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Evolution of sensitivity to trail-following pheromones in termites
Evoluce citlivosti ke stopovacím feromonům u termitů

Master's thesis

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Declaration:

I hereby declare that the work presented in this thesis has been carried out by me, unless otherwise stated. It is entirely of my own composition and has not, in whole, or in part been submitted to apply for any other academic degree.

Prague, 8. 8. 2021 Barbora Záková

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Abstract

Eusocial insects evolved a sophisticated intraspecific communication, dominated by chemical signals, the pheromones. Termites (Isoptera) represent an excellent example in this respect, having a wide range of pheromones, such as trail-following, sex-pairing, alarm, and other pheromones. It is especially the former category of pheromones which is ubiquitous in termites and which was chemically characterized in many taxa across termite phylogeny. This allowed phylogenetic reconstruction of the chemical diversity of trail-following pheromones and calls for searching of evolutionary patterns of the sensitivity to these pheromones in various lineages across the tree of life, including the search for evolutionary scenario of the emergence of specific olfactory receptor proteins. In most species, the trail-following pheromones are represented by mono-, di- and tri-unsaturated fatty alcohols (3*Z*)-dodec-3-en-1-ol (DE), (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (DDE), and (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (DTE).

My overall aim in this thesis was to contribute to the understanding of the evolution of olfactory detection of C_{12} fatty alcohol trail-following pheromones in termites. More specifically, my question was whether evolutionarily more basal clades (*Kalotermes flavicollis* and *Neotermes cubanus* from the family Kalotermitidae), having the ancestral pheromone DE, respond to more modern compounds DDE and DTE, and *vice versa*, whether more advanced lineages (*Reticulitermes flavipes* from the family Rhinotermitidae), having DTE as the trail-following pheromone, respond to the ancestral DE. To this goal, I used behavioural bioassays testing the trail-following activity of individual compounds, and electrophysiological recordings, measuring the olfactory detection of the compounds. My other aim was to establish the technique of single sensillum recording (SSR), which was not previously used in termites, and search for the sensillum specific for the detection of DTE in the modern family Rhinotermitidae. To this goal, I performed the mapping of antennal sensilla in *R. flavipes* using scanning electron microscopy and retrieved candidate olfactory sensilla according to their sensitivity to different C_{12} alcohol pheromones by means of SSR.

My results suggest that evolutionarily older Kalotermitidae have a low, non-specific sensitivity to the modern pheromones DDE and DTE. By contrast, the more recent Rhinotermitidae appear to have retained the sensitivity to the ancestral DE, and potentially have conserved the corresponding DE-responsive pheromone receptor, beside the newly evolved DTE-specific receptor. Both lineages respond weakly to DDE, which only evolved after the diversification of the most recent Termitidae. My results also show that SSR is a suitable technique for the search of specific olfactory sensilla in termites, as evidenced by my observations of DTE-specific sensillum in *R. flavipes*.

Keywords: *Chemical ecology, termites, ethology, pheromones, social insects, electrophysiology, trail-following pheromones, olfactory receptor*

Abstrakt

Společenský hmyz si vyvinul složitou komunikaci, která je dominována chemickými signály, tedy feromony. Termiti (Isoptera) jsou toho dokonalým příkladem, jelikož využívají širokou škálu feromonů, jako stopovací, pohlavní, poplašné a další feromony. Právě první zmíněný typ feromonů byl chemicky charakterizován u relativně mnoha skupin napříč fylogenezí termitů. To umožnilo popsat jejich diverzitu ve fylogenetickém kontextu a vyzývá k hledání evolučních posloupností schopnosti detekce těchto feromonů u různých linií napříč vývojových stromem, včetně hledání evolučního scénáře pro vznik specifických čichových receptorových proteinů. U většiny druhů se jako stopovací feromony vyskytují převážně jednou, dvakrát a třikrát nenasycené alkoholy (3*Z*)-dodec-3-en-1-ol (DE), (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (DDE) a (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (DTE).

Obecným cílem mojí práce bylo přispět k porozumění evoluce čichového vnímání C_{12} alkoholů jako stopovacích feromonů termitů. Konkrétně jsem si kladla otázku, zda evolučně bazálnější linie (*Kalotermes flavicollis* a *Neotermes cubanus* z čeledi Kalotermitidae) mající ancestrální stopovací feromon DE odpovídá na modernější látky DDE a DTE, a naopak, zda pokročilejší linie (*Reticulitermes flavipes* z čeledi Rhinotermitidae) používající jako stopovací feromon DTE odpovídá na ancestrální látku DE. K tomuto účelu jsem používala behaviorální pokusy testující stopovací aktivitu látek a elektrofyziologická měření, stanovující čichovou detekci těchto feromonů. Mým dalším cílem bylo zavést techniku *single sensillum recording* (SSR), která dosud nebyla u termitů používána, a hledat tykadlové senzily specifické pro detekci DTE u moderní čeledi Rhinotermitidae. K tomu účelu jsem zmapovala tykadlové senzily *R. flavipes* pomocí rastrovací elektronové mikroskopie a třídila čichové senzily podle jejich odpovědi na různé C_{12} alkoholy s využitím SSR.

Moje výsledky naznačují, že evolučně starší Kalotermitidae mají nízkou, nespecifickou citlivost k moderním feromonům DDE a DTE. Naopak moderní Rhinotermitidae si podle všeho zachovali citlivost k ancestrálnímu feromonu DE, a je možné, že mají konzervovaný odpovídající DE receptor vedle nově získaného receptoru pro DTE. Obě linie vykazují pouze nízkou reakci na DDE, který se vyvinul až po diverzifikaci nejmodernější čeledi Termitidae. Moje výsledky také ukazují, že SSR je vhodná technika pro hledání příslušných čichových senzil u termitů, jak ukazují moje pozorování senzil specificky odpovídajících na DTE u *R. flavipes*.

Klíčová slova: *Chemická ekologie, termiti, etologie, feromony, společenský hmyz, elektrofyziologie, stopovací feromony, čichový receptor*

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ABBREVIATIONS

AL – antennal lobe

ANOVA – analysis of variance

cAMP – cyclic adenosine monophosphate

DE – (3*Z*)-dodec-3-en-1-ol

DDE – (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol

DTE – (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol

EAG – electroantennography

GC-EAD – gas chromatography–electroantennographic detection

GC/MS – gas chromatography–mass spectrometry

GPCRs – G protein-coupled receptors

Grs – gustatory receptors

iACT – inner antennocerebral tract

imACT – inner-middle antennocerebral tract

Ir – ionotropic receptors

LH – lateral horn

LN – local neuron

mACT – medial antennocerebral tract

oACT – outer antennocerebral tract

OBP – odorant-binding protein

ORN – olfactory receptor neuron

Ors – odorant receptors

PN – projection neuron

SEM – scanning electron microscopy

SSR – single-sensillum recording

SPP – sex-pairing pheromone

TFP – trail-following pheromone

VOCs – volatile organic compounds

1 INTRODUCTION

Eusociality is the most sophisticated and prominent evolutionary novelty of insects. While a diverse range of social behaviour occurs in many insect groups, its advanced form, eusociality, has independently evolved only within a few insect orders. Besides dominant representatives of eusocial insects, i.e. termites and several lineages of aculeate Hymenoptera (ants, wasps, some bees), eusociality has been documented in some aphid families (Hemiptera: Sternorrhyncha: Aphidoidea) and thrips (Thysanoptera), also for the complete list it is necessary to mention the unique occurrence in one eusocial beetle, *Austroplatypus incompertus* (Coleoptera: Curculionidae).

The eusocial colony is defined by the presence of reproductive division of labour, overlapping female generations and cooperative brood care. Although the definition is theoretical and empirical evidence provides a number of transient or disparate situations, all defining features of eusociality presume intense interactions among individuals and a need for effective communication, required to build the nest, to recognize colony affiliation and reproductive status of individuals, to cooperate in offspring care, and all in all to supply and defend the colony. It is therefore in advanced eusocial insects that the largest scale of sophisticated intraspecific communication evolved, which includes visual, vibroacoustic and tactile signalling, but the dominant role is played by the chemical communication, as it is generally the rule in Insecta.

The diversity of chemical communication of eusocial insects consists on the one hand of genetically encoded, albeit environmentally and physiologically modified chemical signals, so-called chemical signatures, which ensure recognition of colony identity and assessment of the relatedness among individuals. These chemical signatures are most often mediated by blends of non-polar substances in the epicuticle of each individual (by cuticular hydrocarbons). On the other hand, social insects produce and recognize a wide spectrum of immutable species-specific signals, the pheromones. According to the purpose and mechanism of action on the recipient, they can be divided into so-called primer pheromones and releaser pheromones. The former pheromone type ensures the division of labour inside the colony by signalling the presence of members belonging to individual castes. Primer pheromones have long-lasting and often irreversible effect on the physiology and development of the receiver and influence its developmental trajectories and fertility. By doing so, the primer pheromones mediate the dominance of the fertile individuals (reproductives) and optimal caste composition of the colonies (e.g. royal pheromones, queen pheromones, soldier pheromones, brood pheromones). By contrast, the releaser pheromones trigger immediate behavioural responses of the recipients. They mediate the encounter of reproductive individuals (sex pheromones), behavioural caste recognition (recognition pheromones), marking of nests, territories, food sources and food routes (marking pheromones, home-marking pheromones, trail pheromones, etc.) or an alarm response (alarm pheromones).

Because termites (Isoptera) represent the social insects with the most elaborate caste systems,

manifested by the presence of several functionally and anatomically specialized sterile castes (workers, soldiers) and multiple types of reproductives (primary and secondary reproductives), it is not surprising that they use most of the pheromone types listed above. My thesis focuses on the evolution of the trail-following pheromones, which are ubiquitous in all termite species and are secreted by a specialized exocrine gland, the sternal gland. Considerable effort has been dedicated during the past decades to the understanding of chemical diversity of termite trail-following pheromones. Our current knowledge covers the chemical identity of these pheromones in all termite families and most important subfamilies, which allows us to reconstruct their evolutionary succession. In particular, I am interested in the trail-following pheromones occurring in the modern termite lineages, in which three primary aliphatic alcohols with twelve carbons and one, two, or three double bonds are the single or dominant components. As the first C_{12} alcohol, (3*Z*)-dodec-3-en-1-ol occurred in the family Kalotermitidae, while the other two, i.e. (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol and (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol, appeared only later in evolution in the families Rhinotermitidae and Termitidae. It is especially the latter compound, which, due to its three double bonds and their specific positions and configurations, represents a unique compound in terms of its molecular structure and possible biosynthesis. At the same time, (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol shows great physiological efficiency, because it elicits behavioural responses at very low quantities compared to other termite trail-following pheromones, and ultimately it became the most successful component of termite pheromones in terms of its dominant presence in the most advanced taxa.

My aim is to test whether modern groups, having (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol as their trail-following pheromone, retained the sensitivity to the evolutionarily older (3*Z*)-dodec-3-en-1-ol, and vice versa, whether more basal taxa, having (3*Z*)-dodec-3-en-1-ol as their pheromone, show a sensitivity to the evolutionarily younger (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol. To this goal, I combine behavioural bioassays with electroantennographic measurements in two species belonging to the more basal family Kalotermitidae (*Kalotermes flavicollis* and *Neotermes cubanus*) and one species from a more modern family Rhinotermitidae (*Reticulitermes flavipes*). Observations from these experiments should provide basic insight into evolution of antennal pheromone receptor genes, such as for instance whether (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol pheromone receptor evolved through a duplication of the existing receptor for (3*Z*)-dodec-3-en-1-ol, and whether both receptors are eventually expressed in the modern family Rhinotermitidae. As my second goal, I independently address these two questions by searching for pheromone receptors specific to these two C_{12} alcohols using single sensillum recordings in *Reticulitermes flavipes*.

2 BACKGROUND

2.1 Systematics and evolution of termites (Isoptera)

As is known nowadays, termites are no longer classified as a separate order; instead, they are considered as the infraorder Isoptera within Blattodea (Lo, et al. 2007). In other words, they are nested within the cockroaches (Inward, et al. 2007a; Legendre, et al. 2008; Ware, et al. 2008) as a sister group to the subsocial xylophagous cockroach of the genus *Cryptocercus* (Cryptocercidae) (Lo, et al. 2004; Buček, et al. 2019).

With the current number of 2977 reported species (Constantino, 2020), termites are split into nine living families, from basal to the most recent: Mastotermitidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Serritermitidae, Stylotermitidae, Rhinotermitidae and Termitidae (Krishna, et al. 2013). Even though these are well-defined morphologically and ecologically, the monophyly and mutual relationships among some families are ambiguous. For instance, Archotermopsidae are sometimes seen as paraphyletic with respect to Hodotermitidae, the same as Rhinotermitidae with respect to Serritermitidae and Termitidae (Bourguignon, et al. 2015; Bourguignon, et al. 2017). The reason for these ambiguities may be due to convergence of individual lineages caused by a specialised way of life (Donovan, et al. 2000).

Traditionally, termites are categorized into lower and higher termites, primarily according to the differences in their microbial ecosystem; the lower termites (Mastotermitidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, Serritermitidae, and Rhinotermitidae) possess in their hindguts eukaryotic cellulolytic flagellates to digest cellulose, mainly parabasalids and oxymonads (Bagnères and Hanus 2015; Nalepa 2020), whereas higher termites (i.e. the single family Termitidae) have lost endosymbiotic flagellates and rely primarily upon bacteria and their own cellulolytic apparatus (Tokuda and Watanabe 2007; Nalepa 2020). Higher termites are not only the most modern, but also the most diversified family, with 83 % of all genera and over 70 % of termite species (Krishna, et al. 2013).

It is the higher termites that carry the huge ecological success of Isoptera in terms of large biomass and dominance among decomposers, especially in the tropics and subtropics, where they complement ecologically the ants as the largest carnivores or predators (Wilson 1992).

2.2 Termite eusociality

All termites are eusocial given the division of labour between reproductive individuals (kings and queens) and helpers. Although in many termite species, the immatures retain the potential of future reproduction, either as winged dispersing reproductives (primary kings and queens) or non-dispersing apterous or brachypterous neotenic reproductives (secondary reproductives), a common feature of all species is the presence of soldiers. These are, with very rare exceptions, completely sterile and thus represent an irreversible crossing of the threshold of eusociality (Myles 1986; Thorne, et al. 2003).

