PAULO FELLIPE CRISTALDO

NEST-SHARING AMONG A TERMITE HOST AND ITS OBLIGATORY INQUILINE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae.*

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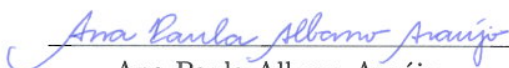
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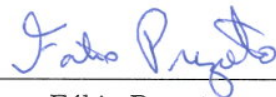Eraldo Rodrigues de Lima
(Coorientador)

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Simon Luke Elliot
(Coorientador)

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Ana Paula Albano Araújo

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Fábio Prezoto

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Og Francisco Fonseca de Souza
(Orientador)

A minha família,
pelo apoio e dedicação ao longo da minha vida.

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SUMÁRIO

RESUMO

CRISTALDO, Paulo Fellipe, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Compartilhamento de ninho entre um cupim hospedeiro e seu inquilino obrigatório**. Orientador: Og Francisco Fonseca de Souza. Coorientadores: Eraldo Rodrigues de Lima e Simon Luke Elliot.

Interações simbióticas ocorrem em todos os grupos de organismos. Este tipo de interação é frequentemente observada em ninhos de insetos sociais, no qual a espécie que coabita o ninho junto com o hospedeiro é geralmente outra espécie eusocial. Especificamente para cupins (Isoptera), os mecanismos envolvidos nesta interação permanecem abertos à investigação. Com o intuito de preencher parte desta lacuna, o objetivo desta tese foi analisar aspectos ecológicos e químicos envolvidos na partição de ninho entre o cupim hospedeiro *Constrictotermes cyphergaster* Silvestri, 1901 (Termitidae: Nasutitermitinae) e seu inquilino obrigatório *Inquilinitermes microcerus* Silvestri, 1901 (Termitidae: Termitinae). Assim, este estudo fornece evidências sobre como os inquilinos conseguem coabitar o ninho junto com os seus hospedeiros. Os resultados mostraram que a colonização do cupinzeiro pelo inquilino obrigatório está relacionada com o desenvolvimento da colônia hospedeira, o que indica que o inquilino precisa da colônia hospedeira ativa e madura para colonizar os ninhos. Para coexistir no ninho hospedeiro, as espécies inquilinas parecem adotar a estratégia de 'evitar conflitos', estabelecendo assim a convivência com o seus hospedeiros no tempo ecológico e evolutivo. Tal estratégia parece ocorrer através de insignificância química da espécie inquilina em seus feromônios de trilha e de alarme. Além disso, foi possível fornecer evidências de que o inquilino obrigatório se beneficia escutando os sinais de alarme do hospedeiro. Além dessas novas evidências sobre a associação obrigatória entre diferentes espécies de cupins em um mesmo ninho, fornecemos resultados importantes para a biologia geral de cupins, como por exemplo: (*i*) a primeira análise completa de comunicação de alarme e (*ii*) importantes características sobre a biologia do inquilino obrigatório *I. microcerus*, ainda pouco estudada.

ABSTRACT

CRISTALDO, Paulo Fellipe, D.Sc., Universidade Federal de Viçosa, February, 2014. **Nest-sharing among a termite host and its obligatory inquiline**. Adviser: Og Francisco Fonseca de Souza. Co-advisers: Eraldo Rodrigues de Lima and Simon Luke Elliot.

Symbiotic interactions are spread throughout all groups of organisms. This kind of association is often observed in nest of social insects, in which the guest species are often other eusocial species belonging to the same phylogenetic group of host species. Specifically for termite (Isoptera), the mechanisms behind symbiotic interaction among termite species that share a common nest remains open to investigation. Given this gap in the study of nest-sharing in termite, in this thesis we focus on ecological and chemical aspects involved in the nest-sharing among a termite host *Constrictotermes cyphergaster* Silvestri, 1901 (Termitidae: Nasutitermitinae) and its obligatory inquiline *Inquilinitermes microcerus* Silvestri, 1901 (Termitidae: Termitinae). In doing so, we aimed to get a better insight about this understudied association, providing evidences on how termite inquilines manage to cohabit the nest along with the termite host species. The results showed that the colonization of termite nest by obligatory inquiline is linked with its host's colony development, which seems to indicate that inquiline needs its host colony active and mature to live in the nest. To live in the nest along with its host colony, inquiline species adopt the 'conflict-avoidance' strategy to establish the coexistence over ecological and evolutionary time. Such 'conflict-avoidance' strategy occurs through chemical insignificance in trail-following and alarm pheromones by inquiline species. In addition, it was possible to provide evidence that inquiline species gains benefit of defense by eavesdropping its host alarm cues. Beyond these insights on termite-termite obligatory association, we provided important findings to termite biology itself, including for instance: (i) the first thorough analysis of termite alarm communication and (ii) important biological features of the poorly studied inquiline *I. microcerus.*

Chapter 1

General Introduction

The ability of organisms to interact with both abiotic and biotic environment has long called the attention of researchers from different fields in biology. In nature it is possible to find countless of complex interactions between all kinds of organisms. The involved species vary greatly in their origins, states and needs, but they share an elementary feature: to find favourable and secure conditions allowing them to survive and reproduce (Darwin, 1859). These complex interactions are often brief (e.g. predation); however, there are many cases where two or more species live in close association for long periods, establishing symbiotic interactions.

The ubiquity of symbiotic relationships is impressive and examples include species of all six kingdoms and in all level of biological organization (Douglas, 1994). Typical examples include gut-inhabited bacteria in mammals (Savage, 1977), vascular plants and arbuscular mycorrhizal fungi (Bonfante & Genre, 2008), termites and their intestinal bacteria, protists and fungi (Radek, 1999). Among these, one of the most conspicuous examples is the association observed among nest builders and their 'symbiotic' species (i.e. species that live inside the nest, in which builder species have built to house their own relatives). Not surprisingly, such association had attracted attention from the times of Aristotle till now: the fascinating behaviour of the European cuckoo's (*Cuculus canorous*) to lay its eggs in the nests of other bird species was known as long ago as the fourth century *BC* (see Birkhead & Monaghan, 2010; Stoddard & Kilner, 2013).

