2008; Vršanský, 2010). In contrast to bees, ants and wasps, termites reveal hemimetabolic ontogeny, and larvae are thus similar to adults in their body shape and diet. The other difference from hymenopteran social insects is diplo-diploid chromosomal arrangement, making the overall relatedness equal among colony members irrespectively of their sex (upon condition of a simple family structure, however the colony fusion events are relatively common among termites, for details see Vargo & Husseneder, 2010). One of the most important differences between termites and social Hymenoptera is the ontogenetic status of the colony members, which are inactive larvae and active imagoes in Hymenoptera, while in termites they are all immature (except of dealate kings and queens; Noirot & Pasteels, 1987), as evidenced by presence of the moulting glands that are in all insects always lost at the imaginal moult (Chapman, 2013).

A caste is in termites defined by morphology and specific behaviour (Šobotník & Dahlsjö, 2017). The basic termite castes comprise workers, soldiers and reproductives. **Workers** are immature (temporarily or permanently sterile and apterous) individuals securing colony well-being in terms of foraging, nest building, care for dependent castes etc. **Soldiers** are specialised defenders whose only function is to protect a colony from various competitors or predators. To do so, they reveal enlarged and strongly sclerotized head, elongated mandibles and/or various defensive glands (Deligne et al., 1981; Prestwich, 1984a; Šobotník et al., 2010c). Soldiers develop usually from workers through a short-term intermediate stage of **presoldier**. Reproductive soldiers are known to exist in Archotermopsidae (Myles, 1986). **Alates** are imagoes that disperse during nuptial flight, form couples and found new colonies. Alates develop through (one or usually more) **nymphal instars** that are brachypterous and progressively increase the wing bud size. Alates shed their wings and change into **primary reproductives, kings and queens**, which can be in some (more often) basal taxa replaced by **secondary reproductives**. According to their ontogenetic origin, they are classified as **adultoids** when derived from winged imagoes, **nymphoids** if originate from nymphs or **ergatoids** if from workers. Sometimes even **preneotenic** can be found when more than one moult is needed to rebuild the body structure from worker to the neotenic. Functional workers split into two categories defined by the ontogenetic potentialities. While true workers lose the ability to develop wings, so-called **pseudergates** are working immatures that may but do not have to develop the wings and attempt establishing own colony. The youngest individuals dependent on the workers are called **larvae**.

## 1.6 Termite ontogeny

Termite caste system is a result of series of moults based in hemimetabolous ontogenesis. The egg-to-imago pathway is shared by all species, and runs through apterous larval and brachypterous nymphal instars (Roisin, 2000; Roisin

& Korb, 2011). Two basic ontogenetic patterns are known to occur in termites, linear and bifurcated scheme (Fig. 7). In both of the ontogeny schemes, age cohorts (**polyethism**) and sex related (**sexual dimorphism**) castes or subcastes can appear ensuring thus diverse body plans within single colony. **Linear ontogeny** consists in straight egg-to-imago developmental line, with possible deviations to soldiers (since the second larval instar) or neotenics (since the fourth larval instar). The older larvae can undergo stationary moults (no change in their appearance) or progressive moults (developing wing buds). Nymphs can

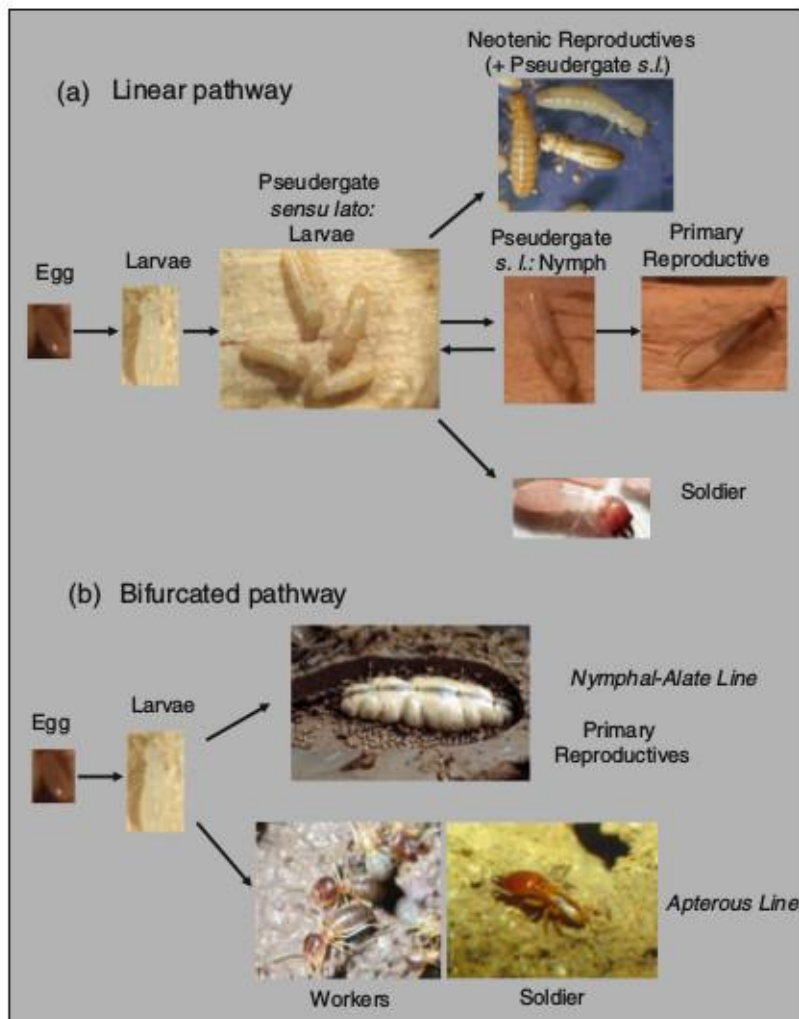


Figure 7: Simplified ontogeny schemes, adopted from Roisin and Korb (2011)



experience regressive, stationary or progressive moults according to the wing size changes (Fig. 8). As far as we know, all these switches are reversible (Roisin, 1990; 1994). Working tasks are performed by pseudergates, older larvae and nymphs. The dependent larvae develop into pseudergates *sensu lato* after several moults, while pseudergates *sensu stricto* originate from nymphs (Roisin & Korb, 2011). Linear ontogeny is linked with instable environments, smaller colony size (maximum thousands of individuals, with the exception of *Psammotermes*), lower polymorphism and lower sex differentiation compared to bifurcated ontogeny (Roisin, 2000). Linear ontogeny occurs in Archotermopsidae, Stolotermitidae, Kalotermitidae, Serritermitidae, and

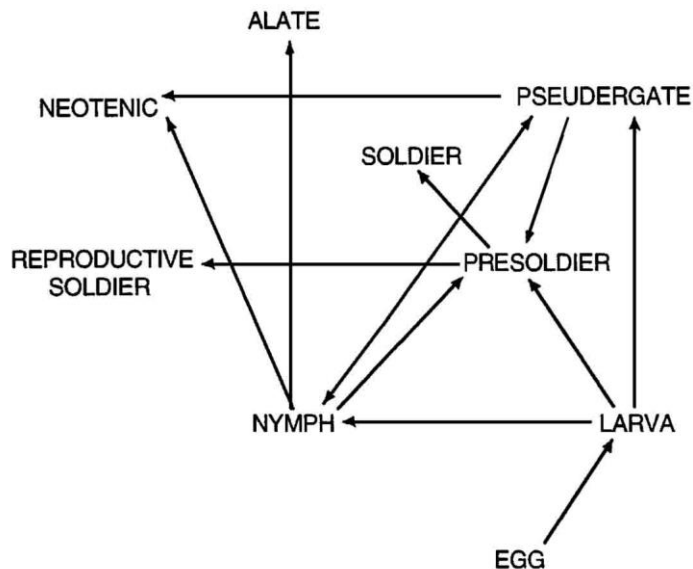


Figure 8: Linear ontogeny type in *Zootermopsis*, edited (Thorne, 1997)

*Prorhinotermes*, *Termitogeton* and *Psammotermes* (“Rhinotermitidae”) (Šobotník & Dahlsjö, 2017). The linear scheme is probably more primitive, similar to cockroach ontogeny where only the soldier caste is evolutionary novelty (Šobotník & Dahlsjö, 2017). The only exception is family Serritermitidae, where all pseudergates are males; females only appear before swarming and they quickly develop into winged imagoes (Bourguignon et al., 2009; Barbosa & Constantino, 2017).

**Bifurcated ontogeny** implies early and irreversible division between two developmental lines, apterous (sterile) and nymphal (fertile) (Roisin, 2000). Nymphal lineage leads towards imagoes via series of nymphal instars with gradually enlarging wing buds ending with winged imagoes or deviating to nymphoid reproductives. Apterous lineage leads to workers and soldiers. The decision point occurs after first or second moult, in very young larval stage. Pseudergates *sensu stricto* may rarely be formed by regression of wing pads in nymphs (Roisin & Korb, 2011). Bifurcated ontogeny is found in *Mastotermes* (Mastotermitidae), Hodotermitidae, Rhinotermitinae, *Reticulitermes* and *Coptotermes* (all “Rhinotermitidae”) and Termitidae (Šobotník & Dahlsjö, 2017).

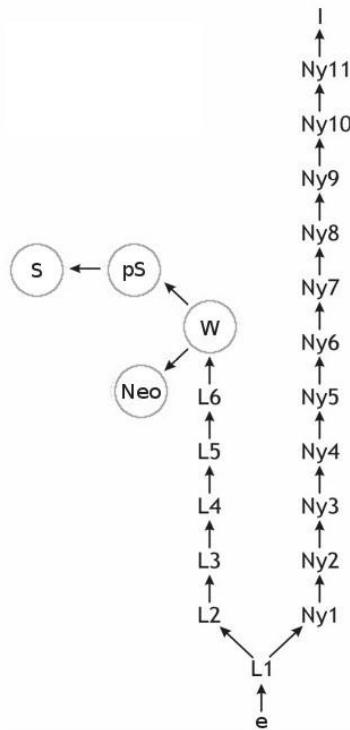


Figure 9: Bifurcated ontogeny in *Mastotermes darwiniensis*, edited (Watson & Sewell, 1981). e – egg, L – larva, Neo – neotenic, Ny – nymph, pS – presoldier, S – soldier, W - worker

Presence of this advanced ontogeny type in *Mastotermes* (Fig. 9; Watson et al., 1977), the mostbasal termite, or in Hodotermitidae (but not in sister taxon – Archotermopsidae: *Zootermopsis*; Bourguignon et al., 2015a) makes the evolution of ontogenetic schemes an interesting and tricky subject. Most

probably, the bifurcated ontogeny type has evolved repeatedly from the simpler linear ontogeny (Shellman-Reeve, 1997). The polymorphism, polyethism and ecological impact is the highest in species with bifurcated ontogeny. There are different sources of polymorphism, and particular subcastes often originate from sexual dimorphism as well as from instar polymorphism. For example, *Acanthotermes acanthothorax* (Termitidae: Macrotermitinae) has 3 soldier morphs, all coming from female apterous lineage at different instars, the largest soldier being derived from the oldest instars, while the male apterous lineage is only giving rise to workers. On the other hand, in case of Nasutitermitinae (Termitidae), the soldiers are small, mobile and originate from males (Roisin, 2000).

The social homeostasis, *i.e.* the species-specific caste proportions, is regulated via primer pheromones or genetic means. The pheromonal regulation of ontogenesis works by negative feedback. If a number of individuals in a caste decreases, the amount of primer pheromone decreases as well, allowing higher number of the missing caste to develop. The only identified primer pheromone is emitted by functional queens (primary or neotenic), and prevents differentiation of additional neotenic within the colony (Matsuura et al., 2010; Matsuura, 2012). The pheromone is a mixture of 2-methyl-1-butanol and n-butyl-n-butyrate, and the same compounds are released also by eggs and trigger the egg-care behaviour (Matsuura, 2012). Also soldier-specific primer pheromone, sesquiterpene (-)- $\beta$ -elemene, was identified in *Reticulitermes speratus* ("Rhinotermitidae") showing inhibitory activity against differentiation of workers into additional soldiers (Mitaka et al., 2017). The source of these pheromones is not known, however, several glands have been speculated to produce it, namely the mandibular glands, the epidermal tegumental gland or the oral gland (see in Chapter 1.9). There is growing body of evidence on genetic regulation of caste development. It is firstly the asexual queen succession, a situation when short-living queen produces parthenogenetic eggs leading to series of neotenic

queens, clones of the founder, with which the founding king reproduces. This phenomenon was studied in *Cavitermes*, *Spinitermes* and *Inquilinitermes* (Termitidae: Termitinae), *Embiratermes* and *Silvestritermes* (Termitidae: Syntermitinae), 3 unrelated *Reticulitermes* species (“Rhinotermitidae”) and *Glyptotermes* (Kalotermitidae) where parthenogenetic eggs give rise to neotenic queens (Matsuura et al., 2009; Vargo et al., 2011; Fougeyrollas et al., 2015; JŠ, pers. comm.). The caste fate is secondly influenced by certain gene alleles that significantly increase a chance of becoming a nymph (Hayashi et al., 2007).

### 1.7 Termite life-types and nesting habits

Life-types of termites are closely linked to their ontogeny and feeding ecology. The simplest life-type, **one-piece type of nesting**, is a strategy where the same piece of wood is the food source and nesting site; the colony can only live until the food is available (Abe, 1987; Shellman-Reeve, 1997). This life-type is connected with linear ontogeny and wood-based diet. The life span of reproductive caste is similar to life span of pseudergates – most common reproductives are neotenics. The colony size is usually small, less than a thousand of individuals. One-piece nesting way-of-life is found in Archotermopsidae, Kalotermitidae, *Glossotermes* (Serritermitidae), *Termitogeton* and *Prorhinotermes* (“Rhinotermitidae”) (Abe, 1987; Shellman-Reeve, 1997; Roisin, 2000).

**Intermediate type of nesting** applies to wood-feeding termites living in the wood items that are connected by (usually) underground galleries. They can find new food sources and increase thus stability of the colony, as well as the colony size and life expectancy of reproductives. This way-of-life is linked with primitive bifurcated ontogeny scheme (*Mastotermes*, most of “Rhinotermitidae”), or with linear ontogeny (Stolotermitidae, Kalotermitidae: *Paraneotermes*,

“Rhinotermitidae”: *Psammotermes*) (Abe, 1987; Shellman-Reeve, 1997; Bourguignon et al., 2012).

**Separate nesting life-type** termites live in centralized nests and they forage outside of it (Fig. 10). They use complex system of interconnected underground or aboveground galleries and feed on plant materials in all stages of decomposition. It occurs in Hodotermitidae, *Coptotermes* (“Rhinotermitidae”) and most of Termitidae (Abe, 1987; Shellman-Reeve, 1997; Roisin & Korb, 2011). Separate nesters are characterized by large colonies, rigid bifurcated ontogeny, low frequency of neotenics, and long life span of reproductive caste (20 to 50 years in Macrotermitinae; Šobotník & Dahlsjö, 2017).



*Figure 10: Nest of Macrotermes michaelseni, JŠ, pers. Archive*

**Inquilinism** denominates strategy when one termite lives entirely inside of nest of separate nester, and feeds either on the nest material itself or on the food stores. It is in fact an analogy to one-piece nesting strategy, as the host nest is the sole food source, and the contacts with the nest builder are always hostile. Inquilines live in small colonies and belong mostly to Termitidae: Termitinae (i.e.

possess bifurcated ontogeny). A notable exception is *Serritermes* (Serritermitidae), the only inquiline among “lower” termites, with linear ontogeny (Abe, 1987; Shellman-Reeve, 1997). Inquilinism evolved mostly from soil-feeding termites (Shellman-Reeve, 1997; Bourguignon et al., 2013; Hellems et al., 2019), except of *Serritermes serrifer* that feeds on the grass and other plant tissues included in the building material of *Cornitermes* (Termitidae: Syntermitinae) nests (Emerson & Krishna, 1975).

### 1.8 Termite defensive strategies

Termites, as eusocial insects represent not only a rich source of nutrient for a plethora of predators, but must also compete for food with other animals within the niche (e.g. other termites, wood feeding insects, earthworms). The defensive strategies can be split into **passive defence**, such as hidden way-of-life, covered galleries and elaborate nest architecture, and the **active defence** taking place when termites get in contact with an opponent. Active defences appear at morphological and behavioural levels, and are the best manifested in a specialised defensive caste, the soldier. The morphological traits include enlarged mandibles, defensive glands and overall high degree of body sclerotization.

Soldiers are crucial for defence of the nest. They represent autapomorphy of termites. They probably evolved only once, being the first caste in the social system (Hare, 1937; Noirot & Pasteels, 1987; Roisin & Korb, 2011). Their weapons are either mechanical or chemical, most commonly combination of both. Mandibles are the most important part of mechanical defence while frontal gland and labial gland are important sources of the chemical defence. Evolution of mandible shapes and adaptations associated with the chemical weapon resulted into soldiers being the most polymorphic caste of termites (Fig. 11). The most primitive type of mandibles, **biting – crushing type** combines

mechanical damage by serrated mandibles with the defensive secretion release. It is found in Mastotermitidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, most of the Kalotermitidae and one “Rhinotermitidae” genus, *Psammotermes* (Deligne et al., 1981; Prestwich, 1984a). **Phragmosis**, special type of head sclerotization and enlargement, allows for blocking the gallery so that no intruders can enter it. The mandibles are shortened and adapted for holding on the gallery wall rather than biting the enemy. When attacked, they are able to close the nest entrance with their specifically evolved heads and further with

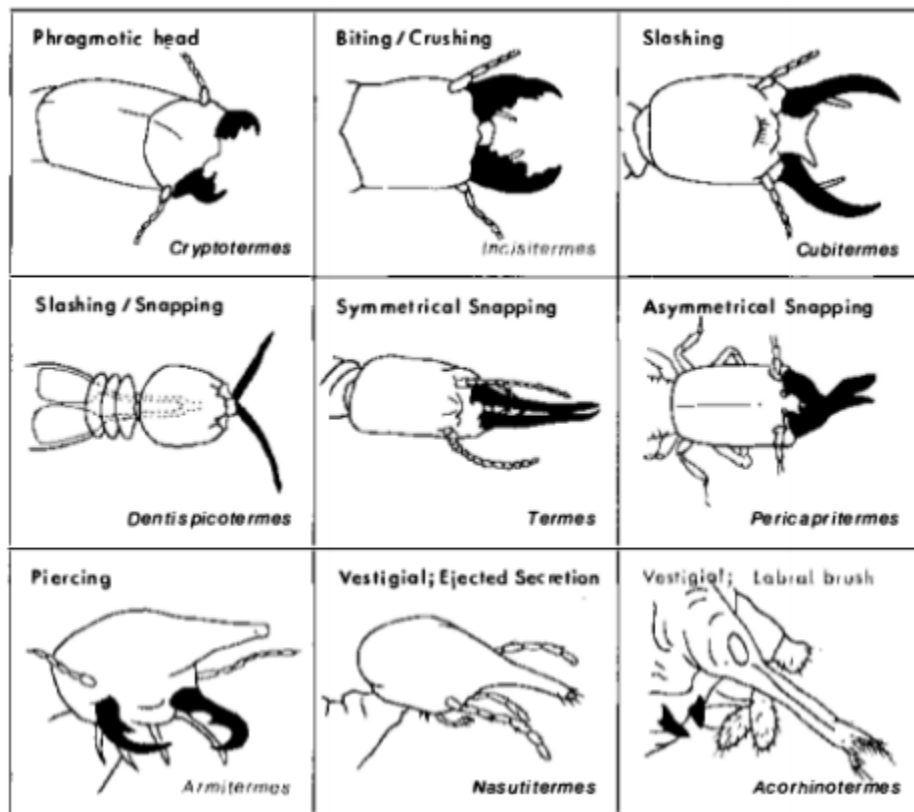


Figure 11: Representation of soldier weapons, adopted from Prestwich (1984a)

mixture of labial gland and frass (Deligne et al., 1981; Prestwich 1984a). It evolved several times (Krishna, 1970), namely in Kalotermitidae (Fig. 12; *Cryptotermes*, *Eucryptotermes*, *Calcaritermes*, *Glyptotermes*; Deligne et al., 1981; Prestwich 1984a; Engel et al., 2009;) and in Termitidae: Apicotermatinae (*Jugositermes*, *Coxotermes*, *Heimitermes*, *Duplidentitermes*, *Indotermes*,

*Euhamitermes*; JŠ, pers. comm.). **Biting – slashing** mandibles reveal a sharp cutting edge and are overlapping more than the crushing type, and thus they inflict more serious damage with smaller muscular power. Since less energy is needed, less space in the head capsule is occupied by mandibular muscles and thus frontal gland evolution could take place (Šobotník et al., 2010b) allowing more secretion to be applied into the wound. This mandible type occurs in most “Rhinotermitidae”, *Glossotermes* (Serritermitidae) and many genera of the family Termitidae (Deligne et al., 1981; Prestwich, 1984a). Mandibles of **piercing** type are pointed with tips oriented against each other, and are used to attach



Figure 12: Phragmotic head of *Cryptotermes* sp., JŠ, pers. archive

the soldier onto an enemy in order to prolong the application of the frontal gland secretion. They occur in Syntermitinae (Termitidae) and *Amitermes*, *Drepanotermes* and *Globitermes* (all Termitidae: Termitinae) (Deligne et al., 1981; Prestwich, 1984a). **Snapping** mandibles are long and slender, and able to accumulate enormous amounts of elastic energy that, when suddenly released, changes into kinetic energy having a devastating impact on the invertebrate enemies. The speed of mandible tip can reach up to 150 m/s, being thus the



fastest movement in animal kingdom (JŠ, pers. comm.). Both mandibles deliver the hit in **symmetric snappers** (Fig. 13), while only the left mandible in **asymmetric** ones (Fig. 14). An intermediate type, the **biting – snapping** mandibles evolved in *Dentispicotermes* and *Orthognathotermes* (Termitidae: Termitinae) (Prestwich, 1984a; JŠ, pers. comm.). Snapping is considered the most efficient means of defence as soldiers do not risk direct contact with the enemy as in other means of defence, and can attack fast and repeatedly without exhausting defensive secretions as in chemically defended soldiers (Deligne et al., 1981). As inferred from phylogenetic studies (Bourguignon et al., 2015a;



Figure 13: Symmetric snapping mandibles in *Termes baculi*, JŠ, pers. Archive



Figure 14: Asymmetric snapping mandibles in *Neocapritermes taracua*. Note also the blue crystals in old worker (right bottom), JŠ, pers. archive

2017), snapping evolved at least 6 times in *Termes*-group, in *Neocapritermes* + *Planicapritermes*, *Pericapritermes*-group, *Dentispicotermes* + *Orthognathotermes*, *Promirotermes* (all Termitidae: Termitinae) and *Roisinitermes* (Kalotermitidae)

The frontal gland of Neoisoptera is a defensive organ of prime importance, and specific adaptations evolved to ease application of secretion onto enemy. The labrum is elongated and terminated by **labral brush** in Rhinotermitinae. Gradual loss of mandibles, the mechanical weapon, is described in “Rhinotermitidae”. *Parrhinotermes*, the basal genus of the group, possesses monomorphic soldiers with the labral brush as well as biting-slashing type of mandibles..



Figure 15: Large soldier possessing labral brush combined with mandibles in *Dolichorhinotermes longilabius*

The soldiers are dimorphic in all other genera, but fully mandibulate only in *Schedorhinotermes*. *Dolichorhinotermes* (Fig. 15) and *Rhinotermes* have mandibulate large soldiers, while the small soldiers (**nasutoids**) lost functional mandibles and rely upon frontal gland secretion only. In *Acorhinotermes*, the most advanced genus, the large soldier caste was lost (Deligne et al., 1981; Prestwich, 1984a). Similar example is the subfamily Nasutitermitinae (Termitidae), in which soldiers evolved mandibles atrophied to plate-like structure, and evolved peculiar head capsule outgrowth, the **nasus** (Fig. 6), with the frontal pore (**fontanelle**) situated on the nasus apex. The secretion is squirt from the fontanelle on the enemy very precisely, even at several cm of distance (Noirot, 1969). Fontanelle can also be missing and the frontal gland secretion released through the body rupture, as in Serritermitidae (Costa-Leonardo & Kitayama, 1991; Šobotník et al., 2010b), *Dentispicotermes brevicarinatus* (Termitidae: Termitinae; JŠ, pers. comm.), *Globitermes sulphureus* (Termitidae:

Termitinae; Bordereau et al., 1997) or *Apilitermes longiceps* (Termitidae: Cubitermitinae; Deligne & DeConinck, 2006).

Exclusively termite workers are responsible for passive defence through construction of nest and closed gallery system protecting the termites not only from enemies but also from microorganisms and disease ensuring air circulation and temperature control (Noirot & Darlington, 2000). **Defensive defecation** denominates intended use of slimy hindgut content that significantly slows down e.g. the attacking ants, giving thus sufficient time to termites to hide away (Lubin & Montgomery, 1981; Prestwich, 1984a). Apart of it, contribution of workers to active defences is negligible, however, they are especially important in soldierless species, and also during conflicts between conspecific colonies defended by chemical means only, as the specific autodetoxification mechanisms make the soldiers useless (Spanton & Prestwich, 1981; Šobotník et al., 2010a). The soldier proportion is low in soil-feeding termites (Haverty 1977), and workers often reveal new adaptations. Workers in *Anoplotermes*-group members (Termitidae: Apicotermitinae) possess the frontal gland (Šobotník et al., 2010a). Termites may even sacrifice themselves during a conflict, and based on an exocrine gland involvement, dehiscence and autothysis is distinguished. **Dehiscence** means body wall rupture without any glandular secretion release, as known from several genera of Apicotermitinae and Termitinae genera (both Termitidae; Prestwich, 1984a). **Autothysis** is body rupture accompanied by release of defensive secretion, known in soldiers of several genera (see above) and certain Termitidae workers (Prestwich, 1984a; Shorter & Ruepell, 2012). Workers of *Ruptitermes* (Termitidae: Apicotermitinae) develop so called “dehiscent glands”, whose secretion is released through the body rupture (Costa-Leonardo, 2004; Poiani & Costa-Leonardo, 2016; details in Chapter 1.9.3). Crystal gland is an autapomorphy of *Neocapritermes taracua* workers (Fig. 14) and other congeneric species (Termitidae: Termitinae; Šobotník et al., 2012; details in Chapter 1.9.3).

Alate imagoes are in high danger, especially during dispersal flight, and the risk is reduced by mass swarms. As the dispersing imagoes emerge from the nest, many predators feed on them, however, they are quickly getting saturated as the swarming is usually synchronised by environmental variables on a large scale (Martius, 2003; Mitchell, 2008). The alates of many Neoisoptera members possess the frontal gland and are thus unpalatable to predators (Šobotník et al., 2010c; Kotalová et al., 2013).

Alarm communication is of a special importance as it allows inter-caste communication and synchronisation of defensive activities. The alarm signals are of two kinds, the vibroacoustic or pheromonal. The alarm signals induce caste-specific responses, with workers being repelled from and soldiers attracted to the source of alarm call (Stuart, 1988; Connétable et al., 1998; 1999; Röhrig et al., 1999; Šobotník et al., 2008). **Vibroacoustic signals** comprise head or abdomen banging in vertical or horizontal direction on the substrate. The sequence of beats is species specific. **Alarm pheromones** originate from a defensive gland, the frontal gland in most cases and the labial glands only in *Mastotermes darwiniensis* (Mastotermitidae), in which the pheromone is (*p*-)benzoquinone (Delattre et al., 2015). Alarm pheromone is sesquiterpene (*E,E*)- $\alpha$ -farnesene in *Prorhinotermes canalifrons* ("Rhinotermitidae"; Šobotník et al., 2008), while it is a mixture of monoterpenes in *Reticulitermes* ("Rhinotermitidae"; Quintana et al., 2003; Delattre et al., 2019). Other monoterpenes are alarm pheromones in various nasutes (Termitidae: Nasutitermitinae), such as  $\alpha$ -pinene and limonene in *Nasutitermes rippertii* and *Velocitermes velox* (Vrkoč et al., 1978; Valterová et al., 1988), carene and limonene in *N. costalis* (Vrkoč et al., 1978),  $\alpha$ -pinene in *N. princeps* (Everaerts et al., 1990) or (1*S*)- $\alpha$ -pinene and myrcene, along with a minor component, (E)- $\beta$ -ocimene in *Constrictotermes cyphergaster* (Cristaldo et al., 2015).

## 1.9 Exocrine glands of termites

Social insects rely on a broad array of volatiles to communicate, defend themselves, and survive in a colony made of closely-related members (Šobotník et al., 2010b; Bordereau & Pasteels, 2011; Rosengaus et al., 2011). Insect exocrine glands may produce pheromones, venoms, antibiotics, silk, lubricants, defensive chemicals or digestive enzymes (Chapman, 2013). Exocrine organs reach higher levels of complexity in social insects compared to their solitary relatives, and as many as 84 different glands were described in ants, 53 in bees and bumblebees, 49 in wasps and 23 in termites (Billen & Šobotník, 2015).