Termites are hemimetabolous insects and their caste differentiation is a matter of postembryonic development, during which morphogenetic programs of different castes can be triggered from the gene pool common to all colony members. In other words, early larvae are multipotential, and the caste-specific developmental trajectories can be switched until late stages (Miura 2005). The differentiation arises as the effect of the environment (nutrition, social stimuli, season, chemical factors), in the form of differential expression of developmental programs of a specific caste (Noirot 1990; Hojo, et al. 2004; Scharf, et al. 2007; Watanabe, et al. 2014). As a crucial factor governing caste differentiation, the actual caste composition of the colony is known to alter the development of immatures (Hayashi, et al. 2007). This social stimulation is mediated by primer pheromones. The environmental stimuli could be perceived by termites themselves or transferred through interactions as allogrooming or trophallaxis (Noirot 1991). Unlike social Hymenoptera, termites are diplodiploid animals, therefore both sexes can be present in each caste (Roisin and Korb 2010), which may offer another level of caste polymorphism due to sex polyphenism.

In general, two main types of postembryonic development and thus resulting caste systems are distinguished, i.e. linear and bifurcated development. The linear developmental model lacks the true worker caste, the work tasks are performed by late larvae (also called false workers, temporary helpers or pseudergates), which only reversibly postpone their development into primary reproductives via nymphal stages. This type of development shows great developmental flexibility: a late larva can become primary or secondary reproductive, develop into a soldier or remain or moult into subsequent pseudergate stages (Roisin 2000; Bourguignon, et al. 2009; Roisin and Korb 2011; Watanabe, et al. 2014). The linear pathway is typically found in the families Stolotermitidae, Archotermopsidae, Serritermitidae and some basal genera of Rhinotermitidae (*Prorhinotermes, Psammotermes, Termitogeton*). The development scheme is depicted in Fig. 1 below.

The bifurcated pathway, characteristic for families Termitidae, Hodotermitidae, Mastotermitidae, and partially Rhinotermitidae, is characterized by the presence of true workers (permanent workers), which irreversibly deviate from the egg-to-alate developmental line at an early larval stage. While the true workers can, in some species, differentiate into neotenic reproductives, they never develop into winged sexuals (Roisin and Korb 2011). This early determination of workers enables a more conspicuous dimorphism, both within the polyphenism and within gender. The sexual dimorphism can result in sexual polyethism (Roisin 2000; Parmentier and Roisin 2003; Roisin and Korb 2011).

Interestingly, the occurrence of the two developmental types does not strongly correlate with the phylogenetic patterns, which for some time, obscured the debate on the evolutionary origin of the true worker caste. Under all available phylogenetic hypotheses, the workers caste would have to evolve multiple times (and would have to be lost multiple times) (Noirot and Pasteels 1988). Nevertheless, the consensus considers the bifurcated pathway (and the true worker caste) as a derived trait. Indeed, the evolution of true workers is accompanied by multiple other aspects of advanced sociality, such as foraging for food, separate nest building, etc., and in the most derived family, Termitidae, true workers

are universal. Therefore, the plausible explanation is that caste systems are tightly linked with ecology rather than phylogeny, which led to the classification of termites as "one-piece" type termites (linear development, no separate nesting, lack of foraging) and "multiple-pieces" type termites (true worker, foraging, separate nesting) (Higashi, et al. 1991).

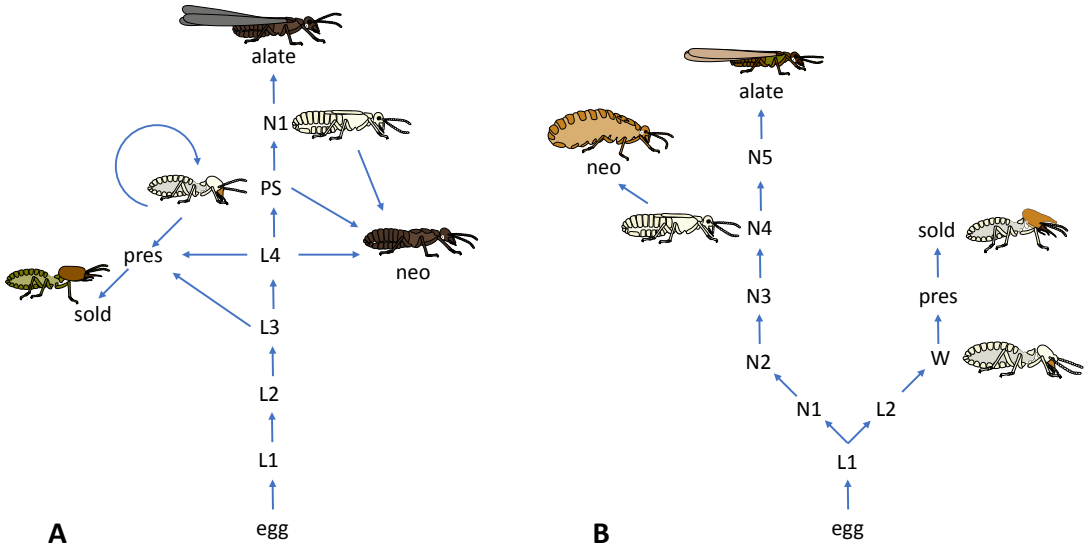

Fig. 1. Two developmental types of termites. **A.** Linear developmental exemplified in *Prorhinotermes simplex* (Rhinotermitidae). **B.** Bifurcated development exemplified in *Embiratermes neotenicus* (Termitidae). W, worker; PS, pseudergate; N, nymph; sold, soldier; pres, presoldier; neo, neotenic reproductive. Based on Roisin (1988, 1992).

2.3 Terminology of castes and developmental stages

Termite caste polyphenism gives rise to various stages and castes with specific terminology according to reproductive potential, role and ontogenetic origin (Nijhout 1999). I summarize below the most important terms, some of which are not common outside the specialized termite literature, mostly based on the terminology by (Thorne 1996).

Larva – apterous food-dependent juvenile with absent signs of morphological specialisation which can develop within imaginal or apterous (worker) lineage (Thorne 1996; Korb and Hartfelder 2008).

Nymph – juvenile having wing buds. Contrary to other hemimetabolous immature stages, isopteran terminology allows using this term only for individuals preceding the imago (Thorne 1996; Korb and Hartfelder 2008). In species with linear development, the nymph can undergo a regressive moult accompanied by a loss of wing buds and become a pseudergate. This is the only case of reversible metamorphosis of brachypterous nymphs in the whole class of Insecta (Nijhout and Wheeler 1982).

Pseudergate (false worker, temporary worker) – late larva occurring only in some lower termites, holding the post of a worker, but maintaining the ability to transform into a presoldier, into a neotenic, but also to proceed through a progressive moult into nymphal stage(s). It can also undergo stationary moult into next pseudergate instar (Thorne 1996; Roisin and Korb 2011; Watanabe, et al. 2014).

Worker (true worker, permanent worker) – individual undertaking most labour within the colony (foraging, feeding, brood and nest care), deviating irreversibly from the development into an alate. In some species, workers can become neotenics (Noirot and Pasteels 1987; Thorne 1996).

Presoldier (white soldier) – short-term transitional stage preceding the soldier. May arise from a larva, a worker or a nymph. Presoldiers lack pigmentation and sclerotization of cuticle on the body and on mandibles (Thorne 1996).

Soldier – sterile, anatomically specialised caste, whose only purpose is to defend the colony. Due to modified mandibles, the soldiers are not able to feed by themselves and are dependent on workers for care (Thorne 1996). The soldier caste is ancestral to all extant termites, only in a few modern lineages it has been lost secondarily (e.g. some Apicotermitinae) (Roisin 2000; Roisin and Korb 2010).

Alate (imago, winged disperser) – adult with fully developed reproductive glands, wings and compound eyes. The only imaginal stage (Thorne 1996). Undergoes dispersal flights and founds new colonies.

Dealate (primary reproductive, primary king and queen) – future kings and queens, which break off their wings after the dispersal and encounter with a potential partner of opposite sex (Thorne 1996).

Secondary reproductive – an ontogenetically diverse category encompassing reproductive individuals, which reproduce in the maternal colony and therefore do not disperse. Has several forms: i) *Adultoid* – a dealate reproducing without leaving the natal nest, ii) *Neotenic* – develops into a fertile state from various stages without reaching the imaginal stage and with underdeveloped adult characteristics (compound eyes, wings): *Ergatoid* – arising from the apterous line, i.e. from workers; *Pseudergatoid* – emerging from a pseudergate; *Nymphoid* (brachypteric neotenic) – differentiates from a nymph usually recognizable by shortened wing buds (Myles 1999).

2.4 Chemical communication of termites

In insects, the chemical senses using olfactory cues represent the dominant mode of perception of the outer world and intra- or interspecific communication. It is even more flagrant in termites, because most of the inhabitants of their colonies (except for winged dispersers) have lost eyesight due to underdeveloped compound eyes. Therefore, chemical communication takes over the visual cues, and only a minor part of the complex communication exchange in the colonies is mediated also by vibratory communication (Costa-Leonardo, et al. 2009; Bordereau and Pasteels 2011; Bagnères and Hanus 2015). Just as in other social insects, chemical communication can be classified into two general categories, i.e. 1) communication using species-specific and conserved semiochemicals secreted by exocrine glands – these signals are widely recognized under the term *pheromones*, and 2) communication using variable and complex chemical mixtures of cuticular hydrocarbons, constituting the non-polar waterproofing layer on insect epicuticle – these signals are known as *signature mixtures* (Wyatt 2010).

Traditionally, pheromones are further classified as *primer pheromones* and *releaser pheromones*. The former pheromones affect physiology, development and caste differentiation of the receivers in the long term. Among them, the *queen (king) pheromones* ensure the reproductive dominance of the reigning reproductives over the non-reproducing nestmates and prevent their differentiation into neotenics, and *soldier pheromones* inhibit the differentiation of immatures into soldiers (Matsuura, et al. 2010; Matsuura 2012; Mitaka, et al. 2017). The primer pheromones thus mediate the establishment of reproductive harmony, social homeostasis and appropriate ratio of different castes in the colonies. By contrast, the *releaser pheromones* elicit rapid behavioural responses of the receivers. Like in other social insects, termites possess a rich repertoire of releaser pheromones, which control the complex social behaviours in their colonies, such as trail-following, sex-pairing, alarm, food-marking, aggregation, egg-recognition and royal recognition pheromones (Bagnères and Hanus 2015; Mitaka and Akino 2021).

The signature mixtures in the form of cuticular hydrocarbons are long-known signals responsible for nestmate recognition in social insect colonies. They convey information about colony identity, caste identity, reproductive status, and other identity aspects of individual colony members. Though in large part genetically determined, the qualitative and quantitative aspects of cuticular hydrocarbon mixtures are not universally conserved within the given species, as documented by dynamic differences among colonies, changes in time, season, geographical variability, etc. These characteristics make them suitable for role in recognition as contact recognition signatures (Clément and Bagnères 1998; Kaib, et al. 2002; Howard and Blomquist 2005). Yet, also among the cuticular hydrocarbons, conserved species-specific compounds occur that are more likely to be classified as pheromones, such as the recently identified king/queen recognition pheromone (Funaro, et al. 2018).

2.4.1 Evolutionary origin of sex-pairing and trail-following behaviour

Trail-following behaviour mediated by trail-following pheromones (TFPs) and formation of bisexual pairs of dispersers (future founders of new colonies) mediated by sex-pairing pheromones (SPPs) are ubiquitous elements of termite behavioural repertoire. As I show below, these two behaviours are tightly linked by their evolutionary origin and in consequence also by the chemistry of the pheromones involved (Bordereau and Pasteels 2011). Termites were originally solitary, therefore formation of pairs of winged imagoes is an ancestral behaviour. It consists of an encounter of a male and a female after the dispersal flight, the formation of a pair, followed by the so-called nuptial promenade, during which the female, followed by the male, searches for a suitable microhabitat to establish a new colony. The encounter is mediated by SPPs, in most species secreted as a long- to mid-range airborne signal by females from sternal or tergal glands in a typical calling posture. Only in basal families both males and females (Termopsidae) or exclusively males (*Hodotermes mossambicus*), produce SPPs (Leuthold and Bruinsma 1977; Bordereau, et al. 2010). The same pheromone may be released by the female during the promenade to ensure the cohesion of the pair. For this purpose, SPP may be applied by the female

from the sternal gland on the substrate in a manner similar to the use of TFPs by the workers (and/or soldiers) to mark the foraging trail during the foraging (Nutting 1969; Quennedey, et al. 2004). The ability of female imagoes to lay a pheromone trail is believed to be co-opted for trail-mediated alarm behaviour performed by the workers (and soldiers) inside the nests, during which the alerted termite lays a pheromone trail from its sternal gland to guide the recruited nestmates to the disturbance site. From these preadaptations, the trail-following behaviour used for orientation in the galleries of the wooden nest is thought to evolve, and, ultimately, also the external foraging outside the nest in socially advanced termites having true workers and nesting site separated from the source of food (Bordereau and Pasteels 2011; Bagnères and Hanus 2015). The assumptions on the evolutionary origin of trail following from sex-pairing communication are supported by the universal presence of sternal gland (which is the ancestral source of SPPs) in all termite castes of all studied species, and also by the very frequent use of the same compounds for both, the SPP and TFP communication.

2.4.2 Pheromone glands

In contrast to Hymenoptera with hundreds of exocrine gland types described, in termites only 17 exocrine glands have been reported (Gonçalves, et al. 2010). The most important exocrine glands are depicted in Fig. 2. Of crucial importance is the *frontal gland*, a unique gland, exclusive to Isoptera, present in winged imagoes, and a source of a great variety of defensive compounds especially in soldiers of the advanced clade Neoisoptera (Stylotermitidae, Serritermitidae, Rhinotermitidae, Termitidae). Several hundred different chemicals have been identified in termite frontal glands, many of them being new to science, some of them playing a secondary role of alarm pheromones or soldier pheromones (Lefeuve and Bordereau 1984; Krasulová, et al. 2012). As stated above, the SPPs and TFPs are secreted mostly from the *sternal gland* and the *tergal glands*, in a few species also from *posterior sternal glands*. Among the least studied, I may list the *labral gland* (Deligne, et al. 1981; Costa-Leonardo and Haifig 2014), the *mandibular gland,* situated at the base of the mandible and present even in *Nasutitermes* soldiers with atrophied mandibles (Noirot 1969), the universally present *labial (salivary) gland* as a source of food-marking pheromone, or, in some species, of defensive chemicals (Noirot 1969; Šobotník 2003; Sillam-Dussès, et al. 2012).