Although cuckoos and their hosts are a classical example of symbiotic interaction among organisms that live in nests, it is only in insects societies of ants, bees, wasps (Hymenoptera) and termites (Isoptera) that this kind of interaction is arguably most remarkable, once their long-lived colonies represent whole ecosystems in themselves providing food and shelter for literally thousands of vertebrate and invertebrate species exhibiting all hues of symbiotic partnerships (see e.g. Brandt et al., 2005; Hughes et al., 2008). Guests of social insect colonies are especially diverse in ant and termite colonies, where they comprise often other social species belonging to the same phylogenetic group of nest the builder (Wilson, 1971; Kistner, 1979). In the case of ants, the symbiotic species are often parasites that employ multiple strategies to overcome barriers to colony invasion, evolving morpho-physiological and behavioural strategies to confront, confound, or avoid completely their host (for

a review see Nash & Boomsma, 2008). Hymenopteran hosts, on their turn, evolve counteracting strategies to detect and face the invader (Brandt et al., 2005).

Differently from current understanding regarding hymenopterans, our comprehension of the mechanisms behind symbiotic interactions among termite species cohabiting a single nest remains open to investigation, only recently being studied under functional (as opposed to pattern-finding) perspective (e.g. Cunha et al., 2003; Costa, 2005; Florencio, 2011; Carrijo et al., 2012; Darlington, 2012; Marins, 2012; Rosa, 2012; Florencio et al., 2013).

In this thesis and its underlying doctoral work, I have focused on ecological and chemical aspects involved in the nest-sharing among a termite host *Constrictotermes cyphergaster* Silvestri, 1901 (Termitidae: Nasutitermitinae) and its obligatory inquiline *Inquilinitermes microcerus* Silvestri, 1901 (Termitidae: Termitinae). In doing so, I aimed to get a better insight about this understudied association, providing evidences on how termite inquilines manage to cohabit the nest along with the termite host species. In the following subsections, I will briefly describe the model system used in the field and lab bioassays and summarize the questions and results addressed in each chapter of this thesis.

The System

Constrictotermes cyphergaster (Fig. 1.1A) is a Neotropical termite species with distribution restricted to South America (Argentina, Bolivia, Brazil and Paraguay) (Mathews, 1977; Krishna et al., 2013). In this species, the nymphal line comprises one larval[1] instar followed by five nymphal instars, and alates. The apterous line includes two larval instars, followed by workers, presoldiers and soldiers. Both workers and soldiers were shown to be monomorphic, having only one instar each and consisting of only male specimens (Moura et al., 2011). An average of 4.5 workers per soldier has been recorded by Cunha et al. (2003) in *C. cyphergaster* nests. Along with other oriental species, *C. cyphergaster* is characterised by an unusual life-style in which the foraging occurs at night in 'open-air' by worker columns protected by

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[1] Termitological jargon for recently hatched young;

numerous soldiers along the paths (Moura et al., 2006b). Their diet is composed by wood in different stages of decomposition, which is feed on the surface of the bark of live trees (Moura et al., 2006b), and on lichens (Bourguignon et al., 2011). The consuption of food per nests has been estimated to be 44.5 kg of plant organic material/ha-year, corresponding to 13 % of the annual production of wood litter in the 'Caatinga' (dry shrubland) biome (Moura et al., 2008).

Nests are often built on trees (Fig. 1.2A), although rocks are also used as nest attachments (Moura et al., 2006b; Vasconcellos et al., 2007). In some Brazilian regions, incipient nests of this species are epigeous (Fig. 1.2B) whereas mature nests are arboreal (Vasconcellos et al., 2007; Cristaldo and DeSouza, *pers. obs.*). Bezerra-Gusmão et al. (2009) observed that in 'Caatinga' biome, this species builds polycalic nests. The nest population can be found all over the nest (Cunha et al., 2003), but the major part is concentrated in the nest core (Cristaldo, *pers. obs.*). There is no distinct royal cell inside the nest. The royal pair and the eggs are found close together, in the inner part of the nest, near the stem of the support tree, where the walls are thicker and compacted but they also can found hiding in the depression of the bark of the tree (Cunha & Brandão, 2002). Active nests can harbor many cohabitants, including one obligatory inquiline and a large number of termitophiles, mainly Staphylinidae (Coleoptera) (Mathews, 1977; Godinho & Lins, 1989; Cunha & Brandão, 2000; Cunha et al., 2003; Vasconcellos et al., 2007). The parts of the nests occupied by the inquiline contain a mass of dark organic material (Mathews, 1977).

