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Analytical chemistry unravels the diversity and function of chemicals used for communication and defence in termite societies

Analytická chemie odhaluje diverzitu a funkci chemických látek užívaných v komunikaci a obraně termitích společenstev

Ph.D. Thesis

Supervisor: Doc. RNDr. Zuzana Bosáková, CSc.
Supervisor-consultant: Doc. RNDr. Irena Valterová, CSc.

Prague 2015
## TABLE OF CONTENTS

### PREFACE & ACKNOWLEDGEMENTS

### ABBREVIATIONS

### ABSTRACT

### ABSTRAKT

### INTRODUCTION

1. Chemical ecology: a fertile field of contemporary science
2. Chemical ecology: basic terms
3. Chemical ecology and social insects
4. Chemical communication in termites
   - 4.1 Trail-following pheromones
   - 4.2 Sex pheromones
   - 4.3 Alarm pheromones
   - 4.4 Fertility signals and primer pheromones
5. Chemical defence in termites
6. Methods in chemical ecology
   - 6.1 Extraction
     - 6.1.1 Solvent extraction
     - 6.1.2 SPME
   - 6.2 Separation
     - 6.2.1 Thin-layer chromatography, column chromatography
     - 6.2.2 Gas chromatography (GC)
     - 6.2.3 Comprehensive two-dimensional gas chromatography (GC×GC)
     - 6.2.4 Preparative GC
   - 6.3 Identification
     - 6.3.1 Kovats retention indices
     - 6.3.2 Mass spectrometry (MS)
     - 6.3.3 Infrared spectroscopy (IR)
     - 6.3.4 Electroantennography (EAG)
   - 6.4 Derivatization
     - 6.4.1 DMDS derivatization
     - 6.4.2 Silylation
   - 6.5 Confirmation and quantification
AIMS ......................................................................................................................... 37
MATERIALS AND INSTRUMENTATION ........................................................ ...... 39
RESULTS AND DISCUSSION .............................................................................. 41
7 Pheromones ....................................................................................................... 41
7.1 Trail-following pheromone of Glossotermes oculatus (ref. Paper A) .......... 41
7.2 Trail-following and sex pheromones in Psammotermes hybostoma
(ref. Paper B) ........................................................................................................... 47
7.3 Trail-following pheromone and sex pheromones in Hodotermpsis sjoestedti
(ref. Paper C) ........................................................................................................... 53
7.4 Alarm pheromone in Termitegeton planus (ref. Paper D) ......................... 58
7.5 Fertility signals in Prorhinotermes simplex ................................................ 62
8 Defensive chemicals .......................................................................................... 68
8.1 Defensive chemicals in soldiers of Psammotermes hybostoma (ref. Paper E)
............................................................................................................................... 68
8.2 Defensive chemistry of the termite Neocapritermes taracua .................... 73
PUBLICATIONS .................................................................................................... 79
Paper A .................................................................................................................. 79
Paper B .................................................................................................................. 89
Paper C .................................................................................................................. 99
Paper D .................................................................................................................. 107
Paper E .................................................................................................................. 115
SUMMARY ........................................................................................................... 125
REFERENCES ...................................................................................................... 127
LIST OF PUBLICATIONS ..................................................................................... 133
I had the opportunity to spend the past five years at the Institute of Organic Chemistry and Biochemistry as a member of the Research Team of Infochemicals, bringing together chemists and biologists with a common interest in the chemical ecology of social insects, especially termites and bumblebees. This thesis results from my collaborations within this multidisciplinary team as well as from international collaborations with chemical ecologists. My main goal and responsibility in this network of collaborators was to unravel the identity of chemicals used in communication and defence by various species of termites. The emphasis was placed on the description of the chemical diversity of these compounds, including the identifications of new structures, as well as on their functional significance in termite societies. As an ultimate goal, we tried to interpret our findings on chemical diversity of termite defensive and communication compounds in an ecological and evolutionary context.

Taking into consideration the unprecedented complexity of chemical communication signals (pheromones) as well as the richness of defensive chemicals produced in minute quantities in very complex matrices, analytical chemistry has a crucial role in studies on chemical ecology of social insects. My participation in the research presented in this thesis covered all aspects of analytical chemistry, ranging from extraction and isolation of compounds, through chromatographic separation and mass spectrometric identification, to derivatization, quantification, and in some cases also a synthesis of candidate compounds. Besides the routine analytical approaches and instrumentation available at the Institute of Organic Chemistry and Biochemistry, I had the opportunity to learn how to operate the two-dimensional gas chromatograph with mass spectrometric detection under the supervision of experienced colleagues. This high-end instrument has revealed to be an excellent tool for separation and detection of ultratrace quantities of analytes in complex mixtures and thus a vital condition for most of the presented studies.

This thesis consists of a brief Introduction to the chemical ecology of social insects with the emphasis on chemical communication and defence in termites together with a concise overview of all applied methods. The introduction is followed by the summary of Aims of this thesis, then the overview of used Materials and Instrumentation, and the chapter Results and Discussion, demonstrating the irreplaceable role of analytical chemistry in the studied topics and providing a detailed insight into the aspects of chemical analysis that were beyond the space limitations of the published papers included in this thesis. In addition to that, this chapter also summarizes the studies which significantly improved my analytical skills although the results have not been
published yet. Those of my results published in peer-reviewed impacted scientific journals are reprinted in the chapter **Publications**, followed by **Summary**. After the list of **References** cited in this thesis, I provide the **List of publications** as an overview of my scientific outputs.

The list of people to whom I feel indebted is very long. First of all, I am grateful to my supervisors, Irena Valterová and Zuzana Bosáková for giving me the possibility to study the Ph.D.

I would like to express my deep gratitude to my current boss, Robert Hanus, whose passion for science got me interested in social insects and biology in general and whose limitless support in sometimes very difficult situations was crucial namely in the final stages of writing this thesis.

I would like to thank my great colleagues, Petr Žáček and Pavel Jiřoš, who introduced me into the mysteries of analytical practice and created a pleasant work environment.

I am very grateful to all my collaborators and to all co-authors of our published papers for professional advice and for the opportunity to be involved in such interesting studies.

I would never finish my Ph.D. without a boundless support of my great family and marvellous friends, Lůca, Terezka, Mišák, Kubík and others.

Last but not least, thank you, Kamil, for making my life so beautiful.
I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere with intention to acquire any other academic degree.

I am aware that any use of the results obtained in this work, beyond the Charles University in Prague and the Institute of Organic Chemistry and Biochemistry AS CR, is possible only with a written consent of these institutions.

In Prague,

Signature
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\alpha$</td>
<td>separation factor</td>
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<tr>
<td>CAR</td>
<td>Carboxen</td>
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<tr>
<td>CI</td>
<td>Chemical Ionization</td>
</tr>
<tr>
<td>CW</td>
<td>Carbowax</td>
</tr>
<tr>
<td>DB-5</td>
<td>5% diphenyl 95% dimethylpolysiloxane</td>
</tr>
<tr>
<td>DB-WAX</td>
<td>column with polyethylene glycol</td>
</tr>
<tr>
<td>DMDS</td>
<td>Dimethyl Disulfide</td>
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<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
</tr>
<tr>
<td>EAD</td>
<td>Electroantennographic detector</td>
</tr>
<tr>
<td>EAG</td>
<td>Electroantennography</td>
</tr>
<tr>
<td>EI</td>
<td>Electron Ionization</td>
</tr>
<tr>
<td>FID</td>
<td>Flame Ionization Detector</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared spectroscopy</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC×GC</td>
<td>Comprehensive Two-Dimensional Gas Chromatography</td>
</tr>
<tr>
<td>HP-Chiral-20B</td>
<td>column with $\beta$-cyclodextrin in (35% phenyl)-methylpolysiloxane</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>High Resolution</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma-Mass Spectrometry</td>
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<tr>
<td>IR</td>
<td>Infrared spectroscopy</td>
</tr>
<tr>
<td>$k$</td>
<td>capacity factor</td>
</tr>
<tr>
<td>KI</td>
<td>Kovats Index</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>$N$</td>
<td>number of theoretical plates</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PA</td>
<td>Polycrylate</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PTV</td>
<td>Programmed Temperature Vaporizing</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>Quadrupole-time-of-flight</td>
</tr>
<tr>
<td>R</td>
<td>resolution</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected Ion Monitoring</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid-Phase Microextraction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>---------------------------</td>
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<tr>
<td>TIC</td>
<td>Total Ion Current</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-Layer Chromatography</td>
</tr>
<tr>
<td>TMSI</td>
<td>Trimethylsilylimidazole</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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ABSTRACT

Analytical chemistry plays a crucial role in studies on chemical ecology and only the development of sophisticated methods enables the detection of biologically active compounds that are usually present in minute quantities and often in very complex mixtures. My thesis is dedicated to the application of modern analytical techniques and instrumentation to unravel the identity, chemical diversity and function of semiochemicals and defensive compounds used by various species of termites.

The first section of this thesis aims at the identity of chemicals used in communication, the pheromones. I studied the chemistry of the trail-following communication in three selected species of termites. Besides the identification of (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol, the most frequent termite trail-following pheromone, as the trail-following pheromone in *Psammotermes hybostoma* (Rhinotermitidae), I participated in the description of two new structures, (10Z,13Z)-nonadeca-10,13-dien-2-one in *Glossotermes oculatus* (Serritermitidae) and syn-4,6-dimethylundecan-1-ol in *Hodotermpsis sjoestedti* (Archotermitidae). We identified (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol to be the female sex pheromone in *Psammotermes hybostoma*, and syn-4,6-dimethylundecanal to be the male sex pheromone in *Hodotermpsis sjoestedti*. I also identified the chemical composition of the complex multi-component alarm pheromone secreted by the soldiers of *Termitogoton planus* (Rhinotermitidae) and the volatile specific to reproductives, dodecane-2,10-diol, in *Protnotermitmes simplex* (Rhinotermitidae).

In the second section, I summarize the studies on the chemistry of termite defensive compounds. In soldiers of *Psammotermes hybostoma*, I detected altogether 33 defensive chemicals belonging mainly to sesquiterpenes and fully identified majority of them. Qualitative and quantitative comparison of defensive blends among colonies clearly distinguished three different chemotypes, corresponding well with the localities of their origin. In workers of *Neosapritermes taracua* (Termitidae), I participated in the description of a previously unknown multi-component defensive mechanism, resulting in the production of benzoquinone(s), converted from hydroquinone(s) through the catalysis by a copper-binding protein.

To conclude, my results highlighted the complexity of chemical defence and communication in termite societies, and contributed to the understanding of the evolution of pheromones and defensive chemicals in these oldest social insects.
ABSTRAKT

Analytická chemie hraje klíčovou roli při studiu chemické ekologie a jen díky sofistikovaným metodám je možné detekovat biologicky aktivní látky, které se obvykle vyskytují v nepatrných množstvích, často jako složky komplexních směsí. Má práce je zaměřena na aplikaci moderních analytických metod a instrumentace při zjišťování identity, chemické diverzity a funkce semiochemikálií a obranných látek používaných různými druhy termitů.

První část mé práce je zaměřena na identifikaci chemických látek používaných při komunikaci, tzn. feromonů. V této části jsem se zabývala studiem chemické komunikace při stopování u tří vybraných termitních druhů. Kromě nejběžnějšího termitího stopovacího feromonu, (3Z,6Z,8E)-dodeka-3,6,8-trien-1-olu, nalezeného u druhu Psammotermes hybostoma (Rhinotermitidae), jsem se podílela na identifikaci dvou nových struktur, (10Z,13Z)-nonadeka-10,13-dien-2-onu u druhu Glossotermes oculatus (Serritermitidae) a syn-4,6-dimethylundekan-1-olu u druhu Hodotermopsis sjoestedti (Archotermitopsidace). Podařilo se nám identifikovat také (3Z,6Z,8E)-dodeka-3,6,8-trien-1-ol jako samičí pohlavní feromon u druhu Psammotermes hybostoma, a syn-4,6-dimethylundekanol jako samičí pohlavní feromon u druhu Hodotermopsis sjoestedti. Identifikovala jsem také chemické složení vícesložkového poplašného feromonu produkovaného vojáky druhu Termiotogeton planus (Rhinotermitidae) a specifickou královskou látku, dodekan-2,10-diol, u druhu Prorhinotermes simplex (Rhinotermiteidae).

Druhá část mé práce shrnuje výsledky dvou studií chemické obrany termitů. U vojáků druhu Psammotermes hybostoma jsem detekovala celkem 33 obranných látek patřících převážně mezi seskviterpeny a většinu z nich plně identifikovala. Kvalitativní a kvantitativní srovnání obranných směsí mezi jednotlivými koloniemi jednoznačně rozlišilo tři rozdílné chemotypy korespondující s lokalitou výskytu. U dělníků druhu Neacapritermes taracua (Termitidae) jsem se podílela na popisu dříve neznámého vícesložkového obranného mechanismu spočívajícího v oxidaci hydrochinonů na benzochinonovy za katalýzy proteinem vázajícím měď.

Mé výsledky zdůrazňují komplexnost chemické komunikace a obrany termitních společenstev a přispívají k pochopení evoluce feromonů a obraných látek u tohoto nejstaršího společenského hmyzu.
INTRODUCTION

1 Chemical ecology: a fertile field of contemporary science

Chemical ecology is a relatively new discipline linking chemistry and biology in order to study interactions between organisms and their environment. These ubiquitous interactions include the chemical communication among animals and plants in both mutualistic and antagonistic contexts on the one hand, and chemical defence against predators, competitors, pathogens and parasites on the other hand. Though the significance of plant- and animal-produced chemicals is known to humans since ages, the beginning of chemical ecology as an independent scientific discipline is dated to the middle of the 20th century [1, 2]. Since, the methods of this newly established discipline have proved to be great tools to explore the wonders of animal and plant biology, as well as to get closer to natural compounds with unusual structures identified in unforeseen sources, with both academic and applied implications [2]. In the past decades, the genetic approaches started to be routinely used as a biological counterpart to the rapidly developing chemical analytics, and thus “the chemical ecology is one of the most fertile research fields of contemporary science” [2].

2 Chemical ecology: basic terms

Chemical signals represent the most ancient and widespread communication tool in animal kingdom. An umbrella term for all chemical compounds acting as mediators in animal communication is semiochemicals (Fig. 1). If the information is transferred within species, the notorious term, pheromone, is used [3]. Pheromones are most often produced in specialized secretory organs, exocrine glands, and can be generally classified according to their mode of action in two basic groups as releaser pheromones on the one hand and primer pheromones on the other hand. Releaser pheromones act directly on the nervous system of the receivers and elicit an immediate behavioural response. Functionally, several categories of releaser pheromones are recognized, such as aggregation, alarm, dispersal, sex, territorial, trail-following pheromones, and others [4]. On the contrary, the primer pheromones do not trigger an immediate change of behaviour; instead, they deeply and often irreversibly alter the physiology, development or reproduction of the receivers.
For interspecific chemical signals the term allelochemicals applies. Allelochemicals are further classified as kairomones, allomones and synomones, based on their significance for the producers and/or receivers: kairomones are signals beneficial for the receivers, allomones are advantageous for the producers, and synomones bring benefits to both of them.

Interestingly, one individual compound can have a multitude of functions within a particular species, depending on concentration, context and presence of other components. In addition, the same compounds can be found to play a variety of different roles in different species, and vice versa, an identical role in sometimes very distant taxa. A great example of this principle, called chemical parsimony, is the use of the same compound as sex pheromone by elephants and moths [5, 6].

![Functional classification of semiochemicals](modified from Wyatt 2014 [6])
3 Chemical ecology and social insects

Social insects are among the most complex and best organized life forms on this planet and have an irreplaceable role in terrestrial ecosystems due to their abundance and ecological significance as pollinators, predators, and decomposers [7]. Insect societies are characterized by highly-developed caste systems underlying an effective division of labour which is controlled by semiochemicals produced in a variety of exocrine glands. Over one hundred different exocrine glands have been described as yet in social insects, situated in various parts of their bodies [8]. For this reason, the social insects are sometimes called “chemical factories” [9]. Exocrine chemicals are involved in practically all aspects of the insect social life from the coordination of everyday tasks to the maintenance of social homeostasis and reproductive dominance of queens and kings [4, 10]. Besides this central role in communication, the exocrine chemicals are extensively used in other activities such as nest building, feeding, and, last but not least, the chemical defence [11, 12]. In short, the success of insect societies is based on chemicals and thus the chemical ecology of social insects has become a busy research field.
4 Chemical communication in termites

Termites, the oldest social insects with the most elaborate social organisation, represent an excellent example of the crucial role of semiochemicals in both communication and defence. The dominant role of chemical communication is obvious from the simple fact that termites, with rare exceptions, are blind and live in a complete darkness of their nests and galleries. Unlike in ants, the system of exocrine glands in termites is rather conservative and simple, and also the chemical diversity of termite pheromones described so far is rather low when compared to social Hymenoptera. Among others, the major exocrine glands in termites and their products are the following: sternal gland – trail-following pheromones, sex pheromones; tergal and posterior sternal glands – sex pheromones; frontal gland – defensive chemicals, alarm pheromones; salivary (labial) glands – secretory products related to nutrition and building, defensive compounds, food-marking pheromones [13, 14, 15]. The general situation of these glands in termite bodies is depicted in Figure 2. In this thesis, I report on the chemistry of pheromone communication in several species of termites of particular interest. Therefore, a brief overview of the chemical diversity of termite pheromones follows in the next chapters. This overview summarizes the state of the art before I started my thesis and does not include our own results, presented in the Results section.

![Fig. 2 Most important exocrine glands in termites](image-url)
4.1 Trail-following pheromones

Foraging for food is regulated by trail-following pheromones. Unlike in ants, these pheromones are produced by a single source, the sternal gland, situated on the ventral side of termite abdomens. The presence of this gland has been confirmed in all castes and all termite species studied in this respect, although the exact location of the gland(s) differs between particular termite lineages [13, 16, 17].

Chemical diversity of termite trail-following pheromones is surprisingly low with only eight different compounds identified to be the major or minor components of trail-following pheromones in more than sixty species from six families studied by 2011 (Fig. 3) [15]. Despite this low diversity, certain phylogenetic trends can be deduced from the distribution of trail-following pheromones on the tree of life. While in basal families, C13, C14 or C18 branched aliphatic aldehydes and alcohols occur, in the more advanced lineages Kalotermitidae + Rhinotermitidae + Termitidae, unbranched mono-, di- or tri-unsaturated alcohols with twelve carbon atoms were identified as trail-following pheromones, in some cases in a combination with diterpene hydrocarbons neocembrene or trinervitatriene. As noted by Bordereau and Pasteels [15], this transition to C12 alcohols is correlated with the changes in the localization and anatomy of the sternal gland in advanced termite families [17].

The quantity of the pheromone per one individual ranges from units to thousands picograms and the observed activity threshold ranges from $10^{-5}$ to 1 ng/cm, highlighting once again the very low active amounts of insect semiochemicals. The great example in this respect is the most frequent trail-following pheromone, (3Z,6Z,8E)-dodeca-3,6,8,-trien-1-ol, occurring sometimes in active concentrations of less than one picogram per one cm of the trail.

In spite of the significant progress in the understanding of the chemical diversity of termite trail-following pheromones in the past decade [18], a few systematic gaps remained to be filled, some of which I studied and included in this thesis. Therefore, I present the results on the chemistry of trail-following pheromones in Glossotermes oculatus (Serritermitidae) (Chapter 7.1), Psammotermes hybostoma (Rhinotermitidae) (Chapter 7.2) and Hodotermopsis sjostedti (Archotermopsidae) (Chapter 7.3).
Fig. 3 Chemical diversity of termite trail-following pheromones and its phylogenetic distribution [based on 15, 19]; state of the art in 2011. The relationships within the terminal group Rhinotermididae + Serritermitidae + Termitidae are not unambiguously resolved. The blue dot indicates the transition to unbranched unsaturated C12 alcohols and/or diterpenes and the switch in the position of the sternal gland. The families studied in this thesis are marked in blue. The structure in violet was proposed but not yet confirmed.
4.2 Sex pheromones

Sex pheromones ensure the pairing and pair cohesion of the alate imagoes, future kings and queens, after the dispersal flight and during the colony foundation. They are produced in sternal glands, posterior sternal glands, or tergal glands or a combination of these sources by females, rarely by males or both sexes [15, 20, 21].

Although the chemistry of sex pheromones is less well-known than that of trail-following pheromones and was studied only in about 20 species, many important characteristics common to both these modes of communication are obvious. First, the sex pheromones demonstrate a low chemical diversity and are mostly composed of single components. Second, the sex pheromones identified in individual species often act as trail-following pheromones or are structurally very similar, with just one additional molecule, the sesquiterpene alcohol (E)-nerolidol, being a sex pheromone component unrelated to the trail pheromones [15, 21] (Fig. 4). When compared with trail-following communication, the specificity of mate attraction results either from (a) different major components, (b) different, sometimes much higher, concentrations of the sex pheromone, or (c) the occurrence of one or more additional, minor components [15]. The widespread use of the sternal gland in courtship behavior as well as the frequent use of the same pheromone for both mate attraction and trail marking (though in different amounts and contexts) support the hypothesis that the trail marking is probably derived from the mate attraction of termite imagoes during dispersal [15, 22].

In this thesis, I present our findings on the sex pheromones in the African sand termite Psammotermes hybostoma (Rhinotermitidae) (Chapter 7.2) and the Asian damp wood termite Hodotermpsis sjoestedti (Archotermopsidae) (Chapter 7.3), both obtained along with our studies on the trail-following communication in these species.

![Sex pheromone components known by 2011](image)

**Fig. 4** Sex pheromone components known by 2011 [15]
4.3 Alarm pheromones

An effective alarm communication is one of the defensive strategies leading to the success of termite societies. In basal termite lineages, the alarm signaling resides in mechanical alarming by body vibrations and drumming of the head against the substrate. On the contrary, in the advanced families Rhinotermitidae, Serritermitidae and Termitidae, in which the soldiers are equipped with the defensive frontal gland, the alarm signaling is more complex and consists not only from the mechanical but also the chemical alarm. The alarm substances are included in the blend of defensive compounds synthesized in the frontal gland and released by excited and/or fighting soldiers.

Even though the chemical alarm has been observed in a number of species, only a few compounds have been unequivocally identified as alarm pheromones, all of them being of terpenoid structure (Fig. 5). Not surprisingly, majority of these compounds are highly volatile monoterpenes and sesquiterpenes [15, 23]. The participation of these compounds in communication is evidenced also by their high enantiomeric purity, frequent in pheromones [24]. However, none of these compounds was as active as the natural extract in eliciting the alarm behaviour which suggests that alarm pheromones are rather multi-component mixtures than just single component signals. It appears that the chemicals evolving primarily as defensive compounds have only secondarily co-opted the function in communication as alarm pheromones, as is the case during the evolution of chemical signals in many other animals [5, 25, 26].

In this thesis, I present our investigations on the alarm communication in a poorly known termite species *Termitogeton planus* (Isoptera: Rhinotermitidae) from West Papua (Chapter 7.4).

![Fig. 5 Components of soldier alarm pheromones confirmed by 2011](image-url)
4.4 Fertility signals and primer pheromones

The hallmark of insect societies is the dominance of the minority of reproductives over the sterile majority of helpers. It is well documented that in advanced social insects, including termites, this dominance is based on chemical signals, the pheromones. By means of these pheromones kings and queens signal their presence and control the fertility of their nestmates. These chemical signals are called primer pheromones, royal pheromones or fertility signals. Despite the importance of these “central molecules” for the function of insect societies, very little is known about their chemical identity. For over half a century, the honey bee was the single exception with the multi-component queen mandibular pheromone being known since late sixties [27, 28].

However, in the past few years, fertility signals and primer pheromones in termites are extensively studied, bringing new insights in this field. Firstly, two studies reported quantitative and qualitative differences between reproductives and sterile individuals in the composition of non-volatile cuticular hydrocarbons present on the body surface and proposed this non-polar blend to be the fertility signal [29, 30]. Secondly, a series of peptides and proteins secreted by kings and queens have been recently described. Their partial characterization suggested that they might be odorant-binding proteins and act as releasers of volatile signal molecules [31]. And finally, in 2010, the very first primer pheromone has been identified in the termite Reticulitermes speratus (Rhinotermitidae) [32]. It is secreted by the present queens and inhibits the development of new neotenic queens. This pheromone is a mixture of two highly volatile compounds, butyl butyrate and 2-methylbutan-1-ol (Fig. 6). In the light of these results, volatiles emitted by kings and queens have been identified in other termite species, such as the queen-specific volatile 1-phenylethanol (Fig. 6) in Nasutitermes takasagoensis (Termitidae) [33]. In this thesis, I present our results on the identification of a king-specific volatile in the termite Prorhinotermes simplex (Rhinotermiteidae) (Chapter 7.5).

![Chemical structures](image)

**Fig. 6** Fertility-related volatiles identified in termite queens
5 Chemical defence in termites

Societies of termites represent a great example of chemically defended social insects, namely due to the presence of a specialized defensive caste of soldiers which is in advanced lineages Rhinotermitidae, Serritermitidae, and Termitidae equipped with a unique defensive organ, the frontal gland. It is in these families that the chemical defence has reached an unprecedented complexity, with nearly four hundred defensive compounds being identified as yet [23] and thus “the termite chemical defences are chemically more novel and variable than in any other insect taxon of comparable species number” [34].

Termite defensive chemicals belong to various chemical classes, such as hydrocarbons, alcohols, aldehydes, ketones, terpenes, quinones, fatty acids, macrocyclic lactones, heterocyclic and aromatic compounds, and others (Tab. 1). A functional diversity of these compounds and their mixtures includes irritants, repellents, glues, antihealants, and contact poisons [23, 34]. In this thesis, I present my results on the chemical diversity of defensive compounds produced by soldiers of the sand termite *Psammotermes hybostoma* (Rhinotermitidae) (Chapter 8.1) and on the unique chemical defence of the workers of the neotropical termite *Neocapritermes taracua* (Termitidae) (Chapter 8.2).

**Tab. 1** Summary of termite defensive chemicals in particular termite families and subfamilies [23]

<table>
<thead>
<tr>
<th>Family</th>
<th>Subfamily</th>
<th>Number of studied species</th>
<th>Chemical classes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastotermitidae</td>
<td>Prorhinotermitinae</td>
<td>4</td>
<td>sesquiterpenes, nitroalkenes</td>
</tr>
<tr>
<td>Rhinotermitidae</td>
<td>Heterotermitinae</td>
<td>10</td>
<td>monoterpenes, sesquiterpenes, diterpenes, alcohol</td>
</tr>
<tr>
<td>Rhinotermitidae</td>
<td>Coptotermitinae</td>
<td>3</td>
<td>hydrocarbons, sesterterpene, triterpene, alcohols, aldehydes, ketones, aromatic compounds, fatty acids, aminosaccharide, ceramides, proteins</td>
</tr>
<tr>
<td>Termitidae</td>
<td>Coptotermitinae</td>
<td>11</td>
<td>hydrocarbons, monoterpenes, sesquiterpenes, aromatic compounds, quinones, macrocyclic lactones, polysacccharide, protein</td>
</tr>
<tr>
<td>Termitidae</td>
<td>Termitinae</td>
<td>25</td>
<td>monoterpenes, sesquiterpenes, diterpenes, ketones</td>
</tr>
<tr>
<td>Termitidae</td>
<td>Syntermitinae</td>
<td>13</td>
<td>hydrocarbons, monoterpenes, sesquiterpenes, ketone, aromatic compounds, heterocycles, macrocyclic lactones</td>
</tr>
<tr>
<td>Termitidae</td>
<td>Nasutitermitinae</td>
<td>30</td>
<td>monoterpenes, sesquiterpenes, diterpenes, alcohols, ketones, aromatic compounds, amide</td>
</tr>
</tbody>
</table>
6 Methods in chemical ecology

The main objective of chemical ecologists is to get ‘the maximum amount of information from the minimum amount of the sample’ [35]. Since semiochemicals often occur in really low quantities, micrograms or less, microscale techniques together with microscale equipment are needed. Prior to any manipulation with the sample, every step should be thought-out to prevent a destruction of often very valuable samples, sometimes prepared for weeks or imported from the field missions. Alongside, the development of sophisticated analytical instruments increases the abilities of analytical chemists.