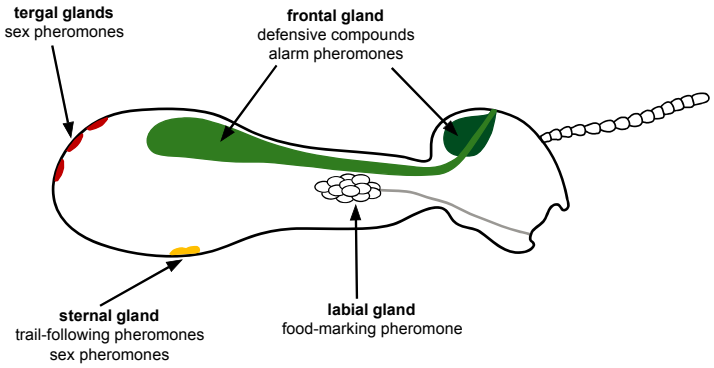

Fig. 2. General scheme of the most important pheromone producing glands in termites.

Sternal glands produce both TFPs in neuters and SPPs in imagoes of most species (Quennedey, et al. 2008). The glandular secretion is stored in the intersternal fold, from which the pheromone is deposited on the substrate in case of trail following, or exposed in a calling posture to the flux of air in case of airborne mate attraction (Noirot 1969). Sternal gland ubiquitous in all studied termite species and castes. In the basal family Mastotermitidae, it occurs in three sections on the third, fourth and fifth sternite; in derived families it is situated under a single sternite (sternite 4 in Stolotermitidae and Hodotermitidae, sternite 5 in others), as depicted in Fig. 3 (Noirot 1969, 1995; Bagnères and Hanus 2015). The epicuticle of the gland is porous, and two cell types have been described in the basal taxa (Mastotermitidae, Hodotermitidae and Kalotermitidae). In the more derived families Serritermitidae and Rhinotermitidae, three classes of cells were observed, whereas in the most modern termites, Termitidae, sternal gland consists of two types of cells. The structure is usually species-specific (Quennedey, et al. 2008). For its cytological diversity, (Noirot 1995) suggests using sternal glands as phylogenetic markers due to serial homology. The cells forming sternal glands are of a mixed origin – class 1 cells are modified epidermal cells, while class 2 are supposed to be modified œnocytes (Noirot and Quennedey 1974). Cockroaches exhibit the same sternal gland arrangement in terms of mixed origin (Sreng 1984).

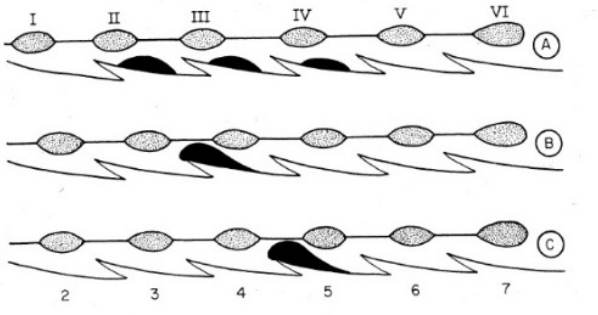

Fig. 3. Sternal glands of termites. **A.** *Mastotermes*. **B.** Stolotermitidae, Hodotermitidae, Archotermopsidae. **C.** Kalotermitidae, Rhinotermitidae, Termitidae. (I–VI) ganglia of the ventral nerve chain; (2 – 7) abdominal sternites. Drawing after Noirot and Noirot-Timothée (1965), systematic assignment of taxa to different schemes based on Quennedey, et al. (2008).

Tergal glands, not characteristic for all species, are present on distal abdominal tergites, although the number varies across species (Noirot 1969; Bordereau, et al. 2002). They occur in some species of advanced termite families (Neoisoptera) and functionally complement or replace the sternal gland in the production of SPPs for female calling and possibly also in ensuring the pair cohesion during the nuptial promenade (Bordereau and Pasteels 2011). Tergal glands are formed by epidermal thickening and consist of two cell types (Noirot 1969; Bordereau and Pasteels 2011). They occur typically only in alates and newly differentiated neotenics, not necessarily in both sexes, and their secretion acts as an SPP (Noirot 1969; Bordereau and Pasteels 2011). The pheromone is released by exposition of one or more glands in a calling posture manifested by raised abdomen (Hanus, et al. 2009; Bordereau and Pasteels 2011).

2.4.3 Trail-following pheromones

The best documented communication component is the trail-following behaviour performed during foraging, the collective food gathering (Wilson 1971; Bordereau and Pasteels 2011). Foraging consists of exploration, which is accompanied by trail marking. TFP is deposited on the substrate by pressing the abdomen against the substrate (Casarin, et al. 2009; Bordereau and Pasteels 2011). The recruitment itself follows once the food is found, whilst the marked trail is strengthened by a more attractive signal; it is not clear whether only the quantity of the pheromone or even the chemical composition is crucial (Oloo and Leuthold 1979; Traniello 1982; Affolter and Leuthold 2000).

For basal taxa, the so-called one-piece termites, the TFP serves for orientation in the corridors inside the nest, which the individuals do not leave, and the life of the whole colony is therefore conditioned by the size of the inhabited piece of wood. Along with increasing efficiency of pheromone communication and the advanced caste differentiation of socially advanced termites, a new evolutionary event has arisen multiple times independently – the occurrence of external foraging. Such species (Mastotermitidae, Hodotermitidae, Stylotermitidae, most Rhinotermitidae, Termitidae) build galleries to forage outside the nest and their food territory may reach up to thousands of square meters. Only in some taxa, the workers forage freely without covered galleries. This applies, for instance, to Hodotermitidae, and the lichen-feeding higher termite genera *Constrictotermes* and *Hospitalitermes* (Nasutitermitinae) (Mori 1987; Miura and Matsumoto 1997). Even though the foraging and food transport to the nest relies on the behavioural abilities of workers, the foraging columns are usually protected by soldiers. In some species it has been documented that the soldiers are those who initiate the foraging and serve as scouts, searching for food and recruiting workers to the food sources (Traniello 1981).

2.4.4 Chemistry of trail-following and sex-pairing pheromones

In contrast to the chemical richness of termite-produced defensive chemicals (Šobotník et al. 2010),

termites remain conservative in the diversity of their TFPs and SPPs; across all termite families, less than a dozen of compounds have been identified as TFP and SPP components – primarily aliphatic aldehydes, unsaturated aliphatic alcohols and diterpene hydrocarbons (Bordereau and Pasteels 2011; Mitaka and Akino 2021).

In basal families (Mastotermitidae, Archotermopsidae and Stolotermitidae) C_{13} or C_{14} branched saturated or unsaturated aliphatic alcohols or aldehydes occur mostly as a single-component TFP (Sillam-Dussès, et al. 2007; Bordereau, et al. 2010; Lacey, et al. 2011; Bagnères and Hanus 2015), in *Hodotermes mossambicus* an unidentified C_{18} branched aldehyde is proposed (Bordereau and Pasteels 2011). A significant evolutionary transition in TFP chemistry took place in Kalotermitidae and Neoisoptera. Along with the shift in sternal gland position from sternite 4 to sternite 5, a transition to unbranched unsaturated C_{12} alcohols, specifically (3*Z*)-dodec-3-en-1-ol (DE), (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (DDE) and (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (DTE) occurred, in a combination with diterpene hydrocarbons in some taxa (Matsumura, et al. 1968; Laduguie, et al. 1994; Peppuy, et al. 2001; Robert, et al. 2004; Sillam-Dussès, et al. 2005; Sillam-Dussès, et al. 2009a; Bordereau and Pasteels 2011; Florencio, et al. 2011; Sillam-Dussès, et al. 2011; Cristaldo, et al. 2014; Wen, et al. 2014; Sillam-Dussès, et al. 2020). An exception is the family Serritermitidae, using the unsaturated C_{19} ketone (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one as a TFP (Hanus, et al. 2012; Sillam-Dussès, et al. 2021).

TFPs were for some species considered as single-component pheromones. Only relatively recently the frequent two-component nature of TFPs has been unveiled using a combination of modern sampling techniques (especially solid phase microextraction) and electrophysiology (first used in Sillam-Dussès, et al. 2009b). Recent studies even attribute different functions to different TFP components with respect to the recruitment and orientation qualities of TFPs. For instance, DE in the two-component TFP of *Odontotermes formosanus* (Termitidae: Macrotermitinae) appears to have an orientation effect, while DDE elicits both orientation and recruitment, and the actual composition of the TFP has been found to be variable depending on the context of trail deposition (Wen, et al. 2014). The most frequently detected components of TFPs are listed in Fig. 4.

The chemistry of SPPs has been fully experimentally elucidated in roughly 20 species. Its significant feature is its similarity with the chemistry of TFPs. In some species, the same compounds act as both TFPs and SPPs, the differences between the two being only in quantities, context, and way of pheromone secretion. In other species, additional components have been identified in SPPs, which are absent in TFPs, or the SPP components are not identical, while still being structurally related to TFP. Thus, branched aliphatic aldehydes act as an SPP in the basal Archotermopsidae, while one or two C_{12} alcohols DE, DDE, and/or DTE are found in Kalotermitidae plus Neoisoptera, sometimes combined with the diterpene hydrocarbons neocembrene or trinervitatriene; in Nasutitermitinae, these diterpenes can even be the single SPP components (Bordereau, et al. 1991; Laduguie, et al. 1994; Bordereau, et al. 2002; Peppuy, et al. 2004; Robert, et al. 2004; Hanus, et al. 2009; Bordereau, et al. 2010;

Bordereau, et al. 2011; Lacey, et al. 2011; Sillam-Dussès, et al. 2011; Wen, et al. 2012; Wen, et al. 2015; Dolejšová, et al. 2018). Species-specificity of SPPs is low and the reproductive isolation among sympatric species is believed to be achieved by other means (timing of dispersal, contact recognition, etc.). Nevertheless, in some sympatric species complexes, the SPPs differ in quantity and activity of different components in a manner that can be responsible for reproductive isolation (e.g. Bordereau, et al. 2011 and Dolejšová, et al. 2018).

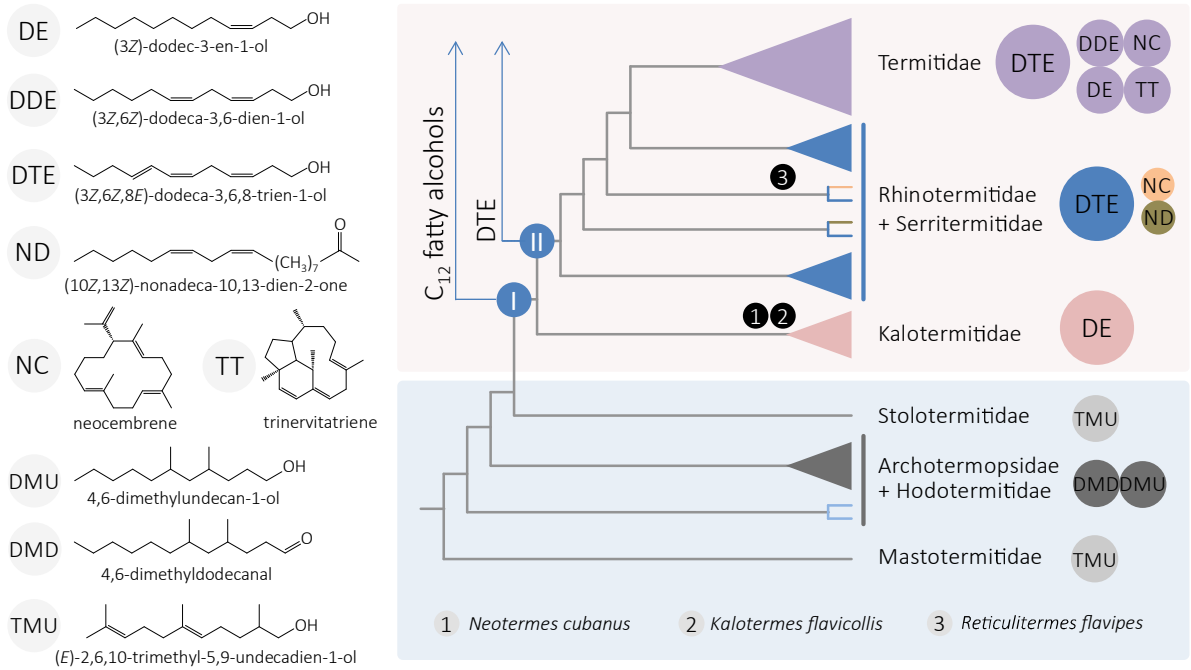

Fig. 4. Evolution of pheromone diversity in termites. The figure shows the structures of trail-following pheromone components (left) and maps their occurrence in individual families on phylogenetic tree (right). Phylogeny is simplified from Inward, et al. (2007b). Important evolutionary transitions are highlighted as I and II. Numbers 1–3 indicate the species used in my thesis.