Exocrine glands are in insects derived from epidermis (with the exception of dehiscent glands, see below), and can be classified either according to localization, e.g. cephalic, thoracic, abdominal, or according to their function, e.g. defensive, digestive, pheromone-producing etc. Both classifications co-exists and are not mutually exclusive, however, the most common classification is based on the secretory cells ultrastructure. Three classes of secretory cells have been defined according to the means of secretion discharge through the cuticle (Fig. 16). The class I. secretory cells are adjoining to the cuticle, through which their secretion passes. The cuticle is usually impermeable and therefore the glandular cells produce during the moult cuticle of more or less modified structure, with enlarged pores and tiny holes through the epicuticle (Noirot & Quennedey, 1974; Quennedey, 1998). The apical part of class I. cells is lined with microvilli that facilitate the exocytosis of vesicles taking always place at the



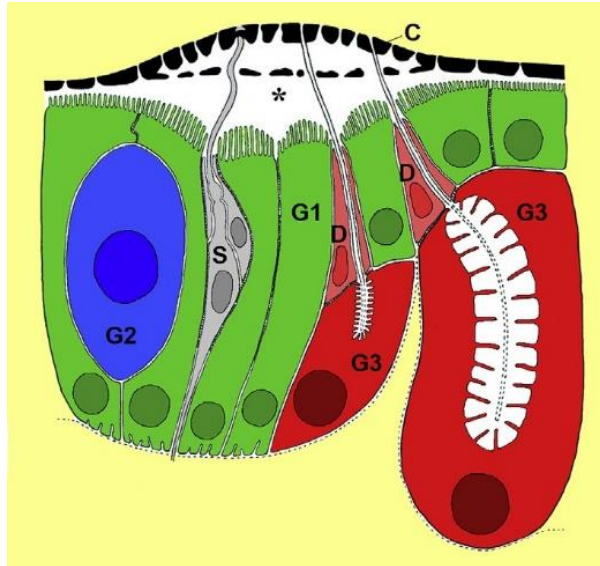


Figure 16: Scheme of a sternal gland in “Rhinotermitidae” showing all 3 classes of secretory cells. Adopted from Billen and Šobotník (2015). \* - subcuticular space, c – cuticle, D – duct cells, G1 – secretory cells class I, G2 – secretory cell class II, G3 – secretory cells class III

microvilli bases, where the buffer zone is needed due to fusion of vesicles with plasma membrane. The microvilli may also reveal central channel, through which the secretion of smooth endoplasmic reticulum passes and is released at their tips (Quennedey, 1998). Class II. secretory cells are from the cuticle separated by class I. cells, through which the secretion is released to the exterior (Noirot & Quennedey, 1974). Later research revealed that class II. cells represent modified oenocytes (Noirot & Quennedey, 1991; Quennedey, 1998). Class III. secretory cells (or bicellular secretory units) are separated from the cuticle by a layer of epidermal cells (unmodified or secretory), and are connected to the exterior by specialised cuticular duct supported by a duct cell (Noirot & Quennedey, 1974). In some cases, one more cell is present in between the secretory and canal cells, either second secretory or canal cell (Quennedey, 1998). The secretion of class III. cells is released into the extracellular lacuna lined with microvilli, to which the terminal porous part of the canal (the receiving canal) is inserted, and the secretion is led by the conducting canal made of continuous cuticle to the exterior (Noirot & Quennedey, 1974; Quennedey, 1998). A gland as a single

functional unit can be formed by one or more classes of secretory cells.  
Examples are given in Tab. 1.

Tab. 1: List of all exocrine glands studied in termites

Gland	Type of secretory cells	Exceptions	Castes	Taxonomic distribution	Key reference
Labral gland	Class I.	Class I-III. in 4 species	All castes	all termites	Palma-O'Neill et al., 2018
Frontal gland	Class I.	Class III. not for defensive purpose	Soldiers, reproductives, workers	Neoptera	Quenney, 1984
Labial gland	Class I.		All castes	all termites	Šobotník & Weyda, 2003
Mandibular gland	Class III.		All castes	all termites	Šobotník & Hubert, 2003
Accessory mandibular glands	unknown		Soldiers, reproductives, larvae	2 spp. <i>Zootermopsis</i>	Greenberg & Plavcan, 1986
Intramandibular gland	unknown		Soldiers	<i>Machadotermes inflatus</i>	Déline et al., 1981
Clypeal gland	Class I. and III.		Imagoes	Neoptera	Křížková et al., 2014
Hypopharyngeal gland	unknown		Workers, soldiers, reproductives	Mastotermitidae, Hodotermitidae, Kalotermitidae	Brossut, 1973
Oral gland	Class I.		Workers, soldiers, reproductives	"Rhinotermitidae", Termitidae	Synek et al. 2019
Rostral gland	unknown		Soldiers	2 spp. Termitidae	Déline, 1983
Nasus gland	Class I., II., and III.		Soldiers	<i>Angularitermes</i>	Šobotník et al. 2015
Dehiscent gland	Fat body		Workers	<i>Ruptitermes</i>	Polani & Costa-Leonardo, 2016
Crystal gland	Class III.		Workers	<i>Neocapritermes taracua</i>	Šobotník et al., 2012
Lateral thoracic glands	Class III.		Workers, soldiers, reproductives	3 spp. Termitidae	Gonçalves et al., 2010
Tarsal glands	Class III.		Workers, soldiers, reproductives	Kalotermitidae, "Rhinotermitidae", Serritermitidae, Termitidae	Costa-Leonardo et al., 2015
Sternal glands	Class I. and II.	Class I-III. in <i>Hodotermitopsis</i> , "Rhinotermitidae", Serritermitidae	All castes	all termites	Quenney et al., 2008
Posterior sternal glands	Class I. and III.	Class III. only in Mastotermitidae, class I., II. and III. in "Rhinotermitidae"	Imagoes	Mastotermitidae, Archotermopsidae, "Rhinotermitidae", Macrotermiinae	Quenney et al., 2004; Ampion & Quenney, 1981
Tergal glands	Class I., class III.	Class III. only in Mastotermitidae, class I., II. and III. in "Rhinotermitidae"	Imagoes	all termites	Ampion & Quenney, 1981; Šobotník et al., 2005
Posterior tergal glands	Class III.		Imagoes	<i>Corritermes</i>	Costa-Leonardo & Halfig, 2010
Abdominal pleural glands	unknown		Imagoes	<i>Cubitermes fungifaber</i>	Ampion, 1980
Spermathecal gland	Class III.		Female reproductives	Archotermopsidae, Kalotermitidae, "Rhinotermitidae", Serritermitidae and Termitidae	Raina et al., 2007
Epidermal tegumental gland	Class I. and III.		Neotenic reproductives	<i>Prorhinotermes simplex</i>	Šobotník et al., 2003
Integumental glands	Class III.		All castes	<i>Kalotermes flavicollis</i>	Leis & Sbrenna, 1983



### 1.9.1 Glands location and distribution

As many as 23 glands have already been described in termites (Tab. 1), and these are following: frontal gland (Quennedey, 1984; Šobotník et al., 2004; 2010a; 2010c; Kutalová et al., 2013), labral gland (Deligne et al., 1981; Palma-Onetto et al., 2018; 2019), clypeal gland (Křížková et al., 2014), hypopharyngeal gland (Brossut, 1973), epidermal tegumental glands (Šobotník et al., 2003), integumental glands (Sbrenna & Leis, 1983), mandibular glands (Lambinet, 1959; Noirot, 1969; Cassier et al., 1977), accessory mandibular glands (Greenberg & Plavcan, 1986), mandibular base glands (Quennedey, 1984), nasus gland (Šobotník et al., 2015), rostral gland (Deligne, 1983), labial glands (Noirot, 1969; Billen et al., 1989; Kaib & Ziesmann, 1992; Sillam-Dussès et al., 2012), tarsal glands (Bacchus, 1979), lateral thoracic glands (Gonçalves et al., 2010), dehiscent glands (Costa-Leonardo, 2004; Poiani & Costa-Leonardo, 2016), crystal glands (Šobotník et al., 2012; 2014), sternal gland (Ampion & Quennedey, 1981; Quennedey et al., 2008), posterior sternal glands (Quennedey et al., 2004; Šobotník et al., 2005), pleural abdominal glands (Ampion, 1980), tergal glands (Ampion & Quennedey, 1981), posterior tergal glands (Costa-Leonardo & Haifig, 2010), spermathecal gland (Raina et al., 2007) and oral gland (Synek et al., 2019). Most termite species possess not less than these 5 glands: the sternal gland, the labial glands, the mandibular glands, the labral gland, spermathecal gland and tergal glands (JŠ, pers. comm.). The presence of other exocrine organs is restricted to specific termite lineages, or to certain castes (Palma-Onetto, 2018). Many of the glands occur only in a termite lineage and not others, such as mandibular base glands of soldiers of *Machadotermes* (Termitidae: Apicotermitinae; Quennedey, 1984), nasus gland of soldiers of *Angularitermes* (Termitidae: Nasutitermitinae; Šobotník et al., 2015) rostral gland of soldiers of *Verrucositermes* (Termitidae: Nasutitermitinae; Deligne, 1983), crystal gland of *Neocapritermes taracua* workers (Termitidae: Termitinae; Šobotník et al. 2014) or dehiscent glands in *Ruptitermes* workers (Termitidae: Apicotermitinae; Costa-

Leonardo, 2004; Poiani & Costa-Leonardo, 2016). The distribution of the clypeal gland, the lateral thoracic glands and the oral gland is yet not understood (Gonçalves et al., 2010; Křížková et al., 2014; Synek et al., 2019).

## 1.9.2 Cephalic glands

### 1.9.2.1 Labral gland

The labral gland occurs in soldiers, imagoes and workers of all termite species studied so far, including the basal lineages of termites and even in the wood roach *Cryptocercus* (Palma-Onetto 2018; 2019). Labral gland epithelium occurs on the ventral side of labrum and on the apicodorsal side of hypopharynx. It consists of class I. secretory cells; class III. secretory cells were observed only in few species and their secretion is released together with the class I. cells. Class I. secretory cells are similar in their ultrastructure across all studied species. The most common secretory organelle is the smooth endoplasmic reticulum (sER), while smaller amounts of rough endoplasmic reticulum (rER) were observed as well.

In 2019, Palma-Onetto et al. executed detailed observation of the labral gland secretion use, showing that it impregnates the surface after encounter of an alien. They have shown that the secretion has a communication function rather than defensive as was proposed in previous studies (Deligne et al., 1981; Quennedey, 1984). From the evolutionary perspective, we can say that the labral gland is an important feature common to all castes of *Cryptocercus* and termites, and it is thus expected to play a vital, although yet-unknown, function in the daily life of termites since they evolved from the cockroach ancestor. For detailed structure of the gland in soldiers see Chapter 4.3 of this thesis.

### 1.9.2.2 Frontal gland

The frontal gland is an unpaired gland, found in all Neoisoptera (Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae; Deligne et al., 1981; Quennedey, 1984; Prestwich, 1984a; Costa-Leonardo & Kitayama, 1991; Wu et al., 2018). It is typical for soldiers, but it was also observed in the nymphs (Noirot, 1969), presoldiers (Prestwich, 1984b; Lelis & Everaerts, 1993; Bordereau et al., 1997; Šobotník et al., 2004), imagoes (Holmgren, 1909; Noirot, 1969; Šobotník et al., 2004; Piskorski et al., 2009), as well as in workers (Šobotník et al., 2010a).

The frontal gland is an epithelial invagination of the frons, forming a part of the frontal weapon in soldiers. It opens through the frontal pore (fontanelle) and it possess a sac-like reservoir (Prestwich, 1984a). Frontal gland occurs in most alates of “Rhinotermitidae” and Serritermitidae (Šobotník et al., 2010c), while frequently lacking in Termitidae (Kutalová et al., 2013). In soldiers of “Rhinotermitidae” and Serritermitidae, it reaches far into the abdomen. In Termitidae soldiers, the reservoir is cited to be confined to the head, although it may fill up most of the whole body volume, e.g. in *Globitermes*, *Dentispicotermes*, and perhaps some others (JŠ, pers. comm.). Frontal gland is much reduced in volume in Macrotermitinae, and the labial glands took over the defensive function. Snapping soldiers in Termitinae were mentioned to lose the frontal gland and rely solely on mechanical protection (Deligne et al., 1981; Quennedey, 1984), however, certain snapping lineages possess well developed (e.g. Termitidae: Termitinae: *Pericapritermes*) or truly gigantic (Termitidae: Termitinae: *Dentispicotermes*) frontal gland (JŠ, pers. comm.). The reservoir is emptied by contraction of the mandibular muscles, as evidenced e.g. by large size of mandibular muscles in Nasutitermitinae soldiers missing functional mandibles. Tentorial-fontanellar muscle opens fontanelle and allows pass-through of the secretion (Šobotník et al., 2010c).

The frontal gland is most commonly made of class I. cells forming the reservoir, except for *Coptotermes* and *Heterotermes* (both “Rhinotermitidae”), in which class III. secretory cells are present as well (Quennedey, 1984; Šobotník et al., 2010c). Often, there are class III. secretory cells that are isolated from the gland itself and are scattered around the fontanelle (Šobotník et al., 2004). Their function is probably not connected to the defensive purpose of the gland (Šobotník et al. 2010b). The secretion of the frontal gland is so diverse that even conspecific colonies do not share the same profile of defensive compounds (Chuah et al., 1990; Chen et al., 1999; Zhang et al., 2006; Krasulová et al., 2012). The defensive chemicals of termite soldiers fall into various classes, such as terpenoids, ketones, aldehydes, alcohols, hydrocarbons, macrolactones and nitroalkenes. Nitroalkenes and sesquiterpene hydrocarbons were found in alates of *Prorhinotermes* (“Rhinotermitidae”) having a saccular frontal weapon (Piskorski et al., 2009). No details on secretion of Stylotermitidae or Serritermitidae were published so far.

The functional classification of the frontal gland secretions is as follows (Prestwich, 1984a): i) greases - nonpolar and nonvolatile semisolid chemicals, associated with slashing mandibles of Termitidae having antihealing properties; ii) irritants - volatile terpenoid (often monoterpenes or sesquiterpenes) materials associated with slashing or piercing mandibles, causing disorientation, topical toxicity, ceasing of attack and beginning of grooming behaviour in ants; iii) glues or incapacitating agents stiffening after air exposure, characteristic for Nasutitermitinae; iv) contact poisons - lipophilic, highly reactive chemicals forming free radical interfering with opponent’s metabolism. If contact poisons are produced, termites reveal presence of specific autodegradation mechanisms preventing them from poisoning by own secretion (Spanton & Prestwich, 1981).

Although the frontal gland is distributed among castes more commonly than ever thought, there is still a gap in knowledge about its presence in workers, and

function in imagoes and workers. Unlike soldiers, workers of Apicotermitinae and imagoes of about half species studied so far possess relatively small frontal gland present as an epithelial thickening devoid of reservoir (Deligne et al., 1981; Noirot & Darlington, 2000; Šobotník et al., 2010c; Kotalová et al., 2013). Saccular frontal gland exists imagoes of basal groups (“Rhinotermitidae”, Termitidae: Macrotermitinae, Termitidae: Forminitermitinae: *Foraminitermes*; Šobotník et al. 2010c), in presoldiers (Prestwich, 1984b; Lelis & Everaerts, 1993; Šobotník et al., 2004), but it disappeared e.g. in large soldiers of *Rhinotermes* (“Rhinotermitidae”; Prestwich & Collins, 1982). If frontal gland lacks reservoir, its secretion is usually produced as proteinaceous biocrystals, that may have antimicrobial or antiseptic function (Deligne et al., 1981; Zhao et al., 2004; Šobotník et al., 2010a, JŠ, pers. comm.). Also components of soldier’s secretions may have antimicrobial function, such as antibacterial trinervitadienes of *Nasutitermes trioidae* (Zhao et al., 2004) or monoterpenes  $\alpha$ -pinene and limonene (common among termites; Šobotník et al., 2010b) that inhibit the fungal growth (Rosengaus et al., 2000).

### 1.9.2.3 Labial glands

The labial (or salivary) glands are common to insect except for few groups (Chapman, 2013). Termites share the general organisation of labial glands with all other Polyneoptera (Noirot, 1969). These paired organs can be found in all castes and developmental stages, although they are becoming active in secreting only since the second half of second instar (Šobotník & Weyda, 2003). The original function of the gland is related to digestion, mostly by producing saliva including digestive enzymes, and also the water management (Chapman, 2013).

Secretory cells of the labial glands are arranged into series of acini (sing. *acinus*) and a reservoir, often called water sac, made of flat epidermal cells with no secretory function. Acini and the water sacs are connected by branching duct creating a grape-like structure (Noirot 1969; Kaib & Ziesmann, 1992), and fusing

in the posterior head (Šobotník & Weyda, 2003). The cells of distinct acinar and water sac duct reveal characteristics of transporting epithelium (see Berridge & Oschman, 1972), and the common outlet opens in the salivarium, at the labium base, and the secretion is spat from the mouth (Noirot, 1969). These glands originate during embryogenesis as invaginations of the epidermis near labium, however, they are found in posterior thorax and/or anterior abdomen, because of volume constraints (Chapman, 2013). The water sacs often fill considerable part of the abdomen cavity, similarly to the frontal gland reservoir, which they are sometimes mistaken with (as in Termitidae: Termitinae: *G. sulphureus*; Noirot, 1969).

Šobotník and Weyda (2003) studied ultrastructural ontogeny of the labial glands in *Prorhinotermes simplex* ("Rhinotermitidae"). Labial glands are always formed by secretory cells of class 1. The ducts evacuate the water or the primary secretion, while water sacs only store either water or labial gland secretion (in some soldiers) (Grube & Rudolph, 1999). Two fundamental types of secretory cells form the acini, central and parietal cells. Central cells are derived from class I secretory cells and secrete mostly proteinaceous secretion. The product is in workers enriched with food-marking pheromone, and in soldiers largely replaced by production of defensive compounds, often of quinone nature (Šobotník et al., 2010b). Parietal cells are smaller than the central cells, and located at acini periphery. They are responsible for pumping water inside of the acinus lumen (Berridge & Oschman, 1972). The water flow through acinus allows the secretion originating in central cells to enter acinar ducts, where it is modified into the final form by selective transport of certain compounds from the duct to the hemocoel. The control over the labial gland secretion release takes place through direct innervation of the central cells changing the apical plasma membrane permeability, combined with water flow created by parietal cells (Šobotník & Weyda, 2003).

Three types of central cells have been described so far (Šobotník & Weyda, 2003). Each caste has different combination of central cell types making the gland very versatile and the secretion diverse (Šobotník & Weyda 2003; Billen et al., 1989; Kaib & Ziesmann, 1992). Type I. central cells probably produce the food-marking pheromone in workers; they do not occur in other castes. Type II. cells produce proteinaceous secretion, supposedly the digestive enzymes, and can be divided into two subtypes, IIa occurring in all castes and developmental stages, and type IIb occurring only in workers and young primary kings and queens. Type III central cells are probably responsible for production of soldier-specific defensive secretion, and the cells of the same ultrastructure occur rarely also in workers, where they represent an early stages of type I. cells development (Šobotník & Weyda, 2003).

Labial glands play distinct roles in particular castes. The secretion is in workers always involved in feeding activities, namely by production of cellulases and other digestive enzymes. Apart of the digestive enzymes, workers' labial glands secrete also hydroquinone acting as food-marking pheromone and *p*-arbutin (glycosylated hydroquinone) being phagostimulative (Reinhard et al., 2002). The secretion is also used as a cement for building nests and galleries, and serves as a food for dependent castes (Noirot, 1969; Grassé, 1982). As the frontal gland presence is an important autapomorphy of Neoisoptera, the soldiers of basal groups (Mastotermitidae, Hodotermitidae, Archotermopsidae, Stolotermitidae and Kalotermitidae) lack it and their defensive secretion is produced by the labial glands only (Sillam-Dussès et al., 2012). The authors found that soldiers produce always defensive compounds in their labial glands, as well as workers of soldierless species (Sillam-Dussès et al., 2012). Compounds structurally similar to hydroquinone, specifically benzoquinone and toluquinone, have been identified in the soldiers' labial gland secretion of *Mastotermes* (Moore, 1968), *Macrotermes* (Maschwitz et al., 1972), *Hypotermes* (Maschwitz & Tho, 1974), and *Odontotermes* (Wood et al., 1975). Both compounds are toxic and also

irritating against a broad spectrum of opponents (Blum, 1981; Eisner et al, 2005). Mycostatic effect is known for 4-hydroxydihydrofuran-2(3H)-one found in workers and soldiers of *Labiotermes labralis* (Termitidae: Termitinae; Sillam-Dussès et al., 2012; Teoh & Mashitah, 2012).

#### *1.9.2.4 Mandibular glands*

This paired cephalic gland is common in many insect groups (Chapman, 2013); it thus occurs in all termite species, their castes and developmental stages (Holmgren, 1909; Noirot, 1969; Brossut, 1973; Cassier, 1977; Šobotník & Hubert, 2003). Mandibular glands are located at the mandible base. It comprises of class III. secretory cells (Šobotník & Hubert, 2003), from which the duct cells emerge, forming a central duct that leads its secretion laterally to the oral cavity. Opening of the duct is situated close to the joint of mandible base and head capsule (Noirot & Noirot-Timotheé, 1969; Brossut, 1973). Ultrastructure shows class III. cells with rER outnumbering the sER (Cassier, 1977; Deligne et al., 1981). The system of vacuoles in the secretory cells and therefore the functionality of the glands depends on the termite caste and age.

The function of mandibular glands is only hypothesized, as there is no direct evidence of any purpose yet. The size of the glands in particular caste is one of the keys for interpreting the function. The gland reveals the highest activity in neotenic reproductives of *Kalotermes flavicolis* (Kalotermitidae), and the function was thus expected to produce primer pheromones preventing other colony members to become reproductive (Cassier, 1977). In *Prorhinotermes simplex* ("Rhinotermitidae"), the largest glands can be found in pseudergates and alates, smaller in soldiers and much smaller in larvae. Authors thus suggested that the secretion can lubricate the mandibular condyli and protect them from wearing out (Šobotník & Hubert, 2003). In *Hodotermopsis sjostedti* (Archotermopsidae), the glands are biggest in the soldier caste, and produce a protein specific to soldiers, what suggests a defensive function (Miura et al.,



1999). The defensive function was also suggested in workers of *Constrictotermes rupestris* (Termitidae: Nasutitermitinae; Constantino & Costa-Leonardo, 1997). Mandibular glands may produce aggregation pheromone in certain cockroaches (Brossut, 1970; 1979), and the same might be true also in termites, also because the localisation of the gland opening is not optimal for digestive function (Noirot & Noirot-Timothee, 1969; Cassier, 1977; Greenberg & Plavcan, 1986). No data on the secretion composition have been published so far.

#### *1.9.2.5 Accessory mandibular glands*

*Zootermopsis angusticollis* and *Z. nevadensis* (Archotermopsidae) are the only termite species where the accessory mandibular glands have been observed so far (Greenberg & Plavcan, 1986). Accessory mandibular glands are found in close vicinity of mandibular glands, the opening is located just 400 µm laterally to the opening of the latter. The gland represents an invagination of intersegmental cuticle and is shaped into a three-fingered glove (Greenberg & Plavcan, 1986). The secretion of accessory mandibular glands consists of hydrocarbons with unknown function. No other observation (e.g. ultrastructure) was published so far.

#### *1.9.2.6 Intramandibular glands*

This paired gland has only been found on mandible bases of *Machadotermes inflatus* soldiers (Termitidae: Apicotermitinae). As the published observations are based exclusively on SEM, the only known fact is that the secretion is released via multiple pores on the surface mandibular base (Deligne et al., 1981).

#### *1.9.2.7 Clypeal gland*

Clypeal gland appears in most Neoisoptera imagoes, except for *Aparatermes* sp. and *Anoplotermes janus* (both Termitidae: Apicotermitinae), where it was

probably lost secondarily (Křížková et al., 2014; Chapter 4.2 of this thesis). It is located on dorsal side of the head, reaching from the posterior part of the labrum to anteclypeus. It consists of class I. and class III. secretory cells. Class I. cells are more common, they possess microvilli in the apical part, large amounts of rER and smaller amounts of sER in the cytoplasm. Secretion occurs in a form of numerous electron-dense granules and less common electron-lucent vesicles. Ultrastructure of class III. cells is similar to class I. cells, and the most common secretory organelle is rER. Duct cells are extraordinary in this gland, since they carry multiple conducting canals, or alternatively, the duct is coiled and therefore viewed as multiple cross-sections (Křížková et al., 2014). For more details, view Chapter 4.2 of this thesis.

Electron-dense granules and rER on one hand indicate that the secretion is probably proteinaceous. SER is on the other hand associated with volatile secretion (Percy-Cunningham & MacDonald, 1987; Tillman et al., 1999). We can only hypothesize that the possible functions may comprise signalling the reproductive status of the king and queen, or marking the oral side of the reproductives, easing workers to feed them. Reproductives are known for not begging actively for food (Kawatsu, 2013), and this might be the signal for the workers to commence feeding or a lead to find the oral region (Křížková et al., 2014).

#### *1.9.2.8 Hypopharyngeal gland*

Hypopharyngeal gland was found in workers, alates and soldiers of the “lower” termites. This paired gland is located inside of the hypopharynx, its openings are slit-like (Brossut, 1973). Neither ultrastructure nor the secretion nature have been studied so far.

#### 1.9.2.9 Oral gland

This newly described gland is the smallest of all termite glands (Synek et al., 2019). As it was observed in workers and soldiers of 3 unrelated species of wood- and soil-feeding termites from “Rhinotermitidae” and Termitidae, it is probably widespread among Neoisoptera, or even in all termites (Synek et al., 2019). The gland is located in anterior pharynx, just behind the mouth. It is split into two secretory regions located ventrally and dorsally, respectively. The gland is formed by only some 30 to 60 class I. secretory cells, and the sER prevails among the organelles. Inclusions in the cells occur in the form of electron-lucent vacuoles, free lipid droplets, and protein granules (Synek et al., 2019).

The chemical nature of the secretion has not been studied, and its function remains thus hypothetical. As sER is an organelle known for lipidic substance production and for pheromone synthesis (Percy-Cunningham & MacDonald, 1987; Tillman et al., 1999), one possibility is that the secretion can serve as a lubricant to ease the swallowing of the particulate food (Synek et al., 2019). Lüscher (1961) proposed that primer pheromone is produced by the royal couple and distributed among colony members through mutual contact. Oral gland of workers and soldiers is hypothesized to produce this type of pheromone that would ensure equilibrium of the castes via trophallaxis (Synek, 2019).

#### 1.9.2.10 Rostral gland

Rostral gland (or rostral system) was observed in soldiers of *Verrucositermes hirtus* (Termitidae: Nasutitermitinae; Deligne, 1983) and *Embiratermes festivellus* (Syntermitinae; Costa-Leonardo & Barsotti, 1996). Although the ultrastructure of this gland was never studied, the SEM observations suggest that it is formed by class III. secretory cells that emerge in groups of 2 – 7 on top of protruberances on the head. These pyramidal structures are located on dorsal and lateral part of the head and the nasus (Deligne, 1983). The function of these diffused cells is not known. According to Deligne et al. (1981), function of class III. secretory cells

on the nasus of Nasutitermitinae consists in production of the secretion that protects the soldier from being entangled by own sticky secretion.

#### *1.9.2.11 Nasus gland*

Basal part of the nasus in *Angularitermes* (Nasutitermitinae) soldiers has rugose surface with multiple cuticular pits bearing one or more outlets of secretory cells at the bottom (Šobotník et al., 2015). The gland consists of two layers, the ectal made of class I. and class III. secretory cells, and the ental made by class II. secretory cells. This combination of all 3 types of secretory cells is very rare, it has only been found in sternal, posterior sternal and tergal glands of some termites and cockroaches (Ampion & Quennedey, 1981; Quennedey et al., 2008; Šobotník et al., 2003; 2005). The class I. cells reveal relatively little activity compared to the two other cell types. Class II. cells release lipid droplets to intercellular space, and class III. cells intake this secretion and release it to the exterior. This is an exceptional phenomenon as only class I. cells were previously reported to accept and release the secretion from class II. cells (Quennedey, 1998; Quennedey et al., 2008; Šobotník et al., 2003; 2005). Find additional details in Chapter 4.1 of this thesis.

The secretion of the nasus gland is, according to the ultrastructural observations lipid-like and proteinaceous. The lipid-like substances could act as a defence allomone since the behavioural tests revealed that *Angularitermes* produces contact poison or irritant (Šobotník et al., 2015). However, the hypothesis of solvent production for frontal gland secretion is still in the game as was noted for the rostral system (Deligne et al., 1981; Šobotník et al., 2005).