Inquilinitermes spp. are medium to small termite species occurring in South America where they are obligate inquilines in the nests of *Constrictotermes* spp., probably deriving their food from the nest material (Mathews, 1977). *Inquilinitermes microcerus* Silvestri, 1901 (Termitidae: Termitinae) (Fig. 1.1B) is only found in *C. cyphergaster* nests, where their galleries are of dark grey material, distinct from the *C. cyphergaster* nests (Mathews, 1977). The size of *I. microcerus* colonies are smaller than the builder termite, varying from 160 to 10,840 individuals (Cunha et al., 2003). The colonies are restricted to certain portions of the nest, usually close to its core (Cunha et al., 2003). There is surprisingly little information in the literature about their biology and ecology.
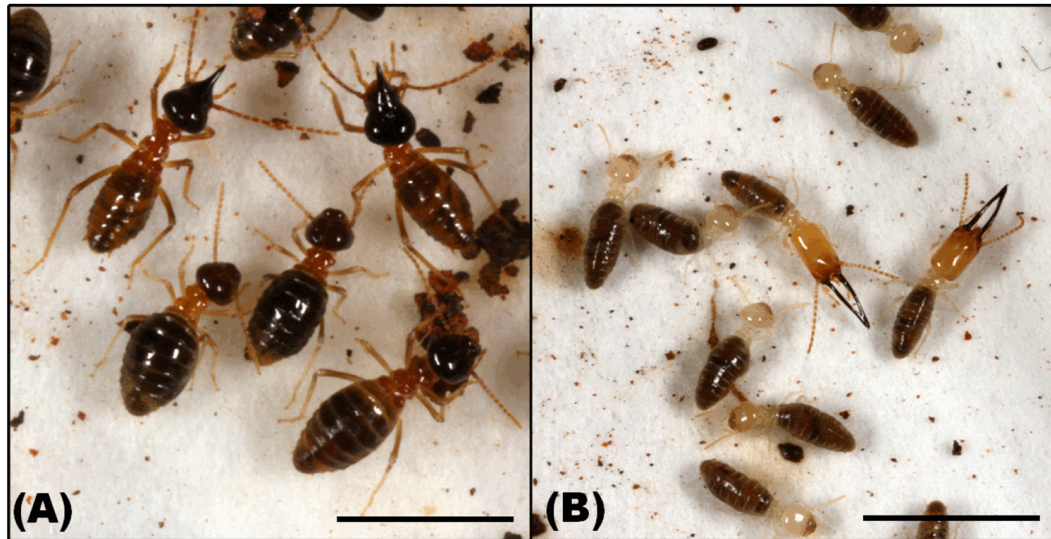
Figure 1.1: The system used in the field and lab bioassays. **A:** The host species *Constrictotermes cyphergaster*; **B:** The obligatory inquiline species *Inquilinitermes microcerus*. Scale bars represents 5 mm. Photos: J. Šobotník.

Figure 1.2: *Constrictotermes cyphergaster* nests in Sete Lagoas-MG, Brazil. **A:** Arboreal nest built on tree; **B:** Incipient nests built on ground - epigeous nests. Photos: A. Marins.

Overview of Chapters

The first chapter is the General Introduction that provides to readers an overview of the central topic of the thesis and the specific questions addressed in the following chapters. The subsequent chapters (from chapter 2 to chapter 5) are composed by manuscripts, in which it was tested some ecological and chemical aspects involved in the nest-sharing among a termite host and its obligatory inquiline. The chapter 6 is a General Conclusion of all results obtained from this thesis. The chapter 7 provides the references used in the present thesis. Below, I will briefly summarize the questions and results addressed in each manuscript (chapters 2 to 5).

Chapter 2: Termitarium volume as a determinant of invasion by obligatory termitophiles and inquilines in the nests of *Constrictotermes cyphergaster* (Termitidae, Nasutitermitinae)

In chapter 2, we analyzed one specific aspect of the dynamics of termite nest colonization by obligatory termitophiles and inquilines, *i.e.*, whether cohabitants were more likely to invade a nest when it reached a critical nest size. Our results indicated that the invasion of *C. cyphergaster* nests by obligatory termitophiles and inquilines was dependent on nest size. There appeared to be a critical nest size above which nests were more prone to invasion. Above this size, there was a significantly higher likelihood of finding obligatory cohabitants. Termitophile species were observed in nests ≥ 2.2 L, whereas inquiline species were only occur in nests ≥ 13.6 L. This may indicate that the obligatory cohabitants studied here did not occupy *C. cyphergaster* nests at random and that they were dependent on features that made these nests suitable for cohabitation, which are linked to colony development. Such findings provide us research pathways for a better understanding of nest-sharing in termites, for example: how termite inquilines manage to cohabit the nest along with the termite host species. This manuscript was published in 2012, in the journal *Insectes Sociaux*.

Chapter 3: Mutual use of trail-following cues by a termite host and its inquiline

In the chapter 3, we studied the trail-following behaviour in *C. cyphergaster* and its

obligatory inquiline, *I. microcerus* (Termitidae: Termitinae). Using behavioural experiments and chemical analyses, we determined that the trail-following pheromone of *C. cyphergaster* is made of neocembrene and (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol. Although no specific compound was identified in *I. microcerus*, workers were able to follow the above compounds in behavioural bioassays. Interestingly, in choice tests, *C. cyphergaster* prefers conspecific over heterospecific trails while *I. microcerus* shows the converse behaviour. In no-choice tests with whole body extracts, *C. cyphergaster* showed no preference for, while *I. microcerus* clearly avoided, heterospecific trails. This seems to agree with the hypothesis that trail-following pheromones may shape the cohabitation of *C. cyphergaster* and *I. microcerus* and reinforce the idea that their cohabitation is based on conflict-avoiding strategies. This was the first study evaluating chemical communication between two closely associated termite species. This manuscript was published in 2014, in the *PLoS ONE*.

Chapter 4: How do you say 'SOS'? Alarm transmission in advanced termite *Constrictotermes cyphergaster* (Blattodea: Termitoidea: Termitidae: Nasutitermitinae)

Here, in chapter 4, we studied the alarm transmission in the host termite *C. cyphergaster*. Observations of *C. cyphergaster* in natural situation revealed the importance of alarm communication, which attract predominantly soldiers towards the source of disturbance. The major source of chemical alarm is the frontal gland of soldier, which produces a rich mixture of terpenoid compounds. Extensive testing led to identification of the alarm pheromone made of abundant monoterpenes *(1S)-α*-pinene and myrcene, along with minor compound, monoterpene *(E)-β*-ocimene. The vibratory alarm signals consist in series of beats produced predominantly by soldiers in response to various endangering stimuli. Our results show that there obviously are different levels of alarm signals: lower excitement is shared with nest-mates rather using vibratory communication, while the alarm pheromone is released during more endangering situations. Such issue allowed us to understand how the alarm communication behave in the host species without presence of its obligatory inquiline species.