Current knowledge on termite phylogeny and chemistry of TFPs and SPPs allows the reconstruction of evolution of termite pheromone communication, as exemplified in Fig. 4. Chemical ecology of termite TFPs and SPPs is an excellent example of chemical parsimony, where a few compounds or combinations thereof may be used for multiple purposes, depending on context, quantities, qualitative ratio of components, glandular origin, etc. (Bordereau and Pasteels 2011). Among the TFPs and SPPs, the unsaturated linear alcohols (C_{12}-OHs) with one, two or three double bonds (DE, DDE, DTE) greatly dominate, being virtually ubiquitous in Kalotermitidae plus Neoisoptera. The biosynthetic origin of DE and DDE is intuitive, since these very probably represent products of several cycles of β-oxidation of common fatty acids, oleic and linoleic acid, followed by reduction to alcohols. However, the structure of the third compound, DTE, is intriguing, because there is no direct precedent of 3*Z*,6*Z*,8*E* relative double bond topology and configuration in insect pheromones. DTE stands out also due to its dominant presence in TFPs and SPPs of advanced termites and due to its great biological efficiency: the activity threshold for DTE to elicit the trail following drops down to less than 10^{-5} ng/cm of the trail in some species (while usually being 10^{-1}–10^{-3} for other C_{12} alcohols) and when it

occurs in mixtures, it usually represents a quantitatively minor component with high biological activity. Thus, DTE evolved as an extraordinarily efficient and universal termite attractant. Its specificity may hypothetically be ascribed to the unusual 3*Z*,6*Z*,8*E* stereochemistry of the double bonds allowing for high selectivity of corresponding odorant receptors. Therefore, my thesis gives special emphasis on the biological activity and olfactory detection of DTE.

2.5 Antenna, the main olfactory sensory organ

Except for the basal group Protura, secondarily having olfactory receptors in the first tarsal segments, the whole subphylum Hexapoda dispose of a pair of antennae equipped with an assemblage of sensilla. Antennae, which can be segmented or annulated, arise from the antennal socket, *fossa antennalis*, on the third head segment, which shields the deuterocerebrum (Minelli 2017). Antennal socket is surrounded by a sclerite (torulus) with an attached joint (antennifer) providing the articulation between the first antennal segment and the head capsule. Typical antenna of Insecta (=Ectognatha) consists of three regions: (i) *the scape* (basal segment), attached to the head and controlled by the extrinsic antagonistic muscles (levators and depressors) coming mostly from the tentorium, (ii) *the pedicel* (second segment), and (iii) *the flagellum*, composed of multiple flagellomeres. Unlike the flagellum, the first two segments are musculated. Pedicel houses the Johnston's organ containing sensory cells allowing to detect the motion of the flagellum, used to perceive the wind, the flow of air during flight and thus to measure the velocity of the insect, and body orientation with respect to the ground, used for instance during the honey bee waggle dance (Greggers, et al. 2013). For the chemosensation, there are various types of sensilla evolutionarily and developmentally derived from macrotrichia, the simplest sensory organ of cuticular origin formed via the secretion of epidermal cells of two types (trichogen and tormogen).

The antenna itself can be found in various morphological types according to the biology of the species. In termites, antennae are moniliform and the number of flagellomeres increases during the individual development, together with sensillar distribution = heterochrony (Minelli 2017). All termite castes possess the same nine types of sensilla (basiconicum, campaniformium, capitulum, chaeticum I, chaeticum II, chaeticum III, marginal, trichodeum I, and trichodeum II), although the antennal morphology and sensillar composition varies (Krishna 1990; Castillo, et al. 2021). The entire sensillar set of termites is depicted in detail in Fig. 5 below.

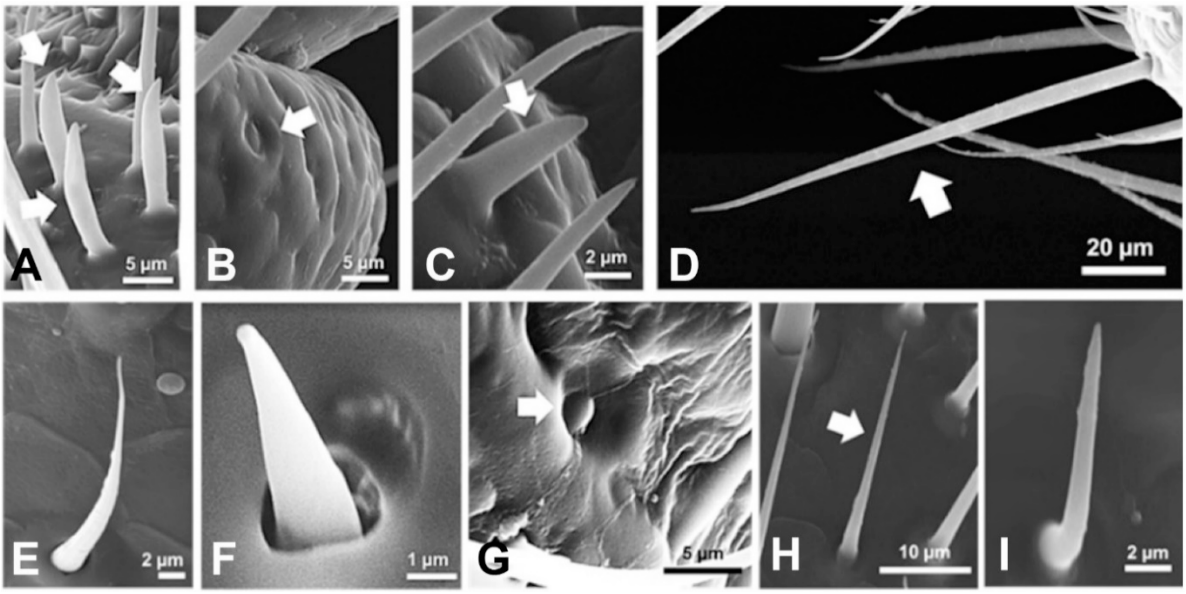

Fig. 5. Nine morphological types of sensilla identified in *Coptotermes formosanus* (Rhinotermitidae). **A.** Sensillum basiconicum. **B.** Sensillum campaniformium. **C.** Sensillum capitulum. **D.** Sensillum chaeticum I. **E.** Sensillum chaeticum II. **F.** Chaeticum III. **G.** Sensillum marginal. **H.** Sensillum trichodeum I. **I.** Sensillum trichodeum II. Modified from Castillo, et al. (2021).

2.5.1 Insect antenna and olfactory perception

Stimuli needed to control the chemotaxis, hygrotaxis or anemotaxis are perceived by antennal sensors. Olfaction alters several key behaviors, such as navigation towards the food source, potential mate or suitable egg-laying site. Antennae are the primary olfactory organs, though olfactory sensilla can also be found on maxillary palps and other body parts. Sensory hairs (sensilla) cover these appendages and occupy a wide range of morphological subtypes adapted to different environments. The cuticular wall of sensilla is hollow and porous, which allows odorants to pass through – see the Fig. 6 (Pask and Ray 2016). To facilitate transport of odorant molecules, often hydrophobic, there are odorant-binding proteins (OBPs) in aqueous sensillar lymph. Biological response to odorants (= interface between chemistry and biology) is mediated by olfactory receptor neurons (ORNs), whose dendrites run out to the sensilla. A single olfactory receptor type on dendritic membrane is usually expressed in each sensillum (Marin, et al. 2002; Wong, et al. 2002), although in some cases two receptors coexist (Goldman, et al. 2005).

ORN axons project to the part of antennal lobe, glomerulus, their axons are connected with the dendrites of projection neurons (Wong, et al. 2002). Projection neurons transport the olfactory signal from the antennal lobe to the mushroom body or lateral horn and usually innervate a single glomerulus (Dobritsa, et al. 2003).

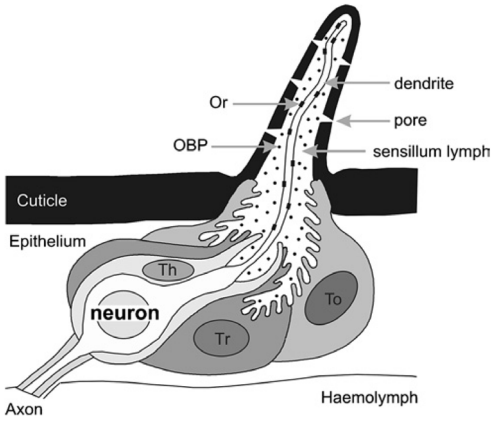

Fig. 6. General organisation of insect olfactory sensillum. ORNs are bipolar cells surrounded by accessory cells (To: tormogen, Th: thecogen, Tr: trichogen cells). Odorant binding proteins (OBPs) are present in sensillum lymph, olfactory receptors (Ors) are anchored in dendrite membrane. From Jacquin-Joly and Merlin (2004).

Insect olfactory receptors are of three types: *the odorant receptor family* (Ors) is the first identified and the most studied, *ionotropic receptors* (Irs) are related to ionotropic glutamate receptors, and *gustatory receptor family* (Gr), detecting CO_2, along with other water-soluble (Jones, et al. 2007; Robertson and Kent 2009).

Ors, found only in insects, most likely evolved from Gr family (Robertson, et al. 2003). Although Ors show significant divergency, one member of the family is strongly conserved across many species – Or coreceptor (Orco) (Pask and Ray 2016). It is also the closest relative to the Gr receptor family (Robertson 2003). In the species studied so far, Orco is universally expressed as a single homolog in the majority of ORNs and has a different role from the Or genes: Orco serves as a co-receptor to individual Or proteins and is important for their localization on the dendritic membrane of ORNs (Vosshall, et al. 2000; Pitts, et al. 2004; Missbach, et al. 2014). Ors are coupled with G proteins (GPCRs) and have seven transmembrane domains (Benton, et al. 2006). The Or structure is shown in Fig. 7.

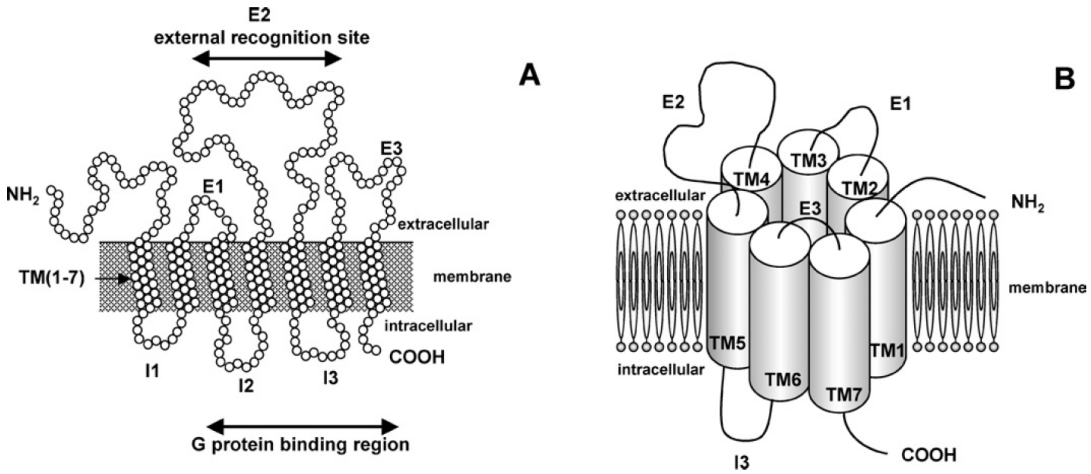

Fig. 7. Generalized olfactory receptor structure. **A.** amino acid representation with seven transmembrane domains (TM), extracellular loops (E) and intracellular loops (I). **B.** three-dimensional representation of Or in the membrane (Jacquin-Joly and Merlin 2004).

2.5.2 Mechanism of action of olfactory receptors

ORNs are depolarized by binding of a specific odorant (first messenger) to the corresponding receptor and by stimulation of a G protein, which leads to activation of adenylate cyclase. This enzyme increases the intracellular concentration of adenosine monophosphate – cAMP (second messenger), which opens the ion channels (Firestein, et al. 1991). The permeability of these channels is controlled by voltage – at the resting potential ion channels are closed and opening takes place when the voltage reaches the threshold membrane potential value. During membrane depolarization, when the channels open, there is an increase in the intracellular concentration of sodium cations. Induced change of electrochemical gradient leads to the formation of an action potential and the opening of other channels that conduct electrical excitation. After transpolarization, the ion channels are closed and sodium cations transported to the extracellular space. By activating the potassium channels, the membrane potential is stabilized to the rest value (Firestein, et al. 1991; Stengl, et al. 1992). The general scheme of insect olfactory system is depicted on Fig. 8.

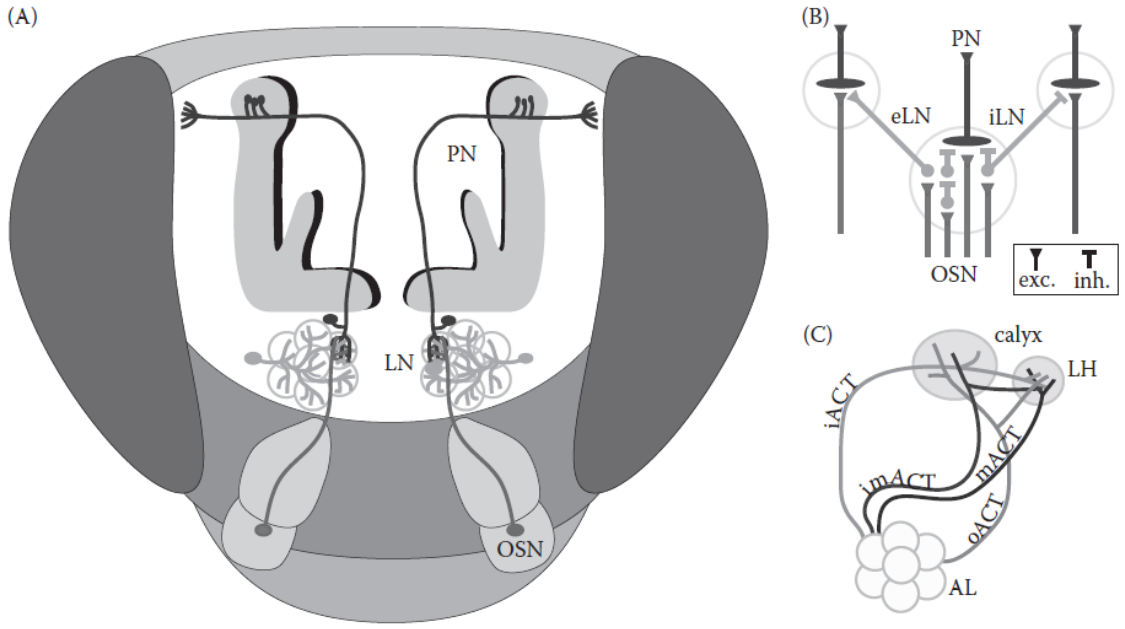

Fig. 8. Olfactory system in *D. melanogaster*. **A.** Schematic view of the fly head and its cut-open brain. ORNs (=OSN) are positioned on the antennae and project into the antennal lobe, where they interact with local neurons (LNs) and synapse onto projection neurons (PNs), leading to mushroom bodies and the lateral protocerebrum. **B.** Scheme of synaptic connectivity with ORNs input to a glomerulus, where they contact directly PNs or indirectly via LNs. The action can be excitatory or inhibitory and the connection is provided within a glomerulus or across glomeruli. **C.** Schematic view of the half brain (midline is left) and the four PN tracts (iACT, imACT, mACT and oACT) that connect the antennal lobe (AL) to the mushroom body calyx and the lateral horn (LH). From Galizia and Sachse (2010).

