Structure, function and evolution of the labral and frontal glands in termites

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Structure, function and evolution of the labral and frontal glands in termites

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Structure, function and evolution of the defensive exocrine glands in termites

Valeria Palma Onetto

A collaboration between the Termites Research Team at the Czech University of Life Sciences and the Laboratoire d’Ethologie Expérimentale et Comparée at the Université Paris 13.

Dédicace

I dedicate this work to all that people who have been my support during these hard years. To my friends for hearing my sorrows and laments. To those colleagues who without knowing me much have taken the time to speak and support me: Aleš, Cecilia and Rebeca. And, those others who became closer and provided extense conversations, knowledge and confidence: Eliska, Katka and Tomáš.

I would like to thanks especially to David, my supervisor, for always providing nice words, advice, constructive criticism and all the tools I may have needed during my PhD.

And finally, to the person without who it would have been impossible, to my girlfriend: Anais. Who got me up in the most difficult moments, heard all my sorrows, read my e–mails when I was not strong enough to do it by myself, encouraged me to follow my objectives and gave me the biggest reason to continue on it.

My parents will never read it, they do not even understand English, but I still want to say: I am sorry. I am sorry for not have been there in these years, where things were not easy for you. I am sorry for let my sadness and pressure overcome my feelings and not had taken the first available flight when it was needed. I am sorry for letting you alone when you needed where to hold.

This work is also for you, although it does not replace those moments I have missed.

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1. General Introduction

Angularitermes pinocchio
Photo by Aleš Buček

> *"Our whole life is but a greater and longer childhood"*
>
> Benjamín Franklin

1. General Introduction

Social insects represent an important part of our lives. Ants, bees and wasps are easily recognisable by almost everyone even kids, we can find references about them in the Holy Bible, and they comprise about the 75 percent of the world's insect biomass (Wilson, 1971). Insects societies have long intrigued and fascinated people, as they also hold a special place in the biophilia (defined by E.O. Wilson as an innate and genetically determined affinity of human beings with the natural world), due the parallelism of their lives with ours. Their colonial life with a central family life, division of labor, communication and the mutualistic peace interweaves with strife and conflicts, have indisputable similarities with the achievements and ideals of our own society. Among all social insects, the one which fits most closely with humans may be termites, which present among their individuals: parents, alloparents, builders, soldiers, biochemical–genomic engineers and children. All of these individuals settled in an extended nuclear family that expand and defend their homes (Howard & Thorne, 2011). Althought these parallelisms and the fact that termites are one of the most (if not the most) abundant insects on Earth (overweighting bees and wasps), termites have received negligected attention in comparison with other social insects.

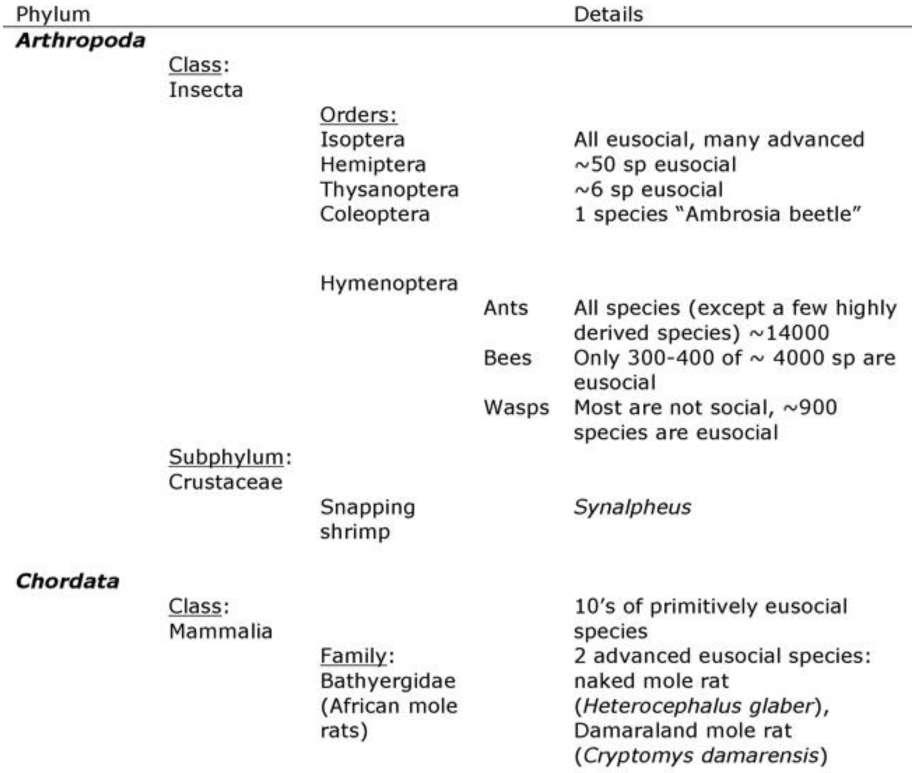
1.1 Eusocial organisms

Although many animals exhibit social behaviors, such as aggregating in large numbers at times or parental care, these behaviors do not mean an animal is social. In fact, biologists refer to true social animals as *eusocial*. By definition, eusocial animals share the following four characteristics: life in groups of the adults, cooperative care of juveniles (individuals care for brood that is not their own), reproductive division of labor (not all individuals get to reproduce), and overlap of generations (Wilson, 1971).

The term "eusocial" was introduced for the first time by Suzanne Batra in 1968, who used it to describe nesting behavior in Halictine bees. She observed colonies which were founded by a single individual and described the essential cooperative behavior of the bees and how the activity of one labor division influenced the activity of another. In 1969, Charles Michenes would expand Batra's classification with a study aimed to investigate the different levels of animal sociality and defined by three main characteristics the concept of eusociality: i) Egg–layers and worker–like individuals among adult females" (division of labor), ii) The overlap of generations (mother and adult offspring), iii) Cooperative work on the cells of the bees' honeycomb. But it was not until 1971 when E. O. Wilson extended the terminology to include other social organisms which comprehend the following three features:

1) Reproductive division of labor (with or without sterile castes)
2) Overlapping generations
3) Cooperative care of young

Moreover, a crucial evolutionary interrogant has arised with the success of the eusocial colonies and it is the origin and persistence of a sterile caste in them, whose existence is the last thing we would expect to be promoted by natural selection and has been a headache for biologists since Darwin who declared in *The Origin of Species* the paradox to be the most important challenge to his theory during the realization of his evolutionary theory. This solution to this paradox can be approached in many different ways, where the most influential one is undoubtedly the Hamilton's inclusive fitness theory (1964). Hamilton presented a kin selection theory which explains that if a gene promoting altruistic behavior has copies of itself in others, helping those others survive ensures that the genes will be passed on. The phenomenon is mathematically described by $c < br$, where r is the degree of relatedness between donor and recipient of the altruistic behavior, b the reproductive benefit to the recipient and c the retroductive cost to the altruist donor.



Phylum				Details
Arthropoda				
	Class: Insecta			
		Orders:		
		Isoptera		All eusocial, many advanced
		Hemiptera		~50 sp eusocial
		Thysanoptera		~6 sp eusocial
		Coleoptera		1 species "Ambrosia beetle"
		Hymenoptera		
			Ants	All species (except a few highly derived species) ~14000
			Bees	Only 300-400 of ~ 4000 sp are eusocial
			Wasps	Most are not social, ~900 species are eusocial
	Subphylum: Crustaceae			
		Snapping shrimp		*Synalpheus*
Chordata				
	Class: Mammalia			10's of primitively eusocial species
		Family: Bathyergidae (African mole rats)		2 advanced eusocial species: naked mole rat (*Heterocephalus glaber*), Damaraland mole rat (*Cryptomys damarensis*)

Table 1. Eusocial animals. Table taken from Plowes, 2010

When thinking about eusocial animals we may immediately think about insects, but eusociality has also arisen three different times among some crustaceans that live in separate colonies (Duffy et al. 2000, Duffy & MacDonald 2010) and two times in mole–rats (Burda et al. 2000; O'Riain et al. 2008). While in a more controversial view (Gintis, 2012; Dawkins 2012; Pinker 2016), E. O. Wilson (2012) suggested humans as a eusocial species. However, the abundance of social animals reaches its peak in the phylum Arthropoda. Inside this phylum, the order Hymenoptera is the largest and most well–known animal group eusocial species, although most of them are not eusocial (Ross & Matthews 1991, Nowak et al. 2010). In fact, the whole concept of eusociality is primarily based on observations on Hymenopteran taxa, leading to a serie of mismatches when applying to other organisms, specially the diploid ones (Nowak 2010). In social Hymenoptera, females arise from fertilized diploid eggs and males arise from unfertilized haploid eggs, a system called haplodiploidy and which may contribute to kin selection, favoring altruistic behavior in this group (Plowes 2010). Diploid organisms' sex determination system would not provide especially high relatedness between some individuals of the group, having all individuals approximately the same fitness.

Among diploid social organisms, termites are probably the most remarkable group. We cannot think of an individual in a termite colony as a standard solitary insect. If you separate it from the colony, it will die (Eggleton 2011). Termites reveal the highest overall caste diversity (Choe & Crespi 1997; Thorne 1997) and each caste lacks some element that is present in a solitary insect, forming them all what is call as a "superorganism". A superorganism is defined as a social unit of eusocial animals, where division of labour is highly specialised and where individuals are not able to survive by themselves for extended periods (Hölldobler & Wilson 2008). And even though they are diploid, they are still eusocial. Within a single termite colony, you can find individuals at various stages of the termite life cycle, generations of termites overlap, and there is a constant supply of new adults prepared to assume responsibility for the colony's care (Nalepa 1994). In termites, two additional hypotheses have been proposed.

Some theories emerged to explain the evolution of eusociality in termites. One theory that used to have weight is the existence of relatedness asymmetry inside the colony, mainly ligated to two mechanisms: (i) The Chromosomal Linkage Hypothesis (Lacy, 1980), which establishes that much of the termite genome is sex–linked; (ii) cycles of inbreeding and outbreeding that would increase the relatedness between workers and the parents' offspring, favoring their evolution (Bartz 1979). In the first, siblings of the same sex would be related somewhat above 0.5, but siblings of different sex would have a relatedness less than 0.5

(Lacy, 1980). Termite workers might then bias their cooperative brood care towards their own sex. In that way, this hypothesis proposes that workers of a colony would only care for the offspring of their same sex (Thorne, 1997). However, several studies have undermined both theories (reviewed in Thorne 1997 and Howard & Thorne 2011). A strongest theory is the Symbiont Transfer Hypothesis (Cleveland et al. 1934, Nalepa 1984), which points out the dependence of termites on their symbiotic communities in their guts, which must be recovered after each molt by interactions with other termites, preventing thus the solitary way–of–life (Thorne, 1997).

1.2 The termites

Known commonly as “white ants”, termites are eusocial insects, with a broad range of morphological forms and diets.

Termites are often compared with the social Hymenoptera. Nevertheless, they differ in their evolutionary origins having big differences in life cycle (Howard & Thorne 2011). In eusocial Hymenoptera, workers are exclusively female, the males (drones) are haploid and develop from unfertilised eggs, while females are diploid and develop from fertilised eggs. On the other hand, termites are diploid individuals in all sexes and castes (Howard & Thorne 2011).

A colony of termites is established by a couple of imagoes, which become the royal couple (king and queen). They copulate and give birth to immatures individuals, which are small white, unsclerotised and essentially helpless. Once growing up, these immatures individuals will become workers, which undertake the most labour within the colony, being responsible for foraging, constructing, food storage, and brood and nest maintenance (Eggleton 2011). Some workers can go through further moulting and become soldiers which defend their colony against predators, or alate imagoes which will fly away from their colony to pair and establish a new one (Eggleton 2011). This description of caste structure is just a simplified and basic one, given that some species may have no soldiers, no true workers, present neotenics or even parthenogenesis (Eggleton 2011, Howard & Thorne 2011, Bourguignon et al. 2012, Fougeyrollas et al. 2015, Fougeyrollas et al. 2017). However, all termite’ species have at least one sterile caste that is pre–determined during the immature stages and follow the three main statements of the eusociality (Boomsma 2009).

All these castes and individuals living inside the colony will conform the animated part of it, but a colony in fact is conformed also by an inanimate part. The inanimate part of the colony is the structure built by them, which can be just a few tunnels to huge and sophisticated structures (Eggleton 2011).

As well as a sophisticated system of castes and differentiate building strategies, termites present a highly variable diet. They are detritivores generalists, consuming dead plants of all decomposition levels (Donovan et al. 2000; Hyodo et al. 2008). Termites rely primarily upon symbiotic microbes which inhabit predominantly the anterior part of the hindgut (Eggleton 2011). They can be protozoa, bacteria or flagellate protists which help termites to digest the cellulose they consume, allowing them to absorb the final products for their own use (Slaytor, 1992; Ikeda–Ohtsubo and Brune, 2009). Flagellates symbionts are absent in new individuals, being the workers which pass them to others through proctodeal trophallaxis. In other words, the immatures are fed by secretions from the anus, which contain the symbionts and alimentary particles (Ohkuma & Brune 2011). Most evolutionary advance termites possess cellulase enzymes, therefore they do not count with flagellates but they rely primarily on bacteria. In these advance termites, the workers fed the immatures only through stomodeal trophallaxis, method that is also present in older evolutionary species and consists in feeding from glands located in the thorax (normally the labial glands) through the mouth (McMahan 1969, Qiu–Ying et al. 2008).

One special case is the symbiosis between the termites and fungi living outside their body, inside the nest. These termites from the group Macrotermitinae maintain a “garden” of *Termitomyces* which is nourished by excrement, then the termites will eat it and their spores will pass through the intestines until complete a cycle by germinating in the fresh faecal pellets (Aanen et al. 2002; Mueller and Gerardo, 2002). This fungus farming system allowed these termites, originally from the rainforest, to colonise the African savannah and other new environments across Africa and Asia (Roberts et al. 2016).

Feeding preferences of termites are variable, and can present fluctuations between species, the taxa or even the season (Donovan et al, 2001; Allen et al. 1980). Donovan and others (2001) classified termites according to the degree of degradation (humification gradient) of the food they consume, mandibles development and guts structure: **Group I,** feeds on dead wood and grass and have relatively simple guts; **Group II,** feeds on wood, grass, leaf litter and microepiphytes and have more complex guts; **Group III** feeds on soil–like material with recognisable plant material in it; **Group IV** feeds on soil–like material with a high proportion of silica and no recognisable plant material. Bourguignon and others (2011) have showed later that this classification is merely structural, while the basics split lays between wood–feeders (lower termites: Groups I and II) and soil–feeders (Higher termites: Groups III and IV), being these lasts the most advanced evolutionary termites.

Phylogeny

The phylogeny of termites has been debated for a long time. The most common view classifies them as the infraorder Isoptera or as the epifamily Termitoidae within the order Blattodea (cockroaches).

Originally, termites were placed as an order, but in 1934 Cleveland and others have suggested them to be closely related to wood–feeding cockroaches according to their gut flagellates. This suggestion became stronger when morphological and phylogenetics studies supported the closeness between termites and cockroaches (McKittrick 1960; Inward et al. 2007; Eggleton et al. 2007; Legendre et al. 2008; Ware et al. 2008). Termites also share some behavioural features with their sister group, the cockroaches of the genus *Cryptocercus* (Lo et al. 2000, Grimaldi and Engel 2005, Ohkuma et al. 2009). The oldest unambiguous termite fossils date to the early Cretaceous, predating those of ants and bess by approximately 35 million years (Thorne et al. 2000, Engel et al. 2007). In the other hand, the last common ancestor of *Cryptocercus* and termites lived probably in the Jurassic (Vrsanky and Aristov 2014, Bourguignon et al. 2014).

About 3,106 species of termites are currently described (Krishna et al. 2013), with perhaps hundreds more still to be described. They are separated in 9 families which can be split in two groups: “lower” termites, comprising basal families (Mastotermitidae, Archotermopsidae, Stolotermitidae, Hodotermitidae, Kalotermitidae, Stylotermitidae, Serritermitidae and Rhinotermitidae), predominately feeding on wood; and “higher” termites, harboring the family Termitidae, which consume a wide variety of soft–materials (including faeces, humus, grass, leaves and roots) (Radek 1999, Engel et al. 2009). The gut in the lower termites contains different species of bacteria along with protozoa as symbionts, while higher termites only have a few species of bacteria with no protozoa (Breznak and Brune 1994).

Higher termites originated 42–54 million years ago in Africa and later dispersed between the continents at least 24 times in two main periods (Bourguignon et al. 2017). Eight subfamilies are recognised in Termitidae: Macrotermitinae, Sphaerotermitinae, Foraminitermitinae, Apicotermitinae, Termitinae, Syntermitinae, Cubitermitinae and Nasutitermitinae (Krishna et al. 2013). However, this subfamily–level classification is still unsatisfactory (e.g. see Kambhampati and Eggleton 2000; Inward et al. 2007b), particularly with respect to the subfamily Termitinae (Inward et al. 2007b). Although termites phylogeny has been highly debated and mostly disentangled (for review see Eggleton 2001), recent phylogenies mostly agree on basic pattern of termite phylogenetic tree (Miura et al. 1998, Lo et al. 2000, Donovan et al. 2000, Thompson et al. 2000, Austin et al. 2004, Inward et al. 2007a,b, Legrendre et al. 2008, Engel et al. 2009, Cameron et al. 2012, Bourguignon et al.

2015), the tree of Bourguignon and others (2015) being the most accepted and apparently accurated today (Fig.2).

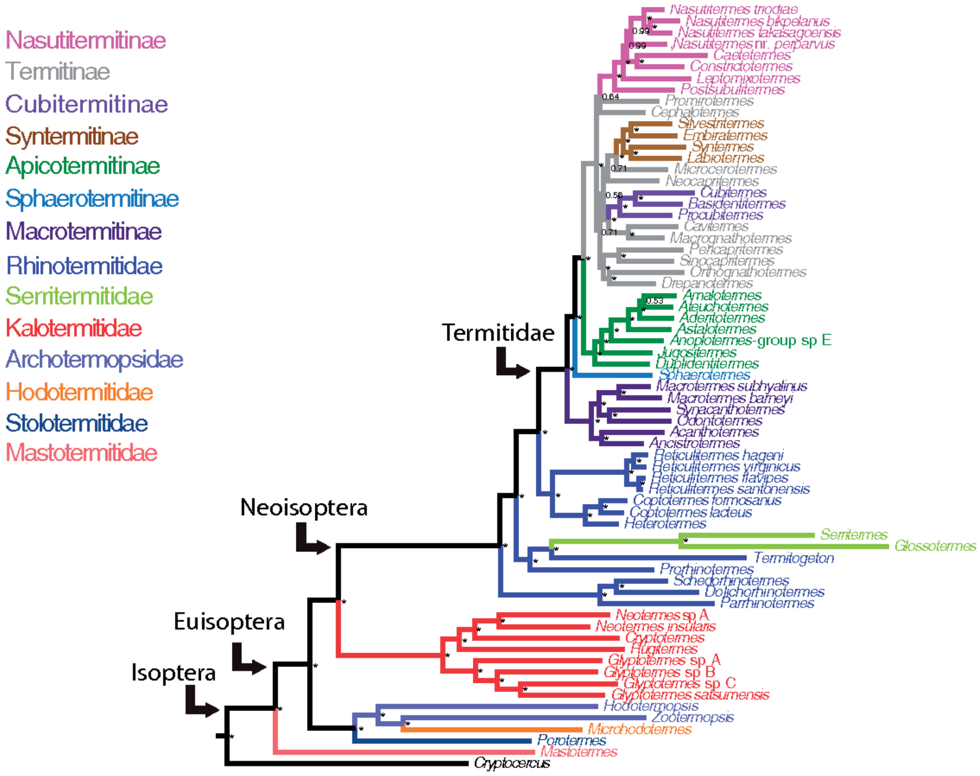


Figure 1. Phylogenetic relationships among termites, including their closest relative, the cockroaches of the genera *Cryptocercus*. Bourguignon et al. 2015.

Economical impact

Even thought termites are not as well–known as other social insects like Hymenopterans, most people are aware of termites. They are economic pests, specially in tropical and subtropical environments where they destroy crops, forests along with wood and wooden structures of human buildings (Meyer et al. 1999). In fact, they cause a significant economic loss of about US $ 22 billion to US $ 40 billion annually worldwide (Su 2002, Rust & Su 2012). In the USA for example, they cause more economic damage than fire and flood combined (Eggleton 2011). Most tropical crops are susceptible to termite attacks worldwide. Among the most remarkable damage termites can cause are those to eucalyptus (Fonseca 1949, Wood & Pearce 1991, Werner et al. 2008, Faragalla and Al Qhtani 2013), coconuts and palms (Aisagbonhi 1985, Logan & El–Bakri 1990, Mariau et al. 1992, Tang et al. 2006), fruit trees (Stansly et al. 2001, Constantino 2002, Ahmed and Qasim 2011, Faragalla and Al Qhtani 2013, Tomar 2013), sugarcane (Novaretti and Fontes 1998, Ahmed et al. 2007; Haifig et al. 2008, Alam et al. 2012), rice (Fonseca 1949, Mill 1992, Dario & Villela–Filho 1998, Agunbiade et al. 2009; Oyetunji et al. 2014), maize (Fernandes & Alves 1992, Nkunika

1994, Mill 1992, Constantino 2002; Faragalla and Al Qhtani 2013), wheat (Ahmed et al. 2004; Pardeshi et al. 2010; Rathour et al. 2014), sorghum (Logan 1991), sunflower (Ashfaq and Aslam 2001; Sileshi et al. 2009), groundnut (Johnson & Gumel 1981; Johnson et al. 1981, Wood et al. 1987, Wood & Pearce 1991), coffee (Kranz et al. 1981, Cowie & Wood 1989, Neves & Alves 1999), tea (Singha et al. 2011), cotton (Wood et al. 1987), tobacco (Shah and Shah 2013), pastures (Sands 1973, Cowie and Wood 1989, Mariconi et al. 1994, Fernandes et al. 1998) and tuber crops (Sands 1973, Tomar 2013).

Apart of their voracity linked to their populous colonies, termites are successful pests due to their capacity to invade new countries or even continents. Currently, 28 species of termites are known to be invasive. Most of them are important invasive pests in urban areas, although 6 of them have colonized natural forests habits (Evans et al. 2013). All these species share some characteristic in common: they all feed on wood, live and construct their nests inside of the alimentary source and easily produce secondary reproductives (Evans et al. 2013). Although the economical cost of invasive termite species has not been calculated, it is known that invasive insects cost a minimum of US$70.0 billion per year globally and the most expensive insect is purportedly a termite: *Coptotermes formosanus,* with an estimated cost higher than US $30.2 billion per year globally (Su 2002; Bradshaw et al. 2016). The genus *Coptotermes* is also one of the most spreaded termites' genera, which along with the genus *Cryptotermes* can be found in Africa, Asia, Europe, Oceania and America (Evans et al. 2013). These two genera plus *Heterotermes* (presented in Africa, Asia and America) represent the main invasive group of termites around the world (Evans et al. 2013). However, termites' impact is not always negative. They play an important role in the decomposition of litter on the ground, the regulation of soil structure, soil organic matter and nutrient cycling, water dynamics, soil erosion, plant growth, restoration of degraded lands, production of greenhouse gases, and overall biodiversity (see Holt and Lepage 2000, Jouquet et al. 2011, Bottinelli et al. 2015, Jouquet et al. 2016, Khan et al. 2018, Govorushko 2018, for reviews), including an important role as buffers of ecosystems against climate change (Bonachela et al. 2015). Termites also possess an economical importance as alimentary source (Figueirêdo et al. 2015). Forty–three species are known to be used as food for humans or to feed livestock in Africa, Asia, and North and South America (De Figueirêdo et al. 2015). However, the economical equivalence of these impacts has not been determined yet.

Termites' abundance

Termites are highly abundant in terms of biomass in warm terrestrial ecosystems, where they may represent 40% to 65% of the overall soil macrofaunal biomass (Loveridge and Moe

2004). They can exceed 6,000 individuals per square meter in tropics (Lee and Wood 1971, Eggleton et al. 1996), revealing comparable abundance to another remarkable group: the ants (Holldöbler & Wilson 1990). Higher termites are the most abundant group comprising 83% of termite genera and about 70% of the species (Krishna et al. 2013), especially the subfamily Termitinae which can represent 80% of the total termites' individuals in tropics (Eggleton et al. 1996). Due to their abundance, termites represent an important food source for a wide variety of predators: invertebrate (spiders, scorpions, mites, centipedes, true bugs, beetles, ants, wasps) and vertebrate (frogs, salamanders, lizards, birds, mammals) (Redford & Dorea, 1984).

1.3 Defense mechanisms of termites

Termites are vulnerable insects of soft body that have overcome high rates of predation and competition becoming one of the most ecologically success organisms (Deligne et al. 1981). They protect themselves through passive and active defence mechanisms, these include: a cryptic lifestyle characterized by a hidden way of life and the construction of defensive structures (Korb, 2011), the development of soldiers (Haverty, 1977) and glands that produce defensive compounds (Prestwich, 1984; Šobotník et al., 2010a).

The nest

Living in a protective nest is a strategy that all social insects share (Howard & Thorne 2011). It promotes the evolution of social cooperation during its construction and defense (Charnov 1978, Andersson 1984, Alexander et al. 1991, Crespi 1994, Wilson 2008), as well as by encouraging relatives to stay in close proximity (Hamilton 1978). Their main function is to protect the colony against enemies and hostile environmental conditions (Noirot & Darlington 2000, De Visse et al. 2008), but it is also a valuable storage for food reserves (Myles 1988; Starr 1991; Breed et al. 2004, Korb 2011).

Nests can be: i) fully underground galleries; ii) an epigeal protruding above the soil surface, which can wind up into very hard mounds of over 8 meters; iii) an arboreal construction, but always connected to the ground via shelter tubes; iv) a gallery system inside wooden structures such as logs, stumps and the dead parts of trees, where the colony develops (Noirot & Darlington 2000). This last is the most primitive way of nesting and provides a two–fold function, due to the importance of the nest not only for protection but also as food source (Abe 1987).

Termites build their nests primarily using their faeces, which are relatively inert to pathogens, are cheap to produce, are a good structural material (Eggleton 2011), and partly digested plant matter (arboreal nests) or soil (subterranean and epigeal nests) (Eggleton 2011).

The soldiers

Soldiers are the first truly altruistic caste present in termites (Hare 1937; Thorne et al. 2003). They are highly diverse, the most of all castes, diversifying over time to plentiful morphs and shapes, which are easily usable to identify genera or even species (Prestwich 1984). The evolution of a soldier caste represents an autapomorphy of termites (Hare 1937, Noirot & Pasteels 1987, Roisin & Korb 2011) and is a defining character of termites. In spite of being ancestral to all extant termite lineages, soldiers are not present in all species, being secondarily lost in the unrelated genera *Anoplotermes*, *Invasitermes*, *Orientotermes* and *Protohamitermes* (Sands 1972, Ahmad 1976; Miller 1984).

The soldiers in a colony have only one function: to defend the colony (Eggleton 2011). They are formed by differentiation of workers through an intermediate presoldier stage (Noirot 1985, Henderson 1998). It seems probable that soldiers are in the colony to defend the colony mainly from ants, so their morphology adaptations would be in reponse to this pressure (Eggleton 2011). Vertebrate predation may also be important, but soldiers cannot represent a real threat to them and they are generally not killing entire colonies, while ants do (Leal & Oliveira 1995).

Relative to workers, soldiers have a reduced digestive tract, long and strong legs, and a highly sclerotised head that usually large along with powerful, highly modified mandibles (Koshikawa et al. 2002, Eggleton 2011). According to Prestwich et al. (1984), soldiers mechanical defences can be separated in 9 types, but they can be summarized in 6 main categories:

a) Biting–crushing mandibles (Fig. 3A). Present in most of lower termites (Deligne et al. 1981), they are robust mandibles rich in dentition intended to hurt the opponent by squeezing or piercing them.

b) Phragmotical head (Fig. 3B). It is a modified highly sclerotized (especially in the rostrum) head cylindrically shaped with short mandibles, which occurs in some Kalotermitidae (Deligne et al. 1981). These heads are used as stoppers to plug holes that could be created during foraging activities or to allow the exit of the alates and thus prevent the entry of predators into the termite nest.

c) Biting–slashing mandibles (Fig. 3C). In this case, the termites possess slend, straight and long mandibles with a great angular motion. This mode is frequent in termites and can be observed in most Rhinotermitidae, Serritermitidae and Termitidae (Prestwich 1984). The use of these mandibles is usually coupled with the injection of greasy, irritating, toxic, or viscous materials into the wound of the enemy.

d) Biting–piercing mandibles. These are slender, inwardly curved mandibles with prominent marginal teeth (Mill 1982). It is common in some basal Termitinae (e.g. *Amitermes*), Syntermitinae (e.g. *Armitermes, Rhynchotermes*), and major soldiers of higher rhinotermitines (e.g. *Rhinotermes*) (Prestwich 1984). As well as for biting–slashing mandibles, these may be accompanied by chemicals entering the wound, normally from the frontal gland (Prestwich 1979, Pretwich and Collins 1982).

e) Snapping mandibles. This kind of mandibles is characterized by a long and slender shape unable to bite, but with the property of releasing energy stored into a single moving mandible, increasing its kinetic energy imparted at impact, killing or knocking down the enemy by a powerful strike (Deligne et al. 1981, Prestwich 1984, Seid et al. 2008). Until recently, snapping mandibles were thought to be present only in some termitines (Deligne et al. 1981, Prestwich 1981) and have evolved several times independently within this subfamily Termitinae (Bourguignon et al. 2017). This year, a new genus of snapping termites has been discovered, it is *Roisinitermes*, from the Kalotermitidae family (Scheffrahn et al. 2018).

f) Nasute (Fig. 3D). Most evolutionary advanced families of termites have developed a mandibular regression, where the space in the head which was normally used for the mandibular muscles is replaced by a huge reservoir for defensive secretions (the frontal gland) which are ejected through a nasute, entangling and incapacitating smaller enemies, and causing scratching and cleaning behaviour in larger ones (Prestwich 1984). This adaptation is characteristic of the Nasutitermitinae subfamily but something similar can be observed in smaller soldiers of Rhinotermitidae. In these small soldiers, mandibles are reduced to grabbing or carrying devices, there is no nasute but a labral brush which may look physically similar, but their defense is in fact accomplished by topical application of lipophilic contact poisons stored in massive abdominal reservoirs of the frontal gland (Prestwich 1984).

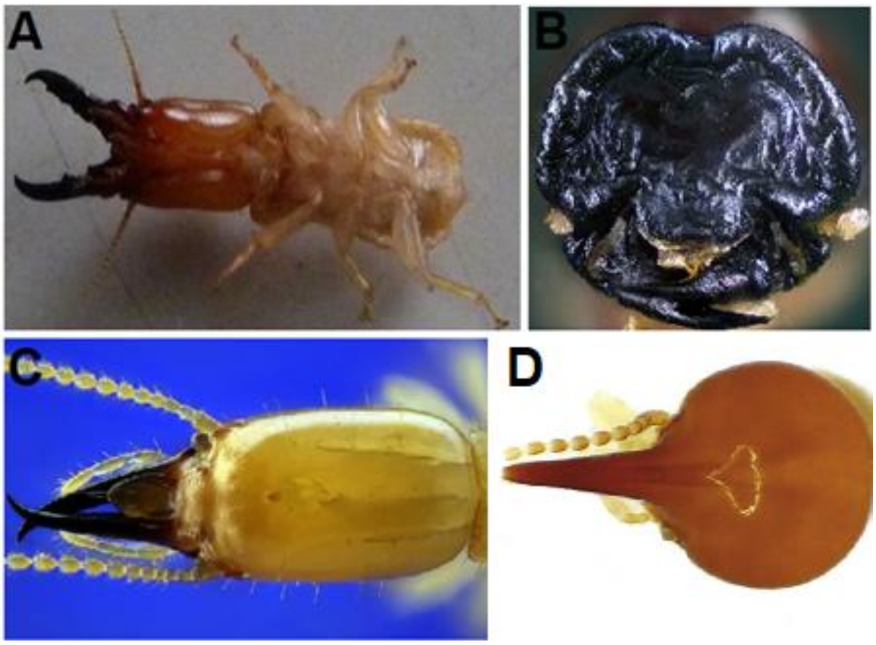


Figure 2. A) Ventral view of the whole body of a *Neotermes chilensis* soldier, showing its biting–crushing mandibles. B) Rostrum of the phragmotic head of *Cryptotermes cavifrons* soldier. C) Dorsal view of the full head of *Heterotermes* sp. Note the thin biting–slashing mandibles. D) Dorsal view of the head of *Nasutitermes longinasus* large soldier, note the well developed nasute. Photos B and C belong to David Mora del Pozo. Photo D was taken from Syaukani (2011).

Defensive strategies in other castes

The highest rates of predation against termites occurs when they are realizing activities out of the nest, such as during the nuptial flight or during foraging activities (Dial & Vaughan 1987; Lepage 1991, Korb & Salewski 2000, Korb and Schneider 2007). During foraging activities, as well as in the colony in general, workers outnumber soldiers considerably (with exception of some Nasutitermitinae species) with proportions which run from 4: 1 to 400: 1 (Haverty 1977). During the nuptial flight, the termite imagoes leave the nest and flight for a variable time and then land on the ground to search for a mate (Eggleton 2011). They are bad flyers and most of termites are depraded by invertebrate and vertebrate predators during these flights, including humans who attract them with lamps and eat them after removing their wings (Nyakupfuka 2003). In fact, Korb and Schneider (2007) have determined that the probability of successfully founding of a nest in *Cryptotermes secundus* is less than 1%.

Although soldiers are an especially developed defensive caste, workers and imagoes are not defenseless (Prestwich 1984). Workers have a primary role in passive defense, building a nest which is the first barrier against predators (Eggleton 2011), but they also present many other defensive roles. One of these roles is conducting detoxification mechanisms to defend conspecifics from chemicals used to attack other termites or ants (Spanton & Prestwich, 1982). More direct defensive strategies include abdomen rupture by dehiscense contaminating the opponent (Sands 1982) or by autothysis realeasing toxic compounds from inside their bodies (Costa–Leonardo, 2004; Šobotník et al. 2010b, 2012; Bourguignon et al. 2015; Poiani & Costa–Leonard, 2016), and defensive defecation on the enemy (Prestwich 1984). In the same way, workers of soldierless species are known for presenting more aggressiveness compared to other workers (Sands,1972; Šobotník et al. 2010a).

Imagoes have also developed several defensive strategies to overcome predation. Among these, an important one is the existence of synchronous nuptial flights which along with reducing endogamy (Roisin 1999, Aguilera–Olivares 2015), act as a defensive strategy increasing the probability of survival by increasing the number of termites flying (Nutting 1969, Nutting and Haverty 1976, Thorne 1983, Jones et al. 1988, Bordereau et al. 1991, Nalepa et al. 2001). Another important defensive mechanism from alate imagoes is the development of the frontal gland. Indeed, Šobotník and others (2010c) described how wasps removed the head of *Coptotermes testaceus* (a termite with a large frontal gland) prior storing them in the nest, while alates of *Anoplotermes s.lat*. spp. (a species with tiny frontal gland) were not.

1.4 Chemical defenses

Exocrine glands are group of cells that produce and secrete substances onto an epithelial surface by way of a duct or epithelial modification (Young et al. 2013). Insects have a wide variety of glandular cells and organs which produce a variety of secretions, creating complex exocrine glandular systems that coordinate different social interactions or activities, including foraging, building, mating, defense, and nestmate recognition. If the glands itself may be not that well–known, their secretions certainly are. Everyone is familiar with sweat, silk or venom, all of them results of glandular secretions. Particularly important although not specially known is their involvement in the production of antibiotics, lubricants, and digestive enzymes (Billen & Šobotník 2015).

In 1974, Noirot and Quennedey formulated a classification for the exocrine glands, which has been widely accepted and became universally used. Glandular cells are classified as: (i) class 1, the cells are adjoined directly to the cuticle which need to be cross to the release of the secretion; (ii) class 2, the cells are not in direct contact with the cuticle, they are surrounded by class 1 cells through which the secretion must run before crossing the cuticle; (iii) class 3, the cells compound units formed by one to several secretory cells isolated from the cuticle plus one or two cells that surround a conducting duct that carries the secretion to the exterior (Fig. 3).