Although there is no general and universal procedure for solving all the tasks, the basic process consists of a series of steps and techniques, namely extraction, separation, identification, confirmation, and finally quantification of biologically active compounds. At each step, the results of chemical approaches are confronted with the knowledge on the biology and chemical ecology of the studied model and related organisms. Given the richness of methods used in chemical ecology, I consider below in more details only the techniques relevant for this thesis that I used to solve the analytical problems reported in my results.

6.1 Extraction

Before anything else, it is necessary to stress that there is no universal extraction method applicable in all cases. Suitable extraction time, temperature and other parameters must be considered for each task separately. In all cases, the attention must be given to the purity of samples, since the risk of contamination may prevent the usefulness of the samples for bioassays, especially when analysis aims at trace compounds [36].

6.1.1 Solvent extraction

The most common and easily accessible technique is a solvent extraction. To choose a suitable solvent, the volatility and solubility of expected analytes as well as the compatibility with subsequent analytical methods and biotests has to be taken in account. At the same time, the effective solvent should not extract undesired compounds, e.g. fatty acids from insect tissues, which complicate the subsequent work. To increase the effectiveness of an extraction, it is recommended to extract more times with lower volume of the solvent than extract once with the final volume. Since the sample should be very often concentrated after the extraction, used
volume of the solvent should be reduced to minimize the loss of volatiles as a result of a very long evaporation [35].

6.1.2 SPME

With regard to the volatility of many semiochemicals and defensive chemicals, Solid-Phase Microextraction (SPME) has been proved to be an appropriate extraction method. This technique was developed in 1990 [37] in response to the rising interest in rapid and solvent-free sampling techniques. Fused silica fibers with different thickness of various adsorbents are used to extract substances either from the headspace or directly from solutions and then thermally desorbed in the GC injector or eventually, after modifications, in HPLC. Despite the fact that the instrumentation is rather simple, the method optimization for the quantitative extraction of analytes from complex matrices is a tricky task. The first step is to choose a suitable fiber according to chemical properties of targeted compounds. Nowadays there are several types of SPME fibers commercially available, differing in the stationary phase: polydimethylsiloxane (PDMS), polyacrylate (PA), carbowax (CW) or their mixtures with divinylbenzene (DVB) and/or carboxene (CAR). If the phase is bonded, the fiber is stable with all organic solvents, if the phase is non-bonded, the fiber should only be used with solvents that are miscible with water. Some types of the fibers are available with different film thickness, influencing the sensitivity of the fiber [38].

Another crucial parameter is the time of the absorption. In an ideal case, the distribution equilibrium between the matrix and the fiber coating would be reached but in practical experiments with complicated matrices the time must be determined experimentally. It is necessary to point out that the relationship between the concentration of the sample and the response is linear only until the saturation of the sorbent. For the desorption time applies that it is a compromise between an elimination of all adsorbed compound and the fiber destruction. The extraction may also be influenced by stirring or by the temperature increase [38]. The extraction is either performed from headspace (Fig. 7) or by a direct extraction from solutions or from the surface. The direct extraction is recommended for polar compounds, however, it makes the calibration difficulties and the manipulation should be very careful because of the fragility of fibers.
A great advantage of the SPME is that it solves the problem of incompatibility of water samples with gas chromatography and enables the in vivo sampling which is more efficient for the study of mechanisms of action and which in many cases would not be possible by other methods. Last but not least, the in vivo SPME extraction is not destructive to the samples, such as living insects or plants; these can then be kept alive and used repeatedly for the extraction. Especially the analyses of volatile emissions and drugs are of a great interest nowadays. Among other advantages of this method belong a reusability of SPME fibers and a possibility of direct derivatization on fibers. Disadvantages are irretrievability of injected samples and difficult quantification caused by different affinity of particular compounds to various fibers [35]. In these cases, the calibration with standards is essential. The role of functional groups on effectiveness of the extraction could be demonstrated by the study of Bartelt [39] in which the calibration factor - the absorbed amount on PDMS fiber depending on headspace concentration - of 71 volatile compounds (C₁-C₁₆) with different functional groups were compared. On the one hand, calibration factor increased with an increasing retention index, on the other hand decreased with increasing temperature. The polar compounds were adsorbed more than hydrocarbons with the same retention index. Surprisingly, lower concentrations of nitrogen- and hydroxy compounds evinced higher calibration factors. No significant differences between 0.15-5 minutes injection time were observed.

6.2 Separation

6.2.1 Thin-layer chromatography, column chromatography

Thin-layer chromatography (TLC) is a simple method used for the primary screening of the extract complexity as well as for the preparative purposes when the required compound could be scraped off the TLC plate and used for following experiments [40]. TLC is also commonly used for a determination of an appropriate solvent system for the separation of individual substances. This solvent system is subsequently used in the second above mentioned method, column chromatography, in which extracts are purified and separated on silica or alumina into fractions. In practice, a gradient elution over an isocratic is preferred, especially for complex samples [41].
6.2.2 Gas chromatography (GC)

Taking into consideration the necessity to communicate often over long distances, the volatility of semiochemicals is one of their main characteristics and for this reason the gas chromatography is often the best analytical tool. GC is useful for all the compounds which could be transferred to the gas phase at GC temperatures and which are thermally stable. Analytes are separated on the basis of their volatility and affinity to the stationary phase and a very high resolution enables the detection of hundreds of analytes during one analysis. At the same time, the GC separation offers a high reproducibility between GC instruments provided that they use the same column. The aim of any separation is to get the best resolution (R) which is dependent on three parameters:

\[
R = \sqrt{\frac{N}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k}{k + 1}}
\]

\(\alpha\) is a separation factor which is related to interactions between stationary phase and analytes, \(N\) is the number of theoretical plates expressing the column efficiency and the combination of these two factors is decisive for the separation of two individual substances on a particular column. Then, \(k\) is a capacity factor which signifies the ability of the column to keep analytes [35, 42].

In view of the fact that biological activity is often related to the chirality of the compounds, special columns enabling the separation of particular enantiomers are often used for this purpose. The combination of this separation method with mass spectrometric detectors enables the identification of unknown compounds.

6.2.3 Comprehensive two-dimensional gas chromatography (GC×GC)

The comprehensive two-dimensional gas chromatography is a very efficient technique for analyses of such complex mixtures as natural extracts, which formerly required multistep sample preparation together with an intricate instrumentation. This method was developed in the last decade of the 20th century [43] as a type of multi-dimensional techniques. In comparison with conventional 2D-GC, when only the chosen fraction was redirected into the second column, all the compounds are submitted for a separation in both dimensions. The use of columns with different polarities enables a clear differentiation between particular classes of compounds and a separation of normally inseparable compounds. At the same time, the retention behaviour in the two-dimensions may be an important guideline for the identification of unknown compounds based on their physico-chemical properties.
While the injector and columns do not differ from those used in other GC techniques, the essential constituent of the GC×GC setup is the interface between the two columns, the modulator. There are two classes of modulators: mass conservation type and sampling type which is based on valves. The accumulation of analytes in the modulator can be performed thermally or cryogenically [44]. Generally, the sample is separated in the first column and then divided into small fractions in a modulator, where is subsequently refocused and then undergo a very rapid separation in the second column (Fig. 8). Since all the fractions created in the modulator must be separated in the second dimension during one modulation period (2-8 second), the second column is usually short and narrow with a low film thickness. If the instrument is equipped with two ovens, it is possible to increase the sensitivity by using different temperature programs.

Since GC×GC requires a very fast detector, it is traditionally combined with a FID detector or with a more convenient TOF detector (Fig. 8). This coupling represents a very useful tool for chemical ecology issues. The outcome of a GC×GC analysis is a set of second-dimension chromatograms that are stacked side by side to create a 2D chromatogram with x and y axes representing the retention time on the first and the second columns, respectively. The signal intensity is depicted by colour scales and for a better demonstration of quantities the three-dimensional plots are sometimes used (Fig. 9).

![Fig. 8 Scheme of GCxGC/MS instrument (Leco, Pegasus 3D) used in our laboratory](image-url)
6.2.4 Preparative GC

Preparative GC is a great tool for isolation and purification of analytes from complicated mixtures which would be often impossible to separate by other techniques. The main constraint of the method was the loss of efficiency of the chromatographic column when larger amounts were used. In 1956 first reports on special preparative columns with higher diameters were described, however a more efficient solution appeared to be repetitive injections of small volumes using classical analytical columns until the required quantity is reached [46]. In response to the increasing interest in injecting larger volumes, a programmed temperature vaporizer (PTV) was developed as the most versatile interface [47].

In contrast to the classical gas chromatographic system, only a small part of the effluent after the separation goes directly to the detector (FID or TCD) and the rest of the effluent leads to the fraction collector where analytes condensate due to the cooling by liquid nitrogen. The synchronization of the detector response and the collector enables an isolation of particular
compounds (Fig. 10). To increase the column capacity, capillary columns with a larger internal diameter are used (e.g. 0.53 mm instead of traditional 0.25 mm).

![Fig. 10 Setup of preparative GC](image)

**Fig. 10** Setup of preparative GC

### 6.3 Identification

#### 6.3.1 Kovats retention indices

Kovats retention indices (KI), relative to the retention times of linear alkanes, represent an easy and useful characteristic of the retention behaviour of individual compounds which is reproducible on other GC instruments equipped with the same column. Taking into consideration that in practice we are working with complicated matrices containing compounds of different volatilities, the programmed temperature analyses are preferred to isothermal ones. In that type of analyses, dependence of a retention time on the number of carbons is more likely linear than logarithmic therefore the standard equation [35] could be simplified as follows:

\[
KI = 100y + 100(z - y)\frac{t_{r,x} - t_{r,y}}{t_{r,z} - t_{r,y}}
\]

where \(t_r\) represents the retention time, \(x\) means an analyte and \(y\) and \(z\) stand for the linear alkanes eluting before and after the analyte, respectively. For a simplification, also the actual retention time can be used instead of the adjusted retention time because the retention time of the compound without interaction with stationary phase is a constant [35]. It is necessary to stress that only indices obtained by the column with the same stationary phase could be compared.
6.3.2 Mass spectrometry (MS)

An excellent tool for chemical identification is a coupling of chromatographic methods with mass spectrometry detection system. At first, the mixture is separated and particular compounds are subsequently ionized and fragmented. Gas chromatography is usually coupled with Electron Ionization (EI) which provides an extensive fragmentation. According to fragmentation patterns, the unknown compound can be classified or in some cases even fully identified. One of the advantages of EI is a large library of mass spectra. However, sometimes dramatically different compounds can be fragmented in the same way; it is therefore necessary to take into account the other compounds characteristics such as retention indices. Due to the high energy of electrons (usually 70 eV), the molecular ion of the compound can be missing in the fragmentation pattern, depending on the stability of functional groups. Therefore, a softer ionization method, chemical ionization (CI), is used for molecular weights confirmation. CI has better sensitivity in comparison with EI [35].

Sophisticated instruments facilitate also measuring with a high-resolution (HR) which gives us the information about an exact mass and an elementary composition of the fragments. Knowing the molecular formula enables to calculate the degree of unsaturation expressed as the total number of rings plus double bonds \((r+db)\) which is another important information on the way to final identification. Having the general formula \(C_xH_yN_zO_n\), the equation is:

\[
 r + db = x - \frac{y}{2} + \frac{z}{2} + 1
\]

The final structure can be in many cases suggested only from the mass spectrum based on typical losses and basic cleavages [48].

The sensitivity varies for different mass analyzers and different classes of compounds. In general, picogram quantities of analytes enable a useful fragmentation. While searching for a particular compound, the sensitivity can be increased by replacing of the total ion current (TIC) measuring by the single ion monitoring (SIM).

6.3.3 Infrared spectroscopy (IR)

Infrared spectroscopy (IR) is a very useful complementary tool for the structure elucidation. The great advantage of this method is its versatility allowing to study various kinds of samples. The other advantages are quite a low quantity of the sample required for the analysis as well as non-destructiveness [49]. Although the first spectrometers started to be used in 1940, the boom of this method took place once it has been combined with Fourier-Transformation (FT), leading
to higher quality spectra and shortening of the total running time. This method measures the vibrations of the atoms induced by infrared radiation. The FT-IR has an irreplaceable role in identification of unknown compounds by determination of functional groups as well as differentiation of geometrical isomers.

IR spectrum consists of molecular vibrations and vibrations of functional groups and could be divided into three parts: near-infrared (13000 - 4000 cm$^{-1}$), mid-infrared (4000 - 400 cm$^{-1}$) and far-infrared (400 - 100 cm$^{-1}$). While near-infrared region is more useful for quantitative analyses and far-infrared for a detection of compounds containing heavy atoms (organometallic, inorganic, halogen compounds), the mid-infrared region is the most helpful for the molecular structure determination. This region, the mid-infrared, can be divided into X-H stretching region (4000 - 2500 cm$^{-1}$; X= more massive atom e.g. P, Si), triple-bond region (2500 - 2000 cm$^{-1}$), double-bond region (2000 - 1500 cm$^{-1}$) and fingerprint region (1500 - 600 cm$^{-1}$).

For chemical ecologists, especially the connection with gas chromatography opens new prospects.

6.3.4 Electroantennography (EAG)

In chemical ecology, the biological activity of the compound is a crucial information; therefore the coupling of the gas chromatography with an electroantennographic detector has a great potential. The origin of electroantennography is dated to the fifties of the twentieth century when the first experiments measuring electrical responses of the insect antenna to different stimuli were performed [50]. This method is based on determining the voltage oscillation between the tip and the base of the antenna as a result of the electric depolarization of olfactory neurons. The amplitude of the response is influenced by many factors such as the nature of the stimulus and its concentration, the condition of the antenna, its life time etc. Also the temperature and the humidity of the experimental environment play an important role. Once the antenna is saturated, the response can not be increased by the increase in concentration [51].

While performing the GC-EAD, the effluent is splitted into two directions, one of them goes to the classical FID detector and the second to the antenna which is placed outside the oven, connected by the heated transferline (Fig. 11). All the compounds which activated the antennal receptor cells are shown in the electroantennogram. Signals from both detectors are recorded in parallel which enables the identification of biologically active compounds.
6.4 Derivatization

In some cases, a simple chemical reaction leading to a modification of the original compound can facilitate the analysis by enhancing of a separation, increasing of detectability or by indicating the presence of particular functional group at specific position in the molecule. Up to now, there were described many derivatization methods compatible with GC conditions that can be used with slight modification also in a microscale.

6.4.1 DMDS derivatization

In our practice we often face the problem with the double bonds rearrangement in EI conditions (70 eV). For this purpose, the derivatization with dimethyl disulfide (DMDS), which does not require a previous isolation of the studied compound, appears to be a good solution. In case of monounsaturated compounds, major fragments result from the cleavage of the bond between the carbons binding methylthio groups (Scheme 1). It is interesting to point out that Buser et al. [52] observed a prolongation in retention times when the double bond was further from a functional group as well as a relationship between intensity of the fragment after the loss of the functional group and the chain length.
In case of diunsaturated compounds, the reaction with DMDS catalyzed by iodine reveals either linear or cyclic polythioethers, depending on the distance between the two double bonds, which are preferentially fragmented next to the methylthio group [53]. Arising fragments easily reveal the original placement of the double bonds. The only exception represents conjugated dienes where methylthiogroups are bound to the ring which prevents the fragmentation of the chain and the most abundant fragment arises from the loss of two molecules of methanethiol (Scheme 2).
The study of unsaturated terpenes and some branched compounds [54] showed that the most intense and therefore more stable fragment is the one with more substituted carbon atom while the fragment with corresponding unsubstituted one was in majority of cases missing. Moreover, the double bond conjugated to a functional group appeared to be resistant to DMDS derivatization which can also be very useful information on the structure of the molecule.

The DMDS derivatization was also studied in triunsaturated compounds, namely alkatrienes and one methyl ester, with maximally 3 methylene groups separating particular double bonds [55]. Scheme 3 depicts the favoured fragmentation.

![Scheme 3 DMDS derivatization of triunsaturated compounds](image)

**6.4.2 Silylation**

Another example of derivatization is the determination of the position of hydroxy groups. The analysis of alcohols by GC is complicated because of their polarity which causes an undesirable tailing on commonly used GC columns. Silylation, which substitutes the hydrogen from a hydroxy group by a trimethylsilyl group, can increase the volatility and at the same time localize the position of hydroxy groups. While performing the derivatization, any water in samples must be avoided because it would react with the reagent with higher preference earlier than the proper analytes. There is a large set of silylation reagents of which trimethylsilylimidazole (TMSI) is selective for hydroxy groups (GC derivatization). Basic fragmentation is depicted in Scheme 4 (based on Sharkey [56]).
6.5 Confirmation and quantification

Ideally, the target compound would be characterized by nuclear magnetic resonance (NMR) spectroscopy. However, the compounds used for communication are usually in insufficient quantities for NMR. Therefore the final proof that the identification was correct can be obtained by the comparison with a natural or synthetic standard. If the identified compound is not commercially available, the suggested structure has to be synthesized or extracted from natural sources. Of course, the stereochemistry and chirality of the compound should not be omitted because it is often critical for its biological activity. The biological effect of the compound can be verified by electroantennography or by a suitable bioassay.

The final step of our studies is the quantification of biologically active compounds. The most suitable detector for this purpose is the Flame Ionization Detector (FID) which responds linearly across a wide concentration range. However, in practice, given the laboratory equipment and a very limited amount of the target compounds in natural samples, the quantity is usually calculated from mass spectrometer responses. To get the relative quantity of several compounds and at the same time to minimize external factors like inaccuracy during sample injection, the internal standard with different properties, which could be easily found in the chromatogram, is used. Because of differences in response factors for various compounds, this procedure may result in an inaccuracy in estimating absolute quantities. Therefore the method of calibration curve or external standard using the identical compound is preferred.
AIMS

The aim of this thesis is to unravel the chemistry of pheromone communication and chemical defence in societies of termites, with emphasis on poorly known species from phylogenetically important lineages. The expected results should contribute to a better understanding of the chemical diversity of exocrine chemicals used by termites on the one hand and of the evolution of chemical communication and defence in termites on the other hand.

The main part of this thesis was performed in the frame of a research project entitled ‘Biology, chemical ecology, and phylogeny of critical termite genera from families Rhinotermitidae and Serritermitidae’ and focused on the genera *Glossotermes*, *Psammotermes* and *Termitogeton*. In these genera, I studied and included in this thesis the following topics:

- trail-following communication of *Glossotermes oculatus* (Serritermitidae) (Chapter 7.1, Paper A)
- trail-following pheromone, sex pheromone and chemical defence in *Psammotermes hybostoma* (Rhinotermitidae) (Chapters 7.2, 8.1, Papers B, E)
- alarm communication in *Termitogeton planus* (Rhinotermitidae) (Chapter 7.4, Paper D)

Beside the main project I was involved in two other projects on the chemical ecology of termites, performed in collaboration with biologists from foreign laboratories. In this thesis, I report my results on the following two topics:

- trail-following pheromone and sex pheromone in *Hodoterms sjoestedti* (Archotermopsidae) (Chapter 7.3, Paper C)
- chemical defence by workers of *Neocapritermes taracua* (Termitidae) (Chapter 8.2, manuscript in preparation)

Last but not least, I participated in the search for chemical fertility signals emitted by the kings and queens. In this thesis, I report on the following topic:

- identification of a fertility related volatile compound in *Prorhinotermes simplex* (Rhinotermitidae) (Chapter 7.5, manuscript in preparation)
MATERIALS AND INSTRUMENTATION

Biological material
Colonies of *Glossotermes oculatus* and *Neocapritermes taracua* were collected in French Guiana, *Psammotermes hybostoma* in Egypt, *Hodotermpsis sjostedti* in Vietnam and *Termitogeton planus* in West Papua. *Prorhinotermes simplex* originated from Cuba and is bred in the laboratory since 1964.

Chemicals
Reagents and synthetic standards were purchased from Sigma-Aldrich or Fluka, solvents from Penta or Merck. Standards of trail-following pheromones were kindly supplied by Christian Bordereau (Dijon, France).

Instrumentation
One-dimensional GC-MS (quadrupole mass analyzer): Focus GC, Thermo Scientific DSQ II.
High-resolution MS data: Waters GCT Premier with time-of-flight mass analyzer.
Chiral separation: gas chromatograph HP 6850 Series (Agilent) with FID detector.
Two-dimensional comprehensive gas chromatograph coupled with mass detector (time-of-flight mass analyzer) GC×GC/TOF-MS: Pegasus 4D, Leco Corporation, United States.
Preparative gas chromatograph: 6890N gas chromatograph (Agilent) equipped with a liquid nitrogen cooled EPC PTV inlet (Gerstel, Mühlheim, Germany) and FID detector. Fractions collected in Preparative fraction collector (Gerstel).
Gas chromatograph coupled with electroantennographic detector (GC-EAD): GC-5890A Hewlett-Packard. EAD-termite antenna connected via two glass Ag/AgCl electrodes to universal AC/DC 10XProbe (Syntech, Hilversum, The Netherlands).
NMR spectra: Bruker Avance 500 MHz.
FTIR spectra: gas chromatograph Agilent 6850 combined with Nicolet 6700FT-IR spectrometer (Thermo Scientific).
RESULTS AND DISCUSSION

7 Pheromones

7.1 Trail-following pheromone of *Glossotermes oculatus* (ref. Paper A)

The termite family Serritermitidae is very outstanding in many aspects of its biology and at the same time, nothing has been known on the chemistry of pheromones. Therefore, new findings could have been expected, including the identification of new pheromones not described yet in termites or even new natural compounds. This chapter reports on our results on the trail-following communication in the South-American species *Glossotermes oculatus*, published in Chemical Senses in 2012 (Paper A) enriched with more details from the chemical point of view.

Prior to the chemical identification, bioassays with whole body extracts and sternal gland extracts of *Glossotermes* workers were performed in order to observe the trail-following behaviour of this species and to confirm that the sternal gland is a source of the trail-following pheromone. Based on this confirmation, a biologically active hexane extract of sternal glands from 300 workers was analyzed by means of two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/TOF-MS, LECO, Pegasus 3D) giving rise to a very rich chromatogram (Fig. 12). Bearing in mind the low diversity of trail-following pheromones across all termite families, the first logical step was to search for characteristic fragments of already known trail-following pheromones. Because we did not detect any of these expected fragments, we concluded the trail-following pheromone probably consisted of a new compound or a mixture of compounds.

![Fig. 12 A. 2D-chromatogram of the sternal gland extract of *G. oculatus* workers](image_url)

**B. 3D visualization**
Because of the complexity of the extract, difficulties caused by the great abundance of fatty acids in the extract and the failure of our attempts of electroantennographic detection, we decided to fractionate the extract and test the biological activity of the fractions. Firstly, we used column chromatography and obtained 11 fractions (hexane:ether from 10:0 to 0:10). Two of these fractions were effective in eliciting the trail-following behaviour therefore we merged them and separated by means of preparative GC in three steps (Table 2).

Tab. 2 Sequential fractionation of the sternal gland extract and the activity of workers expressed by a travelled distance in bioassays

<table>
<thead>
<tr>
<th>Fraction</th>
<th>LRI</th>
<th>Distance (cm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>700-900</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>F2</td>
<td>900-1100</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>F3</td>
<td>1100-1300</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>F4</td>
<td>1300-1500</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>F5</td>
<td>1500-1700</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td><strong>F6</strong></td>
<td>1700-3000</td>
<td>6.33 ± 1.07</td>
</tr>
<tr>
<td><strong>Step 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F6.1</td>
<td>1700-1850</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>F6.2</td>
<td>1850-1970</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td><strong>F6.3</strong></td>
<td>1970-2180</td>
<td>6.6 ± 0.97</td>
</tr>
<tr>
<td>F6.4</td>
<td>2180-3000</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td><strong>Step 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F6.3.1</td>
<td>1970-2200</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>F6.3.2</td>
<td>2020-2060</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td><strong>F6.3.3</strong></td>
<td>2060-2130</td>
<td>3.53 ± 0.61</td>
</tr>
<tr>
<td>F6.3.4</td>
<td>2130-2180</td>
<td>&lt; 1.5</td>
</tr>
</tbody>
</table>

From this series of fractionations we obtained one active retention window (Fig. 13) where we found a single candidate compound (KI = 2075 on DB-5) with m/z 278. EI-MS spectrum of this molecule is shown in Fig. 14.
The loss of 58 yielding the fragment m/z 220 is created by McLafferty rearrangement (Scheme 5) and together with an easily recognizable fragment m/z 58 indicated a carbonyl group at C2. Natural isotopic contributions suggested an unbranched hydrocarbon chain so that the predicted molecular formula was C_{19}H_{34}O with 3 degrees of unsaturation. The literature search showed a similarity of the fragmentation pattern with linoleic acid and its derivatives which brought us to the conclusion that our candidate compound may be an acetyl derivative of linoleic acid, commonly occurring in termites. Therefore we proposed the structure (10Z,13Z)-nonadeca-10,13-dien-2-one (hereafter referred to as nonadecadienone). The stereochemistry was suggested in accordance with the natural linoleic acid.
To confirm our hypothesis, the nonadecadienone was synthesized from commercially available methyl (9Z,12Z)-octadeca-9,12-dienoate (linoleic acid methyl ester) similarly as described in detail by Adams [57] (Scheme 6). The comparison of the chromatographic behaviour and mass spectra of the synthetic compound with our candidate compound from a real extract manifested a perfect match.

Although we have proposed the double bonds locations on the basis of the position in naturally occurring linoleic acid, we decided to confirm these positions empirically by comparing DMDS derivatives of synthetic and natural compounds. According to Vicenti et al. [53], there were just two possibilities of the final molecular mass (m/z 466 and m/z 404) depending on the distance between the double bonds, provided that our proposed structure had two double bonds and a carbonyl group at C2. The DMDS derivative of our candidate compound had molecular mass 404 (Fig. 15). Then I sketched all the possible structures using ChemBioDraw Ultra 12.0 to study their fragmentation. The MS spectrum showed a fragment m/z 357, suggesting the loss
of CH$_3$S' and m/z 309, representing the double loss of CH$_3$S'. In addition, the occurrence of the fragments m/z 225, 203, 201 and 131 demonstrated the position of the double bonds by typical cleavage at the places where the DMDS was incorporated. Thus, we confirmed the double bond positions at C10 and C13.