2.6 Techniques in functional characterization of insect Ors

2.6.1 Electrophysiology

Besides classical methods of insect chemical ecology – bioassays such as olfactrometer, wind tunnel or Y-test, where the direction of movement towards to the source of volatile substances is monitored

in living insect (Hare 1998), modern techniques such as electrophysiological recordings such as electroantennography (EAG) and single sensillum recording (SSR) are suitable for both qualitative and quantitative assessment of the olfactory response to specific odours, for example, in the identification of pheromones and VOCs host plants (Masson and Mustaparta 1990). The first studies have been undertaken in the middle of the last century with basic equipment which remained unaltered to this day (Schneider 1957a; Olsson and Hansson 2013). The foundations of electroantennography were laid by Dietrich Schneider, who tested in male silkworms (*Bombyx mori*) their response to the female sex pheromone, bombykol (Schneider 1957b).

The electroantennographic setup consists of an anti-vibration table, a microscope with long working distance, a grounding block, a micromanipulator with a ground electrode, a motorized micromanipulator with recording electrode and amplifier. The setup is placed in a Faraday cage to eliminate external noise The basic construction of electrophysiological setup is pictured below.

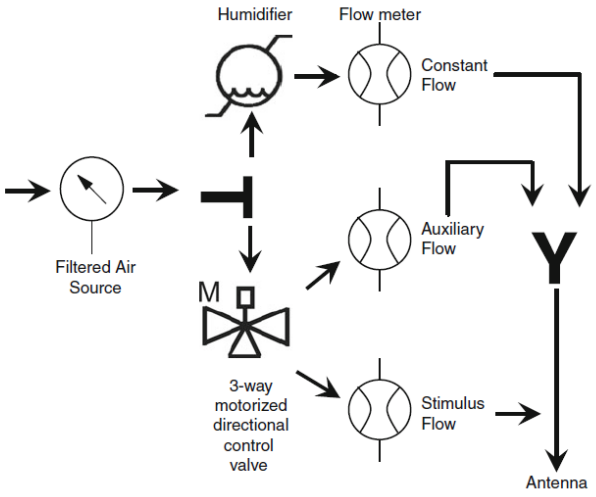

Fig. 9. Scheme of EAG/SSR setup. Arrows indicate direction of the airflow (Olsson and Hansson 2013).

The principle of EAG consists in the connection of the proximal and distal ends of the antenna to conductive electrodes by means of glass capillaries containing physiological saline solution. The connected sensor is then exposed to a stream of air (effluent) containing the test substance, which may be a volatile substance or a pheromone. EAG represents the sum of action potentials on membrane ORNs (Roelofs 1984). The physiological response is understood as a change in the electrical potential at the membrane of ORNs, which manifests itself in the antennograph as a reduction in electrical resistance. Other interpretations suggest that EAG records OSNs, lying in series and the resulting amplitude is adequate to antennal length (Kaissling 1971). Further investigations lead to opinion that EAG signal results from the initial negative drop in potential emerged from OSNs between electrodes combined with electronic potential slowly spreading from surrounding regions of the recording electrode (Nagai 1981). It is also necessary to mention the existence of factors altering the antennal response such as the condition of animal tested, or the position of the electrode and the resulting

strength of the connection (Nagai 1983). Either way, this technique is considered as a reliable way of quick evaluation the biological activity of odorants. For a more efficient identification of ligands a coupled system with gas chromatography (GC-EAD) is used, where the GC column separates individual fractions of volatiles, which are continuously released on the sensor (antenna) and in parallel, the analytes are sent to the GC-connected detector, which allows their chemical identification. A sample of the test substances may be mixed with a solvent containing hydrocarbons, usually alkanes of known number of carbons in their chain, which serve as markers for better orientation within the recording (Masson and Mustaparta 1990).

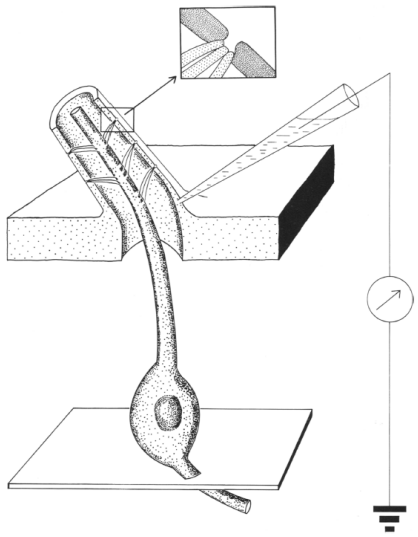

Fig. 10. A single-sensillum recording scheme. The olfactory receptor neuron (ORN) is located among the epidermal cell layer between the cuticle and the basement membrane. The dendrite innervates the cuticle hair, where the finest branches are in contact with the air. The neurite penetrates the basement membrane and proceeds via the antennal nerve to the deuterocerebrum. The recording electrode (tungsten or silver wire) penetrates the cuticle at the base of sensilla. The reference electrode is positioned in the haemolymph space (Boeckh, et al. 1965).

Whereas EAG is designed for measuring the electrical impulses outside the cell at the level of the entire antenna, by contrast, SSR provides a detail view on olfactory sensitivity of individual olfactory sensilla and allows for assignment of the ligand specificity of individual ORNs expressed therein. Likewise, EAG, the SSR setup consists of similar components. The electrode (tungsten or silver wire) penetrating the wall of the sensilla is directly in contact with the sensory lymph (Fig. 10). It is used for more detailed quantitative evaluation of physiological response to odorant stimuli via extracellular measuring. In this case, action potentials arise from OSNs within a single sensillum, where the measuring electrode comes in direct contact with sensillar lymph. The action potential amplitude is differentiated according to individual OSN with varying diameter (Hansson, et al. 1994). This allows to assess the sensitivity and selectivity of single OSN carrying specific receptors which projects onto same region of insect cerebrum (Vosshall, et al. 2000). This method can be used for characterization of the identity of individual Ors, responsible for the detection of specific odorants, including pheromones, thus mapping the overall sensitivity of the olfactory receptor to specific odor (Hallem, et al. 2004; Hallem and Carlson 2006). Thanks to this technique, it is possible to measure the change in

electrical potential even for such structures as short trichoid sensors or plate-like pores and other planar structures (Boeckh 1962; Masson and Mustaparta 1990; Olsson and Hansson 2013).

2.6.2 Drosophila „Empty neuron"

This technique involves a generation of *D. melanogaster* with a mutant ORN, ab3A, which lacks endogenous Ors (Or22a and Or22b). As a result, such empty neuron does not biologically respond to odors. When these receptors are transfected with the candidate receptors of the studied model and their ligand specificity can be studied using electrophysiological techniques described above. This system is used to characterize the Ors, Irs and Grs. In this way, it is possible to analyze how the quality, quantity and durability of the odorant are coded in the receptor repertoire (van Naters and Carlson 2007; Mathew, et al. 2013). It is performed *in vivo*, measures receptor-specific properties of the action potential and can be used to test various receptors. The disadvantage is the time required to generate transgenic lines and possible incompatibility of the receptor with the *Drosophila* neuron (Kreher, et al. 2008; Pask and Ray 2016).

2.6.3 Activity imaging in vivo from neurons

Calcium cations play an important role in signal transduction as secondary messengers. In this method, fluorescently detected Ca^{2+} ions are used. Fluorescent indicators bind to calcium cations in a cell or whole tissue cultures (in case of insects in glomerulus or antenna). Calcium reflux in response to olfactory stimuli can be observed using a fluorescence or confocal microscope. This method allows examination of ORNs *in vivo* (Grienberger and Konnerth 2012; Pask and Ray 2016).

2.6.4 Expression in cell systems

Modified cultures of *Xenopus laevis* oocytes are used to study Ors. The first Or thus expressed was Or43a from *D. melanogaster*. The oocyte with expressed receptors in its membrane is exposed to solutions and the change in electrical potential is measured using a two-electrode voltage clamp or patch-clamp. This technique provides a robust receptor response, however, it is limited to hydrophilic ligand molecules (Wang, et al. 2009; Pask and Ray 2016).

Identification of odorant ligands can also be performed by expression of Ors of known sequence in human kidney or hamster ovary cells. Such cultures are exposed to DMSO, methanol or odorants solubilized by OBPs (Montagné, et al. 2015). The physiological response can be verified by electrode clamp and calcium imaging (Pask and Ray 2016).

2.6.5 In silico screening

Advances in bioinformatics allowed to develop a method for projecting ligands of Ors, Irs, but also Grs *in silico*. The three-dimensional structures of known ligands are analyzed by a software, using which the common structural features of active substance are identified (Boyle, et al. 2013; Pask and Ray 2016).

3 MATERIAL AND METHODS

3.1 Studied species

I studied three termite species, i.e. *Kalotermes flavicollis*, *Neotermes cubanus* (both Kalotermitidae), and *Reticulitermes flavipes* (Rhinotermitidae) – see Fig. 11.

Colony of *K. flavicollis* has been collected in 2006 on Brač Island (Croatia), and since then is kept in a glass vivarium at 26°C and 60% relative humidity and fed with slices of pine wood.

Multiple colonies of *N. cubanus* are kept in laboratory at the same conditions as that of *K. flavicollis*. These colonies arose from a fragmentation and subsequent dispersals of winged imagoes from one colony originally collected in 1988 in Topes de Collantes (Cuba). The native TFP of both species, like other Kalotermitidae, has been identified as DE (Sillam-Dussès, Sémon, et al. 2009).

Three laboratory colonies of *R. flavipes* originate from collection of wild colonies at Oléron island (France) in 2002. They are kept in glass vivaria at 26°C and 60% r.h. and fed with slices of spruce wood. TFP of *R. flavipes*, like in most other Rhinotermitidae, has been identified to be DTE (Tai, et al. 1969).

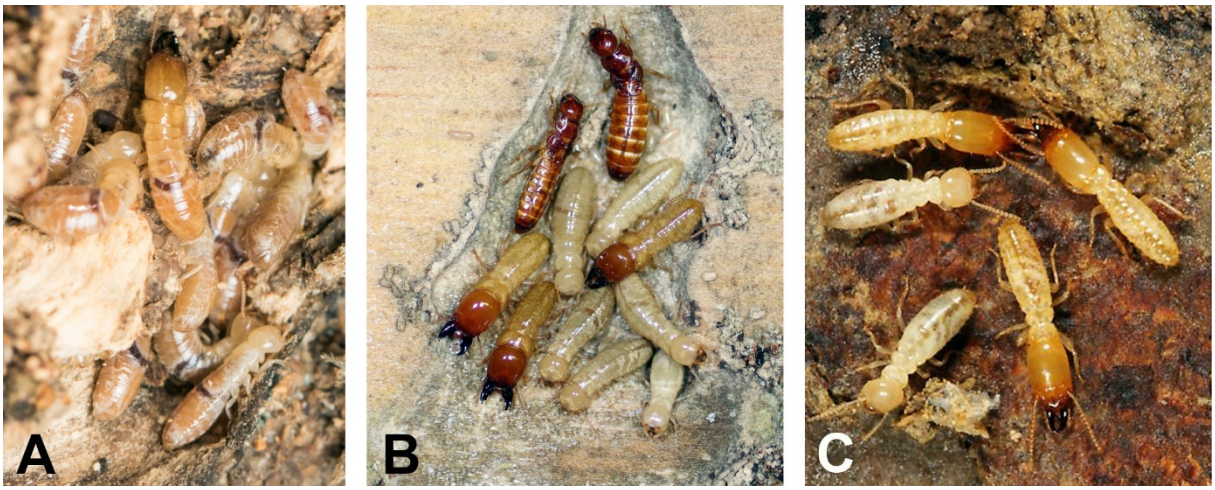

Fig. 11. Termite species studied in this thesis. **A.** *Kalotermes flavicollis*. **B.** *Neotermes cubanus*. **C.** *Reticulitermes flavipes*.

3.2 Pheromone standards

I tested the following termite TFPs: (3*Z*)-dodec-3-en-1-ol (DE), (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (DDE) and (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (DTE) – Fig. 12. All compounds originated from the library of pheromones of my home laboratory. I used either *de novo* prepared dilutions of the compounds from a pure form of these alcohols, or previously made stock dilutions, checked for purity prior to my experiments using gas chromatography with mass spectrometric detection. Pheromone dilution in all my experiments were made into GC/MS grade *n*-hexane.

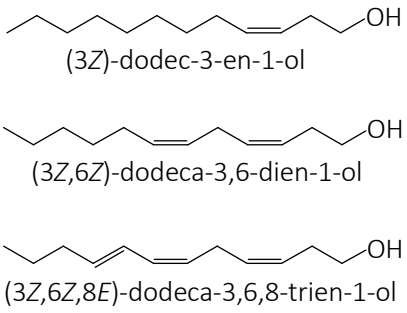

Fig. 12. Structures of the pheromones used.

3.3 Trail-following bioassay

3.3.1 Design of the experiments

For trail-following bioassays, I used the traditional open field Y bioassay, previously established for testing termite TFPs (Hare 1998; Bordereau and Pasteels 2011). It consists in releasing individual workers from a resting arena onto a 15 cm diameter Whatman n°1 filter paper. The opening of the resting arena, allowing a worker to leave it, is placed at the base of a Y-shaped figure, marked using piercing of the paper at every 1 cm of the trail. The basic stem was 3 cm long, while the branches were 7 cm long, at an angle of 60° between the two branches and between each branch and the stem. The design is depicted on Fig. 13.