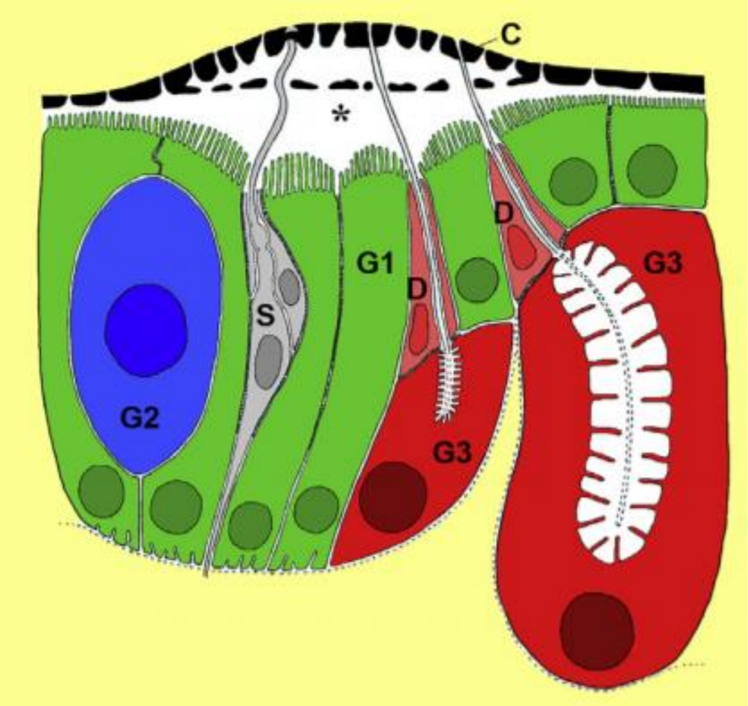


Figure 3. Classification of insect exocrine glands, based on a rhinotermitid sternal gland. Scheme taken from Billen and Šobotník (2015), made after Noirot and Quennedey (1974). Abbreviations: C, cuticle; D, duct cells; G1, secretory cells class 1; G2, secretory cells class 2; G3, secretory cells class 3; S, campaniform sensilla. The asterisk indicates a subcuticular space.

The life in colonies of social insects is a promoter of exocrine developments, as they are used extensively to coordinate different social interactions or activities, including foraging, building, mating, defense, and nestmate recognition (Costa–Leonardo & Haifig 2010, Billen 2011).

As many as 149 exocrine glands have been described for social insects so far, from which 84 can be found in ants, 53 in bees and bumblebees, 49 in wasps and only 20 in termites (Billen & Šobotník 2015).

Exocrine glands in termites

Termites possess 20 glands spread all over their body, but not necesarilly present in all castes or species, and they generally consist of epidermal cells of ectodermal origin with secretory capacities (Blum 1985, Costa–Leonardo & Haifig 2010).

Trail–following and sex pheromones are the most studied exocrine secretions in termites, followed closely by defensive secretions. Trail–following pheromones are secreted to mark the path between the nest and the foraging area. They are used by all termite species studied so far and are secreted by the **sternal gland** of workers and soldiers, being their action much stronger in workers (Howard et al. 1976; Sillam–Dussès et al. 2005, 2007, 2009a, 2009b, 2010, 2011, Bordereau et al 2010, Costa–Leonardo & Haifig 2010, Bordereau & Pasteels 2011). Sex pheromones are released by imagoes of one sex (usually females) in order to attract the opposite sex (Pasteels 1972, Bordereau et al. 2010). They are usually produced by the **tergal glands**, sometimes by the **posterior sternal glands** (both occurring exclusively in termite imagoes and always involved in mate attraction; Noirot 1969) or **sternal glands** (present in all species, castes and developmental stages) (Bordereau & Pasteels 2011, Sillam–Dussès et al. 2011). In the case of defensive secretions, they are known for being mainly produced by the labial and the frontal glands. The **labial glands** (also called salivary glands) are a large paired organ, made of numerous cells arranged in clumps (called acini) along with paired reservoirs (called water sacs) (Sillam– Dussès et al. 2012), that can be found in all castes and developmental stages of all termite species (Noirot 1969). The defensive function of these glands is restricted to soldiers, while in workers they are used as food–marking pheromone and as phagostimulant (Noirot 1969, Sillam– Dussès et al. 2012). On the other side, the **frontal gland** represents a fully defensive organ incomparable among insects (Noirot, 1969). It is present in almost all imagoes and soldiers from all species of termites in Neoisoptera clade (Rhinotermitidae + Serritermitidae + Termitidae) (Deligne et al. 1981, Prestwich 1984, Šobotník et al. 2010a, 2010c, 2010d, Kutalová et al. 2013). It is always present in Neoisoptera soldiers and it occurs as a large reservoir, sometimes extending deep into the abdomen (Rhinotermitidae genera) but normally restricted to the head (all other Neoisoptera families) (Prestwich 1984, Quennedey 1984). It is present in all Neoisoptera imagoes but *Protermes* sp. and *Microtermes toumodiensis,* as an epithelial thickening (all basal Neoisoptera groups) or as an epithelial with reservoir (Termitidae except Foraminitermitinae and Macrotermitinae) (Prestwich 1984, Šobotník et al. 2004, Šobotník et al. 2010c, Kutalová et al. 2013). This gland is also present in almost all workers from soldierless species, where it always occurs as an epithelial thickening (Šobotník et al. 2010d). When the gland has reservoir, it is always accompanied by an opening called “fontanelle”. In those cases where there is no reservoir, just a modified cuticle allows the secretions to go out of the body. Frontal gland compounds can be chemicals of diverse nature, but they all have been found to act as a defensive secretion in soldier, with functions such as: contact poisons, repellents or irritants, entangling and incapacitating agents, anti–healing compounds, or alarm pheromones (Piskorski et al. 2007, 2009; Šobotník et al. 2010b). There are only few cases where the frontal gland is not accompanied by a fontanelle; in these cases, the secretion is released through autothysis (Deligne & DeConinck 2006, Bordereau et al. 1997, Šobotník et al. 2010b). The function of the gland in imagoes with reservoir has been only investigated in *Prorhinotermes simplex* and its function seems analogous to soldiers (Piskorski et al., 2007, 2009). The function of the frontal gland when it is present as an epithelial thickening remains unknown.

There are many other glands in termites, plenty of them presenting unknown or speculative function. Among them, we can find: the **mandibular gland**, located at the ventral mandibular condyle and present in all castes and developed stages (Šobotník & Hubert 2003); the **tarsal glands**, always located on the first and second tarsomere of the leg, sometimes also on the third tarsomere or the distal part of the tibia and present in most termite species (Bacchus 1979, Soares & Costa–Leonardo 2002, Šobotník & Weyda 2002); the **clypeal gland** present at the clypeus of alate imagoes of Rhinotermitidae, Serritermitidae and Termitidae species (Křížková et al. 2014); the **tegumental glands** described in *Kalotermes* and *Prorhinotermes* neotenics (Sbrenna & Leis 1983, Šobotník et al. 2003); the **lateral thoracic glands** described in 3 Termitidae species (Gonçalves et al. 2010); and the **labral gland**, which had been described in few random observations in soldiers (Deligne et al. 1981, Quennedey 1984, Šobotník et al. 2010b).

1.5 Motivation and objectives of my thesis

Termites are fundamental organisms for humans both in their positive and negative aspects,and learning about their chemical defensive mechanisms provides fundamental information for a better understanding of their evolution and behaviour. This project was facilitated through a collaboration between the Termites Research Team, Czech University of Life Sciences (Czech Republic), and the Laboratoire d'Ethologie Expérimentale et Comparée (LEEC), Université Paris 13 (France), with the support of a Université Paris 13 doctoral fellowship.

The presented studies were done under the supervision of David Sillam–Dussès, leader expert on termite pheromones, whose close collaboration allowed me to learn fundamentals of termite communication. At the same time, I took the best from collaboration with my co–supervisor, Jan Šobotník, who is authority in the field of insect exocrine organs, their structure, function and evolution.

My Ph.D. aimed straight on disentangling the evolutionary processes leading to the current development of the frontal and labral glands in termites. Three main aims were raised (corresponding to Chapters 2, 3 and 4). The first aim was:

- *To Disentagle the distribution of the labral glands in termite soldiers*. This study represented the first attempt to describe the gland occurrence in a representative set (28 species) of termite soldiers across all termites (Paper 1). I examined the gland presence in members of all termite families (except for Stylotermitidae, whose material is not available) and most of Termitidae subfamilies. The results were published in the *Biological Journal of the Linnean Society* (IF: 2.3).

The results of this research were that soldiers from all termite species possess the labral gland. In addition to personal observations of its occurrence in imagoes as well, these results suggested that further research should be perform to understand the evolutionary routes of this gland. Hence, our second aim appeared:

- *To determine the evolution of the labral gland of termites.* The study was carried out among workers and imagoes in a representative set of termite species and the closest relative, the woodfeeding cockroach *Cryptocercus punctulatus*, using the histological procedures (Paper 2). The gained observations allowed us to describe the evolution of the labral gland across extant termite taxa. The resulting manuscript has been published in one leading ecological journal, *Biological Journal of the Linnean Society*.

The research about the frontal gland in our study presented two main aims:

- *To unravel the evolution of frontal gland in termite workers:* I executed comparative study of the frontal gland in workers of 37 species across Neoisoptera representatives using histological procedures, and the gland secretory activity was evaluated using methods of transmission electron microscopy in 8 species.

- *To perform a phylogenetic analysis of the frontal gland evolution in Neoisoptera*: I mapped the evolutionary routes leading to the observed diversity of the gland in soldiers, workers and alate imagoes on a robust phylogenetic tree, which allowed me to describe the general trends in the gland structure and use in particular termite taxa.

These two objectives were joined together in a larger manuscript (Paper 3), which I hope it will be published in a leading biological science journal, such as *Proceedings of the Royal Society London B*.

2. General methods

Photo by Aleš Buček

2. General Methods

2.1 Animals of study

For my thesis I used living termite species which were obtained predominantly on the existing material from my supervisors, but I also realised some necessary field trips (in China, Ecuador and French Guiana) which were covered by my supervisors. At the same time, my supervisors already disposed of a set of fixed samples to be used for optical and electron microscopy, and they also provided me additional material from their field works or through existing network of their collaborators. The detail of the species and their place of origin can be found in the supplementary tables of my manuscripts.

2.2 Histology

Histological procedures were done at the laboratory of the Termites Research Team (TRT) of the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czech Republic. There, all the equipment for fixation and embedding was available, as well as a Reichert Ultracut ultramicrotome which I used for sectioning of the samples.

More details about fixative used and fixation methods are provided in the Materials and Methods of each manuscript presented in this thesis.

2.3 Microscopy

Nikon Ni–E optical microscope equipped with a Nikon DS–Fi1c camera was usually used to identify presence/absence of the gland. It was available at the TRT in Prague and the software used for controlling the microscope and for taking and measuring the pictures was Nis–elements AR.

When it was needed to use Transmission Electron Microscope or Scanning Electron Microscope, a Jeol 6380 LV scanning electron microscope and a Jeol 1011 transmission electron microscope were available at the Laboratory of Electron Microscopy of the Faculty of Sciences, Charles University in Prague, Czech Republic. Mirek Hyliš, the technician in charge of them, provided me with assistance and collaboration.

2.4 Behavioural test

Behavioural experiments were performed at the Laboratoire d'Ethologie Expérimentale et Comparée of the Université Paris 13 (France) and at the TRT in Prague. In both cases, they were carry out in rooms with controlled temperature and humidity.

2.5 Others

Other experiments or details are described in each specific manuscript.

Paper 1: The labral gland in termite soldiers

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Résumé

Le succès évolutif des termites repose en grande partie sur un système de communication complexe géré par un riche ensemble de glandes exocrines. Pas moins de 20 glandes exocrines différentes sont connues chez les termites. Bien que certaines de ces glandes soient relativement bien connues, seules des observations anecdotiques existent pour d'autres. La glande labrale est l'une des glandes exocrines qui n'a retenu jusqu'à présent qu'une attention négligeable. Dans cette étude, nous avons examiné la structure et l'ultrastructure du labrum chez des soldats de 28 espèces de termites. Nous confirmons que la glande labrale est présente dans toutes les espèces de termites et comprend deux régions sécrétrices situées sur la face ventrale du labrum et à la partie dorso–apicale de l'hypopharynx. Le labrum des Neoisoptera a une pointe hyaline, qui a été ensuite perdue chez les Nasutitermitinae, les *Microcerotermes* et des espèces à soldats qui claquent. L'épithélium de la glande est généralement constitué de cellules sécrétrices de classe 1, avec en plus des cellules sécrétrices de classe 3 chez certaines espèces. Une caractéristique commune des cellules sécrétrices est l'abondance de réticulum endoplasmique lisse, un organite connu pour produire des sécrétions lipidiques et souvent volatiles. Nos observations suggèrent que la glande labrale est impliquée dans la communication plutôt que dans la défense, comme suggéré précédemment. Notre étude est la première à fournir une image complète de la structure de la glande labrale chez les soldats parmi tous les taxons de termites.

Mots–clefs: glande exocrine, hypopharynx, labrum, Termitoidae, ultrastructure, Isoptera

Abstract

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.

Keywords: exocrine gland, hypopharynx, labrum, Termitoidae, 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 & 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 & Haifig, 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 & Haifig, 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.

Materials 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–µm sections using a Reichert Ultracut ultramicrotome and stained with Azure II for analysis with optical microscopy.

Histology

The samples were dehydrated using a 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 µm 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 xylen. 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 sphaerothorax*) 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 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).

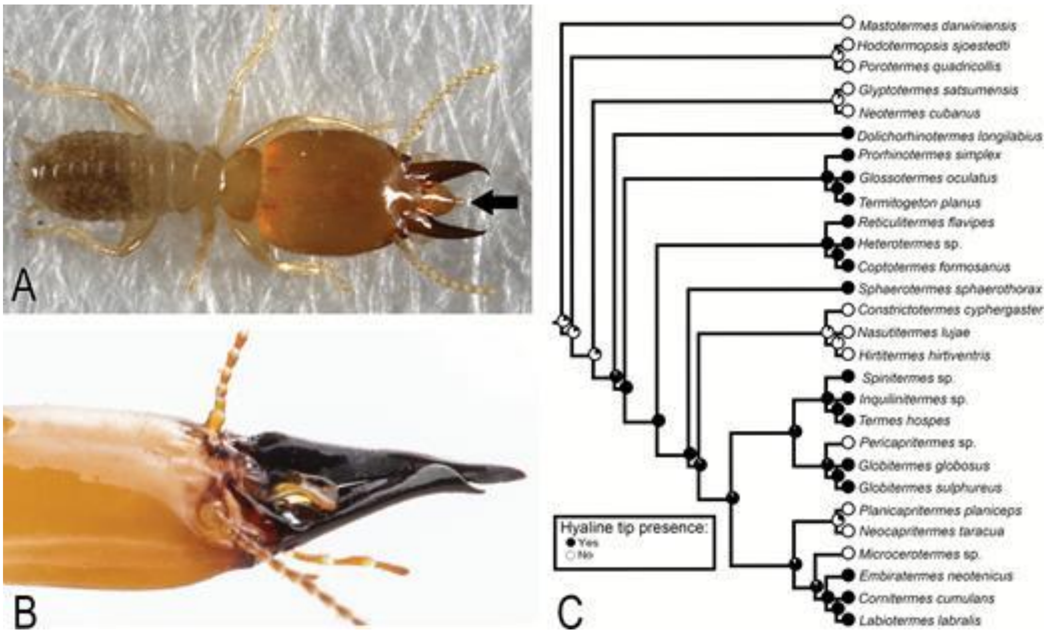

Figure 1. (A) *Sphaerotermes sphaerothorax* soldier. Arrow marks the hyaline tip of the labrum. (B) Head of *Neocapritermes taracua* soldier. (C) Phylogenetic tree showing the evolution of the hyaline tip in soldier caste termites. The presence or absence of the hyaline tip is marked by black or white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

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 µm 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 *Neotermes cubanus*, *Glossotermes oculatus*, *Neocapritermes taracua*, *Spinitermes* sp. and *Labiotermes labralis*. The same pattern was also observed along the midline of the labrum in Prorhinotermes simplex, the basal half of the labrum in *Coptotermes formosanus* (Fig. 2A, B) and *Sphaerotermes 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).

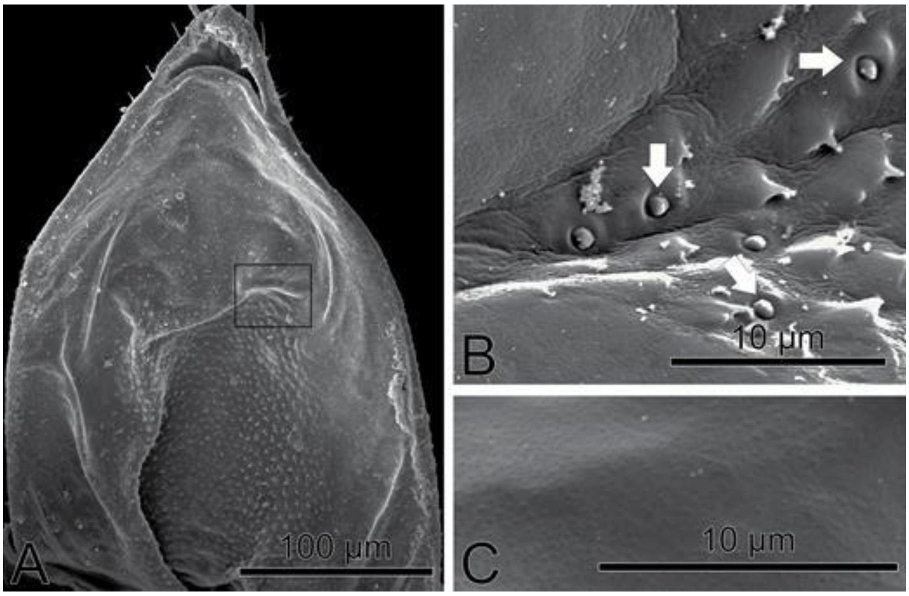

Figure 2. Labral gland development. (A) Micrograph of the ventral side of the labrum of *Coptotermes formosanus*; the small rectangle indicates the sector where the micrograph in B was taken. (B) Region with a group of sensillae (marked with white arrows) in *C. formosanus* labrum. (C) High–magnification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

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 overlaying cuticle with no reservoir.

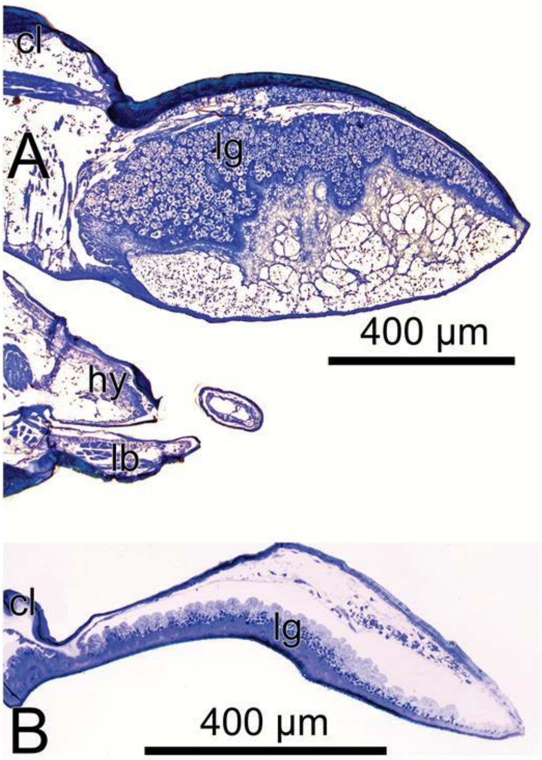

Figure 3. Sagittal sections of the forehead of *Psammotermes hybostoma* medium soldier (A) and *Neocapritermes taracua* soldier (B), showing the secretory epithelium in hypopharynx. Abbreviations: cl, clypeus; hy, hypopharynx; lb, labium; lg, labral gland.

The labral gland secretory epithelium varied in thickness among species, most commonly ranging between 20 and 30 µm. The thinnest epithelium was found in *Nasutitermes lujae* (2 µm) and the thickest epithelium was found in the large soldiers of *Psammotermes hybostoma* (147 µm) (Table S1). Hypopharyngeal thickness varied between 4 and 30 µm. The ultrastructural features were nearly identical between the labral and hypopharyngeal regions of the labral gland in all species. The shape and overall size of the labral gland were diverse and not proportional to the size of the labrum. While some labral glands covered the entire labrum, others covered less than half of the labral ventral area.

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

Transmission 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 Noirot & 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 Labiotermes labralis) (Fig. 4A) and showed frequent pinocytotic activity (Fig. S3D). The nucleus of the class 1 cells was basally located and elliptic or slightly irregular in shape. The largest dimension of the nucleus was 5 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.

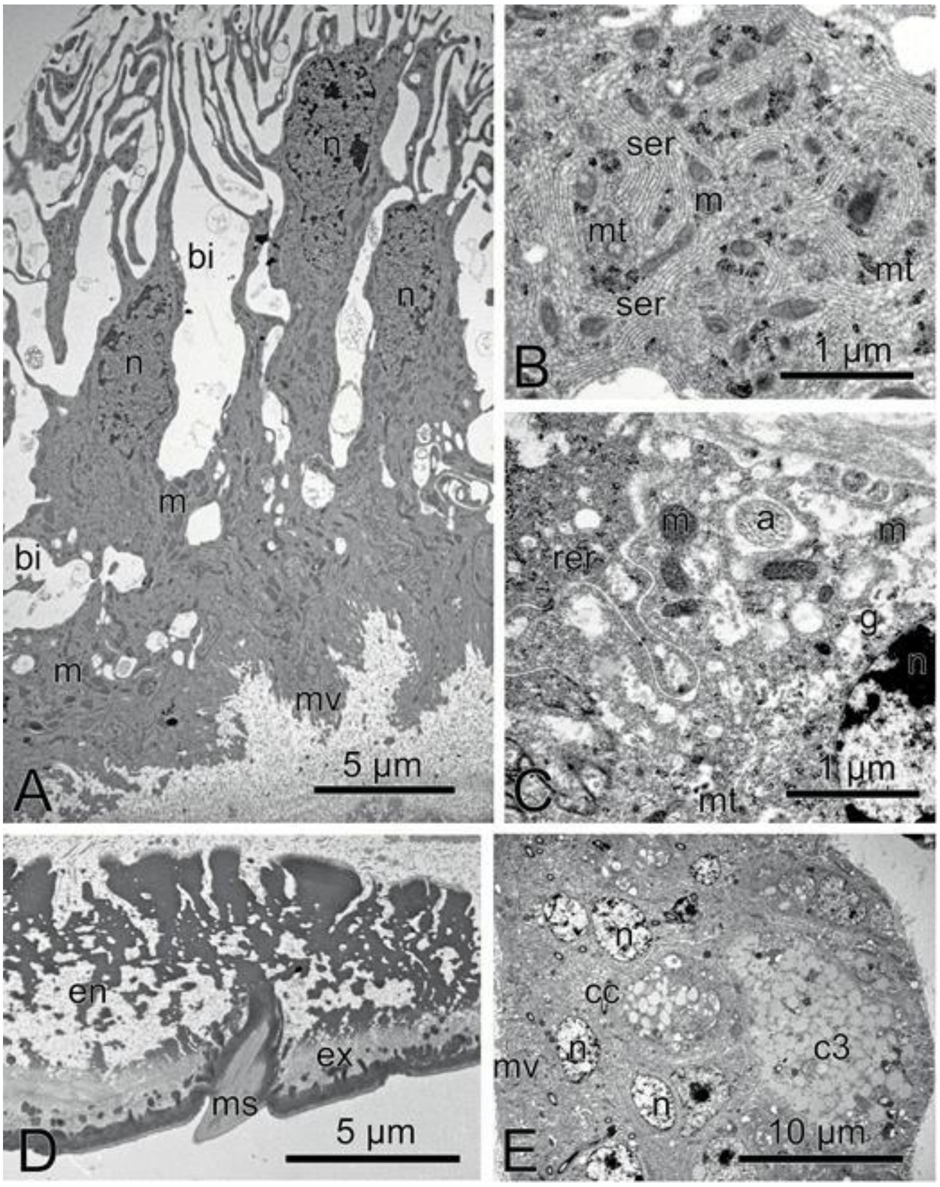

Figure 4. Ultrastructure of the labral gland in soldiers. (A) Overall development of the labral gland in Labiotermes labralis. Note the development of the apical microvilli and basal invaginations. (B) Detail of labral gland secretory cell class 1 cytoplasm in Neocapritermes taracua showing well–developed 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.

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 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.

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 µm 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 & Haifig, 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 & Haifig's (2014) study on the labral gland in Cornitermes cumulans, that additional data on the labral gland appeared. In addition to the presence of the labral gland in termite soldiers, it was also recently observed in some imagoes (Křížková et al., 2014) and certain workers (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 & Haifig, 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 (Deligne 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

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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:

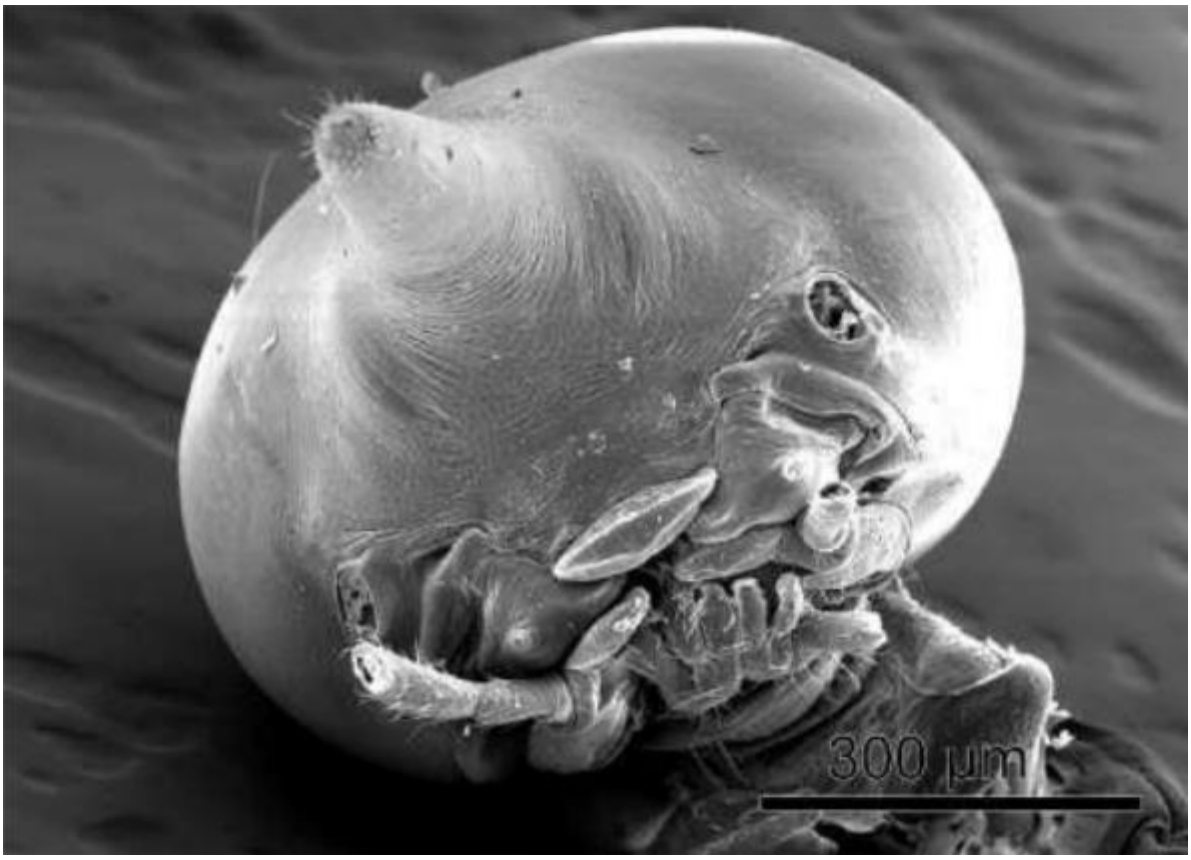


Figure S1. Scanning electron micrograph of the mouth parts of *Nasutitermes lujae,* with antennae and part of the maxillary palp removed.

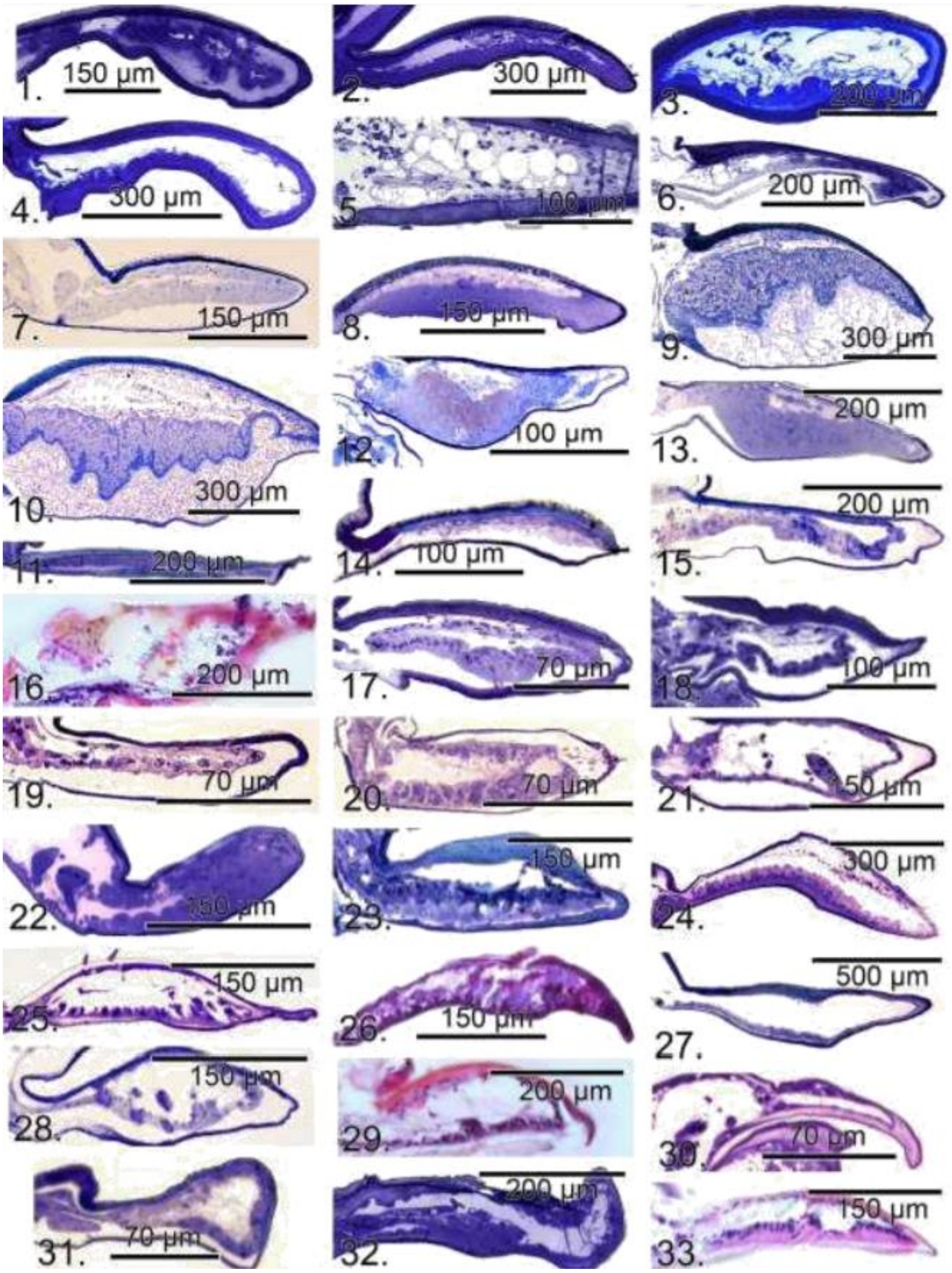


Figure S2. Labral gland development. Sagital sections of the labrum in: (1) *Mastotermes darwiniensis*, (2) *Hodotermopsis sjoestedti*, (3) *Neotermes cubanus* small soldier, (4) *Neotermes cubanus* large soldier, (5) *Dolichorhinotermes longilabius* small soldier, (6) *Dolichorhinotermes longilabius* 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.

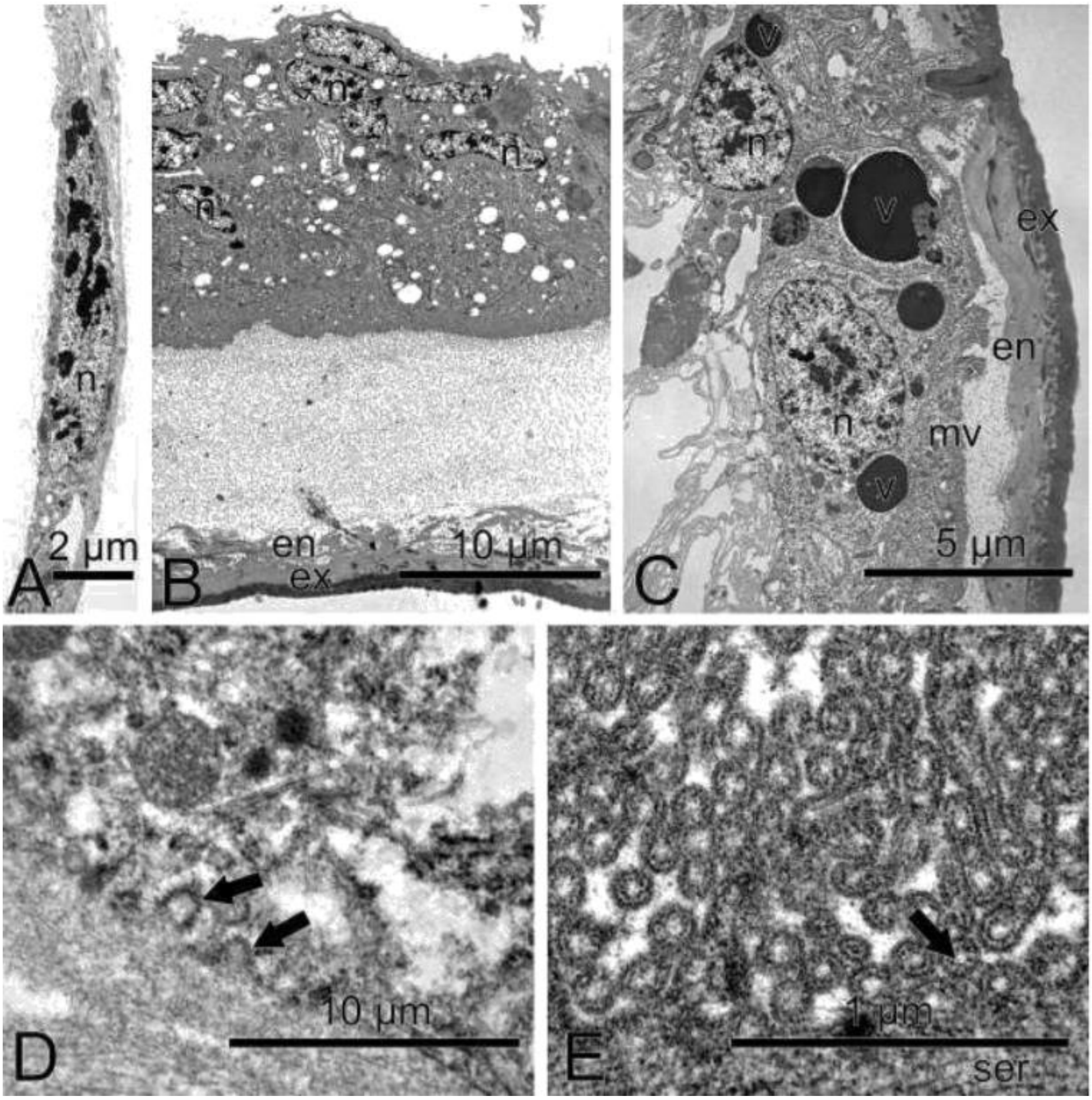


Figure S3. Ultrastructure of the labral gland in soldiers. (A) Non–modified epithelium surrounding the labral gland in *Hirtitermes* sp. (B) Labral gland development in *Hirtitermes* sp. (C) Labral gland development in *Nasutitermes lujae*. Note the highly electron–dense vesicles. (D) Pinocytotic activity 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.