The last step to complete this study was to quantify the amount of nonadecadienone per one worker's sternal gland. For this purpose we used three different approaches. Firstly, GC-MS on quadrupole DSQ II with tricosan-12-one as internal standard was used (Fig. 16). The quantity of the pheromone was estimated to be 1.7 ± 0.4 ng/gland. Secondly, the two-choice bioassay based on decision between a sternal gland extract and our synthetic pheromone estimated the quantity in one worker to range between 0.2 and 1 ng/gland. Finally the electroantennographic analyses determined the quantity to be 1 - 20 ng per one gland. All these estimates are in a good agreement taking into consideration that we were working with a biological material.

**Fig. 15** DMDS of nonadecadienone
To conclude, we succeeded in the identification of a new termite trail-following pheromone with unexpected structure in comparison with C12 unsaturated alcohols occurring in all other advanced termite families and this result underlines the remote phylogenetic position of the family Serritermitidae.

Fig. 16 Comparison of GC chromatograms
7.2 Trail-following and sex pheromones in *Psammotermes hybostoma* (ref. Paper B)

This chapter summarizes our investigations on the trail-following pheromone and the sex pheromone in the sand termite *Psammotermes hybostoma* (Rhinotermitidae) from Egypt, which were published in the Journal of Chemical Ecology in 2011 (Paper B).

The first part of this study was focused on the search for the trail-following pheromone. Since *Psammotermes* has a high level of size polymorphism in workers, only the small workers were chosen for the study of the trail-following behaviour, based on a previous study by Clément [58] showing that larger workers may have different functions for the colony than the foraging for food. Bearing in mind the low quantities of termite trail-following pheromones per one individual, the analysis was performed using GC×GC/TOF-MS (LECO, Pegasus 3D) with a very concentrated sample representing an extract of 50 sternal glands in 1 μl of hexane. Using of such a concentrated sample was possible only because of the low amount of fatty acids in the sternal gland extract in comparison with whole body extracts. Based on the retention indices and a typical fragmentation of the known termite trail-following pheromones, (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol (hereafter named dodecatrienol, m/z 180, KI = 1525 on DB-5) was found in the sternal gland extracts of *Psammotermes hybostoma* when searching for a diagnostic fragment m/z 91 (Fig. 17, 18).

![Fig. 17 GC×GC chromatogram of the sternal gland extract of *P. hybostoma*, a white circle marks the peak of dodecatrienol](image-url)
Fig. 18 EI-MS spectrum of (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol

A. extract of sternal glands, B. synthetic standard

Although the detection of dodecatrienol is not surprising from the phylogenetic point of view, since it is the most frequent termite trail-following pheromone, its chromatographic detection is difficult due to the minute active quantities of the compound and thus its low amounts in the glands. Indeed, this was the first time we had detected this compounds by chromatographic methods which underlines once again the effectiveness of modern analytical methods. For the first identification of this compound in 1968 [59] 385 g of termite material together with 15 kg of wood material containing the same compound were used. The stereochemistry was solved a year later when two isomers of dodecatrienol with the same chemical properties were synthesized and the (Z,Z,E)-isomer was 1000× more effective in eliciting of the trail-following behaviour than (Z,E,Z)-isomer and corresponded greatly with the natural compound [60]. The role of this substance in eliciting of trail-following behaviour was confirmed by Bordereau et al. [61] when they detected dodecatrienol in sternal gland extracts and proved that this compound is synthesized directly by termites.

Obviously, the noticeable fragment m/z 31 is caused by a β-cleavage and indicates a primary alcohol which was subsequently confirmed by FTIR. The molecular mass 180 was confirmed by chemical ionization and the suggested molecular formula was C_{12}H_{20}O with 3 degrees of unsaturation. The structure of dodecatrienol was confirmed by NMR. Solving the MS
spectrum, the major fragment is m/z 91, typical for a tropylium and usually indicative of an aromatic compound. However, a detailed inspection revealed the lack of the m/z 77 typical for the aromatic ring. Based on rules of fragmentations I suggested the possible formation of the fragment m/z 91. Firstly, the dehydration reaction generates alkene m/z 162 from the ionized molecule. The subsequent cyclization creates 1-pentylenhepta-1,3,5-triene which is unstable and therefore fragmented into tropylum ion and pentyl radical (Scheme 7) [48].

![Scheme 7 Suggested formation of the ion m/z 91](image)

Figure 19 shows all the three C12 alcohols described as termite trail-following pheromones and demonstrates the higher stability of the molecular mass with the higher level of unsaturation. Another interesting aspect is the retention shift between dodecadienol and dodecatrienol as indicated in Table 3.

**Table 3** Retention indices of a series of C12 alcohols with different number and topologies of double bonds [based on 62 and own results].

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI on DB-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodecienol</td>
<td>1470</td>
</tr>
<tr>
<td>(2E)-dodecienol</td>
<td>1469</td>
</tr>
<tr>
<td>(3E)-dodecienol</td>
<td>1451</td>
</tr>
<tr>
<td>(3Z)-dodecienol</td>
<td>1457</td>
</tr>
<tr>
<td>(3Z,6Z)-dodecadienol</td>
<td>1438</td>
</tr>
<tr>
<td>(2E,4E)-dodecadienol</td>
<td>1523</td>
</tr>
<tr>
<td>(3Z,6Z,8E)-dodecatrienol</td>
<td>1525</td>
</tr>
</tbody>
</table>
The second part of this study aimed to identify the female sex pheromone of *Psammotermes hyboitoma* produced in the tergal and sternal glands of the swarming females. Prior to the proper analysis, hexane extracts of female alate heads were measured by means of GC-MS with quadrupole mass analyzer to check whether their cuticular hydrocarbons profiles are identical, indicating that they belong to the same species and population (Fig. 20).
Fig. 20 Cuticular hydrocarbon profiles of female alates from different localities

Then the hexane extracts of sternal and tergal glands were analyzed using GC×GC/TOF-MS and by the injection of 25 gland equivalents in 1µl of hexane we once again determined the presence of dodecatrieneol in both type of extracts (Fig. 21).

Fig. 21 GC×GC chromatograms of female tergal and sternal glands
To conclude, we identified (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol as the trail-following pheromone and at the same time as the sex pheromone in the sand termite *Psammotermes hybostoma*. Given an inaccuracy in quantification of this compound by two-dimensional GC caused by tailing, the quantity was estimated by electroantennography to be 1 - 10 pg in the sternal gland of workers and 1 pg in the sternal gland and 10 pg in the tergal glands of females. These results highlighted once more the pheromone parsimony in termites and confirmed the close relation of *Psammotermes* with *Prorhinotermes* species which was suggested by phylogenetic studies in spite of very different life histories of these two genera.
7.3 Trail-following pheromone and sex pheromones in *Hodotermopsis sjoestedti*  
(ref. Paper C)

This chapter summarizes a study on the trail-following pheromone and the sex pheromone in *Hodotermopsis sjoestedti* (Archotermopsidae) on which I had the opportunity to collaborate with researchers in chemical ecology from Australia, France and Japan. The results of the study were published in the Journal of Insect Physiology in 2011 (Paper C). The Indomalayan species *H. sjoestedti* was chosen as a representative of basal termites from the poorly known family Archotermopsidae, seldom studied with respect to the chemistry of pheromones. All pheromone compounds identified as yet differed from those known in more advanced termite families.

For both purposes, extracts were prepared by rubbing of the surface of appropriate glands with SPME fibers (PDMS/DVB). For the study of the trail-following pheromone, sternal glands of 20 - 50 workers were extracted, for the sex pheromone 10 - 20 reproductives were used. The SPME fiber was injected directly into GC-MS.

The trail-following pheromone was identified by the comparison of compounds detected on the sternal gland surface and on the control surface on the dorsum of abdomen. This experiment revealed the presence of one compound specific for the sternal gland with KI = 1468 on non-polar column DB-5 and KI = 1937 on polar DB-WAX. The EI-MS spectrum is shown in Fig. 22.

**Fig. 22** EI-MS spectrum of *H. sjoestedti* trail-following pheromone

Based on a significant fragment m/z 31, the presence of primary alcohol group was expected which was subsequently confirmed by FT-IR. Using chemical ionization with methane and ammonia, the molecular mass of the compound was estimated to be 200 which supported the idea of a primary alcohol where the loss of hydroxy group is preferred. The molecular
formula was proposed to be \( C_{13}H_{28}O \), suggesting an absence of rings and double bonds in the molecule. The fragmentation pattern indicating an aliphatic compound with a branched chain and a similarity with 4,6-dimethyldecane-1-ol which was the precursor in the synthesis of the 4,6-dimethyldecanal, the trail-following pheromone of a related species \textit{Zootermopsis nevadensis} [21]. Therefore, we hypothesized the structure of the candidate compound as 4,6-dimethylundecane-1-ol. Subsequent synthesis following the procedure described by Ghostin et al. [63] yielded a blend of \textit{syn} and \textit{anti} diastereomers (Scheme 8). To identify the correct diastereomer used by the termites, the GC profiles of the synthetic mixture and the natural extract were compared (Fig. 23).

\begin{center}
\textbf{Scheme 8} Synthesis of 4,6-dimethylundecan-1-ol
\end{center}
At the first sight, Figure 23 might have indicated the presence of both diastereomers in the glandular extracts, matching by their retention time with the synthetic syn and anti diastereomers. However, the MS spectrum of the second eluting compound showed no similarities with the candidate compound. On the basis of these results, we suggested that the trail-following pheromone of *H. sjoestedti* is syn-4,6-dimethylundecan-1-ol. To confirm this hypothesis, the synthetic mixture was separated by means of preparative GC and both diastereomers independently or in a mixture were tested in biotests. Indeed, the bioassays showed the same activity of the syn isomer as in case of the natural extract and thus confirmed that it is the trail-following pheromone of *H. sjoestedti*. Since syn-4,6-dimethylundecan-1-ol has two chiral carbon atoms, there were two possible final structures (Fig. 24).

Similarly to the trail-following study, the sex pheromone of *H. sjoestedti* was studied from extracts obtained using SPME of sternal and tergal glands of reproductives. The specific compound of male reproductives with KI = 1406 on DB-5 and KI = 1668 on DB-WAX was detected on the surface of their sternal glands. The EI-MS spectrum is shown in Figure 25.
According to the MS spectrum, the molecular mass of this compound was estimated and later confirmed by chemical ionization to be 198. FT-IR indicated the presence of an aldehyde group, so the molecular formula \( \text{C}_{13}\text{H}_{26}\text{O} \) was proposed, suggesting one degree of unsaturation. In addition, the fragmentation pattern and retention index indicated a branched and saturated molecule. Having compared the MS spectrum with that of the male-specific compound in the related species *Zootermopsis nevadensis*, syn-4,6-dimethyldodecanal [21], we hypothesized the structure of the male sex pheromone in *H. sjoestedti* to be syn-4,6-dimethylundecanal. This compound was easily prepared by the oxidation of the previously prepared trail-following pheromone, 4,6-dimethylundecan-1-ol. Although it was not possible to use a bioassay due to the lack of live termites, the retention behaviour as well as MS spectrum confirmed that the diastereomer syn-4,6-dimethylundecanal is the male sex pheromone. And just as in the case of the trail-following pheromone, there were two possible final structures (Fig. 26).

![Fig. 26 Two possible final structures of syn-4,6-dimethylundecanal](image)

The situation in female reproductives appeared to be much easier since their specific compound corresponded to the female sex pheromone of *Zootermopsis nevadensis*, (5E)-2,6,10-trimethylundeca-5,9-dienal (Fig. 27) with molecular mass 208 and the retention index on non-polar Equity 5 was 1520 and 1875 on DB-WAX [21].
Since this compound has one chiral carbon, the two possible final structures were as follows (Fig. 28):

(S,E)-2,6,10-trimethylundeca-5,9-dienal  (R,E)-2,6,10-trimethylundeca-5,9-dienal

Fig. 28 Two possible final structures of (5E)-2,6,10-trimethylundeca-5,9-dienal

Taking into consideration that sex-specific pheromones in both sexes were previously described only in *Zootermopsis*, our results underlined the relation of the taxa *Hodotermopsis* and *Zootermopsis* which were recently classified as sister genera and separated from other lineages in a distinct family Archotermopsidae [64].
7.4 Alarm pheromone in *Termitogeton planus* (ref. Paper D)

This chapter summarizes our investigations of the chemical composition of the defensive secretion of soldiers in a poorly known termite species *Termitogeton planus* (Isoptera: Rhinotermitidae) from West Papua and on the biological role of the secretion in eliciting the alarm behaviour. The results of the study were published in 2014 in the Journal of Chemical Ecology (Paper D).

In the first step, twenty-four samples of soldier defensive secretion of *T. planus* collected from nine different colonies were analyzed by means of GC-MS with quadrupole mass analyzer (DSQ II) equipped with non-polar ZB-5MS column. For these analyses, two types of samples were prepared, i.e. extracts of 40 - 120 soldier heads prepared directly in the field and extracts of individual soldier heads prepared in the laboratory in Prague. All the samples were prepared in the same way using 10 μl of distilled hexane per individual for an extraction time of 12 hours at 4°C. For the purposes of relative quantifications, the internal standard, 1-bromodecane (40 ng/μl), was coinjected with the samples. Haloalkanes are generally favoured internal standards for their recognizable spectra. In addition, 1-bromodecane elutes at a different retention time (KI = 1356 on ZB-5MS) than all the compounds detected in the extracts. As a control, body extracts of 10 workers from each colony were analyzed, allowing us to clearly discriminate soldier-specific defensive chemicals.

**Tab. 4** summarizes the identity and relative proportion of soldier defensive compounds from all studied colonies.

<table>
<thead>
<tr>
<th>RI</th>
<th>TP-1</th>
<th>TP-2</th>
<th>TP-3</th>
<th>TP-4</th>
<th>TP-5</th>
<th>TP-6</th>
<th>TP-7</th>
<th>TP-8</th>
<th>TP-9</th>
<th>TP-10</th>
<th>TP-11</th>
<th>ng/ind.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonane</td>
<td>900</td>
<td>9.4</td>
<td>14.9</td>
<td>11.2</td>
<td>11.1</td>
<td>7.9</td>
<td>6.8</td>
<td>7.4</td>
<td>10.3</td>
<td>6.5</td>
<td>18.1</td>
<td>15.8</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>940</td>
<td>71.1</td>
<td>66.0</td>
<td>70.5</td>
<td>72.8</td>
<td>75.3</td>
<td>78.3</td>
<td>74.2</td>
<td>75.5</td>
<td>75.3</td>
<td>51.4</td>
<td>35.5</td>
</tr>
<tr>
<td>(1S)-(+)β-Pinene</td>
<td>986</td>
<td>11.1</td>
<td>12.1</td>
<td>11.5</td>
<td>10.0</td>
<td>10.2</td>
<td>9.9</td>
<td>11.1</td>
<td>11.3</td>
<td>10.2</td>
<td>7.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Myrcene</td>
<td>991</td>
<td>0.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.5%</td>
<td>ND</td>
<td>0.6</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Decane</td>
<td>1000</td>
<td>&lt;0.5%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>1.4</td>
<td>2.7</td>
</tr>
<tr>
<td>α-Phellandrene</td>
<td>1011</td>
<td>1.4</td>
<td>1.3</td>
<td>2.0</td>
<td>1.4</td>
<td>1.4</td>
<td>1.0</td>
<td>1.4</td>
<td>0.9</td>
<td>1.2</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>α-Terpine</td>
<td>1022</td>
<td>2.5</td>
<td>1.6</td>
<td>2.9</td>
<td>1.5</td>
<td>1.8</td>
<td>1.5</td>
<td>2.8</td>
<td>0.9</td>
<td>1.9</td>
<td>4.0</td>
<td>4.9</td>
</tr>
<tr>
<td>(R)-limonene</td>
<td>1036</td>
<td>2.7</td>
<td>1.8</td>
<td>1.5</td>
<td>1.9</td>
<td>2.3</td>
<td>1.7</td>
<td>2.3</td>
<td>0.7</td>
<td>2.5</td>
<td>2.9</td>
<td>4.2</td>
</tr>
<tr>
<td>(E)-β-Ocimene</td>
<td>1048</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>15</td>
</tr>
<tr>
<td>γ-Terpine</td>
<td>1062</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>10</td>
</tr>
<tr>
<td>Teprinolene</td>
<td>1092</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.5%</td>
<td>0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Undecane</td>
<td>1100</td>
<td>1.2</td>
<td>2.3</td>
<td>&lt;0.5%</td>
<td>1.3</td>
<td>1.2</td>
<td>0.8</td>
<td>0.6</td>
<td>&lt;0.5%</td>
<td>1.3</td>
<td>12.0</td>
<td>26.2</td>
</tr>
</tbody>
</table>
In contrast to some other termite species, we did not observe any qualitative or quantitative intraspecific variability in the detected analytes, probably due to the small geographical area of the sampling. Therefore, we continued with a detailed analysis especially focused on the identity of monoterpene hydrocarbons, previously confirmed as alarm pheromones in several other termite species [65, 66]. For this reason, the temperature program was set from 50˚C to 120˚C at 8˚C/min rate and then to 320˚C at 15˚C/min rate, which enabled us to separate structurally very similar monoterpene hydrocarbons (Fig. 29).

[Graph showing GC chromatogram with peaks for different hydrocarbons labeled: (1R)-α-pinene, (1S)-β-pinene, myrcene, α-phellandrene, α-terpinene, (R)-limonene, (E)-β-ocimene, γ-terpinene, and terpinolene.]

**Fig. 29** GC chromatogram of the defensive secretion of *Termitogeion planus* soldiers.

For precise separation and identification, some of the samples were analyzed also by means of GC×GC/TOF-MS (Leco, Pegasus III) and the separation in the second dimension revealed the presence of an alkene in trace quantities (Fig. 30).
Finally, we identified 4 saturated or unsaturated hydrocarbons together with nine monoterpene hydrocarbons (Tab. 4), which were confirmed by their retention indices and fragmentation patterns with literature [62] and with commercially available standards (with exception for α-terpinolene). In view of the fact that enantiomeric purity is essential for eliciting of alarm behaviour [24, 65, 67], we analyzed the natural extracts using GC-FID (HP 6850 Series) with a chiral column HP-CHIRAL-20B (30 m, id 0.25 mm, 0.25 μm phase thickness) and compared with commercially available standards.

Afterwards, the absolute quantification of particular compounds was performed using the major compound (1S)-α-pinene (50 ng/ul) as an external standard. The quantities of monoterpene hydrocarbons calculated per one individual are summarized in Tab. 4.

Subsequently, 8 synthetic standards of monoterpenes were mixed together (α-terpinolene was unfortunately not available) in relative proportions corresponding to those in the authentic extracts and used in alarm bioassays. Since the synthetic mixture elicited the same alarm reaction as the natural extract, we continued with searching for the responsible compound(s). The lack of testing individuals did not allow us to try all possible combinations of monoterpenes, therefore we tested the mixtures prepared from 4 major monoterpenes, 4 minor monoterpenes and
the dominant monoterpene (1S)-α-pinene (Fig. 31). Although the reactions of the termites to the mixture of the four major compounds and (1S)-α-pinene were stronger, the mixture of minor compounds was also active in eliciting the alarm behaviour. We concluded that the alarm pheromone of *T. planus* is a multi-component signal combining major as well as minor components from the defensive secretion of soldiers.

![Fig. 31 The natural extract compared with synthetic mixtures](image-url)

Such a multi-component chemical signalling consisting of several relatively simple molecules which together form a unique blend is often preferred to single-component pheromones in a variety of insects and other animals [68, 69] and represents what is called the chemical language [70].
7.5 Fertility signals in *Prorhinotermes simplex*

This chapter summarizes our investigations on chemicals produced by the reproductives of the termite *Prorhinotermes simplex* (Rhinotermitidae), expected to act as recognition cues of the reproductives, and potentially, as primer pheromones regulating the fertility of the nestmates. To this purpose, we selected a species previously studied in our laboratory in detail for all aspects of its biology and chemical ecology, the lower termite *Prorhinotermes simplex*. Based on the previous investigations of my colleagues, we were searching for small, volatile signalling compound, very likely quite polar, released from a binding protein secreted on the body surface of the kings and queens.

With this initial information my first task was to develop a suitable extraction method compatible with GC. Given the hypothesis that the desired molecule is released from a binding protein on the body surface, we started with water extraction of bodies of kings and queens using a 20 minute wash in 10 μl of ultrapure water per individual. A water extract of 57 secondary kings and a control extract of 63 workers were stirred and heated at 70 °C for one hour for protein denaturation, then extracted using a red SPME fibre (SUPELCO, PDMS, 100 μm, non-bonded), submerged directly into the water for 1 hour. Then the fibre was let to dry for 30 minutes and injected into a GC×GC/MS (Leco, Pegasus 3D). Only the use of a two-dimensional setup enabled us to find a compound specific for reproductives with KI = 1610 (on DB-5) co-eluting in one-dimensional arrangement with tetradecanal, commonly occurring on the body surfaces of various termites (Fig. 32).

![Fig. 32 GC×GC chromatogram showing the first detection of the candidate king-specific compound](image-url)
Since the candidate compound was detected in trace quantities only, we tried to increase
the efficiency of the extraction procedure by prolonging the warming period to 4 hours which
doubled the yield of the analyte in the extract. With the new samples prepared from 30 secondary
kings and 60 workers we tested another parameter, the time of the extraction by SPME. Samples
were again heated at 70 °C for 1 hour but extracted for 12 hours. By this experiment we reached
seven times better yield of the candidate compound. However, we were still facing the problem
of a coelution of the candidate compound with other analytes. Bearing in mind the assumed
volatility of the putative king signal, acting on large distances within the colony, we decided
to extract the candidate compound from the headspace. Three secondary kings were extracted
using a gray SPME fibre (SUPELCO, DVB/CAR/PDMS coating, 50/30 μm, bonded) for 12
hours at laboratory temperature and the compound of our interest was found even in higher
quantity (Fig. 33) than in water samples, which supported the hypothesis of its information role.

![Headspace SPME of 3 secondary kings](image)

**Fig. 33** Headspace SPME of 3 secondary kings

Since the SPME extraction is a one-shot extraction method, we were searching for an extraction
protocol allowing a repeated use of a sample. Finally, we succeeded in developing an effective
method of extraction of the king and queen bodies using dichloromethane for 16 hours
in freezer. Afterwards, the samples were measured by GC-MS with a quadrupole mass analyzer,
allowing more precise quantification than the two-dimensional setup.

Fig. 34 shows a comparison of 1D chromatograms of male and female reproductives and
workers prepared by the described method. The previously detected candidate compound
specific to reproductive castes is marked with an asterisk.
The quantity of the candidate compound in male reproductives appeared to be almost 40 times higher than in females. At the first sight, it is also obvious that the extraction by dichloromethane was much more selective than previously described techniques and thus more suitable for our purpose. Subsequently, we proceeded to the identification of the unknown compound of the EI-MS spectrum depicted in Fig 35.

The analysis on Q-TOF (Waters) determined the molecular mass of our compound to be m/z 202 and the elemental composition C\textsubscript{12}H\textsubscript{26}O\textsubscript{2}. The m/z 31, typical for alcohols, together with results from infrared spectroscopy suggested that the compound bears two hydroxy groups. To localize the position of the hydroxy groups, using TMSI we prepared a silylated derivative of the compound as well as the silylated derivative of a commercial standard of a primary diol – dodecane-1,12-diol. The shift of the retention time suggested a branching of our compound.
EI-MS spectrum of the compound after derivatization (Fig. 36) showed among the dominant fragments the fragments m/z 117 and 131. Based on these observations we proposed the molecular structure dodecane-2,10-diol.

![EI-MS spectrum of the silylated compound](image)

**Fig. 36** EI-MS spectrum of the silylated compound

The target compound was synthesized by one-pot procedure described by Gries et al. [71]. 1,2-epoxypropane and 1,2-epoxybutane were opened by Grignard reagent pentan-1,5-bis(magnesium bromide) and the reaction produced a desired diol 2 as the major product together with two other expected diols 1 and 3 (Scheme 9) with KI = 1510 and 1710. The obtained diols were separated by column chromatography with ether/hexane gradient (Fig. 37).

![Synthesis of diols](image)

**Scheme 9** Synthesis of diols
Fig. 37 Separation of individual diols by column chromatography

Fig. 38 Mass spectra of undecane-2,10-diol and tridecane-3,11-diol and their silylated derivatives

Figure 38 depicts changes in the mass spectrum related to the position of the hydroxy group. Interestingly, the intensity of the fragment m/z 31 is rather low in the case of undecane-2,10-diol.
Finally, the compound from the natural extract was compared with the synthetic dodecane-2,10-diol using GC×GC/MS, GC-IR and both analyses revealed a perfect match. In addition, EI-MS spectra of silylated derivatives of both, natural and synthetic compounds, were identical. In the next step, we will selectively prepare the four possible isomers of dodecane-2,10-diol (Fig. 39) and design a suitable bioassay to investigate the biological role of the compound in termite colonies.

Fig. 39 Possible isomers of dodecane-2,10-diol
8 Defensive chemicals

8.1 Defensive chemicals in soldiers of *Psammotermes hybostoma* (ref. Paper E)

This chapter summarizes our investigations on the defensive chemistry of soldiers in the termite *Psammotermes hybostoma* (Rhinotermitidae), studied in the frame of the research project aiming at important understudied genera from the families Rhinotermitidae and Serritermitidae. The results were published in the *Journal of Chemical Ecology* in 2012 (Paper E).

In this study, defensive secretions from the frontal gland of soldiers from nine colonies and five different localities from the Nile Valley and Egyptian Western Desert were analyzed in order to identify the defensive chemicals and compare their intracolonial, intercolonial and geographical chemical diversity.

Firstly, 10 workers from all studied colonies were extracted by shaking in hexane (10 µl per individual) for 20 minutes at laboratory temperature and subsequently analyzed by GC-MS with quadrupole mass analyzer. Since we did not observe any qualitative differences between the profiles of cuticular hydrocarbons among the nine samples, we concluded that all selected colonies belonged to the same species, *Psammotermes hybostoma* (Fig. 40).

![Fig. 40 Comparison of cuticular hydrocarbon profiles from nine studied colonies](image)

In the next step, extracts of workers and soldiers were compared in order to highlight chemicals specific to the caste of soldiers (Fig. 41).
Altogether, we detected 33 soldier-specific compounds, belonging mainly among sesquiterpene hydrocarbons and their oxygenated derivatives. Even though there are two brilliant books devoted to mass spectra of sesquiterpenes [62, 72], the identification of this type of compounds remains rather complicated because of only slight differences in their retention indices and only minute differences in their fragmentation patterns resulting from their structural similarity. Moreover, one compound often has several synonymous trivial names attributed by different authors, making the comparison of literary data even more complicated. Since standards of sesquiterpenes are in most cases not commercially available, majority of *P. hybostoma* chemicals was identified based on the comparison with literary data and when natural or synthetic standards were available, they were used for a final confirmation. In some cases, we were confirming the identity of the sesquiterpenes using a comparison with natural sources, extracted using SPME headspace extraction. Orange peel or ginger rhizome (10 g) was closed in a large vial (volume 3.5 ml) and grey SUPELCO SPME fibre (50 µm, DVB/CAR/PDMS coating) was inserted through the septum. Samples were heated in the water bath to 50 °C for 40 minutes as suggested by Hamm et al. [73] to be an effective method for the extraction of sesquiterpenes. Some other compounds were confirmed by the comparison with a defensive secretion of *Prorhinotermes simplex* from our laboratory colonies; the composition of this secretion was studied and published a few years ago [74]. The other standards were obtained using a relatively simple synthesis from commercially available (Z)-nerolidol according to a procedure described in Svatoš and Attygale [75] (Scheme 10).
The table 5 represents a simplified version of a table included in the Paper E. It shows all soldier-specific compounds identified in extracts in quantity superior to 1%. Even a simple visual inspection of the table allows easily distinguish the different chemotypes, present in our sampling. The most indicative compounds for these chemotypes were β-elemene, valencene and (E)-γ-bisabolone.