To evaluate the sensitivity to the dilutions of individual pheromones, 10 µl of the dilution was deposited on the stem and one of the branches (1 µl/cm) using a 10 µl Hamilton syringe containing 10 µl of the tested solutions, while identical amounts of n-hexane were deposited on the stem and the opposite branch as a control. Individual workers were placed into the resting arena and once they leave it, the length and direction of their motion on the trail is monitored.

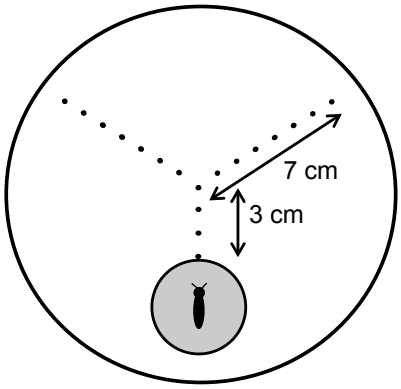

Fig. 13. Scheme of the open field Y trail-following bioassay.

3.3.2 Statistical evaluation

Each experiment was repeated 15 times for each dilution, the filter paper and the directions of the treatment branch and control branch were exchanged every 5 trials. The main rationale of the trail-following bioassay is to detect concentrations for each species and each pheromone at which an efficient trail-following behavior is triggered. To do so, one must define the lower threshold for the

trail-following behavior. Therefore, to state that a solution triggers an efficient trail-following behavior, including the change of the motion direction, I compared the distance travelled on the trail with the lower threshold of at least 3 cm, i.e. the worker not only went in the straight direction along the stem (3 cm), but also made the 60° turn along the branch on which the trail was deposited and travelled further. Therefore, for each treatment, I compared the mean travelled distance of the 15 workers with a fixed value equal to 3. Treatments resulting in a significantly higher mean distance were considered as significant trail-following stimuli. Statistical comparison was performed for each individual treatment using a one-sample t-test with hypothetical value for mean comparison set to 3. GraphPad Prism 8.0 was used for t-test calculations.

3.4 Electroantennography

3.4.1 Design of the experiments

Individual workers (5–6th stage) of the three model species were decapitated and a single antenna with corresponding hemisphere was attached to an electrode, represented by a 4 mm glass capillary filled with Ringer's solution. The capillaries were pulled using a heater to the desired diameter according to the size of the head. Once the antenna on a high impedance amplifier (10^{14}-ohm Syntech) was connected to the EAG setup (when the complementary measuring electrode was attached to the distal segment), an electric circuit was established. Antennal preparation was placed into a stream of clean air, into which odorous stimuli were injected from odour cartridges, i.e. short glass Pasteur pipettes with a 1 cm^2 filter paper impregnated with 10 ml of the test solution. Prior to the experiment, the filter papers were left for 10 minutes at room temperature to let the solvent evaporate. Injection of odour stimulations from the Pasteur pipettes were controlled by a Syntech stimulus controller operated by a foot switch and the maximal negative deflection after the stimulus was measured. The signal was transferred into Syntech EAG software.

Each series of stimulations consisted of a clean air control, hexane control, and a set of DE or DDE or DTE dilutions at doses ranging from 10^{-3} ng to 10^2 ng. At the end of the series, hexane and air control were applied again to verify the sensitivity and viability of the antenna. Each series of stimulations was repeated 15 times with antennae of 15 different workers.

3.4.2 Statistical evaluation

Intensities of antennal responses from each individual series of stimulations (one individual) were related to the values for initial air stimulation (as a measure of overall antennal sensitivity and quality of the circuit). Subsequently, the values were $\log_2$ transformed to reduce the heteroscedasticity typical for EAG responses. After such transformation, the distribution of the 15 responses was controlled with respect to premises for parametric testing (normality, equality of variances) by means of a a Brown-Forsythe test for homogeneity of variances and Wilk-Shapiro test for distribution normality. Then the means were compared using a one-way analysis of variance (ANOVA), followed by Dunnett's test; in

which I compared individually each treatment with the hexane control value. Calculations were performed using GraphPad Prism 8.0.

3.5 Single sensillum recording

As stated above, my aim with using SSR technique was twofold. First, I wanted to establish this technique in termites, since, to the best of my knowledge, it has not been previously used in Isoptera and the antennal anatomy of termites is quite different from those of insect models, in which SSR is routinely applied. And second, I wanted to search for antennal sensilla specifically responding to termite pheromone components, with emphasis on the identification of DTE sensillum, harbouring DTE-specific olfactory receptor. Therefore, intuitively, I selected to work with the *R. flavipes*, since it is the only of my three model species to have DTE as its TFP.

My strategy in the search of DTE-responding sensillum, consisted of two stages. First, I tried to analyse the antennal morphology of terminal antennal segments of workers with respect to the typology and topology of sensilla occurring at these segments, with the aim to retrieve different functional groups of sensilla, i.e. mechanical, olfactory, etc. To do so, I studied the antennae of workers using scanning electron microscopy (SEM), as described below. In the next step, I performed SSR experiments on individual selected candidate sensilla, which I identified on the antennal "map" as olfactory sensors and tried to retrieve them according to their sensitivity to DE, DDE, and DTE with hexane control.

The first technical issue is to fix the living animal in the SSR apparatus for the entire duration of the experiment. To this goal, I inserted the termites into classical 200µl pipette tips with the diameter of the tip opening adjusted using a razor only to let the frontal part of the head with antennae and mouthparts protrude out. A microscope slide, equipped with a double-sided sticky tape, was used to attach the antennae to the surface. A heat-extended borosilicate capillary (A-M Systems) was used to prevent the antennae from moving. Once the thin capillary was inserted between proximal antennal segments, a nylon fibre was laid between distal segments. Insect fixated this way was carried under the objective of an optical microscope of the SSR setup.

I worked with a basic SSR setup as listed above. Tungsten electrode (about 5 cm long, 0.05 cm diameter) was sharpened in KOH dilution under electric current generated by Syntech hardware. The grounding electrode was inserted into the soft part of the insect (in *Drosophila* into the eye; in case of eyeless termites, clypeus is the only penetrable part), using a simple XYZ manipulator. I handled the measuring electrode using a motorized Kleindiek Nanotechnik micromanipulator with six approaching levels, allowing to coordinate the electrode in circular motions in addition to the xyz motion. I inserted the electrode into the base of the target sensillum, where ORN is located. A photograph documenting the electrode inserted in the sensillum is shown on Fig. 14.

Fig. 14. Image from optical microscope capturing the electrode inserted into chemosensory sensillum, most likely s. basiconicum (black arrow).

Once the electric circuit is created, which is recorded by Autospike 3.9 software (Syntech), a clear signal should be visible. A single spike demonstrates the action potential of ORN. These spikes can be extracted from analogue wave and then subjected to classification according to their amplitude and frequency. For evaluation of the recording, when specific odour is applied, the spikes are counted, and the momentary frequency can be plotted. Sometimes the measuring electrode is too wide, bent, or submerged too deep and/or the signal is recorded from several neurons (it can be even possible that the sensillum hosts two ORNs). However, it is possible to select the specific amplitude in the record. Characteristic recording of appropriately connected and stimulated olfactory sensillum is partly depicted in Fig. 15 below.

The odours are applied by glass Pasteur pipettes on 1 cm^2 filter paper. In SSR, higher concentrations of the odour stimulants must be used per one sensillum, contrary to EAG measurement (in the range of order of magnitude higher), which is screening the whole antenna and thus gives the information about summarization of all ORNs. Therefore, based on my previous results with EAG recordings of responses to the three C_{12} alcohol pheromones in *R. flavipes*, I used the range of doses from 10 to 10^3 ng per one stimulation cartridge.

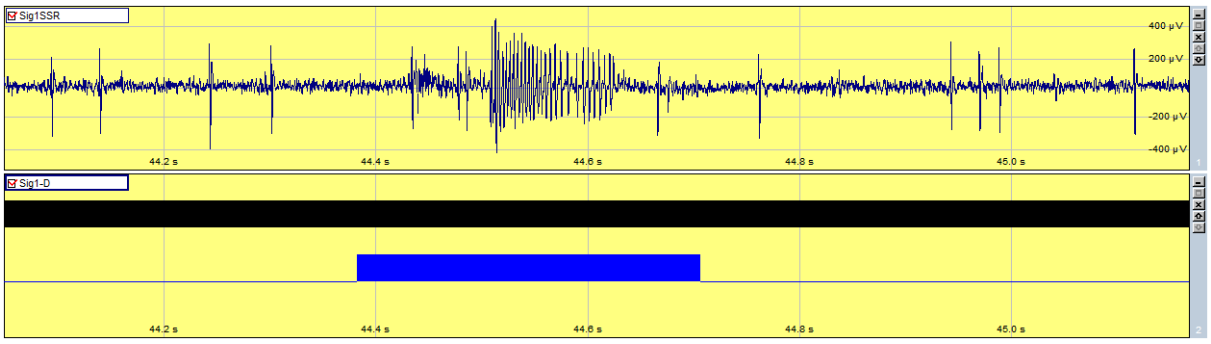

Fig 15. Single sensillum screening of *R. flavipes* with typical neuronal response of sensillum trichoideum II to odorant stimulation. The blue block marks the time interval of odorant stimulation.

3.6 Scanning electron microscopy

Heads of eight *R. flavipes* workers were dehydrated in an ethanol/acetone series (60%, 80%, 90%, 100% ethanol for 2 hours each, followed by aceton overnight). Then, the samples were gold coated, and observed and photographed in a Nova NanoSEM 450 field-emission scanning electron microscope, available in IOCB.

4 AIMS

My overall goal in this thesis is to contribute to the understanding of the evolution of olfactory detection of C_{12} fatty alcohol trail-following pheromone components in termites. More specifically, my question was whether evolutionary basal clades (represented by Kalotermitidae), having the ancestral pheromone DE, respond to more modern compounds DDE and DTE, and *vice versa*, whether more advanced lineages (represented by Rhinotermitidae), having DTE as the pheromone, respond to the ancestral DE.

Obtaining answers to these questions might provide indirect clues about the evolution of the olfactory machinery at the proximate level. For instance, sensitivity of Kalotermitidae to modern pheromones might signal low specificity of the olfactory receptors and/or the preadaptation for the evolution of the modern pheromones DDE and DTE. By contrast, sensitivity of Rhinotermitidae to DE might signal the evolution of DTE detection through duplications and neofunctionalization of the ancestral DE-specific receptor, which remained conserved and expressed in the modern lineages.

My specific aims were the following:

1. Test the responsiveness of workers of *Kalotermes flavicollis*, *Neotermes cubanus* and *Reticulitermes flavipes* to DE, DDE and DTE at behavioural level using standardized trail-following bioassays

2. Test the responsiveness of workers of *Kalotermes flavicollis*, *Neotermes cubanus* and *Reticulitermes flavipes* to DE, DDE and DTE at olfactory level using electrophysiological experiments

3. Map the distribution of different types of sensilla on the antennae of workers of *Reticulitermes flavipes*, retrieve the candidate olfactory sensilla, and develop the technique of single sensillum recording for the subsequent search of DTE-specific, and potentially DDE- and DE-specific sensilla.

5 RESULTS

5.1 Trail-following bioassays

5.1.1 Kalotermes flavicollis

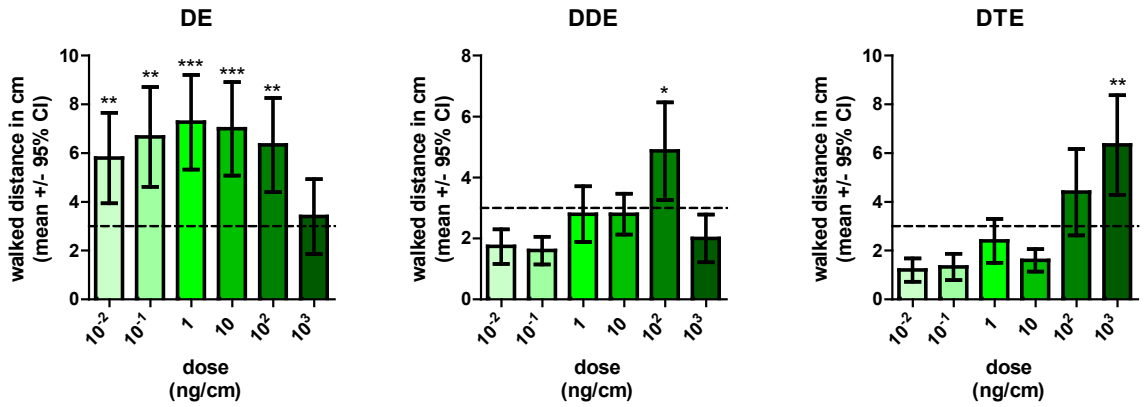

Fig. 16. Mean distance walked by the workers of *K. flavicollis* along the trail made of the three pheromones in the open arena Y bioassay, compared with the value set as a lower threshold for trail-following activity (3 cm). Data was compared with the threshold value using a one-sample t-test for each treatment. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Trail-following bioassays with *K. flavicollis* are summarized in Fig. 16. Workers of this species responded by significant trail-following behaviour to a wide range of concentrations of their native pheromone DE, starting with the lowest tested concentration 10^{-2} ng/cm up to 10^{2} ng/cm, with the peak at 1 ng/cm. At the highest concentration 10^{-3} ng/cm, the trail following activity dropped down to non-significant value, probably due to the saturation of air with the pheromone. This was demonstrated by a rapid onset of the trail following (termites left rapidly the resting arena), accompanied by disoriented running along the trail, but not on the trail itself.

When DDE was tested, the workers did not show any trail following behaviour at low concentrations (10^{-2} and 10^{-1} ng/cm) and took relatively long time to leave the resting arena. At higher concentrations (1–10^{2} ng/cm) they needed a shorter time to leave the arena and showed some elements of trail following, which was marginally significant at 10^{2} ng/cm, but this activity was lost again at 10^{3} ng/cm.

In experiments with DTE, I observed long latency and lack of trail-following behaviour over the lower concentrations (10^{-2}–10 ng/cm). Termites were more active and some of them showed trail-following behaviour at the two highest concentrations; at 10^{3} ng/cm the trail-following activity was significant.