Chapter 5: Foreign language skills by the guest? Eavesdropping of host alarm cues by inquiline termite species

In chapter 5 we investigated whether distinct termite species cohabiting the same nest could eavesdrop each other's alarm cues. Using behavioural bioassays under laboratory conditions, we show that the obligatory inquiline *I. microcerus* responds not only to its own alarm signal but also alarm cues from its host. Conversely their host *C. cyphergaster* is able to perceive only its own alarm signal. Inquilines were never observed helping the defense of experimentally damaged nests in the field, despite perceiving their host alarm. Therefore, it seems plausible to infer that termite inquilines eavesdrop their host alarm in their own benefit, e.g., to evade danger signalled by the host or, at least, to avoid nest regions crowded by defenders at a given time.

Termitarium volume as a determinant of invasion by obligatory termitophiles and inquilines in the nests of *Constrictotermes cyphergaster* (Termitidae, Nasutitermitinae)

Insectes Sociaux

RESEARCH ARTICLE

Termitarium volume as a determinant of invasion by obligatory termitophiles and inquilines in the nests of *Constrictotermes cyphergaster* (Termitidae, Nasutitermitinae)

P. F. Cristaldo · C. S. Rosa · D. F. Florencio · A. Marins · O. DeSouza

Abstract A range of organisms can be found inside termite nests where the degree of association can vary from facultative to obligatory dependence. Studies of the dynamics of nest invasion are still unresolved, so how and when cohabitants enter termite nests remain open questions. This study analyzed one specific aspect of the dynamics of termite nest invasion by obligatory termitophiles and inquilines, i.e., whether cohabitants were more likely to invade a nest when it reached a critical nest size. We collected 36 *Constrictotermes cyphergaster* nests of different sizes and sampled their cohabitant fauna. Our results indicated that the invasion of *C. cyphergaster* nests by obligatory termitophiles and inquilines was dependent on nest size. There appeared to be a critical nest size above which nests were more prone to invasion. Above this size, there was a significantly higher likelihood of finding obligatory cohabitants. Termitophile species were observed in nests ≥ 2.2 L, whereas inquiline species were only occur in nests ≥ 13.6 L. This may indicate that the obligatory cohabitants studied here did not occupy *C. cyphergaster* nests at random and that they were dependent on features that made these nests suitable for cohabitation, which are linked to colony development.

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Introduction

Nesting is a characteristic behavior of many invertebrate and vertebrate animals. Nest structures can provide organisms and their offspring a suitable (thermoregulated) and protected environment (Turner, 2000; Jones and Oldroyd, 2007; Hansell, 2007), while in some cases they also provide a place for food storage (Noirot and Darlington, 2000). However, organisms that live in nests for long periods may by troubled by cohabitation or nest usurpation. Examples include, but are not restricted to, birds (Kronland, 2007), salamanders (Harris et al., 1995), fishes (Mills and Reynolds, 2002), and social insects (Ortolani et al., 2008; Tsuneoka, 2008). Nest invaders have been reported to be parasites, commensals, mutualists, or a combination of these (Hughes et al., 2008).

In the case of social hymenopterans, the mechanisms and dynamics of nest invasion, as well as the interactions between host species and cohabitants, have been well investigated (e.g., Rettenmeyer et al., 2011). In termites, there have been plenty of reports on the identity of nest invaders, but studies on the mechanisms, dynamics, and interactions of nest invasion are still ongoing (but see Redford, 1984; Cunha et al., 2003; Costa, 2005; Carrijo et al., 2012).

Organisms that have been found inside termite nests include birds (Brightsmith, 2000), bats (Dechmann et al., 2004), and a range of arthropods (Cunha and Brandão, 2000; Haddad and Dippenaar-Schoeman, 2002; de Visser et al., 2008; Costa et al., 2009; Carrijo et al., 2012), but especially beetles (Costa and Vanin, 2010), ants (Holt and Greenslade, 1979; Diehl et al., 2005), and other termite species (Redford,

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1984; Cunha et al., 2003; Cunha and Morais, 2010; Darlington, 2012).

In general, termite species that are found in other termite nests are known as inquilines and they can be categorized into two types: (1) obligatory inquilines: species that closely interact with the host and that have lost their nest-building ability; (2) facultative inquilines: species that retain their nest-building abilities but that can also inhabit nests built by other termite species (Mathews, 1977). Other animals, mainly insects, which are associated with termite nests are known as termitophiles (Kistner, 1990). Some of these species can also be categorized as "obligatory" provided they spend at least part of their developmental cycle exclusively within termite nests, e.g., some species of Staphylinidae. Others are not restricted to termite nests, so they fall into the "facultative" category.

Studies of termite cohabitation have revealed a positive relationship between nest size and the number of inquiline species (Domingos, 1983; Redford, 1984; Costa, 2005; Cunha and Morais, 2010) and termitophile species (Carvalho, 2005). To the best of our knowledge, no previous studies have addressed the key aspects of the dynamics of nest invasion. Thus, how and when inquilines and termitophiles enter termite nests are questions that remain open to investigation. In this paper, we focus on one specific aspect of the dynamics of a termite nest invasion by obligatory inquilines and termitophiles, i.e., whether there is a critical nest size above which cohabitants are more likely to invade nests. We addressed this question because it is crucial to future research on the biology of termite cohabitants. If invasion occurs after the nest achieves a specific size, cohabitation must be linked to the host colony's development, and this would allow a large range of hypotheses to be tested. In contrast, the absence of a critical nest size for the establishment of cohabitants must indicate that nest invasion can occur irrespective of colony development, which would lead to completely different research questions. As a model for cohabitation, we used the termite builder *Constrictotermes cyphergaster* Silvestri, 1901 (Termitidae: Nasutitermitinae), its obligatory inquiline *Inquilinitermes microcerus* Silvestri, 1901 (Termitidae: Termitinae), and four species of Coleoptera: Staphylinidae obligatory termitophiles, i.e., *Corotoca melantho* Schiødte, 1853, *Termitocola silvestrii* (Wasmann, 1902), *Spirachtha eurymedusa* Schiødte, 1853, and *Termitoiceus* sp. nov.