For the comparison of intercolonial variability, hexane extracts of 10 soldiers of each colony were analyzed; while to asses the intracolonial variability, samples of 10 individual large soldiers from colony E2 and samples of 10 individual small soldiers from colony A1 were compared. Finally, we evaluated the variability among soldiers of different body sizes in the colony B1, containing all the three soldier categories (small, middle and large). To correct for an accidental volume variations during sample injection, 1-bromodecane (40 ng/μl) was coinjected directly with the sample and the peak areas were calculated in relation to this internal standard. 1-Bromodecane was selected for its easy detectability and because of its short retention time, separated by 30 s from the elution of the first defensive compound.

**Scheme 10** Preparation of 5 sesquiterpenes from (Z)-nerolidol
Tab. 5 List of *P. hybostoma* defensive chemicals

<table>
<thead>
<tr>
<th>RI</th>
<th>Name</th>
<th>Class</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>B1</th>
<th>B1</th>
<th>B1</th>
<th>C1</th>
<th>C2</th>
<th>D1</th>
<th>E1</th>
<th>E2</th>
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<td><em>p</em>-methylacetophenone</td>
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</tr>
<tr>
<td>1394</td>
<td>iso-β-elemene</td>
<td>sesquiterpene</td>
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<td>-</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>3</td>
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<td>-</td>
</tr>
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<td>1402</td>
<td>β-elemene</td>
<td>sesquiterpene</td>
<td>11</td>
<td>8</td>
<td>25</td>
<td>64</td>
<td>62</td>
<td>58</td>
<td>34</td>
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<tr>
<td>1451</td>
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<td>1464</td>
<td>α-helmscapene</td>
<td>sesquiterpene</td>
<td>18</td>
<td>16</td>
<td>8</td>
<td>5</td>
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<td>7</td>
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<tr>
<td>1541</td>
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<td>1648</td>
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<td>-</td>
<td>-</td>
<td>3</td>
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</tbody>
</table>

Figure 42 depicts the fragments of chromatograms highlighting the main differences in defensive chemicals among particular localities. Although the GC profiles markedly varied quantitatively, especially in case of localities A1 and B1, qualitative differentiation indicated three clearly distinct defensive blends = different chemotypes. Whereas two of these chemotypes (AB, DE) each contained some exclusive compounds, lacking in the other, the third one (C) was the mixture of the two others. A comparison of the geographical distribution of the three chemotypes allowed us to make a biogeographical hypothesis on the past events in the distribution of individual populations. What is worth noticing, we were able to recognize particular chemotypes by our own sense of smell.

**Fig. 42** Comparison of defensive secretions among particular localities
The comparison of soldiers of different body sizes within the colony B1 did not reveal any qualitative or quantitative differences in the chemical composition among the soldier categories. On the contrary, the total quantity of the defensive secretion clearly differed among the categories; the maximum amount detected in the small soldiers was 170 µg while in large soldiers it has been estimated to reach up 520 µg. In agreement with these results also the intracolonial variability among 10 individual small soldiers from the colony A1 and 10 large soldiers from the colony E2 was negligible, as clearly indicated in the cluster tree depicted in Figure 43.

![Cluster tree](image)

**Fig. 43 Cluster tree**

To conclude, we had not observed any variations between soldiers of different body sizes neither within colonies. On the other hand, we detected quantitative and qualitative differences among particular localities which were in some cases so distinctive that three significantly different chemotypes corresponding well with the locality of origin were established. These findings prove the possibility of using termite defensive chemistry as a tool for studies on phylogeography.
8.2 Defensive chemistry of the termite *Neocapritermes taracua*

This chapter summarizes our investigations on the defensive chemicals and the functional mechanism of defence in workers of the neotropical termite *Neocapritermes taracua* (Termitidae). This research was conducted as an extension of our previous study on anatomic and behavioural aspects of the defence in this species [76] and will be a part of a future publication on the chemistry of the unusual defensive strategy that we discovered in *N. taracua* [77 in prep.].

Just as the vast majority of other termite species, also *N. taracua* possesses the caste of soldiers, specialized for defensive tasks. However, the large body-sized soldiers of *N. taracua* are not very populous, mainly defend the nest and only rarely were they seen to accompany the workers during their foraging for food out of the nest. Instead, the defence of the foraging parties is carried out by old workers, equipped with a unique two-component chemical defence that they use during a suicidal defensive body rupture. As they age, the workers gradually accumulate a pair of blue crystal-like structures (for simplification hereafter called blue crystals) that are produced by the “crystal gland” [78], secreted outside the body and stored in external pouches. When disturbed, the workers rupture their body wall between blue crystals and labial glands. The mixing of the labial glands content and blue crystals with hemolymph results in a yellow sticky fluid which has been found to be toxic to workers of other termites (Fig. 44). Old workers, equipped with fully developed blue crystals, were observed to be more aggressive to non-nestmates and more ready to perform suicidal body rupture than the young workers without blue crystals. The aim of this research was to investigate chemical aspects of this unique two-component defensive strategy.

![Fig. 44](image_url) Photos of old workers with blue crystals, after the body rupture and after the careful removal of blue crystals
The chemical analysis of the blue crystals was challenging because of their insolubility in majority of solvents and because of their low quantity (about 10 μg/1 worker), limiting the number of suitable analytical methods. Since non-polar solvents were absolutely ineffective and polar solvents only decolorized the crystals, the only possibility appeared to be the dissolution in buffers followed by HPLC analysis. Unfortunately, the solution transformed into a sticky fluid, which damaged HPLC column. In spite of these difficulties, the collaborating biochemists finally determined the blue structures to be copper-containing proteins and the quantity of the copper was estimated by ICP-MS to be 9 ± 2 ng [76]. However, the mechanism of *N. taracua* defence remained unclear.

My task was to unravel the chemical composition of the mixture consisted of blue crystals and labial glands products dissolved in the hemolymph droplet (bursting liquid) resulting from the body rupture. Since solvent extraction revealed to be complicated, I decided to use the SPME headspace extraction to study volatiles emitted by the bursting liquid. Based on the previous tests of solubility, we expected rather polar compounds, therefore the yellow SUPELCO SPME fiber (30 μm, non-bonded PDMS coating) was selected for the detailed analysis. The headspace extraction was performed in a small vial where termites were placed and carefully disturbed with the forceps until they ruptured their bodies and released the bursting liquid. The optimal extraction time was estimated to be only 5 minutes because termites turn black within 15 minutes after the body rupture. Subsequently, SPME fiber was injected directly into GC×GC/TOF-MS (Leco, Pegasus 3D). First experiments were performed with a group of 10 old workers with blue crystals and 10 young workers without the blue crystals. While in the sample of young workers without the blue crystals methyl- and ethylbenzoquinone were detected in moderate quantities, in old workers with the blue crystals we identified very large quantities of benzoquinone and its methyl- and ethyl-derivatives as well as hydroquinone and the corresponding methyl- and ethyl-derivatives (Fig. 45).
These compounds are known insect defensive chemicals [79] and were also detected in labial glands in some termite species [80]. Therefore, we hypothesized that they likely originate in labial glands and are oxidized during body rupture. Five labial glands of workers of three categories (without blue crystals, with small blue crystals and with large blue crystals) were dissected and extracted in methanol (10 μl/gland) overnight at -20°C. Subsequent analyses by means of GC-MS with quadrupole mass analyzer revealed the presence of hydroquinone and both its derivatives which were previously found in the headspace analysis. These compounds were found in all three categories of workers but there was an obvious increase in their quantity along with the increase in size of the blue crystals and thus with the age of the workers (Fig. 46).
Fig. 46 The increasing quantity of hydroquinone and its derivatives with workers age

For the quantification, the calibration curve with commercially available standard of methylhydroquinone was prepared (Fig. 47) and the relative quantity of particular hydroquinones in the three worker categories as well as the absolute quantity of methylhydroquinone were calculated (Tab. 6).

Fig. 47 Calibration curve of methylhydroquinone
Tab. 6 Quantity of hydroquinone and its derivatives in labial glands

<table>
<thead>
<tr>
<th></th>
<th>HQ [%]</th>
<th>m-HQ [%]</th>
<th>e-HQ [%]</th>
<th>m-HQ [ng]</th>
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<tr>
<td>without blue crystals</td>
<td>9.1</td>
<td>26.4</td>
<td>2</td>
<td>88</td>
</tr>
<tr>
<td>with small blue crystals</td>
<td>7.9</td>
<td>44.3</td>
<td>2.6</td>
<td>136</td>
</tr>
<tr>
<td>with blue crystals</td>
<td>14.3</td>
<td>81.4</td>
<td>4.3</td>
<td>240</td>
</tr>
</tbody>
</table>

We also studied the relative quantity of benzoquinone and its derivatives in the bursting liquid of individuals at different age by headspace SPME and subsequent GC×GC/MS analysis using the same conditions described above (Fig. 48).

![Fig. 48](image-url)

**Fig. 48** Relative quantity of benzoquinone and its derivatives in the bursting liquid

Despite the difficulties in the quantification of outputs from two-dimensional gas chromatography, we noticed the same age-dependent trend in the increase of quantity...
of benzoquinones with age as in case of hydroquinone and its derivatives in labial glands. Thus we hypothesized that the defensive mechanism is based on an oxidative conversion of hydroquinone(s) originating in labial glands into benzoquinone(s) catalysed by the blue crystals, made up of copper-binding protein (Fig. 49).

![Chemical reaction diagram](attachment:image.png)

**Fig. 49** Oxidation of hydroquinone by copper-binding protein (according to Li & Trush [81])

Our findings are in agreement with the previously published data where the role of copper ions in enhancing of hydroquinone cytotoxicity has been described [81, 82]. The source of the copper and other aspects of this unique defensive strategy remains unknown.
Nonadecadienone, a New Termite Trail-Following Pheromone Identified in *Glossotermes oculatus* (Serritermitidae)

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Abstract

Within the multitude of chemical signals used by termites, the trail marking by means of pheromones is ubiquitous. Chemistry and biology of the trail-following communication have been described in more than 60 species from all families except for the Neotropical Serritermitidae. The chemical ecology of Serritermitidae is of special interest not only as a missing piece of knowledge on the diversity and evolution of isopteran pheromones but also because it may contribute to the debate on the phylogenetic position of this family, which is still unresolved. Therefore, we aimed in this study to identify the trail-following pheromone of the serritermitid *Glossotermes oculatus*. Based on a combined approach of analytical chemistry, electrophysiology, and behavioral bioassays, we propose (10Z,13Z)-nonadeca-10,13-dien-2-one to be the trail-following pheromone of *G. oculatus*, secreted by the sternal gland of pseudergates. Thus, we report on a new termite trail-following pheromone of an unexpected chemical structure, a ketone with 19 carbons, contrasting with unsaturated alcohols containing 12 carbons as trail-following pheromones in other advanced termite families. In addition to this unique trail-following pheromone, we also describe the sternal gland in pseudergates as an organ of unusual shape, size, and structure when compared with other isopteran species. These results underline the peculiarity of the family Serritermitidae and prompt our interest in the chemistry of pheromones in the other genus of the family, *Serritermes*.

Key words: Glossotermes, Serritermitidae, sternal gland, termites, trail-following pheromone, (10Z,13Z)-nonadeca-10,13-dien-2-one

Introduction

Termites, the eusocial “dwellers in the dark,” represent an excellent example of the prime role of chemical communication in insect societies. Within the multitude of chemical signals used by termites, the trail marking by means of pheromones is ubiquitous, regardless of the differences in foraging and nesting habits of particular species (Bordereau and Pasteels 2011). The sole source of trail-following pheromones, the sternal gland, occurs as a homologous organ in all termite species and castes, despite the variability in its position, size, and ultrastructure among particular isopteran families (Noirot 1969; Ampion and Quennedey 1981; Quennedey et al. 2008). However, its use by foragers in trail marking is probably derived from its ancestral role in mate attraction of termite imagoes during dispersal (Traniello and Leuthold 2000). This is evidenced by the widespread use of the sternal gland in courtship behavior of imagos as well as by the frequent use of the same pheromone for both mate attraction and trail marking, though in different amounts and in different contexts (Bordereau and Pasteels 2011).
The chemistry of termite trail-following and sex pheromones is quite well known, due also to numerous recent contributions using modern methods of extraction and analysis, and has been very recently summarized by Bordereau and Pasteels (2011). Based on our current knowledge, 2 conclusions can be drawn regarding the trail-following pheromones. First, the trail-following pheromones usually consist of a single component; even though this may be due to omissions of tiny amounts of minor components, detected recently in several termite genera only by means of electrophysiology (e.g., see Sillam-Dussès et al. 2009). And second, the evolution of chemical diversity of trail-following pheromones appears to be quite conservative, with only 8 compounds being identified as trail-following pheromones in 6 families and more than 60 species studied up to date. Moreover, the same compounds may occur as trail-following pheromones in phylogenetically distant taxa with a very different foraging ecology. Nevertheless, the relative uniformity of trail-following pheromones within particular families or subfamilies may still be useful to infer various evolutionary conclusions at higher taxonomic levels (see Bordereau and Pasteels 2011).

The Neotropical family Serritermitidae, consisting of 2 genera, Serritermes and Glossotermes (Cancelllo and DeSouza 2005), remains the only isopteran family which has not yet been studied with respect to trail and sex pheromones. Its biology, including chemical ecology, is of special interest due to the debated phylogenetic relationships between Serritermitidae and basal lineages of Rhinotermitidae, with several alternative scenarios being recently proposed, that is, Serritermitidae + (Rhinotermitidae + Termitidae), Rhinotermitidae + (Serritermitidae + Termitidae), or Serritermitidae nested within Rhinotermitidae (see Lo et al. 2004; Ohkuma et al. 2004; Inward et al. 2007; Legendre et al. 2008; Engel et al. 2009).

The biology of the genus Glossotermes has long been unknown; only very recently, we described the caste pattern (Bourguignon et al. 2009) and soldier defensive adaptations (Sobotnik, Bourguignon, et al. 2010). The genus was originally placed within Rhinotermitidae, as the sister group of Psammotermes (Emerson 1950) but was recently transferred to Serritermitidae (Cancelllo and DeSouza 2005). The monophyly of the family is supported by molecular data (e.g., Lo et al. 2004), morphology, and a unique defensive strategy of soldiers through suicidal dehiscence of the frontal gland (Costa-Leonardo and Kitayama 1991; Engel et al. 2009; Sobotnik, Bourguignon, et al. 2010). In this study, we investigated the chemical identity of the trail-following pheromone in Glossotermes oculatus.

Materials and methods

Insects

Colonies of G. oculatus were collected near Petit Saut dam in French Guiana (05°04’N, 52°59’W) from wet rotten logs (Figure 1). Five fragments of colonies (500–2000 individuals each) were collected in January 2010 and transported to Prague in their original portions of wood. They were used for trail-following pheromone identification (histology, preliminary bioassays, chemical analysis, electrophysiology). Three additional colonies were collected in November 2010. They were used for trail-following pheromone confirmation and quantification by means of a set of bioassays, performed at Laboratoire Environnement Hydreco (French Guiana) and by means of gas chromatography–mass spectrometry (GC/MS) and electrophysiology, performed in Prague.

Anatomy of the sternal gland

Optical microscopy of the sternal gland of pseudergates was performed using the protocol and equipment described in Sobotnik, Sillam-Dussès, et al. (2010).

Sternal gland extracts of pseudergates

Cold-immobilized pseudergates were carefully dissected and a portion of the ventral integument containing the sternal gland was submerged into hexane. The sternal glands (30–300 glands) were extracted for 6 h in hexane (1 μL/gland) at 4°C. The extracts were used directly or stored at –18°C. For preliminary bioassays, whole body extracts of pseudergates (WBE) were prepared following the same procedure.

Gas chromatography–mass spectrometry

Chemical identification was carried out using 2D gas chromatography with time-of-flight mass spectrometric detection (GC × GC/TOF-MS, Pegasus 3D; Leco); for details, see Hanus et al. (2009). The temperature program for the first column was 40°C (1 min) to 320°C (5 min) at 7°C/min; the temperature of the second column was set 20°C higher. For quantification of nonadeca-10,13-dien-2-one, 3 different sternal gland extracts of pseudergates (SGE) were measured by means of GC/MS (quadrupole DSQ II; Thermo Scientific) with a DB-5 column (30 m, inner diameter 0.25 mm, 0.25 μm
phase). Temperature program: 80 °C (1 min) to 320 °C (3 min) at 15 °C/min. One gland equivalent (GEq) in 1 μL of hexane was injected in a splitless mode. Prior to injection, tricosan-12-one has been added as internal standard to the SGE at a concentration of 26 ng/μL.

**Column chromatography and preparative gas chromatography**

In order to localize the compound(s) responsible for the trail following in the complex blend detected by GC in the SGE, we fractionated the extract using column chromatography. Columns (Pasteur pipettes) were loaded with 2 g of silica gel and prewashed with 10 mL of pure hexane. One milliliter of SGE was applied on the top of the column and eluted with a series of solvents of increasing polarity (2 mL of hexane:ether from 10:0 to 0:10). Solvent drips were collected into glass vials and concentrated.

Selected fractions were further subjected to preparative gas chromatography. Preparation was performed with a gas chromatograph (AT 6890N; Agilent Technologies) linked to a Preparative Fraction Collector (Gerstel), equipped with a liquid nitrogen cooling system. For GC separation, a HP-5 column (30 m, id 0.53 mm, 0.88 μm phase) was used. The temperature program was 40 °C (1 min) to 300 °C (5 min) at 20 °C/min. The flow rate of carrier gas (helium) was 6 mL/min, the injection volume was 5 μL. The separation intervals of particular fractions were related to linear retention indexes (LRIs) of n-alkanes (C7–C30).

**Chemicals**

(3Z,6Z,8E)-Dodeca-3,6,8-trien-1-ol (dodecatrienol) and (1E,5E,9E,12Z)-1,5,9-trimethyl-12-(1-methylpenylnyl)-1,5,9-cyclooctadecatriene (neocembrene) were kindly supplied by Christian Bordereau (for details, see Sillam-Dussès et al. 2005). Methyl linoleate (purity ≥ 99%), t-butyl acetate (purity ≥ 99%), pure dimethyl disulphate, and other reagents were purchased from Sigma-Aldrich. Solvents were purchased from Penta.

**Synthesis of (10Z,13Z)-nonadeca-10,13-dien-2-one**

The synthesis was analogous to that described in detail by Adams et al. (2010) and performed under an argon atmosphere. A solution of n-butyllithium in pentane (1.2 mL, 2.24 mmol, 3.3 equivalent) was added dropwise to a solution of disopropylamine (300 μL, 2.24 mmol, 3.3 equivalent) in tetrahydrofuran (3 mL) cooled to –78 °C. The mixture was stirred at –78 °C for 15 min, warmed up to 0 °C for 5 min, and cooled down to –78 °C again. After cooling, t-butyl acetate (300 μL, 2.24 mmol, 3.3 equivalent) was added, and the mixture was maintained at –78 °C for 10 min. Methyl (9Z,12Z)-octadeca-9,12)-dioenoate (200 mg, 0.68 mmol, 1 equivalent) in tetrahydrofuran (1.2 mL) was then added, and the reaction mixture was stirred at –78 °C for 1.5 h. Finally, saturated aqueous ammonium chloride (8 mL) was added, and the mixture was warmed to room temperature.

The solution was diluted with water (50 mL) and extracted with ethyl acetate (50 mL). The aqueous phase was washed with ethyl acetate (3 × 20 mL). The organic extracts were combined and washed with aqueous 1 N hydrochloric acid (25 mL) and saturated sodium bicarbonate (50 mL). During this process the product, (10Z,13Z)-nonadeca-10,13-dien-2-one, was formed. Finally, the organic phase was dried over magnesium sulfate and concentrated in vacuo to give a crude oil that was purified by silica gel chromatography (hexane:ether, 8:2). The pure compound (LRi = 2075) was obtained using preparative GC (the same conditions as described above), temperature program: 70 °C (1 min) to 110 °C at 10 °C/min then to 300 °C at 6 °C/min. The flow rate of helium was 6.5 mL/min, and the injection volume was 5 μL.

**Preparation of dimethyl disulfide derivatives**

Dimethyl disulfide (DMDS) derivatives were prepared from the synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one and the SGE of pseudergates. Fifty microliters of the sample in hexane (extract or standard) containing approximately 50 ng of the studied compound were mixed with 50 μL DMDS and 5 μL of iodine solution (60 mg/mL iodine in fresh distilled diethyl ether). The vial was shielded and shaken overnight at room temperature. The reaction was finished after 16 h with adding aqueous sodium thiosulfate (5% in water), the mixture was extracted twice with 200 μL of hexane, concentrated, and injected into GC × GC/TOF-MS.

**Behavioral experiments**

Five colony fragments were used to test the ability of pseudergates to follow the WBE, SGE, particular fractions of these extracts, and standards of termite trail-following pheromones. The ability of pseudergates to follow the WBE, SGE, and the synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one was confirmed in all 3 colonies collected in November 2010; all results reported in this paper (Tables 1 and 3, Figure 4, GC/MS quantification) were recorded on individuals from one colony, collected a few hours before the bioassays. The termites were removed from the wood only a few minutes prior to the experiment, which was carried out at 26 °C under reduced light intensity.

Trail-following bioassays were performed to test the orientation activity of WBE, SGE, neocembrene, dodecatrienol, and (10Z,13Z)-nonadeca-10,13-dien-2-one. Solutions were tested with a “Y open field” bioassay on Whatman No. 1 filter paper (15 cm diameter), with a 120° angle between the branches. An artificial trail was drawn with 10 μL of the solution spread on the stem (3 cm) and one of the branches (7 cm). Ten microliters of hexane were deposited on the stem and the other branch. One termite was placed inside a holding chamber (55 mm Petri dish), with a 2 mm opening located at the base of the Y. The activity
threshold was defined as the minimum concentration that elicited termites to travel a mean distance of more than 3 cm. Each experiment was performed with 30 pseudergates, some of them also with 15 soldiers.

With the same setup, choice tests evaluating the preference of pseudergates for SGE or (10Z,13Z)-nonadeca-10,13-dien-2-one solutions were performed in order to estimate the quantity of the compound in one sternal gland. Each of the compared stimuli was deposited on the stem and on one of the Y branches. This test was repeated with 30 pseudergates; results were evaluated using $\chi^2$-test.

### Electroantennography

The experimental setup is described in Sillam-Dussès et al. (2009). Gas chromatography coupled with electroantennographic detection (GC-EAD) was used to identify the physiologically active compounds in the SGE, using the antenna of a pseudergate. To confirm the identity of the pheromone, a solution of (10Z,13Z)-nonadeca-10,13-dien-2-one was used. The times of antennal responses were related to the retention times of $n$-alkanes (C8–C22) in order to calculate their LRI standards of trail-following pheromones of Rhinotermitidae (see Bordereau and Pasteels 2011), that is, dodecatrienol (10–13)–nonadecadienone (ng/cm) and neocembrene (10–2–10 ng/cm). None of the tested concentrations was effective in eliciting trail-following behavior in pseudergates. The analysis of SGE using GC × GC/TOF-MS did not allow us to detect any of the known termite trail-following pheromones based on characteristic fragment ions of their mass spectra. We concluded that the trail-following pheromone of $G. oculatus$ is a new compound, not described as yet in termites.

### Table 1

<table>
<thead>
<tr>
<th>Source of the trail-following pheromone</th>
<th>Whole body extract (Geq/cm)</th>
<th>Sternal gland extract (Geq/cm)</th>
<th>Nonadecadienone (ng/cm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻¹</td>
<td>1</td>
<td>10⁻¹</td>
<td>0.5</td>
</tr>
<tr>
<td>Pseudergates</td>
<td>7.8 ± 3.3</td>
<td>8.7 ± 2.8</td>
<td>9.1 ± 1.2</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soldiers</td>
<td>—</td>
<td>9.3 ± 2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

The distance traveled by one pseudergate or one soldier on trails made of whole body extract or sternal gland extract of pseudergates or a series of nonadecadienone concentrations was measured (mean in centimeter ± standard deviation). Hexane was used as a control. GEq, sternal gland equivalent; n, number of repetitions.

### Results

#### Source of the trail-following pheromone

In the trail-following bioassays, WBE elicited the trail-following behavior in pseudergates as well as in soldiers (Table 1). SGE elicited the trail following in pseudergates at both tested concentrations, 0.1 and 0.5 GEq/cm of the trail (Table 1). We concluded that the compound(s) responsible for the trail following is (are) secreted by the sternal gland.
elicit trail-following in pseudergates at 1 GEq/cm, the distance traveled being 6.8 ± 0.9 and 8.4 ± 0.87 cm, respectively. These 2 fractions were merged and further fractionated in 3 steps using preparative GC. At each step, all obtained fractions were tested with respect to their trail-following activity; the active fraction was further fractionated. The LRIs and trail-following activity of particular fractions are summarized in Table 2.

A single behaviorally active fraction F6.3.3 (LRI = 2060–2130) was obtained in the third step of fractionation. This fraction was subjected to a detailed GC × GC/TOF-MS analysis, revealing the presence of a prominent peak (LRI = 2075), corresponding to an unknown compound, a candidate for the trail-following pheromone (Figure 3). The electron ionization (EI) mass spectrum of this compound can be interpreted as follows. The primary losses of masses 15, 43, and 60 from the inferred molecular ion of m/z 278, as well as the base peak of m/z 43, indicated that the molecule contained a terminal acetyl group. Furthermore, the molecular mass and natural isotopic contributions suggested that the acetyl group was attached to a hydrocarbon ligand containing 2 double bonds (C11H19S, C11H15S, C10H19S2, C9H15S2, C9H17S3, C7H15S). These results led us to hypothesize that the molecule might be biosynthesized as an acetyl derivative of linoleic acid (linoleyl methyl ketone), and, indeed, the fragmentation pattern at lower masses is similar to those from linoleic acid and its derivatives. Thus, we tentatively designed a molecular structure of the unknown compound to be (10Z,13Z)-nonadeca-10,13-dien-2-one and proceeded to the synthesis of the compound.

**Confirmation and quantification of the trail-following pheromone**

Once the synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one was purified, we compared its chemical properties with those of the candidate compound from the SGE. The retention characteristics and EI mass spectra of both compounds matched perfectly (see Figure 3). In order to localize the positions of the double bonds, DMDS derivatives of the compounds were analyzed using GC × GC/TOF-MS. The derivatives of both compounds corresponded in their retention characteristics as well as in their EI mass spectra, containing the molecular ion m/z 404 and expected characteristic fragments m/z 357, 309, 225, 201, 203, 155, and others (see Figure 3). The fragments 225 [M – C11H15S – CH3SH]+, 203 [M – C11H15S2]1+, 201 [M – C10H19S2]1+, and 155 [M – C9H17S2 – CH3SH]1 are indicative of the double bonds in positions 10 and 13 (e.g., see Vicenti et al. 1987).