### 1.9.3 Thoracic glands

#### *1.9.3.1 Dehiscent glands*

This paired defensive organ has been described as a gland in 2004 by Costa-Leonardo, and later renamed into dehiscent organ by Poiani and Costa-Leonardo (2016). It occurs only in workers of Neotropical genus *Ruptitermes* (Termitidae: Apicotermitinae). *Ruptitermes* belongs to *Anoplotermes*-group whose members are all soldierless, and therefore the missing defensive function was adopted by the workers (Sands, 1982).

The dehiscent glands are located in the vicinity of the labial glands, in the posterior thorax and anterior abdomen, where the glands are visible through the body wall as whitish regions. The dehiscent glands are the sole exocrine organ in all insects that is not of epidermal but mesodermal origin (Poiani & Costa-Leonardo, 2016), what also means that it does not fit into any class of secretory cells defined by Noirot & Quennedey (1974). Each gland is formed by hundreds of units that produce the secretion into the central vacuole. There is neither duct nor aperture, and the secretion is released by autothysis, i.e. body wall rupture facilitated by action of specialised muscles attached to the dehiscent glands (Costa-Leonardo, 2004).

The units are elliptical in shape, held together by connective tissue that separates the gland from the hemocoel. The cells in mature gland have low number of organelles, nuclei are mostly lost, and they are mostly filled up with secretion. However, the same cells are clearly fat body adipocytes in larvae, showing various degree of restructuring into the secretory organs in worker caste (Poiani & Costa-Leonardo; 2016).

The composition of the secretion was identified as a mixture of protein with high molecular weight (Poiani & Costa-Leonardo, 2016). The secretion is clearly

defensive, stiffening after the air exposure, entangling intruders, mostly ants, which often die when they get contaminated (Howse, 1984; Mill, 1984).

#### 1.9.3.2 *Crystal glands*

Crystal glands are paired organ specific to workers of *Neocapritermes taracua* (Termitidae: Termitinae). Colonies of this soil-feeding termite consist of mostly workers; the soldier caste only reaches less than 1 % of the colony population (Krishna & Araujo, 1968). Foraging workers possess two-component defence made of labial gland acini producing granules budding off to the hemocoel, and the crystal glands producing blue crystals stored in pouches formed by metanotal outgrowths overlying the first abdominal tergite. Both components get mixed by the self-sacrifice behaviour and create a drop of bursting liquid with toxic and incapacitating activity (Šobotník et al., 2012).

Labial gland granules are surprisingly produced by parietal cells of acini that are normally responsible for water transport only. The dominant component of the granules are series of relatively harmless hydroquinone analogues, while the blue crystals are made exclusively of specific laccase BP76 that quickly converts the hydroquinones into toxic benzoquinones giving the final secretion the toxic properties (Bourguignon et al., 2015b). The granules are gradually increasing in size over the life of worker, and only the largest granules may bud off to the *hemocoel*, although they stay connected to the acini supposedly by basement membrane properties (Šobotník et al., 2014).

Crystal glands (Fig. 14) are located in the anterior part of each pouch (Šobotník et al., 2012; 2014). They are made of bicellular units, i. e. class III. secretory cells and corresponding duct cells. The secretory cells reveal age-dependent changes. They reveal relatively low secretory activity in young workers lacking the blue crystals, as evidenced by relatively low volume of rER. The secretory activity is peaking in mid-aged workers, while it decreases again in old workers with large

blue crystals (Šobotník et al., 2012; 2014). The younger workers are found predominantly inside of the nest, while older workers rather tend to go out to forage for food, an activity by far more dangerous compared to house-keeping. Another important difference between young and old workers consists in increased aggressiveness and proneness to dehiscence in the latter. Such a complex case of polyethism was never described in any other termite (Šobotník et al., 2012).

#### *1.9.3.3 Lateral thoracic glands*

Lateral thoracic glands were observed in meso- and meta-thoracic pleurae of workers and soldiers of three unrelated Termitidae species, suggesting much broader distribution of it. Each gland consists of approximately 100 class III. secretory cells and their respective duct cells. SER and Golgi apparatus are the most common organelles (Gonçalves et al., 2010). Secretion is most probably non-proteinaceous, suspected from pheromonal function, as the gland reveals presence of no reservoir (Gonçalves et al., 2010).

#### *1.9.3.4 Tarsal glands*

Tarsal glands are found in workers and soldiers of some Kalotermitidae (Fauchaux, 1994), "Rhinotermitidae" (Bacchus, 1979), Serritermitidae (Costa-Leonardo, 1994) and in Termitidae with the exception of *Anoplotermes*-group members (Soares & Costa-Leonardo, 2002; Costa-Leonardo et al., 2015). They are located on all legs, on ventral surface of proximal tarsomeres and distal tibia (Costa-Leonardo et al., 2015; Šobotník et al., 2015). The gland outlets are usually located at sunken pore plates, sometimes placed on cone-shaped protruberances on the tarsomeres (Soares & Costa-Leonardo, 2002). The glands are formed by class III. secretory cells with abundant rER and free ribosomes. The secretory vacuoles are electro-lucent (Costa-Leonardo et al., 2015). The ultrastructural data strongly suggest that the secretion is mostly proteinaceous,

however the function is unknown so far. The position of the glands allows for smearing of the gland product all over the termite body. It could mean that the product plays role in protection against bacteria and fungi (Costa-Leonardo et al., 2015).

#### 1.9.4 Abdominal glands

##### *1.9.4.1 Sternal glands*

The sternal gland is present in all termite species and developmental stages. It can be found in workers or pseudergates, alates, soldiers and presoldiers, nymphs and larvae (Ampion & Quennedey, 1981; Traniello, 1981; Robert et al, 2004; Šobotník & Hubert, 2003; Quennedey et al., 2008). The gland position and ultrastructure varies according to the phylogenetic position of a given species (Quennedey et al., 2008). Mastotermitidae have three glands on the 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> abdominal sternites, Archotermopsidae and Hodotermitidae a single gland on the 4<sup>th</sup> sternite, and all remaining families a single gland on the 5<sup>th</sup> sternite (Quennedey et al., 2008).

Sternal gland is always formed by class I. and class II. secretory cells, class III. cells are present together with the latter two types only in some cases (Quennedey et al., 2008). This situation occurs in *Hodotermopsis* (the only case in Archotermopsidae), in “Rhinotermitidae” and Serritermitidae. The gland reaches the highest complexity in “Rhinotermitidae”. In *Prorhinotermes*, there is just one class III. cells type, whilst in the remaining “Rhinotermitidae”, the middle part consist of class IIIa. cells and the posterior part is formed by class IIIb.cells; the class III. secretory cells subtypes differ by the cell size and the nature of secretory vesicles. The gland is simplified in Termitidae and formed by only class I. and class II. secretory cells. In general, class I. cells are responsible for secretion of proteins, while class II. cells produce lipidic secretion (Quennedey, 1971;



1972). If class III. are present in the gland, they also secrete proteins that are hypothetically used for optimization or stabilization of class I. and II. secretory products (Quennedey et al., 2008).

The sternal glands are known for trail pheromone production (Sillam-Dussès et al., 2007) in workers and soldiers (Leuthold & Lüscher, 1974; Sillam-Dussès et al., 2009) as well as sex pheromone production in alates of certain species (Robert et al., 2004; Sillam-Dussès et al., 2011). On one hand, in many termites, the trail and the sex pheromones are the same compounds that reveal different biological functions according to the context and the pheromone concentrations, with sex pheromones being active at order-of-magnitude higher concentrations (Bordereau & Pasteels, 2011; Sillam-Dussès, 2010). On the other hand, the sex pheromone and trail pheromone can be different chemicals in many species (Leuthold & Bruinsma, 1976; MacFarlane, 1983; Peppuy et al., 2001; 2004). The former case is however a very interesting phenomenon called pheromonal parsimony. Not only that one chemical can have more functions in different concentrations. It is also used for the same purpose within non-related species or species that are isolated by time or location. The pheromones are used in a conservative manner. The chemical nature of trail pheromones and sex pheromones is often conserved within a family. In *Mastotermes darwiniensis* (Mastotermitidae), the pheromone is 14C alcohol, (E)-2,6,10-trimethylundeca-5,9-dien-1-ol, as well as in *Porotermes* (Archotermopsidae) and *Stolotermes* (Stolotermitidae). It is a 13C alcohol or 12C alcohol in Hodotermitidae. In Archotermopsidae and Hodotermitidae it can also be aldehydes (Bordereau & Pasteels, 2011). Neocembrene (C20 hydrocarbon) is usually present in the “higher” termites, however, it is also found in *Prorhinotermes canalifrons* and *P. simplex* (Sillam-Dussès et al., 2005). In general, Kalotermitidae and the “higher” termites have their trail pheromones composed of unbranched C12 alcohols or a C20 diterpene. The “higher” termites, especially Nasutitermitinae, have

pheromones with two components, neocembrene combined with either dodecadienol or dodecatrienol (Bordereau & Pasteels, 2011).

#### *1.9.4.2 Posterior sternal glands*

The posterior sternal glands are found in alate females of *Macrotermes* (Termitidae: Macrotermitinae) on 6<sup>th</sup> and 7<sup>th</sup> sternites (Quennedey et al., 2004), while alate males of *Stolotermes*, *Porotermes* (Archotermopsidae) and *Prorhinotermes* (“Rhinotermitidae”) possess these glands on 8<sup>th</sup> and 9<sup>th</sup> sternites (Ampion & Quennedey, 1981; Šobotník et al., 2005). Both sexes of imagoes possess posterior sternal glands in *Mastotermes* (Mastotermitidae), on 6-9<sup>th</sup> sternites in males, and on 6-7<sup>th</sup> sternites in females (Ampion & Quennedey, 1981).

The ultrastructure of posterior sternal glands was studied only in *Stolotermes*, *Porotermes*, males of *Prorhinotermes* and females of *Macrotermes*, and the glands are always identical to the tergal glands (see below), and thus the same function is expected (Ampion & Quennedey, 1981; Quennedey et al., 2004; Šobotník et al., 2005).

#### *1.9.4.3 Tergal glands*

The tergal glands occur in most termites, but not in the *Cryptocercus* woodroaches (Ampion & Quennedey, 1981). The glands are present only in alate imagoes. In “lower” termites, they are often found in both sexes, while only in females of “higher” termites. As in case of sternal glands, the position of the organs, their number and ultrastructure differs among termite lineages (Ampion & Quennedey, 1981; Quennedey et al., 2008). In Mastotermitidae, the gland is located from the 3<sup>rd</sup> to the 10<sup>th</sup> tergites of both sexes, consisting of class III. secretory cells only. In Archotermopsidae, it is on 8<sup>th</sup> to 10<sup>th</sup> tergite, both sexes, consisting of class I. and class III. secretory cells. In Kalotermitidae, the tergal

glands are present in both sexes on the 9<sup>th</sup> and the 10<sup>th</sup> tergites (also on the 8<sup>th</sup> in Kalotermitidae: *Neotermes*), consisting of class I. and class III. secretory cells in both sexes (Ampion & Quennedey, 1981). In “Rhinotermitidae” and Termitidae, only females possess the tergal glands with the exception of *Prorhinotermes*, where the gland occurs in males although much smaller than in females (Šobotník et al., 2005). The ultrastructure was in detail studied only in *Prorhinotermes*, where the gland is formed by class I., class II. and class III. secretory cells (Šobotník et al., 2005). Class I. cells contain predominantly sER in both sexes, while class II. cells reveal plentiful lipidic inclusions freely in the cytoplasm in males and electron-lucent vacuoles in females. This means that secretion differs between the sexes. Class III. cells are relatively rare and do not differ in their ultrastructure from other secretory cells scattered throughout the body epidermis (Šobotník et al., 2005). In Termitidae, the tergal glands are often reduced into a single gland or even lost, as in many Macrotermitinae. The glands usually consist of class I. and class III. secretory cells in “higher” termites (Ampion & Quennedey, 1981). If the tergal glands are missing, their role is overtaken by enlarged sternal glands or by the lateral tergal glands as in cases of *Pseudacanthotermes spiniger*, *P. militaris* (Bordereau et al., 1991) and *Cubitermes fungifaber* (Ampion, 1980). The sex pheromone can also be produced by both, the tergal and the sternal glands as in *Psammotermes* (Sillam-Dussès et al., 2011).

The sex pheromones belong to 3 chemical classes, C13–C14 aldehydes, C12 alcohols and C20 hydrocarbons. Their chemical structure is very similar to trail-following pheromones described above as well as the evolution in individual families. The same pheromone may even have different glandular sources in different species (Bordereau et al., 1991; Bordereau et al., 2002). When both, tergal and sternal glands are present, the tergal glands were shown to produce a pheromone that is used for long distance attraction of a mate (Bordereau et al., 2002; Leuthold, 1975).

#### *1.9.4.4 Posterior tergal glands*

The posterior tergal glands were only found in alate females of *Cornitermes* (Termitidae: Syntermitinae) (Costa-Leonardo & Haifig, 2010). The authors believe this organ is comparable to epidermal tegumental gland (see below), and was recognized as a novel organ due to the specific position and ultrastructure. It is located on the intersegmental membrane of 8<sup>th</sup> and 9<sup>th</sup> tergite facing tergal glands of the subsequent tergite. Only class III. secretory cells form this gland, and reveal abundant sER, myelin figures and secretory vesicles of various electron-densities (Costa-Leonardo & Haifig, 2010). The secretion is mixed with secretion of tergal glands in a pouch formed by the intersegmental membrane over the next tergite. The nature of the secretion has not yet been studied, however the ultrastructural data suggest its heterogeneous nature made of volatile as well as proteinaceous components. The secretion function is probably related to the tergal glands (Costa-Leonardo & Haifig, 2010).

#### *1.9.4.5 Abdominal pleural glands*

The abdominal pleural glands were described in queens of *Cubitermes fungifaber* (Termitidae: Cubitermitinae) but not in other species of *Cubitermes* (Ampion, 1980; Grassé, 1982). The glands are located on the 3<sup>rd</sup> to 5<sup>th</sup> abdominal segment pleurae. The gland is developed as an epidermal thickening made of class III. secretory cells. The ultrastructure of abdominal pleural glands has not been studied yet, and neither was the secretion. Since tergal glands are absent in this species (Ampion & Quennedey, 1981) and since the abdominal pleural glands are present in alate females only, it is possible that they play role in courtship behaviour.

#### 1.9.4.6 Spermathecal gland

Spermathecal gland was studied in alate and/or dealate females of Archotermopsidae, Kalotermitidae, "Rhinotermitidae", Serritermitidae and Termitidae, suggesting that it is developed in all termites (Costa-Leonardo & Patricio, 2005; Raina et al, 2007). The secretory epithelium inside the spermatheca is lined with cuticle, showing the gland is again of ectodermal origin. It takes shape of finger-like projections into the spermatheca lumen (Costa-Leonardo & Patricio, 2005; Raina et al, 2007). The gland is formed by class III. secretory cells with rER being the most common secretory organelle (Raina et al, 2007). The secretion is released into the spermathecal lumen, possibly providing proteins for the sperm nutrition during their storage between the copulation and egg fertilisation (Costa-Leonardo & Patricio, 2005).

#### 1.9.5 Whole-body glands

##### 1.9.5.1 Epidermal tegumental gland

Epidermal tegumental gland was found in *Prorhinotermes simplex* ("Rhinotermitidae") neotenic reproductives only (Šobotník et al., 2003). Majority of epidermis is modified into this gland with exception of cells underlying cuticular membranes. Class I. and class III. secretory cells are present in this immense gland in equal proportion. Class I. cells contain abundant sER, lower amounts of rER and the secretion in form of electron-dense granules. The class III. secretory cells produce proteinaceous secretion via abundant rER and the cells are often filled up with electron lucent vacuoles. Surprisingly, also the canal cells of class III. secretory cells reveal certain secretory activity, disposing of rER and numerous mitochondria (Šobotník et al., 2003).

Secretion of the gland is formed by two substances – volatile and proteinaceous. The volatile component is synthesized by class I. cells, while the class III. cells and corresponding canal cells excrete proteinaceous part. The gland probably

synthesizes a primer pheromone that helps to maintain the optimal proportion of neotenic reproductives. In case of orphaning, young neotenic were found in the colony in 17 days (Šobotník et al., 2003), therefore production of the pheromone must be continuous. The proteinaceous proportion could serve as an attractant for frequent grooming by other castes (Maistrello & Sbrenna, 1999) ensuring that the secretion would reach all individuals in the colony.

#### *1.9.5.2 Integumental glands*

Integumental glands were described by Sbrenna & Leis (1983) in *Kaloterme flavicollis* (Kalotermitidae). They are present in all castes but reach the highest abundance in soldiers. The gland is formed by secretory cells class III. scattered all over the head, thorax and abdomen of an individual. SER and Golgi complex are the most common secretory organelles. Secretory vesicles occur in two forms – electron lucent vacuoles and granules containing fibrillar material. Secretion is therefore expected to be of dual nature – volatiles, presumably pheromones, originating from the electron-lucent vacuoles, and the matter originating from the granules of unknown characteristic. The two types of secretion granules appear gradually accumulating over time, reaching higher quantity in aged termite soldiers (Sbrenna & Leis, 1983). Authors of the study believe that the secretion serves first for maintenance of the cuticular layers and later as a pheromone with unknown function.

## 2 Materials and Methods

### **Sampling**

Termite material has partially been obtained from the nature (foraging termites or termites from nests) and imported to Prague based on legal procedures, partially from laboratory breeds at Faculty of forestry and wood sciences (CULS) or from breeds of colleagues (Berlin and Paris). Species lists together with the material origin are given in the resulting publications. Altogether approximately 40 species were used for the purposes of the thesis.

### **Direct observations**

Living termites were photographed in order to compare shape of labrum and presence of the hyaline tip, part of the labral gland. Photographing took place in laboratory conditions on filter paper. The termites were cooled down when immobilization was needed.

### **Histology**

Observation of histological sections was performed using optical microscope for measuring various parameters of the glands. According to purpose, we used fixatives Bouin's solution or Duboscq-Brasil mixture. Bouin's solution consists of saturated picric acid, formaldehyde and glacial acetic acid in ratios 30:10:2 (Crookham & Dapson, 1991). Duboscq-Brasil solution contains picric acid 5g, 80 % ethanol 750 ml, formaldehyde 300 ml and acetic acid 75 ml (Crookham & Dapson, 1991). After 24 hours, ethanol series was used to dehydrate the tissues (50 %, 60 %, 70 %, 80 %, 90 % and 100 % ethanol, each concentration applied for 15-20 minutes). Paraffin embedding and sectioning was followed by staining by hemalunpicroindigocarmin or Mallory's trichrome in order to reveal gland parameters described in the respective papers.

### **Resin fixation and transmission electron microscopy (TEM):**

We always used fixative based on phosphate buffer when the material was available in Prague, however, cacodylate-based fixative was used if the fixation had to start in the field, as this approach is less sensitive to changing temperatures and timing of subsequent steps. Standard fixative was prepared as a mixture of phosphate buffer solution (PBS) (0.2 M, pH 7.2), 10 % formaldehyde and 8 % glutaraldehyde in ratios 2:1:1. If cacodylate buffer (0.2 M, pH 7.3) was used, the mixture contained also 8 % glutaraldehyde and distilled water in ratios 2:1:1 (Glauert & Lewis, 1998). Living termites were submerged into a drop of fixative and dissected to head, thorax and abdomen using razor-blade in order to enable the fixative penetrate tissues properly and fast enough. If soil-feeding termites were studied, we extracted the gut during the dissecting as it often contains sand grains disallowing the sectioning. Also the mandibles were removed to ease the sectioning. The washing out the fixative was usually done after 24 hours of fixation, to prevent damage of the tissues. The washing solution consists of 0.1 M PBS or 0.2 M cacodylate buffer and glucose solution (2.7g in 50 ml of water) at ratio 1:1. While PBS-based fixation must be processed immediately, the cacodylate-based fixation can be extended over longer time. The washing solution was changed every 10 days until further steps. Postfixation was done using 1.5 or 2 % osmium tetroxide in the washing buffer. Postfixation was followed by repeated washing with distilled water and dehydration using acetone series (30 %, 50 %, 70 %, 80 %, 90 %, 95 % and 100 %). The samples were then steeped with standard Spurr resin using mixtures with acetone in ration 1:2, 1:1, 2:1 and repeated pure resin. The embedding into hard silicon molds was followed by polymerization at 70°C for 8 hours. Semithin sections (0.5 µm) were cut using Reichert-Jung ultramicrotome, and stained with Azure II or toluidine blue, and inspected using Nikon Eclipse Ni optical microscope. When the target gland was reached, ultrathin sections (40-60 nm) were prepared. These sections were contrasted with uranyl acetate (Watson, 1958) and lead



citrate (Reynolds, 1963) at the Laboratory of Electron Microscopy (Faculty of Sciences, Charles University in Prague), and inspected using a Jeol 1010 transmission electron microscope.

### **Scanning electron microscopy (SEM)**

Freeze-killed or ethanol killed termites were used. Only the heads were studied, and for visualising the labral gland surface, the mandibles, maxillae and labium were removed. Termite heads were dehydrated by ethanol or acetone, cleaned in ultrasound bath and dried using critical point drying at the Laboratory of Electron Microscopy (Faculty of Sciences, Charles University in Prague). To study the nasus gland, alternative way was used: 24 hours long impregnation in hexamethyldisilazane was followed by air drying (Nation, 1983). In both cases, the heads were glued onto an aluminium holder and sputter-coated with gold and observed using a Jeol 6380LV scanning electron microscope.

### **Glands measurements**

All dimensions were measured using Nikon NIS Elements software. Pictures resulting from histology, semithin resin sections or TEM preparation were used for this purpose.

### 3 Results

Three publications are included in the thesis body to present my contribution to termite gland research. The works are mentioned in Chapter 1.9 and discussed below.

- Šobotník, J., Bourguignon, T., Carrijo, T.F., Bordereau, C., Robert, A., Křížková, B., Constantini, J.P. & Canello, E.M. (2015). The nasus gland: A new gland in soldiers of *Angularitermes* (Termitidae, Nasutitermitinae). *Arthropod structure & development*, 44(5), 401-406.

15 % is the proportion of my work on the publication.

- Křížková, B., Bourguignon, T., Vytisková, B. & Šobotník, J. (2014). The clypeal gland: a new exocrine gland in termite imagoes (Isoptera: Serritermitidae, Rhinotermitidae, Termitidae). *Arthropod structure & development*, 43(6), 537-542.

30 % is the proportion of my work on the publication.

- Palma-Onetto, V., Hošková, K., Křížková, B., Krejčířová, R., Pfliegerová, J., Bubeníčková, F., Plarre, R., Dahlsjö, C.A.L., Synek, J., Bourguignon, T., Sillam-Dussès, D. & Šobotník, J. (2018). The labral gland in termite soldiers. *Biological Journal of the Linnean Society*, 123(3), 535-544.

5 % is the proportion of my work on the publication.

### 3.1 The nasus gland: A new gland in soldiers of *Angularitermes* (Termitidae, Nasutitermitinae).



## The nasus gland: A new gland in soldiers of *Angularitermes* (Termitidae, Nasutitermitinae)



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### ABSTRACT

Termites have developed many exocrine glands, generally dedicated to defence or communication. Although a few of these glands occur in all termite species, or represent synapomorphies of larger clades, others are morphological innovations of a single species, or a few related species. Here, we describe the nasus gland, a new gland occurring at the base of the nasus of *Angularitermes* soldiers. The nasus gland is composed of class 1, 2, and 3 secretory cells, a rare combination that is only shared by the sternal and tergal glands of some termites and cockroaches. The ultrastructural observations suggest that the secretion is produced by class 2 and 3 secretory cells, and released mostly by class 3 cells. The base of the nasus has a rough appearance due to numerous pits bearing openings of canals conducting the secretion from class 3 secretory cells to the exterior. We tentatively assign a defensive function to the nasus gland, although further research is needed to confirm this function. Although the gland is described only from species of *Angularitermes*, other genera of Nasutitermitinae also present a rough nasus base, suggesting the presence of a similar, possibly homologous, gland.

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### 1. Introduction

The ecological success of social insects is evidenced by their dominance in most terrestrial biotopes, especially in tropical and subtropical ecosystems where they make up a large part of the animal biomass (Fittkau and Klinge, 1973; Ellwood and Foster, 2004). Because of their abundance, social insect colonies strongly compete with each other and attract a wide variety of predators (Hermann, 1984). Strong competition and predation are the main factors that have triggered the evolution of sophisticated and fine-tuned defence systems.

The soldier caste is present in all termites, except for a few advanced taxa in which it was secondarily lost (Hare, 1937; Roisin and Korb, 2011). Fully devoted to colony defence, soldiers play an essential role in the colony life, enhancing the reproductive capability of king and queen (Higashi et al., 2000). The soldier weaponry

is diverse and many morphological and chemical adaptations are specific to a few species or genera (Hare, 1937; Deligne et al., 1981; Prestwich, 1984; Quennedy, 1984). Crushing mandibles are pleiomorphic and some termite lineages evolved slashing, reaping and/or snapping mandibles, with or without associated defensive glands. Alternatively, soldier mandibles are reduced in minor soldiers of Rhinotermitinae, which possess an elongated brush-like labrum used to apply the frontal gland secretion onto predators (Quennedy and Deligne, 1975), and in all Nasutitermitinae (Termitidae), which rely almost exclusively upon the frontal gland secretion they squirt out by the fontanelle located at the tip of the nasus (Hare, 1937; Deligne et al., 1981; Prestwich, 1984; Šobotník et al., 2010a). The frontal gland of Nasutitermitinae soldiers secretes species-specific mixtures of defensive compounds dominated by monoterpenes and diterpenes, frequently mixed with other classes of chemicals (Prestwich, 1984; Šobotník et al., 2010a). This secretion primarily acts as glue, entangling and incapacitating enemies, but it is also irritant, repellent, and has an alarm function (Deligne et al., 1981; Prestwich, 1984; Šobotník et al., 2010a).

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Termites possess a rich set of exocrine organs, whose development varies among species and castes and are in general distributed all over the body. The glands play diverse functions, including defensive (frontal, labial, labral glands) (Prestwich, 1984; Šobotník et al., 2010a) and pheromone-producing (frontal, labial, sternal, tergal glands) (Reinhard et al., 2002; Šobotník et al., 2010a; Bordereau and Pasteels, 2011), but for many their function remains unknown.

The genus *Angularitermes* Emerson (Nasutitermitinae) is widely distributed in South America, from the Guyana Shield to Southeast Brazil (Constantino, 1998). Six species have been described so far, all characterized by intricate microsculptures at the base of the nasus (Mathews, 1977; Canello et al., 1996; Carrijo et al., 2011). Sands (1957) was the first to report the “rugose” appearance of the base of the nasus of *Angularitermes* and *Eutermellus* soldiers. Later on, Deligne (1983) hypothesized that the rugosity may represent a glandular system (“rostral system”), which occurs in species of the genera *Velocitermes*, *Tenuistrotermes*, *Nasutitermes*, *Trinervitermes*, *Verrucositermes* and *Spatulitermes*. In this paper we describe the structure of a new exocrine organ on the nasus of *Angularitermes* using light microscopy as well as scanning and transmission electron microscopy. Our combined approach provides evidence of a new and complex exocrine organ that we name the “nasus gland”.

## 2. Materials and methods

### 2.1. Scanning electron microscopy (SEM)

Two specimens of *Angularitermes nasutissimus* (Emerson, 1925), collected in the Nouragues Nature Reserve, French Guiana (see Bourguignon et al., 2011a), were studied using SEM. Soldiers were dehydrated in a conventional ethanol series, impregnated for 24 h in hexamethyldisilazane, air dried and gold coated. Digital images were taken with a Philips XL 30 ESEM.

### 2.2. Optical microscopy

Foraging soldiers of *Angularitermes pinocchio* Canello and Brandão, 1996 were collected in the litter of a Cerrado (Brazilian Savannah) in Jataí Reserve, Luiz Antônio, São Paulo, Brazil. They were fixed in Duboscq-Brasil mixture for 24 h and embedded into paraffin after dehydration with ethanol. The serial longitudinal sections (7 µm) of three soldiers were stained with hemalun picroindigocarmin.

### 2.3. Transmission electron microscopy

Soldiers of *Angularitermes coninasus* Carrijo and Rocha, 2011 were collected from a nest in the Amazon forest, Porto Velho, RO, Brazil. Living soldiers of *A. coninasus* were submerged into a drop of fixative (cacodylate buffer [0.2 M sodium cacodylate in water, pH 7.05]: glutaraldehyde 8%: distilled water = 5:3:2) and cut into parts using a razor blade. Soldiers were fixed for approximately 20 h and then displaced into washing buffer (cacodylate buffer: glucose solution [2.7 g in 50 ml of water] = 1:1). Washing buffer was replaced every 10 days until further steps, as explained in Šobotník et al. (2010b). Semithin (0.5 µm) and ultrathin (gold, silver and grey) sections of three soldiers were prepared using a Reichert-Jung ultramicrotome, and observed with a Nikon Eclipse Ci optical microscope combined with a Jenoptik ProgRes CT3 camera or a JEOL 1011 transmission electron microscope, respectively.