Materials and methods

Terms definition

The term "colony" will be used to refer to the biological structure of termite individuals living together, which includes the reproductive pair, workers, soldiers, nymphs (semi-mature young), larvae (termitological jargon for recently hatched young), and eggs. This is intended to be distinct from the "nest", which refers to the physical structure built by termites. "Inquiline" refers to a termite species that establishes its colonies within a nest previously built by other termite species. Here, "termitophile" will refer to four staphylinid species found inside the study nests. The inquiline and termitophile species investigated in this study are considered to be "obligatory" cohabitants because they are not found outside termite nests.

Builder species

Constrictotermes cyphergaster is a common termite species in Brazil, Paraguay, Bolivia, and Northern Argentina (Mathews, 1977; Godinho and Lins, 1989; Moura et al., 2006a). The nest volume and population size or biomass have a linear positive correlation (Vasconcellos et al., 2007). *Constrictotermes cyphergaster* forages at night in exposed columns (Moura et al., 2006a) and feeds mainly on wood in different stages of decomposition, on the surface of the bark of live trees (Moura et al., 2006b), and on lichens (Bourguignon et al., 2011); their consumption per nests has been estimated to be 44.5 kg of plant organic material/ha-year, corresponding to 13 % of the annual production of wood litter (Moura et al., 2008). The nymphal line comprises one larval instar, followed by five nymphal instars, and alates. The apterous line includes two larval instars, followed by workers, presoldiers and soldiers. Both workers and soldiers were shown to be monomorphic, having only one instar each and consisting of only male specimens (Moura et al., 2011). An average of 4.5 workers per soldier has been recorded by Cunha et al. (2003) in *C. cyphergaster* nests.

In the study site, this species builds fragile nests with weak walls made from a thin layer of soil. However, this species has been observed to build nests with hard clay walls in other Brazilian regions (DeSouza, pers. obs.). Nests are often built on trees, although rocks are also used as nest attachments (Moura et al., 2006b; Vasconcellos et al., 2007). In some Brazilian regions, incipient nests of this species are epigeous whereas mature nests are arboreal (Vasconcellos et al., 2007; Cristaldo and DeSouza, pers. obs.). Bezerra-Gusmão et al. (2009) observed that in "Caatinga" (dry shrubland) biome, this species builds polycalic nests. According to Mathews (1977), the interior of the nest consists of fairly thin-walled irregular cells with a gray/buff-colored lining of fecal origin; these cells are large near the outside of the nest but smaller towards the center; they interconnect by small termite-sized holes and do not lead into each other like the much more convoluted galleries of *Nasutitermes* nests. The nest population can be

found all over the nest (Cunha et al., 2003), but the major part is concentrated in the nest core (Cristaldo, pers. obs.). There is no distinct royal cell inside the nest. The royal pair and the eggs are found close together, in the inner part of the nest, near the stem of the support tree, where the walls are thicker and compacted but they also can found hiding in the depression of the bark of the tree (Cunha and Brandão, 2002). Active nests can harbor many cohabitants, including one of two obligatory inquilines, i.e., *Inquilinitermes microcerus* and *Inquilinitermes fur* Silvestri, 1901, and a large number of termitophiles, mainly Staphylinidae (Coleoptera) (Mathews, 1977; Godinho and Lins, 1989; Cunha and Brandão, 2000; Cunha et al., 2003; Vasconcellos et al., 2007). The parts of the nests occupied by cohabitants contain a mass of dark organic material (Mathews, 1977); however, the Staphylinidae species are also found in the middle parts of the nest in close association with the colony (Cristaldo and Rosa, pers. obs.).

Inquiline species

Inquilinitermes spp. are medium to small termite species occurring in South America where they are obligate inquilines in the arboreal nests of *Constrictotermes* spp., probably deriving their food from the nest material (Mathews, 1977). *Inquilinitermes microcerus* is only found in *C. cyphergaster* nests, where their galleries are of dark grey material, distinct from the *C. cyphergaster* nests (Mathews, 1977). The size of *I. microcerus* colonies are smaller than the builder termite, varying from 160 to 10,840 individuals (Cunha et al., 2003). The colonies are restricted to certain portions of the nest, usually close to its core (Cunha et al., 2003).

Termitophile species

Coleoptera, mainly Staphylinidae, have more termitophiles forms than any other group, perhaps more than all other groups put together (Seevers, 1957; Kistner, 1969, 1982; 1990; Grassé, 1986; Jacobson et al., 1986). Despite the large number of reports, there is surprisingly little in the literature about their biology and how they are collected (Kistner, 1969, 1990). The Aleocharine subfamily is by far the most successful group of cohabitant in nests of social insects, especially in ant and termite nests (Seevers, 1957, 1965 and references). One of the most extraordinary features of termitophile Staphilynidae is the ability of many species to achieve physogastry. Sometimes, this physogastry is followed by subsequent secondary sclerotization of some or all of the expanded membrane. This is a feature of the Corotocini tribe (Jacobson et al., 1986), here represented by *Corotoca melantho*, *Spirachtha eurymedusa* and *Termitoiceus* sp. nov. (Seevers, 1957; Kistner, 1982; Jacobson et al., 1986; Kistner, 1990). The abdomen of some Coro-

tocini can be further modified to include the presence of several pairs of membranous lobes that look like legs (*S. eurymedusa*) and according to Kistner (1979) this feature is used by beetles to integrate themselves into the termite society. Another interesting feature is the viviparity in *Corotoca*, and perhaps in *Spirachtha* (Seevers, 1957). All these features allow these species to engage close relationships with their termites hosts, enough to be classified as "True Guests" (Kistner, 1979). These three Corotocini species were found only in *Constrictotermes* spp. nests, so far (Seevers, 1957; Fontes, 1977; Jacobson et al., 1986). Although all Corotocini termitophiles exhibit some degree of physogastry, the members of the tribe Termitonannini (here represented by *Termitocola silvestrii* (Wasmann 1902)) in the majority of cases are limuloid (Seevers, 1957; Kistner, 1969). *Termitocola silvestrii* have been found associated exclusively with *C. cyphergaster* (Seevers, 1957; Kistner, 1979; Cunha and Brandão, 2000).