Behavioral bioassays, summarized in Table 1, clearly show that (10Z,13Z)-nonadeca-10,13-dien-2-one elicits the trail-following behavior in both pseudergates and soldiers, the activity threshold being 10−2 ng/cm of the trail for both.
Figure 3  Two-dimensional chromatograms of (a) the extract of sternal glands of pseudergates before fractionation, with dashed lines indicating the LRI interval 2060–2130, (b) behaviorally active fraction F.6.3.3 of the sternal gland extract of pseudergates, and (c) a solution of synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one, after synthesis and before purification. All samples were coeluted with n-alkane standards (nC7–nC30). EI mass spectra of (d) the candidate compound from the sternal gland extract, (e) the synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one, (f) DMDS derivative of the synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one, and (g) DMDS derivative of the candidate compound. Numbering: 1, candidate compound (10Z,13Z)-nonadeca-10,13-dien-2-one; 2, methyl (9Z,12Z)-octadeca-(9,12)-dienoate (linoleic acid methyl ester).
castes. The distance traveled on the trail lengthened with increasing doses of the compound; we did not observe any decrease in the trail-following activity at high concentrations, up to 10 ng/cm of the trail in pseudergates.

In the subsequent GC-EAD experiment (rate 70 °C/min), we observed a consistent response of pseudergate antenna to the SGE at the retention time corresponding precisely to the LRI of (10Z,13Z)-nonadeca-10,13-dien-2-one as well as a response to the synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one (9°09’, LRI = 2075).

From these evidences, we concluded that the only or at least the major component of the trail-following pheromone of G. oculatus is nonadeca-10,13-dien-2-one. This is congruent with our initial hypothesis on the biosynthesis of this compound from the naturally occurring linoleic acid with 2 double bonds in cis configuration. We proposed therefore (10Z,13Z)-nonadeca-10,13-dien-2-one as G. oculatus trail-following pheromone.

From the choice trail-following experiment (see Table 3), we estimated the amount of the compound in a sternal gland of a pseudergate to be between 0.2 and 1 ng. The subsequent quantitative analysis of 3 independent SGEs by means of GC-MS estimated this quantity to be approximately 1.7 ± 0.45 ng per sternal gland. As the very last step, we attempted to quantify the amount of the pheromone by means of EAG. The approximate quantity estimated by this method was 1–20 ng (Figure 4).

Discussion

The Neotropical family Serratitermitidae remained the only isopteran family which had not been studied with respect to trail and sex pheromones. In the present study, we propose that (10Z,13Z)-nonadeca-10,13-dien-2-one is the trail-following pheromone of the serritermitid G. oculatus or at least the major component of this pheromone. The activity threshold was estimated to be 10⁻² ng of the compound per centimeter of the trail for both tested castes, pseudergates and soldiers.

The quantity of the pheromone in the sternal gland of 1 pseudergate was estimated using 3 independent assays to range from 0.25 to 1 ng of a compound from a sternal gland extract as well as to the synthetic (10Z,13Z)-nonadeca-10,13-dien-2-one (9°09’, LRI = 2075). From these evidences, we concluded that the only or at least the major component of the trail-following pheromone of G. oculatus is nonadeca-10,13-dien-2-one. This is congruent with our initial hypothesis on the biosynthesis of this compound from the naturally occurring linoleic acid with 2 double bonds in cis configuration. We proposed therefore (10Z,13Z)-nonadeca-10,13-dien-2-one as G. oculatus trail-following pheromone.

From the choice trail-following experiment (see Table 3), we estimated the amount of the compound in a sternal gland of a pseudergate to be between 0.2 and 1 ng. The subsequent quantitative analysis of 3 independent SGEs by means of GC-MS estimated this quantity to be approximately 1.7 ± 0.45 ng per sternal gland. As the very last step, we attempted to quantify the amount of the pheromone by means of EAG. The approximate quantity estimated by this method was 1–20 ng (Figure 4).

In the early steps, we failed to detect a consistent physiological response to the sternal gland extract by means of GC-EAD, probably due to the loss of activity of the antennae at the late elution time of nonadeca-10,13-dien-2-one (23°10⁹)

with the given temperature program. In consequence, the laborious methods of fractionation had to be used combined with trail-following bioassays. Indeed, at the faster temperature program, applied in the final experiment, the antennae of pseudergates responded well to nonadeca-10,13-dien-2-one contained in the sternal gland extract as well as to the synthetic compound (elution time 9°09’).

Our recent studies indicated that the presence of minor components in termite trail-following pheromones may be much more common than previously thought, the knowledge about them being limited due to their barely detectable quantities (Sillam-Dussé et al. 2009, 2010). The most frequent minor component of termite trail-following pheromones is dodecatrienol,

### Table 3 Two-choice trail-following bioassays with sternal gland extract and (10Z,13Z)-nonadeca-10,13-dien-2-one presented to pseudergates at 2 concentrations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EAG response (%)</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sternal gland extract (GEq/cm)</td>
<td>10⁻¹</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Nonadecadienone (ng/cm)</td>
<td>10⁻²</td>
<td>10⁻¹</td>
<td></td>
</tr>
<tr>
<td>nP</td>
<td>29</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>—</td>
<td>27</td>
</tr>
<tr>
<td>22</td>
<td>—</td>
<td>8</td>
<td>30</td>
</tr>
</tbody>
</table>

Values indicate the number of choices made by one pseudergate when given a choice between a trail made of sternal gland extract or (10Z,13Z)-nonadeca-10,13-dien-2-one. GEq, sternal gland equivalent; n, number of repetitions; P, p value (χ²-test).
which has a very strong physiological activity even at very low quantities (see also Hanus et al. 2009), and only units of picograms may be present in one forager (see Bordereau and Pasteels 2011). However, in the case of Glossotermes, we did not observe any trail-following activity of dodecatrienol, despite the broad range of tested concentrations. In conclusion, we did not identify any minor component of the trail-following pheromone, even though their presence cannot be excluded.

In proposing the molecular structure of nonadecadienone based on GC/MS, a question arised about the position and geometry of the 2 double bonds in the molecule. Using DMDS derivatization and GC × GC/MS, we situated the 2 double bonds in positions 10 and 13. Unfortunately, the geometry of these double bonds could not be empirically studied. Nevertheless, the most parsimonious and obvious hypothesis is a biosynthetic origin of nonadeca-10,13-dien-2-one as an acetyl derivative of naturally occurring and abundant linoleic acid (linoleyl methyl ketone), therefore with cis double bonds. Moreover, it has been suggested previously that (3Z,6Z)-dodeca-3,6-dien-1-ol, the trail-following pheromone and sex pheromone of Ancistrotermes pakiitanicus, is biosynthesized from a linoleic acid ligand (Robert et al. 2004). Thus, we conclude that the trail-following pheromone of G. oculatus is very probably (10Z,13Z)-nonadeca-10,13-dien-2-one.

The description of nonadeca-10,13-dien-2-one as the trail-following pheromone of G. oculatus is surprising in 2 aspects. Firstly, it is not in agreement with the major evolutionary trend inferred from available data by Bordereau and Pasteels (2011). The authors have noted a transition to unbranched and unsaturated alcohols with 12 carbon atoms and/or a diterpene (neocembrene or trinervitatriene) in the advanced group Kalotermitidae + Rhinotermitidae + Termitidae (within which Serritermitidae have undoubtedly branched); a phenomenon concurrent with the development of the sternal gland on the fifth sternite. Secondly, the occurrence of a C19 ketone is interesting from the functional point of view, as this compound has the lowest volatility and the highest molecular weight of all termite trail-following pheromones described so far. In this respect, it is worth noting that the effective physiological quantities of the pheromone (activity threshold and content in one sternal gland) are relatively high (compared with those observed for C12 alcohols and diterpenes), given the small body size of Glossotermes, and only units of picograms may be present in one forager (see Bordereau and Pasteels 2011). However, in the case of Glossotermes, we did not observe any trail-following activity of dodecatrienol, despite the broad range of tested concentrations. In conclusion, we did not identify any minor component of the trail-following pheromone, even though their presence cannot be excluded.

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In conclusion, the occurrence of nonadeca-10,13-dien-2-one as the trail-following pheromone of G. oculatus represents a unique peculiarity of this genus. None of the relevant scenarios of relationships among Rhinotermitidae, Serritermitidae, and Termitidae can explain the unexpected presence of a C19 ketone instead of a C12 alcohol, even though there are possible similarities in the biosynthesis of these alcohols and nonadeca-10,13-dien-2-one (Robert et al. 2004; Bordereau and Pasteels 2011). These results underline once again the special biology of the 2 serritermitid genera, Serritermes and Glossotermes, together with the unique defensive strategy of their soldiers (Costa-Leonardo and Kitayama 1991; Šobotník, Bourguignon, et al. 2010) and the lack of true workers in Glossotermes (Bourguignon et al. 2009). It would be of great interest to investigate the chemical ecology and caste system of Serritermes but also to identify the trail-following pheromone in Termitogeton, a poorly known rhinotermitid genus lacking true workers (Parmentier and Roisin 2003) and considered in some studies (e.g., Inward et al. 2007) as a sister clade to Serritermitidae.

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### Acknowledgements

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### References


Abstract Within the complex network of chemical signals used by termites, trail pheromones and sex pheromones are among the best known. Numerous recent papers map the chemical identity and glandular origin of these pheromones in nearly all major isopteran taxa. In this study, we aimed to describe the sex pheromone and the trail pheromone of a poorly known sand termite, *Psammotermes hybostoma*. We identified \((3Z,6Z,8E)\)-dodeca-3,6,8-trien-1-ol (dodecatrienol) as the sex pheromone released by tergal and sternal glands of female imagos and, at the same time, as the trail pheromone secreted from the sternal gland of workers. We conclude that chemical communication in *Psammotermes* does not differ from that of most other Rhinotermitidae, such as Reticulitermes, despite the presence of a diterpene as a major component of the trail pheromone of *Prorhinotermes* to which *Psammotermes* is presumed to be phylogenetically close. Our findings underline once again the conservative nature of chemical communication in termites, with dodecatrienol being a frequent component of pheromonal signals in trail following and sex attraction and, at the same time, a tight evolutionary relationship between the trail following of working castes and the sex attraction of imagos.

Key Words Sex pheromone · Trail pheromone · *Psammotermes hybostoma* · Termites · Rhinotermitidae · Tergal glands · Sternal gland · GC-EAD · Dodecatrienol · Timber pest

Introduction

Devoid of visual orientation, termites have reached a high level of social and functional complexity, with semiochemicals being involved in many aspects of their life. Within the network of chemical signals used by termites, the trail pheromones and the sex pheromones are among the best known. Numerous recent papers map the chemical identity and glandular origin of these pheromones in all but one (Serritermitidae) of the extant isopteran families using modern methods of chemical extraction (solid phase micro-extraction) and electrophysiological experiments (Sillam-Dussès et al., 2009; reviewed in Bordereau and Pasteels, 2011).

This progress in chemical ecology allows us to discern several particularities of termite trail and sex pheromones, contrasting with the situation in social hymenopterans. First, only a few glands are involved in the trail-following communication and sexual attraction in termites. Trail pheromones are always produced by the same homologous gland, the sternal gland of workers (but the gland is present in all castes and developmental stages) (Quennedey et al., 2008). The sex pheromones are secreted from the sternal gland and/or the tergal glands and/or the posterior sternal glands of female, or rarely from male imagos or both sexes (reviewed in Pasteels and Bordereau, 1998; Bordereau and Pasteels, 2011). Second, the chemical diversity of pheromonal components is surprisingly low, with only 8 compounds being identified as trail pheromones in more than 60 species studied, and only 9 compounds confirmed so far to participate in sexual attraction in 25 species studied.
et al., 1968; Tai et al., 1969; Tokoro et al., 1989, 1991; (dodecatrienol) as their trail pheromone (e.g., Matsumura). However, the subfamily Prorhinotermitinae displays another situation in Termitidae (Bordereau et al., 2002, 2010; Sillam-Dussès et al., 2010): the trail pheromone consists of components could have been overlooked in the past. Nevertheless, despite the conservative nature of both trail and sex pheromones and their glandular origins, their chemical ecology still represents a useful comparative tool at higher taxonomical levels. It is particularly true for the situation within the paraphyletic family Rhinotermitidae, within which both Serritermitidae and Termitidae probably evolved (see recent molecular phylogenetic studies, e.g., Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007; Legendre et al., 2008). Most rhinotermitids studied so far use the unsaturated alcohol (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol (dodecatrienol) as their trail pheromone (e.g., Matsumura et al., 1968; Tai et al., 1969; Tokoro et al., 1989, 1991; Wobst et al., 1999; Sillam-Dussès, 2004), and also as the sex pheromone produced by the sternal or tergal glands of females (Laduguie et al., 1994a,b; Hanus et al., 2009). However, the subfamily Prorhinotermitinae displays another combination of glands and compounds, similar to the situation in Termitidae (Bordereau et al., 2002, 2010; Sillam-Dussès et al., 2010): the trail pheromone consists of the diterpene neocembrine (major component) and dodecatrienol (Sillam-Dussès et al., 2009); the sex pheromone is once again dodecatrienol, but it is secreted by female tergal glands (Hanus et al., 2009). This striking singularity of Prorhinotermites promoted our interest in the chemistry of sex and trail pheromones in other basal rhinotermitid genera, namely Psammotermes, which is suggested to be a sister clade to Prorhinotermites in several recent phylogenies (Austin et al., 2004; Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007).

Species in the genus Psammotermes, Desneux, 1902, which are important timber pests, inhabit arid areas of the Old World, namely Sahara and Arabia (P. hybostoma Desneux, 1902), South Africa (P. allocerus Silvestri, 1908), Madagascar (P. voeltzkowi Wasmann, 1910), and South-West Asia (P. rajasthanicus Roonwal & Bose, 1960). However, the most basic aspects of Psammotermes biology remain unknown, such as the caste system (only a pronounced size polymorphism of workers and soldiers has been noted; Clément, 1952; Roonwal, 1988) or the chemistry of the defensive frontal gland of soldiers. Together with Stylotermitinae and Termotectorinae, Psammotermites is among the last rhinotermitid subfamilies that have not yet been studied with respect to the trail and sex pheromones. In this study, we report the results of our investigations on the source and chemical identity of the trail pheromone and the sex pheromone in the sand termite Psammotermites hybostoma.

Methods and Materials

Insects Large portions of Psammotermites hybostoma colonies were collected from tamarisk wood at the periphery of Sohag, Egypt and brought to Prague, Czech Republic. The colonies were held under laboratory conditions in their original wood at 26°C and a low relative humidity. Some of the colonies contained alate imagos of both sexes waiting for their dispersal flight. These were kept in the colonies and used in the study of the sex pheromone. Males were eventually dealated prior to behavioral experiments. The readiness of males to respond to female sex pheromone has been tested by observation of tandem behavior. Within the polymorphic worker caste, the “large workers” represented a minority of individuals, their number being insufficient for the purpose of this study. Moreover, Clément (1952) questioned the participation of “large workers” in colony tasks. Therefore, only individuals that could be classified as “minor workers” were included in trail pheromone analysis.

Histology of Exocrine Glands Semithin resin sections were prepared with male and female dealate imagos and with workers (see Šobotník et al. (2010) for details of fixation procedure). Sections (1 μm) were cut using an Ultracut Reichert-Jung, stained with Azur II solution, and studied using a Carl Zeiss Amplitude optical microscope combined with a Canon EOS 500D camera.

Pheromone Standard (3Z,6Z,8E)-Dodeca-3,6,8-trien-1-ol (dodecatrienol) was kindly supplied by Christian Bordereau (Dijon, France).

Glandular Extracts The following hexane extracts were prepared after dissection of females and workers: 250 sternal glands of workers, 500 sternal glands and 500 tergal glands of female imagos, 500 female bodies devoid of the sternal and tergal glands. Extracts were diluted with hexane to two concentrations, i.e., 1 and 10 gland equivalents per 10 μl. These extracts were used in the sex attraction bioassays, and trail-following bioassays. Concentrated glandular extracts were used for electrophysiological experiments and gas chromatography (GC).
Electrophysiological Experiments Electroantennographic bioassays (EAG) and gas chromatography coupled with electroantennographic detection (GC-EAD) were performed with antennae of males and workers as described in Sillam-Dussès et al. (2009). Concentrated extracts of sternal and tergal glands of female imagos, concentrated extracts of sternal glands of workers, and a solution of a standard of dodecatrienol were used in GC-EAD. The applied solutions were co-eluted with a series of n-alkanes (C8-C22) in order to calculate the linear retention indices (LRI) of particular compounds. A DB-5 column (30 m×20.25 mm i.d., 0.25 μm film; J&W Scientific, Folsom, CA, USA) was used, programmed from 40°C (2 min) to 270°C (10 min) at 50°C/min. Injector and detector temperatures were 220°C and 250°C, respectively. Glandular extracts and dodecatrienol (0.1–100 pg per stimulation) were tested in EAG, with hexane and air being used as controls.

Gas Chromatography The extract of tergal and sternal glands of females, and sternal glands of workers were analyzed by two-dimensional GC with mass spectrometric time of flight detection (GC × GC/TOF-MS, Pegasus 3D, Leco, St. Joseph, MI, USA). A nonpolar DB-5 column as above was used in the first column, and a polar BPX-50 column (2 m×0.1 mm i.d., 0.1 μm film; SGE, Melbourne, Australia) was used as the second column. The first GC was programmed from 40°C (1 min) to 320°C at 7°C/min, and the second column was set at 340°C. 1 μl Samples were injected in a splitless mode; injector temperature was 250°C; and helium (1.0 ml/min) was used as carrier gas. Separation time in the second dimension was 4 sec with a 0.6 sec hot pulse. Detector conditions: The EI source for the TOF-MS operated at temperature 220°C using 70 eV ionization, and an acquisition rate 50 spectra/s.

Sex Attraction Choice Bioassays Two dealate male imagos were introduced at the same time in a 15 cm Petri dish lined with Whatman No. 1 filter paper. The dish contained two folded pieces of filter paper (1 cm²), treated with 10 μl of hexane extract of female bodies (devoid of sternal and tergal glands) or sternal and/or tergal glands extracts of females (always 1 female equivalent per paper) or various concentrations of dodecatrienol standard or 10 μl of hexane as control. Two males were used because a single male deprived of tactile communication displays an unstable behavior. The time spent by one or two males in contact with particular pieces of paper was measured during 5 min. Results obtained from 15 replicates for each treatment combination were statistically analyzed by using Mann-Whitney U test.

Trail-following Choice Bioassays Trail-following bioassays were performed to test the orientation activity of hexane extracts of sternal glands. Sternal gland extracts of females were used in tests with males, worker sternal gland extracts in tests with workers, and dodecatrienol standard solutions. Solutions were tested with a ‘Y open field’ bioassay on Whatman No.1 filter paper discs (15 cm diam), with a 120° angle between each branch. On the stem (3 cm) and one of the branches (7 cm), an artificial trail was drawn with a syringe containing 10 μl of sternal gland extract (1 μl/cm of the trail, i.e., 0.1 or 1 gland equivalent per cm of the trail). Ten μl of hexane were deposited on the stem and the other branch of the Y. One termite was placed inside a holding chamber (55 mm Petri dish), with a 2-mm opening located at the base of the Y. The distance travelled by the insect on the trail was measured. The activity threshold was defined as the minimum concentration that elicited termites to travel a mean distance of more than 3 cm, the maximum response being 10 cm. Each experiment was repeated with 15 or 30 individual termites. Choice tests were performed with the same experimental setup to compare the preference of termites between gland extracts and dodecatrienol. Each of the compared stimuli was deposited on the stem and on one of the Y branches of the experimental arena. This test was repeated with 30 individual termites, and results were evaluated using χ²-test.

Results

Origin of the Female Sex Pheromone – Microscopy Semi-thin sections of P. hybostoma female imagos revealed the presence of three well developed tergal glands in the anterior parts of the 8th, 9th, and 10th tergites, and of one sternal gland in the anterior part of the 5th sternite (Fig. 1). Tergal glands consist of two common cell types, and these are probably class 1 and class 3 secretory cells (sensu Noirot and Quennedey, 1974, 1991). Tergal glands are between 130 and 180 μm long, and up to 50 μm thick. We did not observe the tergal glands in P. hybostoma males. The sternal gland of imagos is about 250 μm long and 35 μm thick, and its external reservoir is quite large (see Fig. 1).

Origin of the Female Sex Pheromone – Behavior Calling behavior was not observed in either sex in the field or the laboratory, but tandems (a female followed by a male) were formed immediately after encountering the opposite sex (Fig. 2). In sex attraction bioassays, males preferred the mixed extract of sternal and tergal glands of females to the extracts of whole female body devoid of these glands (63±16 sec vs. 3±1 sec, P<10⁻⁵). Males clearly preferred the extract of sternal glands and, even more, the extract of tergal glands of females to hexane controls (Table 1). No significant preference was observed when the extract of
tergal glands and the mixed extract of tergal and sternal glands were exposed to the males (72±18 sec vs. 54±9 sec, N=15, P=0.56). In addition, trail-following bioassays showed that males were able to consistently follow the trails made with sternal gland extract of females (Table 2). All these results indicate that the components of the female sex pheromone are located in both the sternal gland and the tergal glands of female imagos.

**Chemical Nature of the Female Sex Pheromone**

In GC-EAD experiments, injections of extracts of 6 female tergal glands elicited a single significant and reproducible major anteninal response in males to a compound eluting at 7.21 min (Fig. 3a). Co-injection of the extract with a series of hydrocarbons revealed that the LRI of the putative active compound was approximately 1525. The retention index was characteristic for dodecatrienol. Indeed, the injection of 10 ng of dodecatrienol resulted in a clear FID peak at the same retention time, and a corresponding antennal response (Fig. 3c). Subsequently, the presence of dodecatrienol in the tergal glands was confirmed by an analysis of the extract (25 gland equivalents) by GC × GC/TOF-MS.

The extract of female sternal glands elicited a single antennal response of males at the retention time identical to that observed for the tergal gland extract and dodecatrienol standard (Fig. 3b). Analysis of this extract (25 gland equivalents) by GC × GC/TOF-MS confirmed the presence of the dodecatrienol in the sample. We concluded that dodecatrienol is a component of the female sex pheromone of *P. hybostoma*, secreted by sternal and tergal glands.

**Biological Activity of Dodecatrienol**

Sex attraction bioassays showed that males are highly attracted to dodecatrienol at 10 pg per paper and higher (Table 1). In the
choice test, males preferred dodecatrienol at 100 pg/paper and higher to tergal glands extract; this indicates that approximately 10 pg of dodecatrienol are present in the tergal glands of one female (Table 1).

In trail-following bioassays, the trail-following activity of males was elicited by dodecatrienol at a concentration 10 pg/cm and higher (Table 2). In the choice trail-following test, males preferred to follow the trail made with dodecatrienol at 100 pg/cm and higher to the extracts of sternal gland (1 gland equivalent per cm). This indicates that between 10 and 100 pg of dodecatrienol are present in the sternal gland of one female (Table 3).

**Quantification of the Female Sex Pheromone** In addition to the sex attraction and trail-following bioassays (see above), the dose-response relationship of the EAG response to dodecatrienol standard solutions compared to glandular extracts was used to estimate the quantity of dodecatrienol in sternal and tergal glands of females. EAG amplitude increased with increasing concentrations of dodecatrienol; dodecatrienol was estimated to be approximately 1 pg in the sternal gland, and 10 pg in tergal glands of one female (Fig. 4). The results for the two methods used are summarized and compared in Table 5.

**Origin of the Trail Pheromone of Workers – Microscopy** The presence of a single sternal gland located at the anterior part of the 5th sternite of worker was confirmed, as in all Rhinotermitidae studied so far (Fig. 1). The gland is approximately 170 μm long and 50 μm thick. It does not differ in overall size from the gland of female imagos, but its shape is bilobed, as in workers of other rhinotermitids (see Ampion and Quennedey, 1981).

**Origin of the Trail Pheromone of Workers – Behavior** - Workers followed trails made of the sternal gland extract of workers at both concentrations tested (5.1±1 cm at 10^{-1} gland equivalent/cm, N=15; 6.9±0.7 cm at 1 gland equivalent/cm).

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**Table 1** Sex attraction bioassays with dodecatrienol or female gland extracts

<table>
<thead>
<tr>
<th>Hexane</th>
<th>Tergal gland extract</th>
<th>Sternal gland extract</th>
<th>Dodecatrienol (pg/paper)</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 TGE/paper</td>
<td>1 SGE/paper</td>
<td>1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>13±3</td>
<td>110±22</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16±3</td>
<td>–</td>
<td>58±17</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>9±2</td>
<td>–</td>
<td>14±3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6±1</td>
<td>–</td>
<td>–</td>
<td>39±5</td>
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<td>–</td>
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<tr>
<td>6±3</td>
<td>–</td>
<td>–</td>
<td>68±16</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6±1</td>
<td>–</td>
<td>–</td>
<td>43±9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8±1</td>
<td>–</td>
<td>110±13</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8±1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>154±23</td>
<td>15</td>
</tr>
<tr>
<td>–</td>
<td>57±21</td>
<td>–</td>
<td>24±4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>11±4</td>
<td>–</td>
<td>47±17</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>15±4</td>
<td>–</td>
<td>80±18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>17±3</td>
<td>–</td>
<td>75±11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>9±2</td>
<td>–</td>
<td>–</td>
<td>92±14</td>
<td>15</td>
</tr>
</tbody>
</table>

a The time spent by two dealate male imagos on papers impregnated with hexane, female tergal gland extract, female sternal gland extract, or dodecatrienol at 6 different concentrations was measured (mean in sec ± S.E.M.). TGE – tergal gland equivalent, SGE – sternal gland equivalent, N – number of repetitions, P value (Mann-Whitney U test), NS – non-significant.

**Table 2** Trail-following bioassays with dodecatrienol or female sternal gland extracts

<table>
<thead>
<tr>
<th>Hexane</th>
<th>Sternal gland extract (SGE/cm)</th>
<th>Dodecatrienol (pg/cm)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-1}</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>1.9±0.6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>5.7±0.85</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>–</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a The distance travelled by one dealate male imago on trails made of sternal gland extract or dodecatrienol was measured (mean in cm ± S.E.M.). SGE – sternal gland equivalent, N – number of repetitions.
Therefore, we concluded that the trail pheromone of workers is secreted by their sternal gland.

**Chemical Nature of the Trail Pheromone of Workers** Injection of concentrated extract of sternal glands from 5 workers into the GC-EAD setup resulted in a single antennal response of workers, matching the retention characteristics of dodecatrienol as described above (Fig. 3d, g). The workers responded identically to the extracts of the female sternal and tergal glands, suggesting the presence of the same active component, dodecatrienol, in the glands (Fig. 3e, f). In addition, workers followed trails made of the tergal gland extract of female imagos (10⁻¹ female tergal gland equivalent per cm), which confirms the identity of the female sex pheromone and the worker trail pheromone. The presence of dodecatrienol in the sternal gland extract of workers was confirmed using GC × GC/TOF-MS (50 gland equivalents injected). Thus, we concluded that dodecatrienol is a component of the trail pheromone of *P. hybostoma* workers.

**Table 3** Trail-following choice bioassays to female sternal gland extract and dodecatrienol

<table>
<thead>
<tr>
<th>Sternal gland extract</th>
<th>Dodecatrienol (pg/cm)</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SGE/cm</td>
<td>10</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

Values indicate the number of choices made by one dealate male imago when given a choice between a trail made of female sternal gland extract or dodecatrienol. SGE = sternal gland equivalent, N = number of repetitions, P value (χ²-test).