### 2.4. Behavioural observations

Individuals of *A. coninasus*, collected from a single colony in Rondônia, Brazil, and workers of *Procornitermes lespeii* collected from different logs in a fragment of forest in São Paulo city, Brazil, were used. Soldiers of *A. coninasus* were confronted with *Procornitermes lespeii* workers, which have similar body size. Each treatment consisted in three *A. coninasus* soldiers placed in a Petri dish (ø 55 mm) with three *P. lespeii* workers. The rate of survival of *P. lespeii* workers was recorded after 18 h. We used nail enamel in order to prevent the release of secretion from the frontal and/or nasus glands. We carried out five treatments, namely: frontal gland blinded, nasus gland blinded, frontal and nasus glands blinded, no gland blinded, and a control consisting of *P. lespeii* workers alone. Each treatments were replicated five times with different individuals.

## 3. Results

### 3.1. Scanning electron microscopy

The basal part of the soldier nasus of *Angularitermes* contains plentiful small pits. The pits occur in two groups laterally located on the nasus. Their density is high near the head capsule and decrease toward the tip of the nasus (see Fig. 1A). The pits are rounded and generally irregular in shape, although some are regular and oval. The regular pits are about 5 µm in diameter and always contain a single hole of about 0.5 µm in diameter (i.e. the outlet of the conducting canal of class 3 secretory cell *sensu* Noirot and Quennedey, 1974; see below), while the irregular pits are larger (7–10 µm across) and obviously resulted from partial fusion of simple pits (between 2 and 4, see Fig. 1B). Although the pit interior is smooth, the cuticle between the pits reveals plentiful tiny depressions corresponding to epicuticle perforations (Fig. 1B, SF5).

### 3.2. Optical microscopy

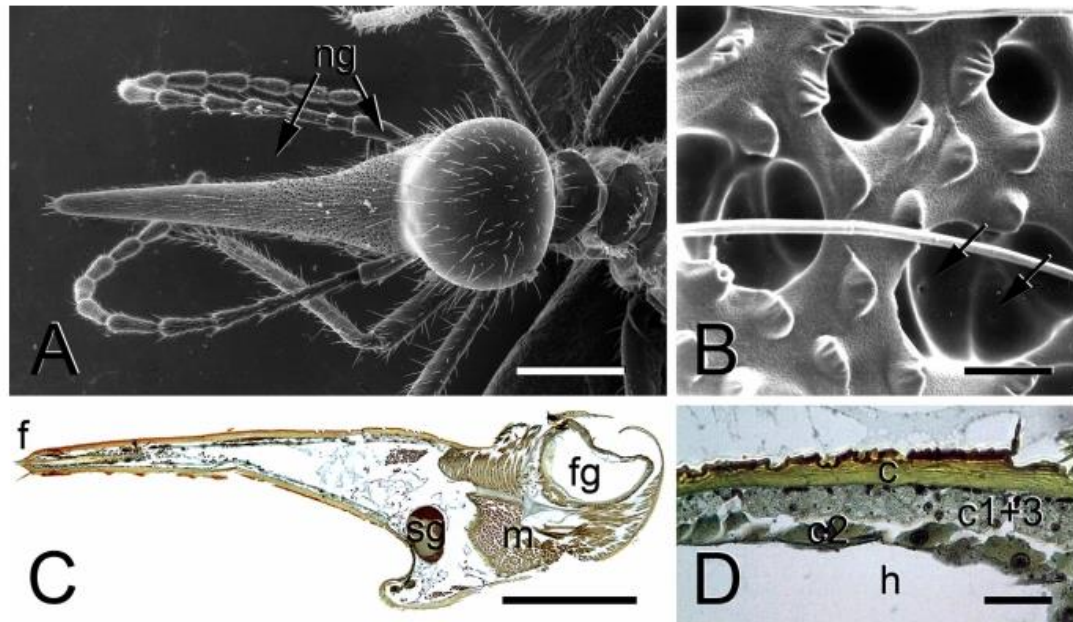
Both histological and semithin resin sections reveal the same glandular tissue associated with cuticular pits (Fig. 1C, D). The glandular cuticle is between 30 and 40 µm thick and consists of epicuticle, thin exocuticle (5–10 µm thick) and thick endocuticle (22–33 µm thick). The pits are up to 7 µm deep, and the thickness of both cuticular layers decreases in the pit, particularly for the exocuticle (Fig. 1D).

The glandular epithelium is bulky (up to 60 µm thick) and its cells form two layers, of which the ectal one is continuous and formed by smaller cells with highly vacuolised cytoplasm, while the ental layer is formed by larger spaced cells containing dense cytoplasm (Fig. 1D).

### 3.3. Ultrastructure

The nasus gland consists of secretory cells of class 1, 2 and 3 (according to the classification of Noirot and Quennedey, 1974), with all cell types being approximately of the same abundance. TEM observations of the cuticle reveal a thin epicuticle made of inner (about 30 nm) and outer (about 10 nm) epicuticular layers. The cuticular modifications allowing the secretion to flow outside through the epicuticle consist of enlarged pore canals (their width was very variable, from 200 nm to about 1.2 µm), and tiny epicuticular pores (about 30 nm; Fig. SF5). The outlets of canal cells connected to class 3 secretory cells are always situated on the bottom of depressions, and these depressions are always made at the expense of exocuticle, reducing endocuticular layer thickness only slightly.





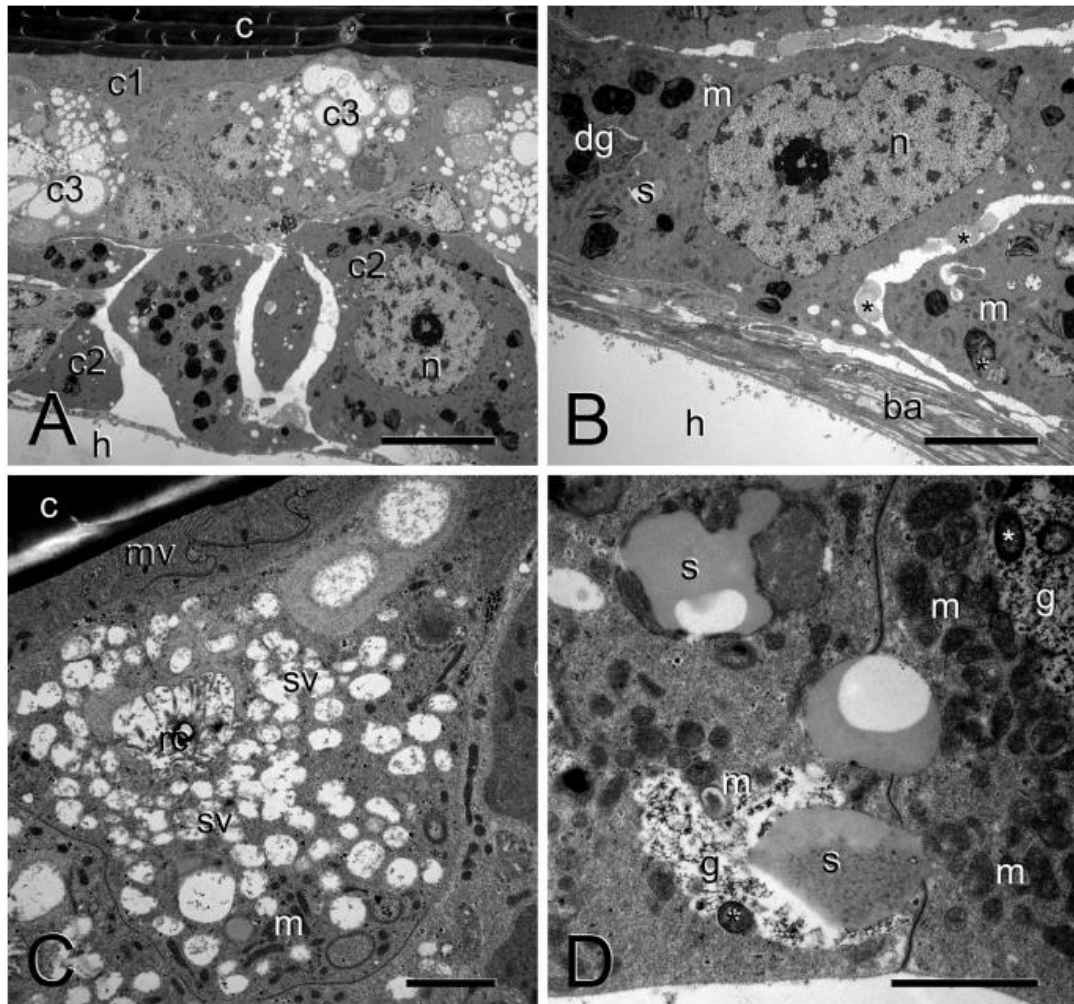
**Fig. 1.** Basic anatomy of the nasus gland. **A:** SEM micrograph of *Angularitermes nasutissimus* soldier head. Note the extent of the nasus gland marked by arrows. Scale bar 500  $\mu\text{m}$ . **B:** Detail of the glandular cuticle and the pits corresponding to class 3 secretory cells. Arrows mark outlets of canal cells. Note that the pit on top-right is regular and contains a single outlet, top-left contains 2, mid-left 3 and bottom-right 4 outlets. Note also the small depressions among the pits, which correspond to epicuticular punctures. Scale bar 10  $\mu\text{m}$ . **C:** Oblique histological section of *Angularitermes pinocchio* soldier head. Scale bar 500  $\mu\text{m}$ . **D:** Parasagittal histological section of *Angularitermes pinocchio* soldier head showing the structure of the nasus gland and overlying cuticle with section through numerous pits. Scale bar 50  $\mu\text{m}$ . **Abbreviations:** c, cuticle; c1+3, layer formed by secretory cells class 1 and 3; c2, layer formed by secretory cells class 2; f, fontanelle; fg, frontal gland reservoir; h, haemocoel; m, mandibular muscles; ng, nasus gland; sg, suboesophageal ganglion.

Class 1 cells are attached to the cuticle, class 3 cells occur entally to them, and class 2 cells are the most entally situated (Fig. 2A). The whole gland is basally covered with a single basement lamina between 30 and 70 nm thick that is locally thicker (up to 200 nm) due to strengthening with collagen fibres. Intercellular junctions connect neighbouring class 1 cells and comprise an apical zonula adherens followed by a long septate junction; the latter junction also connects neighbouring class 3 cells and canal cells to respective class 3 secretory cells, and canal cells to neighbouring class 1 cells (Figs. SF2, SF3, SF4). Intercellular spaces are very large, especially in the ental parts of the gland, and often contained nerves (bundles of tens of axons covered with glial cells; Fig. 2B). Individual axons (without any sheet cells) are frequently observed between secretory cells throughout the gland, including in the ectal parts. The axons often contained typical electron-dense grains of neurosecretion. Two kinds of sensilla containing either 1 or 4 sensory dendrites can also be observed in the nasus gland (Figs. SF11, SF12).

The class 1 secretory cells form a continuous layer, 2–6  $\mu\text{m}$  thick, with sparse basal projections reaching up to 15  $\mu\text{m}$  entally (Fig. 2A). Locally, they possess bundles of short microvilli (up to 1  $\mu\text{m}$  long), a few mitochondria and small nuclei (about 5  $\mu\text{m}$  in the largest dimension), which are ovoid and slightly irregular, with numerous large condensations. Secretory organelles comprise low amounts of rough endoplasmic reticulum (ER) around the cell nucleus and free ribosomes scattered throughout the cytoplasm (Figs. SF3, SF4). Inclusions comprise low amounts of glycogen rosettes, scarce lucent vesicles and electron-dense granules. Lipid-like droplets are observed among the secretory cells and sometimes also freely in the cell cytoplasm (Fig. SF3).

Class 3 secretory cells are basal to class 1 cells, located in the mid part of the glandular epithelium. They are much larger than class 1 cells, cubic in shape, reaching up to 20  $\mu\text{m}$  in their largest dimension. Each extracellular reservoir is lined with spaced microvilli between 0.5 and 1.5  $\mu\text{m}$  long (Fig. 2C). Each receiving canal (between 450 and 550 nm in diameter) made of several (3–5) layers of porous epicuticle is inserted into a reservoir, and ectally changes into conducting canal (about 450 nm in diameter) made of outer and inner epicuticle, similar to, and continuous with, the glandular epicuticle. The nuclei of class 3 secretory cells are slightly irregular, oval in shape, filled predominantly with dispersed chromatin, up to 8  $\mu\text{m}$  in diameter. Mitochondria (up to 1  $\mu\text{m}$  long) are very abundant and located predominantly in the cell periphery. Secretory organelles comprise variable amounts of rough ER and Golgi apparatus (Figs. SF6, SF7). Inclusions comprise glycogen rosette aggregations, rare irregular electron-dense granules (up to 3  $\mu\text{m}$  large), and electron-lucent vesicles containing variable amounts of small electron-dense particles considerably differing in size among cells (Fig. 2D). Lipid droplets (0.5–2.5  $\mu\text{m}$  in diameter) are frequently observed in the intercellular spaces between class 3 cells, and seem to be incorporated by the secretory cells (Fig. 2D). Within the cells, the droplets are located either freely in the cytoplasm or inside larger vesicles (these droplets often seem to be dissolving), but infrequently they can also be observed within the extracellular reservoir. The class 3 cells probably reveal certain secretory cycles, presumably starting in cells containing lower amounts of smaller lucent vesicles (less than 1  $\mu\text{m}$  in diameter) and considerably higher volume of cytoplasm, secretory organelles, and glycogen and lipid droplets. As the process of inclusions





**Fig. 2.** Ultrastructure of the nasus gland in *Angularitermes coninassus*. **A:** TEM micrograph of the nasus gland showing basic features of secretory cells class 1, 2, and 3. Scale bar 10  $\mu\text{m}$ . **B:** Ental part of the nasus gland showing the position of the class 2 secretory cells. Asterisks mark the secretion located within intercellular space. Scale bar 5  $\mu\text{m}$ . **C:** Ectal part of the nasus gland showing the position of the class 1 and 3 secretory cells. Scale bar 2  $\mu\text{m}$ . **D:** Basal part of two neighbouring secretory cells class 3 showing the intake of lipid-like droplets from the intercellular spaces. Asterisks mark bacteria within secretory cells. Scale bar 2  $\mu\text{m}$ . **Abbreviations:** ba, bundle of axons; c, cuticle; c1, secretory cell class 1; c2, secretory cell class 2; c3, secretory cell class 3; dg, electron-dense granule; g, glycogen; h, hemocoel; m, mitochondria; mv, microvilli; n, nucleus; rc, receiving canal; s, secretion within the cell; sv, secretory vesicle.

accumulation continues, cells are filled predominantly with large electron-lucent vesicles (up to 7  $\mu\text{m}$  in diameter) and a very low amount of cytoplasm and organelles. The release of inclusions is observed rarely, but is probably controlled by free axons running among secretory cells (Fig. SF7). Secretory class 3 cells often contain *Bacillus*-shaped bacteria (about 300 nm in diameter and up to 1  $\mu\text{m}$  long) located either freely in the cytoplasm, but more often occurring within the glycogen aggregations (Fig. 2D).

The class 2 secretory cells are located entally in the gland, and are extraordinarily large, sometimes up to 35  $\mu\text{m}$  in their largest dimension. There is an extensive system of intercellular spaces and

lacunae among the class 2 cells, which reveal neither intercellular junctions nor attachment to the basement membrane. The nuclei are very large (up to 13  $\mu\text{m}$  in the largest dimension), rounded in shape, slightly irregular, and filled only with dispersed chromatin and a few small chromatin aggregates (Fig. 2A). Secretory organelles comprise abundant smooth ER, and low amounts of rough ER and free ribosomes scattered throughout the cells (Figs. SF9, SF10). Inclusions comprise only angular electron-dense granules containing variable amounts of electron-lucent gaps and rare lipid-like droplets, which seem to be excreted into intercellular spaces (Fig. 2B). Class 2 cells also often contain *Bacillus*-shaped bacteria



located always freely in the cytoplasm, but otherwise identical to those observed in class 3 cells.

### 3.4. Defensive behaviour

All workers of *P. lespesii* died during the tests with non-treated *A. coninasus* soldiers (i.e. both glands opened), while all survived when confronted with *A. coninasus* soldiers from which both glands were sealed (or without soldiers). Sealing the nasus gland (frontal gland opened) reduced the survival to 40%, and sealing the frontal gland (nasus gland opened) led to 73% survival. These results were not significant due to high variability and low number of repetitions.

Untreated soldiers of *A. coninasus* repeatedly sprayed the frontal gland secretion on *P. lespesii* workers, partially incapacitating them, and often getting entangled too. Shaking (longitudinal oscillatory movements [LOMs] *sensu* Howse, 1965) was often performed by soldiers in the opponent vicinity, and less frequently by workers.

## 4. Discussion

The termite set of exocrine organs is rich compared to that of solitary insects, and comprises 20 known glands, including the nasus gland. Termite glands can be classified by function, ontogenetic origin, volatility of their secretion, or other features. However, they are generally classified based on their ultrastructural features: glands made of class 1 cells (frontal, labral, and sternal glands in *Mastotermes*; Ampion and Quennedey, 1981; Quennedey, 1984; Quennedey et al., 2008; Šobotník et al., 2004, 2010b; Costa-Leonardo and Haifig, 2014), class 3 cells (mandibular base glands in *Machadotermes*, and mandibular, lateral thoracic, tarsal, tergal, and pleural glands; Lambinet, 1959; Ampion, 1980; Ampion and Quennedey, 1981; Quennedey, 1984; Costa-Leonardo and Haifig, 2010; Gonçalves et al., 2010; Costa-Leonardo et al., this issue), class 1 + 2 cells (sternal and posterior sternal glands; Ampion and Quennedey, 1981; Quennedey et al., 2008), class 1 + 3 cells (frontal gland in *Coptotermes* and *Heterotermes*, labral gland in *Glossotermes*, and clypeal, tergal, and tegumental glands; Ampion and Quennedey, 1981; Quennedey, 1984; Šobotník et al., 2003, 2010b; Křížková et al., 2014), class 1 + 2 + 3 cells (sternal, posterior sternal, and tergal glands; Ampion and Quennedey, 1981; Quennedey et al., 2008; Šobotník et al., 2005). In a few rare cases, however, this classification is impossible because the secretory cell type is unclear: (i) the secretory cells formally belong to class 1 cells but are highly modified for exclusion of large amounts of water-carried proteinaceous secretion (labial glands; Billen et al., 1989; Šobotník and Weyda, 2003); (ii) cells are of unknown ultrastructure (hypopharyngeal glands; Brossut, 1973); (iii) cells are disconnected from the cuticle and are of unknown origin (dehiscent glands in *Ruptitermes*; Costa-Leonardo, 2004).

The cellular organization of the nasus gland differs from other termite glands in several respects. Based on our observations, class 2 cells release their secretion in the form of lipid droplets freely into enlarged intercellular spaces, from where they seem to be accepted and released mostly by class 3 cells. This contrasts with other termite glands, in which only class 1 cells accept the secretion of class 2 cells (Quennedey et al., 2008; Šobotník et al., 2005). While class 3 cells are large and show high secretory activity, as evidenced by populous mitochondria and secretory vesicles, class 1 cells are obviously less active and differ from regular epidermal cells only in their volume, the presence of microvilli, scarce secretory organelles, vesicles and lipid droplets. Our observations therefore suggest that the secretion consists of a combination of non-polar (lipid-

like) compounds, produced by the class 2 cells and with allomone function, and of proteins produced by class 2 and 3 cells.

We observed bacteria in the cytoplasm of class 2 and 3 secretory cells but we did not study them in detail. The ultrastructural observations revealed morphological similarities between these bacteria and the genus *Wolbachia*, a common parasite of many insects (Sacchi et al., 2010; Chagas-Moutinho et al., 2015). However, more work would be needed to determine their function and their phylogenetic position with satisfactory precision.

Based upon our behavioural observations, we believe that the nasus gland produces a contact poison, similarly to the labial glands of *Mastotermes* and *Macrotermitinae* (Termitidae) or the frontal gland of *Rhinotermitidae* (Prestwich, 1984; Šobotník et al., 2010a). We have repeatedly observed *A. coninasus* soldiers hitting the opponents with the head during LOMs, and as this behaviour was selectively performed in the opponent vicinity, it may serve to deliver the secretion. However, the main weapon remains the frontal gland, as in other *Nasutitermitinae* (Prestwich, 1984; Šobotník et al., 2010a). *A. coninasus* is a soil-feeder (Bourguignon et al., 2011b) that we always observed in closed gallery systems. When encountered, workers tended to hide away while soldiers went out, kept their nasi up and scanned the surroundings with their antennae. This activity quickly increased in response to any kind of disturbance, e.g. air currents or presence of opponents (unpublished observation). Thus, the nasus gland secretion might also play a repellent or irritant role. Another possible function of the nasus gland may consist in purging the frontal gland secretion, which can lethally entangle nasute soldiers. Such a function was already hypothesized by Quennedey (1984), who claimed that the "rostral system" of *Trinervitermes* (identical class 3 secretory cells emptying their secretion either to frontal gland duct or to nasus surface) produces a solvent preventing the frontal gland secretion from adhering to the body cuticle. This assumption remains to be tested.

*Angularitermes* occupies a basal position in the *Nasutitermitinae* (Inward et al., 2007). They occur throughout South American rainforests and open formations ('cerrado' vegetation), but generally at a low abundance (e.g. Bourguignon et al., 2011a; Canello et al., 2014). They are largely outnumbered by other termites, which are devoid of nasus gland. The presence of nasus gland is obvious in *Angularitermes* soldiers but was not previously studied, probably because of the rarity of *Angularitermes* and other *Nasutitermitinae* that could possibly be endowed with a nasus gland. The presence of organs similar to the nasus gland has already been speculated in *Eutermellus*, *Spatulitermes*, and *Hirtitermes*, but no additional data are available. The glandular system is different in *Verrucositermes*, as the class 3 secretory cells orifices are grouped on tubercles on the nasus, the head and the antennae (Deligne, 1983; Quennedey, 1984; Canello et al., 1996). Additionally, the phylogeny of *Nasutitermitinae* is still not adequately resolved to determine if these putative glands may have a common origin. Future histological and phylogenetic studies are needed to resolve the origin of the nasus gland in *Nasutitermitinae*, although this is hampered by the sample rarity.

## 5. Conclusion

The nasus gland is a morphological novelty that evolved in soldiers of *Angularitermes*. It is the first gland composed of class 1, 2, and 3 cells (*sensu* Noirot and Quennedey, 1974) except for the sternal glands of some termites (Quennedey et al., 2008) and cockroaches (Sreng, 1985); and for the posterior sternal and tergal glands of termite (Šobotník et al., 2005) and cockroach (Sreng,



1985) imagoes. Unlike these, the nasus gland likely plays a defensive role, because of its presence on the nasus of *Angularitermes* spp. soldiers.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.asd.2015.08.006>.

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Figure SF1: Parasagittal histological section of the nasus gland. Note the arrow pointing at non-modified head epithelium.



Figure SF2: TEM picture showing development of approximately apical half of the nasus gland secretory epithelium. Note the centrally placed class 3 cell surrounded by class 1 cells.

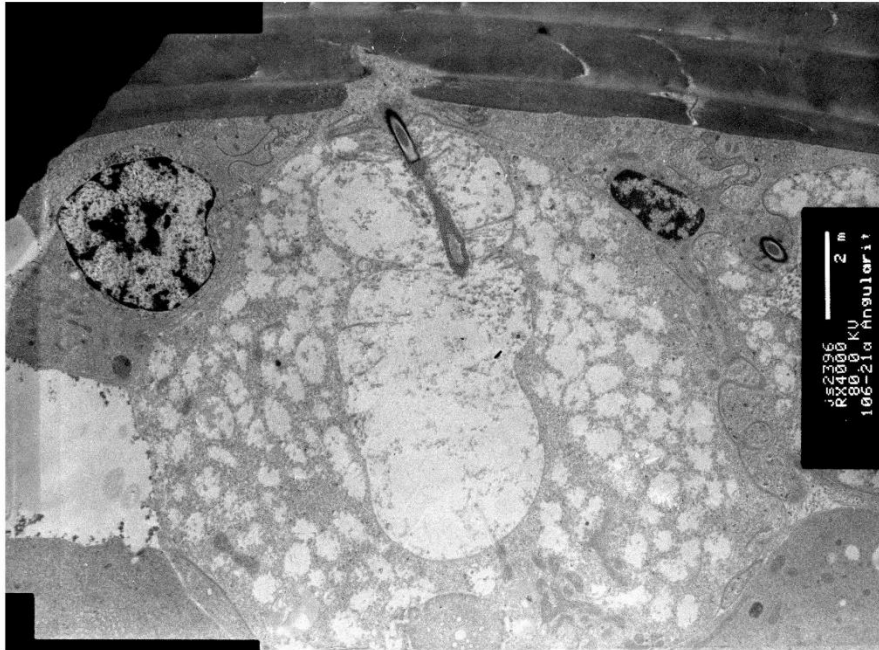




Figure SF3: TEM picture showing development of class 1 cell containing electron-lucent and electron-dense vacuoles, and lipid droplets located freely in the cytoplasm. Also note presence of apical microvilli and mitochondria. Secretory organelles are relatively poor, but numerous free ribosomes are present in the cytoplasm.

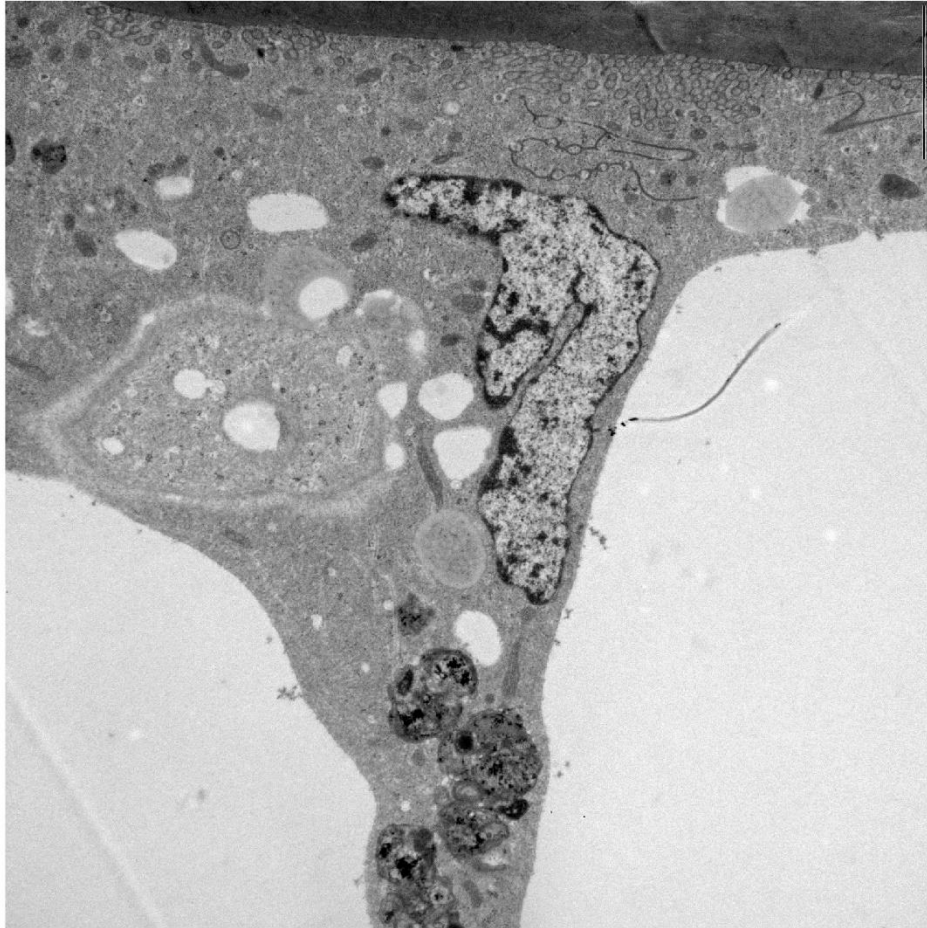


Figure SF4: TEM picture showing intercellular junctions (apical zonula adherens followed by septate junction) and a lipid droplet located in-between 2 neighbouring cells. Note well-developed apical microvilli. Note the microtubules of cytoskeleton and ribosomes located freely in the cytoplasm.



Figure SF5: Epicuticular pore in the nasus gland cuticle. The size roughly corresponds to SEM observations.