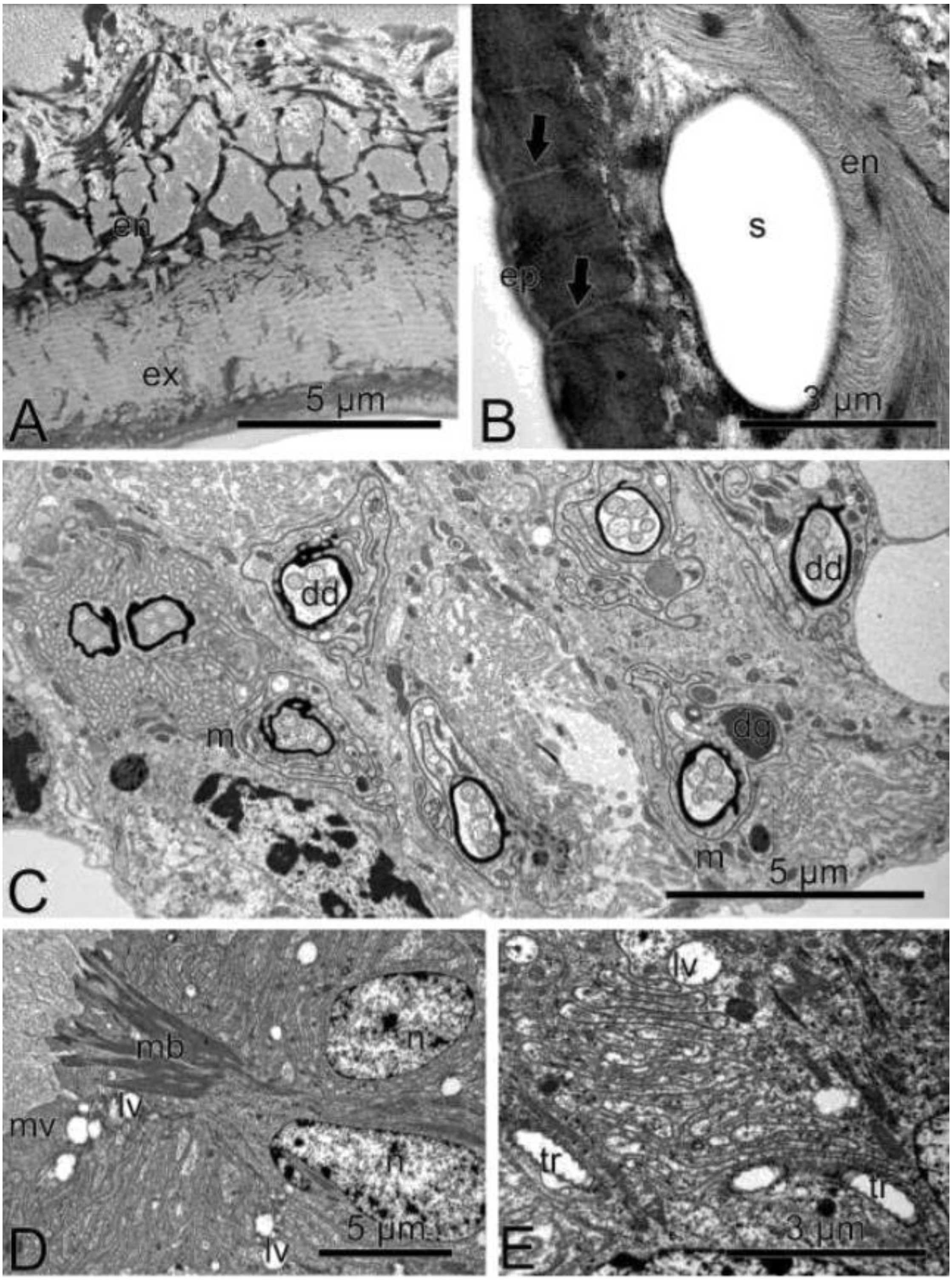
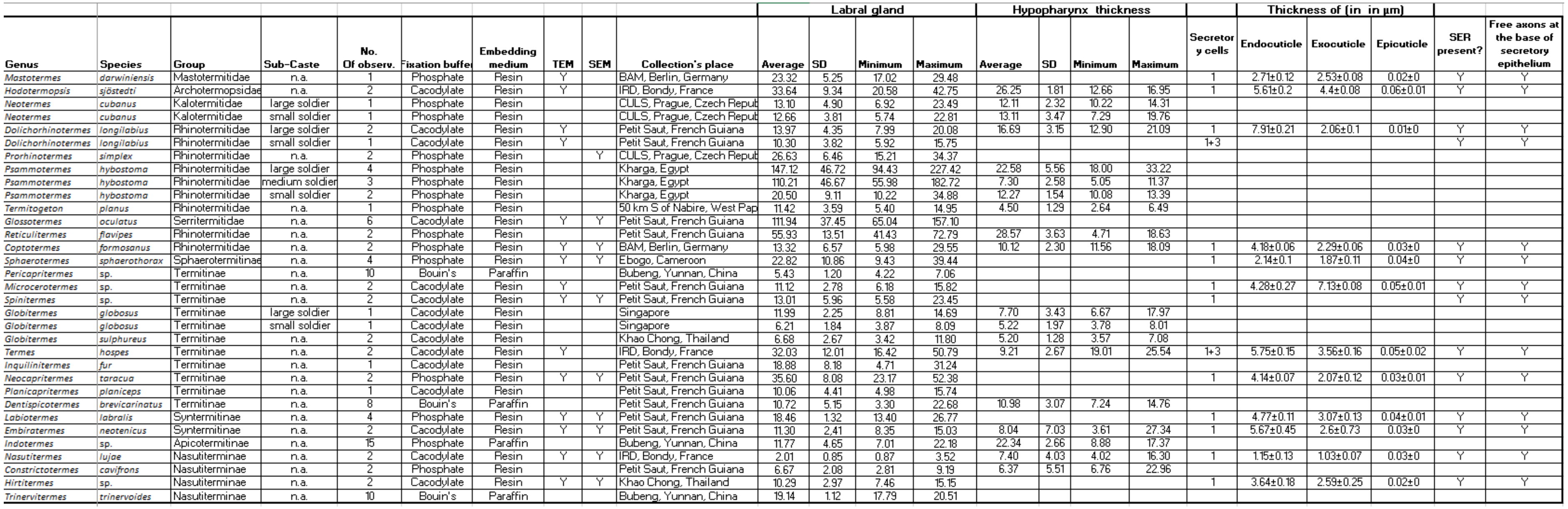


Figure S4. Ultrastructural features in the labral gland. (A) Highly modified cuticle underlying the labral gland in *Neocapritermes taracua*. (B) Detail of apical glandular cuticle at the tip of the labral gland in *Nasutitermes lujae* showing epicuticular pores allowing secretion out from the body. (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 (µm). 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.



										Labral gland				Hypopharynx thickness					Thickness of (in in µm)				
Genus	**Species**	**Group**	**Sub-Caste**	**No. Of observ.**	**ixation buffe**	**Embedding medium**	**TEM**	**SEM**	**Collection's place**	**Average**	**SD**	**Minimum**	**Maximum**	**Average**	**SD**	**Minimum**	**Maximum**	**Secretory cells**	**Endocuticle**	**Exocuticle**	**Epicuticle**	**SER present?**	**Free axons at the base of secretory epithelium**
Mastotermes	*darwiniensis*	Mastotermitidae	n.a.	1	Phosphate	Resin	Y		BAM, Berlin, Germany	23.32	5.25	17.02	29.48					1	2.71±0.12	2.53±0.08	0.02±0	Y	Y
Hodotermopsis	*sjöstedti*	Archotermopsidae	n.a.	2	Cacodylate	Resin	Y		IRD, Bondy, France	33.64	9.34	20.58	42.75	26.25	1.81	12.66	16.95	1	5.61±0.2	4.4±0.08	0.06±0.01	Y	Y
Neotermes	*cubanus*	Kalotermitidae	large soldier	1	Phosphate	Resin			CULS, Prague, Czech Repub	13.10	4.90	6.92	23.49	12.11	2.32	10.22	14.31						
Neotermes	*cubanus*	Kalotermitidae	small soldier	1	Phosphate	Resin			CULS, Prague, Czech Repub	12.66	3.81	5.74	22.81	13.11	3.47	7.29	19.76						
Dolichorhinotermes	*longilabius*	Rhinotermitidae	large soldier	2	Cacodylate	Resin	Y		Petit Saut, French Guiana	13.97	4.35	7.99	20.08	16.69	3.15	12.90	21.09	1	7.91±0.21	2.06±0.1	0.01±0	Y	Y
Dolichorhinotermes	*longilabius*	Rhinotermitidae	small soldier	1	Cacodylate	Resin	Y		Petit Saut, French Guiana	10.30	3.82	5.92	15.75					1+3				Y	Y
Prorhinotermes	*simplex*	Rhinotermitidae	n.a.	2	Phosphate	Resin		Y	CULS, Prague, Czech Repub	26.63	6.46	15.21	34.37										
Psammotermes	*hybostoma*	Rhinotermitidae	large soldier	4	Phosphate	Resin			Kharga, Egypt	147.12	46.72	94.43	227.42	22.58	5.56	18.00	33.22						
Psammotermes	*hybostoma*	Rhinotermitidae	medium soldier	3	Phosphate	Resin			Kharga, Egypt	110.21	46.67	55.98	182.72	7.30	2.58	5.05	11.37						
Psammotermes	*hybostoma*	Rhinotermitidae	small soldier	2	Phosphate	Resin			Kharga, Egypt	20.50	9.11	10.22	34.88	12.27	1.54	10.08	13.39						
Termitogeton	*planus*	Rhinotermitidae	n.a.	1	Phosphate	Resin			50 km S of Nabire, West Pap	11.42	3.59	5.40	14.95	4.50	1.29	2.64	6.49						
Glossotermes	*oculatus*	Serritermitidae	n.a.	6	Cacodylate	Resin	Y	Y	Petit Saut, French Guiana	111.94	37.45	65.04	157.10										
Reticulitermes	*flavipes*	Rhinotermitidae	n.a.	2	Phosphate	Resin			Petit Saut, French Guiana	55.93	13.51	41.43	72.79	28.57	3.63	4.71	18.63						
Coptotermes	*formosanus*	Rhinotermitidae	n.a.	2	Phosphate	Resin	Y	Y	BAM, Berlin, Germany	13.32	6.57	5.98	29.55	10.12	2.30	11.56	18.09	1	4.18±0.06	2.29±0.06	0.03±0	Y	Y
Sphaerotermes	*sphaerothorax*	Sphaerotermitinae	n.a.	4	Phosphate	Resin	Y	Y	Ebogo, Cameroon	22.82	10.86	9.43	39.44					1	2.14±0.1	1.87±0.11	0.04±0	Y	Y
Pericapritermes	sp.	Termitinae	n.a.	10	Bouin's	Paraffin			Bubeng, Yunnan, China	5.43	1.20	4.22	7.06										
Microcerotermes	sp.	Termitinae	n.a.	2	Cacodylate	Resin	Y		Petit Saut, French Guiana	11.12	2.78	6.18	15.82					1	4.28±0.27	7.13±0.08	0.05±0.01	Y	Y
Spinitermes	sp.	Termitinae	n.a.	2	Cacodylate	Resin	Y	Y	Petit Saut, French Guiana	13.01	5.96	5.58	23.45					1				Y	Y
Globitermes	*globosus*	Termitinae	large soldier	1	Cacodylate	Resin			Singapore	11.99	2.25	8.81	14.69	7.70	3.43	6.67	17.97						
Globitermes	*globosus*	Termitinae	small soldier	1	Cacodylate	Resin			Singapore	6.21	1.84	3.87	8.09	5.22	1.97	3.78	8.01						
Globitermes	*sulphureus*	Termitinae	n.a.	2	Cacodylate	Resin			Khao Chong, Thailand	6.68	2.67	3.42	11.80	5.20	1.28	3.57	7.08						
Termes	*hospes*	Termitinae	n.a.	2	Cacodylate	Resin	Y		IRD, Bondy, France	32.03	12.01	16.42	50.79	9.21	2.67	19.01	25.54	1+3	5.75±0.15	3.56±0.16	0.05±0.02	Y	Y
Inquilinitermes	*fur*	Termitinae	n.a.	1	Cacodylate	Resin			Petit Saut, French Guiana	18.88	8.18	4.71	31.24										
Neocapritermes	*taracua*	Termitinae	n.a.	2	Phosphate	Resin	Y	Y	Petit Saut, French Guiana	35.60	8.08	23.17	52.38					1	4.14±0.07	2.07±0.12	0.03±0.01	Y	Y
Planicapritermes	*planiceps*	Termitinae	n.a.	1	Cacodylate	Resin			Petit Saut, French Guiana	10.06	4.41	4.98	15.74										
Dentispicotermes	*brevicarinatus*	Termitinae	n.a.	8	Bouin's	Paraffin			Petit Saut, French Guiana	10.72	5.15	3.30	22.68	10.98	3.07	7.24	14.76						
Labiotermes	*labralis*	Syntermitinae	n.a.	4	Phosphate	Resin	Y	Y	Petit Saut, French Guiana	18.46	1.32	13.40	26.77					1	4.77±0.11	3.07±0.13	0.04±0.01	Y	Y
Embiratermes	*neotenicus*	Syntermitinae	n.a.	2	Cacodylate	Resin	Y	Y	Petit Saut, French Guiana	11.30	2,41	8.35	15.03	8.04	7.03	3.61	27.34	1	5.67±0.45	2.6±0.73	0.03±0	Y	Y
Indotermes	sp.	Apicotermitinae	n.a.	15	Phosphate	Paraffin			Bubeng, Yunnan, China	11.77	4.65	7.01	22.18	22.34	2.66	8.88	17.37						
Nasutitermes	*lujae*	Nasutiterminae	n.a.	2	Cacodylate	Resin	Y	Y	IRD, Bondy, France	2.01	0.85	0.87	3.52	7.40	4.03	4.02	16.30	1	1.15±0.13	1.03±0.07	0.03±0	Y	Y
Constrictotermes	*cavifrons*	Nasutiterminae	n.a.	2	Phosphate	Resin			Petit Saut, French Guiana	6.67	2.08	2.81	9.19	6.37	5.51	6.76	22.96						
Hirtitermes	sp.	Nasutiterminae	n.a.	2	Cacodylate	Resin	Y	Y	Khao Chong, Thailand	10.29	2.97	7.46	15.15					1	3.64±0.18	2.59±0.25	0.02±0	Y	Y
Trinervitermes	*trinervoides*	Nasutiterminae	n.a.	10	Bouin's	Paraffin			Bubeng, Yunnan, China	19.14	1.12	17.79	20.51										

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Paper 2: The labral gland in termites: Evolution and function

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Résumé

Les termites sont des contributeurs importants au fonctionnement de l'écosystème. Ils sont très abondants dans les habitats tropicaux et subtropicaux et représentent une ressource importante pour un large éventail de prédateurs. Leur succès évolutif repose en grande partie sur une vie dans des colonies peuplées avec un système de communication complexe contrôlé par un riche ensemble de glandes exocrines dont les sécrétions sont impliquées dans de nombreux aspects de la vie des termites. On sait que jusqu'à 20 organes exocrines différents sont connus chez les termites. Parmi eux, la glande labrale représente l'un des organes largement sous–étudiés. Ici, nous avons examiné la structure de la glande labrale chez des ouvriers de 28 espèces et des imagos de 33 espèces représentants tous les taxons de termites, ainsi que chez la blatte xylophage *Cryptocercus*. La glande labrale est présente chez toutes les espèces et comprend deux régions de sécrétion situées respectivement sur la face ventrale du labrum et la partie dorso–apicale de l'hypopharynx. L'épithélium de la glande est constitué de cellules sécrétrices de classe 1 avec une abondance de réticulum endoplasmique lisse, de longues microvillosités avec un canal à l'intérieur, qui libèrent les sécrétions à travers une cuticule modifiée. Nos observations suggèrent que la glande labrale est impliquée dans la communication défensive après la rencontre avec un étranger.

Mots–clefs: glande exocrine, Isoptera, Termitoidae, ultrastructure, évolution, développement

Abstract

Termites are important contributors to ecosystem functioning. They are highly abundant in tropical and sub–tropical habitats, and represent an important resource for a wide range of predators. Their evolutionary success is driven largely by a life in populous colonies with a complex communication system controlled by a rich set of exocrine glands whose secretions are involved in many aspects of termite life. As many as 20 different exocrine organs are known to occur in termites. Among them, the labral gland represents one of the largely understudied organs. Here we examined the structure of the labral gland in workers of 28 species and imagoes of 33 species cross all termite taxa, and in Cryptocercus wood roach. The labral gland is present in all species, and comprises two secretory regions located on the ventral side of the labrum and the dorso–apical part of the hypopharynx, respectively. The epithelium of the gland consists of class 1 secretory cells with an abundance of smooth endoplasmic reticulum, long microvilli with a channel inside, which release secretions through a modified cuticle. Our observations suggest that the labral gland is involved in defensive communication after encounter with an alien.

Keywords: exocrine gland, Isoptera, Termitoidae, ultrastructure, evolution, development

Introduction

Termites are the most important decomposers of dead plant material of prime importance in both, natural and urban areas. Their impact on tropical lands can hardly be estimated: they ingest 50%–100% of raw plant production biomass in tropical forests (Bignell & Eggleton, 2000); they importantly participated in the termination of terrestrial carbon reserves accumulation after their adaptive radiation at the beginning of Tertiary (Engel et al., 2009); and they significantly contribute to the world's atmospheric carbon dioxide and methane release (Sugimoto et al., 2000). Termites are often called ecosystem engineers due to their dramatic impact on the land environments consisting in nutrients release from the dead vegetal matter, soil aeration, transport of tons of materials per hectare and year, and increase of the soil heterogeneity and net productivity (Jouquet et al., 2006; Eggleton, 2011; Evans et al., 2011).

The importance of termites is reflected by their abundance, often exceeding 1,000 individuals per square meter in tropics (Eggleton et al., 1996; Dahlsjö et al., 2014). Due to their enormous amounts, termites represent an important food source for a wide variety of predators (Deligne et al., 1981; Redford & Dorea, 1984). The selection pressure resulted in an arm race leading to improved defensive abilities of termites, expressed the best in a specialized caste of defenders, the soldiers (Haverty, 1977; Deligne et al., 1981; Krishna et al., 2013). However, not only soldiers participate in defensive activities and workers are particularly important for their ability to construct underground or above ground galleries and nests, having a primarily protective function (Eggleton, 2011). Termite colony members, in general, live in safe closed system of chambers and galleries, but the alate imagoes are an exception since they leave the maternal nest at one moment and establish new colonies by their own, what represents the riskiest moment of their life, during which most of the alates are eaten by non–specialized predators or outcompeted by older colonies (Nutting, 1979). However, the defensive mechanisms have been almost exclusively studied in soldiers (for a review see Šobotník et al., 2010a), and defensive abilities are with few exceptions (e.g. Thorne, 1982; Sands, 1982; Piskorski et al., 2009; Šobotník et al., 2012; Bourguignon et al., 2015) completely neglected in other castes.

Exocrine glands can have multiple functions, producing among others pheromones, defensive chemicals, antibiotics, lubricants, or digestive enzymes (Chapman, 2013). They are organs of fundamental importance in all insects, reaching the peak in abundance and diversity in social insects (Billen & Šobotník, 2015). Social insects live in a complex societies and use a broad network of chemical signals produced by as many as 149 different glands described so far. While ants possess altogether 84 exocrine glands producing mostly infochemical signals (Hölldobler & Wilson, 1990; Billen & Šobotník, 2015), only 20 exocrine glands have been described in termites so far.

Some termite exocrine glands are present in all castes, although they might be inactive in larval instars (Šobotník & Hubert 2003, Šobotník & Weyda 2003), while others are limited to only some species and castes. These might be related to sexual behaviour occurring in winged imagoes, or defensive glands in soldiers, workers, or imagoes. The frontal gland is the most studied termite organ and a defensive organ of prime importance in termites, which occurs in most Neoisoptera (Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae) soldiers and imagoes, and also in some workers (Piskorski et al., 2009; Prestwich and Collins, 1982; Quennedey, 1984; Šobotník et al., 2004, 2010b, 2010c; Wu et al. 2018). Another important organ, the labial glands, is gland universally present in termites (Noirot, 1969; Billen et al., 1989; Šobotník & Weyda, 2003). Its function in workers is connected to feeding (Noirot, 1969; Reinhard et al., 2002; Fujita et al., 2008) and constructing behaviour (Noirot, 1969; Reinhard et al., 2002), while in soldiers and workers of soldierless species it produces defensive secretions (Sillam–Dussès et al., 2012).

Workers have developed different means of defence, protecting them during foraging activities or during invasion into the nest (Deligne et al., 1981; Prestwich, 1984; Šobotník et al., 2012; Bourguignon et al., 2015; Poiani & Costa–Leonardo, 2016). The most important contribution of workers to the colony defence is making up the passive defences, such as constructing the system covered galleries and nest fortification (Šobotník et al., 2010a). Termite workers are often directly engaged in the nest defences (Thorne, 1982; Binder, 1988), and this contribution is of a special interest in: (*i*) conflicts of conspecific colonies defended primarily by soldier–produced toxins due to presence of specific auto–detoxification mechanisms (Spanton & Prestwich, 1982), (*ii*) soldierless species in which workers are considerably more aggressive compared to soldiered species (Sands, 1982; Šobotník et al., 2010a), (*iii*) dehiscence mechanisms when the whole abdomen ruptures and its content contaminates the opponent (Sands, 1982), and (*iv*) autothysis as a body rupture connected to the release of the toxic compounds from inside of the body (Costa–Leonardo, 2004; Šobotník et al., 2010a, 2012; Bourguignon et al., 2015; Poiani & Costa–Leonardo, 2016).

The labral gland is an important member of a set of termite secretory organs, studied in details only in soldiers so far (Deligne et al., 1981, Quennedey 1984, Šobotník et al., 2010d; Costa–Leonardo & Haifig 2014; Palma–Onetto et al., 2018). It was first discovered on the ventral side of the labrum in *Macrotermes bellicosus* (Deligne et al., 1981), later observed also on the dorsal side of the hypopharynx in the same species (Quennedey 1984), and finally reported to occur in all termite soldiers (Palma–Onetto et al., 2018). The gland epithelium consists in soldiers of class 1 secretory cells in most representatives, with additional class 3 secretory cells in few species only (Šobotník et al., 2010d; Costa–

Leonardo & Haifig, 2014; Palma–Onetto et al., 2018). Here, we describe the occurrence, structure, and ultrastructure of the labral gland in a representative set of termite workers and imagoes, as well as in the wood roach *Cryptocercus punctulatus*.

Material and Methods

Scanning Electron Microscopy, Optical microscopy and Transmission Electron Microscopy

The observations of the labrum and hypopharynx were made using optical, scanning electron microscopy (EM) and transmission EM. We examined workers (including sub–castes if present) of 28 species and imagoes of 33 species representing most of extant termite taxa (see Krishna et al., 2013). We also examined nymphs and female adults in the cockroach *C. punctulatus,* member of the sister group of termites (Lo et al., 2000; Inward et al., 2007; Bourguignon et al., 2015). The procedures used for optical, TEM and most of the SEM pictures, are well–established in our lab, and correspond to protocol provided in details by Šobotník and Weyda (2003) and Palma–Onetto and others (2018). Important data are summarised in Supplementary tables S1, S2 and S3. In addition, we also used a scanning electron microscope FEI Helios NanoLab 660 G3 UC with focused ion beam milling equipped for cryo–imaging and correlative light–electron microscopy.

Behavioural experiments

We performed two kinds of bioassays. In the first experimental set–up, we ran arena tests in *Glossotermes oculatus* and *Coptotermes testaceus* groups of 5 workers and 2 soldiers, to which we introduced a single intruder, a worker of a different termite species or an ant. The behaviour resulting from subsequent encounters was recorded and specific behavioural patterns analysed later on. The tests were performed under dimmed artificial light, and Canon EOS 60D, 6D or 5D SR cameras, combined with Canon EF 100 mm f/2.8L Macro IS USM lenses were used.

In the second experimental set–up, labral extracts were prepared by dissecting 60 labra of *Prorhinotermes canalifrons* soldiers (4 replicates), which were then extracted in 400 µm of either hexane or methanol (2 repetitions for each solvent), and used in behavioural tests (repeated 6 times for each stimulus). These tests consisted in placing groups of *P. canalifrons* (2 soldiers and 8 workers) in a Petri dish lined with a filter paper split into two sectors: labral extracts (6 labra equivalents in 40 µl of solvent) *vs*. control 1 (6 legs equivalents in 40 µl of solvent; the leg extracts prepared the same way like the labral) or control 2 (40 µl of pure solvent). The same bioassay was performed 6 times using groups of *Reticulitermes flavipes* made of 2 soldiers and 8 workers, to test a possible effect on another termite species. The number of termites on each sector was recorded using the above–mentioned equipment after 10 minutes since the introduction of the termites in the Petri dish. The number of termites choosing the sector treated with labral extracts in was compared with the one in solvent by t–student test (Norusis, 1990). In order to see any preference for a sector, T–student tests were used if the comparison between sectors from the same Petri dish was normal, Mann Withney U test was used if it was not normal (Norusis, 1990).

Chemical analyses

Chemical analyses using samples of 100 labra or 100 legs (as control) extracted in methanol or hexane were carried out using a 6890N gas chromatograph (Agilent, Santa Clara, CA, USA) coupled to a 5975B quadrupole mass spectrometer and equipped with a fused silica capillary column HP5ms (30 m x 0.25 mm, 0.25 µm, Agilent). The carrier gas was helium at 1 mL/min. The injector was operated in split mode (10:1) at 200°C; the injected volume was 1 µL. The temperature program: 40°C (2 min), then 8°C/min to 200°C, then 15°C/min to 320°C (3 min). Standard 70 eV mass spectra were recorded in the mass range of 25 – 600 Th; 4 min solvent delay was used. Temperatures of the transfer line, ion source and quadrupole were 280°C, 230°C and 150°C, respectively. Profiles of labra and legs extracts were compared to determine some specific compounds from the labral gland.

Results

Scanning Electron Microscopy

The labrum of a worker or an imago was most often oval shaped (Fig. 1), broadly attached to the clypeus. The labrum usually did not differ much in size among species and castes, being approximately 2.7 times shorter compared to the head length (distance between clypeo–frontal boundary and posterior margin of head), with the exception of *Termes hospes* and *Microcerotermes* sp. workers, the small worker of *Pseudacanthotermes militaris* and *Coptotermes testaceus* imagoes, in which the labrum was about 3.2 – 5 times shorter than the head (Tabs S1 and S2).

The dorsal side of the labrum was covered by smooth rectangular plates of about 10 µm in size mixed with few hair–like sensillae. The ventral faces of the labrum and of the hypopharynx were made of four regions of similar appearance for workers and imagoes of all studied taxa, and these were as follows (see Fig. 1): (*a*) a smooth region in the apical zone along the midline of the labrum, which looked like a wrinkled structure with numerous pores of about 30–50 nm in diameter (Fig. 1A, 1B and 1D); (*b*) a basal zone in the midline extending forward around the zone “*a*”, made of many irregular hair–like structures (supposedly acanthae according to TEM observation), ranging in length between 5 to 25 µm; (*c*) two lines of sensillae (numerous chemoreceptors with usually 4 dendrites and relatively few campaniform sensillae located predominantly in the basal parts of the sector) encircling the "*b*" zone (Figs 1A and 1B) on ventral labrum and missing in the hypopharynx; (*d*) lateral regions, composed by irregular scales ranging in size between 2 and 4 µm (Fig. 1).

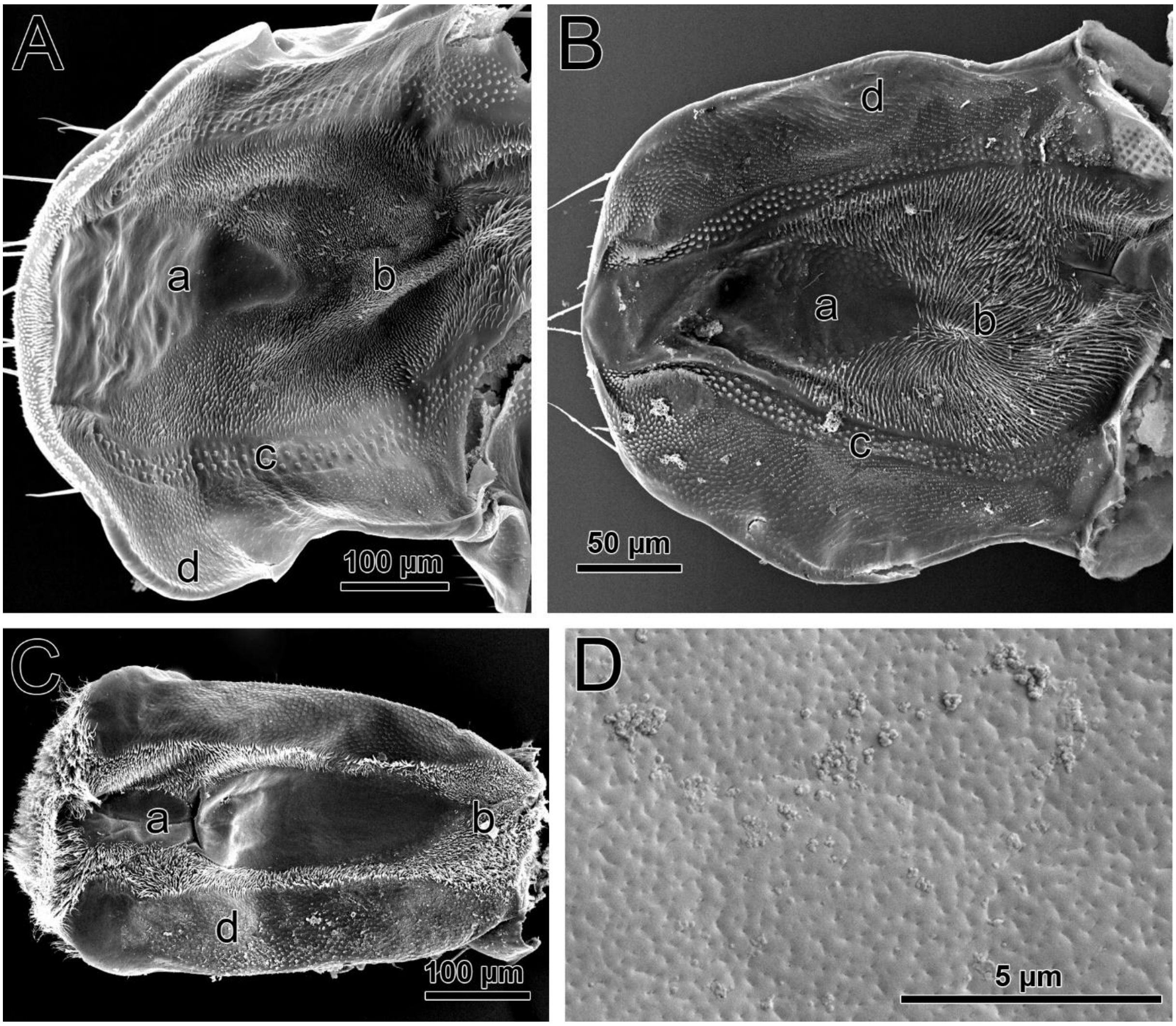

Figure 1. Labral gland development using Scanning Electron Microscopy. (A) Ventral side of labrum in *Embiratermes neotenicus* female imago. (B) Ventral side of labrum in *Pseudacanthotermes militaris* worker. (C) Dorsal side of the hypopharynx in *Acanthotermes acanthothorax* female imago. (D) Detailed view on region "*a*" with pores through the epicuticle in labrum of *Microcerotermes* sp. female imago. The sectors are abbreviated as follows: a, zone with small porosities located at the apex of the labrum along the midline; b, zone formed with many irregular hair–like structures located partially around zone "a"; c, two lines of sensillae located the "b" zone; d, region with scales of irregular shape located at the labrum margins.

Optical microscopy

The labral gland was found in workers and imagoes of all studied species. It was located at the ventral side of the labrum with extension to the dorsal side at the labrum apex, and in the dorso–apical region of the hypopharynx (Figs 2A and 2B). It appeared as a thickened epithelium composed by columnar cells (Fig. 2). The thickness of the secretory epithelium was in general about 15 – 30 µm (on average 17.98 µm) in workers. The thinnest epithelium was found in *P. militaris* small worker (7.80 µm) and the thickest in *Mastotermes darwiniensis* (29.22 µm) worker (Tab. S1). In imagoes, the labral gland thickness was on average 18.21 µm; the thinnest epithelium was found in *Nasutitermes* sp. (8.27 µm) and the thickest in *Neocapritermes araguaia* (32.65 µm) (Tab. S2). The thickness of the labral gland differed slightly between sexes in imagoes, without a clear picture. The hypopharyngeal part of the epithelium was in general significantly thinner, usually between 8 to 15 µm thick, with the exception of the “lower” termite workers, in which the thickness of the secretory epithelium was similar in the labral and hypopharyngeal portions of the gland.

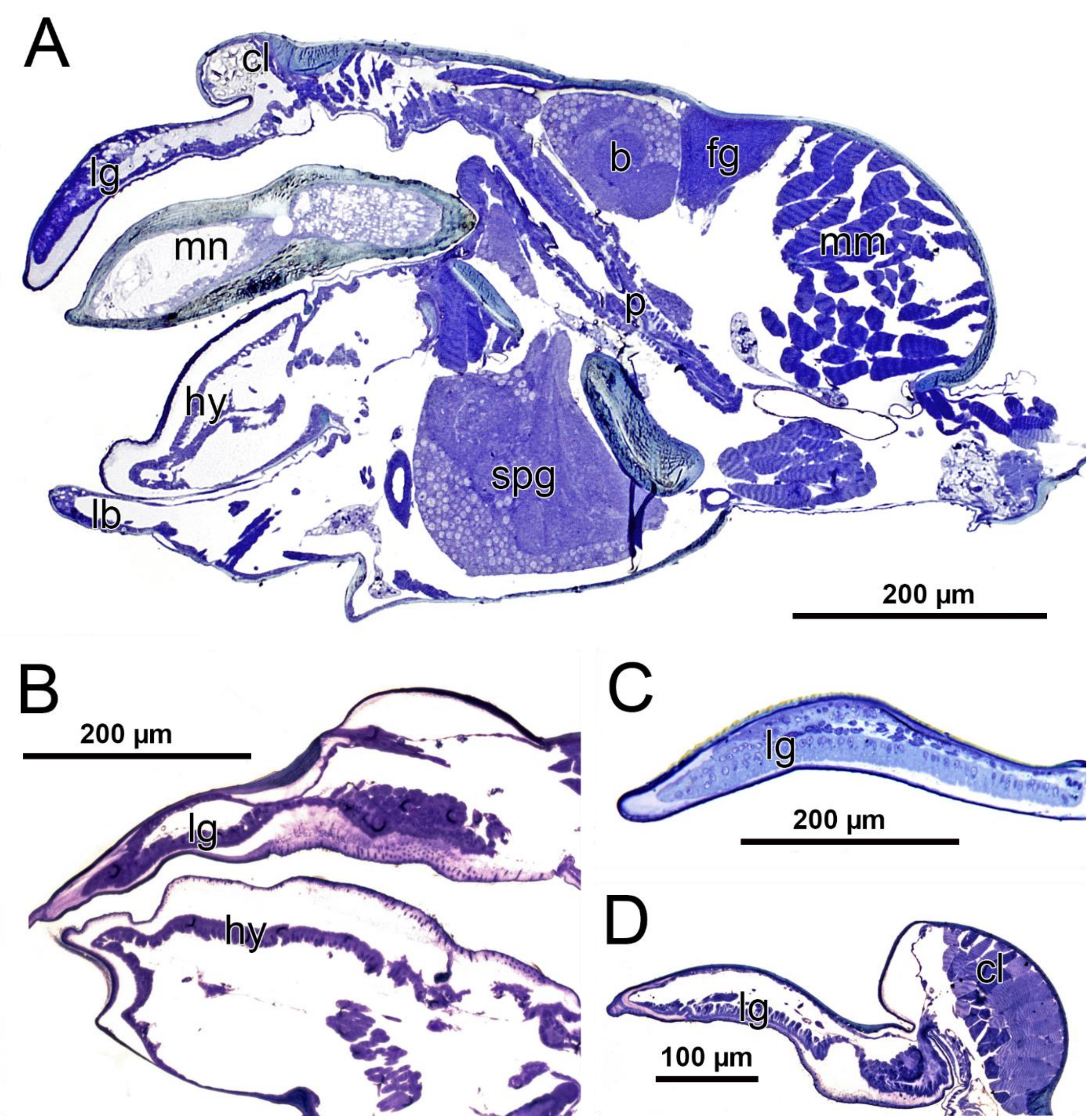

Figure 2. Sagittal sections of the labral gland. (A) Head of *Termitogeton planus* worker. (B) Labrum and

hypopharynx of *Globitermes sulphureus* worker. (C) Labrum of *Coptotermes testaceus* male imago. (D) Labrum of *Neocapritermes taracua* worker. Note the secretory epithelium of hypopharynx in figures A and B. Abbreviations: b, brain; cl, clypeus; fg, frontal gland; hy, hypopharyngeal portion of the labral gland; lb, labium; lg, labral gland; mn, mandible; mm, mandibular muscles; p, pharynx; spg, subesophageal ganglion.

Transmission Electron Microscopy

The secretory cells of the labral gland were always of class 1 (according to the classification of Noirot & Quennedey 1974), and their ultrastructure was nearly identical in the labral and hypopharyngeal regions of the labral gland in all castes and therefore our description is based on the observation of both parts of the gland. There often was an abundance of class 3 secretory cells (according to the classification of Noirot & Quennedey 1974) on the dorsal face of the labrum, but these cells never mixed with the labral gland epithelium, unlike in some soldiers (Palma–Onetto et al., 2018), and always released their secretion to the dorsal side of the labrum (Fig. 3A). Class 1 and class 3 secretory cells were very different, and also easily distinguished from the non–modified epidermal cells (Fig. 3A); the latter were much thinner (typically about 0.5 µm) and contained virtually no secretory organelles (Fig. S1B).