Fig. 3 Electroantennographic recordings of male (left) and worker (right) antennae exposed to the extracts of female tergal and sternal glands, worker sternal glands, and dodecatrienol standard solution. Upper curve (blue) – FID, lower curve (black) – EAD. Note the antennal response of both, males and workers, at the retention time of 7.21 in all experiments. In c and f, the tested solutions are co-eluted with *n*-alkane standards (C8–22). Y scale (mV per division): a – EAD=0.1, FID=4; b – EAD=1, FID=3; c – EAD=1, FID=25; d – EAD=1, FID=5; e – EAD=0.1, FID=4; f – EAD=0.5, FID=4; g – EAD=1, FID=10.

Fig. 4 EAG responses of males to air control, glandular extracts of females, and various concentrations of dodecatrienol (mean ± S.E.M.). Data were normalized to hexane stimulations and log transformed to reduce heteroscedasticity. Data normality was controlled using Levene’s test (P=0.09). Bars marked with different letters indicate significant differences (P<0.05) among treatments, calculated by means of ANOVA (F(4,8,16.83)=74.5, P<10⁻⁴) with subsequent post-hoc comparison using HSD for unequal N. Numbers at the base of each bar represent the number of observations for each treatment. SGE = sternal gland equivalent; TGE = tergal gland equivalent.
Biological Activity of Dodecatrienol

Dodecatrienol was highly active in eliciting the trail-following of workers; the activity threshold being 1 pg/cm, with maximum activity observed at 10 pg/cm. A decrease in the trail-following activity was observed at 100 pg/cm and higher (Table 4). In the choice trail-following test, workers preferred dodecatrienol at a concentration 100 pg/cm to sternal gland extract (1 gland equivalent per cm), which suggests that approximately 10 pg are present in one gland.

Quantification of the Trail Pheromone of Workers

In addition to the trail-following choice bioassays (see above), the EAG dose-response experiments were performed to estimate the quantity of dodecatrienol in sternal glands of workers. The threshold activity was observed at 1 pg of dodecatrienol; the quantity of dodecatrienol in the sternal gland of one worker was estimated to be between 1 and 10 pg (Fig. 5). The results of the two approaches are compared in Table 5.

Discussion

Our study demonstrates that (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol (dodecatrienol) is the major component of the sex pheromone of female imagos and of the trail pheromone of workers in the sand termite Psammotermes hybostoma. The female sex pheromone is produced by the tergal glands and the sternal gland, and the trail pheromone is secreted by the sternal gland of workers. The identity of the two pheromones has been studied and confirmed using a combination of behavioral bioassays, electrophysiology, and chemical analysis.

For the first time, a termite sex pheromone secreted by two different glandular sources, the tergal glands and the sternal gland, has been chemically characterized. Previous studies implicated multiple glands in termite sexual attraction, such as the sternal gland and the tergal glands in Trinervitermes bettonianus (Termiidae: Nasutitermitinae) (Leuthold, 1975, 1977) and Kalotermites flavicollis (Kalotermitidae) (Wall, 1971), or the tergal and the posterior sternal glands in Macrotermes annandalei and M. barneyi (Termitidae: Macrotermitinae) (Peppuy et al., 2004), but the nature of the sex pheromones has not been identified in these species. The participation of the female tergal glands in the sexual attraction was obvious from their occurrence in histological sections; the female imagos being the only caste and sex with these glands present (see also Ampion and Quennedey, 1981). The role of the sternal gland and its secretion was less obvious but also likely, based on their histology: the gland is smaller than in Reticulitermes (Rhinotermitidae: Heterotermitinae), in which it is the sole source of the sex pheromone, but at the same time it is significantly larger than in the related Prorhinotermes, in which it does not participate in the sexual attraction and is vestigial (Šobotnik and Hubert, 2003; Hanus et al., 2009).

Table 4 Trail-following bioassays with dodecatrienol

<table>
<thead>
<tr>
<th>Dodecatrienol (pg/cm)</th>
<th>10⁻¹</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1±0.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>30</td>
</tr>
<tr>
<td>3.8±0.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>30</td>
</tr>
<tr>
<td>5.8±0.6</td>
<td>–</td>
<td>–</td>
<td>3.4±0.6</td>
<td>–</td>
<td>30</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.9±0.5</td>
<td>–</td>
<td>30</td>
</tr>
</tbody>
</table>

The distance travelled by one worker on trails made of dodecatrienol is measured. Another trail, made of hexane, is used as a control and is never followed. N = number of repetitions.

Table 5 Comparison of the quantity of dodecatrienol in exocrine glands involved in sex attraction and trail following based on EAG experiments and behavioral bioassays

<table>
<thead>
<tr>
<th>Caste</th>
<th>Gland</th>
<th>Dodecatrienol (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EAG</td>
</tr>
<tr>
<td>Female imago</td>
<td>Tergal</td>
<td>10</td>
</tr>
<tr>
<td>Female imago</td>
<td>Sternal</td>
<td>1</td>
</tr>
<tr>
<td>Worker</td>
<td>Sternal</td>
<td>1–10</td>
</tr>
</tbody>
</table>
What would be the purpose of having two types of glands secreting the same compound for sexual attraction? Courtship in termites usually consists of calling behavior and tandem behavior, both associated with secretion of a sex pheromone. In Psammotermes, we did not observe the typical calling posture of females with the tergal glands exposed (see e.g., Hanus et al., 2009). However, the readiness of females to call might have been affected by the laboratory conditions. Alternatively, the females can secrete their sex pheromone without the characteristic calling posture. Nevertheless, the males are attracted by the females, and immediately after an encounter they form the typical tandem following the female tergal region with their heads and antennae. Therefore, we presume that females use their tergal glands to attract males at a short distance and to ensure the tandem cohesion, while they lay an odor trail with their sternal gland that prevents an accidental tandem separation, as described in other termite species (Nutting, 1969; Bordereau and Pasteels, 2011). Thus, interestingly, the same compound, dodecatrienol, would be used as a short-range airborne sex pheromone and as a trail pheromone. Nevertheless, although dodecatrienol was the only active compound identified, we cannot exclude that other, undetected compounds are involved.

Dodecatrienol has been confirmed as the major or only component of the sex pheromone in numerous termite species; it is produced by the tergal glands in Syntermitinae (Bordereau et al., 2002), and by the sternal gland in the macrotermite Pseudacanthotermes (Bordereau et al., 1991) and in the rhinotermid Reticulitermes (Laduguie et al., 1994a). In Prorhinotermes, probably the closest relative of Psammotermes, we also have recently ascribed the function of sex pheromone to dodecatrienol secreted from the tergal glands of females (but not from the vestigial sternal gland), as evidenced by bioassays and GC-EAD experiments (Hanus et al., 2009). However, we were not able to detect the compound using GC-MS, probably due to its very low concentrations in the tergal glands (units of picograms). In Psammotermes females, the content of the compound in the sternal gland and the tergal glands, and its active concentrations, also are very low (picograms) (see Table 5). Such low concentrations of dodecatrienol also have been observed in Reticulitermes (Laduguie et al., 1994a), and contrast with usually high concentrations of sex pheromones in other species (see Bordereau and Pasteels, 2011).

We also have demonstrated that dodecatrienol, secreted from the sternal gland of workers, is the trail pheromone used to mark foraging trails. Even though we cannot exclude the presence of multiple components, we observed no GC-EAD evidence of other compounds being involved in trail following. This finding is not surprising given that the sternal gland is a universal source of trail pheromones in termites, and dodecatrienol is used as a trail pheromone in all Rhinotermitidae studied (Sillam-Dussès et al., 2009; Bordereau and Pasteels, 2011). The sternal gland of workers does not differ significantly in size from the sternal gland of females, but it differs in having a bilobed shape, as in workers of other rhinotermitids (Ampion and Quennedey, 1981). The estimated quantity of dodecatrienol in one gland (1–10 pg), and the estimated activity threshold (1 pg/cm), correspond to values observed in some Reticulitermes species (see Bordereau and Pasteels, 2011).

The activity threshold appears to be only slightly higher in females than in workers, as well as the content of dodecatrienol in the corresponding glands (Table 5). This is, once again, a similarity with Reticulitermes, i.e., R. santonensis (Laduguie et al., 1994a,b; Wobst et al., 1999). On the other hand, it contrasts with the dramatic differences between the amounts of dodecatrienol used by workers and females in the termitids Pseudacanthotermes spiniger (Bordereau et al., 1991, 1993) and Cornitermes bequaerti (Bordereau et al., 2002).

In summary, the chemistry of trail-following and sex attraction in Psammotermes, with dodecatrienol being used for both purposes, differs little from that of other subterranean rhinotermitids, despite the extremely different climatic conditions in which Psammotermes lives. Probably the most obvious adaptation to arid environment is the unprecedented speed of motion of both workers and imagos, probably to minimize the time spent in hot and arid areas during foraging and imaginal dispersal; their velocity is comparable to that of open-air foraging termites (pers. obs.). Interestingly, workers of Psammotermes do not easily follow their own sternal gland extracts. The orientation effect of the trail pheromone is weak in Psammotermes, while this is not the case in all other Rhinotermitidae studied, Prorhinotermes included. This weak orientation effect may be the due to the unprecedented speed of the individuals tested.

The surprising constancy of pheromonal signaling with ecological conditions has already been noted in distant taxa sharing the same pheromones, such as Kalotermitidae and Macrotermitinae (see Bordereau and Pasteels, 2011). The only singularity of Psammotermes within Rhinotermitidae is the production of the sex pheromone by both the sternal and the tergal glands of female imagos. On the other hand, the genus Prorhinotermes, supposedly a close relative of Psammotermes (Austin et al., 2004; Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007), remains the sole genus of Rhinotermitidae with a diterpene (neocembrene) used as the major component of the trail pheromone, in addition to the minor component dodecatrienol (Sillam-Dussès et al., 2005, 2009).

Our findings underline the conservative nature of chemical communication in termites, with dodecatrienol being the most frequent component of pheromonal signals in trail-following and sex attraction in Rhinotermitidae and Termitidae. At the same time, they highlight the tight evolutionary relationship
between the trail following of working castes and the sex attraction of imagos, the ancestral life forms of termites; the former has supposedly developed from the latter during the social evolution of Isoptera (Traniello and Leuthold, 2000, Bordereau and Pasteels, 2011).

Acknowledgments The authors are grateful to Jarmila Titzenthalenová for technical help in experiments. This research was supported by the Czech Science Foundation (No. P506/10/1570) and by the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague (project No. Z40550506). We are grateful to two anonymous reviewers for comments on the manuscript.

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Z,E)-3,6,8-dodecatrien-1-ol, a major component of trail-
following pheromone in two sympatric termite species Reticuliter-
Chemical communication in termites: syn-4,6-dimethylundecan-1-ol as trail-following pheromone, syn-4,6-dimethylundecanal and (5E)-2,6,10-trimethylundeca-5,9-dienal as the respective male and female sex pheromones in Hodotermopsis sjoestedti (Isoptera, Archotermopsidae)

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1. Introduction

Recent reviews have highlighted the conserved nature of the pheromones involved in ecology of foraging and dispersal in termites (Bordereau and Pasteels, 2011; Pasteels and Bordereau, 1995). Nevertheless, a clear division is observed between the basal termites (Bordereau and Pasteels, 2011; Pasteels and Bordereau, 2011) and, in contrast, a clear difference between the pheromones of the taxa Hodotermopsis and Zootermopsis and, in contrast, a clear difference between the pheromones of the taxa Hodotermopsis and Zootermopsis and, in contrast, a clear difference between the pheromones of the taxa Hodotermopsis and Zootermopsis...
Archotermopsidae (Bordereau et al., 2010; Sillam-Dussès et al., 2007). The Archotermopsidae Zootermopsis are the only termites where sex-specific pheromones have been demonstrated (Pastoels, 1972; Stuart, 1975). The Archotermopsidae well illustrate the pheromonal parsimony of termites, i.e. the secretion of multifunctional pheromones. For example, in Z. nevadensis, syn-4,6-dimethyl-7-dodecanal is used both as a male sex pheromone and a trail-following pheromone (Bordereau et al., 2010).

Data reported on pheromones of the basal termites are limited to a very low number of taxa (Bordereau and Pasteels, 2011). It appeared interesting to investigate another representative of the new family Archotermopsidae, the Asian termite Hodotermopsis sjoes-tedti, to compare it to Z. nevadensis. Here we report our results obtained on its trail-following pheromone and sex-pairing pheromones.

2. Material and methods

2.1. Insects and rearing

Hodotermopsis has an Indo-Malayan distribution. This damp-wood termite is distributed throughout East Asia, from the Sats-urnan Islands in Japan to Northern Vietnam (Matsumoto et al., 1990). For a long time, two species were recognized, Hodotermopsis sjoes-tedti Holmgren (1911) in Vietnam and H. japonicus Holmgren (1912) in Japan (see Snyder, 1949). However, H. sjoes-tedti was later recognized as a synonym of H. japonicus (Takematsu, 1996; Huang et al., 2000).

Termites for our study were collected in Tam Dao national park (Vietnam) located at 80 km north from Hanoi. In this park, H. sjoes-tedti causes severe damage to pine trees in which it forms populous colonies of a few thousands individuals of large size. H. sjoes-tedti is of the 'one piece nest' ecological type (Abe, 1987). However, this termite is able to use, at least under rearing conditions, shelter tubes to move from one piece of wood to another (Bordereau and Pasteels, 2011). Pieces of wood containing termites (pseudergates, soldiers, nymphs, alates and possibly neotenics) were accumulated on the fibre before thermal desorption in a gas chromatographic injector. Ten to twenty alates were used per analysis for sex pheromones, 20–50 pseudergates for trail-following pheromone. The analyses were repeated three times for dea-lates, five times for pseudergates.

2.3. Chemical analyses

Pheromones were extracted with the solid phase microextraction (SPME) technique. Sternal glands of cold anaeasthetized termites were rubbed under a stereomicroscope with a microtitre syringe containing 1 μl of extract or solvent per cm of trail. The dis-tance travelled by insects was measured; the activity threshold for an extract being defined as the minimum concentration eliciting termites to travel a mean distance of more than 3 cm (ii) in a ‘T–maze’ guided situation, giving a choice between two branches of a T tunnel formed in a perspex block set on a Whatman No.1 filter paper. Trails were 10 cm long in both situations, the Y stem and the T base were 3 cm long, the branches 7 cm long. One termite was placed inside a holding chamber consisting of a small plastic vial. Fifteen to thirty replicates of each bioassay were run. Each termite and each trail were used only once to prevent any effects from behavioural conditioning or trail reinforcement. Results were ana-lyzed with a χ² test for ‘T-maze’ bioassays.

Sex attraction bioassays could not be carried out as alates were no longer available when sex-specific compounds of sternal glands of alates were identified.

2.4. Synthesis and characterization of standards

NMIR spectra were determined in CDCls solutions on a Bruker Avance 500 MHz spectrometer operating at 499.5 MHz for ¹H. Chemical shifts are expressed in δ (ppm) scale relative to tetra-methylsilane. High-resolution MS (EI) data were obtained using a Waters GCT Premier GC–MS/TOF system.

(5E) and (5Z) isomers of 2,6,10-trimethylundeca-5,9-dienal were synthesized by the Darzens condensation of ethyl chloroacetate with geranylacetone (Fluka) and nerylacetone (Fluka) respectively, followed by hydrolysis of the intermediate ester and decarbonylation (Kulesza and Gora, 1969). 4,8-Dimethylundecan-1-ol (I) was prepared analogously to the procedure described in detail for 4,6-dimethylhexadecan-1-ol by Gostin et al. (2011). The key step was the Wittig reaction between 4-methyl-5-oxopentanoic acid being synthesized from commercial hexyltriphenylphosphonium bromide (Bondinell et al., 1968) followed by subsequent hydrogenation and reduction steps (Fig. 1).
4,6-Dimethylundecan-1-ol (2) was prepared from 4,6-dimethylundecan-1-ol by a standard pyridinium chlorochromate (PCC) oxidation (Corey and Suggs, 1975). 4,6-Dimethyldecanal was prepared as described in Ghosh et al., 2011.

2.4.1. (E,Z)-Methyl 4,6-dimethylundec-5-enoate (4)

A suspension of iodide 3 (2.44 g; 5 mmol) in anhydrous tetrahydrofuran (15 mL) was added to n-butyllithium in hexane (2.4 mL, 1.4 M, 5.5 mmol) and the resulting mixture was stirred at 20 °C for 2 h. Then, a solution of methyl 4-methyl-5-oxo-pentanoate (1.4 g, 10 mmol) in anhydrous tetrahydrofuran (2 mL) was added at −78 °C. The reaction mixture was stirred 1 h at −78 °C and 24 h at 20 °C. Pentane was added, the precipitate removed from the mixture by filtration and solvents were evaporated. Purification of the residue by flash-chromatography (Merck Kiesegel 60; hexane/Et 2O, 98:2) afforded the ester 4 (605 mg, 2.67 mmol, 53%) as a colourless oil. 1H-NMR: δ 7.87 (d, J = 9.44 Hz, 1H), 3.65 (s, 3H), 2.34–2.35 (m, 3H), 1.97 (m, 2H), 1.80 (d, J = 1.25 Hz, H-C=6 E or Z), 1.70 (m, 2H), 1.29–1.33 (m, 6 H), 0.98 (d, J = 6.7 Hz, H-C=4), 0.90 (t, J = 6.8 Hz, 3H).

2.4.2. Methyl 4,6-dimethylundecanoate (5)

Methyl 4,6-dimethylundec-5-enoate (4) (226 mg, 1.00 mmol) in anhydrous methanol (5 mL) was hydrogenated 18 h on Pd/C (10%, 4.5 mg) under hydrogen atmosphere at 20 °C. The solvent was evaporated in vacuo and purification of the residue with flash-chromatography (Merck Kiesegel 60; hexane/Et 2O, 98:2) afforded the saturated compound 5 (220 mg, 0.97 mmol, 97%). 1H-NMR: δ 3.64 (s, 3H), 2.27–2.35 (m, 2H), 1.88 (m, 2H), 1.64 (m, 2H), 1.21–1.32 (m, 10 H), 0.94–0.97 (m, 5H), 0.88 (t, J = 6.8 Hz, 3H).

2.4.3. 4,6-Dimethylundecan-1-ol (1)

Ester 5 (114 mg, 0.50 mmol) in anhydrous diethyl ether (2 mL) was added at 0 °C to suspension of Li[AlH₄] (160 mg, 4.21 mmol) in anhydrous diethyl ether (3 mL). After stirring for 1.5 h at 0 °C, the reaction was quenched with an ice-cold brine and the aqueous layer extracted with diethyl ether. The ethereal extracts were combined, evaporated in vacuo and purified by flash-chromatography (Merck Kiesegel 60; hexane/ethylacetate 9:1) to yield alcohol 1 (90 mg, 0.45 mmol, 90%) as a colourless oil. 1H-NMR: δ 3.82 (t, J = 6.8 Hz, 2H), 1.69 (m, 2H), 1.18–1.52 (m, 14H), 0.94–0.96 (m, 6H), 0.87 (t, J = 6.7 Hz, 3H). EIMS, m/z [%]: M⁺ not present, 199, 154 (12), 139 (32), 111 (80), 98 (74), 83 (44), 69 (100), 57 (61), 41 (21). HRMS: for C₁₃H₂₆O [M+1]⁺ calculated 199.2062; found 199.2058.

2.4.4. 4,6-Dimethylundecan-2-ol (2)

Alcohol 5 (20 mg, 0.10 mmol) in anhydrous dichloromethane (200 µL) was added at 0 °C to suspension of PCC (215 mg, 1 mmol) and NaOAc (21 mg, 0.25 mmol) in anhydrous dichloromethane (1 mL). After stirring for 30 min at 0 °C, the reaction mixture was diluted by Et₂O (5 mL) and filtered through a small plug (in Pasteur pipette) from layers of neutral alumina, charcoal and cellulite. The colourless filtrate was evaporated in vacuo and purified by flash-chromatography (Merck Kiesegel 60; hexane/Et₂O 98:2) to yield aldehyde 2 (15 mg, 0.076 mmol, 76%) as a colourless oil. 1H-NMR: δ 9.72 (t, 1H), 2.38–2.42 (m, 2H), 1.68 (m, 2H), 1.19–1.56 (m, 14H), 0.94–0.97 (m, 6H), 0.87 (t, J = 6.7 Hz, 3H). EIMS m/z (%): 198, 139 (47), 127 (33), 109 (82), 98 (100), 83 (72), 69 (37), 57 (92), 43 (27). HRMS: for C₁₃H₂₅O calculated 198.1984; found 198.1979.

3. Results

3.1. Pseudergates and the trail-following pheromone

For pseudergates of Hodotermopsis collected in Vietnam, the GC profiles after SPME of the sternal gland cuticle and the abdominal tergal cuticle showed common cuticular hydrocarbons from C₁₅ to C₂₀ and a compound specific to the sternal gland surface (Fig. 2). This compound had a LRI of 1937 on a polar column (DB™-Wax). The most abundant molecular parent ion in the PCI (methane) mass spectrum was that of m/z 199, but the most abundant molecular parent ion in the PCI (ammonia) mass spectrum was that of m/z 218, which demonstrated that the molecular mass of the compound was actually 200. The EI mass spectrum (Fig. 3) showed that the aliphatic chain was branched and suggested the presence of a primary alcohol group (m/z 31). GC-FTIR confirmed a saturated alcohol (absence of a stretching band characteristic of ethylenic bonds).
unsaturation and presence of a band characteristic of a primary alcohol group. The EI fragmentation pattern showed a number of features common to those of dimethylalkan-1-ols, especially to 4,6-dimethylundecan-1-ol which had been previously synthesized to elucidate the Zootermopsis pheromone (4,6-dimethylundecanal) (Bordereau et al., 2010). From the LRI and the EI mass spectral characteristics of 4,6-dimethylundecan-1-ol, the structure 4,6-dimethylundecan-1-ol was suggested for the specific compound of H. sjoestedti. This structure was then confirmed by synthesis. The LRI of the synthesized syn and anti diastereomers, 1935 and 1945, respectively on a DB™-Wax column, showed that the specific compound was the diastereomer syn-4,6-dimethylundecan-1-ol. The quantity of 4,6-dimethylundecan-1-ol secreted at the surface of the sternal gland was estimated at 1 ng per pseudergate.

In addition to the compound syn-4,6-dimethylundecan-1-ol, pseudergates of H. sjoestedti also secreted traces of 4,6-dimethylundecan-1-ol, the compound previously identified as the trail-following pheromone of Z. nevadensis (Bordereau et al., 2010). syn-4,6-Dimethylundecan-1-ol was also shown to be a major specific compound of the sternal gland surface of H. sjoestedti pseudergates collected in Japan. However, sesquiterpene hydrocarbons were also present in tiny amounts at the surface of the sternal gland of these Japanese Hodotermopsis. Cuticular hydrocarbon profiles appear to be similar in pseudergates from both geographic origins.

### 3.1.2. Biological activity

Trail-following bioassays showed that pseudergates of Hodotermopsis collected in Vietnam could equally follow trails made of sternal glands of pseudergates coming from Vietnam or Japan, and vice versa.

The biological activity of the compound 4,6-dimethylundecan-1-ol was first tested with ‘open field’ trail-following bioassays. Results are summarized in Table 1. Trails made with a mixture of syn + anti-4,6-dimethylundecan-1-ol were followed by pseudergates from 10⁻¹ ng/cm but the mean walked distance was moderate. With syn-4,6-dimethylundecan-1-ol, the activity threshold was obtained at 10⁻² ng/cm, and the average distance walked by termites was significantly higher, especially at 10⁻¹ ng/cm. In contrast, with the diastereomer anti-4,6-dimethylundecan-1-ol, the activity threshold was only obtained at 1 ng/cm, i.e. at a concentration 100 times higher than with the syn diastereomer.

Under experimental conditions of ‘T-maze’ trail-following bioassays, the diastereomer syn-4,6-dimethylundecan-1-ol was even 1000 times more active than the anti diastereomer. The compound syn-4,6-dimethylundecan-1-ol at 10⁻¹ ng/cm was as active as an extract of 10⁻¹ sternal gland indicating an approximative quantity of 1 ng pheromone per individual (Table 1).

‘Open field’ trail-following bioassays showed that the second specific compound of the sternal gland surface of H. sjoestedti pseudergates, the aldehyde syn-4,6-dimethylundecan-1-ol, was only moderately active at higher concentrations (Table 3.1). At lower concentrations, it did not elicit trail-following in ‘open field bioassays’ nor in ‘T-maze’ bioassays (Tables 3.1 and 3.2). A mixture of syn-4,6-dimethylundecan-1-ol and syn-4,6-dimethylundecanal at the respective concentrations of 10⁻² ng/cm and 10⁻⁴ ng/cm did not elicit a higher trail-following than syn-4,6-dimethylundecan-1-ol alone (respective mean walked distances: 8.1 ± 0.6 cm vs 8.6 ± 0.5 cm, n = 30).

### Table 1

**Open field** trail-following bioassays.

<table>
<thead>
<tr>
<th>Hexane</th>
<th>syn + anti-4,6-Dimethylundecan-1-ol (ng/cm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻¹</td>
<td>10⁻²</td>
<td>10⁻³</td>
</tr>
<tr>
<td>0</td>
<td>1.1 ± 0.3</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>2.0 ± 0.4</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>6.8 ± 0.7</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>6.6 ± 0.7</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>5.4 ± 0.8</td>
<td>–</td>
</tr>
</tbody>
</table>

### Table 2

**‘T-maze’** trail-following bioassays with 4,6-dimethylundecan-1-ol.

<table>
<thead>
<tr>
<th>Hexane</th>
<th>Sternal gland</th>
<th>syn-4,6-Dimethylundecan-1-ol</th>
<th>anti-4,6-Dimethylundecan-1-ol</th>
<th>n</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>30</td>
<td>S+++</td>
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<tr>
<td>13</td>
<td>–</td>
<td>17</td>
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<td>30</td>
<td>NS</td>
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<td>9</td>
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<td>–</td>
<td>30</td>
<td>S+++</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>12</td>
<td>–</td>
<td>30</td>
<td>NS</td>
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<td>–</td>
<td>–</td>
<td>12</td>
<td>–</td>
<td>30</td>
<td>NS</td>
</tr>
</tbody>
</table>
male alates collected in Vietnam revealed a major compound specific to the sternal gland surface. Two minor compounds secreted by the sternal gland of male alates were also secreted by the sternal gland of pseudergates. In alates collected in Japan, besides these compounds, the sternal gland of both male and female alates secreted sesquiterpene hydrocarbons (Figs. 4 and 5).

### 3.2. Male alates

A comparison of GC profiles of SPME extracts from the sternal gland surface and the abdominal tergal and intertergal surface of male alates from Japan. The arrows indicate the specific compounds of the sternal gland. The dotted arrow indicates a compound present in male alates (syn) and in pseudergates (p). The asterisks indicate isomers of farnesal.

Table 3.2

<table>
<thead>
<tr>
<th>Hexane</th>
<th>syn-4,6-Dimethylundecanal</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻¹</td>
<td>10⁻²</td>
<td>10⁻¹</td>
</tr>
<tr>
<td>0</td>
<td>1.7 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>0</td>
<td>1.0 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>0</td>
<td>1.0 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>0</td>
<td>1.0 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

### 3.2.1. Compounds common to male alates and pseudergates

These compounds have been identified by their LRIs and EI-MS characteristics as syn-4,6-dimethylundecanal and syn-4,6-dimethylundecan-1-ol.