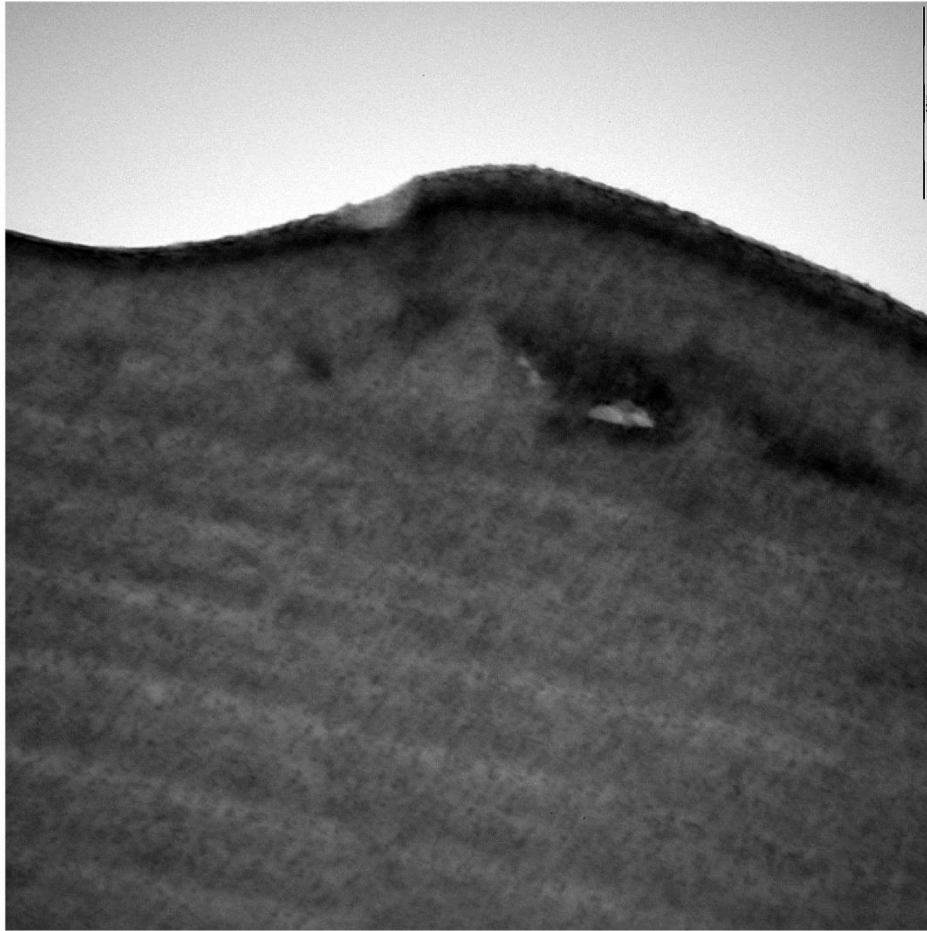


Figure SF6: TEM picture showing well-developed Golgi apparatus and smooth ER in the class 3 cell.

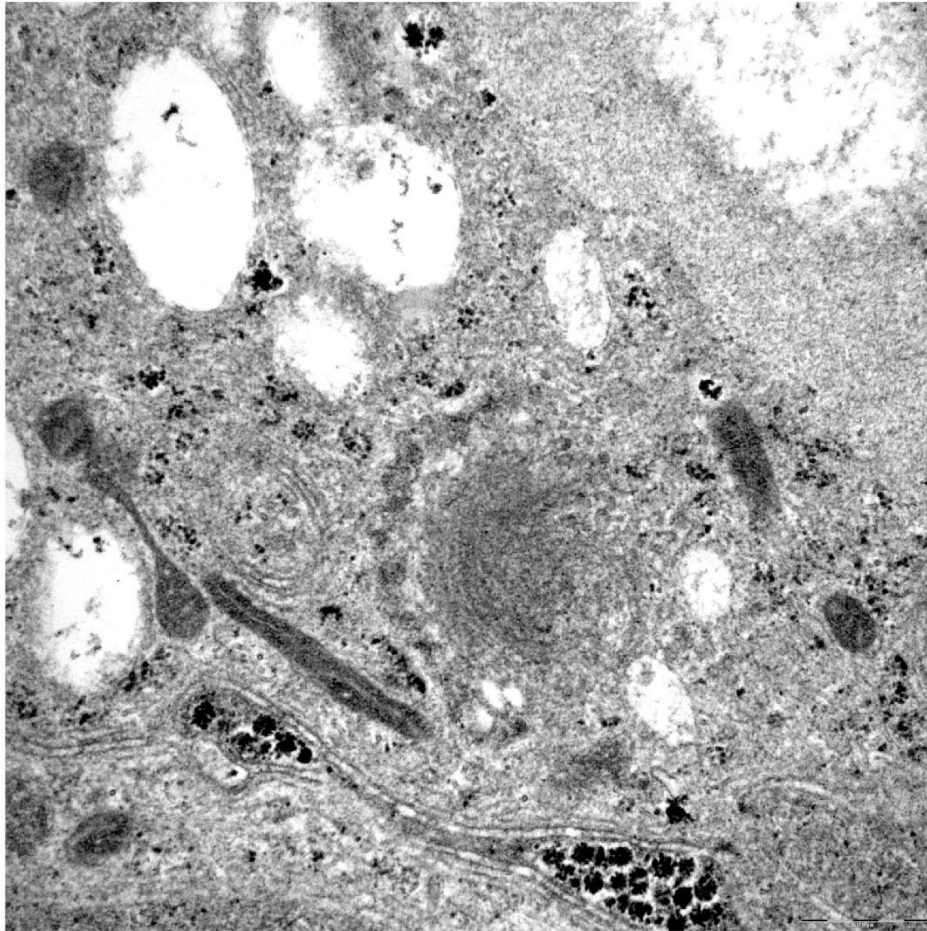




Figure SF7: TEM picture showing smooth ER, glycogen, lipid droplet and lucent vesicles in two neighbouring class 3 secretory cells. Note the presence of two free axons in-between the cells.

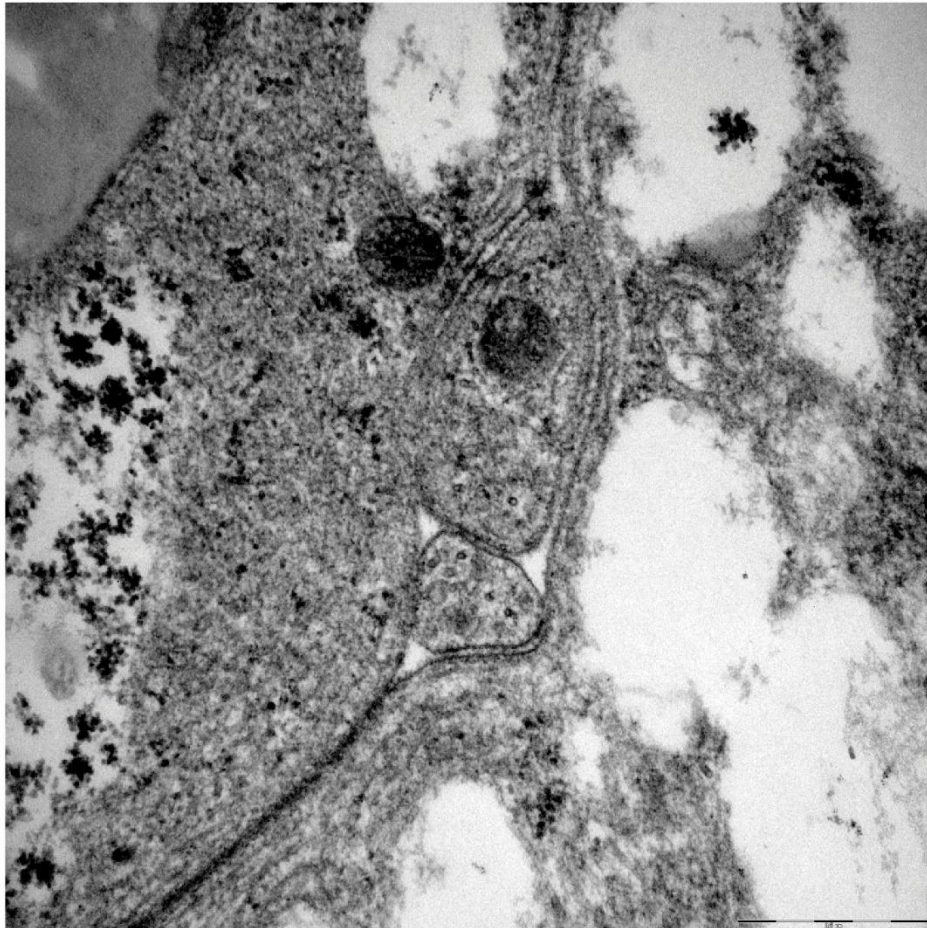




Figure SF8: TEM picture showing canal cell containing the conducting canal running through the cuticle.

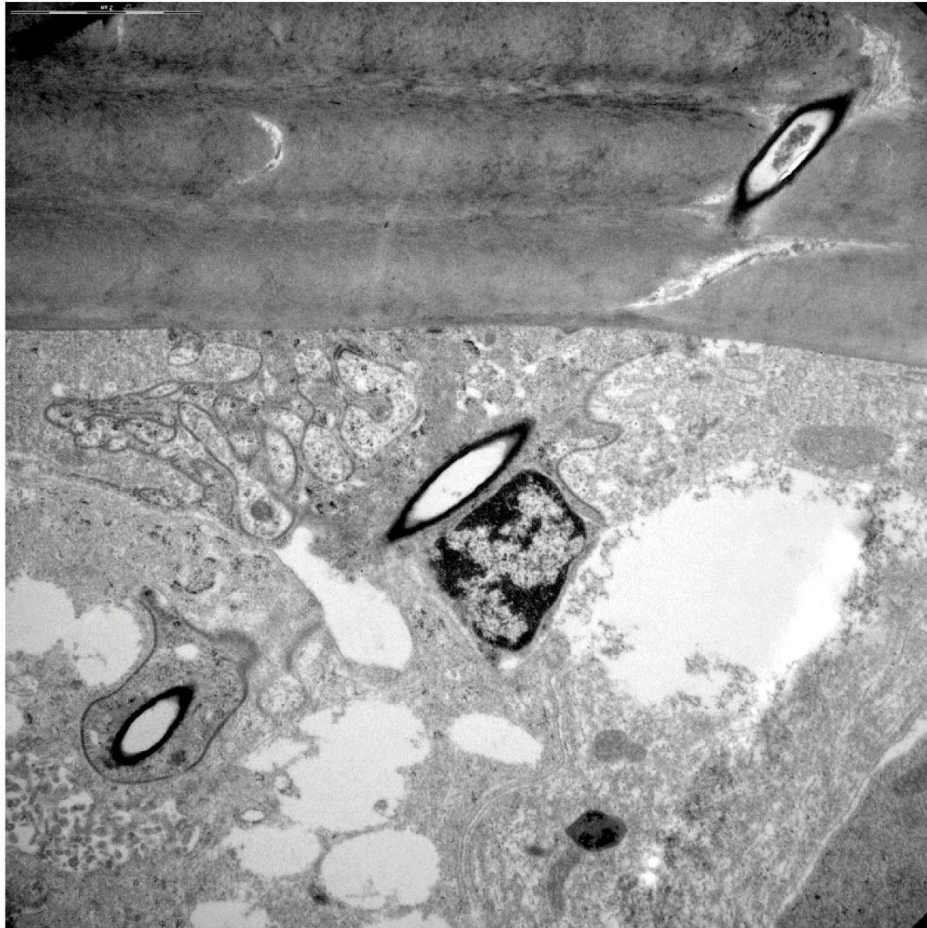


Figure SF9: TEM picture showing cytoplasm of class 2 secretory cell with well-developed smooth ER.

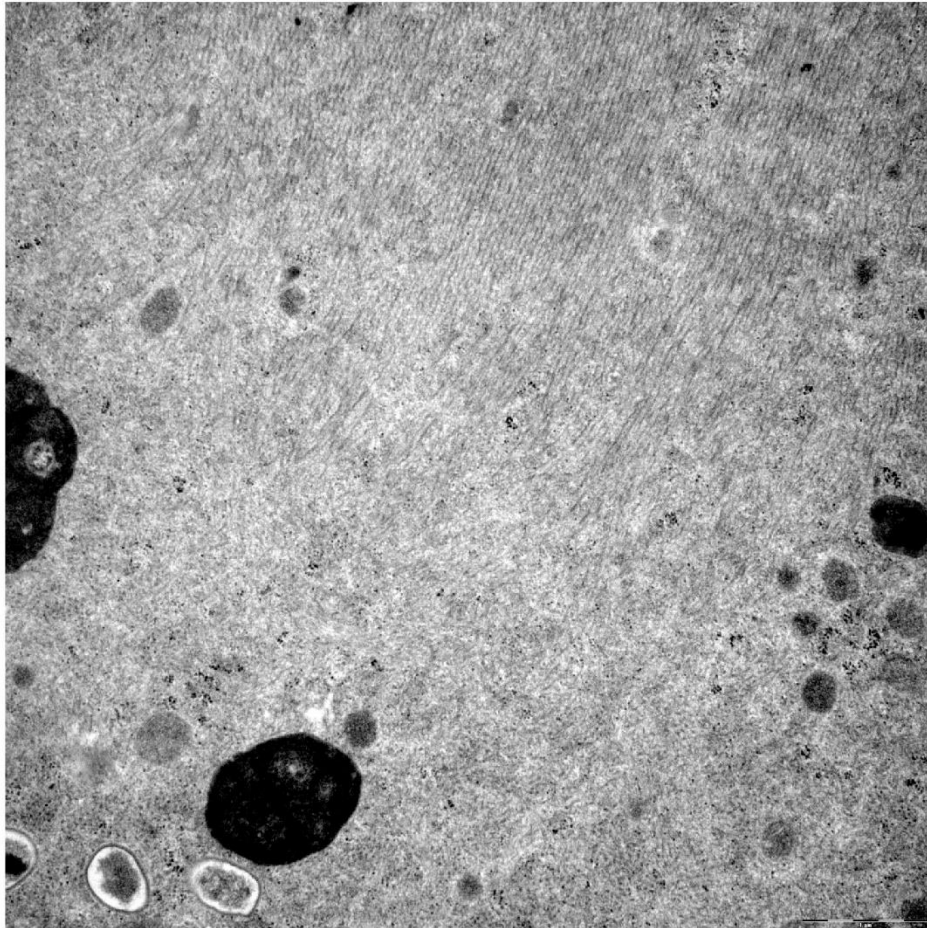


Figure SF10: TEM picture showing cytoplasm of class 2 secretory cell with well-developed rough ER and many free ribosomes.

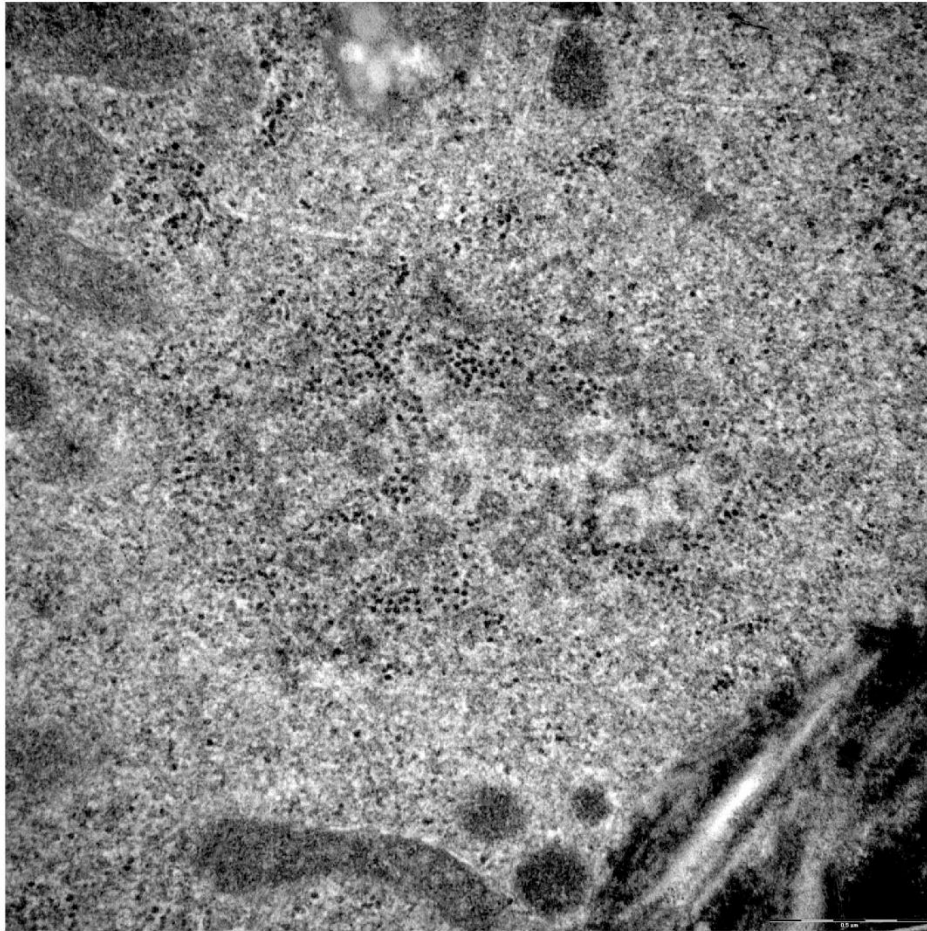


Figure SF11: Section through a basal part of mechanoreceptive seta possessing a single receptor neuron.

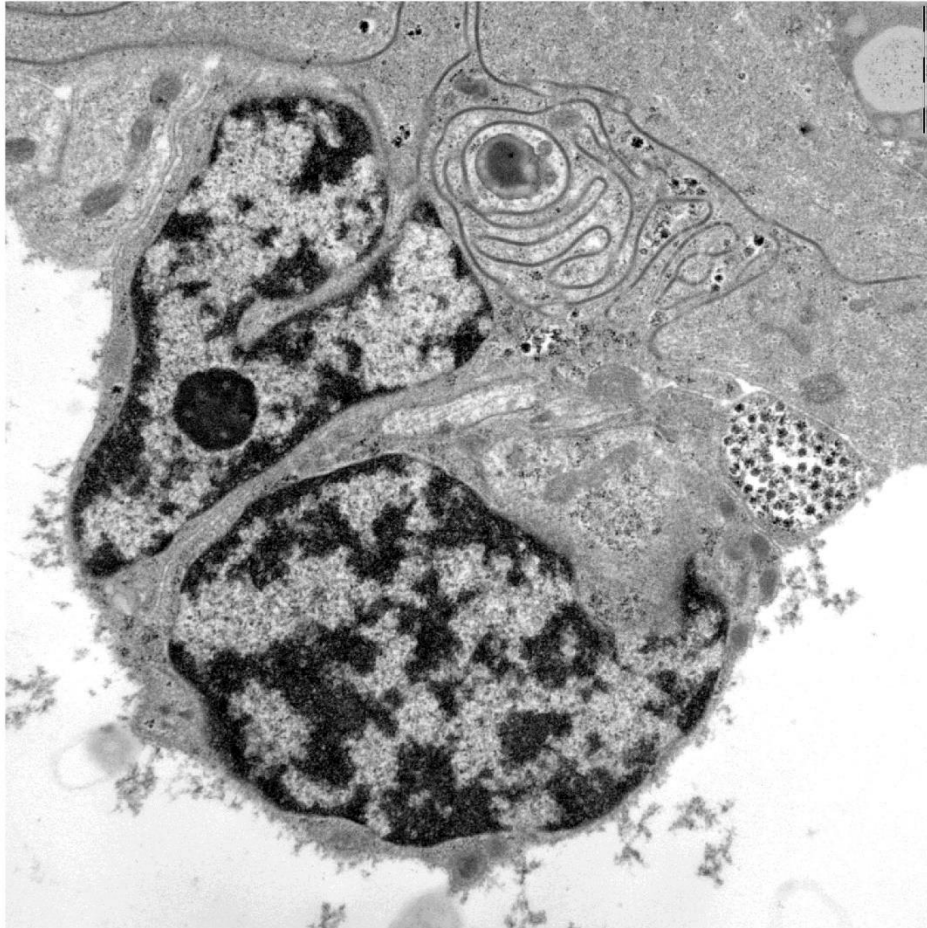
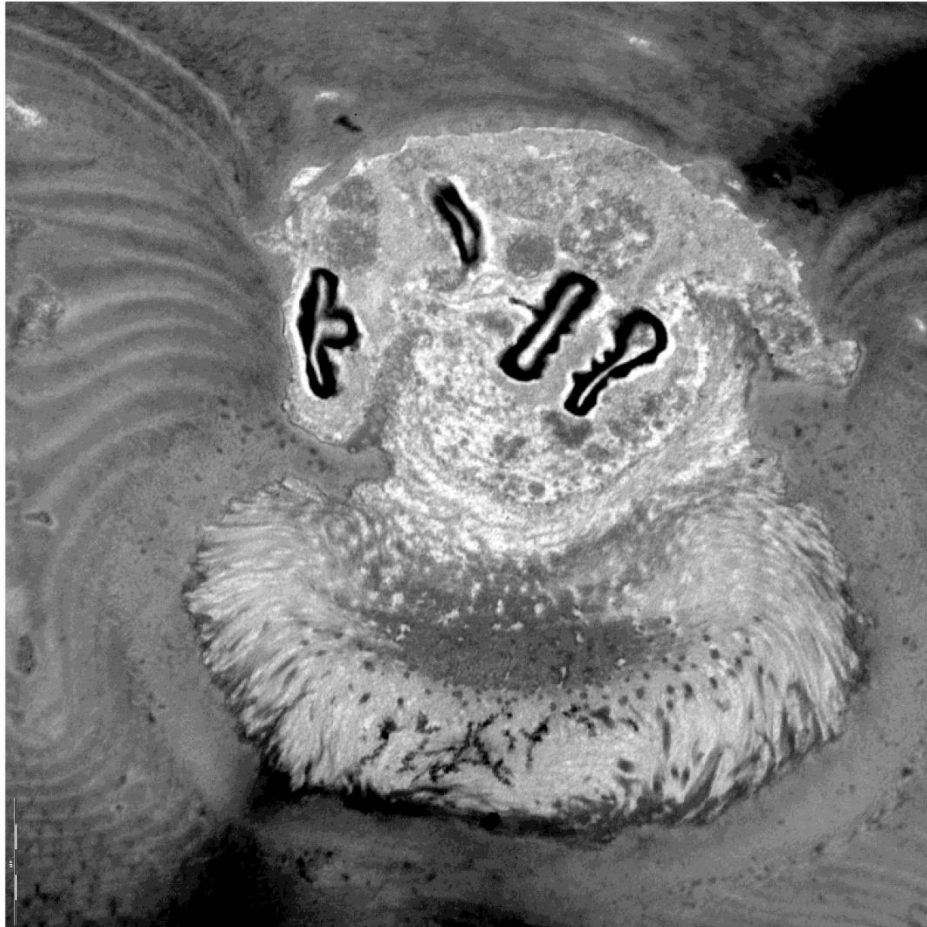


Figure SF12: Section through a chemoreceptive seta possessing four receptor neurons.



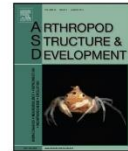
### 3.2 The clypeal gland: a new exocrine gland in termite imagoes (Isoptera: Serritermitidae, Rhinotermitidae, Termitidae).





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## The clypeal gland: A new exocrine gland in termite imagoes (Isoptera: Serritermitidae, Rhinotermitidae, Termitidae)

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## ABSTRACT

Social insects possess a rich set of exocrine organs producing diverse pheromones and defensive compounds. This is especially true for termite imagoes, which are equipped with several glands producing, among others, sex pheromones and defensive compounds protecting imagoes during the dispersal flight and colony foundation. Here, we describe the clypeal gland, a new termite exocrine organ occurring in the labro-clypeal region of imagoes of most Rhinotermitidae, Serritermitidae and Termitidae species. The clypeal gland of *Coptotermes testaceus* consists of class 1 (modified epidermal cell) and class 3 (bicellular gland unit) secretory cells. Ultrastructural features suggest that the gland secretes volatile compounds and proteins, probably after starting the reproduction. One peculiar feature of the gland is the presence of multiple secretory canals in a single canal cell, a feature never observed before in other insect glands. Although the function of the gland remains unknown, we hypothesize that it could produce secretion signalling the presence of functional reproductives or their need to be fed.

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## 1. Introduction

Living in society brings new challenges that require the evolution of plentiful behavioural, physiological and morphological adaptations. In social insects, tasks are partitioned among castes – categories of morphologically distinct individuals – that, for some of them, specifically evolved new devices with various functions. Many of these novelties are involved in communication or defence, and can be extremely exuberant, such as the defensive adaptations present in termite soldiers. New sophisticated means of communication are in part achieved through the evolution of new exocrine glands, whose diversity is particularly high in social insects. For example, 75 different exocrine glands have been described in ants (Billen, 2009a), of which 20 occur only in the legs (Billen, 2009b).

Unlike the diverse set of exocrine glands described in ants, only 20 exocrine glands have been described in termites; including the 17 glands listed in Gonçalves et al. (2010), the hypopharyngeal gland described by Brossut (1973), the crystal gland described by Šobotník et al. (2014) and the posterior tergal glands (Costa-Leonardo and Hafig, 2010). In comparison, only 11 exocrine glands have been described in cockroaches (see Brossut, 1973;

Brossut and Sreng, 1980; Abed et al., 1993), the group to which termites belong (Lo et al., 2000). Although the exact function of many of these glands remains unclear, known functions are generally related to defence and communication. The sternal and tergal glands produce the trail and sex pheromones, respectively (Bordereau and Pasteels, 2011). The frontal gland, present in all Neoisoptera, produces defensive compounds and alarm pheromones (Šobotník et al., 2010a). The secretion of the workers' labial glands reveals several functions connected to feeding (including food marking pheromone, phagostimulant, digestive enzymes and food for dependent castes), but also serves as a cement used for nest building (Noirot, 1969; Reinhard et al., 2002); soldiers produce certain part of the defensive secretions in their labial glands (Sillam-Dussès et al., 2012). Finally, several of the other described exocrine glands are specific to a few species, such as the defensive glands of *Ruptitermes* or *Neocapritermes* involved in the self-sacrifice of its carrier through autothysis (e.g. Costa-Leonardo, 2004; Šobotník et al., 2012, 2014).

Among the different castes occurring in termite colonies, imagoes are the one with the highest diversity of exocrine glands. Imagoes need to signal their reproductive status and produce the primer pheromones, which avert nest-mate reproduction (Ampion, 1980; Leis and Sbrenna, 1983; Šobotník et al., 2003; Matsuura et al., 2010). Unlike other termite castes living in closed gallery systems, which provide suitable environment and protection against

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desiccation and predators, alate imagoes are directly exposed to predators when they leave the parental nest to found their own colonies (Nutting, 1969). In response, alate imagoes of many termite species have developed defensive glands, such as the frontal gland (well-developed especially in soldiers), which is an active secretory organ producing defensive and repellent secretion (Piskorski et al., 2009; Sobotník et al., 2010b; Kutalová et al., 2013).

Most studies on glands and the secretion they produce were conducted on workers (labial and sternal glands) and soldiers (frontal and labial glands). In contrast, imaginal glands have only occasionally been studied in details (tergal glands). On the 126 studies dealing with composition of defensive secretions in termites, only two focused on imagoes (see Sobotník et al., 2010a). To a lesser extent, this is also true for structural works; larvae, presoldiers and reproductives being the least studied termite castes. Here, we describe a new termite gland, the clypeal gland, which consists of a thickened secretory epithelium located in the head of alate imagoes. We described in detail the clypeal gland of the termite pest species *Coptotermes testaceus* (Rouland-Lefèvre, 2011), and report on the gland occurrence in a representative set of 39 species from the families Rhinotermitidae, Serritermitidae and Termitidae.

## 2. Material & methods

We sampled imagoes of *C. testaceus* (Linnaeus, 1758) during a dispersal flight that took place near Petit Saut dam (French Guiana, N 05°04', W 52°59'), on the 31st of January 2010. Workers and soldiers of the same species were collected in nearby forest two days later. Living termites were submerged into a drop of fixative (mixture of 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2), cut into three pieces (head without mandibles, thorax + the first two abdominal segments, rest of abdomen), and stored at 4 °C. 24 h later, the samples were submerged into washing buffer (0.1 M cacodylate buffer with addition of 5% glucose), which was replaced every 10–15 days until subsequent steps. The samples were post-fixed in 1.5% OsO<sub>4</sub> in 0.1 M cacodylate buffer for 2 h, dehydrated with a standard ethanol series (50%, 75%, 100%), embedded into standard Spurr resin and polymerized for 8 h at 70 °C. Semithin sections (0.5 µm thick) were stained with toluidine blue and studied using a Nikon Eclipse Ci optical microscope combined with a Jenoptik ProgRes CT3 camera. Ultrathin sections (50–80 nm) were stained according to the standard protocol developed by Reynolds (1963) and observed using a Jeol 1011 transmission electron microscope (TEM). Two male and two female imagoes were treated, as well as two workers and two soldiers. The labro-clypeal region of the head was observed in imagoes of 13 Rhinotermitidae, 1 Serritermitidae and 25 Termitidae species (Table 1) using optical microscopy. The gland was considered to be present when the thickness of the glandular epithelium largely exceeded that of the non-glandular epithelium (more than 5 µm for the gland and less than 1 µm for the non-modified epidermis). Direct confirmation of the gland presence using TEM was only available for a female imago of *Termes* sp. B.