The labral gland secretory cells were columnar (Figs 3A, 3B and 3C), and their cytoplasm contained abundant smooth endoplasmic reticulum (ER), scattered rough ER, small secretory vesicles, abundant mitochondria, populous microtubules orientated predominantly apico–basally, glycogen granules, and sometimes also myelin figures. While the microtubules are scattered throughout the secretory cells in most representatives, they appear grouped into bundles in in Glyptotermes sp. workers. Apical microvilli were well developed throughout the gland (Fig. 3D), however, they were longer in the middle part of the epithelium compared to the margins. The microvilli were up to 1.3 µm long and about 80 nm thick, slightly shorter in workers than in imagoes, and always with a central channel of about 30 nm diameter in termite imagoes and about 40 nm in workers (Tab. S3, Figs 3D and S1D). Numerous small vesicles were observed at the microvilli bases (Fig. 3D). These vesicles were generally electron–lucent when occurring at the base of microvilli, but sometimes they appeared more electron–dense deeper in the cells, like in both sexes of G. oculatus alate imagoes, in female alate imagoes of Heterotermes tenuis, and in workers of Thoracotermes sp. Lipid–like droplets were observed only rarely, but they were more common in C. formosanus imagoes, Nasutitermes lujae workers and P. militaris large workers. The basal parts of the secretory cells differentiated into invaginations typically about 5 µm deep (up to 12 µm in workers of Neocapritermes taracua and C. formosanus) with frequent formation of pinocytotic vesicles (Figs 3C, S1C). Free axons were commonly observed to be inserted within the basal invaginations. The basal parts of secretory cells were covered by basement membrane (of about 100 nm thick) sometimes strengthened by clusters of collagen fibres (up to 1.5 µm thick then). There was no junction between the neighbouring secretory cells in the basal parts, while there always were zonulae adherens followed by septate junctions in the apical parts. The nuclei were elliptic in shape, located at the cell bases, and usually about 5 µm long (up to 7 µm in N. taracua worker and G. oculatus male alate imagoes). The nuclei contained predominantly dispersed chromatin with few aggregates only.

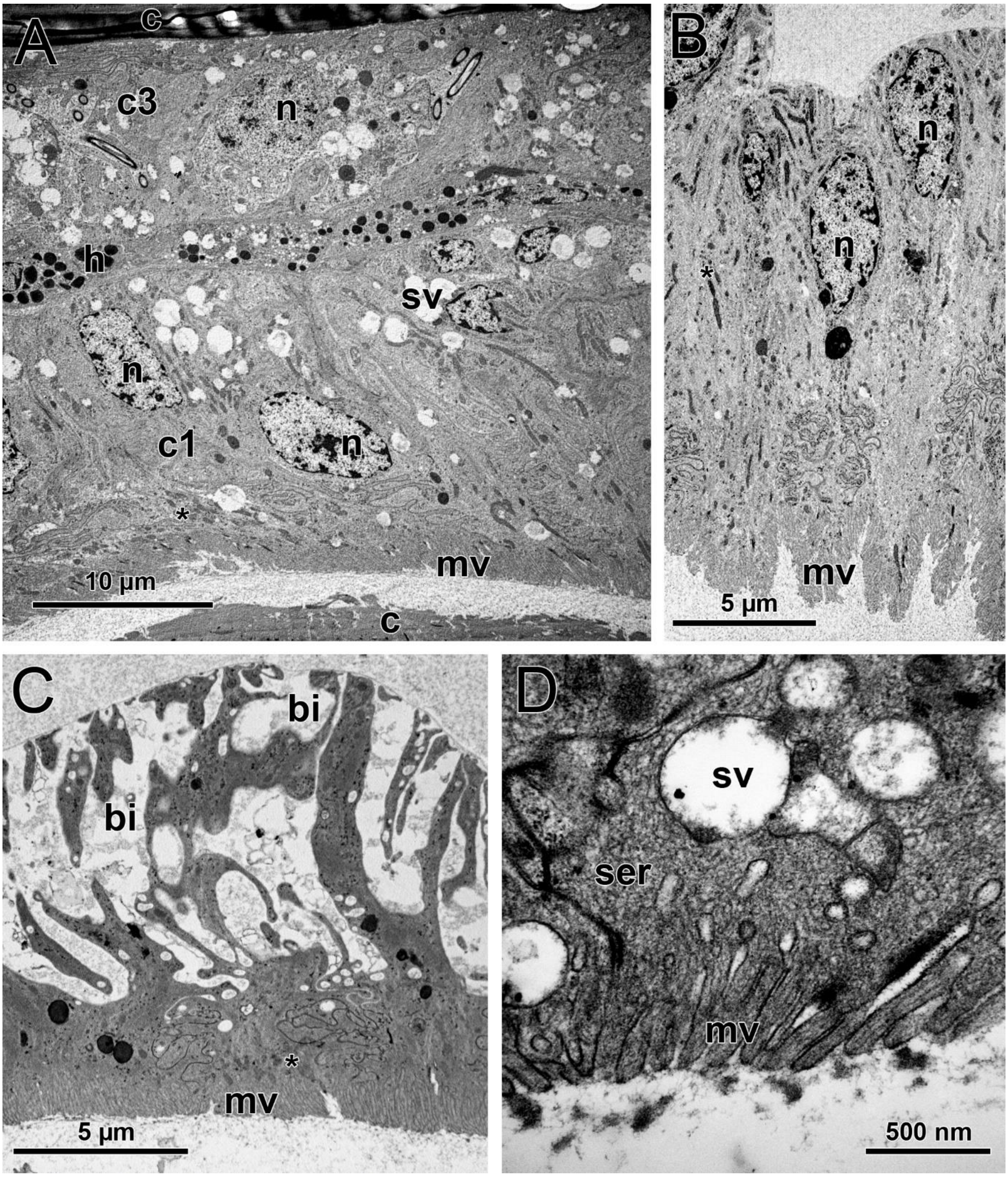

Figure 3. Ultrastructure of the labral gland in termites. (A) The middle part of the labrum in *Coptotermes*

testaceus female imago, showing the labral gland made of class 1 secretory cells at the bottom, and the class 3 secretory cells occurring at the dorsal side of the labrum. (B) The labral gland secretory epithelium in *C. testaceus* female imago. (C) The labral gland secretory cells in *Coptotermes formosanus* worker. Note the well–developed invaginations reaching near the cell apices. (D) Detailed view on the apex of labral gland secretory cells in *Pseudacanthotermes militaris* worker showing well–developed smooth endoplasmic reticulum. Abbreviations: bi, basal invaginations; c, cuticle; c1, class 1 secretory cell; c3, class 3 secretory cell; h, hemocytes; mv, microvilli; n, nucleus; ser, smooth endoplasmic reticulum; sv, secretory vesicle. The asterisks indicate the mitochondria in the cell cytoplasm.

The cuticle overlying the labral gland was highly modified for the secretion evacuation, and always thicker in imagoes compared to the workers (on average 6.5 µm and 4 µm, respectively; Fig. 4A). The cuticle was formed by endocuticle of helicoidal structure, exocuticle and a thin epicuticle (about 30 nm thick; Table S3, Fig. 4B). The modifications of the glandular cuticle were highly pronounced especially in the smooth middle part of the ventral labrum. These modifications included increased number of pore canals, which widened towards the cuticle base (Fig. 4B) and plentiful epicuticular pores. The cuticle of the hypopharyngeal portion of the gland was very similar, although the endocuticle was slightly thicker than in the labrum. There was no reservoir and the secretion was stored only in the space between the secretory epithelium and the cuticle, or inside the porous cuticle.

The labral gland was also observed in *C. punctulatus* nymphs and female imagoes. The epithelium of the labral gland keeps the same characteristics, although the microtubules predominantly occur in large bundles (Fig. S1F). Important difference were shallower basal invaginations and shorter microvilli lacking the central channels inside.

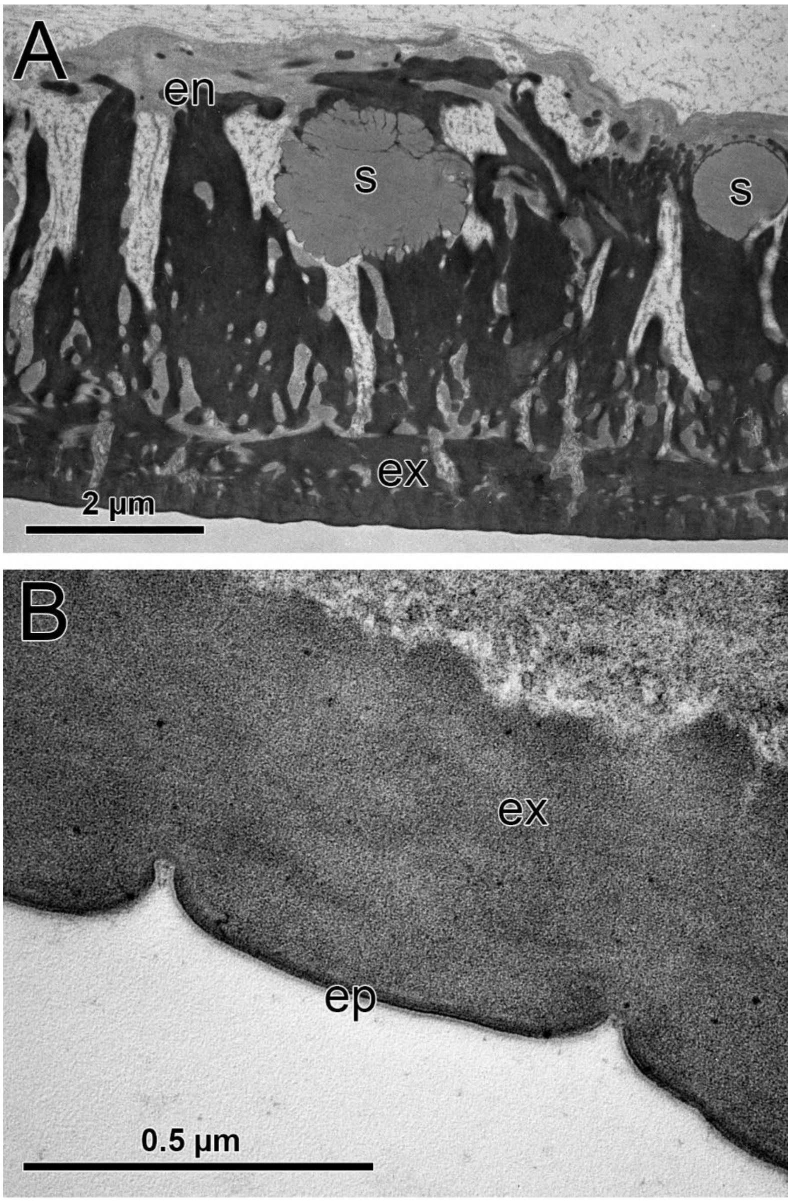

Figure 4.
Cuticle of the labral gland. (A) Highly modified cuticle underlying the labral gland in *Coptotermes testaceus* male imago. Note the enlarged pore canals. (B) Detail of the apical cuticle underlying the labral gland of *Coptotermes testaceus* female imago. Note the distinct layers of the epicuticle. Abbreviations: en, endocuticle; ep, epicuticle; ex, exocuticle; s, secretion.

Behavioural experiments

First, we observed the potential use of the labral gland in soldiers of *G. oculatus* and *C. testaceus* after encounter with an alien, termite or ant worker. In fact, right after the encounter, the soldiers changed their behaviour by walking backwards while rubbing the labrum against the substrate (see Supplementary Video S1).

In the second experimental bioassay, the number of workers and soldiers of *Prorhinotermes canalifrons* gathered at the two sectors did not differ between the labral extract vs. the legs extract, neither using methanol (p=0.869) nor hexane (p=0.355) nor pure solvent (p=0.325 for methanol, p=0.614 for hexane). Interestingly, the number of workers and soldiers of *R. flavipes* avoiding the sector treated with the labral glands extracts was higher compared to controls irrespectively of solvent, either legs extract (p<0.0001) or solvent (p<0.0001).

Chemical analyses

No specific compounds were detected in the labra extracts, the profiles of these extracts and of the legs extract did not differ much (data not shown).

Discussion

The labral gland is an integral part of the labrum in termites, occurring in soldiers (Palma–Onetto et al., 2018), workers and imagoes. While its structure is well–known in soldiers (Palma–Onetto et al., 2018), only anecdotal information about its presence in some imagoes has previously been published (Křížková et al., 2014). In the present study, we described for the first time the labral gland in workers and imagoes of a set of representative termite species and in nymphs and adults of the wood roach *C. punctulatus*.

The labrum and the labral gland share the same characteristics in all species and castes studied so far. The common features are a higher degree of sclerotization of the dorsal side of the labrum, which is in general more pronounced in soldiers, occurrence of class 3 secretory cells at the dorsal side of labrum but rarely also within the labral gland, and the presence of labral gland made of class 1 secretory cells on the ventral side of labrum and on the dorsal side of hypopharynx. The secretory cells are also quite similar in their ultrastructure, showing well–developed apical microvilli with a central channel (lacking in *C. punctulatus*), numerous vesicles of different electron densities, abundant smooth and rough ER, cuticle modified for secretion release, and innervation of the secretory cells through axons running freely within the basal invaginations (Palma–Onetto et al., 2018). At the same time, there are also considerable differences between termite soldiers on one hand, and workers and imagoes on the other: (i) the hyaline tip, present in soldiers of many advanced species is missing in other castes, (ii) the shape of the labrum is highly variable in soldiers while it is almost the same in all workers and imagoes, and (iii) the overall development of microvilli and basal invaginations is lower in workers and imagoes (Palma–Onetto et al., 2018). These observations suggest that the labral gland has the same function in all castes, but plays a more important function in soldiers. We also cannot exclude that the secretion is used in a different context by workers and imagoes, as the rubbing of labral gland secretory openings against the substrate was observed exclusively in the soldier caste. Even though

C. punctulatus presented some differences in the labral gland structure in comparison to termites like shorter microvilli devoid of a central channel, the presence of microtubule bundles was shared particularly between *C. punctulatus*, and *M. darwiniensis* and *Hodotermopsis sjoestedti* soldiers (Palma–Onetto et al., 2018), and *Glyptotermes* sp. workers, suggesting to be a common feature in basal taxa inherited from cockroach ancestors.

The hyaline tip, a transparent and extensible apical part of the labrum, is probably an evolutionary novelty occurring in some soldiers of Rhinotermitidae and Termitidae. Our mapping of ancestral characters (Palma–Onetto et al., 2018) suggests that the hyaline tip evolved in a common ancestor of Rhinotermitidae and Termitidae, and was subsequently lost at least in four independent occasions. These cases include (*i*) all soldiers of Nasutitermitinae in which the whole labrum is highly reduced in size as well as all other mouth parts, (*ii* and *iii*) in snapping soldiers, represented by two independent lineages, *Pericapritermes* and *Neocapritermes*, in which the labrum is highly modified, and (*iv*) in *Microcerotermes* without a clear reason apart of the general small size of labrum in soldier caste (Palma–Onetto et al., 2018). At the same time, workers and imagoes of lineages reveal similarly–developed labra without hyaline tip, even in taxa with highly modified labra in the soldiers caste However, the secretory cells ultrastructure is always similar, although the overall size of the labral gland is much larger in soldiers having the secretory epithelium approximately twice as thick, apart of larger size of the labrum in general (see Palma–Onetto et al., 2018).

An interesting question is the way how the labral gland secretion release is controlled. It seems clear that the release from the secretory cells is under neuronal control, similarly to sternal gland secretion in Mastotermitidae, Archotermopsidae and Kalotermitidae (Quennedey, 1969; Quennedey, 1975; Quennedey et al., 2008), the nasus gland of *Angularitermes* soldiers (Šobotník et al., 2015), the salivary glands of different insects (Whitehead, 1971; Lange et al. 1988; Ali et al. 1993; Ali and Orchard, 1996; Ali, 1997), including termites *Kalotermes flavicollis* (Alibert, 1983) and *Prorhinotermes simplex* (Šobotník & Weyda 2003). After the release from secretory cells, the secretion is supposedly evacuated from the body by pressing the labrum (and hypopharynx) against the substrate, and the pressure is probably controlled by the groups of campaniform sensillae similarly to the trail pheromone release from the sternal gland (Stuart & Satir, 1968; Quennedey et al., 2008). The chemoreceptors are clearly more populous within the “c” area, but it remains unknown if these receptors are also involved in the control of the secretion release or if they play rather gustatory function.

The labral gland does not form any specific reservoir, and the secretion is stored only in the space between the secretory epithelium and the overlying cuticle, as well as within the cuticle itself. The absence of a reservoir, a feature characteristic of defensive glands (Chapman, 2013), excludes the potential defensive function of the labral gland, in contrast to previous speculations (see e.g. Deligne et al., 1981 or Quennedey, 1984). The gland also reveals a very similar structure in all castes and species, which indicates that it is not linked to defensive function. In addition, the high abundance of smooth ER, an organelle notoriously known for producing secretions of lipidic and volatile nature, typical for pheromone–producing glands (Percy–Cunningham & MacDonald, 1987; Nakajima, 1997; Tillman et al., 1999; Alberts et al., 2002), provides additional evidence for communicative function rather than strictly defensive function.

We repeatedly observed soldiers wiping the labrum against the substrate after encountering a threat (heterospecific termite or an ant worker), and the observed behaviour (moving backwards combined with wiping the labrum against the surface) suggests that the soldiers are warning their nestmates using the labral gland secretion. Unfortunately, this function was not proven by our experiments irrespectively of the settings, and only the avoidance of heterospecifics (which can be considered as potential competitors or enemies) to the labral gland extracts was statistically significant. However, the effect can also be due to the other compounds dissolved from the labra, such as cuticular hydrocarbons as species–recognition cues (Howard & Blomquist, 1982, 2005) or frontal gland secretion which inevitably contaminates all body parts of termite soldiers (Piskorski et al., 2007, unpublished observations). Therefore, the function of the secretion should be rigorously tested in the future, especially since we could not detect any labral gland–specific compounds, probably due to the small quantity of the secretion linked to the small gland size and the absence of a reservoir.

Conclusion

The labral gland has been thought to have a defensive function (Deligne et al., 1981; Quennedey, 1984). However, Palma–Onetto et al. (2018) suggested that according to the gland morphology, structure and ultrastructure, it may play a communicative function rather than defensive function. The presence of the labral gland in other castes and in the closest relative of termites, the wood roach *C. punctulatus*, as well as the occurrence of the same basic features of the gland structure and ultrastructure, reinforce its alternative function and suggest an essential role of the gland in colony survival and success. Moreover, our observations of the behaviour suggest that the gland produces volatiles secreted in response to a threat. A better understanding of the labral gland function in termites and cockroaches is needed to enhance the knowledge of termite chemical communication behaviour.

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Supplementary materials

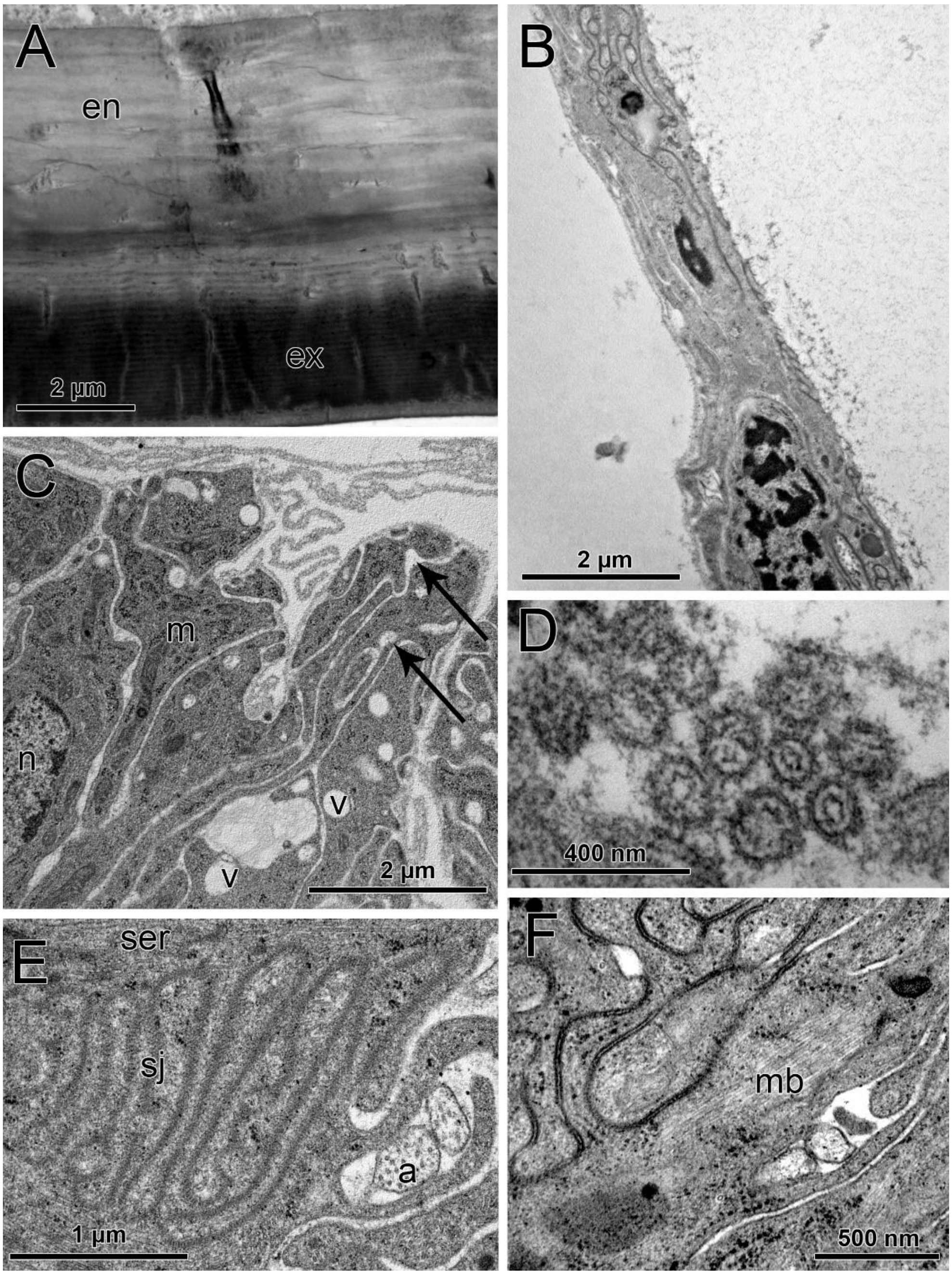


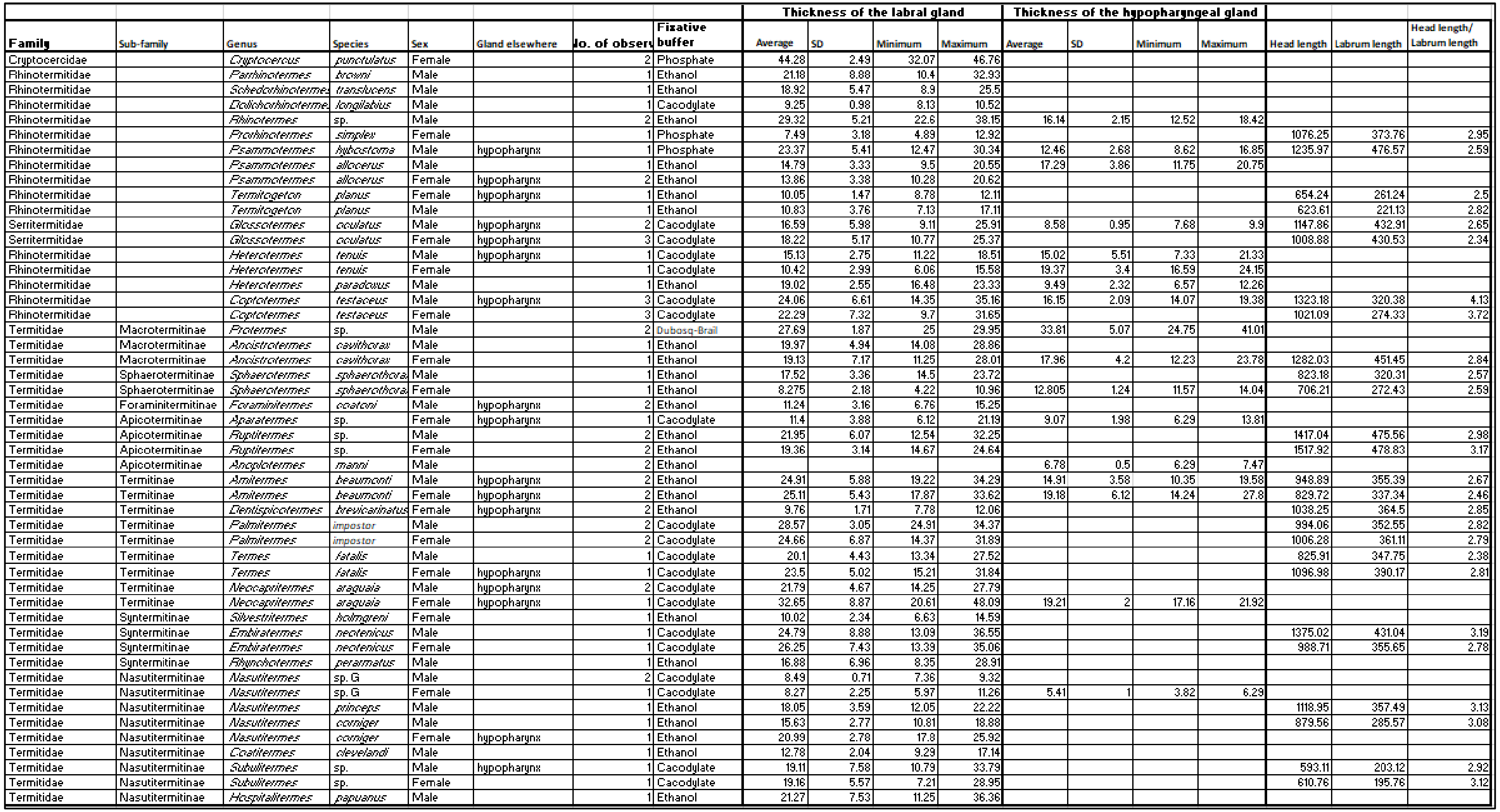
Figure S1. Ultrastructure of the labral gland. (A) Non–modified cuticle at the dorsal side of the labrum in *Embiratermes neotenicus* male imago. (B) Non–modified epithelium surrounding the labral gland in *Coptotermes formosanus* worker. (C) Pinocytotic activity at the cell base in the labral gland epithelium in the male imago of *Glossotermes oculatus*. Arrows indicate the pinocytotic activity at the base of the cell. (D) View of the central channels in the microvilli of *Coptotermes testaceus* female imago, allowing secretion release from secretory cells. (E) Detail of labral gland basal part in the worker of *Neocapritermes taracua* showing free axons located within the basal invagination. (F) Large microtubule bundle running through secretory cells in the wood roach *Cryptocercus punctulatus*. Abbreviations: a, axon; en, endocuticle; ex, exocuticle; m, mitochondria; mb, microtubule bundle; n, nucleus; ser, smooth endoplasmic reticulum; sj, septate junction; v, vesicle.

								Labral gland thickness				Hypopharyngeal gland thickness						
Family	**Sub-family**	**Genus**	**Species**	**Sub-caste**	**Gland elsewhere**	**observations**	**Fixative buffer**	**Average**	**SD**	**Minimum**	**Maximum**	**Average**	**SD**	**Minimum**	**Maximum**	**Head length**	**Labrum length**	**Head lenght/ Labrum length**
Cryptocercidae		*Cryptocercus*	*punctulatus*	nymph		2	Phosphate	16.41	2.09	13.54	18.46					752.72	338.38	2.22
Mastotermitidae		*Mastotermes*	*darwiniensis*		hypopharynx	3	Phosphate	29.22	11.35	18.95	50.23	29.17	13.28	12.5	42.59	1493.29	553.49	2.7
Archotermopsidae		*Hodotermopsis*	*sjoestedti*		hypopharynx	3	Cacodylate	29.03	6.8	18.72	39.49	45.59	7.31	35.19	55.82	2008	967.5	2.08
Kalotermitidae		*Cryptotermes*	sp.		hypopharynx	1	Cacodylate	11.7	4.04	7.22	18.69	8.04	2.34	5.1	11.31	904.59	330.71	2.74
Rhinotermitidae		*Dolichorhinotermes*	*longilabius*		Hypopharynx	2	Cacodylate	22.94	8.06	12.45	34.34	19.02	3.85	13.44	24.99	936.28	359.59	2.6
Rhinotermitidae		*Prorhinotermes*	*simplex*			3	Phosphate	16.14	5.42	9.1	24.18					894.3	314.15	2.85
Rhinotermitidae		*Termitogeton*	*planus*		Hypopharynx	2	Phosphate	9.86	1.71	7.03	13.64	6.08	1.58	4.35	9.51	606.88	230.9	2.63
Rhinotermitidae		*Reticulitermes*	*lucifugus*			1	Phosphate	21.62	7.78	15.78	34.96							
Rhinotermitidae		*Coptotermes*	*formosanus*			3	Phosphate	13.74	3.6	8.81	19.17					925.61	330.32	2.8
Serritermitidae		*Glossotermes*	*oculatus*		Hypopharynx	1	Cacodylate	8.45	2.74	3.63	13.99	12.39	5.41	3.83	17.84	880.6	312.52	2.82
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	small worker		1	Cacodylate	7.8	1.15	6.66	8.95							
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	large worker	Hypopharynx	2	Cacodylate	38.82	13.53	20.83	55.27	23.855	5.8	14.82	30.98	826.4	315.95	2.62
Termitidae	Sphaerotermitinae	*Sphaerotermes*	*sphaerothorax*			1	Phosphate	21.38	4.51	12.81	30.13					878.18	292.41	3
Termitidae	Termitinae	*Microcerotermes*	sp.		Hypopharynx	3	Cacodylate	20.78	3.9	14.75	25.36	11.17	4.7	6.6	19.25	1051.08	276.42	3.8
Termitidae	Termitinae	*Spinitermes*	trispinosus		Hypopharynx	1	Cacodylate	11.58	3.6	5.31	17.55	2.826	0.76	1.44	3.63	802.09	278.5	2.88
Termitidae	Termitinae	*Globitermes*	*sulphureus*		Hypopharynx	1	Cacodylate	16.48	2.37	11.1	19.07	15.39	3.21	11.09	18.76			
Termitidae	Termitinae	*Termes*	*hospes*		Labium	1	Cacodylate	18.29	3.44	14.99	25.63					694.9	212.5	3.27
Termitidae	Termitinae	*Neocapritermes*	*taracua*			2	Phosphate	16.89	5.28	6.54	23.88					1298.88	470.6	2.76
Termitidae	Apicotermitinae	*Aparatermes*	*cingulatus*			5	Cacodylate	26.46	7.5	16.58	36.19							
Termitidae	Apicotermitinae	*Anoplotermes*	sp. Q		Hypopharynx	2	Cacodylate	10.49	3.12	6.28	15.65	15.06	7.21	4.51	34.43	724.96	284.3	2.55
Termitidae	Apicotermitinae	*Jugositermes*	tuberculatus			3	Phosphate	26.66	13.62	7.31	55.27	24.24	3.69	19.87	30.53	1109.82	425.22	2.61
Termitidae	Apicotermitinae	*Tonsuratermes*	*major*			1	Phosphate	8.3	5.12	4.89	19.63	15.59	3.55	11.18	20.73	1134.36	389.81	2.91
Termitidae	Syntermitinae	*Silvestritermes*	sp.		Hypopharynx	1	Cacodylate	24.97	1.79	21.58	27.59							
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*			2	Cacodylate	17.98	4.22	12.18	25.53					950.13	330.59	
Termitidae	Cubitermitinae	*Thoracotermes*	sp.			1	Phosphate	16.58	9.71	5.80	26.22							
Termitidae	Cubitermitinae	*Furculitermes*	sp.			1	Phosphate	14.91	4.88	6.64	23.41					863.3	319.21	2.7
Termitidae	Cubitermitinae	*Cubitermes*	sp.			1	Phosphate	16.69	8.33	6.86	42.95							
Termitidae	Nasutitermitinae	*Hirtitermes*	sp.			2	Cacodylate	20.77	6.87	10.39	27.99	31.75	1.98	29.94	35.05			
Termitidae	Nasutitermitinae	*Nasutitermes*	*lujae*			3	Cacodylate	25.07	8.34	11.31	42.97					1146.62	286.66	4

Table S1.

List of termite workers subjected to our analyses, with additional information and secretory epithelium measures. Blank spaces indicate lack of information.

All measurements are in µm.



								Thickness of the labral gland				Thickness of the hypopharyngeal gland						
Family	Sub-family	Genus	Species	Sex	Gland elsewhere	**lo. of obser**	**Fixative buffer**	Average	SD	Minimum	Maximum	Average	SD	Minimum	Maximum	Head length	Labrum length	Head length/ Labrum length
Cryptocercidae		*Cryptocercus*	*punctulatus*	Female		2	Phosphate	44.28	2.49	32.07	46.76							
Rhinotermitidae		*Parrhinotermes*	*browni*	Male		1	Ethanol	21.18	8.88	10.4	32.93							
Rhinotermitidae		*Schedorhinoterme*	*translucens*	Male		1	Ethanol	18.92	5.47	8.9	25.5							
Rhinotermitidae		*Dolichorhinoterme*	*longilabius*	Male		1	Cacodylate	9.25	0.98	8.13	10.52							
Rhinotermitidae		*Rhinotermes*	sp.	Male		2	Ethanol	29.32	5.21	22.6	38.15	16.14	2.15	12.52	18.42			
Rhinotermitidae		*Prorhinotermes*	*simplex*	Female		1	Phosphate	7.49	3.18	4.89	12.92					1076.25	373.76	2.95
Rhinotermitidae		*Psammotermes*	*hybostoma*	Male	hypopharynx	1	Phosphate	23.37	5.41	12.47	30.34	12.46	2.68	8.62	16.85	1235.97	476.57	2.59
Rhinotermitidae		*Psammotermes*	*allocerus*	Male		1	Ethanol	14.79	3.33	9.5	20.55	17.29	3.86	11.75	20.75			
Rhinotermitidae		*Psammotermes*	*allocerus*	Female	hypopharynx	2	Ethanol	13.86	3.38	10.28	20.62							
Rhinotermitidae		*Termitogeton*	*planus*	Female	hypopharynx	1	Ethanol	10.05	1.47	8.78	12.11					654.24	261.24	2.5
Rhinotermitidae		*Termitogeton*	*planus*	Male		1	Ethanol	10.83	3.76	7.13	17.11					623.61	221.13	2.82
Serritermitidae		*Glossotermes*	*oculatus*	Male	hypopharynx	2	Cacodylate	16.59	5.98	9.11	25.91	8.58	0.95	7.68	9.9	1147.86	432.91	2.65
Serritermitidae		*Glossotermes*	*oculatus*	Female	hypopharynx	3	Cacodylate	18.22	5.17	10.77	25.37					1008.88	430.53	2.34
Rhinotermitidae		*Heterotermes*	*tenuis*	Male	hypopharynx	1	Cacodylate	15.13	2.75	11.22	18.51	15.02	5.51	7.33	21.33			
Rhinotermitidae		*Heterotermes*	*tenuis*	Female		1	Cacodylate	10.42	2.99	6.06	15.58	19.37	3.4	16.59	24.15			
Rhinotermitidae		*Heterotermes*	*paradoxus*	Male		1	Ethanol	19.02	2.55	16.48	23.33	9.49	2.32	6.57	12.26			
Rhinotermitidae		*Coptotermes*	*testaceus*	Male	hypopharynx	3	Cacodylate	24.06	6.61	14.35	35.16	16.15	2.09	14.07	19.38	1323.18	320.38	4.13
Rhinotermitidae		*Coptotermes*	*testaceus*	Female		3	Cacodylate	22.29	7.32	9.7	31.65					1021.09	274.33	3.72
Termitidae	Macrotermitinae	*Protermes*	sp.	Male		2	Dubosq-Brail	27.69	1.87	25	29.95	33.81	5.07	24.75	41.01			
Termitidae	Macrotermitinae	*Ancistrotermes*	*cavithorax*	Male		1	Ethanol	19.97	4.94	14.08	28.86							
Termitidae	Macrotermitinae	*Ancistrotermes*	*cavithorax*	Female		1	Ethanol	19.13	7.17	11.25	28.01	17.96	4.2	12.23	23.78	1282.03	451.45	2.84
Termitidae	Sphaerotermitinae	*Sphaerotermes*	*sphaerothora*	Male		1	Ethanol	17.52	3.36	14.5	23.72					823.18	320.31	2.57
Termitidae	Sphaerotermitinae	*Sphaerotermes*	*sphaerothora*	Female		1	Ethanol	8.275	2.18	4.22	10.96	12.805	1.24	11.57	14.04	706.21	272.43	2.59
Termitidae	Foraminitermitinae	*Foraminitermes*	*coatoni*	Male	hypopharynx	2	Ethanol	11.24	3.16	6.76	15.25							
Termitidae	Apicotermitinae	*Aparatermes*	sp.	Female	hypopharynx	1	Cacodylate	11.4	3.88	6.12	21.19	9.07	1.98	6.29	13.81			
Termitidae	Apicotermitinae	*Ruptitermes*	sp.	Male		2	Ethanol	21.95	6.07	12.54	32.25					1417.04	475.56	2.98
Termitidae	Apicotermitinae	*Ruptitermes*	sp.	Female		2	Ethanol	19.36	3.14	14.67	24.64					1517.92	478.83	3.17
Termitidae	Apicotermitinae	*Anoplotermes*	*manni*	Male		2	Ethanol					6.78	0.5	6.29	7.47			
Termitidae	Termitinae	*Amitermes*	*beaumonti*	Male	hypopharynx	2	Ethanol	24.91	5.88	19.22	34.29	14.91	3.58	10.35	19.58	948.89	355.39	2.67
Termitidae	Termitinae	*Amitermes*	*beaumonti*	Female	hypopharynx	2	Ethanol	25.11	5.43	17.87	33.62	19.18	6.12	14.24	27.8	829.72	337.34	2.46
Termitidae	Termitinae	*Dentispicotermes*	*brevicarinatus*	Female	hypopharynx	2	Ethanol	9.76	1.71	7.78	12.06					1038.25	364.5	2.85
Termitidae	Termitinae	*Palmitermes*	*impostor*	Male		2	Cacodylate	28.57	3.05	24.91	34.37					994.06	352.55	2.82
Termitidae	Termitinae	*Palmitermes*	*impostor*	Female		2	Cacodylate	24.66	6.87	14.37	31.89					1006.28	361.11	2.79
Termitidae	Termitinae	*Termes*	*fatalis*	Male		1	Cacodylate	20.1	4.43	13.34	27.52					825.91	347.75	2.38
Termitidae	Termitinae	*Termes*	*fatalis*	Female	hypopharynx	1	Cacodylate	23.5	5.02	15.21	31.84					1096.98	390.17	2.81
Termitidae	Termitinae	*Neocapritermes*	*araguaia*	Male	hypopharynx	2	Cacodylate	21.79	4.67	14.25	27.79							
Termitidae	Termitinae	*Neocapritermes*	*araguaia*	Female	hypopharynx	1	Cacodylate	32.65	8.87	20.61	48.09	19.21	2	17.16	21.92			
Termitidae	Syntermitinae	*Silvestritermes*	*holmgreni*	Female		1	Ethanol	10.02	2.34	6.63	14.59							
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	Male		1	Cacodylate	24.79	8.88	13.09	36.55					1375.02	431.04	3.19
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	Female		1	Cacodylate	26.25	7.43	13.39	35.06					988.71	355.65	2.78
Termitidae	Syntermitinae	*Rhynchotermes*	*perarmatus*	Male		1	Ethanol	16.88	6.96	8.35	28.91							
Termitidae	Nasutitermitinae	*Nasutitermes*	sp. G	Male		2	Cacodylate	8.49	0.71	7.36	9.32							
Termitidae	Nasutitermitinae	*Nasutitermes*	sp. G	Female		1	Cacodylate	8.27	2.25	5.97	11.26	5.41	1	3.82	6.29			
Termitidae	Nasutitermitinae	*Nasutitermes*	*princeps*	Male		1	Ethanol	18.05	3.59	12.05	22.22					1118.95	357.49	3.13
Termitidae	Nasutitermitinae	*Nasutitermes*	*corniger*	Male		1	Ethanol	15.63	2.77	10.81	18.88					879.56	285.57	3.08
Termitidae	Nasutitermitinae	*Nasutitermes*	*corniger*	Female	hypopharynx	1	Ethanol	20.99	2.78	17.8	25.92							
Termitidae	Nasutitermitinae	*Coatitermes*	*clevelandi*	Male		1	Ethanol	12.78	2.04	9.29	17.14							
Termitidae	Nasutitermitinae	*Subulitermes*	sp.	Male	hypopharynx	1	Cacodylate	19.11	7.58	10.79	33.79					593.11	203.12	2.92
Termitidae	Nasutitermitinae	*Subulitermes*	sp.	Female		1	Cacodylate	19.16	5.57	7.21	28.95					610.76	195.76	3.12
Termitidae	Nasutitermitinae	*Hospitalitermes*	*papuanus*	Male		1	Ethanol	21.27	7.53	11.25	36.36							

Table S2.