5.1.2 Neotermes cubanus

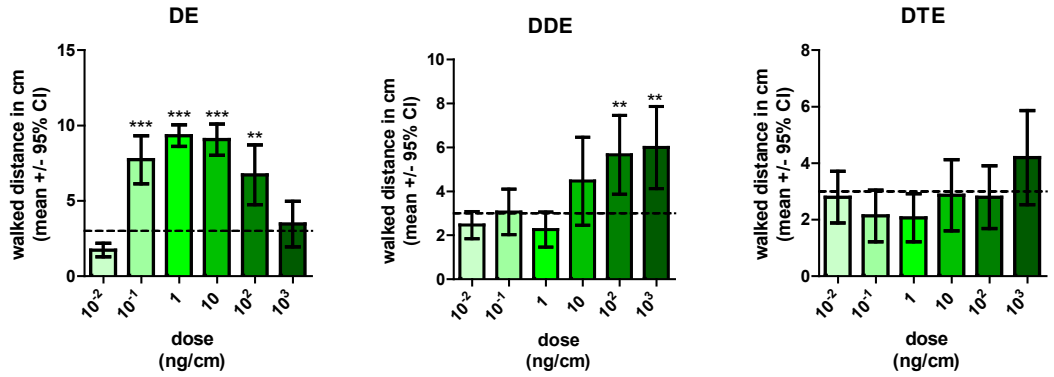

Fig. 17. Mean distance walked by the workers of *N. cubanus* along the trail made of the three pheromones in the open arena Y bioassay, compared with the value set as a lower threshold for trail-following activity (3 cm). Data was compared with the threshold value using a one-sample t-test for each treatment. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

In experiments with *N. cubanus*, my observations and obtained results were very similar to those shown above for *K. flavicollis*, as depicted in Fig. 17. Significant trail-following activity spanned the range of concentrations from 10^{-1} to 10^{2} of the native pheromone DE, with a peak at 1 ng/cm, at which the mean travelled distance along the trail reached almost the maximum value of 10 cm (almost all workers walked up to the end of the pheromone trail). Thus, the only difference in comparison to *K. flavicollis* was the absence of trail-following activity at the lowest concentration 10^{-2} ng/cm.

DDE was inactive up to 1 ng/cm, started to elicit trail-following at 10 ng/cm and higher, with values for 10^{2} and 10^{3} ng/cm being significant.

Likewise, DTE only showed some trail-following activity at the highest concentration used (10^{3} ng/cm), which was not, however, evaluated as significant.

5.1.3 Reticulitermes flavipes

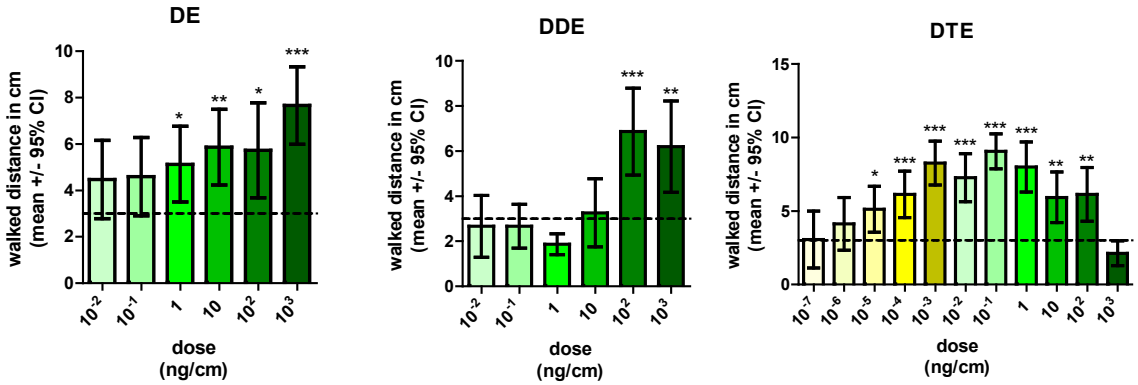

Fig. 18. Mean distance walked by the workers of *R. flavipes* along the trail made of the three pheromones in the open arena Y bioassay, compared with the value set as a lower threshold for trail-following activity (3 cm). Data was compared with the threshold value using a one-sample t-test for each treatment. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Results for *R. flavipes*, summarized in Fig. 18, were very different from those obtained for the two species of Kalotermitidae. The native pheromone DTE was significantly active in eliciting trail-following behaviour over a broad range of concentrations starting as low as at 10^{-5} ng/cm, having a peak at 10^{-1} ng/cm and dropping down to non-significant value at 10^{3} ng/cm.

DDE elicited trail-following behaviour only at the two highest concentrations (10^{2} and 10^{3} ng/cm). And finally, DE was relatively active at all concentrations (mean value was above 4 for all treatments), with the values for 1–10^{3} ng/cm being significantly higher than the threshold value.

5.2 Electrophysiological experiments

Electrophysiological experiments with the three species followed the trends similar to those observed in trail-following bioassays, even though the concentrations used in the bioassays cannot be directly compared to those applied in EAG, because the latter technique usually needs higher doses than EAG (when we compare ng/cm of the trail and ng per EAG stimulation cartridge).

5.2.1 Kalotermes flavicollis

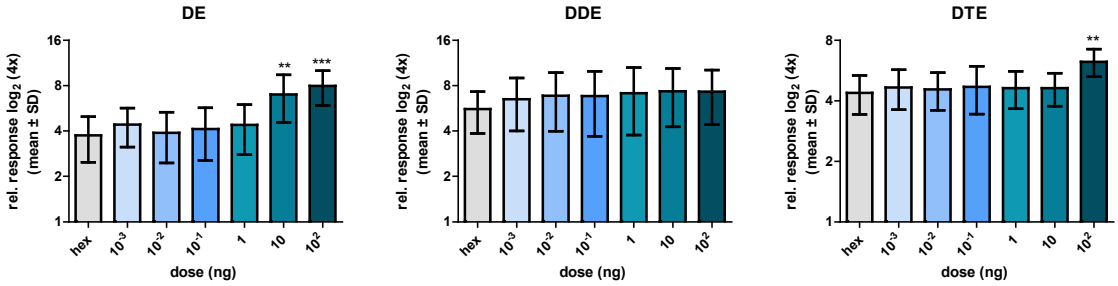

Fig. 19. Relative antennal responses of workers of *K. flavicollis* to a series of decadic dilutions of the three pheromones, compared to control hexane stimulations. Means were compared using one-way ANOVA, followed with Dunnett's test, comparing each treatment separately with the hexane control. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Relative EAG responses of *K. flavicollis* to the three tested compounds are shown in Fig. 19. The EAG measurements with the native pheromone DE revealed highly significant responses for the doses 10 and 10^{2} ng DE. By contrast, I did not record any significant responses to DDE over the range of doses applied. In case of DTE, only the highest dose of 10^{2} ng elicited a significant response when compared to hexane control stimulations.

5.2.2 Neotermes cubanus

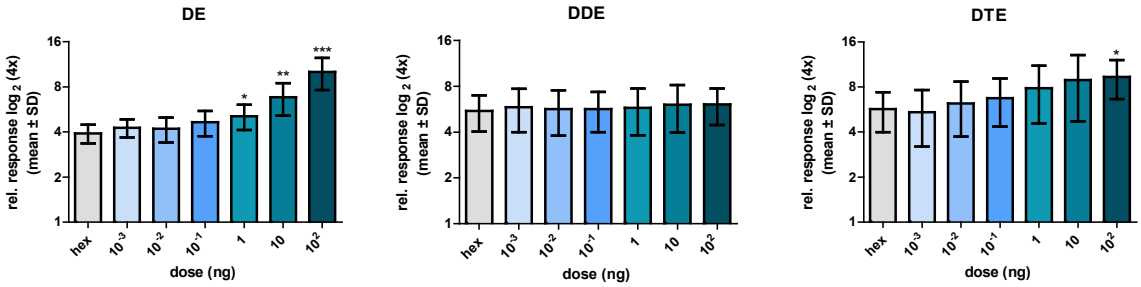

Fig. 20. Relative antennal responses of workers of *N. cubanus* to a series of decadic dilutions of the three pheromones, compared to control hexane stimulations. Means were compared using one-way ANOVA, followed with Dunnett's test, comparing each treatment separately with the hexane control. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

The EAG responses to the native pheromone DE in *N. cubanus* showed an increasing trend, starting to be significantly higher than the hexane control at doses 1 ng and higher. As in case of *K. flavicollis*, DDE did not show any significant responses at the doses applied. DTE responses were characteristic by a slow gradual increase of mean values along the increasing set of doses, with the highest, 10^2 ng, being significantly higher than the control stimulations.

5.2.3 Reticulitermes flavipes

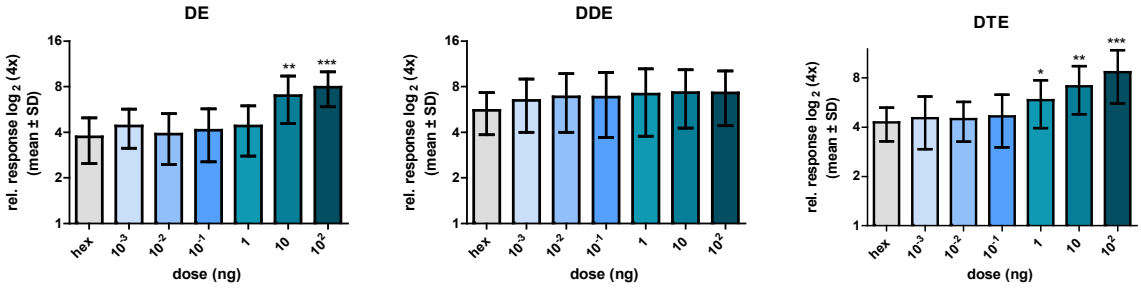

Fig. 21. Relative antennal responses of workers of *R. flavipes* to a series of decadic dilutions of the three pheromones, compared to control hexane stimulations. Means were compared using one-way ANOVA, followed with Dunnett's test, comparing each treatment separately with the hexane control. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

In *R. flavipes*, I observed a steep increase in EAG responses at doses 1, 10, and 10^2 ng of the native pheromone DTE. By contrast, and like in the two Kalotermitidae, I did not record any significant responses, nor increasing trends, in experiments with DDE. As for DE, the two highest doses (10 and 10^2 ng) significantly higher responses elicited significantly higher responses than the hexane control.

5.3 Mapping of antennal sensilla in R. flavipes

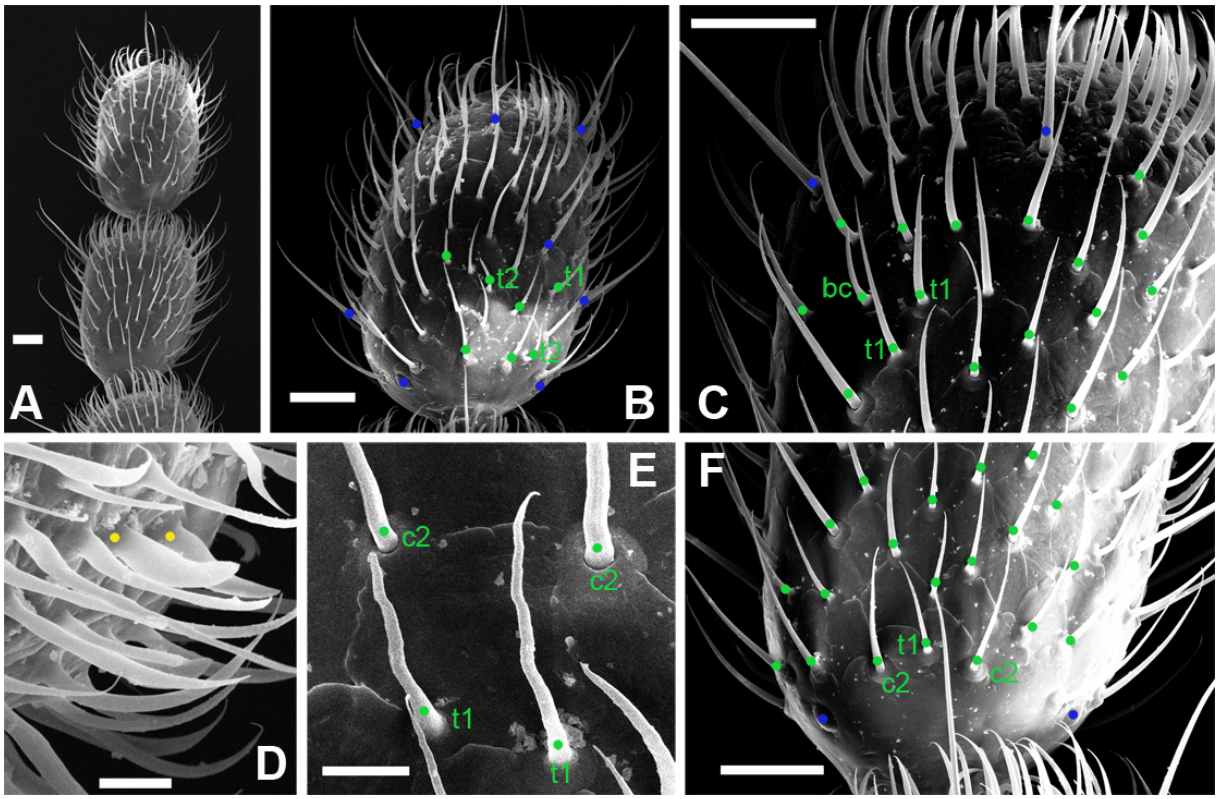

Fig. 22. SEM map of the last two antennal segments in workers of *R. flavipes*. **A.** General view of the last two segments. **B.** General view of the last segment. **C.** General view of the distal part of the subterminal segment. **D–E**. Detailed view of the subterminal segment. **F.** General view of the basal part of the subterminal segment. Scale bars represent 20 µm in A, B, C and F, and 5 µm in D and E. Spots with different colours represent different types of sensilla: Yellow dots mark hygro/thermoreceptive sensillum (s. capitulum); blue dots mark mechanosensory sensilla (spherical, bud-like sensillum campaniformium and long, movable s. chaeticum I are present) and green dots represent chemosensory – olfactory sensilla (bc, basiconicum; t1, trichoideum I; t2, trichoideum II; c2, chaeticum II). Morphological differences between s. trichoideum I and s. chaeticum II are in detail depicted in E.