## 3. Results

The gland presence was tentatively confirmed in all Neoisoptera species available for our study with the exception of *Aparatermes* sp. and *Anoplotermes janus* (for details see Table 1). Although its size varies among species (see Fig. 1), in most of them it covers the dorsal posterior side of the labrum and anteclypeus to the large group of muscles stretched between the postclypeus and pharynx.

The clypeal gland is located on the dorsal part of the labrum and anteclypeus in male and female alate imagoes of *C. testaceus*, and the gland is absent in workers and soldiers. It is made of class 1 and 3 secretory cells (*sensu* Noirot and Quennedey, 1974). The secretory

epithelium is about 10 µm (5–15 µm) thick (Fig. 2A) and the gland is surrounded by thinner unmodified epidermal cells (about 0.5 µm thick; not shown). The overlaying cuticle reveals structural modifications saturating the secretion discharge from the body, consisting namely in enlarged pore canals within the endocuticle (to a lesser extent also in the exocuticle, see inset in Fig. 2A) and the epicuticle perforated with tiny pores. The endocuticle is between 3 and 6 µm thick, while the exocuticle is between 0.5 and 2 µm thick but completely missing at the labro-clypeal articulation. The epicuticle consists of 30–60 nm thick inner epicuticle and a typical trilaminar outer epicuticle of about 20 nm thick. The gap between secretory cells layer and the cuticle is present predominantly in the gland centrum near the labro-clypeal articulation, where it may reach up to 6 µm thick, but diminishes marginally. The gland is covered by a basal lamina made of a single layer about 50 nm thick.

Class 1 secretory cells (Fig. 2B, C) are columnar, with basally located elongated nuclei (about 6 µm in the longest dimension). Junctions between neighbouring class 1 cells comprise apical zonula adherens followed by smooth and perforated septate junctions; plasma membranes are devoid of junctions in the basal half of the secretory epithelium thickness. Microvilli are abundant but relatively short (usually between 0.7 and 1.2 µm), with or without central ductule. Secretory organelles comprise fewer smooth and more abundant rough endoplasmic reticulum (ER) (Fig. 2C) and large but infrequent Golgi apparatus. Elongated mitochondria (up to 2 µm long) are moderately populous and scattered throughout the cells. Secretory vesicles are of two basic kinds, the most common are (i) electron-dense granules coated in plasma membrane between 0.7 and 1 µm in diameter, and (ii) small (about 200 nm in diameter) and rare electron-lucent vesicles originated from the pinocytotic activity at the cell bases.

Class 3 secretory cells (or bicellular secretory units) are less frequent compared to class 1 cells. They are oval in shape (10–15 µm in their longest dimension), squeezed between class 1 cells, without any contact neither to the apex nor to the basis of the secretory epithelium (Fig. 2D). Their nuclei are regular, ovoid in shape, about 4.5 µm in diameter. The dominant secretory organelles comprise rough ER and Golgi apparatus, located basally in the cells. Their central part is occupied by large (0.7–1.7 µm in diameter) electron-lucent secretory vesicles and end apparatus consisting of a long coiled receiving canal (about 600 nm thick, made of several perforated epicuticular layers altogether about 200 nm thick) placed within extracellular reservoir lined with short (0.4–1.2 µm) and spaced microvilli. The cytoplasm also contains abundant electron-dense granules between 0.5 and 1 µm in diameter, bound in plasma membrane similarly to those observed in class 1 cells. Mitochondria are moderately abundant, elongated (up to 1.7 µm), located predominantly at the cell apices.

Canal cells are poor in organelles and contain irregular small (up to 7 µm in the longest dimension) and flat nuclei with numerous large chromatin aggregates. The cells are connected to secretory cells with a perforated septate junction only. Conducting canals (about 400 nm in diameter) are made of outer and inner epicuticle (about 80 nm thick together), continuous with the body cuticle. Surprisingly, canal cells contain several sections of conducting canal(s) (up to 8 sections of canals; see Fig. 2A), and this observation can be explained either by the presence of more than a single canal in a canal cell, or by the coiled arrangement of a single conducting canal.

The ultrastructure of the secretory epithelium was very similar in a *Termes* sp. B female.

## 4. Discussion

The clypeal gland is a new member of the termite exocrine gland set and occurs in the head of alate imagoes of Rhinotermitidae,



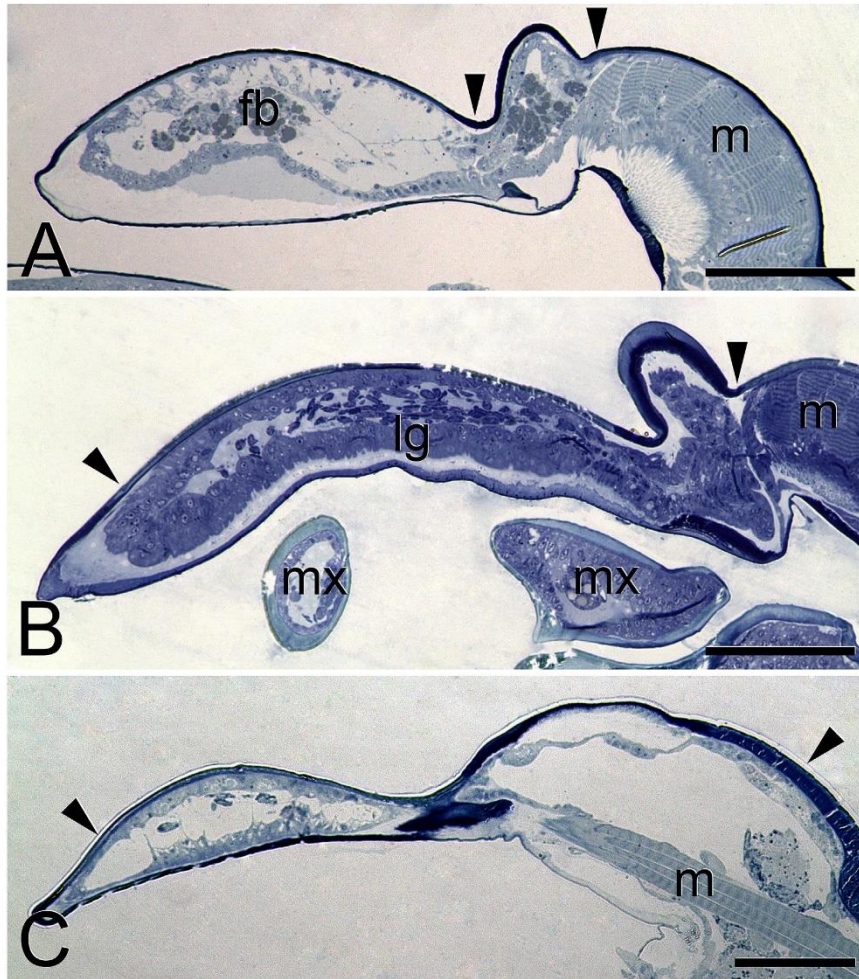
**Table 1**

List of species used in this study, presence/absence of the clypeal gland and number of observations.

Species	Group	Sex	Presence of the gland	No. of observations
<i>Glossotermes oculatus</i> Emerson, 1950	Serritermitidae	Male	+	2
		Female	+	3
<i>Prorhinotermes simplex</i> (Hagen 1858)	Rhinotermitidae: Prorhinotermitinae	Male	+	1
		Female	+	1
<i>Psammotermes allocerus</i> Silvestri, 1908	Rhinotermitidae: Psammotermitinae	Female	+	1
<i>Psammotermes hybostoma</i> Desneux, 1902	Rhinotermitidae: Psammotermitinae	Male	+	2
		Female	+	3
<i>Termitogeton planus</i> (Haviland, 1898)	Rhinotermitidae: Termitogeninae	Female	+	1
<i>Reticulitermes lucifugus</i> (Rossi, 1792)	Rhinotermitidae: Heterotermitinae	Male	+	1
		Female	+	1
<i>Heterotermes tenuis</i> (Hagen, 1858)	Rhinotermitidae: Heterotermitinae	Male	+	3
		Female	+	2
<i>Heterotermes paradoxus</i> (Froggatt, 1898)	Rhinotermitidae: Heterotermitinae	Male	+	2
		Female	+	1
<i>Coptotermes formosanus</i> Shiraki, 1909	Rhinotermitidae: Coptotermitinae	Male	+	1
		Female	+	1
<i>Coptotermes testaceus</i> Linnaeus, 1768	Rhinotermitidae: Coptotermitinae	Male	+	2
		Female	+	2
<i>Parrhinotermes browni</i> (Harris, 1958)	Rhinotermitidae: Rhinotermitinae	Female	+	1
<i>Schedorhinotermes translucens</i> (Haviland, 1898)	Rhinotermitidae: Rhinotermitinae	Male	+	1
		Female	+	1
<i>Dolichorhinotermes</i> sp.	Rhinotermitidae: Rhinotermitinae	Male	+	1
<i>Rhinotermes marginalis</i> (Linnaeus, 1758)	Rhinotermitidae: Rhinotermitinae	Male	+	2
<i>Ancistrotermes cavithorax</i> (Sjoestedt, 1899)	Termitidae: Macrotermitinae	Male	+	1
		Female	+	1
<i>Microtermes toumodiensis</i> Grassé, 1937	Termitidae: Macrotermitinae	Male	+	1
<i>Protermes</i> sp.	Termitidae: Macrotermitinae	Male	+	1
<i>Sphaerotermes sphaerotherax</i> (Sjoestedt, 1911)	Termitidae: Sphaerotermitinae	Male	+	2
		Female	+	2
<i>Foraminitermes coatoni</i> Krishna, 1963	Termitidae: Foraminitermitinae	Male	+	1
		Female	+	1
<i>Anoplotermes janus</i> Bourguignon et al., 2010	Termitidae: Apicotermitinae	Male	–	1
<i>Anoplotermes</i> -group gen. sp.	Termitidae: Apicotermitinae	Male	+	2
<i>Aparatermes</i> sp.	Termitidae: Apicotermitinae	Male	–	1
		Female	–	1
<i>Astalotermes quietus</i> (Silvestri, 1914)	Termitidae: Apicotermitinae	Female	+	1
<i>Longusitermes manni</i> (Snyder, 1922)	Termitidae: Apicotermitinae	Male	+	1
<i>Ruptitermes</i> sp.	Termitidae: Apicotermitinae	Male	+	1
		Female	+	1
<i>Amitermes beaumonti</i> Banks, 1918	Termitidae: Termitinae	Male	+	3
		Female	+	2
<i>Dentispicotermes brevicarinatus</i> (Emerson, 1950)	Termitidae: Termitinae	Female	+	1
<i>Microcerotermes</i> sp.	Termitidae: Termitinae	Male	+	2
<i>Neocapritermes araguaia</i> Krishna and Araujo, 1968	Termitidae: Termitinae	Male	+	2
		Female	+	1
<i>Pericapritermes papuanus</i> Bourguignon and Roisin, 2008	Termitidae: Termitinae	Male	+	1
<i>Termes</i> sp. B	Termitidae: Termitinae	Male	+	4
		Female	+	3
<i>Embiratermes neotenicus</i> (Holmgren, 1906)	Termitidae: Syntermitinae	Male	+	1
	Termitidae: Syntermitinae	Female	+	2
<i>Rhynchotermes perarmatus</i> (Snyder, 1925)	Termitidae: Syntermitinae	Male	+	1
<i>Silvestritermes holmgreni</i> (Snyder, 1926)	Termitidae: Syntermitinae	Female	+	1
<i>Grallatotermes grallator</i> (Desneux, 1905)	Termitidae: Nasutitermitinae	Female	+	1
<i>Hospitalitermes papuanus</i> Ahmad, 1947	Termitidae: Nasutitermitinae	Male	+	1
		Female	+	1
<i>Nasutitermes corniger</i> (Motschulsky, 1855)	Termitidae: Nasutitermitinae	Male	+	1
<i>Nasutitermes princeps</i> (Desneux, 1905)	Termitidae: Nasutitermitinae	Male	+	1
<i>Subulitermes</i> sp.	Termitidae: Nasutitermitinae	Male	+	2

Serritermitidae and Termitidae. In *C. testaceus* and *Termes* sp. B, it consists of classes 1 and 3 secretory cells (according to Noirot and Quennedey, 1974). It represents an active secretory organ, as evidenced by the presence of (i) secretory organelles (rough and smooth ER, Golgi apparatus), (ii) microvilli at the cell apices, (iii) secretion inside and outside of the secretory cells, (iv) more mitochondria in the secretory cells than in the surrounding unmodified epidermal cells, and (v) structural modification of the overlying cuticle allowing the secretion penetration to the exterior. One peculiarity of the clypeal gland is the numerous sections of conducting canal(s) associated with a single canal cell. The presence of

several canals within a single canal cell is not excluded based on our observations, but this would represent a unique feature of the clypeal gland. An alternative explanation is that a canal cell possesses a single coiled conducting canal, allowing the gland to grow considerably bigger in mature reproductives, as in the tegumental glands of *Prorhinotermes* neotenicus (Sobotník et al., 2003). This feature is anyway truly remarkable, because other less frequent modifications of bicellular secretory units are different, e.g. two secretory cells releasing their products into the same canal (for review see Quennedey, 1998), a single canal leading through several canal cells (for a review see Quennedey, 1998; or Pekár and



**Fig. 1.** Development of the clypeal gland. **A.** Sagittal section of the fore head of *Glossotermes oculatus* female. **B.** Sagittal section of the fore head of *Coptotermes testaceus* male. **C.** Parasagittal section of the fore head of *Nasutitermes* sp. male. The approximated extent of the gland is delimited by arrowheads. Scale bars represent 100  $\mu\text{m}$  in all figures. Abbreviations: fb, fat body; lg, labral gland; m, muscle; mx, maxilla.

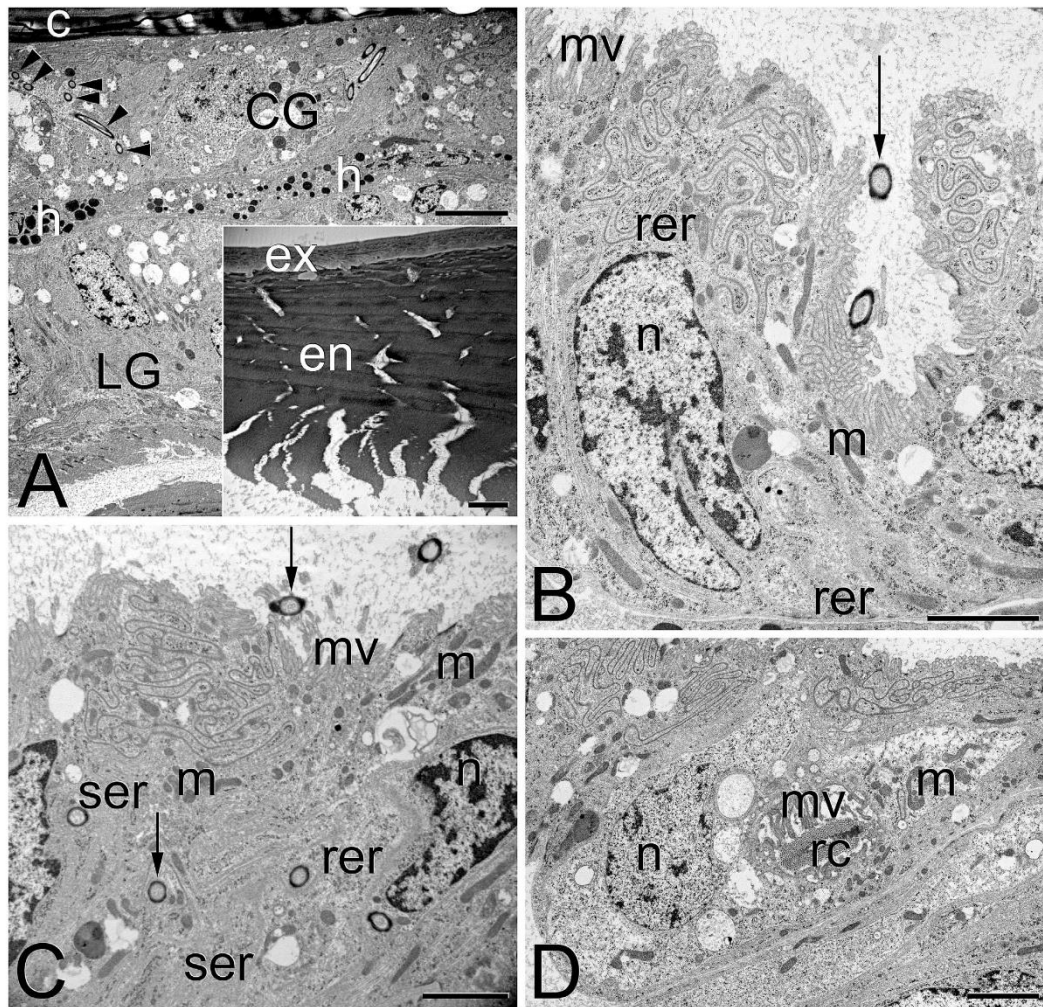
(Sobotník, 2007) or certain secretory function of the canal cell itself (Sobotník et al., 2003).

The function of the clypeal gland is unknown and can only be speculated based on the ultrastructural features of secretory cells. The secretion is probably partially proteinaceous due to the presence of rough ER and numerous electron-dense granules (likely of proteinaceous nature) in class 1 and class 3 secretory cells. Class 1 secretory cells are also rich in smooth ER which is well-known for the production of volatile compounds (Percy-Cunningham and MacDonald, 1987; Tillman et al., 1999). As the gland occurs in alate imagoes only, and that volatile secretion can provide a pheromonal signal, one putative function of the clypeal gland is that it produces the pheromones signalling the reproductive status of the royal couple. Kings and queens are attractive for sterile castes (Maistrello and Sbrenna, 1999, personal observation), and thus

reproductive-specific glands can provide information about their identity. Another possible function of the clypeal gland secretion is marking the oral side of the reproductives to ease worker feeding. Reproductives do not beg actively for food like larvae and soldiers do and therefore must possess alternative mechanisms to be fed by workers (Kawatsu, 2013, personal observation). If it is so, the proteins can further enhance the signal, inducing worker grooming and easing the feeding of reproductives. Other functions known for the exocrine organs in social insects, like nest-mate recognition or recruitment, can be excluded because the gland occurs in alate imagoes only.

The clypeal gland, as observed in detail in *C. testaceus*, reveals only a low secretory activity, as evidenced by a moderate number of mitochondria and low amounts of secretion released from secretory cells (in fact, although the secretion observed inside and





**Fig. 2.** Ultrastructure of the clypeal gland in *Coptotermes testaceus*. **A.** Cross section of the basal part of the labrum showing the clypeal and the labral glands. Arrowheads indicate 6 canals running in a single canal cell. Scale bars represent 5  $\mu\text{m}$  or 1  $\mu\text{m}$  in the inset, respectively. **B.** Appearance of class 1 secretory cell. Arrow indicates conducting canal. Scale bar represents 2  $\mu\text{m}$ . **C.** Detail of apical part of class 1 secretory cell. Arrows indicate conducting canals within canal cells. Scale bar represents 2  $\mu\text{m}$ . **D.** Appearance of class 3 secretory cell. Scale bar represents 2  $\mu\text{m}$ . Abbreviations: c, cuticle; CG, clypeal gland; en, endocuticle; ex, exocuticle; h, haemocyte; LG, labral gland; m, mitochondria; mv, microvilli; n, nucleus; rc, receiving canal; rer, rough endoplasmic reticulum; ser, smooth endoplasmic reticulum.

outside the secretory cells look very similar, no observation of the secretion release was made). These facts support our view that, irrespectively of the speculated function, the gland reaches its maturity only in older, reproductively active imagoes, which were not observed in this study.

The overall structure of the clypeal gland also suggests that it does not have any defensive function, because defensive glands are generally equipped with a reservoir in which the secretion accumulates (Chapman, 1998). Also, the protection from parasites or pathogens does not conform with the gland position, because such glands are expected to be located where the secretion can easily be applied onto the substrate or other body parts (e.g. tarsal or sternal

glands), or alternatively where pathogens can easily enter the body (weak intersegmental membranes, articulations etc.).

The clypeal gland occurs in most species studied here and is thus expected to belong to the basic equipment of termite imagoes, and its absence in *A. janus* and *Aparatermes* sp. is probably the result of secondary loss. Future work should investigate whether the clypeal gland also occurs in more basal termites, which were not studied here.

In a paper describing a new lateral thoracic gland, Gonçalves et al. (2010) named 17 glands known in termites, but the hypopharyngeal gland (*sensu* Brossut, 1973) was omitted from this list. Three new glands were discovered since then; namely, the crystal



glands (Šobotník et al., 2012, 2014), the posterior tergal gland (Costa-Leonardo and Haifig, 2010) and the clypeal gland described here. At the same time, some of the glands considered as distinct secretory organs by Gonçalves et al. (2010) do not deserve their own denomination, especially (i) the cibarial gland and (ii) the tibial gland. The term cibarial gland was introduced by Quennedy (1984), who discovered that the secretory epithelium on the ventral side of *Macrotermes* soldier labrum, previously described as labral gland (Deligne et al., 1981), also occurs on the dorsal side of hypopharynx. He therefore suggested renaming the whole secretory organ, which consists of identical secretory cells in two different body parts. Tibial gland is a term introduced by Gonçalves et al. (2010) to refer to the tibial part of the tarsal gland. Tarsal glands presence is highly variable among taxa, but they may occur on the distal side of the tibia, and on the 1st, 2nd and 3rd tarsomere (Bacchus, 1979; Soares and Costa-Leonardo, 2002), and the ultrastructure of the secretory cells is identical between tibia and tarsal segments at least in *Reticulitermes lucifugus* (Šobotník and Weyda, 2002). In conclusion, our opinion is that termite glandular set comprises 19 distinct known exocrine organs, that are epidermal tegumental glands, bicellular unit tegumental glands, frontal gland, labral gland, mandibular base glands, mandibular glands, labial glands, tarsal glands, lateral thoracic glands, dehiscent glands, tergal glands, sternal glands, posterior sternal glands, spermathecal gland, pleural abdominal glands (reviewed by Gonçalves et al., 2010), hypopharyngeal gland (Brossut, 1973), crystal glands (Šobotník et al., 2012, 2014), posterior tergal glands (Costa-Leonardo and Haifig, 2010) and the clypeal gland described here.

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### 3.3 The labral gland in termite soldiers

## The labral gland in termite soldiers

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The evolutionary success of termites has been 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 comprises two secretory regions located on the ventral side of the labrum and the dorso-apical part of the hypopharynx. The labrum of Neoisoptera has 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.

ADDITIONAL KEYWORDS: exocrine gland – hypopharynx – labrum – Termitoidea – ultrastructure – Isoptera.

### INTRODUCTION

Termites are an important food resource for a range of animals (Redford & Dorea, 1984), and they compete for resources with other wood- and soil-feeding taxa (Šobotník, Jirosová & Hanus, 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 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, the complexity of which is reflected in the development of 20 different signal-producing exocrine organs (Billen &

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Šobotník, 2015). Four glands are found in most termite species: the frontal gland, the sternal gland, the labial glands and the mandibular glands. 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, known only from the soldier caste of three termite species (Deligne, Quennedey & Blum, 1981; Quennedey, 1984; Šobotník et al., 2010b; Costa-Leonardo & Hafig, 2014), and from some imagoes (Křížková et al., 2014).

The labral gland was first described 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 of 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 & Hafig, 2014). The function of the labral gland has not been studied for 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 cameras, combined with Canon EF 100 mm f/2.8L Macro IS USM and Canon MP-E 65 mm f/2.8 lenses, and equipped with the Canon Macro Twin Lite MT-24EX flash. The photographs 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 (0.2 M, pH 7.2 buffer/formaldehyde 10%/glutaraldehyde 8% = 2 : 1 : 1), cacodylate buffer (0.2 M, pH 7.3 buffer/glutaraldehyde 8%/

distilled water = 2 : 1 : 1) and standard Bouin's solution (for details see Supplementary Information, Table S1). For electron microscopy, soldier heads were cut off and the mandibles were removed to facilitate sectioning. The mandibles were left intact in the minor soldiers of Rhinotermitinae and in all Nasutitermitinae. Samples were postfixed using 2% osmium tetroxide, and embedded in Spurr resin. The samples were cut into 0.5- $\mu$ m sections using a Reichert Ultracut ultramicrotome and stained with Azure II for analysis with optical microscopy.

### HISTOLOGY

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

### ELECTRON MICROSCOPY

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

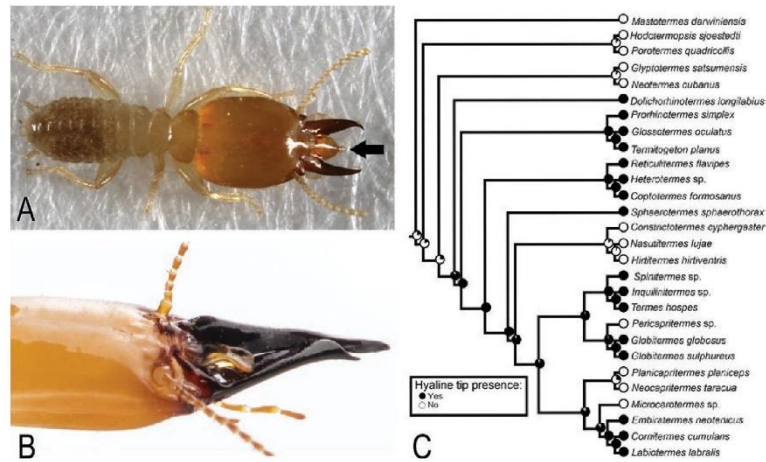
Ultrastructural features were studied in selected samples (see Table S1) using a Jeol 1011 transmission electron microscope, as described by Šobotník, Weyda & Hanus (2003).

### EVOLUTION OF THE HYALINE TIP

We reconstructed the presence of the 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 a transparent



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

inflated apical part termed the '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 species. The hyaline tip has been lost in several lineages, including the snapping soldiers and all Nasutitermitinae (Figs 1C, S1).

#### SCANNING ELECTRON MICROSCOPY

The ventral facies of the labrum were flexible and appeared wrinkled (Fig. 2A), while the dorsal facies were more rigid with a sclerotized cuticle. The ventral side of the labrum generally carried a few tens of sensillae (Fig. 2B), probably acting as contact chemoreceptors [based on combined scanning (SEM) and transmission electron microscopy (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  $\mu\text{m}$  in the largest dimension. 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 *Neoterms cubanus*, *Glossotermes oculatus*, *Neocapritermes taracua*, *Spinitermes* sp. and *Labiatermes labralis*. The same pattern was also observed along the midline of

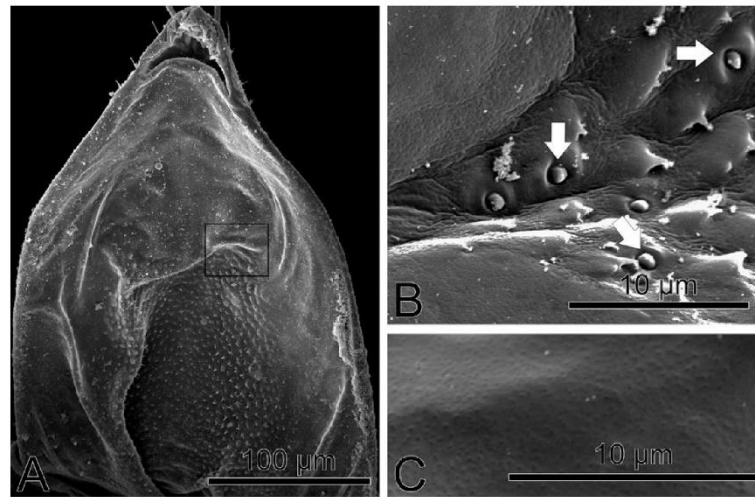
the labrum in *Prorehinotermes simplex*, the basal half of the labrum in *Coptotermes formosanus* (Fig. 2A, B) and *Sphaeroterms sphaerothorax*, and the basal part of the labrum in *Embiratermes neotenicus*. In all specimens, the apical and ventro-lateral part of the labrum possessed numerous pores typically about 30–50 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 (Fig. 3A, B). Labral gland secretions were shown to accumulate in the space between the secretory epithelium and the overlying 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 soldiers of *Psammotermes hybostoma* (147  $\mu\text{m}$ ) (Table S1). Hypopharyngeal thickness varied between 4 and 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.