List of termite imagoes subjected to our analyses, with additional information and secretory epithelium measures. Blank spaces indicate lack of information.

All measurements are in µm.

							Invaginations		Nucleus				Microvilli						Presence of				Epicuticle (10⁻³)						Exocuticle		Endocuticle	
Family	**Sub-family**	**Genus**	**Species**	**Caste**	**Sub-caste or sex**	**Sector**	**Length**	**SD**	**Length**	**SD**	**Width**	**SD**	**Length**	**SD**	**Diameter (10⁻³)**	**SD (10⁻³)**	**Channel diameter (10⁻³)**	**SD (10⁻³)**	**SER**	**Basal axons**	**Chemoreceptors**	**Mechanoreceptors**	**Length**	**SD**	**Outter**	**SD**	**Inner**	**SD**	**Length**	**SD**	**Length**	**SD**
Cryptocercidae		*Cryptocercus*	*punctulatus*	nymph		Labrum middle	3.45	0.26	5.74	1.11	1.8	0.13	0.79	0.051	83,65	14	Absent		Yes	Yes	NV	1	NM									
Cryptocercidae		*Cryptocercus*	*punctulatus*	nymph		Labrum apex	4.54	1.99	4.53	0.42	3.03	0.06	0.92	0.12	90	20	Absent		NV	NV	NV	NV	NM									
Cryptocercidae		*Cryptocercus*	*punctulatus*	nymph		Labrum middle	6.7	0.76	4.93	1.02	1.88	0.57	0.43	0.014	64.56	12	Absent		NV	NV	NV	NV	NM									
Archotermopsidae		*Hodotermopsis*	*sjoestedti*	worker		Labrum middle	3.12	0.04	5.45	1.61	3.68	1.4	NV		NV		NV		NV	Yes	NV	NV	NV		NV		NV		2.12	0.34	1.31	0.065
Kalotermitidae		*Kalotermes*	*flavicollis*	imago	Female	Labrum middle	NV		4.89	1.07	3.16	0.61	NV		86	5	0.03	0	Yes	No	NV	NV	30	5.2	NV		NV		2.74	0.041	3.53	0.11
Kalotermitidae		*Cryptotermes*	sp.	worker		Labrum middle	4.69	0.33	3.9	0.81	2.72	1.03	NV		NV		NV		NV	NV	NV	Yes	20	2	10	0.7	10	3	0.85	0.1	1.09	0.08
Kalotermitidae		*Glyptotermes*	sp.	worker		Labrum apex	5.02	0.16	4.59	0.77	2.08	0.76	0.79	0.18	91.37	10	NV		Yes	No	NV	Yes	70	60	NV		NV		0.35	0.14	3.02	0.3
Rhinotermitidae		*Coptotermes*	*testaceus*	imago	Female	Labrum middle	1.85	0.54	5.74	1.27	2.75	0.92	1.43	0.12	66	4.7	30	0.0043	Yes	No	NV	NV	23	4.3	NV		NV		2.003	0.058	1.25	0.065
Rhinotermitidae		*Coptotermes*	*testaceus*	imago	Male	Labrum middle	NV		5.85	0.48	2.98	0.54	1.18	0.17	NV				Yes	No	NV	NV	NV		NV		NV		NV		NV	
Rhinotermitidae		*Heterotermes*	*tenuis*	imago	Female	Labrum middle	1.92	0.24	5.21	1.35	4.15	0.32	1.83	0.11	96	4.3	30	0	Yes	Yes	NV	NV	30	0	NV		NV		0.82	0.073	0.52	0.078
Rhinotermitidae		*Coptotermes*	*formosanus*	worker		Labrum middle	11.252	0.15	4.62	0.12	2.08	0.43	1.02	0.053	76	6.4	59	0.0037	Yes	No	NV	NV	30	15	NV		NV		1.188	0.052	0.98	0.54
Rhinotermitidae		*Heterotermes*	*tenuis*	imago	Male	Labrum middle	NV		3.94	0.57	2.05	0.28	1.92	0.029	NV		NV		Yes	No	0.8	NV	47	8.2	NV		NV		2.21	0.07	2.65	0.15
Serritermitidae		*Glossotermes*	*oculatus*	imago	Female	Labrum middle	NV		5.18	0.36	3.39	0.66	1.21	0.15	87	4.7	30	0	Yes	No	NV	NV	40	0	NV		NV		1.42	0.069	1.2	0.18
Serritermitidae		*Glossotermes*	*oculatus*	imago	Male	Maxilla	5.43	0.37	4.46	1	2.43	0.82	1.38	0.17	83	5.6	NV		Yes	Yes	NV	Yes	30	0	NV		NV		2.82	0.23	1.67	0.065
Serritermitidae		*Glossotermes*	*oculatus*	imago	Male	Hypopharynx	9.2	1.11	4.75	0.89	1.77	0.43	1.89	0.29	94	8	NV		NV	NV	NV	NV	NV		NV		NV		1.58	0.13	1.29	0.29
Serritermitidae		*Glossotermes*	*oculatus*	imago	Male	Labrum middle	9.99	0.4	7.12	0.55	1.96	0.062	1.39	0.11	93	4.7	NV		Yes	No	NV	NV	36	9.4	NV		NV		9.48	0.55	15.92	0.71
Termitidae	Macrotermitinae	*Ancistrotermes*	*cavithorax*	imago	Male	Labrum middle	NV		5	0.56	1.71	0.26	NV		NV		NV		NV	NV	NV	NV	NV		NV		NV		2.6	0.11	2.51	0.085
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	worker	large worker	Labrum middle	NV		4.83	1.39	3.32	1.27	1.67	0.89	120	15	55	0.012	Yes	Yes	NV	NV	30	1.7	10	1	23	3	2.73	0.16	4.17	0.35
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	worker	large worker	Labrum middle	NV		NV		NV		0.75	0.09	97	8	47	0.004	Yes	No	NV	NV	30	0.2	10	0.8	20	3	1.95	0.2	2.44	0.14
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	worker	large worker	Labrum middle	3.93	0.56	6.91	0.26	4.48	0.04	0.98	0.058	100	13	45	0.006	Yes	No	NV	NV	30	1.7	11	2	24	0.28	2.38	0.165	2.32	0.16
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	worker	large worker	Labrum bottom	NV		3.02	0.31	1.47	0.16	0.48	0.026	96	10	41	0.001	Yes	No	NV	NV	NM						1.84	0.11	NV	
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	worker	small worker	Labrum middle	NV		5.12	1.33	2.31	0.55	0.57	0.086	96	8	35	0.008	Yes	No	NV	NV	36	4	14	3	25	2	1.56	0.25	NV	
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	worker	small worker	Labrum middle	NV		3.69	2.05	1.04	0.25	NV		NV		NV		Yes	No	NV	NV	NM									
Termitidae	Termitinae	*Termes*	*fatalis*	imago	Female	Labrum middle	4.23	0.19	6.355	0.81	3.03	0.67	NV		NV		NV		NV	NV	NV	NV	24	4.9	NV		NV		1.86	0.082	2.38	0.17
Termitidae	Termitinae	*Termes*	*fatalis*	imago	Male	Labrum middle	7.6	0.77	6.9	0.44	3.78	0.14	1.19	0.13	NV		NV		NV	NV	NV	NV	NV		NV		NV		NV		Not visible	
Termitidae	Termitinae	*Neocapritermes*	*taracua*	worker		Labrum middle	12.16	0.54	7.08	0.18	2.48	0.056	1.45	0.045	94.25	15.9	0.04	0	Yes	Yes	NV	Yes	28	4.3	NV		NV		2.31	0.087	3.38	0.25
Termitidae	Apicotermitinae	*Ruptitermes*	sp.	imago	Male	Labrum middle	NV		5.02	0.43	2.58	0.94	NV		NV		NV		Yes	NV	NV	NV	NV		NV		NV		NV		NV	
Termitidae	Apicotermitinae	*Anoplotermes*	sp.	worker		Hypopharynx	3.4	1.02	4.18	0.38	2.53	0.63	1.42	0.21	71.75	8	0.03	0.005	Yes	No	NV	NV	52	0.9	15	1	36	2	2.8	0.42	27	0.45
Termitidae	Apicotermitinae	*Anoplotermes*	sp.	worker		Labrum middle	3.055	0.075	NV		NV		0.75	0.023	106	2.8	0.05	0.0038	Yes	Yes	NV	Yes	30	2	10	0.6	20	3	0.61	0.012	3.39	0.35
Termitidae	Apicotermitinae	*Anoplotermes*	sp. Q	worker		Labrum middle	2.89	0.15	NV		NV		0.96	0.053	101	6.39	0.05	0.0038	Yes	NV	1	Yes	43	15	NV		NV		0.78	0.052	3.92	0.54
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	imago	Female	Labrum middle	NV		4.17	1.23	2.7	0.81	1.63	0.2	NV				No	No	NV	NV	NV		NV		NV		NV		NV	
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	worker		Labrum middle	4.79	0.18	5.04	0.68	2.46	0.8	1	0.05	100	6	0.04	0.007	Yes	No	NV	Yes	30	0.7	8	0.7	20	2	1.18	0.045	0.62	0.003
Termitidae	Cubitermitinae	*Cubitermes*	sp.	worker		Labrum middle	3.54	1.17	4.59	0.046	3.55	0.17	1.53	0.1	120	10	0.05	0.004	Yes	Yes	NV	NV	20	4	NV		NV		2.6	0.2	2.68	0.09
Termitidae	Cubitermitinae	*Thoracotermes*	sp.	worker		Labrum middle	4.53	0.55	4.1	0.02	4.16	0.023	0.7	0.14	80	4	0.024	0.004	Yes	Yes	NV	NV	30	4	10	1	15	1	1.15	0.15	0.78	0.21
Termitidae	Nasutitermitinae	*Subulitermes*	sp.	imago	Female	Labrum middle	NV		2.72	0.25	1.39	0.012	0.95	0.072	98	5.3	NV		NV	NV	NV	Yes	NV		NV		NV		1.58	0.048	1.9	0.054
Termitidae	Nasutitermitinae	*Subulitermes*	sp.	imago	Female	Labrum middle	NV		5.2	0.95	1.83	0.027	0.9	0.039	NV		NV		NV	NV	NV	NV	NV		NV		NV		1.34	0.045	1.58	0.095
Termitidae	Nasutitermitinae	*Subulitermes*	sp.	imago	Male	Labrum middle	NV		5.14	0.47	2.53	0.022	0.84	0.01	0.083	1	NV		NV	NV	NV	NV	30	2.8	NV		NV		0.82	0.021	1.11	0.014
Termitidae	Nasutitermitinae	*Nasutitermes*	*lujae*	worker		Labrum middle	2.001	0.317	5.23	1.08	2.9	1.12	1.45	0.027	100	10	0.029	0.01	Yes	Yes	NV	Yes	20	1	10	0.8	10	1	2.41	0.13	2.26	0.26

Table S3.

List of termite workers and imagoes used for transmission electron microscopy. Abbreviations: NM, not modified cuticle; NV, not visible (due to samples' quality). Blank spaces indicate lack of information. All measurements are in µm.

Video S1

Encounter of *Glossotermes oculatus* with the ant *Solenopsis invicta*. Note the soldier of *G. oculatus* walking backwards while rubbing the labrum against the substrate immediately after the encounter.

To access to this video, check the mp4 file available in the electronic version of this thesis.

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Paper 3: The evolution of the most powerful defensive organ found in termites, the frontal gland, in Neoisoptera

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Résumé

Les termites sont des insectes au corps mou qui constituent la source de nourriture principale pour de nombreux animaux. Pour surmonter cette pression des prédateurs, les termites ont développé différents mécanismes de défense. La défense chimique fournie par les glandes exocrines est l’une des stratégies les plus efficaces et peut être trouvée chez tous les termites. La glande frontale est l'organe défensif le plus puissant des termites. Malgré son potentiel, il a surtout retenu l'attention des soldats et, dans une moindre mesure, de ses imagos, alors qu'il n’existe qu’une seule publication centrée sur sa description chez les ouvriers et qui est restreinte à une seule sous–famille. Afin de brosser un tableau complet de l'évolution de cette glande chez les termites et de préciser son évolution au sein des termites, nous avons étudié la glande frontale de 41 espèces réparties chez tous les Néoisoptères. La glande est présente chez la plupart des ouvriers Néoisoptères avec peu de cas de régression et est toujours faite d'un épithélium formé de cellules de classe 1 sans réservoir. Nos données suggèrent une évolution de la glande frontale spécifique à la caste, ainsi qu'une variation de sa fonction chez les ouvriers.

Mots–clefs: Glande exocrine, évolution, Isoptère, développement, termite.

Abstract

Termites are insects of soft body that are a primary food source for many animals. To overcome this predatory pressure, they have developed different defensive mechanisms. Chemical defense provided by exocrine glands is one of the most successful strategies and can be found in all termites. The frontal gland is the most powerful defensive organ that occurs in termites. In spite of its potential, it has received primarily attention in soldiers and in a lesser degree in imagoes while there is a single publication focusing on its description in workers and it is restricted to one sub–family only. In order to provide a complete picture of the evolution of this gland in termite workers and to clarify its evolution in termites, we studied the frontal gland of 41 species all across Neoisoptera. The gland occurs in most Neoisoptera workers with few cases of regression and was always made of an epithelial formed by class 1 cells without reservoir. Our data suggest a caste–specific evolution of the frontal gland along with a variation of its function in workers.

Keywords: Exocrine gland, evolution, Isoptera, development, termite

Introduction

Termites are social, soft–bodied insects with dominating in tropical lands and provide organic matter decomposition at huge scales (Bignell & Eggleton, 2000), contributing with about 4% and 2% of total global methane and carbon dioxide emissions approximately, respectively (Sanderson, 1996). They present a high abundance that is especially observable in tropics where they can exceed 1,000 individuals per square meter (Eggleton, 2000). Because of this high abundance, termites represent an important food source for a wide variety of predators (Deligne & Quennedey, 1981). To overcome predation pressure, termites have developed different defensive mechanisms, including: elaborated hard and/or hidden nests, behavioural strategies, morphological adaptations and chemical means of colony defence (Prestwich, 1984; Eggleton, 2000; Šobotník et al., 2010a; Palma–Onetto et al., 2018). Among the novel characters, the most remarkable one is the establishment of a specialised defensive caste: the soldier. The soldier presence is a synapomorphy to all termite lineages (Hare, 1937; Noirot & Pasteels, 1987; Roisin, 2000), although it was secondarily lost several times independently in Apicotermitinae (Sands, 1972; Krishna et al., 2013; Bourguignon et al., 2017) and two more times within Termitinae (Ahmad, 1976; Miller, 1984). The soldierless species avoid predation through a hidden way–of–life and other adaptations, such as highest degree of aggressiveness in termite workers (Sands, 1972; Šobotník et al., 2010a), defensive defecation (Prestwich, 1984), defensive compounds in the labial glands (Sands, 1982; Sillam–Dussès et al., 2012), abdominal dehiscence (Sands, 1982; Prestwich, 1984) and autothysis (Sands, 1982; Costa–Leonardo, 2004; Šobotník et al., 2012). At the same time, not only workers of soldierless species are equipped with unprecedented defensive mechanisms, and although defensive strategies are reduced in other species, most of workers' defensive glands still occur also in soldiered species (Mill, 1984; Prestwich, 1984; Šobotník et al., 2012; Sillam–Dussès et al. 2012; Bourguignon et al., 2015; Poiani & Costa–Leonardo, 2016). The soldiers are in general incomparably better fighters compared to workers (see Binder, 1988), but the colony sometimes faces threads that mechanical defenses are unable to solve, such as intraspecific fights of species in which they depend exclusively on chemical weapons and consequently, depending also of specific autodetoxification mechanisms (Spanton & Prestwich, 1982). In other words, optimal colony investments include also workers which are able to participate at active defences, and such universal features became more pronounced in soldierless species.

The best–known termite organ, the frontal gland, is a purely defensive organ. This gland of prime importance in termites is a novelty with no equivalent among other insects (Noirot, 1969). It is a defining character of Neoisoptera, a group gathering the families

Stylotermitidae, “Rhinotermitidae” (polyphyletic group; Miura et al., 1988; Donovan et al., 2000; Kambhampati & Eggleton, 2000; Thompson et al., 2000; Eggleton, 2001; Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007; Bourguignon & Roisin, 2011; Cameron et al., 2012; Bourguignon et al., 2015, 2017), Serritermitidae and Termitidae. In soldiers, the chemical nature of the frontal gland secretions is diverse and may include nitroalkenes, sesquiterpenes and ketones (Vrkoč & Ubik, 1974; Chuah et al., 1990; Hanus et al., 2006; Piskorski et al., 2007). These rich blends act as contact poisons, repellents, irritating compounds, entangling and incapacitating agents, anti–healing compounds, or alarm pheromones (Piskorski et al., 2007, 2009; Šobotník et al., 2010a). Frontal gland presence in termite soldiers is notorious, but considerably less is known about its occurrence in other castes, such as presoldiers (Prestwich, 1984; Lelis & Everaerts, 1993; Bordereau et al., 1997; Šobotník et al., 2004), imagoes (Holmgren, 1909; Feytaud, 1912; Bugnion, 1913; Noirot, 1969; Šobotník et al., 2004; Piskorski et al., 2009; Šobotník et al., 2010b; Kutalová et al., 2013) and workers (Šobotník et al. 2010c).

The frontal gland is always an unpaired organ, epithelial lining of a large saccular reservoir, opening in the posterior frons through the fontanelle (Noirot, 1969; Prestwich and Collins, 1982; Quennedey, 1984; Šobotník et al., 2004, 2010a). Among Neoisoptera, the frontal gland may be absent (*Protermes* sp. and *Microtermes toumodiensis* imagoes; Kutalová et al., 2013), confined to the head as an epithelial thickening (imagoes of *Psammotermes* genus, all imagoes of Termitidae except Foraminitermitinae and Macrotermitinae groups, and workers from soldierless Apicotermitinae species; Holmgren, 1909; Noirot, 1969; Šobotník et al., 2004; Šobotník et al., 2010; Kutalová et al., 2013) or as an epithelial thickening with reservoir (most of the Termitidae soldiers, Serritermitidae imagoes, *Reticulitermes lucifugus, Termitogeton planus* and *Coptotermes* spp. Imagoes; Noirot, 1969; Santos et al., 2005; Santos and Costa–Leonardo, 2006; Šobotník et al., 2010b), or extended in most of the overall body cavity volume (most Rhinotermitidae and Serritermitidae soldiers and imagoes; Noirot, 1969; Šobotník et al., 2004, 2010b).

Althought the frontal gland development and therefore its evolution is well known in soldiers (Deligne et al., 1981; Prestwich, 1984; Quennedey, 1984; Šobotník et al. 2010a) and imagoes (Šobotník et al. 2010b; Kutalová et al., 2013), it has received almost no attention in workers. Thus, the evolutionary routes of the frontal gland in Isoptera remain uncertain. In fact, there is just a single publication dealing with the frontal gland structure in workers of *Aparatermes nr. cingulatus* and most soldierless Anoplotermes–group termites (Šobotník et al. 2010c). However, there is no reason to think that the presence of the frontal gland is only limited to Apicotermitinae and thus the examination of other groups for the presence/absence of this gland is needed in the frame of the evolution of this gland among termites.

Here, we provide a report on the development of the frontal gland in workers of 37 genera across Neoisoptera representatives, in order to shed light on the evolution of this gland in termites.

Materials and Methods

Termite samples

We examined workers of 41 species (Tab. 1), representatives of almost all families and sub–families of termites, collected from across the world. Despite the gland has never been observed in any caste of no–Neoisoptera termites, we decided to check carefully in 5 species among them: *Mastotermes darwiniensis, Hodotermopsis sjoestedti*, *Glyptotermes* sp., *Kalotermes flavicollis,* and *Neotermes cubanus.*

Frontal gland occurrence, structure and ultrastructure through optical and electron microscopies

Whole individuals of 23 species were carefully examined under a Leica Z6 APO optical microscope to detect the presence of the frontal gland (Tab. 1). Images of the heads were taken with a Nikon DS–fi1c digital camera attached to the microscope. Micrographs were stitched using Helicon Focus software.

We dissected and fixed workers from 27 other species, following the protocol described by Palma–Onetto and others (2018). The only exception was *Tonsuritermes tucki* which was kept in ethanol 80% and not post–fixed with osmium. By using both optical and transmission electron microscopies, we have studied structure and ultrastructure of 8 species among them: 2 Rhinotermitinae (*Dolichorhinotermes longilabius* and *Coptotermes formosanus*), 1 Serritermitidae (*Glossotermes oculatus*), 1 Macrotermitinae (*Pseudacanthotermes militaris* large worker), 2 Apicotermitinae (1 undetermined species of the genus *Anoplotermes* and *Tonsuritermes tucki*), 1 Termitinae (*Neocapritermes taracua*), and 1 Nasutitermitinae (*Nasutitermes lujae*).

Measurements of the gland and its relative size

Length (L) of the frontal gland was measured on sagittal sections with the *NIS–Element Advance Research* software. The width (W) was obtained from the pictures of the coronal view of the whole workers head. These parameters were used for frontal gland volume calculation. The frontal gland shape was normally estimated as a cone and for those samples where we counted with sagittal and coronal pictures of the frontal gland, the volume was estimated by the equation $V = \frac{1}{3} \times \pi \times \left(\frac{W}{2}\right)^2 \times L$. In those cases in which the frontal gland was shaped as a group of cells of more or less the same length, the volume was estimated as a cylinder and the volume was calculated by the equation $V = \pi \times \left(\frac{W}{2}\right)^2 \times L$. At last, in termites with a hemispherical frontal gland, the volume was estimated by $V = \frac{2}{3} \times \pi \times \left(\frac{W}{2}\right)^2 \times L$.

Calculations of the relative frontal gland size were performed by comparing the frontal gland volume (V) and head length (HL = distance between clypeo–frons boundary and posterior margin of head) of each specimen using the formula $V/(HL)^3 \times 100.000$.

Because of the presence of workers with blue crystals in *Neocapritermes taracua* (Šobotník et al., 2012), a distinction has been made between workers which possess these crystals and those which not.

Phylogenetic analysis of the frontal gland evolution

The data obtained in this study and data from previous studies were used to create a robust table (Tab. 2) that contains the main points features of the frontal gland in order to build a phylogenetic tree, reconstructed using previously published phylogenetic trees (Bourguignon et al., 2015, 2017). Ancestral state reconstruction was carried out with Mesquite (Maddison & Maddison, 2010) on the different features analysed, using the Mk1 likelihood model and parsimony analyses.

Results

Common features of the frontal gland in workers

The frontal gland was generally observed through the skin as a circular structure located at the ventral side of the head, posteriorly to the brain, behind the posterior Y–shaped junction of the epicranial and frontal sutures, frequently pushing the mandibular muscles backwards (Fig. 1, 2 and 3). It was usually a small epidermal thickening of hemispherical, cylindrical or conical shape made of columnar class 1 cells only and with no reservoir. When the frontal gland was conical, several tentorial fontanellar muscle fibres were always observed stretched at the base of the secretory cells with the largest height. These muscles were not observed when the frontal gland was hemispherical or cylindrical.

The fontanelle, a narrow pore located above the posterior part of the brain leading inside the frontal gland and being used to release its secretion, was not observed at any of the studied species (Fig. 1, 2 and 3).

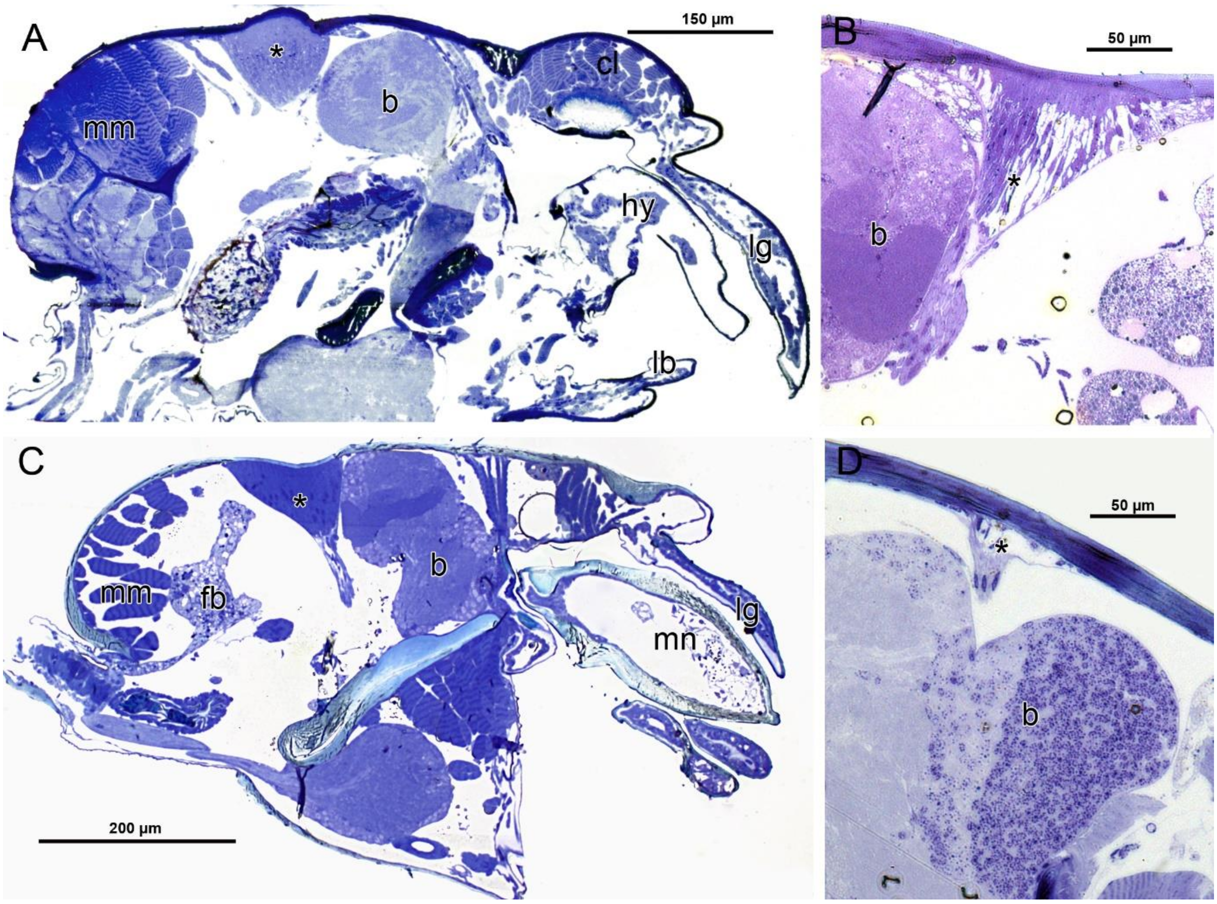

Figure 1. The development of the frontal gland in lower termite workers. (A) Full head of *Glossotermes oculatus.* (C) Full head of *Termitogeton planus*. (D) Forehead of *Dolichorhinotermes longilabius.* Asterisks mark the frontal gland. Abbreviations: b, brain; cl, clypeus; fb, fat body; hy, hypopharynx; lb, labium; lg, labral gland; mm, mandibular muscles; mn, mandible.

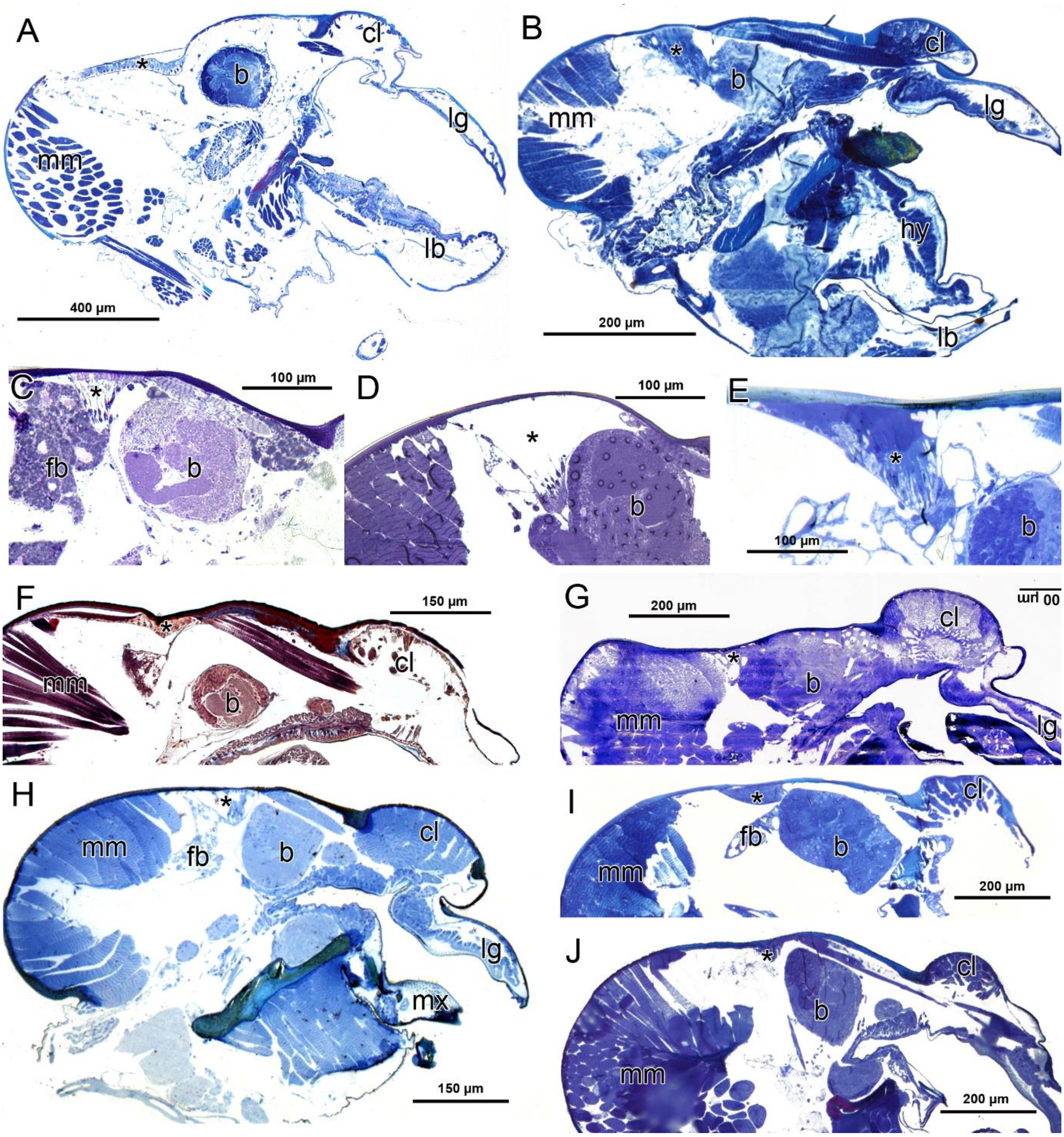

Figure 2. Frontal gland development in higher termites (Termitidae) workers. **A** *Tonsuritermes tucki,* **B** *Planicapritermes planiceps,* **C** *Spinitermes* sp., **D** *Termes hospes,* **E** *Neocapritermes taracua,* **F** *Cubitermes* sp., **G** *Embiratermes neotenicus,* **H** *Microcerotermes* sp., **I** *Globitermes sulphureus,* **J** *Nasutitermes lujae.* The asterisks mark the frontal gland. Abbreviations: b, brain; cl, clypeus; fb, fat body; hy, hypopharynx; lb, labium; lg, labral gland; mm, mandibular muscles; mx, maxillar.

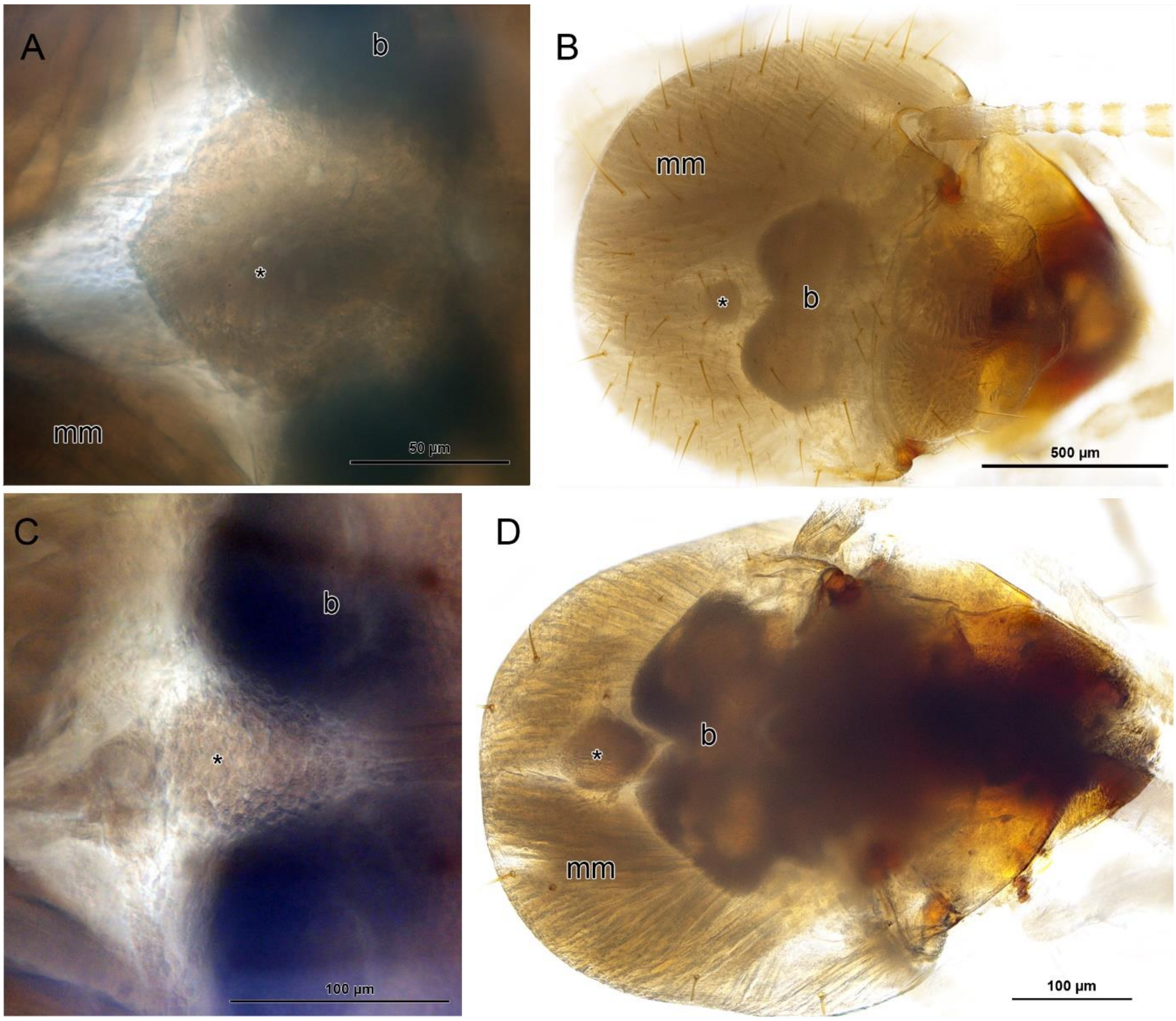

Figure 3. Frontal gland of termite workers as seen through the cuticle using an optical microscope. **A** Frontal gland in *Glossotermes oculatus*. **B** *Jugositermes* sp. **C** *Embiratermes neotenicus*. **D** *Pericapritermes* sp*.* The asterisks mark the frontal gland. Abbreviations: b, brain; mm, mandibular muscles.