Study site

The study was conducted in the "Cerrado" (an environment that is ecologically but not floristically similar to savanna) near Sete Lagoas town (19°27′S, 44°14′W), in the state of Minas Gerais, southeastern Brazil. The altitude varies from 800 to 900 m above sea level at the study site. According to Köppen's classification, the study area lies in Aw (equatorial with dry winter) climate (Kottek et al., 2006). The total accumulated rainfall during 2008 was 1,469 mm while the mean monthly temperature varied from 16.4 to 29.1 °C (Agritempo, 2009). In 2011, the rainfall was 695.3 mm while the mean monthly temperature varied from 16.9 to 29.3 °C (Agritempo, 2012). Natural fire is recurrent in the entire Cerrado and at this particular site. The native biota is considered fire-dependent or at least fire-tolerant (Coutinho, 1990; DeSouza et al., 2003).

Sampling

To test the effect of nest size on the presence of obligatory cohabitants in *C. cyphergaster* nests, we removed 36 nests of different sizes from trees, which were measured and dissected thoroughly to search for cohabitants. The choice of nests was made arbitrarily, in an attempt to sample nests in a continuous range of volume that was manageable for our aim. All cohabitants found in the nest (including the resident builders) were collected with entomological forceps, placed in 80 % alcohol, labeled, and taken to the laboratory for identification. Termites were identified to species level, following Mathews (1977) and specific literature referred to by Constantino (2002). Identifications were confirmed by comparison with the collection of the Isoptera Section of the Entomological Museum of the Federal

University of Viçosa (MEUV), where voucher specimens were deposited. Termitophiles were sent to specialists for identification to the lowest taxonomic level possible.

Sampling was conducted in July 2008 and May 2011, during daylight from 7:30 a.m. to 2:00 p.m. Only undamaged arboreal nests were inspected.

Nest volumes were recorded as suitable descriptors of size. Volumes of termite nests are usually calculated with a formula for the most approximate geometric form (see Coles, 1980; Domingos, 1983; Cunha et al., 2003). However, the wide geometric diversity of termite nest forms (Negret and Redford, 1982), coupled with frequent irregularities in shapes, suggest that this type of approximation will always be prone to significant errors. To minimize this error, nests volumes were calculated based on the Cavalieri principle, by summing up the volumes of several superimposed cylindrical cross sections into which the nest was visually decomposed and the volumes of the hemispherical caps at both ends of the nest (see Fig. 1) in a manner similar to that described in Rodrigues et al. (2004). To improve accuracy, in cases where the nest encompassed its supporting tree trunk, the volume of the trunk was also estimated and subtracted from total estimated nest volume.

Data analysis

The data were analyzed by logistic regression, a form of generalized linear modeling (GLM) under binomial errors, which is suitable for modeling the effects of one or more continuous or categorical explanatory variables on a binary response variable (Logan, 2010). We aimed to determine the effects of the continuous explanatory variable "nest volume" (x-var) on the binary response variable "presence/absence of cohabitants" (y-var).

Logistic models differ from traditional linear regression models because they do not describe the rate of change in the response variable with change in the explanatory variable. Our logistic model describes the likelihood that a given cohabitant would be present in a termitarium of a given size, thereby establishing the likelihood of a relationship between the response and the predictor. A linear model would be inappropriate in this case because it is not bounded by logical probability limits of 0 and 1.

We used a GLM to test whether a sigmoid curve with an asymptote towards 0 and 1 at the y-axis (i.e., a logistic model) fitted the data better than a horizontal line parallel to the x-axis, which intercepted the y-axis at 0.5 (i.e., equal chances of presence and absence). The choice of this sigmoid function (H_1) over a horizontal line (H_0) would establish a relationship between nest volume and cohabitation and also show that there is a critical nest volume above which cohabitants are more likely to be found in the nest (i.e., likelihood >50 %). This critical size would correspond to the inflection point of the sigmoid curve. The likelihood of housing termitophiles/inquilines is related to the nest volume according to the logistic equations:

(a) termitophiles
$$\log(p/q) = -0.4608 + 0.2106v$$

(b) inquilines
$$\log(p/q) = -3.1265 + 0.2296v$$

where (p/q) is the odds ratio of housing/not housing a guest and v is the nest volume, given in liters (L).

Analyses were performed in *R* (R Development Core Team, 2011), followed by residual analysis to check the suitability of the error distribution and model fitting.

Fig. 1 Nest volumes were calculated by summing the volumes of cylinders and hemispherical caps into which the nest was visually decomposed
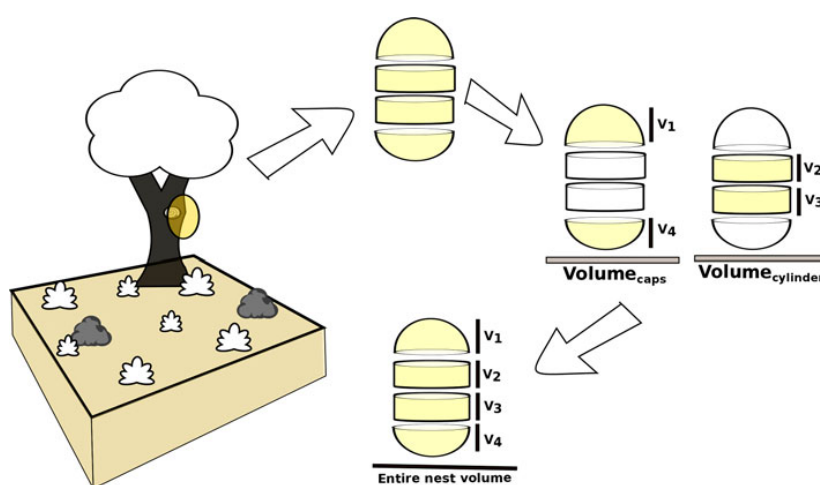
Results