**Figure 2.** Labral gland development. (A) Micrograph of the ventral side of the labrum of *Coptotermes formosanus*; the small rectangle indicates the sector where the micrograph in B was taken. (B) Region with a group of sensillae (marked with white arrows) in *C. formosanus* labrum. (C) High-magnification micrograph of the apical region with epicuticular pores in *Sphaeroterme sphaerotherax* labrum.

Within the four studied species with soldier subcastes, the thickness of the labral gland increased with the size of the soldier morph (Table S1).

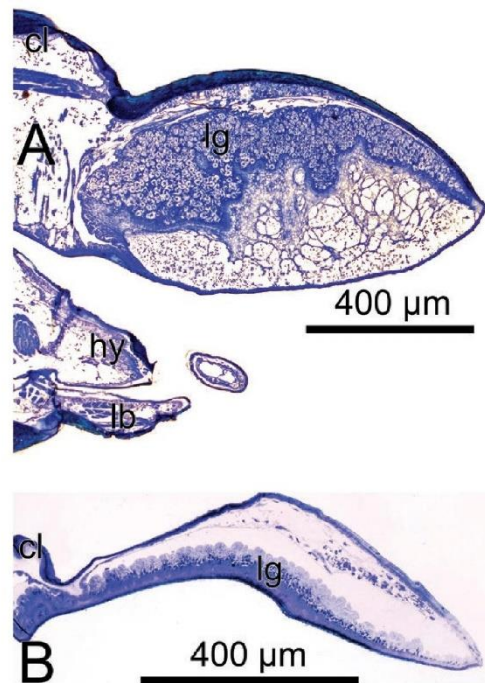
#### TRANSMISSION ELECTRON MICROSCOPY

TEM 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 of Noiro & Quennedey, 1974) that were characterized by an abundance of smooth endoplasmic reticulum (ER), vesicles of different electron densities, abundant mitochondria, numerous microtubules orientated apico-basally, glycogen granules, myelin figures and sparse rough ER mainly located around the nucleus (Fig. 4A–C). The secretory cells could easily be differentiated from the non-modified cells (Fig. S3A) as the latter are thinner and lack the characteristics 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 µm in diameter; Fig. S3B, C) 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 zonulae adherens followed by septate junctions, while the basal parts of the membranes were devoid of any junctions. Basal invaginations were well developed throughout the gland, and on average were about 5 µm deep (up to 20 µm in *Labioterme 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 µm (rarely up to 10 µm) and the nucleus was predominantly filled with dispersed chromatin with few aggregates. Microvilli were well developed, about 1.5 µm in length (rarely up to 3–4 µm), approximately 100 nm thick, and always had a central channel about 40 nm in diameter (Figs 4A, S3C, E). The basal invaginations and microvilli of the hypopharyngeal region of the labral gland were always shorter than those of the labral region. Microvilli were in some cases longer in the central part of the gland than in the gland margins.

The cuticle was in general made up of three layers, the 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



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

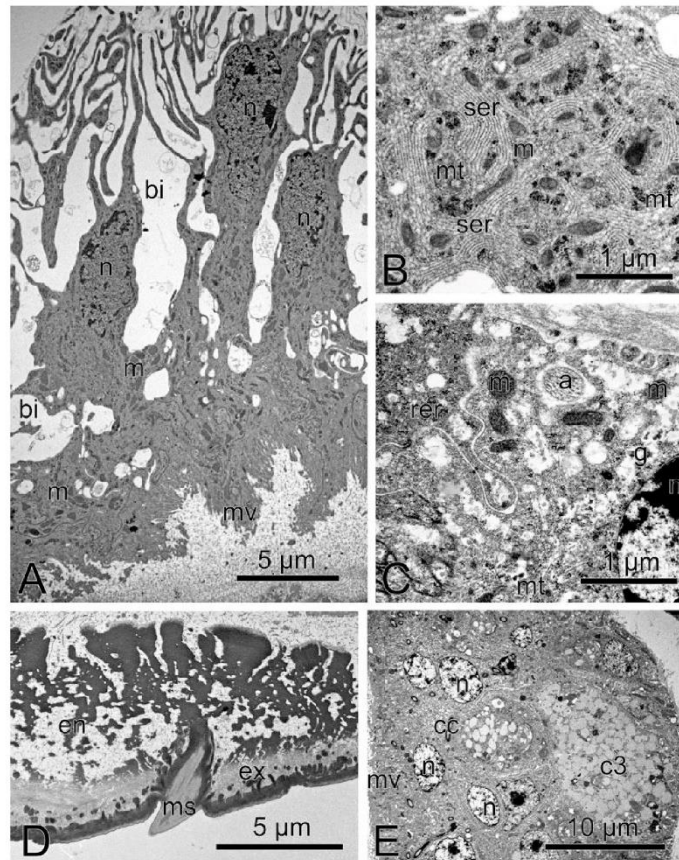
the secretory epithelium, the overlying cuticle and inside the porous cuticle. There was no invaginated reservoir in any of the studied species. The cuticle showed numerous adaptations for release of the secretion, and these were more pronounced towards the labral tip (Fig. 2C, 4D, S4A, B). The cuticular 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 two and five 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 characteristics of the labral gland.

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 cases mixed with class 1 cells (Fig. 4E) in *Glossotermes oculatus*, *Termes hospes*, and in the minor soldiers of *Dolichorhinotermes longilabius*. In *Mastotermes darwiniensis*, by contrast, the class 3 secretory cells were located adjacent to the class 1 secretory cells.





**Figure 4.** Ultrastructure of the labral gland in soldiers. (A) Overall development of the labral gland in *Labiotermes labralis*. Note the development of the apical microvilli and basal invaginations. (B) Detail of labral gland secretory cell class 1 cytoplasm in *Neocapritermes taracua* showing well-developed smooth endoplasmic reticulum. (C) Detail of labral gland secretory cell class 1 cytoplasm in large soldier of *Dolichorhinotermes longilabius* showing a free axon located at the base of the secretory epithelium. (D) Highly modified cuticle underlying the labral gland in *Embiratermes neotenicus*. Note enlarged pore canals ensuring secretion release and the margin of the sensillum. (E) Class 3 secretory cell in *Glossotermes 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.

Class 3 cells did not touch either the apex or 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 approximately 0.4  $\mu\text{m}$  in diameter. The conducting canal comprised

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 lip-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 three 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 by 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 imagos (Křížková *et al.*, 2014) and certain workers (Palma-Onetto V and Šobotník J, our unpublished data). These random observations suggest that the labral gland might be present in all termite castes, pointing to its importance during termite evolution.

The labral gland is split into two secretory regions located in the ventral part of the 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 thus be treated as a single gland. The nomenclatural change from 'labral gland' to 'cibarial gland' proposed by Quennedey (1984), based on 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 traditionally described morphological character. The dorsal side of the labrum is always sclerotized, while the ventral part is always formed by a lucent membranous cuticle. However, species may differ in the level of sclerotization of the dorsal side, especially at the labrum apex. While some soldiers show an 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 termite families studied here. This suggests that the evolution of snapping mandibles did not see a loss of the labral gland and 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 showed many similarities among all studied species. Additionally, the four species with polymorphic soldiers that we studied showed that the labral gland volume increased with sub-caste size and was particularly pronounced in *Psammotermes hybostoma*.

The common features shared by labral and hypopharyngeal parts of the labral glands include: (1) abundance of smooth ER, (2) the presence of apical microvilli with a central channel, (3) well-developed basal invaginations ensuring the intake of precursors from the haemolymph, and (4) 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 overlying 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 have often been detected at the base of the secretory epithelium.

The function of the labral gland is probably 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 glands, with 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 on the ventral side of the labrum is likely to aid in dosage of labral secretions. As all observed receptors contained several



dendrites, a chemosensory function is likely for all species while a mechanoreceptive function remains hypothetical. The idea that the labral receptors respond to mechanical pressure has a functional parallel in the sternal gland, secretion releases from which 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 and on the sclerotized body cuticle (Šobotník *et al.*, 2004; Šobotník, Weyda & Hanus, 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 & Haifig, 2014) and *T. hospes* (presented here). Class 3 cells have not been observed in the hypopharyngeal part of the labral gland in any of above-mentioned species. The ultrastructure of the class 3 secretory cells is uniform in termites, irrespective of their caste (Costa-Leonardo & Shields, 1990; Šobotník *et al.*, 2004) and position in the gland, such as mandibular (Lambinet, 1959; Cassier, Fain-Maurel & Lebrun, 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 on the mandibles and deposited into the wound following bite (Deltigne *et al.*, 1981; Quennedey, 1984; Šobotník *et al.*, 2010b; Costa-Leonardo & Haifig, 2014). However, preliminary observations based on the morphology, structure and

ultrastructure of the labral gland suggest that labral gland secretion has a communicative function.

The presence of a 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. A better understanding of the function of the labral gland in termites is called for to enhance knowledge of termite defence mechanisms and communication behaviour.

#### ACKNOWLEDGEMENTS

Credit for Figure 1B goes to Aleš Buček (OIST, Japan). We thank Mirek Hyliš from the Laboratory of Electron Microscopy (Faculty of Sciences, Charles University in Prague) for his help and support with SEM and TEM. We are grateful to Yves Roisin for constructive criticism of the manuscript. We also thank three anonymous reviewers for their helpful comments and suggestions. Financial support was provided by the project IGA FLD No. A13/17 (Czech University of Life Sciences, Prague).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Scanning electron micrograph of the mouth parts of *Nasutitermes lujae*, with antennae and part of the maxillary palp removed.

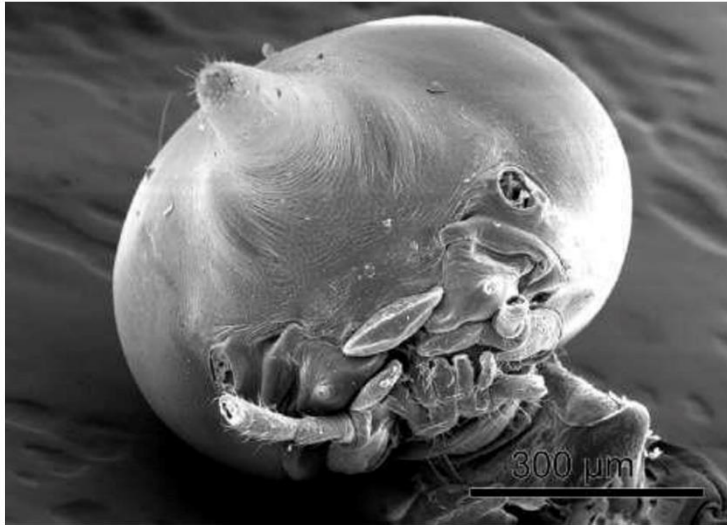
**Figure S2.** Labral gland development. Sagittal sections of the labrum in: (1) *Mastotermes darwiniensis*, (2) *Hodotermopsis sjoestedti*, (3) *Neotermes cubanus* small soldier, (4) *Neotermes cubanus* large soldier, (5) *Dolichorhinotermes longilabius* small soldier, (6) *Dolichorhinotermes longilabius* large soldier, (7) *Prorhinotermes simplex*, (8) *Psammotermes hybostoma* small soldier, (9) *Psammotermes hybostoma* medium soldier, (10) *Psammotermes hybostoma* large soldier, (11) *Termitogeton planus*, (12) *Glossotermes oculatus*, (13) *Reticulitermes flavipes*, (14) *Coptotermes formosanus*, (15) *Sphaerotermes sphaerothorax*, (16) *Pericapritermes* sp., (17) *Microcerotermes* sp., (18) *Spinitermes* sp., (19) *Globitermes globosus* small soldier, (20) *Globitermes globosus* large soldier, (21) *Globitermes sulphureus*, (22) *Termes hospes*, (23) *Inquilinitermes fur*, (24) *Neocapritermes taracua*, (25) *Planicapritermes planiceps*, (26) *Dentispicotermes brevicarinatus*, (27) *Labiotermes labralis*, (28) *Embiratermes neotenicus*, (29) *Indotermes* sp., (30) *Nasutitermes lujae*, (31) *Constrictotermes cavifrons*, (32) *Hirtitermes* sp., (33) *Trinervitermes* sp.

**Figure S3.** Ultrastructure of the labral gland in soldiers. (A) Non-modified epithelium surrounding the labral gland in *Hirtitermes* sp. (B) Labral gland development in *Hirtitermes* sp. (C) Labral gland development in *Nasutitermes lujae*. Note the highly electron-dense vesicles. (D) Pinocytotic activity at the cell base in the labral epithelium in the large soldier of *Dolichorhinotermes longilabius*. Arrows indicate the pinocytotic activity at the base of the cell. (E) View of the central channel present in the microvilli, allowing secretion release from secretory cells. Abbreviations: en, endocuticle; ex, exocuticle; l, lipid-like droplet; mv, microvilli; n, nucleus; v, vesicle.

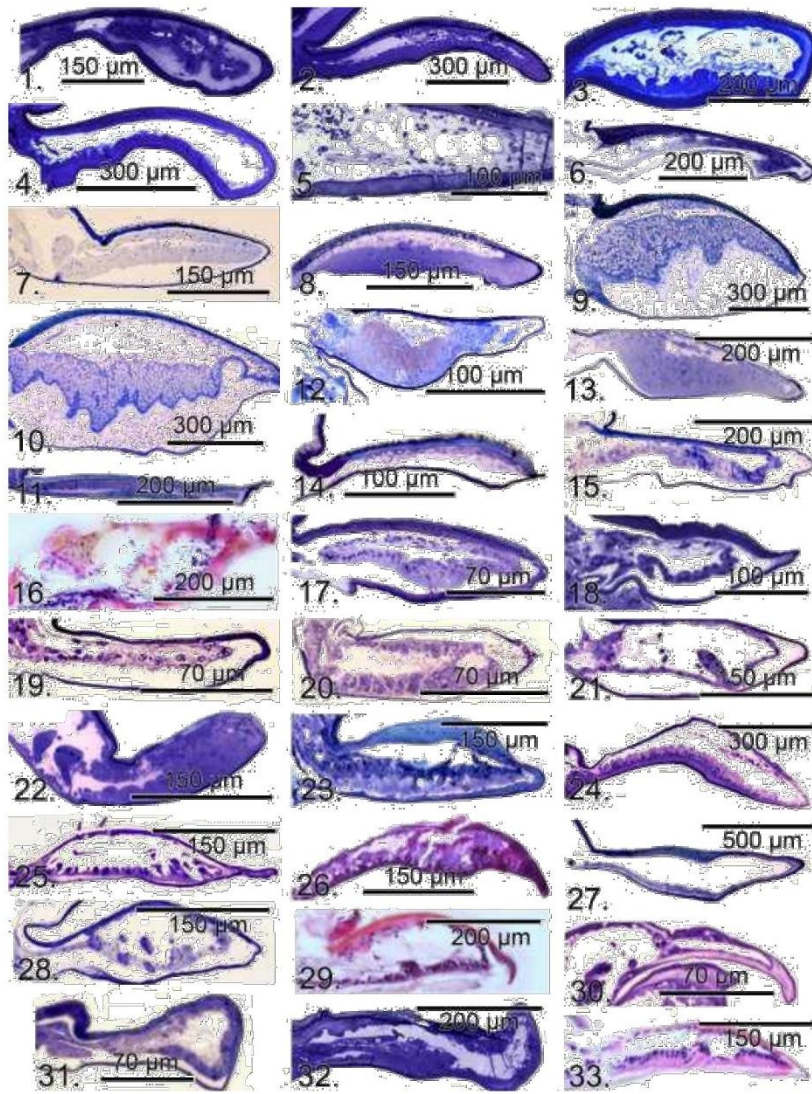
**Figure S4** Ultrastructural features in the labral gland. (A) Highly modified cuticle underlying the labral gland in *Neocapritermes taracua*. (B) Detail of apical glandular cuticle at the tip of the labral gland in *Nasutitermes lujae* showing epicuticular pores allowing secretion out from the body. (C) Chemoreceptors containing four or five axons going through the labral epithelium in *Hirtitermes* sp. (D) Large microtubule bundles running through secretory cells in *Hodotermopsis sjoestedti*. (E) Tracheae going through labral gland cells in *Mastotermes darwiniensis*. Abbreviations: dd, distal dendrite; dg, electron-dense granule; en, endocuticle; ep, epicuticle; ex, exocuticle; lv, electron-lucent vesicle; m, mitochondria; mb, microtubule bundle; mv, microvilli; n, nucleus; s, secretion; ser, smooth endoplasmic reticulum; tr, trachea; v, vesicle.

**Table S1.** List of studied termite species, with indication of the fixation buffer used, collection location, species and subcastes (if any), number of repetitions, and labral and hypopharynx epithelium measures ( $\mu\text{m}$ ). The last four columns provide detail of the cells analysed by TEM, with indication of cell type, thickness of cuticular layers, smooth ER and presence of axons. Abbreviations: n.a., not applicable; Y, yes.

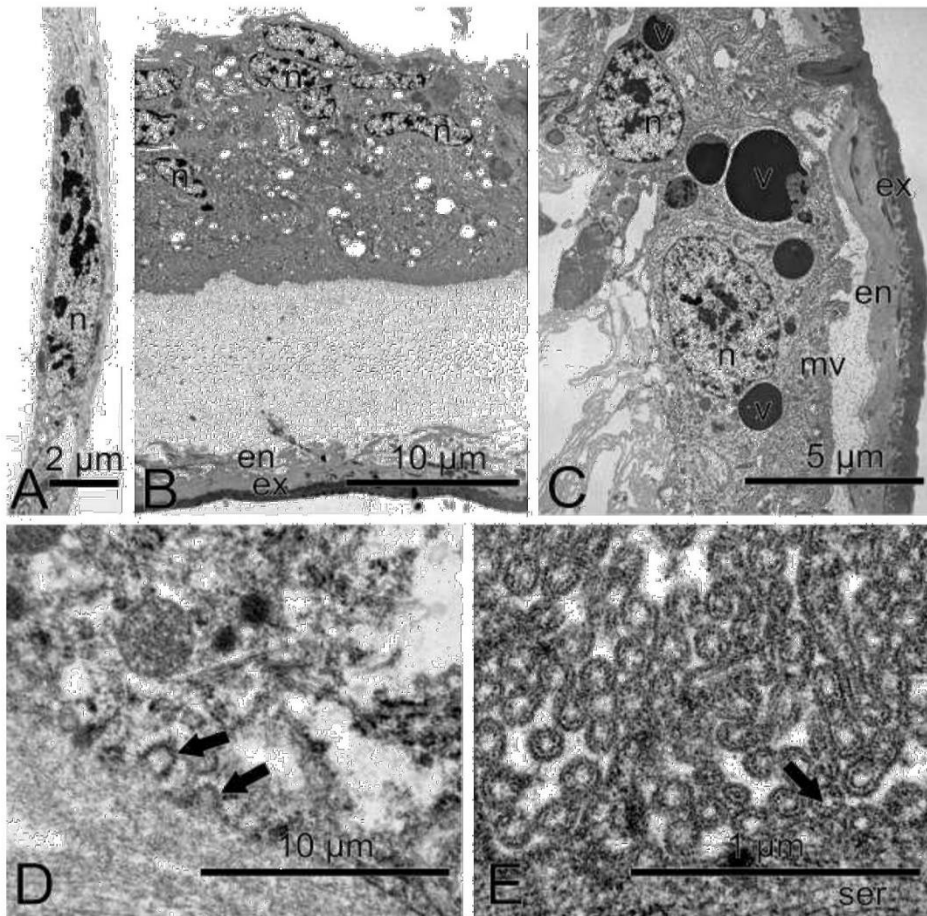
Supplementary materials



**Figure S1.** Scanning electron microscopy picture of the mouth parts of *N. lujae*, with antennae and part of the maxillary palp removed.

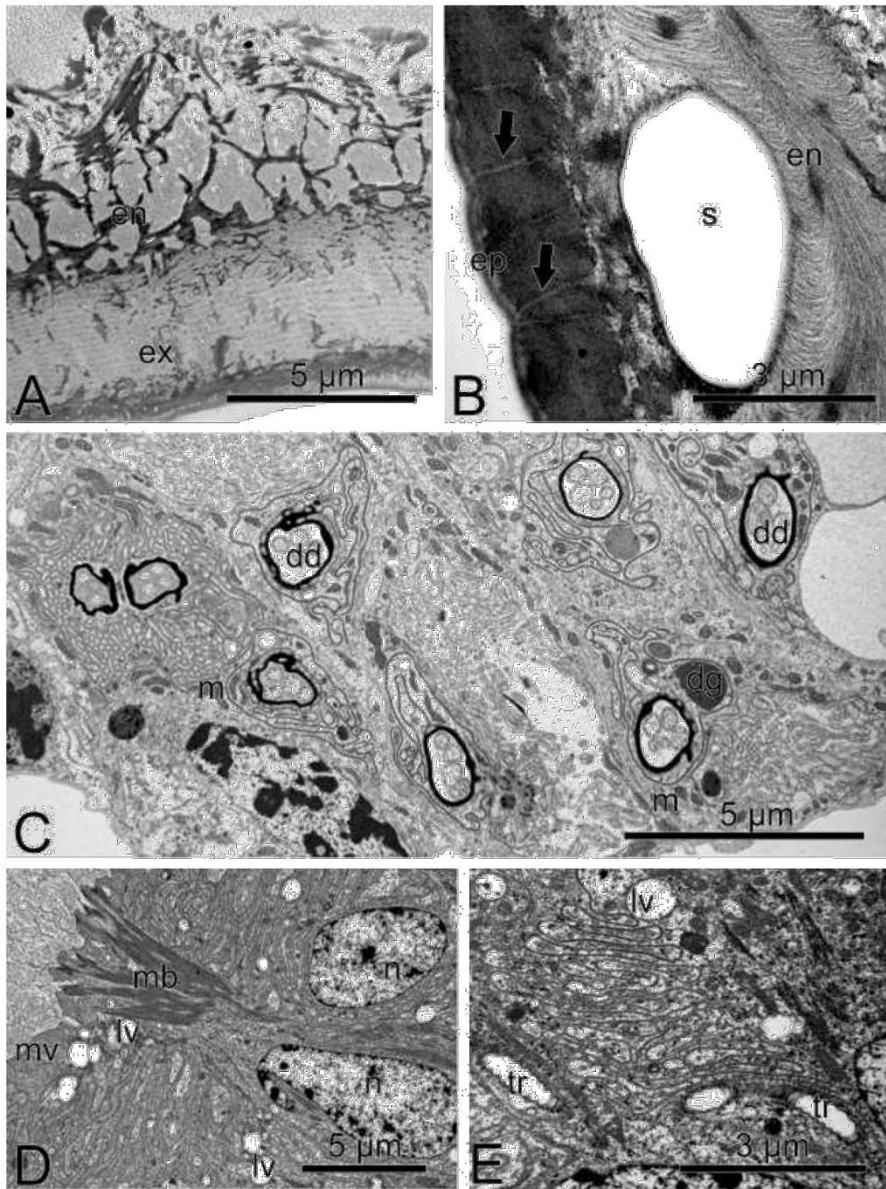


**Figure S2.** Labral gland development. Sagittal sections of the labrum in: 1) *Mastotermes darwiniensis*, 2) *Hodotermopsis sjöstedti*, 3) *Neotermes cubanus* small soldier, 4) *Neotermes cubanus* large soldier, 5) *Dolichorhinotermes longilabius* small soldier, 6) *Dolichorhinotermes longilabius* large soldier, 7) *Prorhinotermes simplex*, 8) *Psammotermes hybostoma* small soldier, 9) *Psammotermes hybostoma* medium soldier, 10) *Psammotermes hybostoma* large soldier, 11) *Termitogeton planus*, 12) *Glossotermes oculatus*, 13) *Reticulitermes flavipes*, 14) *Coptotermes formosanus*, 15) *Sphaerotermes sphaerotherax*, 16) *Pericapritermes* sp., 17) *Microcerotermes* sp., 18) *Spinitermes* sp., 19) *Globitermes globosus* small soldier, 20) *Globitermes globosus* large soldier, 21) *Globitermes sulphureus*, 22) *Termes hospes*, 23) *Inquilinitermes fur*, 24) *Neocapritermes taracua*, 25) *Planicapritermes planiceps*, 26) *Dentispicitermes brevicarinatus*, 27) *Labiotermes labralis*, 28) *Embiratermes neotenicus*, 29) *Indotermes* sp., 30) *Nasutitermes lujae*, 31) *Constrictotermes cavifrons*, 32) *Hirtitermes* sp., 33) *Trinervitermes* sp.



**Figure S3.** Ultrastructure of the labral gland in soldiers. (A) Non-modified epithelium surrounding the labral gland in *Hirtitermes sp.* (B) Labral gland development in *Hirtitermes sp.* (C) Labral gland development in *N. lujae*. Note the highly electron-dense vesicles. (D) Pinocytotic activity at the cell base in the labral epithelium in the large soldier of *D. longilabius*. The arrows indicate the pinocytotic activity at the base of the cell. (E) View of the central channel present in the microvilli, allowing secretion release from secretory cells. Abbreviations: en = endocuticle, ex = exocuticle, l = lipid-like droplet, mv = microvilli, n = nucleus, v = vesicle.





**Figure S4** Ultrastructural features in the labral gland. (A) Highly modified cuticle underlying the labral gland in *N. taracua*. (B) Detail of apical glandular cuticle at the tip of the labral gland in *N. lujae* showing epicuticular pores leading the secretion out from the body. (C) Chemoreceptors containing 4 or 5 axons going through the labral epithelium in *Hirtitermes* sp. (D) Large microtubule bundles running through secretory cells in *H. sjostedti*. (E) Tracheae going through labral gland cells in *M. darwiniensis*. Abbreviations: dd = distal dendrite, dg = electron-dense granule, en = endocuticle, ep = epicuticle, ex = exocuticle, lv = electron-lucent vesicle, m = mitochondria, mb = microtubule bundle, mv = microvilli, n = nucleus, s = secretion, ser = smooth endoplasmic reticulum, tr = trachea, v = vesicle.



**Table S1.**

List of studied termite species, with indication of the fixation buffer utilised, collection's place, species and subcastes (if any), number of repetitions, labral and hypopharynx epithelium measures ( $\mu\text{m}$ ). In the last four columns is possible to find the detail of the cells analysed by TEM, with indication of the cell's type, thickness of cuticular layers, SER and axons presence. Abbreviations: n.a. = not applicable, Y= yes.