The overlaying cuticle, composed by an endocuticle, an exocuticle and an epicuticle, presents about the half of its width in other part of the body. Its shows slight modifications, among which stand out: an endocuticle reduced in size or even absent; an exocuticle which tends to slightly increase its width by a disintegration of the layer, forming extended pore canals which are running through all the layers but are especially wide in the exocuticle; and an epicuticle with multiple perforations (Fig. 4A and 4B). Ectodermal epithelial cells which are usually find under the cuticle of the head were absent in the portion of the head where the frontal gland is. These ectodermal cells, normally flat (about 0.5 – 2 µm length) and wide (with a distance in between cells' nucleus higher than 17 µm), were characterized by the presence of few organelles, no mitochondria, plenty of invaginations, abundant microtubules, big nucleus of about 1.5 µm length and 4 µm width (up to 8 µm in *Coptotermes formosanus*) and a basal lamina of about 500 nm.

High abundance of fat body was observed surrounding the frontal gland (Fig. 1E, 1D, 2C and 2H). This fat body was composed by several adipocytes with their characteristic structure made of abundant lipid droplets, a well–developed Golgi, numerous mitochondria, a basal lamina and plenty of glycogen which can be sometimes present as a membrane–bounded vacuole containing glycogen.

The ultrastructure of the frontal gland was normally characterized by its long and thin cells forming groupings which were observed as a circle from above and as an ovoid or a cone from the sagittal view. All of the secretory cells were equivalent in structure and presented a clear differentiation along the apicobasal axis. The apical sector was formed by short (about 1–2 μm long; up to 3.2 μm long in *Glossotermes oculatus*) tightly packed microvilli of about 80 nm thick in contact with the cuticle (Fig. 4B). Vesicles were located freely on the cytoplasm and were running to the microvilli in which basal exocytosis was observed to occur. The centre of the cell was generally long and characterized by a cytoplasm composed by numerous microtubules oriented apico–basally, the presence of rough endoplasmic reticulum (RER), Golgi apparatus, free ribosomes, lucent vesicles, few myelin figures, mitochondria (Fig. 4C and S3). The basal sector of the cells was characterized by short invaginations and a basement lamina formed by one to four layers which becomes wider at the centre of the gland (Fig. 4D). The position of the nucleus varied from the middle part of the cells to the base of them, was ovoid in shape, about 10 μm long and mainly filled with dispersed chromatin. Neighbouring cells were connected by zonulae adherens at the apex, septate junctions in the central parts and were not connected by anything at the base of the cells.

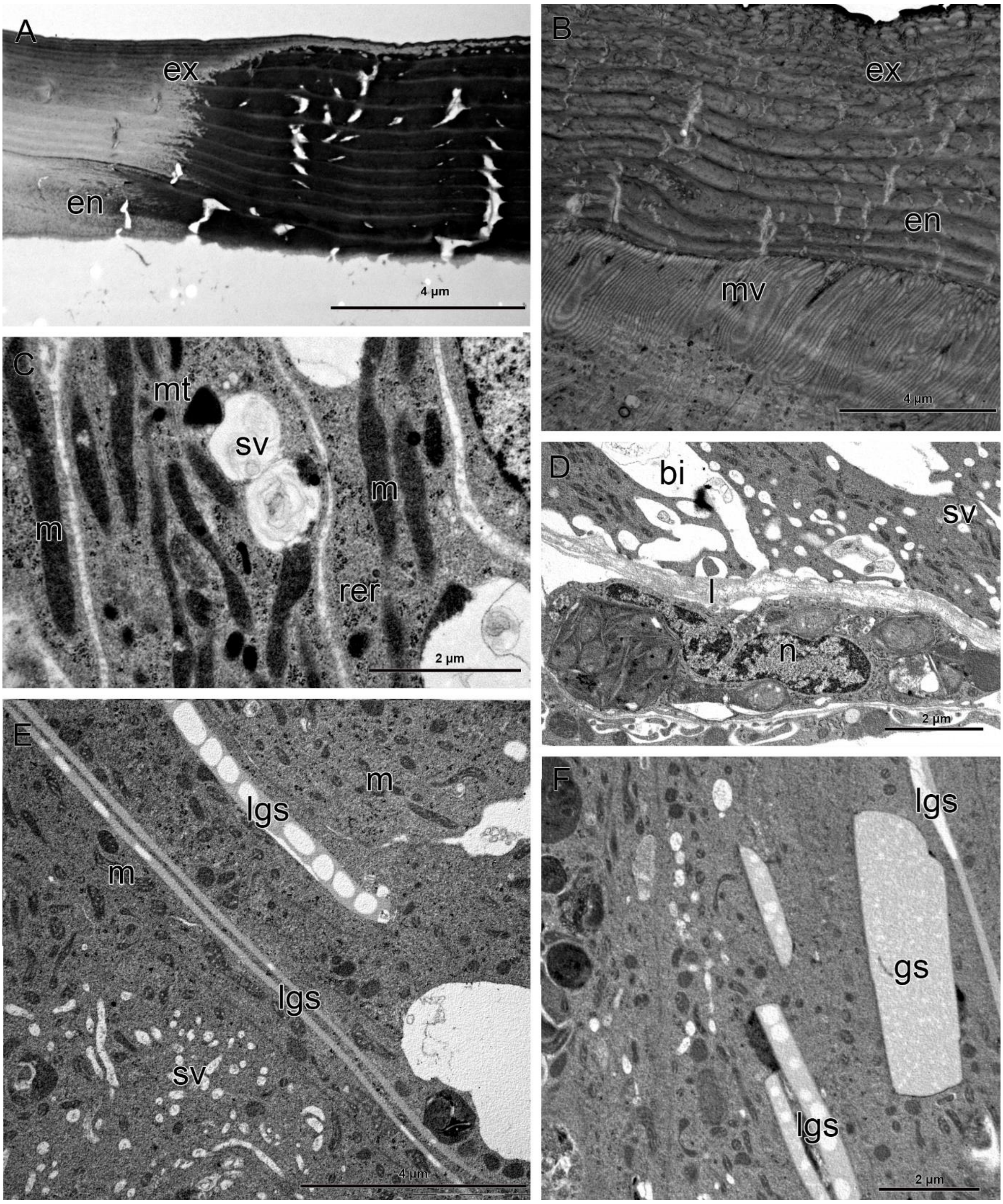

Figure 4. Ultrastructure of the frontal gland in termite workers. Highly modified cuticle overlying the frontal gland in *Dolichorhinotermes longilabius* **A** and *Glossotermes oculatus*. Note enlarged pore canals ensuring secretion release and the well–developed microvilli. **B** Detail of cytoplasm in the mid part of secretory cell of *G. oculatus*. **C** Basal sector of the secretory cell in *Neocapritermes taracua*, showing well–developed basal invaginations and an envelope secretory cell. **D** Detail of cytoplasm in the mid part of secretory cell of *N. taracua,* showing abundant secretory vesicles and especially long thread–like granules of secretion. **E** Mid part of the secretory cell of *N. taracua,* showing differently developed granules of secretion. Abbreviations: bi, basal invagination; en, endocuticle; ex, exocuticle; gs, granule of secretion; l, lamina; lgs, long–thread granules of secretion; m, mitochondria; mt, microtubules; mv, microvilli; n, nucleus; rer, rough endoplasmic reticulum; sv, secretory vesicle.

Systematic survey

The frontal gland was absent in lower termites as well as in its closest relative, the cockroach *Cryptocercus punctulatus*. It was clearly visible in all workers from Neoisoptera species studied, with the exception of *Sphaerotermes sphaerothorax, Pseudacanthotermes militaris* and some Rhinotermitidae.

In Rhinotermitidae, the gland was absent in all species analysed but *Termitogeton planus* and *Prorhinotermes simplex.* Workers of *T. planus* possessed a frontal gland as an epidermal thickening with cone shape, made of few enlarged secretory cells. Its endocuticle changed dramatically above the frontal gland, losing several layers and measuring about the half than in other parts of the head in width. As for *P. simplex,* the frontal gland in the shape of a cone was made of really thin cells with the nucleus located on the middle of them, with abundance of adipocytes on the lateral borders. In the case of *Dolichorhinotermes longilabius,* the presence of the frontal gland could not be completely confirmed, there was something that seems to be an epithelium (destroyed during fixation and too small to be easily located) composed of few cells forming which seems to be a hemispherical–shaped frontal gland. It was slightly displaced towards the anterior part of the head, being located above the middle part of the brain. Its epithelium was then attached by the bottom to several muscle fibres running by the middle of the brain. TEM did not provide a clue about the epithelium due to its disintegration during preparation, but showed that the cuticle above it was highly modified. An epithelial thickening could be observed at the forehead of *Coptotermes formosanus.* TEM showed that it was a cube–shaped structure without microvilli and without modified cuticle above. This epithelial thickening was guessed to be only an accumulation of adipocytes most of the time in contact with epidermal cells which presented several differences among individuals from the same species. The cuticle above this sector did not presents any visible modifications.

The presence of the gland remained unclear in *Serritermes serrifer* (Serritermitidae). Because of the sample quality, we could not certify whether there is or not a frontal gland. In *Glossotermes oculatus*, the gland was large, conical and produced an inflammation over the head. The cells composing this gland were long and thin, all of them were equivalent in structure and showed differentiation along the apico–basal axis. The apical part of the cells was characterized by particularly long microvilli (up to 4 µm long), numerous microtubules orientated apico–basally, small vesicles, rough endoplasmic reticulum (RER) and absence of mitochondria. The central part of the cells was characterized by abundant RER, several long mitochondria (up to 8 µm long), microtubules, scarce smooth ER, numerous electron–luscent vesicles and a nucleus of loose chromatin of about 7–10 µm long and variable width. The basal part of the cells was composed by numerous thin–invaginations which could reach deep in the cell (up to 75 µm long) and showed pinocytotic activity.

In *Pseudacanthotermes militaris* (Macrotermitinae) and *Sphaerotermes sphaerothorax* (Sphaerotermitinae)*,* the workers did not present a frontal gland. The space where the gland could be localized was filled by the brain, fat body and muscles running from the hindhead to the labrum.

In our representative of Syntermitinae, *Embiratermes neotenicus*, workers possessed a very small epidermal thickening of conical shape, made of relatively short cells in comparison with other species, and no visible modification in the cuticle width could be observed. The presence of the gland was then after confirmed by light microscope pictures of the full head of the termite.

Foraminitermes coatoni (Foraminitermitinae) was observed only under light microscope contrast and showed the existence of a frontal gland.

All studied species of Apicotermitinae presented a frontal gland. The gland was very circular in shape from the ventral view and conical from the sagittal view point, made of narrow columnar cells and measured about 100 µm in the longest position in *Jugositermes* sp*.* and *Silvestritermes* sp*.* Its ultrastructure remained the standard features but differed from others groups because of the nuclei located at the basal part of the cells and the presence of another layer of cells located at the bottom of the frontal gland. These cells were enclosed by the basal lamina, presented many invaginations and lysosomes of different stages of development, and enclosed the frontal gland epithelium from the hemolymph. In *Tonsuritermes tucki,* all features varied considerably from other species, even from species of the same group (Apicotermitinae). Its frontal gland appeared as an epidermal thickening of square cells of about 16 µm long and 12 µm wide, with abundant electron–lucent secretory vesicles and mitochondria. The cuticle above the frontal gland was made of two thin layers of modified cuticle, both being very different from the cuticle of other parts of the head.

The frontal gland occurred in all representatives of Termitinae as well (Tab. 1). It appeared as an epidermal thickening conical in shape with long and thin cells, with the exception of *Globitermes sulphurous* in which the gland possessed a hemispherical shape with more compact cells. The cells in the centre of the gland could reach 60 µm in length, whereas the width of the cells was about 3 µm.

The size of the gland was variable among species, the largest one occurred in *Neocapritermes taracua* and the smallest in *Spinitermes* sp. (for details, see Tab. 1)*.* The ultrastructure of the frontal gland was observed only in the Termitinae *N. taracua*. In spite of keeping the basic ultrastructure of the frontal gland, *N. taracua* presented some special features: relatively short mitochondria in comparison with other species which presented conical frontal gland (usually about 600 nm long, up to 1.5 µm long), presence of envelope cells at the basal part of the frontal gland (Fig. 4D), similar to those which can be found at the frontal gland of *Silvestritermes sp.* but containing electron–dense vesicles, well–developed basal invaginations which were then modified into vesicles running across the cytoplasm in the basal half of the cells, and the presence of a strange dense secretion which seems to be biocrystals of tubular shape with regular borders. Generally, this secretion looked like long–thread granules of secretion (LGS), that were more abundant in the middle area of the cell than close to the base (Fig. 4E, 4F). The length of these LGS of about 300 nm wide (reaching up to 700 nm) and about 8 µm long observable, but was estimated to reach up to 60 µm, crossing the cell from the basal part to the central area, almost reaching the apical sector of the cell but never presented in there (Fig. 4E). Its formation seems to come from protein granules and to be present predominantly at the cell basal margins.

All Cubitermitinae species studied possessed a frontal gland. It had a hemispherical shape and its size was relatively small in proportion of the head of the termite.

The presence of a frontal gland in Nasutitermitinae remained unclear. In one specimen of *Nasutitermes lujae,* a wide accumulation of cells appeared at the place where the frontal gland should be found, but the cuticle was actually wider at this localisation than in others. This accumulation was composed of few cells, making difficult their observation under TEM and only ectodermal cells and fat body were observed. Eight other species were observed under light microscope and a frontal gland seemed to be present in half of them (for details see Tab. 1). It is not clear whether the gland was absent in *Postsubulitermes* sp., *Nasutitermes gaigei, Nasutitermes guyanae* and *Nasutitermes* sp. or if it was too small to be visible under light microscope.

Relative size and volume

The overall frontal gland size (evaluated as volume) was rather small, varying generally between 2000 and 6000 µm^3, with the exception of *D. longilabius* were it would measure about 800 µm^3, although the presence of the gland in this specie remains unclear. The largest gland by far occurred in *T. tucki*, while the smallest gland, excluding *D. longilabius,* was found in *Spinitermes* sp*.* The frontal gland diameter averaged 154 µm and varied usually between 90 and 150 µm, getting down to 81 µm in *Spinitermes* sp. and up to 704 µm in the case of *T. tucki*.

The correlation between the diameter of the frontal gland and the length of the head was in general well established (about 1:7), with only two exceptions where its proportion was higher: *Termes hospes* (1:4) and *T. tucki* (1:2).

The relative size of the gland was slightly variable ranging about 0.1 to 0.3, without any tendency inside the groups, but was particularly larger in *Termitogeton planus, Planicapritermes* sp. and *T. hospes.*

Evolution of the frontal gland inferred from its phylogenetic tree

In soldiers, the frontal gland was always a saccular shape organ when it was present. In the Rhinotermitidae species studied, it could extend until the abdomen, with the only exception of *Psammotermes hybostoma* large and medium soldiers. In all the other groups, the frontal gland was confined to the head (Tab. S1), with the exception of *Globitermes sulphureus* and *Dentispicotermes brevicarinatus* (both Termitinae), two species in which the frontal gland did not have any opening and thus the secretion release must be realised by autothysis. Even though the gland is confined to the head, the size of the reservoir was especially big in Nasutitermitinae, where the gland harboured about half of the head size. The chemical nature of the compounds that can be found inside the reservoir is highly variable, but monoterpenes seem to be a standard compound in Nasutitermitinae.

In imagoes, the gland was described to be always present except for *Microcerotermes toumodiensis* and *Protermes* sp. (Tab. S1). It presented a saccular shape in Rhinotermitidae, Macrotermitinae and Foraminitermitinae, while in Sphaerotermitinae and in Termitidae, it was always shaped as an epithelium without reservoir. In all cases where there was a reservoir, the head of the termite presented a frontal opening.

The shape of the cells which conformed the frontal gland seemed to be highly variable and would not represent an evolutionary development.

The frontal gland was in general formed exclusively by class 1 secretory cells in all castes. The presence of class 3 secretory cells was common in species with fontanelle (Grassé 1982; Šobotník et al. 2010), but these cells release their products in the vicinity of the fontanelle and are not part of the gland itself. Exceptions about it are soldiers and alate imagoes of *Coptotermes* genus (Quennedey 1984; Šobotník et al. 2010c) and alates of *Heterotermes* (Šobotník et al. 2010c).

Discussion

The frontal gland is the most powerful defensive mechanism found in termites so far and was well known for occurring as an epithelium with reservoir in soldiers of the most advanced taxa, Rhinotermitidae, Serritermitidae, and Termitidae (Noirot 1969; Quennedey 1984; Costa–Leonardo 1998; Šobotník et al. 2010a, 2010d). In imagoes, its presence has been confirmed in the same groups than for soldiers (Feytaud 1912; Noirot 1969; Šobotník et al., 2004; Piskorski et al. 2009; Šobotník et al., 2010b; Kutalová et al., 2013) but its shape varies among species which have a reservoir (all Serritermitidae, Foraminitermitinae and most of Rhinotermitidae and Macrotermitinae; Šobotník et al., 2010b; Kutalová et al., 2013) and those which do not have a reservoir (one single Rhinotermitidae: *Psammotermes* sp. and all Sphaerotermitinae, Apicotermitinae, Termitinae, Syntermitinae and Nasutitermitinae) (Šobotník et al., 2010b; Kutalová et al., 2013). The only exceptions are *Protermes* sp. and *Microtermes toumodiensis* (both Macrotermitinae) which do not have a frontal gland. Information about the frontal gland in workers was limited to its presence as an epithelial thickening without reservoir in soldierless species of Apicotermitinae (Šobotník et al., 2010c). Regarding the frontal gland presence in workers of other groups, no rigorous investigation had been performed yet. In the present study, we described for the first time the frontal gland in workers of several Neoisoptera species.

The frontal gland was present in 8 of the 11 Neoisoptera groups analyzed (out of 12 Neoisoptera groups existing in the world, lacking only Stylotermitidae representatives) suggesting a common origin about 130 million years ago by the common ancestor of Neisoptera but old Rhinotermitidae species, according to Bourguignon and others (2015).

The frontal gland presence varies among species in Rhinotermitidae and Nasutitermitinae. In Rhinotermitidae, the gland, when present, has always a conical shape. The gland was absent in the clade formed by *Schedorhinotermes* and only a small epithelium was observed in *Dolichorhinotermes*, both from the sister group to the common ancestor of all other Neoisoptera (accorded to Bourguignon et al., 2015), but also in some more recent genera like *Reticulitermes* and *Heterotermes.* In fact, the ultrastructure of the frontal gland in *Dolichorhinotermes longilabius* did not provide clear evidence on the presence of the frontal gland, but we can guess the epithelium at this localization was the gland itself, due to its highly modified cuticle. In the other hand, the frontal gland was present in the clade composed by *Prorhinotermes simplex, Termitogeton planus* and Serritermitidae species, in which the frontal gland was present and shared a similar diameter, shape and proportion head/gland. Interestingly, the four genera composing this clade (*Prorhinotermes, Termitogeton, Serritermes* and *Glossotermes*) also share several behavioral and developmental features (Roisin, 1988; Parmentier & Roisin, 2003; Bourguignon et al., 2009).

Among the Termitidae studied, the frontal gland was absent in workers of Sphaerotermitinae and, its paraphyletic group, Macrotermitinae. Previous studies by Kutalová and others (2013) suggested that the absence of the frontal gland in imagoes of the two Macrotermitinae species studied was probably related to the reduction of the size of the termite, but our study showed there is no frontal gland in workers of a big size species of that group (*Pseudacanthotermes militaris*), suggesting that an absence of this gland could may not be related to the size of the termite head. In all groups which include Sphaerotermitinae as sister–group, the frontal gland was present in workers, with the only exception of Nasutitermitinae in which the frontal gland was quite likely absent or very small (less than 10% of head size). The doubt on the occurrence of this gland is explained by the highly sclerotized head of Nasutitermitinae workers which did not allow easy preparation for TEM and observation under light microscope. All Termitinae and Cubitermitinae presented a frontal gland of conical shape. Surprisingly, the size of the gland in *Neocapritermes taracua* was twice smaller in workers with no blue crystals. Since the blue crystals appear when the termites get older (Šobotník et al., 2012), it suggests that the frontal gland continue its development during ageing.

Considering the evolutionary development of the frontal gland in workers, it is likely that the gland was lost three times in workers: once in the common ancestor of the clade comprising *Termitogeton planus, Prorhinotermes simplex* and Serritermitidae; a second time in *Pseudacanthotermes militaris;* and a third time in *Sphaerotermes sphaerothorax.*

The frontal gland development was not always conserved among related species. In Apicotermitinae, the gland is developed similarly in *Anoplotermes* sp.Q and *Jugositermes* sp. but it was incredibly larger in *T. tucki.* In this last species, the frontal gland covered 50% of the head and was composed by square class 1 cells which release their secretion through a double modified cuticle on the anterior part of the head. The evolutionary routes which led to this particular frontal gland remain unknown but the absence of soldiers in this species may support the need of specific defensive mechanisms in workers, as it has been demonstrated before by modifications in the labial gland of other soldierless species (Sillam–Dussès et al., 2012).

While the frontal gland has no reservoir in workers, this gland has a reservoir of variable size in soldiers and in some imagoes or it is made of a simple epithelium in other imagoes (Noirot, 1969; Prestwich, 1984; Santos et al. 2005; Šobotník et al., 2010b; Kutalová et al., 2013). This data supports the idea of Šobotník et al (2010c) of distinct caste–specific evolutionary routes of the frontal gland, that is the development of this gland in one caste is not an ontogenetic result of the pressure which affects the other castes. Thus, the frontal gland would be an important organ for all castes and species in which it is present.

The fontanelle, described as the frontal gland aperture (Šobotník et al., 2010a) was not observed in any of the species studied. However, some studies have suggested to define this term as any structure, opening or extension on the vertex of any caste, even if it is not an aperture *per se* (Constantini et al., 2018) or as the middle spot in the head of termites (Weesner, 1969; Grassé, 1982). We do not acquire these definitions due to the utility of the term “fontanelle” provides when differentiating an important fact as is the mechanism of releasing the secretion out of the body.

According to our data and previous reports, the structure homology of workers frontal gland with the one found in imagoes and soldiers can be confirmed but there is probably no function homology. The frontal gland is known to have a defensive role in soldiers (Noirot, 1969; Prestwich, 1984; Šobotník et al., 2010a) and likely in imagoes, at least the ones with a frontal gland with reservoir (Piskorski et al., 2009). Indeed, the reservoir allows the accumulation of many chemicals so it is considered as a feature of the defensive function (Chapman, 2013). In termites lacking a frontal gland with reservoir, its function remains uncertain. Some studies have stated that the function of the frontal gland in workers is vestigial (Noirot, 1969; Noirot & Darlington, 2000), while Šobotník and others (2010c) have suggested that it may produce defensive proteinaceous secretions, according to the abundance of RER and secretory inclusions in the cells, and to the absence of SER, feature which was also found in our study. It may also be supported by the absence of a fontanelle, therefore a reservoir would be needed to ensure the contamination of the individual through body rupture in case of a defensive behaviour (Bordereau et al., 1997; Šobotník et al., 2010d). According to this, the frontal gland in workers might have an antibacterial or antifungal function (Rosengaus et al., 2000; Šobotník et al., 2010c), which seems reasonable for workers and funding couples, both of them sharing the same patterns in their frontal glands (Kutalová et al., 2013; Šobotník et al., 2010c). Nonetheless, we cannot exclude other functions for the gland until rigorous studies are performed.

According to the occurrence of the frontal gland in soldiers, imagoes and workers from numerous species belonging to different families, it is clear that this gland is an important organ in termites and it plays a fundamental role in ensuring the colony survival and success. While the structure homology of the gland has been confirmed in all castes, its function may have evolved differently. This may explain why the gland has suffered an extreme reduction of its size in workers, limiting its shape to an epithelial thickening lacking a reservoir.

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Tables

Family	Sub-family	Genus	Species	Sub-caste	llection's coun	Fixative	FG in histology	FG in photos	FG in TEM	Shape	FG diameter	SD	Head lenght	SD	Epithelium lenght	DS	Minimum	Maximum	FG volume	FG/HL	Relative FG Size
Mastotermitid	n.a.	*Mastotermes*	*darwiniensis*	n.a.	Australia	Phosphate bu	No	n.a.	n.a.				3000	50							
Archotermops	n.a.	*Hodotermopsi*	*sjoestedti*	n.a.	Vietnam	Cacodylate bu	No	n.a.	n.a.				2916.67	54.7							
Kalotermitida	n.a.	*Glyptotermes*	sp.	n.a.	Papua New Gu	Phosphate bu	No	n.a.	n.a.				891	44.83							
Kalotermitida	n.a.	*Kalotermes*	*flavicollis*	n.a.	France	Phosphate bu	No	n.a.	n.a.				1195	26.13							
Kalotermitida	n.a.	*Neotermes*	*cubanus*	n.a.	Cuba	Phosphate bu	No	n.a.	n.a.				1784	31.02							
Rhinotermitid	Heterotermitir	*Coptotermes*	*formosanus*	n.a.	China	Phosphate bu	Not clear	Not clear	No				1128	27.86							
Rhinotermitid	Heterotermitir	*Heterotermes*	sp.	n.a.	French Guiana	No fixation	n.a.	No	n.a.												
Rhinotermitid	Heterotermitir	*Reticulitermes*	*lucifugus*	n.a.	Italy	Phosphate bu	No	No	n.a.				1001	15.6							
Rhinotermitid	Prorhinotermi	*Prorhinoterme*	*simplex*	n.a.	Cuba	Phosphate bu	Yes	Not clear	n.a.	Conical	111.09	22.84	800.2	25.4	81.87	30.1	39.41	102.79	2989.95	0.14	0.58
Rhinotermitid	Prorhinotermi	*Termitogeton*	*planus*	n.a.	Papua New Gu	Phosphate bu	Yes	n.a.	n.a.	Conical	113.07	15.28	606.75	3.83	71.53	22.79	10.25	81.71	2419.14	0.19	1.08
Rhinotermitid	Rhinotermitin	*Dolichorhinote*	*longilabius*	n.a.	French Guiana	Cacodylate bu	Not clear	No	No	Conical	76.16	2.07	857.1	11.58	29.94	12.32	9.83	42.51	847.73	0.09	0.13
Rhinotermitid	Rhinotermitin	*Schedorhinote*	sp.	large worker	Cameroon	No fixation	No	No	n.a.				1246	30.02							
Serritermitida	n.a.	*Glossotermes*	*oculatus*	n.a.	French Guiana	Cacodylate bu	Yes	Yes	No	Conical	160.85		883.75	8.38	77.64	28.66	30.91	108.81	4582.78	0.18	0.66
Termitidae	Apicotermitin	*Anoplotermes*	sp. Q	n.a.	French Guiana	Cacodylate bu	Yes	n.a.	Yes	Conical	122.84	15.2	925	9.85	77.62	21.5	50.18	106.44	3423.6	0.13	0.43
Termitidae	Apicotermitin	*Jugositermes*	*tuberculatus*	n.a.	Cameroon	Phosphate bu	Yes	Yes	n.a.	Conical	102.96	20.55	1198.5	13.67	56.05	18.7	14.92	71.73	1933.69	0.09	0.11
Termitidae	Apicotermitin	*Tonsuritermes*	*tucki*	n.a.	Perú	Ethanol	Yes	Yes	n.a.	Cylindrical	703.98	60.41	1359	14.02	28.62	5.54	21.02	35.76	6591.69	0.52	0.26
Termitidae	Cubitermitina	*Basidentiterm*	sp.	n.a.	Cameroon	No fixation	n.a.	Yes	n.a.				857.18	5.43						0	
Termitidae	Cubitermitina	*Cubitermes*	sp.	n.a.	Cameroon	Phosphate bu	Yes	Yes	n.a.	Hemispherica	186.34	6.76	1277.12	16.46	19.94	0.64	19.3	20.58	2008.23	0.15	0.1
Termitidae	Cubitermitina	*Furculitermes*	sp.	n.a.	Cameroon	Phosphate bu	Yes	n.a.	n.a.	Hemispherica	122.46		863.25	11.88	22.65	10.12	7.21	33.94	2176.58	0.14	0.34
Termitidae	Foraminiterm	*Foraminiterme*	*coatoni*	n.a.	Cameroon	No fixation	n.a.	Yes	n.a.				589.22	2.32						0	
Termitidae	Macrotermitir	*Pseudacantho*	*militaris*	small worker	Cameroon	Cacodylate bu	No	n.a.	No				821	41.55							
Termitidae	Macrotermitir	*Pseudacantho*	*militaris*	large worker	Cameroon	Cacodylate bu	No	n.a.	n.a.				1155.7	20.53							
Termitidae	Nasutitermitir	*Agnatotherme*	sp.	n.a.	French Guiana	No fixation	n.a.	Yes	n.a.												
Termitidae	Nasutitermitir	*Atlantitermes*	sp.	n.a.	French Guiana	No fixation	n.a.	Yes	n.a.												
Termitidae	Nasutitermitir	*Nasutitermes*	*gaigei*	n.a.	French Guiana	No fixation	n.a.	No	n.a.				998.57	10.43							
Termitidae	Nasutitermitir	*Nasutitermes*	*guayanae*	n.a.	French Guiana	No fixation	n.a.	No	n.a.												
Termitidae	Nasutitermitir	*Nasutitermes*	*lujae*	n.a.	Congo	Cacodylate bu	Not clear	n.a.	No				1144.25	16.77							
Termitidae	Nasutitermitir	*Nasutitermes*	sp.	n.a.	Cameroon	No fixation	n.a.	No	n.a.												
Termitidae	Nasutitermitir	*Nasutitermes*	sp. A	n.a.	French Guiana	No fixation	n.a.	Yes	n.a.				958.14	9.89							
Termitidae	Nasutitermitir	*Postsubulitem*	sp.	n.a.	Cameroon	No fixation	n.a.	Not clear	n.a.				1144.25	16.77							
Termitidae	Nasutitermitir	*Subulitermes*	sp.	n.a.	French Guiana	No fixation	n.a.	Yes	n.a.				488.9	17.24							
Termitidae	Sphaerotermit	*Sphaeroterme*	*sphaerothorax*	n.a.	Cameroon	Phosphate bu	No	n.a.	n.a.				933.16								
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	n.a.	French Guiana	Phosphate bu	Yes	Yes	No	Conical	129.44		1031	8.28	34.75	7.65	13.05	88.64	3004.26	0.13	0.27
Termitidae	Syntermitinae	*Silvestritermes*	sp.	n.a.	French Guiana	Cacodylate bu	Yes	n.a.	n.a.	Conical	106.47		1003.5	5.77	32.02	2.52	29.77	75.53	2105.65	0.11	0.21
Termitidae	Termitinae	*Globitermes*	*sulphureus*	n.a.	China	Cacodylate bu	Yes	n.a.	n.a.	Hemispherica	169.79		1087.37	15.01	47.6	8.3	33.41	53.7	4774.8	0.16	0.37
Termitidae	Termitinae	*Microcerotern*	sp.	n.a.	French Guiana	Cacodylate bu	Yes	n.a.	n.a.	Conical	140	20.47	943.25	7.91	39.51	19.37	9.81	65.57	2403.65	0.15	0.29
Termitidae	Termitinae	*Neocapriterme*	*taracua*	blue workers	French Guiana	Phosphate bu	Yes	n.a.	n.a.	Conical	183.91		1074.65	12.74	33.6	18.87	8.65	57.86	2786.27	0.17	0.22
Termitidae	Termitinae	*Neocapriterme*	*taracua*	Withe workers	French Guiana	Phosphate bu	Yes	n.a.	Yes	Conical	100		1020.75	10.28	50.56	11.53	12.83	86.53	2265.72	0.1	0.21
Termitidae	Termitinae	*Orthognatote*	sp.	n.a.	French Guiana	No fixation	n.a.	Yes	n.a.												
Termitidae	Termitinae	*Pericapriterme*	sp.	n.a.	Cameroon	No fixation	n.a.	Yes	n.a.				782.77	12.37						0	
Termitidae	Termitinae	*Planicapritern*	sp.	n.a.	French Guiana	Cacodylate bu	Yes	n.a.	n.a.	Conical	126.58		710.63	10.06	70.88	23.46	20.65	108.15	3584.51	0.18	1
Termitidae	Termitinae	*Spinitermes*	sp.	n.a.	French Guiana	Cacodylate bu	Yes	n.a.	n.a.	Conical	81.2		875.75	15.13	36.93	15.99	16.07	56.6	1203.4	0.09	0.18
Termitidae	Termitinae	*Termes*	*hospes*	n.a.	Congo	Cacodylate bu	Yes	n.a.	n.a.	Conical	182.2		758	21.15	65.65	35.74	23.95	123.13	5874.23	0.24	1.35

Table 1. List of studied termite species, with indication of the species collection location, fixation, subcastes (if any), buffer used, number of repetitions, and frontal gland and head measures (μm). Blank spaces indicate lack of information. Abbreviations: n.a.= not applicable.