### 3.2.2. Male sex-specific compound

This compound had a LRI of 1674 on a DB™-Wax polar column, and 1402 on an Equity 5 non-polar column. The FTIR spectrum showed a stretching vibration band νC=O at 1723 cm⁻¹ and a deformation vibration band δνCH at 2725 cm⁻¹, characteristic of an aldehyde group. No stretching band characteristic of ethylenic unsaturation could be observed. These data indicated that the compound was a saturated aldehyde. From the EI mass spectrum (Fig. 6) and PCI mass spectra, the molecular mass was determined as 198. The comparison of EI mass spectra of Zootermopsis (Bordereau et al., 2010) and Hodotermopsis male sex-specific compounds suggested the structure 4,6-dimethylundecanal for Hodotermopsis. In particular, the fragment ions of m/z 168 and 153 for Zootermopsis were shifted to m/z 154 and 139 for Hodotermopsis and with similar relative abundances. Alpha cleavage of the 4,5 bond for both Zootermopsis and Hodotermopsis aldehydes would generate the ion (CH₃–CH–CH₂–CH₂–CHO)⁺ of m/z 127 in each case. Subsequent water loss would lead to the fragment ion of m/z 109. Also, the low mass region below m/z 90 was very similar for Zootermopsis and Hodotermopsis. That the specific male compound of H. sjoestedti was indeed 4,6-dimethylundecanal was confirmed by synthesis. Comparison of the LRIs of the two diastereomers and the male specific compound indicated that the male compound was the diastereomer syn-4,6-dimethylundecanal (LRIs 1679 and 1399 on DB™-Wax polar and DB5 non-polar columns respectively for the synthetic compound).

### 3.3. Female alates

A comparison of GC profiles of SPME extracts from the sternal gland surface and the abdominal tergal and intertergal cuticular surface of female alates collected in Vietnam revealed a major compound and a few minor compounds specific to the sternal gland surface. The LRIs of the major compound were 1875 on a polar column (DB™-Wax), and 1520 on a non-polar column (Equity 5). The FTIR spectrum showed a stretching vibration band νC=O at 1723 cm⁻¹ and a deformation vibration band δνC–H at 2718 cm⁻¹.
characteristic of an aldehyde group. Ethylenic unsaturation was also indicated by the stretching band v_C=C at 1675 cm⁻¹. This FTIR spectrum was therefore very similar to that of the female sex-pairing pheromone of Zootermopsis (Bordereau et al., 2010). The molecular mass and the MS characteristics of the female specific compound of Hodotermopsis were also shown to be identical to those of the female specific compound of Zootermopsis (Bordereau et al., 2010). Thus, the specific compound of the female alates of H. sjoestedti was identified as (E)-4,6-dimethyldodecanal. This compound is also a new chemical structure in termites. We have not been able to test its biological activity on female alates. However, the fact that it is only secreted at the surface of the sternal gland of male alates and that its structure is close to that of the male sex-specific pheromone (syn-4,6-dimethylundecanal) of Z. nevadensis and Z. angusticollis (Bordereau et al., 2010) strongly suggests that it is the male sex-specific pheromone of H. sjoestedti. In female alates, the compound (5E)-2,6,10-trimethylundeca-5,9-dienal was only identified at the surface of their sternal gland. As for males, we could not test its biological activity on male alates, but the same compound has been previously identified as the female sex-specific pheromone of Zootermopsis. Very probably, the compound (5E)-2,6,10-trimethylundeca-5,9-dienal is also a key compound of the female sex specific pheromone of H. sjoestedti. However, it will be necessary to test the biological activity of the farnesal isomers secreted by the female sternal gland. Thus, our study highlights a clear chemical proximity in communication strategies for foraging and dispersal between the Indomalayan termite Hodotermopsis and the nearctic termite Zootermopsis, although these phylogenetically close taxa (Inward et al., 2007; Krishna, 1970; Legendre et al., 2008) have dissimilar developmental pathways for alate differentiation (Miura et al., 2004). This proximal position is reinforced by the secretion of syn-4,6-dimethylundecanal in pseudergates and male alates of both taxa, although the role of this compound remains obscure in H. sjoestedti.

With regard to the behavioural aspects, the presence of sex-specific compounds in alates of H. sjoestedti strongly suggests a reciprocal male–female attraction at the time of dispersal of alates for pairing, which has only been demonstrated until now in Z. nevadensis (Bordereau et al., 2010; Pasteels, 1972). It would be particularly interesting to study the post-flight behaviour of H. sjoestedti in comparison to that of Z. nevadensis, as different strategies of chemical communication seem to have been selected by the two species. Whereas in Zootermopsis, male alates secrete only one compound, syn-dimethylundecanal, as a trail-following pheromone and as a sex pheromone, male alates of Hodotermopsis appear to secrete two compounds, syn-4,6-dimethylundecanal-1-ol as a tail-following pheromone and syn-4,6-dimethylundecanal as a sex pheromone. The chemical communication of Hodotermopsis appears slightly less parsimonious than in Zootermopsis. In contrast, in both taxa, we note the surprising absence of trail-following pheromone in female alates. This could be due to a ‘stay and invite’ strategy by female alates and the absence of an actual nuptial promenade after pairing as has been observed in Zootermopsis (Bordereau et al., 2010; Shellman-Reeve, 1999).

The enantiomeric compositions of the pheromone components have not yet been determined. Nevertheless, our chemical study reveals interesting data with respect to the taxonomy and the classification of termites. Two species of Hodotermopsis, H. sjoestedti and H. japonicus, were recognized by Holmgren (1911, 1912), but Takematsu (1996) and Huang et al. (2000) considered that H. sjoestedti was a synonym of H. japonicus and that there was only one species of Hodotermopsis, H. sjoestedti. Our study principally supports this latter taxonomy. Firstly, we note the presence of similar cuticular hydrocarbons and the total absence of agonistic behaviour between pseudergates collected in Vietnam and in Japan. Secondly, artificial trails made by pseudergates of H. sjoestedti collected in Vietnam were precisely followed by termites collected in Japan and vice versa. Thirdly, alates collected in both areas secreted the same sex-specific compounds, but the trail-following pheromones and sex pheromones are often not species-specific.
in termites. More interesting, we could note some chemical differ-
ence at the surface of the sternal glands of termites from the two
geographic areas. As well as the common compounds, the pseudon-
gates and the alates of the Japanese Hodotermopsis secreted addi-
tional sesquiterpene hydrocarbons. These terpenes did not ap-
pear to be involved in trail-following activity and did not bring
species-specific responses in trail-following. Nevertheless, they
might represent a beginning of a geographic speciation.
In other respects, the trail-following pheromones of Hodotermop-
sis and Zootermopsis are chemically close, whereas they are clearly
different from those of Porotermes and Stolotermitidae (Sillam-Düss-
et al., 2007). This substantiates the creation by Engel et al. (2009)
of the family Archotermopsidae for the taxa Archotermopsis, 
Hodotermopsis and Zootermopsis, and the family Stolotermitidae
for the taxa Stolotermes and Porotermes. It would be very interesting
to study the pheromones of the Himalayan termite Archotermopsis,
considered as the most basal Archotermopsidae.

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Chemical alarm in the termite *Termitogeton planus* (Rhinotermitidae)

Klára Dolejšová · Jana Krasulová · Kateřina Kutalová · Robert Hanus

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**Abstract** Effective defense is a common characteristic of insect societies. Indeed, the occurrence of specialized defenders, soldiers, has been the first step toward eusociality in several independent lineages, including termites. Among the multitude of defensive strategies used by termite soldiers, defense by chemicals plays a crucial role. It has evolved with complexity in advanced isopteran lineages, whose soldiers are equipped with a unique defensive organ, the frontal gland. Besides direct defense against predators, competitors, and pathogens, the chemicals emitted by soldiers from the frontal gland are used as signals of alarm. In this study, we investigated the chemical composition of the defensive secretion produced by soldiers of the termite *Termitogeton planus* (Isoptera: Rhinotermitidae), from West Papua, and the effects of this secretion on the behavior of termite groups. Detailed two-dimensional gas chromatography/mass spectrometry analyses of the soldier defensive secretion revealed the presence of four linear and nine monoterpene hydrocarbons. Soldier head extracts, as well as synthetic mixtures of the monoterpene found in these extracts, elicited alarm behavior in both soldiers and pseudergates. Our results suggest that the alarm is not triggered by a single monoterpene from the defensive blend, but by a multi-component signal combining quantitatively major and minor compounds.

**Keywords** Termites · Soldiers · Frontal Gland · Alarm Pheromone · Rhinotermitidae · Termitogeton

**Introduction**

The multiple independent origins of eusocial organization in unrelated insect lineages are fascinating examples of convergence in the biology and life histories of insects. A trait common to all eusocial taxa is collective defense, manifested by the presence of a powerful sting in most eusocial hymenopterans, and the evolution of specialized defensive morphs, soldiers, in termites, some ants, and eusocial aphids and thrips (Crespi, 1994; Tian and Zhou, 2014). Along with defensive adaptations, alarm communication, recruiting defenders while allowing other nestmates to retreat, also has evolved in insect societies.

The ancestral and ubiquitous mode of alarm communication in termites consists of direct physical contact between alerted individuals and nestmates, combined with a chemical recruitment using a trail pheromone. Alerted termites lay a pheromone trail and actively recruit nestmates by bumping into their body; recruited termites then follow the chemical trail to the site of disturbance (Kettler and Leuthold, 1995; Stuart, 1963). In addition, alerted termites may perform body vibrations that are transmitted to nestmates as substrate-borne signals perceived by the subgenual organs on their legs (Hunt and Richards, 2013). This short-range signaling may be complemented by drumming of the head or abdomen against the substrate (Connétable et al., 1999; Hertel et al., 2011 Kirchner et al., 1994; Röhrig et al., 1999; Stuart, 1988). In socially advanced lineages, the head drumming is predominantly restricted to soldiers and elicits a positive feedback reaction in other soldiers. It has become an effective strategy that allows signal transmission over large distances through a chain of vibrating soldiers (Connétable et al., 1999; Röhrig et al., 1999). Another type of alarm recruitment has evolved in the Neoisoptera clade, comprising the families Serritermitidae, Rhinotermitidae, and Termitidae, having soldiers equipped with a defensive frontal gland. The primary function of this unique
gland is to produce defensive chemicals; the structural and functional diversity of these chemicals has been described in a number of termite species (reviewed in Sohotnik et al., 2010). Secondly, some of these chemicals also serve as alarm pheromones, released when excited and/or fighting soldiers discharge their frontal gland.

This type of short-range alarm recruitment has been documented in several rhinotermitids, such as Prorhinotermes, Schedorhinotermes, and Reticulitermes, as well as in numerous Termitidae, namely many species of Nasutitermitinae (reviewed in Pasteels and Borderèau, 1998; Sohotnik et al., 2010). Nevertheless, the chemical identity of the alarm pheromones is known in only a few species. All previously identified termite alarm pheromones are terpenoids. Monoterpene hydrocarbons elicit alarm in Nasutitermitinae, namely α-pinene in Nasutitermes princeps (Roisin et al., 1990) and a blend of several monoterpenes in N. rippersii (α-pinene, limonene, and β-pinene) and N. corniger (3-carene, limonene, β-pinene, α-pinene, terpinolene) (as N. costalis, Vrkoč et al., 1978). The sesquiterpene hydrocarbon (E,E)-α-farnesene has been described as the alarm pheromone in Prorhinotermes canalicifrons (Sobotnik et al., 2008). The data on Reticulitermes provide a more complex change of alarm pheromone compounds: in R. grosseri, the alarm reaction is elicited by the sesquiterpene hydrocarbon γ-cadinene, in R. banyulensis by the diterpene alcohol geranylgeraniol, in R. flavipes by the monoterpenes hydrocarbons α-pinene, β-pinene, and limonene, and the diterpene alcohols geranyllinalool and geranylgeraniol, and finally in R. lucifugus by the sesquiterpene hydrocarbons germacrene A, germacrene C, β-selinene, γ-selinene, γ-cadinene, and the diterpene alcohol geranyllinalool (Reinhart et al., 2003).

Termitogeton is a rare rhinotermitid genus, divided into two species occurring in Sri Lanka (T. umbilicatus) and in peninsular Malaysia, Borneo, and West Papua (T. planus) (Bourguignon and Roisin, 2011). It is characterized by a noticeably flattened body (Fig. 1), adapted for the life under the bark of damp, decaying tree trunks (Krishna et al., 2013; Parmentier and Roisin, 2003). Within the unresolved internal phylogeny of Rhinotermitidae, the Termitogetoninae subfamily is most often situated in the proximity of Prorhinotermesinae and Psammothorinae subfamilies and theousting family Serritermitidae (Engel et al., 2009; Inward et al., 2007; Legendre et al., 2008, 2013). This placement is corroborated by primitive traits in its biology. Termitogeton lives in small colonies of up to a few thousand individuals and, just like Psammothorinae, Prorhinotermes, and Serritermitidae, it lacks the caste of true workers; the work tasks being carried out by immature stages, larvae and pseudergates (Barbosa, 2012; Bourguignon et al., 2009, 2012; Parmentier and Roisin, 2003). Termitogeton soldiers possess a well-developed frontal gland, even though it is smaller than those of its close relatives, Prorhinotermes, Serritermes, and Glossotermes, and its reservoir is restricted to the head capsule (Quennedey, 1984; Quennedey and Deligne, 1975). Here, we report on the chemistry of the defensive frontal gland of soldiers of Termitogeton planus from West Papua, and the biological significance of its components, with special emphasis on the alarm function of these chemicals.

**Methods and Materials**

**Origin of the Insects** Eleven colonies (or colony fragments) were collected in June 2011 in West Papua, 30 km southeast from Nabire (S 3° 29.213′, E 135° 42.089′–227°). The area of the collection site was 0.1 km²; the distance between the colonies ranged from 5–400 m. Colonies were found under bark and inside the wood of dead, fallen, rotten trunks. They were transported alive to Prague (Czech Republic) in pieces of dissected trunks and kept in aquariums inside the original pieces of wood at 27 °C and elevated humidity. The species was determined to be Termitogeton planus, previously collected and described in the same locality (Bourguignon and Roisin, 2011; Parmentier and Roisin, 2003). Live termites were imported to the Czech Republic with the authorization of the State Phytosanitary Administration (Ref. No. SRS 038564/2011).

**Chemicals and Sample Preparation for Chemical Analyses** Myrcene (purity ≥95 %), α-terpinene (≥95 %), γ-terpinene (≥98,5 %), as well as both enantiomers of α-pinene (both ≥99 %), β-pinene [(S)≥99 %, (R)≥98,5 %], and limonene [(S)≥95 %, (R)=97 %] were purchased from Sigma-Aldrich. α-Phellandrene and (E)-β-ocimene originated from laboratory supplies. Hexane was purchased from Merck and was redistilled prior to use.

The chemistry of the soldier frontal gland was studied in nine colonies. The heads of cold-anesthetized soldiers were dissected in two pieces using micro scissors and submerged in 10 μl of hexane per individual for 12 h at 8 °C. Two types of extracts were prepared. First, for chemical identification and comparison of intercolonial diversity, 40–120 soldier heads from each colony were extracted. Second, to evaluate intracolonial variability, five extracts of individual soldier heads were prepared from three colonies. As a control, extracts of ten pseudergates from each colony were prepared in the same way.

Mixtures of monoterpenes used in behavioral experiments were prepared from commercial standards based on the quantification of individual monoterpenes hydrocarbons in natural extracts, using (1S)-α-pinene as an external standard.
The temperature program was set from 40 °C (1 min) to 60 °C was used to distinguish between monoterpene enantiomers. CHIRAL-20B (30 m, id 0.25 mm, 0.25μm film thickness) was set from 50 °C (1 min) to 320 °C (5 min) at 8 °C.min⁻¹; the secondary medium polarity column (BPX-50; 1.5 m, id 0.1 mm, 0.1μm film thickness) was set 10 °C higher.

For the quantification, GC/MS with a quadrupole mass analyzer (DSQ II, Thermo Scientific) was used. The temperature program for the nonpolar column (ZB-5MS; 30 m, id 0.25 mm, 0.25μm film thickness) was set from 50 °C (1 min) to 120 °C at 8 °C.min⁻¹ and then to 320 °C at 15 °C.min⁻¹. One microliter of an extract (representing approximately 1/10 of a termite equivalent) was injected in a splitless mode. Helium was used as carrier gas, at a constant flow of 1 ml.min⁻¹. 1-Bromodecane (40 ng/μl) was co-injected with samples as an internal standard for quantification.

The primary identification of soldier-specific compounds was based first on a comparison of their retention indices and fragmentation patterns with a MS library (NIST MS Search 2.0) and literature data (Adams, 2007). Retention indices were calculated from the retention times of n-alkanes (nC10–nC30). Final confirmation was performed using commercially available standards, and the absolute quantity of the detected monoterpenes was calculated using (1S)-α-pinene (50 ng/μl) as an external standard.

GC-FID (HP 6850 Series) with a chiral column HP-CHIRAL-20B (30 m, id 0.25 mm, 0.25μm film thickness) was used to distinguish between monoterpeno enantiomers. The temperature program was set from 40 °C (1 min) to 60 °C (13 min.) at 50 °C.min⁻¹, then to 110 °C (12 min) at 10 °C.min⁻¹, and finally at the same rate to 150 °C (50 min). Hydrogen was used as carrier gas. The retention times of commercial standards were compared with monoterpenes from natural extracts.

**Alarm Bioassays** Two colonies of *T. planus* were used in laboratory bioassays testing the behavioral effects of the soldier frontal gland secretion and synthetic solutions of monoterpenes identified in the frontal gland. In each experiment, a group of 15 pseudergates and 3 soldiers was placed into a Petri dish (60 mm ID) lined with a moistened Whatman No. 1 filter paper. The lid of the Petri dish was pierced with three openings (10 × 2 mm) situated symmetrically at the perimeter of the lid (Fig. 2). Pieces of Whatman No. 1 filter paper (8 × 6 mm) were impregnated with hexane solutions of the odorants, as specified below, and the solvent left to evaporate for 1 min. at room temperature. The treated paper then was carefully suspended on an entomological pin inside the opening of the Petri dish, next to a group of termites, while staying out of their direct reach.

The following solutions were applied and tested: i) 4 μl of soldier head extract; ii) 10 μl of a hexane solution of 8 monoterpane hydrocarbons identified in soldier head extracts; iii) 10 μl of a hexane solution of 4 major monoterpene hydrocarbons identified in soldier head extracts, *i.e.*, (1S)-α-pinene, (1S)-β-pinene, α-terpinene, and (R)-limonene; iv) (1S)-α-pinene; v) 10 μl of a hexane solution of 4 minor monoterpene hydrocarbons identified in soldier head extracts, *i.e.*, myrcene, α-phellandrene, (E)-β-octimene, and γ-terpinene; and finally vi) 10 μl of pure hexane as a control. The doses and relative proportions of all the compounds used in the experiments were equivalent to 4 soldiers (Figs 3, 4). Each stimulus was tested 8 times, using 4 groups of termites from each colony. For each experiment, new termites were used from the colonies.

The experiments were performed under red light at 27 °C. Prior to the experiment, groups of the termites were left to adapt in the test Petri dish for 2 h. The behavior of the termites was recorded for 2 min before and 10 min after the insertion of the odorant stimulus using a video camera. The speeds of all three soldiers and five randomly selected pseudergates were tracked in each movie, for 2 min before to 2 min after the insertion of the odorant stimulus, using MouseTracer software. The increase in speed was calculated for each individual as the difference between the mean speed 2 min before and 2 min after stimulus introduction, and was compared first among groups with the same treatment. Since no significant inter-group differences were detected, data from the eight replicates were pooled and used for a comparison among treatments by ANOVA, followed by Tukey’s HSD post-hoc comparison (N=24 and 40 for each treatment for soldiers and pseudergates, respectively). Prior to the analyses, the heteroscedasticity of data was reduced using a logarithmic.
transformation in order to meet the criteria for parametric data treatment, evaluated using Levene’s test.

## Results

### Chemistry of the Frontal Gland

Detailed GC×GC/MS analyses of soldier-specific compounds from head extracts revealed the presence of four linear hydrocarbons, nC9-11 alkanes and nC11 alkene, and nine monoterpene hydrocarbons, (1S)-α-pinene, (1S)-β-pinene, myrcene, α-phellandrene, α-terpinene, (R)-limonene, (E)-β-ocimene, γ-terpinene, and terpinolene (Fig. 3), with (1S)-α-pinene being the most abundant. The quantity of monoterpene hydrocarbons was estimated to be up to 4.5 μg per soldier, representing nearly 68% of the total defensive secretion. We did not observe any qualitative differences or important quantitative differences in the chemical composition of soldier head extracts among soldiers within particular colonies. Similarly, differences in soldier-specific compounds among the nine studied colonies were only quantitative (except for terpinolene, detected as a minor compound in two colonies and not detected in the others) and are considered as only slight intercolonial variations of a single chemotype. The relative patterns of soldier defensive compounds are shown in Fig. 4.

### Alarm Behavior

After two hours of habituation, termites were quiet and clustered in one group: pseudergates fed on the moistened filter paper, while soldiers stayed motionless in the group. Upon stimulation with odorants, the following behavioral sequence was observed in the termite groups: soldiers started to perform a zig-zag movement of the head and antennae, while searching for the direction of the odor source. Subsequently, they started to walk in the direction of the odor source, while scanning space with the antennae. When they reached the area under the treated paper, they evaluated the odor emanating from the paper and then ran rapidly back to the group of nestmates, bumping into every non-alerted nestmate encountered on the way. In addition, alerted soldiers performed characteristic longitudinal vibratory movements in the vicinity of non-alerted nestmates. The excited soldiers also occasionally opened and closed the mandibles and banged heads against the substrate. Pseudergates, alerted by odor or by alarm transmission from nestmates (soldiers), stopped feeding and started to walk in the arena. Just like soldiers, pseudergates were attracted to the odor source and, once excited, also alerted nestmates by direct contact, but were not observed to open the mandibles or bang heads. As a result of the alarm perception and its
active transmission, the entire groups of pseudergates and soldiers dispersed within a few seconds and walked rapidly all around the arena.

**Alarm Bioassays** Even a very careful insertion of an intact paper or hexane-treated paper resulted in slight agitation of the group. As a consequence, even in control treatments (hexane-treated paper), a slight increase in speed of both soldiers and pseudergates was observed (Figs 5, 6). However, a dramatic increase in speed, accompanied by the alarm behavior and active alarm propagation described above, was observed when any of the five other odorant stimuli were introduced, i.e., the soldier head extract, eight soldier monoterpenes, major and minor soldier monoterpenes, and (1S)-α-pinene. The results of the experiment, summarized in Fig. 5, indicate that the difference in speed increase was significant for all treatments and both studied castes when compared to controls ($F_{5,133}=34.6$, $P<10^{-4}$ for soldiers, $F_{5,234}=48.5$, $P<10^{-3}$ for pseudergates). In soldiers, the difference in response to the artificial mixture of eight soldier-specific monoterpenes and the four minor monoterpenes was significant; the other treatments did not differ from each other (Fig. 5a). In pseudergates, we did not detect any significant differences in speed among the five treatments, even though the mixture of eight monoterpenes appeared to elicit the strongest response and the minor monoterpenes the weakest (Fig. 5b).

The temporal dynamics in the speed of soldiers and pseudergates, depicted in Fig. 6, revealed differences among the five treatments. For both soldiers and pseudergates, soldier head extract initiated alarm behavior more slowly than the synthetic monoterpenes, as evidenced by the slope of the speed curves. In addition, soldier head extracts, the mixture of four major monoterpenes and (1S)-α-pinene, appeared to have a stable and long-lasting effect on the behavior of termites, whereas in response to the eight monoterpenes and minor monoterpenes, the fast and intense initial reaction of termites decreased substantially within a few seconds.

**Discussion**

Our results show that monoterpane hydrocarbons produced in the defensive frontal gland of soldiers of *T. planus* are perceived by both soldiers and pseudergates and elicit alarm behavior in groups of termites. This confirms, again, that while the ancestral and widespread function of the frontal gland is chemical defense, its products also can be secondarily used to communicate information on attack or disturbance of the nest. Soldiers, specialized defenders of termite colonies, are the best messengers of such information.

The defensive secretion produced by *T. planus* soldiers consists of a blend of monoterpenes, three non-branched alkanes, and one non-branched alkene ($n$C9–11 alkanes and $n$C11 alkene). Both of these chemical classes occur as defensive chemicals in termite soldiers (Šobotník et al., 2010). Short-chain alkanes have been identified in the rhinotermitid Coptotermes formosanus (Zhang et al., 2006) and the syntermitine genus Silvestritermes holmgreni (formerly known as Armritermes) (Prestwich, 1982). Our unpublished observations suggest that saturated and unsaturated straight-chain hydrocarbons are common in soldiers of the subfamily Syntermitinae. Monoterpane hydrocarbons have been identified in higher termites in a few species from the subfamilies Termitinae (Amitermes group) and Syntermitinae, and in great diversity in numerous species of Nasutitermitinae (Šobotník et al., 2010). Within Rhinotermitidae, monoterpenes occur in several species of Reticulitermes, including most of the compounds identified in the present study (Quintana et al., 2003). However, monoterpenes have not been identified in the putative closest relatives of Termitogeton (i.e., Prohinitermes, Psammothorbes, and Serritermitidae; Krasulová et al., 2012; pers. obs.; Piskorski et al., 2007).

Our results differ dramatically from the observations by Chuah et al. (1990) on the presence of a series of saturated and unsaturated ketones (C12, C16, and C17) with hexadec-1-en-3-one as the major components of the soldier defensive
secretion in *T. planus* from peninsular Malaysia. In addition, these authors did not report any monoterpenes, and they argued that the presence of the vinyl ketones in *Termitogeton* indicates chemical closeness to the subfamily Rhinotermitinae (Šobotník et al., 2010). Whereas, the absence of monoterpenes can be explained by the *in vacuo* evaporation of the solvent used by Chuah et al. (1990), which would result in the loss of the most volatile compounds, the presence of ketones remains puzzling, especially as we did not detect the characteristic MS fragments of any of the compounds reported.

We did not observe any important intercolonial variability or distinct chemotypes of monoterpenes among the colonies studied, perhaps because the area sampled in the present study was too small to detect geographical variability, described in a few other termite species over larger geographical scales (e.g., Bagnères et al., 1990; Krasulová et al., 2012; Perdereau et al., 2010).

When disturbed by a chemical stimulus, soldiers and pseudergates exhibited a series of characteristic alarm behaviors, described also in other termite species, namely attraction to the odor source (e.g., Reinhard et al., 2003), zig-zag running and active alarm recruitment through direct contact (e.g., Kettler et al., 1995; Reinhard and Clément, 2002), and longitudinal vibrations and head banging performed by soldiers (Connétable et al., 1999; Kirchner et al., 1994; Röhrig et al., 1999; Stuart, 1963, 1988).

The finding that soldier-produced monoterpenes are responsible for alarm transmission in *T. planus* is not surprising,

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**Fig. 5** A comparison of increase in speed of soldiers A and pseudergates B of *Termitogeton planus*, in mixed groups, after exposure to various odorant stimuli. Bars represent means and whiskers standard deviations; *N* = 24 soldiers and 40 pseudergates per treatment. Data were log-transformed prior to ANOVA with Tukey’s HSD post-hoc comparison. Bars marked with different letters differ at α = 0.05.