Genus	Species	Group	Sub-Caste	No. Of obsrv.	Fixation-buffer	Embedding medium	TBM	SEM	Collection's place	Labral gland (Lg) thickness (Lg)	LB Minimum	LB Maximum	Hypophan-HLSD	H-Min	H-Max	Secratory cells	Thickness of endocuticle in µm	Thickness of exocuticle in µm	Thickness of epicuticle in µm	SER present ?	Free axons at the base of secretory epithelium
<i>Mesochorus</i>	<i>divinensis</i>	Mesochorini	n.a.	1	Phosphate	Resin	Y	Y	Bonn, Berlin, Germany	23.2	1.72	2.48	35.35	1.81	13.66	16.95	2.31±0.08	0.03±0.0	0.03±0.0	Y	Y
<i>Neodermes</i>	<i>spidei</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	IRD, Bondy, France	31.6	2.08	2.45	25.79	1.81	12.72	14.52	4.4±0.08	0.06±0.01	0.06±0.01	Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	large soldier	1	Phosphate	Resin	Y	Y	CUS, Prague, Czech Republic	31.6	2.08	2.45	25.79	1.81	12.72	14.52				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	small soldier	1	Phosphate	Resin	Y	Y	CUS, Prague, Czech Republic	31.6	2.08	2.45	25.79	1.81	12.72	14.52				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	large soldier	1	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	13.66	3.81	7.95	21.81	3.27	7.26	19.78				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	small soldier	1	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	10.30	3.81	5.92	15.78	3.15	12.90	21.09				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Phosphate	Resin	Y	Y	CUS, Prague, Czech Republic	26.63	6.46	15.21	34.37							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	large soldier	4	Phosphate	Resin	Y	Y	CUS, Prague, Czech Republic	147.12	46.72	94.43	227.42	22.58	5.56	18.00				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	medium soldier	3	Phosphate	Resin	Y	Y	Kharga, Egypt	110.21	46.72	55.98	182.72	7.30	2.58	5.05				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	small soldier	2	Phosphate	Resin	Y	Y	Kharga, Egypt	20.50	9.11	10.22	34.88	12.27	1.54	10.08				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	1	Phosphate	Resin	Y	Y	50 km S of Nabho, West Papua, Indonesia	11.42	3.59	5.40	14.95	4.50	1.29	2.64				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	6	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	111.94	37.45	65.04	157.10							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Phosphate	Resin	Y	Y	Peit, Saut, French Guiana	55.93	13.51	41.43	72.79	28.57	3.63	4.71				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Phosphate	Resin	Y	Y	Peit, Saut, French Guiana	13.32	6.57	5.98	29.55	10.12	2.30	11.56				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	4	Phosphate	Resin	Y	Y	ERM, Berlin, Germany	22.82	10.86	9.43	39.44							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	10	Bouin's	Paraffin	Y	Y	Bubeng, Yunnan, China	5.43	1.20	4.22	7.06							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	11.12	2.78	6.18	15.82							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	13.01	5.96	5.58	23.45							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	large soldier	1	Coccolate	Resin	Y	Y	Singapore	11.99	2.25	8.81	14.69	7.70	3.43	6.67				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	small soldier	1	Coccolate	Resin	Y	Y	Singapore	6.21	1.84	3.87	8.09	5.22	1.97	3.78				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	Khao Chong, Thailand	6.68	2.67	3.42	11.80	5.20	1.28	3.57				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	IRD, Bondy, France	32.03	12.01	16.42	50.79	9.21	2.67	10.01				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	18.88	8.18	4.71	31.24							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	1	Phosphate	Resin	Y	Y	Peit, Saut, French Guiana	35.60	8.08	23.17	52.38							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	1	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	10.06	4.41	4.98	15.74							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	8	Bouin's	Paraffin	Y	Y	Peit, Saut, French Guiana	10.72	5.15	3.30	22.68	10.98	3.07	7.24				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	4	Phosphate	Resin	Y	Y	Peit, Saut, French Guiana	18.46	1.32	13.40	26.77							Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	11.30	2.41	8.35	15.03	8.04	7.03	3.61				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	15	Phosphate	Paraffin	Y	Y	Bubeng, Yunnan, China	11.77	4.65	7.01	22.18	22.34	2.66	8.88				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Coccolate	Resin	Y	Y	Peit, Saut, French Guiana	2.01	0.85	0.87	3.52	7.40	4.03	4.02				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	2	Phosphate	Resin	Y	Y	Peit, Saut, French Guiana	6.67	2.08	2.81	9.19	6.37	5.51	6.76				Y	Y
<i>Neodermes</i>	<i>obovatus</i>	Neodermatini	n.a.	10	Bouin's	Paraffin	Y	Y	Bubeng, Yunnan, China	19.14	1.12	17.79	20.51							Y	Y

## 4 Discussion

Interaction among nest-mates is a key characteristic of eusocial insect. Exocrine glands provide the semiochemicals used in various aspects of colonies' daily-life, which cannot be fully understood without disentangling a gland function and context of a secretion use. Cephalic glands studied in this thesis serve probably for defence (nasus gland), communication with the reproductives (clypeal gland) and signalling a threat (labral gland). Our observation on the cephalic glands in numerous termite species and their castes, and *Cryptocercus*, represents an important contribution in the fields of termite anatomy

### 4.1 The nasus gland

This novel gland was described from soldiers of *Angularitermes* that belongs to the rare taxa difficult to collect (Canello et al., 2014; Bourguignon et al., 2011), being distributed over South American rainforests and cerrado (Carrijo et al. 2011). The first relevant observation comes, from the superficial description based on scanning EM images (Canello et al., 1996), and it strongly suggested presence of an exocrine gland. However, the material paucity combined with the need of getting living termites to a well-equipped lab for fixation of tissues caused almost 20 more years needed to confirm the glandular nature of the tissue.

The ultrastructure of nasus gland is unique, comprising of all 3 classes of secretory cells. Only three abdominal glands were found with the same combination of secretory units: sternal, posterior sternal, and tergal glands (Ampion & Quennedey, 1981; Quennedey et al., 2008; Šobotník et al., 2005). Class I. cells, located apically, do not reveal own secretory activity. They differ from ordinary epidermal cells only by presence of microvilli and scarce organelles, secretory vesicles, and lipid droplets. Even if their volume is greater

than of epidermal cells, number of mitochondria is approximately the same and therefore functionally, the gland is formed by class II. and class III. secretory cells only. The exceptional feature of the nasus gland is that secretion of class II. secretory cells is accepted and released by class III. secretory cells. Class II. secretory cells are located most entally. Their most abundant organelle is sER, producing lipid droplets. The secretion is released into enlarged intercellular spaces (lacunae). Class II. secretory cells are in fact modified oenocytes; they do not adjoin the cuticle (Noirot & Quennedey, 1974; Noirot & Quennedey, 1991), therefore their secretion is in all other cases (Ampion & Quennedey, 1981; Quennedey et al., 2004; Šobotník et al., 2005; Quennedey et al., 2008) released by passage through class I secretory cells. In case of the nasus gland, class I. secretory cells are not active therefore class III. cells have overtaken their function. Class III. secretory cells, located in between class I and II cells, accept the lipid-like secretion by direct penetration through the plasma membrane, and release it through channel continuous with the body epicuticle. The size of class III. cells is much greater than that of class I. secretory cells; mitochondria are numerous, secretion consists of rare electron-dense granules and frequent electron-lucent vesicles, suggesting prevailing lipidic secretion. Both, class II. and class III. secretory cells contain also free ribosomes and variable amount of rER, producing thus proteinaceous secretion. The ultrastructure suggests that the secretion of nasus gland is a volatile lipid-like material and in smaller proportion also proteinaceous.

This novel exocrine gland has not been observed in any other species yet, however similar nasus structure with marked holes or protuberances has been reported in other rare Nasutitermitinae, such as *Verrucositermes hirtus* (Deligne, 1983), *Velocitermes*, *Tenuirostritermes*, *Nasutitermes*, *Trinervitermes*, *Spatulitermes* and *Eutermellus* (all Termitidae: Nasutitermitinae; Deligne, 1973). It is hard to judge as no additional data are available for the moment. *Angularitermes* is however positioned among other Neotropical

Nasutitermitinae (Bourguignon et al., 2017), what opens an interesting question: Is there an unrecognized power making unrelated species evolve repeatedly the similar organs? In fact, the older generations used to group genera based on morphological similarities, what is worthy in some cases but misleading in others, resulting into polyphyletic nature of several traditional groups of termites (Inward et al. 2007b, Bourguignon et al. 2015a; 2017; Buček et al., 2019).

Behavioural studies were conducted to find function of the nasus gland secretion. The main obstacle is that *Angularitermes* is a rare termite, and the living termites are extremely sensitive to all kinds of manipulation. Therefore the number of observations was not statistically representative, unfortunately. However, workers of *Procornitermes lespesii* that were confronted with *A. coninasus* soldiers were often killed, when either frontal gland, nasus gland or both glands were in action (otherwise blinded with nail polish). We therefore believe that the nasus gland is an active player in the combat, and the dead workers of *P.lespesii* after confrontation with *A. coninasus* soldiers with blinded frontal gland suggest a function of contact poison production. Alternative explanation by Quennedey (1984) suggests that the rostral gland prevents soldiers from getting stuck in own secretion. All Nasutitermitinae soldiers produce terpenoid secretion by their frontal glands (Šobotník et al., 2010b), and the lipidic secretion of the rostral gland may indeed eliminate the stickiness of the secretion by being comparably nonpolar but more fluid, however, another question rises afterwards: Why only few rare unrelated genera share this novelty, being absent in majority of Nasutitermitinae species? The lipidic secretion is proposed to bear communication function rather than defensive (Percy-Cunningham & MacDonald, 1987; Tillman et al., 1999) which is in contrast to observed behaviour.

Our experiences suggest that behavioural tests at larger scale can resolve the nasus gland function, however, enormous amount of work would have to be performed, not speaking about the effort possibly put into identification of the



major components of the frontal and nasus glands secretion. However, study of the ultrastructures in the above-mentioned organs in other soil-feeding Nasutitermitinae can help understanding the function of the nasus gland and other organs.

## 4.2 The clypeal gland

This gland was observed in many imagoes on anterodorsal part of head, reaching roughly from the posterior part of the labrum to anteclypeus. It occurs in most of studied Neoisoptera species, except for *Aparatermes* sp. and *Anoplotermes janus* (both Termitidae: Apicotermitinae), where it was probably lost secondarily. Both sexes of alate imagoes possess the gland, which probably remains active in functional reproductives. Surprisingly, the clypeal gland was neglected by other researchers, probably because termite imagoes are not a frequent subject to the research, which mostly focuses at workers and soldiers. Nevertheless, alate imagoes reveal glands specific to this caste that are often but not always linked to sexual behaviour, as the functional king and queen may need other secretions in a colony. Clypeal gland was observed in 40 species of Neoisoptera at the level of optical microscopy, and only in *Coptotermes testaceus* ("Rhinotermitidae") using TEM.

The clypeal gland consists of class I. and class III. secretory cells showing moderate or low activity according to relatively few mitochondria observed in the secretory cells. Ducts supported by duct cells accompanying the class III. secretory cells reveal unique structure in the clypeal gland, as the number of ducts exceeds one per a single duct cell. Such feature has never been observed, however, an alternative explanation is that the gland is in immature state in swarming imagoes and the ducts are coiled inside the duct cell. The increased activity would be evidenced by enlargement of the secretory cells, thickening the epithelium and stretching the ducts. Ultrastructure of the clypeal gland in

functional reproductives is needed to answer this question. Both types of secretory cells produce proteinaceous as well as lipid-like secretion. The function of the gland remains unknown, and we can only speculate based on the structural observations. Defensive function of the gland can be excluded due to lack of reservoir. The glandular product cannot be applied to the body, and thus antimicrobial activity is not probable either. The secretion can however be applied on walls of the nest or it can be a direct signal for nest-mates. We expect that the gland serves for communication, and the signal can either show the reproductive status of the royal couple (primer pheromone; Ampion, 1980; Sbrenna & Leis, 1983; Šobotník et al., 2003; Matsuura et al., 2010) or signalling the workers where the oral region of king and queen is. The caste of reproductives does not forage and they neither actively beg for food (Kawatsu, 2013). Blind worker should therefore know if king or queen need to be fed and where the clypeus is. If the clypeal gland becomes active only in functional reproductives, it would be extremely difficult to get additional information about the structure and function of it. The structural studies require repeated observations and considerably more material would be needed to identify the secretion and for behavioural tests, and at the same time, every colony contains only a single royal couple. The gland was only studied in Neoisoptera, and further work is needed to prove presence or absence of the gland in basal termite families.

### 4.3 The labral gland

The labral gland was observed in all studied termite soldiers, workers and imagoes, as well as in the wood roach *Cryptocercus* (Palma-Onetto et al., 2018; 2019). The gland is composed of two regions, i.e. ventral part of labrum and apicodorsal part of hypopharynx, sharing the same ultrastructures in the secretory cells. Palma-Onetto et al. (2019) also inspected defensive behaviour in

several termite species, and could show that the labral gland secretion is applied on the surface after encounter with an alien, so the function is clearly communicative, although it remains unclear whether the signal is used to repel nest-mates (pheromone) or aliens (allomone). At the same time, our attempts to establish a functional behavioural bioassay or to identify labral gland specific substances were not successful.

The gland was firstly observed by Deligne et al. (1981) in the labrum of *Macrotermes* soldier, and named labral gland, while Quennedey (1984) found it in the hypopharynx of *Macrotermes* soldier, and named it cibarial gland. These two secretory regions were by later authors treated as one (in *Glossotermes* soldiers; Šobotník et al., 2010d) or two glands (in *Cornitermes* soldiers; Costa-Leonardo & Haifig, 2014). The nomenclatoric ambiguities were resolved only recently, and both parts should be called labral gland due to identical ultrastructure of both regions (Palma-Onetto et al., 2018). The function was originally considered defensive (without any evidence), and the secretion was speculated to impregnate the mandibular blades. While working on the clypeal gland, localised in the same body region (forehead), Křížková et al. (2014) noticed the presence of labral gland in termite alate imagoes. We have observed the gland in soldiers of 28 termite species, workers of 28 species and imagoes of 33 species from almost all termite families. In other words, the long neglected structure became synapomorphy of all termites and subsocial *Cryptocercus* woodroaches (Palma-Onetto et al., 2018, 2019).

The ultrastructure of the gland is identical between labral and hypopharyngeal parts, made up of the class I. secretory cells. The class III. secretory cells are common at the dorsal side of labrum, as a singular isolated cells in soldiers and workers or as a part of clypeal gland in imagoes (Palma-Onetto et al., 2018, 2019; Křížková et al., 2014). The class III. cells never mix with class I. cells of the labral gland in workers and imagoes (Palma-Onetto et al., 2019), while they sometimes do so in soldiers, for example in minor soldiers of *Dolichorhinotermes*

*longilabius* ("Rhinotermitidae"), in *Termes hospes* (Termitidae: Termitinae; Palma-Onetto et al., 2018), *Glossotermes oculatus* (Serritermitidae; Šobotník et al., 2010d) and *Cornitermes cumulans* (Termitidae: Syntermitinae; Costa-Leonardo & Haifig, 2014). Class III. secretory cells contain large amounts of rER, some Golgi apparatus and variable amount of moderately electron-lucent vesicles. RER is mostly connected with proteinaceous secretion (Pollard et al., 2016) that could serve as mechanical protection to the outer-most layer of the cuticle (Chapman, 2013). The most prominent features of the labral gland ultrastructures is as follows: i) abundant sER; ii) apical microvilli with a central channel; iii) well-developed basal invaginations ensuring nutrient transport into secretory cells; and iv) modified cuticle in apical part of the labral gland, ensuring release of the secretion out from the body. The secretion is stored under and inside of the cuticle. Sensory organs associated with the labral gland are numerous (Costa-Leonardo & Haifig, 2014), and based on our observations, they are chemoreceptive sensillae with probably mechanoreceptive function as well.

Secretory organelle responsible for volatile secretion, the sER, was the most numerous one suggesting the gland secretes lipid-like volatile substances that can be used as pheromones. This theory is supported by absence of reservoir associated with the gland and presence across all different defensive strategies of soldiers, even in the case of rupturing termites. Behavioral tests were not performed in the present study, however it was executed later (Palma-Onetto et al., 2019). It has been shown that soldiers wipe the labrum on surface in case of encounter of heterospecific termite. However the labral extract applied on petri dish with workers and soldiers did not induce any significant response. Chemical structure was studied in the latter work and no substance was detected.

Labrum of many soldiers is apically terminated by a hyaline tip. The labrum sclerotization in general decreases towards the apex, and in some cases the hyaline tip is clearly visible, being an important taxonomic character. Our reconstruction of ancestral states suggests that the hyaline tip is common to all

Neoisoptera while all the basal lineages lack it. It has later on been lost at least 4 times independently, namely in i) all Nasutitermitinae soldier having highly reduced labra; ii) and iii) snapping soldiers of *Pericapritermes*, and *Neocapritermes* + *Planicapritermes* whose labra are modified in shape and iv) in *Microcerotermes* with small labrum in soldiers. The functionality and presence of the labral gland is however unchanged across all the species.

#### 4.4 Glands, communication and related behaviour in cockroaches and termites

Termites are inner group of cockroaches (Lo et al., 2000; Inward et al., 2007a; Bourguignon et al., 2015a; Buček et al., 2019), with which they share numerous traits associated with glands, communication and pheromones. Both groups rely mostly or purely on non-visual means of orientation, and have in common three types of behaviour (Bell et al., 2007). It is firstly the **vibroacoustic communication** that plays important role in the group communication including alarm propagation in termites and Cryptocercidae. Other use of vibrational signals include courtshipping males of cockroaches, e.g *Periplaneta americana* (Blattodea: Blattidae; Bell et al., 2007). The second behaviour common to both groups is ability of **following a scent trail**. The trail-following pheromone originates from the sternal gland in termites, while cockroaches mark their trails rather with fecal pellets enriched by aggregation pheromones. Rectal pad cells were suggested to be the source of the pheromone released along with the fecal pellets (Ishii & Kuwahara, 1967; 1968). An alternative source may occur in *Periplaneta americana* that possesses sternal glands for this purpose (Brousseau-Gaury, 1976). The third common type of behaviour is **kin recognition** known to occur in all termites and several groups of cockroaches, always based on cuticular hydrocarbons (Roth & Willis, 1952; Howard & Blomquist, 1982; 2005). The dermal glands are spread all over the abdominal integument in at least

Oxyhaloinae (Blaberidae; Sreng, 1984) and “Rhinotermitidae” (Šobotník et al., 2005), supposedly being responsible for the hydrocarbons production. Apart of the communication, the two groups also share thigmotactic strategy, nesting and building behaviour, the ability to transport food, aggregation behaviour, elaborate brood care, hygienic behaviour, allogrooming, and antennal cropping. The **nesting** and **building** behaviour is one of the important characteristics shared by termites, *Cryptocercus* and some other wood- and soil-dwelling cockroaches. The need of stable symbiont transmission within the family units, protecting the eggs and biparental brood care shaped the common ancestor of Cryptocercidae + termites into the eusocial way-of-life (Nalepa, 1984; Bell et al., 2007; Eggleton, 2011; Evangelista et al., 2019). Other interesting features shared between cockroaches and *Mastotermes*, the basalmost termite of all recent taxa, are mentioned in the Chapter 1.2.1.

Termites and cockroaches are members of Polyneoptera, which all share homologous mandibular and labial glands of similar structural and ultrastructural organisation and function (Chapman, 2013). Other glands shared in termites and cockroaches comprise labral, tergal and sternal glands (Brossut, 1973; Palma-Onetto et al., 2019). Mandibular glands of two species (*Blaberus craniifer* and *Eublaberus distanti*) secrete an aggregation pheromone (Brossut, 1970; 1979). All other cephalic glands in cockroaches only have putative function, and they are expected to play a role in communication, production of antimicrobial agents or joint protection (Brossut, 1973; Bell et al., 2007). In general, the cephalic glands were rarely studied (and this is why I decided to study them in termites); more attention has been paid to frontal gland in termite soldiers (reviewed by Šobotník et al., 2010b), or abdominal glands in cockroaches (Roth, 1969; Stuart, 1961; 1969; Peppuy et al., 2001). The tergal glands and sternal glands of cockroaches are used for chemical defence and deterrence, trail pheromone deposition and courtship behaviour (Brossut, 1983, Bordereau & Pasteels, 2011).



The defensive glands in cockroaches are usually located at pleurae and associated with spiracles (Roth & Alsop, 1978).

Termites are eusocial cockroaches derived from their oldest lineages. The most common ancestor of termites and cockroaches was an odd-looking ancient animal that diverged early in the Cretaceous (Evangelista, 2019; Buček, 2019). Comparing thus the group of termites to recent Blattodea does not seem reasonable after all. However, presence of 5 glands in most termite species (the labial glands, the mandibular glands, the labral gland, spermathecal gland, the sternal gland and tergal glands) was proposed (JŠ, pers. comm.). These glands are present also in cockroaches, however all of them might not be in *Cryptocercus* (Brossut, 1973), and we can thus anticipate they played an important role in communication before the lineages split. The evolution and structure of termite glands is described mainly in Chapter 1.9.

## 5 Conclusion

My work contributed to understanding evolution of termite communication and maintenance of integrity of their colonies. Termites are important part of global ecosystems, since their role in organic matter decomposition is truly immense. Wood digestion makes them notable pests in tropical and subtropical regions. Primary tropical rainforests are highly exploited with secondary forests or plantations taking their place. Processes in the primary tropical forests are slowly being disentangled, and termites are one of its key players. These processes will be essential for the future restoration of the ecosystem where termites will surely take a part. Tropical soil management would give a chance not only to nature protection but also to promoting its fertility in long term perspective, and it is impossible without termites.

Glandular system of termites evolved in fact de novo, there are only 5 glands out of 23 that are common to termites and cockroaches. The eusocial family life demanded more complex communication tools than those found in termite ancestors. The thesis has three parts in which structure and ultrastructure of novel or understudied exocrine glands was described and discussed. Part one revealed a gland common to one genus only, the nasus gland in *Angularitermes*. Part two concerns the clypeal glands present in imagoes of almost all Neoisoptera, and part three relates to labral gland found in all termites including their sister group, Cryptocercidae wood roaches.

### **Part I, the nasus gland**

The nasus gland is found on the base of nasus in genus *Angularitermes* (Termitidae: Nasutitermitinae) only. Similar structure may appear also in other Nasutitermitinae that were not studied due to the material paucity. Its function is probably related to the defence. It is used in contact with intruders that show

higher mortality rate when both, frontal and nasus glands are functional. However the true mechanism of its function remains unclear. It can be a defensive organ or gland acting as a neutralizer of the toxic and entangling frontal gland secretion. Ultrastructure of the nasus gland is unique in presence of all three classes of secretory cells. The class I. cells show low activity, class II. cells and class III. cells are active in producing both lipidic and to smaller extent also proteinaceous secretion. The product of class II. secretory cells is transported via class III. cells towards the cuticle which is exceptional as this function is usually performed by class I. cells. The rostral system of *Verrucositermes hirtus* and of other Nasutitermitinae remain to be studied in order to answer the question whether there is a selective pressure for evolution of this structure in unrelated species within the subfamily.

### **Part II, the clypeal gland**

The clypeal gland is an organ common to almost all Neoisoptera imagoes, with only two exceptions known so far. It occurs on dorsal side of head (labrum and anteclypeus). The clypeal gland probably becomes active after the colony is founded, and not when alate imagoes go on their nuptial flights. Position of the gland can have explanatory function for its use. It can either be source of primer pheromone or serve as a signal for workers to feed their king or queen. However behavioural tests with the series of royal couples are difficult or impossible to conduct. The low activity of the clypeal gland in alate imagoes is indicated by low activity of the secretory cells (class I. and class III.). A peculiar character is the canal cells that carry more than a single canal, or, alternatively, the canal is coiled inside of the cell and pre-prepared for enlargement of the glandular volume. This can only be decided by future studies dealing with functional reproductives from mature nests.

### **Part III – Labral gland**

This gland is found in all termite species and all of their castes studied so far. It consists of two secretory regions located in the ventral part of labrum and the dorsal surface of hypopharynx. This gland was previously known to occur only in soldiers of few termite genera, while this study revealed that it is an autapomorphy of Cryptocercidae and Termitoidae. We have shown that the gland is most active in the soldier caste, pointing to defensive purpose of the gland. However, as no reservoir is associated with labral gland to store the defensive secretion, the production of toxins, as previously suggested, seems quite unlikely. Apart of chemical and mechanical defences, soldiers can protect the colony also by alarm communication. The labrum was smeared over the surface when soldiers were confronted with an enemy. Behavioural experiments were used to support hypothesis on the defensive communication function of the labral gland secretion, however no significant results were obtained. Soldiers of many species possess a hyaline tip on apex of labrum associated with the gland. We have shown that it is a synapomorphy for “Rhinotermitidae” and Termitidae and it was repeatedly lost in some of their lineages. It happened most probably due to spatial limitations and it had no effect on actual presence of the gland. The gland is functionally made mostly of class I. secretory cells that share attributes of ultrastructure common to all termites. Presence of numerous sER synthesizing lipidic substances is one of them.

## 6 References

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## 7 Supplementary material

S1. Nomenclature of species and genera mentioned in the text of thesis. In alphabetical order, adopted from Krisha (2013)

*Acanthotermes acanthothorax* (Sjöstedt, 1898)  
*Acorhinotermes* Emerson, 1949  
*Amitermes* Silvestri, 1901  
*Anacanthotermes* Jacobson, 1905  
*Angularitermes* Emerson, 1925  
*Anoplotermes* Mueller, 1873  
*Anoplotermes janus* Bourguignon & Roisin, 2010  
*Aparatermes* Fontes, 1987  
*Apicotermes* Holmgren, 1912  
*Archotermopsis wroughtoni* (Desneux, 1904)  
*Blaberus craniifer* Burmeister, 1838  
*Calcaritermes* Snyder, 1925  
*Cavitermes* Emerson, 1925  
*Constrictotermes* Holmgren, 1910  
*Constrictotermes cyphergaster* (Silvestri, 1901)  
*Constrictotermes rupestris*  
Constantino, 1997  
*Coptotermes* Wasmann, 1896  
*Coptotermes formosanus* Shiraki, 1909  
*Coptotermes testaceus* (Linnaeus, 1758)  
*Cornitermes* Wasmann, 1897  
*Cornitermes cumulans* (Kollar, 1832)  
*Coxotermes* Grassé & Noirot, 1955  
*Cryptotermes* Banks, 1906  
*Cryptotermes brevis* (Walker, 1853)  
*Cubitermes* Wasmann, 1906  
*Cubitermes fungifaber* (Sjöstedt, 1896)  
*Dentispicotermes* Emerson, 1949  
*Dentispicotermes brevicarinatus* (Emerson, 1950)  
*Dolichorhinotermes* Snyder & Emerson, 1949  
*Dolichorhinotermes longilabius* (Emerson, 1925)  
*Drepanotermes* Silvestri, 1909  
*Duplidentitermes* Emerson, 1959  
*Embiratermes* Fontes, 1985  
*Embiratermes neotenicus* (Holmgren, 1906)  
*Embiratermes festivellus* (Silvestri, 1901)  
*Eucryptotermes* Holmgren, 1911  
*Eublaberus distantii* (Kirby, WF 1903)  
*Euhamitermes* Holmgren, 1912  
*Eutermellus* Silvestri, 1912

*Foraminitermes* Holmgren, 1912  
*Globitermes* Holmgren, 1912  
*Globitermes globosus* (Haviland, 1898)  
*Globitermes sulphureus* (Haviland, 1898)  
*Glossotermes* Emerson, 1950  
*Glossotermes oculatus* Emerson, 1950  
*Glyptotermes* Froggatt, 1897  
*Heimitermes* Grassé & Noirot, 1955  
*Heterotermes* Froggatt, 1897  
*Hodotermes* Hagen, 1853  
*Hodotermopsis* Holmgren, 1911  
*Hodotermopsis sjoestedti* Holmgren, 1911  
*Inquilinitermes* Mathews, 1977  
*Kalotermes* Hagen, 1853  
*Kalotermes flavicollis* (Fabricius, 1793)  
*Labiotermes labralis* (Holmgren, 1906)  
*Macrotermes* Holmgren, 1910  
*Macrotermes michaelsoni* (Sjöstedt, 1914)  
*Machadotermes* Weidner, 1974  
*Machadotermes inflatus* Weidner, 1974  
*Mastotermes* Froggatt, 1897  
*Mastotermes darwiniensis* Froggatt, 1897  
*Microcerotermes* Silvestri, 1901  
*Nasutitermes* Dudley, 1890  
*Nasutitermes rippertii* (Rambur, 1842)  
*Nasutitermes triodiae* (Froggatt, 1898)  
*Neocapritermes* Holmgren, 1912  
*Neocapritermes taracua* Krishna and Araujo, 1968  
*Orthognathotermes* Holmgren, 1910  
*Paraneotermes* Light, 1937  
*Parrhinotermes* Holmgren, 1911  
*Pericapritermes* Silvestri, 1914  
*Periplaneta americana* (Linnaeus, 1758)  
*Planicapritermes* Emerson, 1949  
*Planicapritermes planiceps* (Emerson, 1925)  
*Porotermes* Hagen, 1858  
*Procornitermes lespesii* (Müller, 1873)  
*Promirotermes* Silvestri, 1914  
*Prorhinotermes* Silvestri, 1909  
*Prorhinotermes canalifrons* (Sjöstedt, 1904)  
*Prorhinotermes simplex* (Hagen, 1858)  
*Protohamitermes* Holmgren, 1912  
*Psammotermes* Desneux, 1902  
*Reticulitermes* Holmgren, 1913  
*Reticulitermes speratus* (Kolbe, 1885)  
*Rhinotermes* Hagen, 1858  
*Roisinitermes* Scheffrahn, 2018  
*Ruptitermes* Mathews, 1977

*Serritermes* Wasmann, 1897  
*Serritermes serrifer* (Hagen and Bates, 1858)  
*Schedorhinotermes* Silvestri, 1909  
*Silvestritermes* Rocha & Cancelli, 2012  
*Spatulitermes* Coaton, 1971  
*Sphaerotermes sphaerotherax* (Sjöstedt, 1911)  
*Spinitermes* Wasmann, 1897  
*Stolotermes* Hagen, 1858  
*Tenuirostritermes* Holmgren, 1912  
*Termes* Linnaeus, 1758  
*Termes hospes* (Sjöstedt, 1900)  
*Termitogeton* Desneux, 1904  
*Termitogeton planus* (Haviland, 1898)  
*Trinervitermes* Holmgren, 1912  
*Velocitermes* Holmgren, 1912  
*Verrucositermes* Emerson, 1960  
*Verrucositermes hirtus* Deligne, 1983  
*Zootermopsis* Emerson, 1933  
*Zootermopsis angusticollis* (Hagen, 1858)  
*Zootermopsis nevadensis* (Hagen, 1874)