Family	Sub-family	Genus	Species	References	Sub-caste	1	2	3
Mastotermitidae		*Mastotermes*	*darwiniensis*	1; pers. observ.		0	n.a.	n.a.
Hodotermitidae			spp.	1; pers. observ.		0	n.a.	n.a.
Archotermopsidae			spp.	1; pers. observ.		0	n.a.	n.a.
Kalotermitidae		*Kalotermes*	spp.	1; 3; pers. observ.		0	n.a.	n.a.
Kalotermitidae		*Neotermes*	sp.	2; pers. observ.		0	n.a.	n.a.
Rhinotermitidae	Heterotermitinae	*Coptotermes*	*formosanus*	3		2	3	3
Rhinotermitidae	Heterotermitinae	*Coptotermes*	*lacteus*	14; 1		2	3	3
Rhinotermitidae	Heterotermitinae	*Coptotermes*	spp.	15; 5; 1; 3		2	3	3
Rhinotermitidae	Heterotermitinae	*Coptotermes*	*testaceus*	1; pers. observ.		2	3	3
Rhinotermitidae	Heterotermitinae	*Reticulitermes*	*flavipes*	5; 18		2	3	3
Rhinotermitidae	Heterotermitinae	*Reticulitermes*	*lucifugus*	19; 18		2	3	3
Rhinotermitidae	Heterotermitinae	*Reticulitermes*	spp.	3; 17		2	3	3
Rhinotermitidae	Prorhinotermitinae	*Prorhinotermes*	*simplex*	8		2	3	3
Rhinotermitidae	Prorhinotermitinae	*Prorhinotermes*	spp.	3; 5; 9		2	3	3
Rhinotermitidae	Prorhinotermitinae	*Termitogeton*	*planus*	3; 10; pers. observ.		2	2	3
Rhinotermitidae	Psammotermitinae	*Psammotermes*	*hybostoma*	16; pers. observ.	Small	2	3	3
Rhinotermitidae	Psammotermitinae	*Psammotermes*	*hybostoma*	16; pers. observ.	Medium	2	1	3
Rhinotermitidae	Psammotermitinae	*Psammotermes*	*hybostoma*	16; pers. observ.	large	2	1	2
Rhinotermitidae	Rhinotermitinae	*Dolichorhinotermes*	*longilabius*	3; pers. observ.	small	2	3	3
Rhinotermitidae	Rhinotermitinae	*Dolichorhinotermes*	*longilabius*	3; pers. observ.	large	2	3	3
Rhinotermitidae	Rhinotermitinae	*Dolichorhinotermes*	*tenebrosus*	5	small	2	3	3
Rhinotermitidae	Rhinotermitinae	*Rhinotermes*	spp.	7; 3; 5; pers. observ.	small	2	3	1
Rhinotermitidae	Rhinotermitinae	*Schedorhinotermes*	*putorius*	1; 4	small	2	3	3
Rhinotermitidae	Rhinotermitinae	*Schedorhinotermes*	*putorius*	1; 4	large	2	3	3
Rhinotermitidae	Rhinotermitinae	*Schedorhinotermes*	sp.	1; 3	small	2	3	3
Rhinotermitidae	Rhinotermitinae	*Schedorhinotermes*	spp.	4; 5; 3; 6	small	2	3	3
Serritermitinae		*Glossotermes*	*oculatus*	11		2	3	3
Serritermitinae		*Serritermes*	*serrifer*	12; 13		2	3	3
Termitidae	Cubitermitinae	*Apilitermes*	*longiceps*	34		2	1	1
Termitidae	Cubitermitinae	*Cubitermes*	spp.	3		2	1	1
Termitidae	Cubitermitinae	*Proboscitermes*	sp.	1; 3		2	1	1
Termitidae	Foramitermitinae	*Foramitermes*	spp.	3		2	1	1
Termitidae	Macrotermitinae	*Macrotermes*	spp.	3		2	1	1
Termitidae	Macrotermitinae	*Macrotermes*	*subhyalinus*	20	small	2	1	1
Termitidae	Macrotermitinae	*Macrotermes*	*subhyalinus*	20	large	2	1	1
Termitidae	Nasutitermitinae	*Atlantitermes*	sp.	25		2	2	1
Termitidae	Nasutitermitinae	*Constrictotermes*	*cyphergaster*	25		2	2	1
Termitidae	Nasutitermitinae	*Diversitermes*	*diversimilis*	25		2	2	1
Termitidae	Nasutitermitinae	*Nasutitermes*	spp.	3; 7; 22; 25; 26; 36		2	2	1
Termitidae	Nasutitermitinae	*Subulitermes*	*microssoma*	25		2	2	1
Termitidae	Nasutitermitinae	*Trinervitermes*	*trinervius*	1		2	2	1
Termitidae	Nasutitermitinae	*Velocitermes*	spp.	3; 14; 22; 25; 35		2	2	1
Termitidae	Sphaerotermitinae	*Sphaerotermes*	*sphaerothorax*	pers. observ.		2	1	1
Termitidae	Syntermitinae	*Cornitermes*	*cumulans*	23		2	1	1
Termitidae	Syntermitinae	*Cornitermes*	*cumulans*	25		2	1	1
Termitidae	Syntermitinae	*Embiratermes*	*chagresi*	3		2	1	1
Termitidae	Syntermitinae	*Embiratermes*	*festivellus*	27		2	1	1
Termitidae	Syntermitinae	*Embiratermes*	*heterotypus*	25		2	1	1
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	3; 26		2	1	1
Termitidae	Syntermitinae	*Procornitermes*	*araujoi*	23		2	1	1
Termitidae	Syntermitinae	*Rhynchotermes*	*nasutissimus*	23		2	2	1
Termitidae	Syntermitinae	*Silvestritermes*	*euamignathus*	28; 25; 3		2	1	1
Termitidae	Syntermitinae	*Silvestritermes*	*holmgreni*	14; 1; 3		2	1	1
Termitidae	Syntermitinae	*Syntermes*	*dirus*	22; 21		2	1	1
Termitidae	Syntermitinae	*Syntermes*	*grandis*	21		2	1	1
Termitidae	Syntermitinae	*Syntermes*	*nanus*	22		2	1	1
Termitidae	Syntermitinae	*Uncitermes*	*teevani*	3; 24		2	1	1
Termitidae	Termitinae	*Cavitermes*	sp.	32		2	1	1
Termitidae	Termitinae	*Cavitermes*	*tuberosus*	32; 33		2	1	1
Termitidae	Termitinae	*Dentispicotermes*	*brevicarinatus*	31		2	3	3
Termitidae	Termitinae	*Drepanotermes*	*rubriceps*	14; 3		2	1	1
Termitidae	Termitinae	*Globitermes*	*sulphureus*	29; 30		2	3	3
Termitidae	Termitinae	*Inquilinitermes*	*inquilinus*	32		2	1	1
Termitidae	Termitinae	*Microcerotermes*	sp.	29; pers. observ.		2	1	1
Termitidae	Termitinae	*Pericapritermes*	sp.	2		2	1	1
Termitidae	Termitinae	*Spinitermes*	*brevicornutus*	27		2	1	1
Termitidae	Termitinae	*Spinitermes*	*trispinosus*	32		2	1	1
Termitidae	Termitinae	*Termes*	*aff. fatalis*	32		2	1	1
Termitidae	Termitinae	*Termes*	sp.	2		2	1	1

Table S1. The frontal gland in soldiers. Review of information plus personal observations of the frontal gland data, indicating the species that have been analyzed in previous studies, the reference of the study, the frontal gland development (Absent=0, epithelial thickening=1, saccular=2), frontal opening presence (absent=0, present=1), frontal gland size (confined to the head=1, extending to the thorax=2, extending to the abdomen=3) and cell shape (cubic=1, columnar=2, squamous=3) for termite soldiers. Abbreviations: n.a.= not applicable.

Family	Sub-family	Genus	Species	References	1	2	3
Rhinotermitidae	Heterotermitinae	*Coptotermes*	*formosanus*	57	2	1	3
Rhinotermitidae	Heterotermitinae	*Coptotermes*	*testaceus*	57	2	1	3
Rhinotermitidae	Heterotermitinae	*Heterotermes*	*paradoxus*	57	2	2	1
Rhinotermitidae	Heterotermitinae	*Heterotermes*	sp.	7	2	1	3
Rhinotermitidae	Heterotermitinae	*Heterotermes*	*tenuis*	57	2	1	1
Rhinotermitidae	Heterotermitinae	*Heterotermes*	*venestus*	pers. observ.	2	1	1
Rhinotermitidae	Heterotermitinae	*Reticulitermes*	*lucifugus*	57	2	1	1
Rhinotermitidae	Prorhinotermitinae	*Prorhinotermes*	*simplex*	58	2	1	1
Rhinotermitidae	Prorhinotermitinae	*Termitogeton*	*planus*	57	2	1	2
Rhinotermitidae	Psammotermitinae	*Psammotermes*	*allocerus*	57	1	1	1
Rhinotermitidae	Psammotermitinae	*Psammotermes*	*hybostoma*	57	1	1	1
Rhinotermitidae	Rhinotermitinae	*Dolichorhinotermes*	*longilabius*	57	2	3	1
Rhinotermitidae	Rhinotermitinae	*Parrhinotermes*	*browni*	57; pers. observ.	2	2	3
Rhinotermitidae	Rhinotermitinae	*Rhinotermes*	spp.	7; 57; pers. observ.	2	3	1
Rhinotermitidae	Rhinotermitinae	*Schedorhinotermes*	*translucens*	57	2	3	1
Serritermitidae		*Glossotermes*	*oculatus*	57	2	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*janus*	59	1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	sp.	59; pers. observ.	1	1	2
Termitidae	Apicotermitinae	*Aparatermes*	sp.	59	1	1	2
Termitidae	Apicotermitinae	*Astalotermes*	*quietus*	59	1	1	2
Termitidae	Apicotermitinae	*Longustitermes*	*manni*	59	1	1	1
Termitidae	Apicotermitinae	*Ruptitermes*	sp.	59; pers. observ.	1	1	2
Termitidae	Cubitermitinae	*Cubitermes*	*fungifaber*	1	1	1	1
Termitidae	Foramitermitinae	*Foramitermes*	*coatoni*	59	2	1	1
Termitidae	Macrotermitinae	*Ancistrotermes*	*cavithorax*	pers. observ.	2	1	2
Termitidae	Macrotermitinae	*Macrotermes*	*bellicosus*	1	2	1	2
Termitidae	Macrotermitinae	*Microtermes*	*toumodiensis*	59	0	n.a.	n.a.
Termitidae	Macrotermitinae	*Odontotermes*	*horni*	60	2	1	2
Termitidae	Macrotermitinae	*Odontotermes*	*pauperans*	59	2	1	2
Termitidae	Macrotermitinae	*Protermes*	sp.	59	0	n.a.	n.a.
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	59	2	1	2
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*spiniger*	59	2	1	2
Termitidae	Nasutitermitinae	*Diwaitermes*	*kanehirae*	59	1	1	1
Termitidae	Nasutitermitinae	*Grallatotermes*	*grallator*	59	1	1	1
Termitidae	Nasutitermitinae	*Hospitalitermes*	*papuanus*	59	1	1	2
Termitidae	Nasutitermitinae	*Nasutitermes*	*chaquimayensis*	7	1	1	1
Termitidae	Nasutitermitinae	*Nasutitermes*	*princeps*	59	1	1	1
Termitidae	Nasutitermitinae	*Subulitermes*	sp.	59	1	1	1
Termitidae	Sphaerotermitinae	*Sphaerotermes*	*sphaerothorax*	59	1	1	1
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	59	1	1	2
Termitidae	Syntermitinae	*Rhynchotermes*	*perarmatus*	59	1	1	2
Termitidae	Syntermitinae	*Silvestritermes*	*holmgreni*	59	1	1	1
Termitidae	Syntermitinae	*Syntermes*	*molestus*	59	1	1	2
Termitidae	Syntermitinae	*Syntermes*	sp.	pers. observ.	1	1	2
Termitidae	Termitinae	*Amitermes*	*beaumonti*	59	1	1	1
Termitidae	Termitinae	*Crepititermes*	*verruculosus*	59	1	1	1
Termitidae	Termitinae	*Dentispicotermes*	*brevicarinatus*	59	1	1	2
Termitidae	Termitinae	*Ephalotermes*	*argutus*	59	1	1	2
Termitidae	Termitinae	*Microcerotermes*	sp.	pers. observ.	1	1	1
Termitidae	Termitinae	*Neocapritermes*	*araguaia*	59; pers. observ.	1	1	2
Termitidae	Termitinae	*Pericapritermes*	*odontomachus*	59	1	1	2
Termitidae	Termitinae	*Pericapritermes*	*papuanus*	pers. observ.	1	1	2
Termitidae	Termitinae	*Protocapritermes*	*odontomachus*	pers. observ.	1	1	2
Termitidae	Termitinae	*Termes*	*fatalis*	59; pers. observ.	1	1	2
Termitidae	Termitinae	*Termes*	*hospes*	1	1	1	2
Termitidae	Termitinae	*Termes*	sp. B	59	1	1	2

Table S2. The frontal gland in imagoes. Review of information plus personal observations of the frontal gland data, indicating the species that have been analyzed in previous studies, the reference of the study, the frontal gland development (Absent=0, epithelial thickening=1, saccular=2), frontal opening presence (absent=0, present=1), frontal gland size (confined to the head=1, extending to the thorax=2, extending to the abdomen=3) and cell shape (cubic=1, columnar=2, squamous=3) for termite imagoes. Abbreviations: n.a.= not applicable; pers. Observ. = personal observations.

Family	Sub-family	Genus	Species	References	Sub-caste	1	2	3
Mastotermitidae		*Mastotermes*	*darwiniensis*	This study		0	n.a.	n.a.
Archotermopsidae		*Hodotermopsis*	*sjoestedti*	This study		0	n.a.	n.a.
Kalotermitidae		*Glyptotermes*	sp.	This study		0	n.a.	n.a.
Kalotermitidae		*Kalotermes*	sp.	This study		0	n.a.	n.a.
Kalotermitidae		*Neotermes*	*cubanus*	This study		0	n.a.	n.a.
Rhinotermitidae	Heterotermitinae	*Coptotermes*	*formosanus*	This study		0	n.a.	n.a.
Rhinotermitidae	Heterotermitinae	*Reticulitermes*	*lucifugus*	61		0	n.a.	n.a.
Rhinotermitidae	Prorhinotermitinae	*Prorhinotermes*	*simplex*	This study		1	1	2
Rhinotermitidae	Prorhinotermitinae	*Termitogeton*	*planus*	This study		1	1	2
Rhinotermitidae	Rhinotermitinae	*Dolichorhinotermes*	*longilabius*	This study		1	1	1
Rhinotermitidae	Rhinotermitinae	*Schedorhinotermes*	sp.	This study		0	n.a.	n.a.
Serritermitidae		*Glossotermes*	sp.	This study		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*banksi*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*distans*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*fumosus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*gracilis*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*gripunctatus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*hagemi*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*jheringi*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*manni*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*meridianus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*nr. subterraneus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*pacificus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*parvus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	sp. AF	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	sp. AR	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	sp. K	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	sp. Y	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Anoplotermes*	*turricola*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Aparatermes*	*cingulatus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Aparatermes*	*nr. cingulatus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Astalotermes*	*quietus*	61; pers. observ.		1	1	2
Termitidae	Apicotermitinae	*Grigiotermes*	*bequaerti*	61		1	1	2
Termitidae	Apicotermitinae	*Grigiotermes*	*nr. metoecus*	61		1	1	2
Termitidae	Apicotermitinae	*Jugositermes*	*tuberculatus*	This study		1	1	2
Termitidae	Apicotermitinae	*Ruptitermes*	*arboreus*	61		1	1	2
Termitidae	Apicotermitinae	*Ruptitermes*	*proratus*	61		1	1	2
Termitidae	Apicotermitinae	*Ruptitermes*	*reconditus*	61		1	1	2
Termitidae	Apicotermitinae	*Ruptitermes*	*xanthochiton*	61		1	1	2
Termitidae	Apicotermitinae	*Silvestritermes*	sp.	This study		1	1	2
Termitidae	Apicotermitinae	*Tonsuritermes*	*tucki*	This study		1	1	1
Termitidae	Cubitermitinae	*Basidentitermes*	sp.	This study		1	1	1
Termitidae	Cubitermitinae	*Cubitermes*	sp.	This study		1	1	1
Termitidae	Cubitermitinae	*Furculitermes*	sp.	This study		1	1	1
Termitidae	Foramitermitinae	*Foramitermes*	*coatoni*	This study		1	1	1
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	This study	small	0	n.a.	n.a.
Termitidae	Macrotermitinae	*Pseudacanthotermes*	*militaris*	This study	large	0	n.a.	n.a.
Termitidae	Nasutitermitinae	*Nasutitermes*	*guyanae*	This study		1	1	1
Termitidae	Nasutitermitinae	*Nasutitermes*	*lujae*	This study		0	n.a.	n.a.
Termitidae	Sphaerotermitinae	*Sphaerotermes*	*sphaerothorax*	This study		0	n.a.	n.a.
Termitidae	Syntermitinae	*Embiratermes*	*neotenicus*	This study		1	1	2
Termitidae	Termitinae	*Globitermes*	*sulphureus*	This study		1	1	2
Termitidae	Termitinae	*Microcerotermes*	sp.	This study		1	1	2
Termitidae	Termitinae	*Neocapritermes*	*taracua*	This study		1	1	2
Termitidae	Termitinae	*Orthognathotermes*	sp.	This study		1	1	2
Termitidae	Termitinae	*Pericapritermes*	sp.	This study		1	1	2
Termitidae	Termitinae	*Planicapritermes*	sp.	This study		1	1	2
Termitidae	Termitinae	*Spinitermes*	sp.	This study		1	1	2
Termitidae	Termitinae	*Termes*	*hospes*	This study		1	1	2

Table S3. The frontal gland in workers. Review of information about the frontal gland in termite workers plus the data from this study indicating the species that have been analyzed in previous studies, the reference of the study, the frontal gland development (Absent=0, epithelial thickening=1, saccular=2), frontal opening presence (absent=0, present=1), frontal gland size (confined to the head=1, extending to the thorax=2, extending to the abdomen=3) and cell shape (cubic=1, columnar=2, squamous=3). Abbreviations: n.a.= not applicable.

Paper 4: *Tonsuritermes*, a new soldierless termite genus and two new species from South America (Blattaria: Isoptera: Termitidae: Apicotermitinae)

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Résumé

Un nouveau genre, *Tonsuritermes* Cancello & Constantini gen. nov., découvert en Amérique du Sud est décrit. Les principales caractéristiques morphologiques du nouveau genre sont une glande frontale et des protibia remarquables avec deux rangées de soies en forme de colonne vertébrale. Deux nouvelles espèces de *Tonsuritermes* sont décrites: *T. tucki* Cancello & Constantini sp. nov. et *T. mathewsi* Cancello & Constantini sp. nov. Des comparaisons, des mesures, une carte, l'histologie de la glande frontale et une illustration de tous les aspects morphologiques fondamentaux sont fournis.

Mots–clefs: Glande frontale, Néotropiques, fontanelle, histologie, Apicotermitinae

Abstract

A new genus, *Tonsuritermes* Cancello & Constantini gen. nov., is described from South America. The main morphological features of the new genus are a remarkable frontal gland and protibia with two rows of spine–like bristles. Two new species of *Tonsuritermes* are described: *T. tucki* Cancello & Constantini sp. nov. and *T. mathewsi* Cancello & Constantini sp. nov. Comparisons, measurements, a map, histology of the frontal gland, and illustration of all fundamental morphological aspects are provided.

Keywords: Frontal gland, Neotropics, fontanelle, histology, Apicotermitinae

Introduction

The ecological relevance of the Apicotermitinae is validated by their abundance and diversity. In South America, this subfamily of Termitidae represents more than 30% of the species in termite assemblages in Amazonia (Ackerman *et al*. 2009; Bourguignon *et al.* 2011; Palin *et al.* 2011), in French Guiana (Davies 2003), and in the savannas and grasslands of central Brazil (Carrijo *et al.* 2009; Oliveira *et al.* 2013). Similar findings are also reported for Brazil's Atlantic Forest (Cancello 1994, 2014). Despite its importance, the taxonomy of the group is still problematic, and in the last years some efforts have been made to further elucidate the group (Bourguignon *et al*. 2010; Scheffrahn 2013; Acioli & Constantino 2015; Bourguignon *et al.* 2016; Scheffrahn *et al.* 2017).

The neotropical Apicotermitinae are characterized by the absence of the soldier caste, so all the taxonomy of the group is based on workers and imagoes. Although there are many earlier species described from imagoes only, it is now evident that workers possess the robust morphology necessary for species discrimination. Many studies have shown the importance of the digestive tube characters for termite taxonomy, studied mainly in the worker caste (Sands, 1972; Noirot, 1995, 2001). Despite this recent trend, external morphology of workers is still poorly studied. For instance, the frontal gland, that is a synapomorphy of the clade comprising the families Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae (Deligne *et al.* 1981; Prestwich 1984; Krishna *et al.* 2013), was extensively studied in soldiers but neglected in workers (see Šobotník *et al.* 2010a, b).

In this paper, we described a new genus including two new South American species of Apicotermitinae with a remarkable frontal gland.

Material and methods

The terminology used to describe worker mandibles follows Sands (1998, Fig. 5), while worker digestive tube descriptions follows Noirot (2001). Terms used for pilosity are comparative: bristles are stiff hairs with well marked bases; spine–like bristles are shorter and thicker than bristles; hairs are shorter and finer than bristles, without conspicuous bases.

Strictly speaking, what is referred to as "fontanelle" is the frontal gland aperture in soldiers (Šobotník *et al.* 2010a). However, this name also refers to the whitish region of the head of workers and imagoes. The peculiarity of this region among different castes was historically reported in the literature (Banks 1920, p. 2; Weesner 1969, p. 25), and some authors named it the “middorsal spot” in some worker and imagoes (Torre–Bueno 1989, p.452; Weesner 1969, p. 31). Classical works, however, such as Grassé (1982, p.27), use the term fontanelle for all castes.

Therefore, will be defined as the fontanelle any structure, opening, or extension on the vertex of all castes even if not being the aperture of the frontal gland *per se*. Adoption of a new terminology may be used in the future, if new studies corroborate the non–homology of these structures in different castes (i.e. embryological and/or gene expression studies).

The samples were stored in an 80% ethanol solution before examination. Worker heads of *Tonsuritermes tucki* sp. nov. with mandibles removed were embedded into Spurr resin, polymerised, and sectioned into 0.5 µm slides using a Reichert Ultracut ultramicrotome. The same samples were used for ultrastructural study, using a Jeol 1011 Transmission Electron microscope.

Images of the head capsule and digestive tube were taken with a Leica DFC 295 digital camera attached to a Leica M205C stereomicroscope. Specimens were placed in a plastic Petri dish containing 80% ethanol hand sanitizer. A mirror was placed underneath the dish to highlight pilosity. Mandibles and enteric valves were mounted on slides with PVA Mounting Medium (BioQuip #6371A) and the images were taken with a Leica DM750 compound microscope attached to a Leica ICC50HD camera.

All images were composed of multiple photomicrographs taken at different focal planes that were merged with Helicon Focus 6 software. Measurements were taken with a micrometric reticule on the eyepiece of a stereomicroscope.

The following morphometric characters were measured as defined by Roonwal (1970) and indicated in parenthesis: *for alates*—maximum diameter of compound eyes (48); inter–eye distance (52); maximum diameter of ocellus (55); minimum diameter of ocellus (56); eye–ocellus distance (57); length of pronotum (65); width of pronotum (68); length of forewing with scale (73); *for alates and workers*—length of the head to lateral base of mandibles (5); width of head (17); maximum diameter of fontanelle aperture (26); length of the pro– and metatibia (85). We also measured the maximum width of the protibia which is often enlarged in apicotermitine workers. The distribution map was created using QGis 2.14. The list of material examined is sorted by country (uppercase), state or province and locality. Collection data is organized as follows: date, name of the collector, collection, and collection number.

The institutional collections acronyms cited in this paper are: MZUSP – Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil; UFG – Universidade Federal de Goiás, Goiás, Brazil; UF – Fort Lauderdale Research and Education Center, University of Florida, Davie, Florida, United States.

Taxonomy

Tonsuritermes Cancello & Constantini gen. nov.

Type–species. *Tonsuritermes tucki*, by present designation.

Diagnosis. Worker and imago. Fontanelle very large in dorsal view, ranging from 1/4 to 3/4 the diameter of the head capsule (Figs. 1B; 2A, C, E); two rows of spine–like bristles on the inner face of the protibia (Fig. 3A–C).

Description. Imago (Figs. 1; 3A, E–G, I). Head capsule trapezoidal in dorsal view, flattened dorsoventrally in profile (Fig. 1A, B). Two frontal marks located in dorsal view between the postclypeus and ocellus, above the antenna insertion (Fig. 1B, arrows), larger than the ocelli, and two smaller triangular marks between the frontal marks and the fontanelle (Fig. 1B). Fontanelle massive rounded, slightly depressed, occupying 1/2 of the head capsule in dorsal view (Fig. 1B). Eyes rounded, smaller in diameter than the fontanelle. Ocellus small, elliptical, separated from the eye margin by its diameter. Postclypeus moderately inflated, with median line conspicuous (Fig. 1A, B). Labrum with hyaline distal margin. Left mandible (Fig. 3I) with apical tooth much more prominent than M1+2, incision conspicuous, M3 triangular with margins forming acute angle with the tip, point of molar tooth not hidden by molar prominence; molar prominence moderately developed; molar region without ridges. Right mandible (Fig. 3I) with apical tooth much more prominent than M1, M2 triangular with margins forming an obtuse angle with the tip; molar plate moderately developed; molar region without ridges. Pronotum narrower than head without eyes (Fig. 1B, Table 1), anterior margin straight (in Fig. 1B, the image became slightly deformed during the stacking process), lateral margins convex, converging posteriorly. Wings ornamented with asteroid micrasters (Fig. 3F– G). Profemur subcylindrical. Protibia thin, not inflated (Fig. 3A). Females generally larger than males (Table 1). Pilosity of head capsule with a dense coverage of uniform bristles and short hairs. Labrum with two long bristles and several hairs. Pronotum with bristles and short hairs, mainly along the margins. Tergites and sternites with short bristles and short hairs covering the center of the plates. Procoxa with 4–5 thick bristles. Profemur densely covered by bristles of different lengths (Fig. 3A). Protibia with two rows of 10–12 thick bristles, with the femur, resembling a weakly “raptorial” leg (Fig. 3A). Margin and veins of wings densely covered with hairs (Fig. 3E, F). Coloration of head capsule dark brown, frontal marks and triangular marks slightly lighter than the rest of the head capsule, with poorly defined margins. Fontanelle concolorous with the head capsule (Fig. 1B). Pronotum slightly lighter than the head capsule (Fig. 1B).

Worker. Mono– or dimorphic (Figs. 2; 3B–D, H, 4, 5). Head capsule flattened dorsoventrally in profile, rounded in dorsal view. Fontanelle margins well delineated, depressed within vertex, occupying 1/4 to 3/4 of the head capsule (Fig. 2A,C, E, see histological discussion below). Antenna with 14 articles. Postclypeus moderately to highly inflated. Left mandible (Fig. 3H) with apical tooth longer than M1+2, cutting edge between M1+2 and incision, incision conspicuous, M3 triangular with lateral sides forming an acute angle with the tip; molar tooth not hidden by molar prominence; molar prominence well developed; molar region concave, without ridges. Right mandible (Fig. 3H) with apical tooth longer than M1; M2 triangular with margins forming an acute angle with the tip; molar plate well developed; molar region concave, without ridges. Pronotum in lateral view (Fig. 2B, D, F) with anterior lobe much longer and forming right angle with posterior lobe. Mesonotum and metanotum subretangular. Thoraco–abdominal glands or dehiscent organs absent. Profemur with ventral surface forming a groove between the two rows of bristles. Protibia with ventral face strongly flattened, resembling an interlocking raptorial leg (Fig. 3B, C). Digestive tube (Fig. 4) with inner mixed segment vestigial (Fig. 4C); P1 with uniform diameter throughout, forming an inverted 'C' in ventral view; P2 not armed, composed of six symmetrical cushions covered with faint polygonal scales (Fig. 4E–F) and 3–5 small short triangular spines in the proximal region of the cushions (Fig. 4F). Enteric valve seating tubular, without lobes. Paunch with P3a pyriform and P3b forming an S–shaped, isthmus conspicuous in almost all the specimens; P4 of uniform width, passing under mesenteron in the sagital line and making a 180º loop in the left side of body right before the P5. Pilosity of head capsule covered by bristles of variable orientation and length. Pronotum with long bristles with variable orientation, in greater number on margin of the anterior lobe and in the rounded regions in the posterior lobe. Tergites and sternites with short bristles in the center of the plates, in variable orientations. Procoxa with 7–11 thick bristles. Profemur with two rows of bristles less striking and organized than the protibia (Fig. 3B, C). Protibia with two well marked rows of spine–like bristles (thick and short) on inner margin; number of spine–like bristles varying from six to 16 (Fig. 3B, C). Head capsule coloration whitish yellow; fontanelle lighter (Fig. 2A, C, E, 3D).

Comparisons. *Tonsuritermes*, with its massive worker and imago fontanelle, differs from all known New World Apicotermitinae. *Tonsuritermes* is close to *Aparatermes* Fontes, 1986 (worker width of head of *A. abbreviatus* = 1.16 mm (mean), Fontes 1986) and *Ruptitermes* Mathews, 1977 in size (worker width of head of *R. reconditus* (Silvestri, 1901) = 1.13 mm (mean), Acioli & Constantino 2015). In Snyder's (1926) description for *Anoplotermes grandifons*, he highlights the fontanelle as a diagnostic feature of the species with a description based only on a wingless female: *'Fontanelle a very prominent hyaline oblong depression, slightly on a bias, 0.25 mm in length and 0.20 mm in width'.* The fontanelle of the imago of *Tonsuritermes tucki* is not hyaline and has the maximum diameter between 0.34–0.36 mm. Snyder does not make any mention of bristles or spine–like bristles in the legs. Efforts were made to examine the type material of *A. grandifons*, but without success. Thus, for the difficulty of comparisons using only the description of the imagoes we considered our samples different from *A. grandifons*. The protibia and profemur with thick spines, although also a very distinct characteristic in relation to the other described termites in the subfamily, has been observed in other groups of termites not related to the genus and not yet described (JC, unpublished data).

Histological discussion. The large region of frons in *Tonsuritermes tucki* workers is formed by a frontal gland of unique structure, even compared to other members of the *Anoplotermes*–group studied previously (Šobotník *et al*. 2010a) (see Fig. 5A–C). This observation was corroborated by transmission electron microscopy that revealed the glandular nature of the tissue. The most unusual feature is the cuticle overlying the frontal gland, which is made of two discrete layers of modified cuticle, both being very different from normal head cuticle (see Fig. 5B). The two–layered glandular cuticle is a unique character not shared by any other termite gland studied so far, just slightly reminding the two cuticles occurring in presoldiers of *Prorhinotermes simplex* (Hagen, 1858) (Šobotník *et al*. 2004). The glandular cells were severely damaged by the 80% alcohol, and the specialised secretory organelles (e.g. microvilli, endoplasmic reticulum or Golgi apparatus) could not be observed. We also cannot confirm the existence of the second cell layer and the envelope cells observed in *Aparatermes* (Šobotník *et al*. 2010a). However, locally the cell remanent contained electron–lucent secretory vesicles and abundant mitochondria. We hope to acquire the living material in the future, allowing us to describe the ultrastructure of this peculiar secretory organ in detail.

Etymology. From Latin *tonsura* (“a clipping, trimming”). “Tonsure” is named after Franciscan monks’ haircut, which the fontanelle resembles, particularly in the workers. The idea for the name came from a note written by Filippo Silvestri (MZUSP 1199, 03.i.1909) for a sample of this genus he examined where he indicated a possible name for the species: "magnotonsura".

Distribution. Neotropical region: Brazil, Colombia, French Guiana, Paraguay, Peru (Fig. 6).

***Tonsuritermes tucki* Cancello & Constantini sp. nov**.
(Figs.1; 2A–D, 3A–B, D–I, 4, 5, 6)

Holotype. Worker type 1 from lot MZUSP 6480 (in a separate vial with the remaining sample).

Type–locality. BRAZIL. Santa Catarina: Campos Novos, lat 27.40S, long 51.22W.
Type–repository. MZUSP

Paratypes. BRASIL. Bahia: Andaraí, lat 12.8072S, long 41.3313W, 13–14.xii.1990, EM Cancello & MT Ponte coll., MZUSP 10367; Goiás: Caldas Novas, Parque Estadual da Serra de Caldas Novas, lat 17.7927S, long 48.7038W, 23.iii.2008, DE Oliveira coll., UFG 1740, 1741; Minas Gerais: Poços de Caldas, Morro do Ferro (Norte), lat 21.9166S, long 46.5166W, 18.ix. 1967, RL Araujo coll., MZUSP 0456; Mato Grosso do Sul: Aquidauana, lat 20.4711S, long 55.7872W, 29.v.2012, AR Abot coll., MZUSP 27373; Rio de Janeiro: Santa Maria Madalena, Parque Estadual do Desengano, lat 21.9522S, long 42.0148W, 24.xi.2016, JP Constantini coll., MZUSP 26687; Rondônia: Porto Velho, Abunã, lat 9.5970S, long 65.3645W, 09.iii.2010, TF Carrijo & RG Santos coll., MZUSP 13039; Jaci Paraná, lat 9.0245S, long 64.2530W, 16.ix.2010, TF Carrijo & RG Santos coll., MZUSP 17193; lat 9.4502S, long 64.3674W, 12.i.2011, RG Santos & CY Mandai coll., MZUSP 17196; lat 9.0293S, long 64.2499W, 07.i.2011, RG Santos & CY Mandai coll. , MZUSP 17197; lat 9.4526S, long 64.3900W, 20.i.2010, TF Carrijo & RG Santos coll., MZUSP 17198; Nova Mutum Paraná, lat 9.2869S, long 64.7445W, 09.i.2011, RG Santos & CY Mandai coll. , MZUSP 17194, 17195; (same holotype sample), 23.xii.1975, RL Araujo coll., MZUSP 6480, (imago, workers); São Paulo: São Paulo, lat 23.53S, long 46.62W, 3.i.1909, Luederwaldt coll., MZUSP 1199. COLOMBIA. Meta: San Juan de Arama, lat 3.34639N, long 73.8894W, vii.1992, LO Sánchez coll., MZUSP 24480. FRENCH GUIANA. Régina: Nouragues Nature Reserve, lat 4.0833S, long 52.6833W, T Bourguignon coll.; PARAGUAY. Cordillera: Vallenzuela, lat 25.5833, long 56.8667W, 04.i.1992, L Cabello & B Barrios coll., MZUSP 10855. PERU. Madre de Dios: Tambopata, Research Lodge, lat 13.13700S, long 69.61200W, 09.ix.2015, L Carnohan, UF PU1104.

Diagnosis. Dimorphic worker with fontanelle of two sizes; head capsule with two lengths of well marked bristles and bristles on tergites uniformly oriented backwards.

Imago. As described for the genus.

Worker. Dimorphic (Fig. 2A–D). Head capsule with two lengths of well marked bristles. Bristles on tergites uniformly oriented backwards. Postclypeus moderately inflated. Protibia inflated. Worker (type 1, W1) with fontanelle occupying 3/4 of the cephalic capsule in dorsal view. Worker 2 (type 2, W2) with fontanelle occupying 1/4 to 1/2 of the cephalic capsule in dorsal view.

Etymology. In reference to the character Friar Tuck, the supposed Franciscan monk of the legend of Robin Hood.

Biological notes. Collected foraging in galleries in the ground, at base of trees, among litter and sticks and under rotting log.

Comments. We described as two types of workers because there are two sizes of fontanelle. To know if it is the same worker with the fontanelle changing over time, it would be necessary to do a developmental analysis, and this is out of the scope of the present paper. For taxonomic purposes, it is important to know that two types of workers can be found in the same sample.

Tonsuritermes mathewsi Cancello & Constantini sp. nov.

(Figs. 2E–F; 3C; 6)

Holotype: worker from lot MZUSP 12268, (in a separate vial with the remaining sample).
Type–locality: BRAZIL. Mato Grosso: Chapada dos Guimarães, lat 15.4605S, long 55.7497W.
Type–repository: MZUSP

Paratypes. BRASIL. Goiás: Anápolis, lat 16.3266S, long 48.9527W, DE Oliveira coll., UEG 4075; same data of holotype sample: 04.viii.2009, TF Carrijo coll., MZUSP 12268, 12615, 27374; 04.viii.2009, MR Rocha coll., MZUSP 23920.

Diagnosis. Monomorphic worker; head capsule with long bristles of almost the same size and bristles of tergites scattered.

Imago. Unknown.

Worker. Monomorphic (Fig. 2E–F). Head capsule with long bristles of almost the same size. Bristles of tergites scattered. Postclypeus hyper–inflated. Fontanelle 1/4 diameter of head capsule in dorsal view. Protibia strongly inflated, with a groove in the inner surface of the profemur.

Comparison and remarks. A huge variation at the head pilosity, number of spine–like bristles on the protibia, and form and size of the fontanelle were observed among the samples of the new genus, which makes the distinction of the two species quite challenging. Despite the similarity between *Tonsuritermes tucki* and *Tonsuritermes mathewsi*, the latter, with monomorphic workers, is restricted to the Brazilian savannah, the Cerrado, while the dimorphic *T. tucki*, was collected primarily in forests and was sampled mostly (but not only) in forest formations. The head of *T. tucki* has bristles of two sizes, and those on tergites are all pointing backwards, while in *T. mathewsi* the bristles are same size, long on the head, and in the tergites, the bristles are pointing in multiple directions. The postclypeous of *T. mathewsi* is more inflated than in *T. tucki*. The workers of *T. mathewsi* are larger than *T. tucki* (Tab. 2) and the fontanelle is on average lower in *T. mathewsi* than W2 of *T. tucki*. In addition, the protibia of *T. mathewsi* is more inflated, and the groove in the inner surface of the profemur is deeper.

Etymology. *Tonsuritermes mathewsi* is named in honour of A. G. Anthony Mathews for his important contribution to the taxonomy and biology of the termites of Mato Grosso, Brazil (Mathews 1977).

Biological notes. Some samples were collected from derelict nests built by undetermined species of *Cornitermes* Wasmann, 1897 in cerrado vegetation.