Of the 36 termite nests built by *C. cyphergaster*, 21 (70 %) contained inquilines and termitophiles, nine (25 %) contained only termitophiles, while six (16.7 %) contained no cohabitants. There were two species of inquilines, i.e., *I. microcerus* and *Heterotermes longiceps* Snyder, 1924 (Rhinotermitidae: Heterotermitinae). Of these, only the first is known to be an obligatory inquiline. *Heterotermes longiceps* was found in a single nest alongside *I. microcerus* and staphylinid termitophiles. Among the termitophiles, there were four Staphylinidae species, i.e., *Corotoca melantho*, *Termitocola silvestrii*, *Spirachtha eurymedusa*, and *Termitoiceus* sp. nov.. *Corotoca melantho* was the most frequent and it was present in all 30 nests containing termitophiles, while *T. silvestrii* was found in eight nests, *S. eurymedusa* in three nests and *Termitoiceus* sp. nov. in only one nest. Nest volumes ranged from 0.42 to 51.30 L with an average of 19.49 L. The distribution of termitophile and inquiline species along the nest sizes was summarized in the Supplementary Material 1. The nest size was correlated with the presence of termitophile ($\chi^2 = 20.756$, $df = 35$, $p < 0.001$) and inquiline species ($\chi^2 = 21.294$, $df = 35$, $p < 0.001$). The analyses indicated a critical
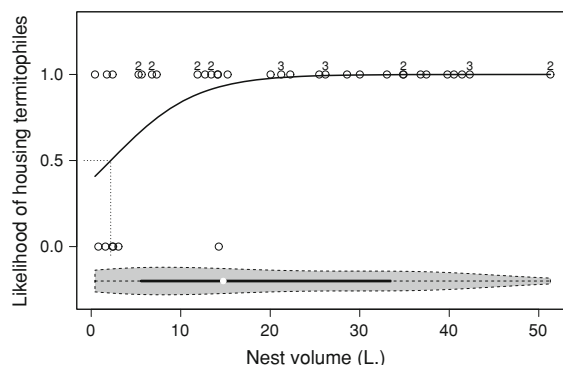
Fig. 2 Effect of nest volume on the presence of four species of staphylinid termitophiles in *Constrictotermes cyphergaster* nests ($\chi^2 = 20.756$, $df = 35$, $p < 0.001$). Sete Lagoas, MG, Brazil, 2008–2011. Logistic regression with Generalized Linear Models under binomial errors and logit link function. On *y*-axis, *0* indicates the absence of termitophiles whereas *1* indicates their presence. Each *dot* refers to a single termitarium. At the *upper set of dots* (i.e., at $y = 1$) *superscript digits* indicate the number of termitophile species inhabiting that given termitarium; absence of digits indicates a single termitophile species. Likelihood of housing termitophile species is related to nest volume according to the logistic equation: Log $(p/q) = -0.4608 + 0.2106v$; where (p/q) is the odds ratio of housing/not housing termitophiles and *v* is the nest volume, given in liters (L). The critical nest volume above which the likelihood of housing termitophiles exceeds 50 % is represented by a *dotted line* (2.2 L). At the *bottom*, a violin plot depicts for all inspected nest volumes, the median (*white dot*) and its associated first and third quartiles (*black bar*) as well as the density trace (*grey area*)
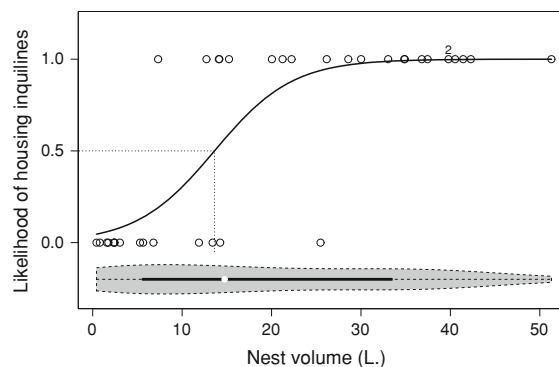
Fig. 3 Effect of nest volume on the presence of *Inquilinitermes microcerus* (Isoptera: Termitidae) inquiline in *Constrictotermes cyphergaster* (Isoptera: Termitidae) nests ($\chi^2 = 21.294$, $df = 35$, $p < 0.001$). Sete Lagoas, MG, Brazil, 2008-2011. Logistic regression with Generalized Linear Models under binomial errors and logit link function. On *y*-axis, 0 indicates the absence of inquilines whereas *1* indicates their presence. Each *dot* refers to a single termitarium. At the *upper set of dots* (i.e., at $y = 1$) *superscript* digits indicate the number of inquiline species inhabiting that given termitarium; absence of digits indicates a single inquiline species. Likelihood of housing inquiline species is related to nest volume according to the logistic equation: Log $(p/q) = -3.1265 + 0.229v$; where (p/q) is the odds ratio of housing/not housing inquilines and *v* is the nest volume, given in liters (L). The critical nest volume above which the likelihood of housing inquiline exceeds 50 % is represented by a *dotted line* (13.6 L.). At the *bottom*, a violin plot depicts for all inspected nest volumes, the median (*white dot*) and its associated first and third quartiles (*black bar*) as well as the density trace (*grey area*)

volume above which cohabitants were more likely to be found in *C. cyphergaster* nests, and this volume was smaller for termitophiles than inquilines. Termitophiles were more likely to be found in nests $\geq 2.2 \pm 0.10$ L (Fig. 2) whereas inquilines were more likely to be found in nests $\geq 13.6 \pm 0.07$ L (Fig. 3).