While recording the SEM micrographs, I tried to capture the entire surface of the terminal antennal segments of workers, and to compare the presence and distribution of individual types of sensilla with the general typology of insect sensilla, as well as with two recently published papers on sensillar typology in workers of the termite *Coptotermes formosanus* (also from the family Rhinotermitidae) (Fu, et al. 2020).

I succeeded in identifying seven out of nine sensillar types reported by (Castillo, et al. 2021), namely s. capitulum (hygro/thermoreception), s. campaniformium and long movable s. chaeticum I (mechanosensors), and four types of chemosensory sensilla, i.e. s. basiconicum, s. trichoideum I, s. trichoideum II, and chaeticum II. These sensilla are marked in colours in Fig. 22.

The sensilla retrieved as chemosensory were the prime candidates for the search of olfactory (pheromone detecting) sensilla in my SSR experiments.

5.4 Single sensillum recordings

Indeed, upon establishing a reliable electric contact of the SSR setup with these candidate sensilla, we succeeded in obtaining electrophysiological records indicating their function as olfactory detectors of airborne signals. This applies more specifically to sensillum basiconicum, sensillum trichoideum I and trichoideum II (see Figs 15 and 22). For some of them, notably sensillum trichoideum I, we obtained, in some of our experiments, specific responses to DTE, as shown in Fig. 23.

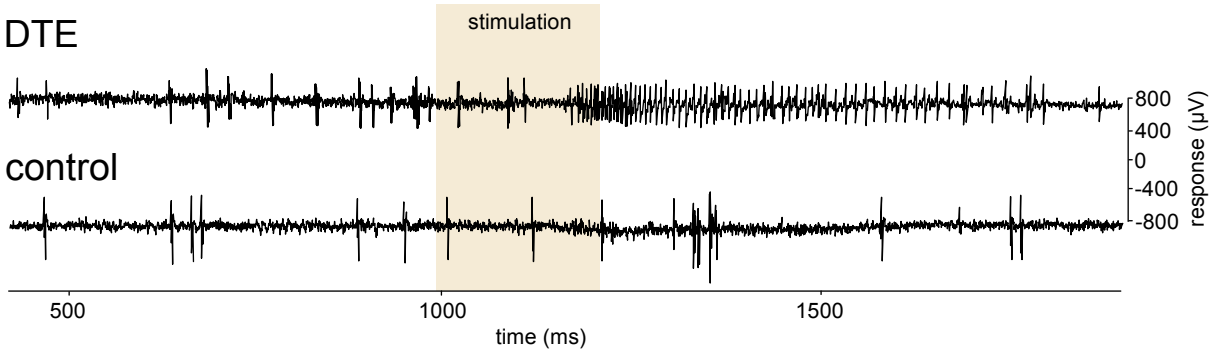

Fig 23. Single sensillum recording from a sensillum trichoideum I using 10^2 ng DTE (top) and control solvent stimulation (bottom). Characteristic burst of spikes following the stimulation time window indicates a specific response of the sensillum to DTE.

6 DISCUSSION and CONCLUSIONS

The main goal of my thesis was screening of the sensitivity to two most important C_{12} alcohol trail-following pheromones DE and DTE, with emphasis on the evolution of perception from more basal taxa (Kalotermitidae), in which the ancestral DE occurred, to the advanced lineage of Rhinotermitiadae, in which the most recent and biologically very efficient molecule DTE evolved. In parallel, I tried to develop the method of single sensillum recording in termites with the ultimate goal to search for specific sensillum responsible for the perception of these C_{12} alcohol pheromones. In this field, I focused on the receptor of DTE in the rhinotermitid *R. flavipes*.

General motivation for my work, as a part of a larger project on the evolution of termite pheromone communication at the level of biosynthesis and olfactory detection (No. 20-17194S, Czech Science Foundation), was to better understand the evolution of the detection of DTE. It is an outstanding molecule in terms of its structure, combining three double bonds in *cis* and *trans* positions. This makes it rare among other known natural compounds presumably derived from fatty acids, in which such a combination of double bonds is uncommon. DTE is exceptional also in terms of its biological activity: one gram of this TFP would be enough for a trail 10^{19} kilometres long, and the threshold quantities needed to elicit the trail-following activity in some Rhinotermitidae is by several order lower than in the case of the other C_{12} alcohols (Bordereau and Pasteels 2011). The same is applied also when we consider its role of a component of SPP emitted by female dispersers, in which it sometimes occurs in active amounts that are barely detectable by modern instrumentation (e.g. *Prorhinotermes simplex*, Hanus et al., 2009).

My behavioural experiments using the traditional open field Y bioassay revealed to be a relatively simple but reliable and robust tool to establish the sensitivity of different taxa to individual pheromones, and in most instances, it provided results which were in line with those previously reported using the same bioassays. When I tested the native pheromone DE in Kalotermitidae, I observed the lower threshold concentrations, peak activity values and saturation concentrations comparable to the observations reported by Sillam-Dussès et al. (2009a) and Sillam-Dussès (2004). In *K. flavicollis*, the peak value was observed by Sillam-Dussès (2004) to be 10^{-1} ng/cm, the lower threshold 10^{-2} ng/cm and the loss of activity through saturation was not reached at 10 ng/cm. In my experiments, the peak was observed at 1 ng/cm, higher threshold by 10^{3} ng/cm, and the lower threshold was not observed since I only started at 10^{-2} ng/cm and did not use the lower concentrations. But I can estimate, based on the shape of the activity curve, that 10^{-3} ng/cm would elicit an activity, which would not be significantly higher than the selected threshold value. The results for *N. cubanus* were roughly comparable to *K. flavicollis*, except for the higher value of the lower threshold and an overall better trail-following behaviour: *N. cubanus* workers at the peak activities walked a mean distance nearing the maximum of 10 cm and the SD of the trail-following activity was low, when compared to the maxima in *K. flavicollis* (nearing 8 cm) and the corresponding larger SD values.

Similar differences in trail-following behaviour can be seen between *Kalotermes* and *Neotermes* in Sillam-Dussès (2004).

Response of *R. flavipes* to its native pheromone DTE showed a very large range of active concentrations and a very low activity threshold, dropping down to 10^{-5} ng/cm, accompanied by a good trail-following efficiency at peak concentrations, reaching the walking distances between 9 and 10 ng/cm. These results are in line with observations on some other Rhinotermitidae and especially *Reticulitermes* (reviewed e.g. by Bordereau and Pasteels 2011), and underline once again the great sensitivity of *Reticulitermes* to DTE at the behavioural level. This can be explained by the biology of this genus when compared to Kalotermitidae, because *R. flavipes* belongs among the socially advanced termites with well-developed foraging for food on large territory. Hand in hand with this ability, also the olfactory capacities are expected to be advanced so as to allow the exploration of the diversified feeding territory and to allow an efficient trail following. This phenomenon can indirectly be documented by a rich repertoire of sensilla on *R. flavipes* antennae, when compared to one-piece type Kalotermitidae, spending the whole colony life inside the nesting piece of wood (Fig. 24) (Roisin and Korb 2011). An alternative and intuitive hypothesis is that Kalotermitidae and Rhinotermitidae differ in body sizes, the latter being substantially smaller, and thus also the quantities of pheromone produced and needed for biological activity can be expected to be smaller. Yet, the differences in pheromone sensitivity in three orders of magnitude recall the big efficiency of DTE and its corresponding pheromone receptor. How much all these factors contribute to interspecific differences might hypothetically be answered in the future once the pheromone receptors for the two alcohols will be discovered in the respective species and compared in the same expression system.

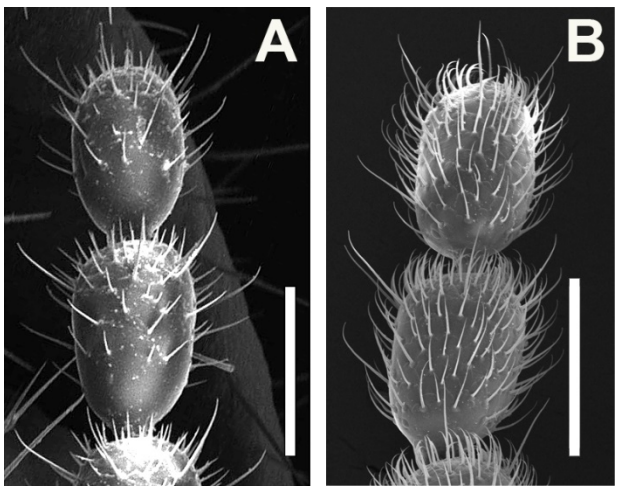

Fig. 24. Detailed SEM view on the last two antennal segments. **A.** *K. flavicollis*. **B.** *R. flavipes*. Scale bars show 100 µm.

Behavioural response to non-native pheromones differed between Kalotermitidae and *Reticulitermes*. DDE revealed to be mostly inactive, and only showed a significant activity at the two highest concentrations 10^2 in *K. flavicollis* and 10^2 and 10^3 ng/cm in *N. cubanus* and *R. flavipes*. These concentrations are very high and are not much biologically relevant with respect to the efficiency of

native pheromones and usually reported quantities and activities in different species (Bordereau and Pasteels 2011). Moreover, the mean walked distance never was longer than 7 cm. Therefore, I consider these responses to be rather non-specific. This view would agree with the evolutionary scenario of the origin of DDE as TFP and/or SPP as late as in higher termites, the most basally in Macrotermitinae (Robert, et al. 2004). Therefore, the two families studied here (Kalotermitidae and Rhinotermitidae) are evolutionarily older than the first occurrence of DDE. Interestingly, in some previous reports on *K. flavicollis*, DDE revealed to be more active in the Y assay than in my experiments, having an important trail-following activity already at 1 ng/cm of the trail, even though, just like in my experiments, the trail-following activity never reaches the high values in terms of the mean walked distance (Sillam-Dussès 2004; Sillam-Dussès et al., 2009a).

The activity of DTE in Kalotermitidae was only observed to come at concentrations 10^2 and 10^3 ng/cm in *K. flavicollis*, being significant only at the higher treatments. In *N. cubanus*, I observed an increase, moreover a non-significant one, only at 10^3 ng/cm. Therefore, also in this case, the behavioural response only took place at an overdose concentration, far higher than biologically relevant range. Once again, to my surprise, my observations somewhat differ from the previous report on *K. flavicollis* (Sillam-Dussès 2004; Sillam-Dussès et al., 2009a), which showed a significant activity of DTE already at 1 ng/cm.

DE was clearly more effective in eliciting trail-following activity in *R. flavipes* than was DTE in Kalotermitidae, significant activities being recorded over the range of concentrations $1–10^3$ ng/cm. Moreover, the mean values were reaching over 4 cm already for the two lowest tested concentrations 10^{-2} and 10^{-1} ng/cm. This suggests sensitivity of this species to DE across biologically relevant concentrations, and may signal the conservation of the response to DE in the modern lineage of Rhinotermitidae.

In summary, the results obtained in the trail-following bioassays suggest that while the older taxa do not show a preadaptation for behavioural response to the more modern pheromones, the modern Rhinotermitidae retain the capacity to detect the ancestral pheromone DE. This may hypothetically signal that also the olfactory receptor for DE remained conserved in the rich set of olfactory sensilla of *R. flavipes* and prompts the future search for this receptor, beside the newly evolved receptor specific to DTE.

Also, the EAG technique revealed to be suitable to distinguish between the sensitivity of different species to different pheromones. It is known that termites are not the best models in terms EAG responses when compared to established models such as Lepidoptera. Therefore, there were long omitted by electrophysiological studies. The first successful electrophysiological study of termite pheromones was performed in my home laboratory in 2009 (Sillam-Dussès, et al. 2009b). The listed article and following studies pinpointed an important advantage of EAG and GC-EAD: thanks to electrophysiology, pheromone components occurring in minute quantities can be detected, which are not detected by chemical analyses of termite extracts or unexpected chemicals can be attributed the

role of pheromone components (e.g. Sillam-Dussès, 2011; Hanus et al. 2012; Wen, et al. 2012, 2014, 2015; Dolejšová, et al. 2018). In fact, prior to the use of electrophysiology, termite TFP were considered to be single-component pheromones.

At the same time, it must be noted, that physiology of termite olfaction, probably also due to the anatomy of termite antennae, does not provide strong EAG responses, and high doses of odorants must usually be used to obtain solid responses. This also applied to my research, in which I which I was using a range of doses 10^{-3}–10^{2} ng, which revealed in most cases to be insufficient to detect the saturation maximum and peak doses, and rather allowed me to identify the lower threshold of sensitivity to individual tested pheromones when compared to solvent control. I observed, that native pheromones elicit significant responses at 1 or 10 ng and higher in the three species. From non-native pheromones, DDE did not show any activity at any tested dose. DTE was significantly detected by Kalotermitidae only at the highest dose 10^{2} ng. By contrast, DE showed highly significant responses in *R. flavipes* already at the dose of 10 ng. In summary, in spite of partial differences, EAG observations were in line with those obtained from the behavioural bioassays and may lead to identical conclusion: while the most derived DDE does not seem to show adaptive response, DTE in Kalotermitidae elicited limited but significant response, and DE in *Reticulitermes* showed significant response at relatively low doses. Thus, the search for the DE pheromone receptor in Rhinotermitidae is a meaningful future step also when considering the EAG results.

Techniques of SSR have not yet been successfully applied in termites. Therefore, I was satisfied to see, that this approach is functional and that we were able to retrieve from the repertoire of different sensilla those that are candidate for olfactory detectors and finally to obtain reliable SSR signals proving their olfactory function. This has been facilitated by the recent publication on classification of termite sensilla (Fu, et al. 2020; Castillo, et al. 2021). We also successful in obtaining convincing responses to DTE identifying individual sensilla specific to this compound. As a logical next step, the hypothesis on the conservation of a receptor for DE in Rhinotermitidae should be tested by searching for DE-specific sensilla in *R. flavipes*. It should be noted that during previous work of mine and my colleagues from CULS, specific responses to DE were already obtained; yet, a more rigorous future work is needed to verify these observations. To this goal, I would like to carry on in my SSR experiments, in the frame of the project which also includes deorphanization techniques to unambiguously identify the pheromone receptor proteins and genes.

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