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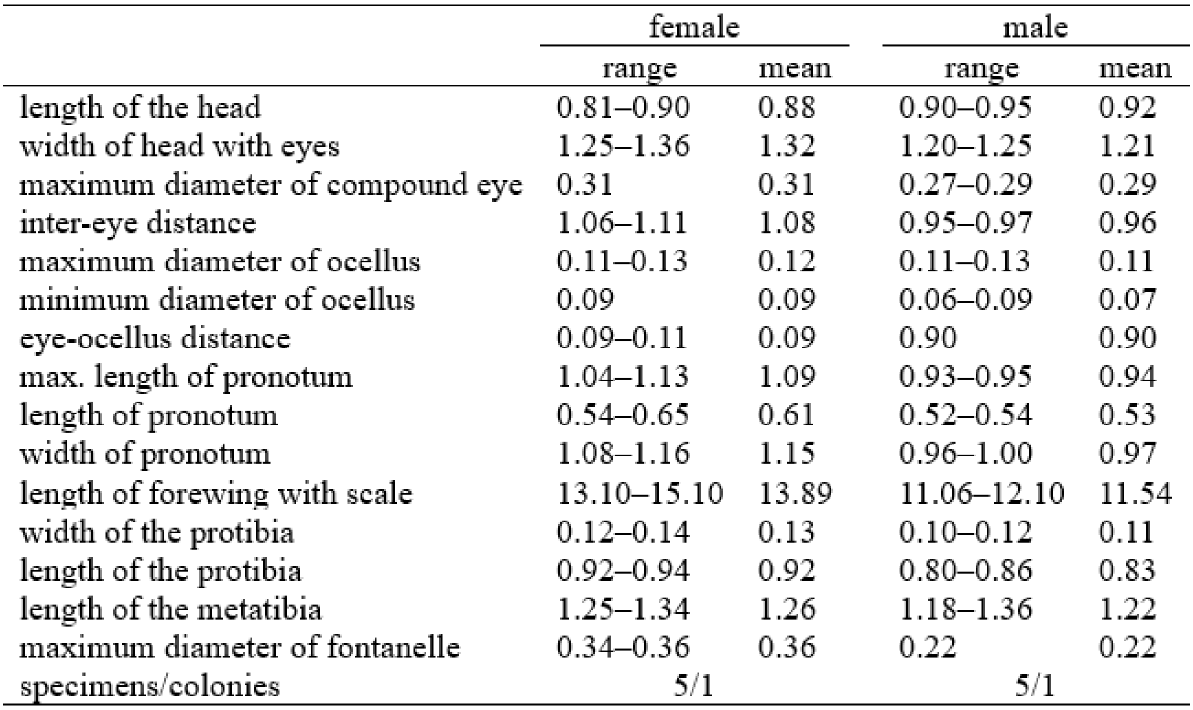
Tables and Figures



	female		male	
	range	mean	range	mean
length of the head	0.81–0.90	0.88	0.90–0.95	0.92
width of head with eyes	1.25–1.36	1.32	1.20–1.25	1.21
maximum diameter of compound eye	0.31	0.31	0.27–0.29	0.29
inter-eye distance	1.06–1.11	1.08	0.95–0.97	0.96
maximum diameter of ocellus	0.11–0.13	0.12	0.11–0.13	0.11
minimum diameter of ocellus	0.09	0.09	0.06–0.09	0.07
eye-ocellus distance	0.09–0.11	0.09	0.90	0.90
max. length of pronotum	1.04–1.13	1.09	0.93–0.95	0.94
length of pronotum	0.54–0.65	0.61	0.52–0.54	0.53
width of pronotum	1.08–1.16	1.15	0.96–1.00	0.97
length of forewing with scale	13.10–15.10	13.89	11.06–12.10	11.54
width of the protibia	0.12–0.14	0.13	0.10–0.12	0.11
length of the protibia	0.92–0.94	0.92	0.80–0.86	0.83
length of the metatibia	1.25–1.34	1.26	1.18–1.36	1.22
maximum diameter of fontanelle	0.34–0.36	0.36	0.22	0.22
specimens/colonies	5/1		5/1	

Table 1. Measurements (mm) of imagoes of *Tonsuritermes tucki*, sp. nov.

	Tonsuritermes tucki				*T. mathewsi*	
	worker type 1		worker type 2			
	range	mean	range	mean	range	Mean
length of the head	0.79–1.12	0.92	0.72–0.96	0.86	0.84–1.08	0.98
width of head	0.96–1.24	1.13	0.96–1.20	1.09	1.05–1.34	1.25
length of the protibia	0.66–0.84	0.73	0.64–0.78	0.70	0.80–0.90	0.85
width of the protibia	0.14–0.18	0.15	0.13–0.18	0.15	0.14–0.18	0.16
length of the metatibia	0.84–1.10	0.97	0.81–1.08	0.96	1.03–1.32	1.15
max. diameter of fontanelle	0.5–0.76	0.67	0.12–0.48	0.31	0.16–0.26	0.20
spine–like bristles	6–14		6–16		11–15	
specimens/colonies	57/18		49/18		38/5	

Table 2. Measurements (mm) and number of spine–like bristles on inner margin of protibia of workers of *Tonsuritermes tucki*, sp. nov., and *T. mathewsi*, sp. nov.

Figure Legends

Figure 1. *Tonsuritermes tucki* sp. nov., imago head and thorax. A– lateral view, B– dorsal view. Arrows point to the frontal marks. Specimen from lot MZUSP6480.

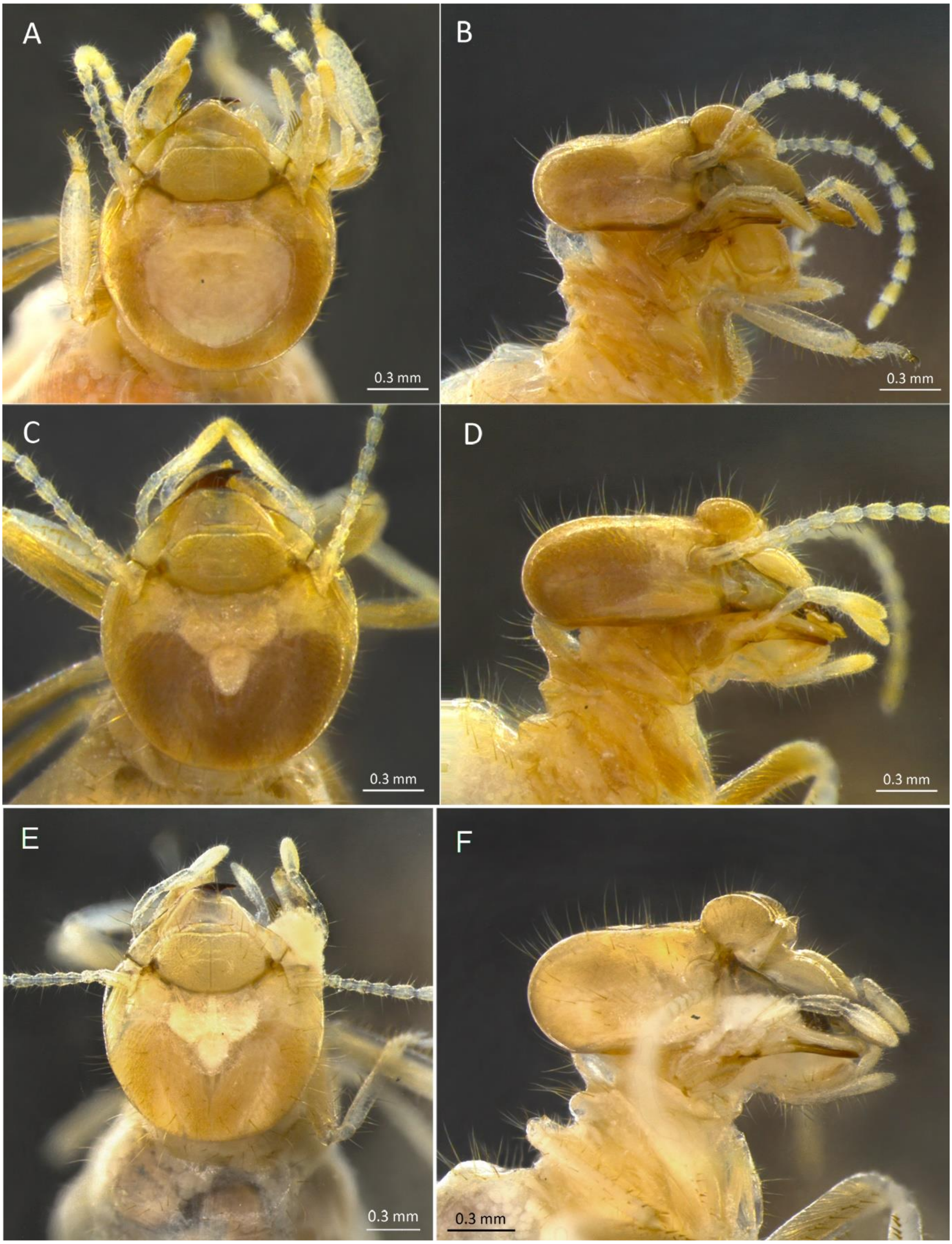


Figure 2. *Tonsuritermes*, workers head and thorax. A– worker type 1 of *T. tucki,* sp. nov., dorsal view; B– same as A in lateral view; C– worker type 2 of *T. tucki,* sp. nov., dorsal view; D– same as C in lateral view. E– *T. mathewsi* sp. nov., dorsal view; F– same as E in lateral view. Specimens from lots MZUSP6480 (A–D) and MZUSP12268 (E–F).

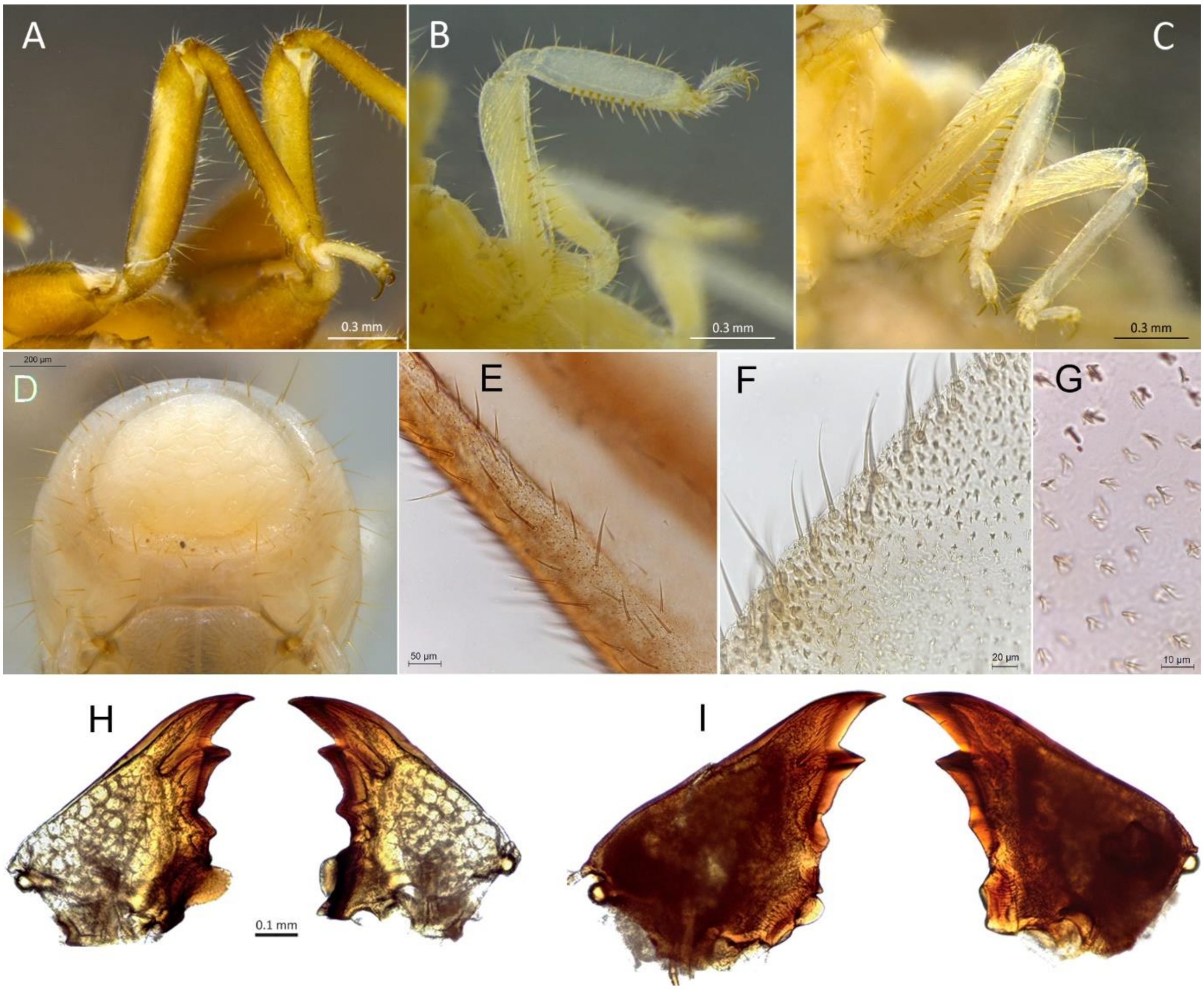


Figure 3. *Tonsuritermes*, workers and imago. A– foreleg of the imago of *T. tucki,* sp. nov.; B– foreleg of the worker of *T. tucki*; C– foreleg of the worker of *T. mathewsi*, sp. nov.; D– detail of the texture and surrounding pilosity of the glandular opening, worker type 1 of *T. tucki*; E– detail of external margin of the wing of *T. tucki*; F– detail of inner margin of the wing; G– micrasters on membranous area; H– worker mandibles; I– imago mandibles. Specimens from lots MZUSP6480 (A–B, E–I), MZUSP12268 (C) and UF PU1104 (D).

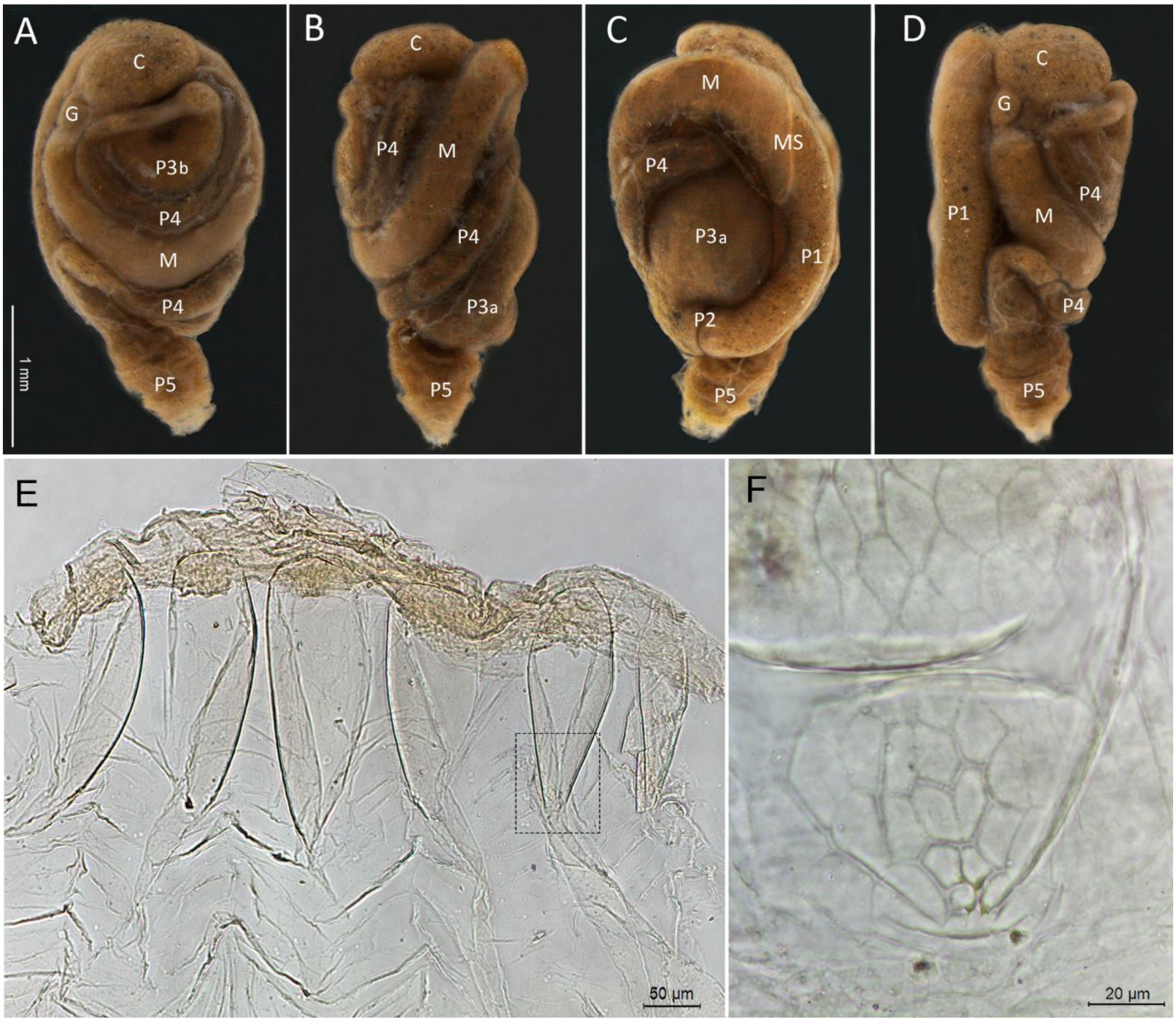


FIGURE 4. *Tonsuritermes tucki* sp. nov., worker. Digestive tube in A– dorsal; B– right; C– ventral; D– left views; E– enteric valve armature; F– enlarged view of the a cushion. Specimens from lots MZUSP6840 (A–E) and MZUSP 10367 (F). Gut regions indicated in figs. A–D: *C*=crop, *G*=gizzard, *M*=mesenteron, *MS*= mixed segment, *P1*= ileum, *P2*= enteric valve, *P3*= paunch, *P4*= colon, *P5*=rectum.

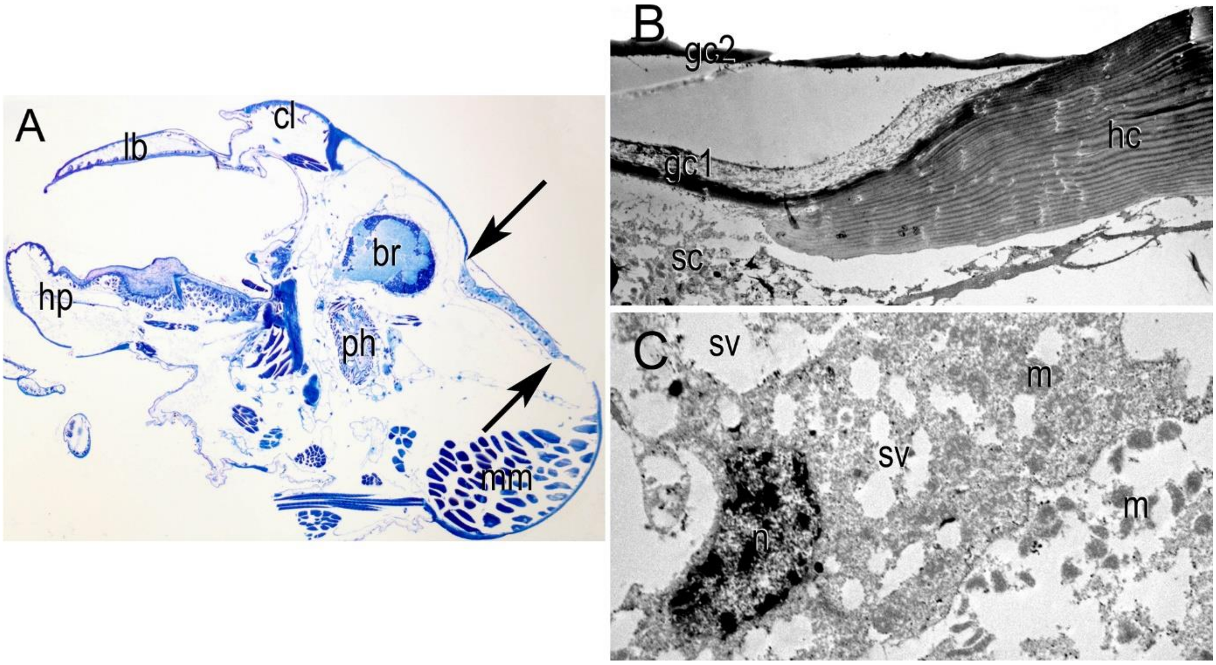


FIGURE 5. *Tonsuritermes tucki* sp. nov., structure of the frontal gland in worker type 1, A– Sagittal section of the head of *Tonsuritermes tucki* sp. nov. worker. The frontal gland is stretched in–between the two arrows. B– Transition between the head cuticle and highly modified glandular cuticle overlying the secretory cells. C– Remains of secretory cell ultrastructures. Abbreviations: br, brain (supraoesophageal ganglium); cl, clypeus; gc1, inner glandular cuticle; gc2, outer glandular cuticle; hc, head cuticle; hp, hypopharynx; lb, labrum; m, mitochondria; mm, mandibular muscles; n, nucleus; ph, pharynx; sc, remains of a secretory cell; sv, secretory vesicle.

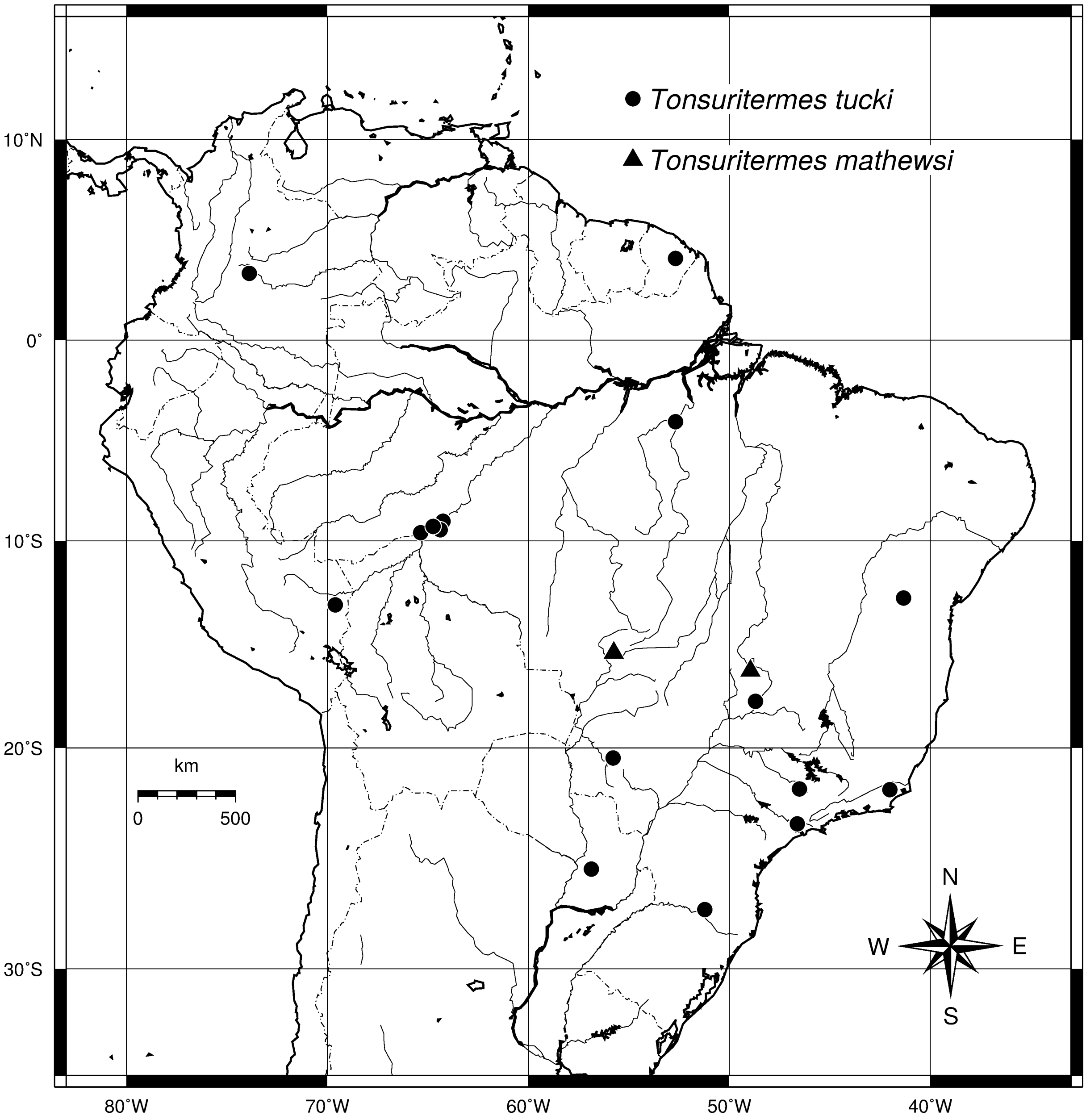


FIGURE 6. Distribution map of the species of *Tonsuritermes* gen. nov.

Neocapritermes taracua soldiers. Photo by Aleš Buček.

3. General Conclusions

3. General conclusions

Social insects' life requires a constant interaction among the nestmates, either to recognize the members of the group, or to communicate important information for the colony, like for example to indicate sources of food, threats, needs, etc. This communication is normally done by chemical signals (pheromones), although some insects like wasps have are able to recognize faces of others individuals (Sheehan & Tibbetts 2011). In insects such as termites were most of invividuals are blind, most of communication is in fact chemical communication, requiring them of a complex communication through pheromones released by exocrine glands (Bordereau & Pasteels, 2011). The exocrine system in termites is extremely well developed and it is frequent to see how one individual of the colony produces chemical substances to elicit a change in the behaviour of the others. In other words, chemicals produced by termites are a reflect of their behaviour as individuals and as colony. In fact, Keller and Nonacs (1993) hypothesized that pheromones may change completely a colony acting as signals that tells immatures if either an altruistic (to become a worker or a soldier) or a selfish (to become a reproductive) pathway would better serve the colony interests. In the same line, pheromones are thought to play a fundamental role in the mechanisms underlying the development and maintenance of eusociality. It has been demonstrated by Harrison and others (2018), who found that the evolution of enzymes involved in the production and perception of pheromones plays an important role for the emergence of eusociality in Isoptera and Hymenoptera.

Normally when confronting a threat, termites perform a mixture of behaviours which includes signals of alarm, information about the threat and defensive mechanisms. Most of these activities are performed solely by pheromones secretions. An effective communication allows the escape of the weakest individuals of the colony (normally reproductives, immatures and workers) and the gathering of the defenders (soldiers) (Šobotník et al. 2010a). The most important alarm pheromone source is the frontal gland, which also plays a fundamental role as defensive pheromone source.

Thanks to modern techniques, new glands can be discovered and old ones can be re–examined. Thus, these new techniques allow us to increase the knowledge about glands, their distribution and evolution. It has changed the way we used to see animals, in particular insects, and provided a wide variety of advantages through the knowledge, such as the use of this communication as a tool for pests control.

The goal of this thesis was to straight on disentangling the evolutionary processes leading to observed development of the labral and frontal glands in termites. The first gland, with a probable communicational role, broadly extended through all termite species; and the second, a well known and extremely powerful arm present in almost all Neoisoptera, being both of them fundamental for the colony success.

The initial part of my thesis focused on my first objective that was to disentagle the distribution of the labral glands in termite soldiers through the analysis of the development of the gland in all castes and most representative groups across termite's taxa. My first study facilitated a deeper understanding of the literature and description of the precense/absence of the labral gland in soldiers. As a result, I was then able to suggest its identification in other castes such as workers or imagoes. Through it, I found out the presence of the labral gland in all termite species and castes examined so far. It confirms that the labral gland is a very conservative organ with an important and also probably conservative role for the colony success. This discovery along with the gland morphology, structure, ultrastructure and behavioural observations suggest a fundamental communicative role of the gland, which is reinforced by the presence of the gland in all species including the closest relative of termites (*Cryptocercus punctulatus*). It should stimulate further questions and studies in the field, thus continuing to fill the gaps in the knowledge regarding the function of the labral gland in termites and its variability among castes.

In relation to our studies about the frontal gland, my aim was to unravel the evolution of the frontal gland in termite workers and then to join it to the current knowledge of its presence in other castes, creating a potent phylogenetic tree which allowed me to discuss its evolution in termites. I found that the frontal gland was also present in all castes from most of Neoisoptera species, with few regresions occurring. However, my results showed that the gland does not possess a conservative development between castes suggesting that it may have taken caste–specific evolutionary routes, suffering an extreme reduction of its size in workers lacking of reservoir in all species. It is probable that the frontal gland has evolved as an auxiliary weapon in a common ancestor of Rhinotermitidae, Serritermitidae and Termitidae, but it faced a swap between the production of few well–defended descendants and the production of larger numbers of poorly defended descendants as in most advanced Termitidae species. In these species, imagoes presented a reduced frontal gland, but the number of individuals conforming the colony was higher than in other species. Moreover, species with reduced frontal gland in imagoes are also well characterized with a bigger proportion of soldiers in the colony and powerful chemical defences weighing up the mechanical ones. In workers, the reduction of its gland does not result so strange due to the fact that worker's role, even the defensive one, is limitated to an enclosed life. Termites deal with predators mainly during foraging activities, moment in which they are well protected by soldiers. It seems that evolution has prioritized the development of alternative defensive ways in workers (as the dehiscense mechanisms found in some soldierless workers or the autothysis observed in *Ruptitermes* or *Neocapritermes taracua* workers; Costa–Leonardo 2004, Šobotník et al. 2012), keeping these reduced frontal glands. Anyway, the functionality of the gland in these species may still be important for the colony, hypothesis that is supported by its broad presence in Neoisoptera workers. This caste–specific evolution is a finding that opens the door to many new questions, such as the function of the gland in workers and the mechanisms which may cause the frontal gland disappearance in some species.

In spite of the ecological and economical importance of termites, they have received scarse attention, especially compare to other social insects as bees, ants and wasps. Not much is known about termites' exocrine glands, which has been shown by the finding of the labral gland in all of them, while it had been just scattered commented in some random species previously. Studies of glands structure, ultrastructure and function are needed to understand termites' ecology, especially due to their constant interaction with humans in many different aspects of our life, like how they affect the air we breath or how they eat our houses. A farther realization of bioassays, quantitative gas chromatography–mass spectrometry and electroantennography analyses will contribute to a better understanding of the communication inside the termite colony and the involvement of other exocrine glands with secretion of pheromones. Understanding termite's chemical communication system will also enhance our understanding about the evolution and social organization of Isoptera.

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5. Declaration

I, Valeria Palma Onetto, confirm that the content of this thesis is the result of my personal endeavour. I confirm that any data, reasoning and conclusions sourced from existing literature are reported exactly as they were found and are cited in full in the References. Finally, I confirm that this thesis has never been reviewed nor published elsewhere.

Valeria Palma Onetto
Paris,

Abstract

Structure, function and evolution of the labral and frontal glands in termites

Résumé

Les termites représentent un groupe d'insectes eusociaux qui vivent dans des colonies contenant des centaines, voire des millions d'individus. Ils sont très abondants, dépassant 6 000 individus par mètre carré sous les tropiques. En raison de leur abondance, les termites représentent une source de nourriture importante pour une grande variété de prédateurs. Les adaptations défensives des termites permettent aux colonies de surmonter avec succès les pressions des prédateurs. Cette réussite s'explique par le développement d'un système de communication complexe opéré par un riche ensemble de glandes exocrines. Pas moins de 20 glandes exocrines différentes sont connues chez les termites. Certaines de ces glandes avaient fait l'objet d'une attention négligeable, n'étant connues que par des observations anecdotiques. L'une d'elles était la glande labrale. Dans cette étude, j'ai examiné la structure et l'ultrastructure du labrum chez des soldats de 28 espèces, des ouvriers de 28 espèces et des imagos de 33 espèces parmi les principaux représentants des termites, ainsi que chez la blatte xylophage *Cryptocercus*. La glande labrale était présente chez toutes les espèces et castes et comprenait deux régions de sécrétion situées respectivement sur la face ventrale du labrum et la partie dorso–apicale de l'hypopharynx. L'épithélium de la glande était constitué de cellules sécrétrices de classe 1, auxquelles s'ajoutaient des cellules sécrétrices de classe 3 chez les soldats de quelques espèces. Une caractéristique commune des cellules sécrétrices était l'abondance de réticulum endoplasmique lisse (un organite connu pour produire des sécrétions lipidiques et souvent volatiles), de longues microvillosités avec un canal à l'intérieur, qui libèrent la sécrétion à travers une cuticule modifiée. D'après ces expériences sur la structure, l'ultrastructure et le comportement, mes résultats suggèrent que la glande labrale est impliquée dans la communication défensive après une rencontre avec un étranger. D'autre part, d'autres glandes sont étudiées de manière approfondie chez certaines castes mais n'ont pratiquement pas fait l'objet d'attention chez d'autres castes. C'est le cas de la glande frontale, organe sans équivalent parmi les autres animaux. La glande frontale est bien connue des soldats et des imagos, mais elle était peu connue chez les ouvriers. Afin de brosser un tableau complet de l'évolution de cette glande chez les termites et, par conséquent, chez les termites, je l'ai étudiée chez 41 espèces supplémentaires sur l'ensemble des néoisoptères. La glande frontale de ces espèces était formée uniquement de cellules sécrétrices de classe 1 et se présentait comme un épithélium sans réservoir dans tous les cas. Mes données suggèrent que la glande frontale aurait des voies d'évolution propres à la caste, avec une forme ancestrale épithéliale avec réservoir chez les soldats et les imagos, mais en n'étant qu'un épaississement épithélial chez les ouvriers. Cette étude a été la première à fournir une image complète de la structure des glandes labrale et frontale à travers tous les taxons et castes des termites, fournissant des informations fondamentales pour améliorer notre compréhension de l'évolution et du comportement social des Isoptera.

Mots–clés: glande exocrine, Isoptera, Termitoidae, ultrastructure, évolution, développement

Abstract

This thesis includes four manuscripts. One is already published, one is under revision, another is in preparation for submission and the last is accepted by the journal but not published yet,

Termites represent a group of eusocial insects that live in colonies containing up to hundreds to millions. They are highly abundant, exceeding in tropics 6,000 individuals per square meter. Due to their abundance, termites represent an important food source for a wide variety of predators. At the same time, termite defensive adaptations allow the colonies to overcome the predator pressures, being extremely evolutionary successful. This achievement is explained by the development of a complex communication system operated by a rich set of exocrine glands. As many as 20 different exocrine organs are known in termites. Some of these organs had received negligible attention being only known by anecdotal observation. One of these was the labral gland. In this study, I examined the structure and ultrastructure of the labrum in soldiers of 28 species, workers of 28 species and imagoes of 33 species across termites' main representatives, and in the wood roach *Cryptocercus*. The labral gland was present in all species and castes, and comprises two secretory regions located on the ventral side of the labrum and the dorso–apical part of the hypopharynx, respectively. The epithelium of the gland consisted of class 1 secretory cells, with an addition of class 3 secretory cells in soldiers of few species. A common feature of the secretory cells was the abundance of smooth endoplasmic reticulum (an organelle known to produce lipidic and often volatile secretions), long microvilli with a channel inside, which releases the secretion through a modified cuticle. According to the structure, ultrastructure and behavioural experiments, my results suggest that the labral gland is involved in defensive communication after encounter to an alien. On the other hand, other glands are extensively studied in some castes but have received almost no attention in other castes. It is the case of the frontal gland, an organ without any equivalent among other animals. The frontal gland is well known in soldiers and imagoes but not much was known about it in workers. In order to provide a complete picture of the evolution of this gland in termite workers and consequently in termites, I studied it in 41 additional species across Neoisoptera. The frontal gland of these species was formed by class 1 secretory cells only, and occured as an epithelial without reservoir in all cases. My data suggest that the frontal gland would have caste–specific evolutionary routes, being its ancestral form epithelial with reservoir in soldiers and imagoes, while epithelial thickening in workers. This study was the first to provide a comprehensive picture of the structure of the labral and frontal gland across all termite taxa and castes, providing fundamental information to enhance our understanding about the evolution and social behaviour of Isoptera.

Keywords: exocrine gland, Isoptera, Termitoidae, ultrastructure, evolution, development

Discipline: Ethology

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Appendix

Publications of the candidate during the course of her PhD

Peer–reviewed journals

Palma–Onetto V., Hošková K., Křížková B., Krejčířová R., Pflegerová J., Bubeníčková F., Plarre R., Dahlsjö C., Synek J., Bourguignon T., Sillam–Dussès D., Šobotník J. 2018. The labral gland in termite soldiers. *Biological Journal of the Linnean Society*, 123(3), 534–544.

Constantini J., Fernandes T., **Palma–Onetto V**., Scheffrahn R., Carnohan L.P., Šobotník J., Cancello E. 2018. *Tonsuritermes*, a new genus and two new soldierless termite species (Blattaria: Isoptera: Termitidae) from South America. *Zootaxa,* 4531(3), 383.

Palma–Onetto V., Pflegerová J., Plarre R., Synek J., Cvačka J., Sillam–Dussès D., Šobotník J. The labral gland gland in termites: evolution and function. *Biological Journal of the Linnean Society*, 126(3), 587–597.

Poster presentations

International conferences

Palma–Onetto V., Pflegerová J., Hošková K., Křížková B., Krejčířová R., Bubeníčková F., Sillam–Dussès D. & Šobotník J. Labral gland in termite soldiers. Enthomology conference, Zoologické dny, Czech Republic, České Budějovice, 11–12 February, 2016.

Palma–Onetto V., Pflegerová J., Hošková K., Křížková B., Krejčířová R., Bubeníčková F., Sillam–Dussès D. & Šobotník J. Labral gland in termites. Enthomology conference, 4 Zoologické dny, Czech Republic, České Budějovice, 09–10February, 2017.

Palma–Onetto V., Sillam–Dussès D. & Šobotník J. The labral gland in termites. International Union for the Study of Social Insects conference, Brazil, Guarujá, 5–10 August, 2018.

National conferences

Palma–Onetto V., Sillam–Dussès D. & Šobotník J. The frontal gland in termites. IFE Student Cours, 2018.

Others

Participant of the course “Termite Biology and Control Class” imparted by University of Florida

Fort Lauderdale Research & Education Center. 19th to 23rd of June. 2017

Participation on the development of the Czech documentary: Svět podle termite (World according to termites). Winner of the Czech Life Sciences Film Festival 2017.