**Fig. 6** The dynamics in speed of soldiers A and pseudergates B of *Termitogeton planus*, in mixed groups, in response to odorant stimuli. The curves represent instantaneous motion speeds for each second of the recordings, pooled from all individuals and all experiments, and converted into running averages of 10 neighboring values.
as monoterpenes have been identified as alarm pheromones in several Nasutitermitinae (Roisin et al., 1990; Vrkoč et al., 1978) and Rhinotermitidae (Reinhard and Clément, 2002). Among the multitude of functions attributed to monoterpenes, sequestered or de novo-synthesized by animals in a diversity of structures, a role in communication is common (Gershenzon and Dudareva, 2007). Their biosynthetic “availability” and optimum volatility makes them suitable as pheromones for fast signal transmission also in termite nests.

Our data suggest that the alarm function in T. planus is not restricted to a single compound from the monoterpene blend, because the four major or four minor monoterpenes, as well as the dominant monoterpene (IS)-α-pinene, all elicited an intense alarm reaction. Unfortunately, due to the lack of termites and vulnerability of termites to experimental manipulations, we were unable to test each of the monoterpenes or their combinations in bioassay. Additionally, we were not successful in comparing the electrophysiological responses of termites to individual monoterpenes; the small size and low survival rate of termites in our experiments hampered electroantennographic analyses. Nevertheless, a few previous studies have shown that termite terpenoid blends act as multi-component alarm pheromones, with a more or less pronounced synergistic effect of quantitatively major and minor compounds, rather than as single-component signals (Reinhard et al., 2003; Roisin et al., 1990; Vrkoč et al., 1978).

Our observations revealed that the temporal dynamics in the responses of pseudergates and soldiers differed when the extract or artificial mixtures were applied. The onset of alarm behavior appeared to be slower in the presence of the extract than for the artificial mixtures. Moreover, when mixtures containing only the minor monoterpenes were applied, alarm reactions decreased to lower values within tens of seconds after the initial rapid increase. This suggests that the soldier head extract differs in its physico-chemical properties from the synthetic blends. This may be caused by the presence of additional components in the soldier head extract, namely nC9-11 alkanes, nC11 alkene, and a small amount of cuticular hydrocarbons. Although the role of short-chain hydrocarbons, accounting for 30% of soldier head extract, is considered to be defensive (Zhang et al., 2006), they also may contribute to the slower evaporation of the monoterpenes. In consequence, the alarm behavior is rapidly triggered by synthetic mixtures, but is not as long-lasting as soldier head extract. This is more obvious in artificial mixtures containing the minor monoterpenes, perhaps due to these compounds evaporating to suboptimal quantities faster than the more abundant major monoterpenes. A similar phenomenon was noticed previously when authentic extracts were compared against synthetic solutions for alarm-eliciting properties (Roisin et al., 1990; Šobotnik et al., 2008).

The richness of the monoterpene fraction observed in the defensive blends of many termite species can be explained by the low selectivity of terpene synthases, which produce complex mixtures of terpenoids (Tholl, 2006). At the same time, terpenoid blends are likely to be synthesized primarily as defensive mixtures; their structural diversity can, therefore, be viewed as an adaptation increasing the spectrum of possible targets (Gershenzon and Dudareva, 2007). The chemical alarm and sensitivity of Termitogeton planus soldiers and pseudergates to several compounds from a rich blend would thus evolve as a secondary function of the chemically diversified defensive secretion.

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Abstract A great diversity of defensive chemicals has been described in termite soldiers equipped with a unique defensive organ, the frontal gland. Along with the functional diversity of these compounds, reflecting the evolutionary history of particular lineages and their defensive strategies, a considerable degree of chemical variability often occurs among species and populations. Thus, the chemistry of termite defense may provide information on the phylogeny and geographic dispersal of species and populations. In this paper, we report on the anatomy of the frontal gland and on the diversity of soldier defensive chemicals in the sand termite, *Psammotermes hybostoma*, from nine colonies and five different localities in Egypt. Using gas chromatography–mass spectrometry, a total of 30 sesquiterpene hydrocarbons, or their oxygenated derivatives, were detected, and the chemical identity of most of them identified. In addition, a ketone, an ester, and a diterpene were identified in some colonies. Within colonies, the chemical composition was stable and did not differ among soldier size categories. However, there were pronounced quantitative and qualitative differences in frontal gland chemicals among colonies and geographic locations. The findings are discussed in a broader comparison with other termite taxa.

Keywords Termites · Chemical defense · Frontal gland of soldiers · Rhinotermitidae · *Psammotermes hybostoma* · Sesquiterpenes

Introduction

Collective defense is a prominent characteristic of insect societies, manifested by defendable nests and defensive adaptations of inhabitants (Crespi, 1994). In termite ancestors, the caste of soldiers evolved as the first altruistic caste, completely sterile and fully devoted to defense (Roisin, 2000). Along with a plethora of anatomic and behavioral adaptations for mechanical defense, termite soldiers also possess efficient chemical weaponry, especially for the advanced families Rhinotermitidae, Serritermitidae, and Termitidae, which are equipped with a unique defensive organ, the frontal gland (Deligne et al., 1981; Prestwich, 1984; Quennedey, 1984; Šobotník et al., 2010).

A fascinating diversity of defensive chemicals produced by this gland has been discovered over the past four decades, with several hundred compounds from various chemical classes, including alcohols, mono-, sesqui-, di-terpenoid hydrocarbons, ketoaldehydes, fatty acids, macrocyclic lactones, heterocyclic and aromatic compounds, having being identified. The functional diversity of these compounds and their mixtures includes use as irritants, repellents, glues, anti-healants, and contact poisons (Prestwich, 1984; Šobotník et
Besides their defensive function, volatiles from the frontal gland also may be involved in signaling alarm by fighting or irritated soldiers (Šobotník et al., 2010). The functional chemistry of the frontal gland secretion co-evolved with structural aspects (anatomy of the glandular reservoir, the frontal pore, shape of the head and mandibles, and related behavior) used in association with defense, into a multitude of defensive strategies, ranging from contact discharge combined with mandibular biting, to non-contact delivery by spraying (Quennedey, 1984; Šobotník et al., 2010). Thus, the chemistry and anatomy of the frontal gland provide information on the evolutionary history of defensive strategies in particular lineages. At the same time, several studies have highlighted that defensive blends often are highly variable at interspecific and intercolonial scales, both in quality and quantity, thus making the frontal gland chemistry an interesting tool for studies on taxonomy and biogeography (see e.g., Goh et al., 1984; Bagneres et al., 1990; Quintana et al., 2003; Perdereau et al., 2010).

**Psammotermes**, Desneux, 1902, is the most arid-adapted isopteran genus, living in arid areas of the Old World, namely Arabia, Sahara, and Sahel (P. hybostoma Desneux, 1902; P. assuanensis Sjöstedt, 1912; P. fuscofemoralis Sjöstedt, 1904; P. senegalensis Sjöstedt, 1924), South Africa (P. allocerus Silvestri, 1908), Madagascar (P. voeltzkowi Wasmann, 1910), and South-West Asia (P. rajasthanicus Roonwal & Bose, 1960). The nesting and foraging habits, as described e.g., by Grassé, (1984) for *P. hybostoma*, are reminiscent of advanced rhinotermitids with populous colonies that inhabit a large polycalic system of hypogeal constructions with peripheral centers in the feeding substrate (i.e., various cellulose-containing material, from dead trees, through wind-blown vegetal debris to animal feces) (Harris, 1970; Grassé, 1984). Despite their wide distribution and economic importance as pests of timber and crops, many aspects of *Psammotermes* biology remain poorly understood, especially the caste system (only a pronounced size polymorphism of workers and soldiers has been noted; Clément, 1952; Roonwal, 1988) and chemical ecology, including the composition of the frontal gland. Therefore, we are investigating some of these aspects, namely chemical communication (Sillam-Dussès et al., 2011), the caste system (Bourguignon, unpublished data), and the chemistry of defensive compounds from the frontal gland.

In this study, we report on the anatomy of the frontal gland and on the chemistry of the frontal gland secretion in soldiers of the sand termite, *Psammotermes hybostoma*, collected from nine colonies in five localities in Egypt. We compare, among colonies and localities, data on soldiers of different body sizes, and discuss results in a phylogenetic context.

**Methods and Materials**

**Insect Origin and Sampling** Nine fragments of *P. hybostoma* colonies were collected at five different localities (A–E, see Fig. 1) in the Nile Valley and Egyptian Western Desert in March 2010. Eight colony fragments were extracted from tamarisk wood, and one from a palm tree, and brought live to Prague. The colonies were held under laboratory conditions in their original wood at 26°C and at a low relative humidity. The colonies are hereafter denominated with the locality code (A–E), followed by the colony number.

Given the pronounced size polymorphism of *Psammotermes* soldiers, we aimed to interpret our findings with respect to body size. The observed variability in soldier size was extreme, with head widths ranging from 0.9 to 2 mm. Therefore, we defined three categories based on the head width (HW): small soldiers (SS; HW<1.1 mm), medium soldiers (MS; HW=1.1–1.5 mm), and large soldiers (LS, HW>1.5 mm) (Fig. 2).

![Fig. 1 Situation of the five localities, A–E, in the Nile Valley and Western Desert](image)
workers from each of the colonies, to compare the profile of chemical diversity among the three categories; iii) extracts of 10 soldiers of each size category from colony B1, for comparing soldiers among the same size category; ii) extracts of 5 soldiers from each colony, for evaluating intracolonial chemical diversity with published data and with synthetic and/or natural standards, as specified in Table 1. The retention indices of particular compounds were calculated using the retention times of n-alkanes (C10–C30).

Optical and Electron Microscopy Five soldiers of each size category were removed from colony B1, submerged in a drop of fixative (2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2), and dissected into head (mandibles carefully removed, thorax, and abdomen. After 24 h at 4°C, the samples were washed in 0.1 M phosphate buffer, post-fixed for 2 h in 1.5 % osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in an ethanol series, and embedded into standard Spurr resin. Subsequently, 1 μm-thick sections were cut with an Ultracut Reichert-Jung, stained with Azure II solution, and studied using a Carl Zeiss Amplival optical microscope equipped with a Canon EOS 500D camera. Ultrathin sections (50–80 nm) were stained using the standard protocol developed by Reynolds (1963) and observed with a JEOL JSM-7401F scanning electron microscope. Entire soldier bodies were desiccated, gold-coated, and observed with a JEOL JSM-7401F scanning electron microscope.

Extracts Soldiers, freshly removed from colonies, were frozen at –20°C for 5 min. In order to open the frontal gland reservoir and extract its content, soldiers were dissected into head and rest of body, and extracted with distilled hexane (20 μl per soldier). The samples were shaken for 10 min, and stored at –20°C. Extracts of workers were prepared in the same way, but without the dissection.

The following extracts were prepared: i) extracts of 10 individual SS from colony A1 and 10 individual LS from colony E2 for evaluating intracolonial chemical diversity among soldiers of the same size category; ii) extracts of 5 soldiers of each size category from colony B1, for comparing chemical diversity among the three categories; iii) extracts of 10 soldiers from each colony, for identification and quantification, and intercolonial comparison; and iv) extracts of 10 workers from each of the colonies, to compare the profile of cuticular hydrocarbons among colonies and, thus, verify that all collections belonged to the same species; these extracts also allowed us to discriminate compounds specific to the frontal gland of soldiers from compounds present on the cuticle.

Gas Chromatography–Mass Spectrometry (GC-MS) Chemical identification and quantification were carried out by GC-MS (quadrupole DSQ II, Thermo Scientific) using a DB-5 column (30 m, id 0.25 mm, 0.25 μm film). The oven-temperature program was 50°C (1 min.) to 320°C (5 min.) at 7°C.min⁻¹. 1-Bromodecane (40 ng/μl) was added as an internal standard. Splitless injection was used, with helium as the carrier gas (1 ml.min⁻¹). Identification of compounds was based on comparison of retention indices and MS fragmentation patterns (70 eV, electron impact ionization) with published data and with synthetic and/or natural standards, as specified in Table 1. The retention indices of particular compounds were calculated using the retention times of n-alkanes.

Solid-Phase Microextraction (SPME) Several standard terpenes were extracted by SPME from natural sources: ar-curcumene and β-elemene were obtained, together with other sesquiterpenes, from ginger rhizome; valencene was obtained from Valencia orange peel. The samples (10 g) were heated at 50°C, and the volatile fraction adsorbed for 40 min. on a grey SUPELCO SPME fiber (50 μm DVB/ CAR/PDMS coating) situated 5 cm above the heated sample. After this, the SPME fiber was injected into a GC and analysis performed under the conditions as for the hexane extracts.

Solvents and Standards β-Bisabolene and (E)-α-bisabolene were synthesized from (Z)-nerolidol (Sigma-Aldrich), using the same experimental conditions as described for synthesis from (E)-nerolidol by Svatoš and Attygale (1997). (Z)-γ-Bisabolene and (E)-γ-bisabolene were obtained from stored termite imagos of Prorhinotermes simplex (Piskorski et al., 2009). Hexane was purchased from Merck and redistilled prior to use.

Statistics Differences in chemical composition of soldier extracts were evaluated using the formula of Nei (1972). The calculation was based on relative abundances of the 33 compounds listed in Table 1; the resulting matrix of Nei distances then was plotted as a cluster tree with the unweighted pair-group average clustering method (performed with Statistica 8).

Results

Anatomy of the Frontal Gland The frontal pore was located on the anterior frons, at the beginning of a shallow groove.
leading to the labrum (Fig. 3). The size of the frontal gland reservoir differed considerably among soldier categories; it reached as far as to the fourth abdominal segment in SS (Fig. 4B), while it was confined to the head in MS and LS (Fig. 4A).

The secretory epithelium consisted entirely of class 1 secretory cells, whereas there was an abundance of class 3 secretory cells around the fontanelle (Figs. 3 and 4A). This epithelium usually was between 5–20 μm thick (Fig. 4) and lined with a highly modified cuticle made of fibrous material, occurring in electron lucent and electron dense patches (see Fig. 4D). A single continuous electron-dense layer (about 25 nm thick) occurred at the cuticle apex. The apical plasma membrane formed irregular projections (Fig. 4D) supported with numerous microtubules. The basement membrane was formed by a single lamina, 0.5–0.75 μm thick, connected to the secretory cells by numerous focal contacts. Intercellular junctions consisted of a single apical zonula adherens, followed by a septate junction; gap junctions were rare. The membranes of neighboring cells were free in their basal halves. Nuclei were centrally located, irregular, about 5 μm in the largest

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Secretory organelles included smooth and rough endoplasmic reticulum. Secretory vesicles were of two types, more abundant lipid-like droplets and less frequent electron-lucent vesicles; both originated from a conversion of large electron-dense granules (up to 3 μm diam.) and were released at the cell apex. Mitochondria were moderately abundant and often located next to the secretory vesicles.

Comparison of Cuticular Profiles, Specific Identity of the Collected Colonies

Cuticular hydrocarbons profiles of workers from all colonies did not differ qualitatively in chemical composition, and only minor quantitative variations were observed (Fig. 5A). These results suggested that all collected colonies belonged to one species, *P. hybostoma*, widely distributed and abundant in the Sahara.

Chemical Composition of the Frontal Gland Secretion

Soldier extracts were rich in volatiles that were lacking in the extracts of workers (Fig. 5B). Altogether, 33 compounds specific to soldiers were detected (Table 1). Most of these were sesquiterpenes (20) or oxygenated sesquiterpenes (10). Sesquiterpenes quantitatively dominated (over 95 % of) the extracts. In addition, a few other compounds, such as *p*-methylacetophenone, hexadecenyl acetate, and (E,E)-geranyllinalool, were identified as minor or trace compounds in some colonies. The method of identification of particular compounds is specified in Table 1. For most compounds, characteristic fragmentation patterns of mass spectra and retention indices allowed unambiguous identification based on published data. However, due to the high similarity of the mass spectra and retention indices of some sesquiterpenes, the identity of several compounds was confirmed using synthetic or natural standards. Nevertheless, the projection of the apical plasma membrane supported with microtubules. The scale bar represents 1 μm. E. The basal part of the secretory cell showing various interstages of secretory vesicle development. The scale bar represents 2 μm. Abbreviations: 1–4, abdominal segment 1–4; b, brain; bm, basement membrane; c, cuticle; fb, fat body; fr, frontal gland reservoir; hg, hindgut; m, mitochondria; mg, midgut; mm, mandibular muscles; n, nucleus; ser, smooth endoplasmic reticulum; sg, suboesophageal ganglion.
identification of β-elemene should be treated with caution, because of the risk of the possible Cope rearrangement of germacrene structures to elemenes under GC conditions (Takeda, 1974).

Total quantities of volatiles in individual soldiers were relatively high, ranging from tens to hundreds of micrograms per soldier, reflecting the relatively large size of *Psammotermes* soldiers. However, quantities were highly variable among individual soldiers, likely because of the differences in body size and in fullness of the gland reservoir. The maximum total quantities roughly corresponded to size differences among soldier categories, large (520 μg), medium (230 μg), and small soldiers (170 μg), and to the estimated sizes of their respective gland reservoirs. Hence, the quantities of particular compounds allowed a reliable relative quantification and comparison among individual soldiers.

**Intracolonial Chemical Variability** Chromatographic patterns of ten individual small soldiers from colony A1 and ten individual large soldiers from colony E2 were similar within each colony; the chemical dissimilarity between each pair of soldiers depicted in Fig. 6 was lower than 0.015 for both colonies. The same applies for the differences in relative abundances among large, medium and small soldiers, evaluated in colony B1. Thus, intracolonial chemical variability within particular size categories, and among the three size categories, can be considered as low.

**Chemical Variability Between Colonies and Localities** While the chromatographic patterns were alike within individual colonies, a higher level of variability was recorded among colonies from the same locality, as shown in Fig. 6 and Table 1. Colonies A1 and A2 were similar, with their
All other colonies were different from each other within the same locality (A1 + A2 vs. A3, C1 vs. C2, and E1 vs. E2), mostly due to quantitative differences in major compounds or qualitative differences in minor or trace components. The highest intercolonial chemical difference was observed between the two colonies from locality C, owing to the quantitative shift in the two major sesquiterpenes, \( \beta \)-elemene and valencene (34 and 19 % in C1 vs. 15 and 31 % in C2). Colonies E1 and E2, extracted from the tamarisk and palm trees, respectively, did not differ more than other pairs of colonies from one locality.

Fundamental differences were observed among localities. Quantitative patterns of relative abundances account for the differences between localities A and B from the Nile Valley, mostly due to the shift between valencene and \( \beta \)-elemene, the former being dominant in locality A (62± 3 %) and the latter being the major compound in locality B (59± 5 %). Chemical differences between localities D and E from the Western Desert were low, a result of slight quantitative variations. In contrast, locality C can be distinguished easily from the two other localities, based on relative abundances of four major sesquiterpenes, \( \beta \)-elemene, valencene, \( \beta \)-bisabolene, and \((E)\)-\( \gamma \)-bisabolene; i.e., 24±5, 25±6, 5±2, and 24±1 %, respectively, in colonies C1+C2, contrasting with 18±4, 4±1, 10±3, and 43±7 % in colonies D1+E1+E2.

A clear distinction can be made between colonies from the Nile Valley and those from the Western Desert, representing two dramatically different chemotypes, hereafter called NV and WD chemotypes. Each of the chemotypes is characterized by the presence of exclusive compounds, such as aristolochene and germacrene A in the NV phenotype, and ar-curcumene, selina-4,11-diene, four bisabolene isomers, and several oxygenated sesquiterpenes in the WD chemotype. The differences between the two chemotypes are apparent from the representative chromatograms depicted in Fig. 5C and peaks listed in Table 1.

**Discussion**

The frontal gland of *Psammotermes hybostoma* is among the smallest of all Rhinotermitidae (Sobotnik et al., 2010). Along the continuum of soldier body sizes, the gland reservoir is developed in two modal forms. In small soldiers, it reaches deep into the abdomen, as in numerous other Rhinotermitidae, while in medium and large soldiers it resembles that of *Termiotogeton*, the only rhinotermitid with the gland situated only in the head (Quennedey, 1984). The ultrastructure of the gland corresponds to the description by Quennedey (1984), and is similar to that in *Prohino- termes simplex* (Sobotnik et al., 2004), especially in the following traits: the structure of the glandular cuticle, irregular projections of the apical plasma membrane, scarce secretary organelles, and the process of secretion formation.

The total amount of defensive volatiles increases with body size; small soldiers may contain up to 170 \( \mu \)g, while the largest soldiers may contain as much as 500 \( \mu \)g. However, the relative quantity of defensive chemicals was much smaller than in most other rhinotermitids, e.g., *Coptotermes* or *Prorhinotermes* (Waller and La fage, 1987; Hanus et al., 2006). Despite these dramatic size differences among soldiers, we did not observe any chemical polymorphism. Therefore, the size variability is unlikely to be linked with a differential use of the frontal gland.

We observed a trend in chemical variability among colonies and localities. Intercolonial variability at one locality was usually low. Very similar chemical composition was recorded in colonies A1 and A2; these two samples likely represented two fragments of one large colony, given the distance of only a few tens of meters between the two sites. Other colonies had slightly different quantitative patterns of volatiles from each other at the same locality. However, the five localities showed two fundamentally different chemotypes. All colonies from the Nile Valley were of the NV chemotype, while all colonies from the Western Desert (25 km from the Nile Valley) were the WD chemotype. By our own sense of smell (unpublished results) we could distinguish samples from each colony (5 soldiers in a Petri dish) with respect to chemotype.
Aside from the two chemotypes, more subtle trends can be seen. In colonies C1 and C2, even though they belonged to the WD chemotype, characteristics of the NV chemotype are apparent, such as the presence of selina-5,11-diene and cis-eudesma-6,11-diene, or a higher proportion of valencene. This introgression of the NV chemotype into the WD chemotype corresponds well with the central position of locality C. Moreover, this locality was situated in the suburbs of a large Kharga oasis with a great probability of a passive transport of colony fragments or alates with human traffic. Colony D1, collected close to C locality but outside the urban area, corresponded much better with colonies E1 and E2.

Interspecific differences in frontal gland chemistry are well known (Sobotnik et al., 2010) and, along with cuticular hydrocarbons, can be used as chemotaxonomic markers (e.g., Bagnères et al., 1990). Conspicuously different chemotypes also have been noted within species (e.g., Valterová et al., 1989) or even within a single chemotype of cuticular hydrocarbons (Bagnères et al., 1990; Perdereau et al., 2010), thus allowing the evaluation of another level of chemodiversity when disentangling the relatedness and dispersal history of populations or sibling species. Our results represent another example of intraspecific polymorphism in biosynthetic pathways of defensive compounds, independent of cuticular hydrocarbon chemotypes and feeding substrate, and subject to genetic variation among populations.

The richness and variability of the sesquiterpenoid secretion suggest that its defensive potential results from the complex blend, rather than from the individual compounds. Among the biological functions attributed to terpenoids, their use as deterrents, repellents and irritants is widespread (e.g., Gershenzon and Dudareva, 2007). Multiple explanations have been proposed for the occurrence of terpenoids in mixtures, including the optimization of physicochemical properties, enlargement of the spectrum of target organisms, decreased likelihood of development of resistance to them, and functional constraints of biosynthetic pathways (Fischbach and Clardy, 2007; Gershenzon and Dudareva, 2007). It is interesting to note that the sesquiterpenes characteristic for each of the two chemotypes typically have a common biogenesis, differing only in the final steps; e.g., the bisabolene isomers and their oxygenated derivatives in WD, or valencene, nootkatol and nootkatone in NV.

Terpenoid mixtures are common in termite frontal glands (see Sobotnik et al., 2010), and their repellent and toxic properties have been confirmed experimentally, especially sesquiterpene hydrocarbons (Scheffrahn et al., 1983) and oxygenated sesquiterpenes (Wadhams et al., 1974; Zhu et al., 2003). A strong toxicity has been shown for the diterpene geranylinalool, frequently found in Reticulitermes sp. (Lemaire et al., 1990) and occurring in trace quantities in Psammotermes. Hence, we hypothesize that the sesquiterpene mixtures of Psammotermes have a repellent and/or irritant function, or participate in unpalatability. In addition, sesquiterpenes can act as alarm pheromones, such as in Prohino termes (Sobotnik et al., 2008) or Reticulitermes (Reinhard et al., 2003). Due to the limited survival of Psammotermes in captivity, we were not able to test their function.

In Rho nitermitidae, sesquiterpenes have been recorded in soldiers of several Reticulitermes species, as well as in soldiers and imagos of Pro rhoh otermes, always as a mixture with other classes of compounds (Quintana et al., 2003; Hanus et al., 2006; Piskorski et al., 2007, 2009). Several recent phylogenetic hypotheses situate Psammotermes as a sister or closely related taxon to Pro rhoh otermes (Austin et al., 2004; Lo et al., 2004; Okumura et al., 2004; Inward et al., 2007). Despite the dramatic differences in ecology, the close relationship of the two genera is supported by similarities in anatomy of the frontal gland (Sobotnik et al., 2004), by the unique shape of the nymphal wing buds (Štys and Sobotnik, 1999, Bourguignon, unpublished data), and by the primitive type of caste development, which lacks an early divergence of a permanent worker caste (Roisin, 1988; Bourguignon, unpublished data).

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References


SUMMARY

The aim of this thesis was to contribute to the knowledge on chemical communication and defence in selected important and poorly studied genera of termites, namely on chemical identity and function of semiochemicals and defensive compounds, and their chemical diversity in a phylogenetic context.

Within a research project focusing on critical termite genera from families Rhinotermitidae and Sertitermitidae, I participated on the identification of a new trail-following pheromone in the serritermitid *Glossotermes oculatus*. The identified structure, (10Z,13Z)-nonadeca-10,13-dien-2-one, differs dramatically from C12 unsaturated alcohols occurring as trail-following pheromones in all other advanced termite families and underlines the remote phylogenetic position of Serritermitidae.

I was involved in the identification of trail-following and sex pheromones in the sand termite *Psammotermes hybostoma* (Rhinotermitidae). Although in both cases we assigned the pheromone role to the most frequent termite pheromone, (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol, it was the first time we have detected this compound by chromatographic methods. In *Psammotermes hybostoma*, also the defensive chemistry has been studied. Altogether 33 defensive chemicals have been detected and majority of them fully identified. Qualitative and quantitative comparison of defensive blend among colonies clearly distinguished three different chemotypes corresponding well with the locality of origin. Under the terms of the same project, I identified the chemical composition of alarm pheromone secreted by soldiers of a poorly known termite species *Termington planus* (Rhinotermitidae). The alarm communication appears to be a multi-component signal, combining major and minor monoterpene components from the defensive secretion.

In collaboration with foreign laboratories, two new pheromones have been identified in the basal termite *Hodotermopsis sjoestedti*, i.e. syn-4,6-dimethylundecan-1-ol as the trail-following pheromone and syn-4,6-dimethylundecanal as the male sex pheromone. Our results underlined the phylogenetic relationship of the genera *Hodotermopsis* and *Zootermopsis* within the family Archotermopsidae.

In workers of the neotropical termite *Neocapritermes taracua* (Termitidae), I participated on the description of multi-component defensive mechanism, resulting in the production of benzoquinone(s), converted from hydroquinone(s) through the catalysis by a copper-binding protein. Last but not least, dodecane-2,10-diol was identified as a fertility-related volatile in *Prorhinotermes simplex* (Rhinotermitidae) and a hypothetic primer pheromone.
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