Discussion

Our results suggest that the invasion of *C. cyphergaster* nests by obligatory termitophiles and inquilines was dependent on nest size (Figs 2, 3). There appeared to be a critical nest size above which nests were more prone to invasion, and above this size there was a significant increase in the likelihood of finding obligatory cohabitants in *C. cyphergaster* nests.

Several reports have shown a positive correlation between nest size and the richness and abundance of cohabitants in termite nests, including nests built by *Armitermes euamignathus* Silvestri, 1901 (Termitidae: Syntermitinae) (Domingos, 1983), *C. cyphergaster* (Cunha et al., 2003), *Cornitermes cumulans* Kollar in Pohl, 1832 (Termitidae: Syntermitinae) (Redford, 1984; Costa, 2005; Carvalho, 2005), *Cornitermes snyderi* Emerson in Snyder, 1949

(Termitidae: Syntermitinae), and *Syntermes grandis* Rambur, 1842 (Termitidae: Syntermitinae) (Cunha and Morais, 2010). The novel finding of our study was that, at least for the cohabitants studied here, this correlation did not hold linearly for all nest sizes, while it dropped to zero cohabitants in nests below a specific size. This may indicate that, rather than randomly invading nests, inquilines and termitophiles can only settle in suitable nests and this suitability is linked to the nest size. Previous studies do not mention this kind of relationship but, rather, report (in some cases only suggest) a linear one, even when dealing with the same termite host species, *C. cyphergaster*. This seems to be more linked to the sampling and analytical procedures than to any biological reason. First, some authors (e.g., Domingos, 1983; Redford, 1984) do not present a formal statistical test of the alleged positive correlation between nest volume and termitophile/inquiline abundance and richness. Rather, they derive this pattern from field observation and accumulated experience. Second, logistic relationships as the one described here need data points referring to "zero values", i.e., termitaria holding only the builder termite and no inquilines or termitophiles, but some authors (e.g. Cunha et al., 2003; Costa, 2005; Carvalho, 2005) focused their work exclusively on guest-bearing termitaria. Third, even when considering these non-invaded termitaria, authors (e.g. Cunha and Morais, 2010) do not explicitly model invader presence as a sigmoidal function of nest volume/size.

Of the various hypotheses that might explain our result, the simplest one is related to the incremental increase in free space as a nest grows. In termite nests, linear and sublinear correlations are known to exist between nest size and its termite builder population (Josens and Soki, 2010). Specifically for *C. cyphergaster* nests, this correlation is linear (Vasconcellos et al., 2007; Josens and Soki, 2010) but their population is bound to fluctuate seasonally (Lepage and Darlington, 2000; Moura et al., 2006b) such that some parts of the nest may remain unoccupied by the resident builders, as it happens with other termite builders (Noirot and Darlington, 2000). It is conceivable that such spaces would be larger and more frequent in bigger nests compared with smaller nests, such that some of these spaces may be largely unpatrolled above a certain nest size. Invaders might therefore more frequently enter in such unoccupied spaces in the case of larger nests, leading to the sigmoid function observed in Figs. 2, 3. This might apply to any free space in nests and also to a mass of dark organic material found in the nests of *C. cyphergaster*, which is known to house inquilines and termitophiles (Mathews, 1977).

A deeper consideration of our results showed that termitophiles and inquilines differed substantially in terms of the critical nest size correlated with their presence in *C. cyphergaster* termitaria. Termitophilous staphylinids were more likely to occupy nests that were on average three times smaller than the nests where *I. microcerus* inquilines also occurred. If nest size correlated with nest age in such a species (as it seems to be), one could safely assume that staphylinids were able to invade *C. cyphergaster* nests earlier than *I. microcerus*. This might lead to hypotheses that link the degree of colony maturation to invasion by cohabitants. Termitophile staphylinids here reported seem not to need to be hosted by a fully mature termite colony, but appear to only require that the colony is established and functional. Among them, *C. melantho* and *S. eurymedusa* present mimetic physogastry and are well accepted by their hosts, being licked and fed by termite workers (Costa and Vanin, 2010; Grassé, 1986). It seems therefore that, as long as a colony holds enough workers, such staphylinids would be able to establish themselves as termitophiles. On the other hand, the limuloid body of *T. silvestrii* seems to obstruct interactions, as it would ease hiding and offer a few contact points a termite could grab if trying to attack the beetle. It appears that, unlike the previous two termitophiles which possess morphogical features enabling interactions with their host, *T. silvestrii* traits help to minimize such contacts. This could mean that *T. silvestrii* does not actually need the termite colony itself but it is linked to some (unknown) feature of the termitarium. If this is so, *T. silvestrii* would be able to invade a nest as soon as it attains some minimal set of typical traits. Associations of organisms with termitaria rather than termites are not a novelty, and are known as "termitariophily", a term coined by K. Berg as early as 1900 (Araújo 1970, p. 536). In short, a termite nest would be suitable to these staphylinids as soon as it attains a size enough to hold (1) a minimum critical amount of workers to keep interactions stable or (2) a minimal set of features which characterizes it as a proper termite nest. As these conditions are not necessarily linked to full colony maturation, small young nests may be suitable to invasion by such beetles, and this would explain the differences in patterns recorded in Figs. 2, 3.

Inquilinitermes microcerus seemed to be much more dependent on the maturity of the host colony, because they were found in larger (and possibly older) nests than those occupied by staphylinids. One particular feature of *C. cyphergaster* nests suggested a link between their maturity and the likelihood of their invasion by *I. microcerus*. These nests originate in the soil, where they first develop an epigeous structure that houses the colony while an arboreal unit is built. When an arboreal unit reaches around 7 L in size, the royal couple moves in after migrating from the epigeous unit, which is then abandoned (Vasconcellos et al., 2007). Thus, if *I. microcerus* preferably occupies *C. cyphergaster* arboreal nests measuring ≥ 13.6 L, as depicted in Fig. 3, they will necessarily invade nests that have been housing a royal couple for a long period and such nests might possibly

be fully mature. This hypothetical interdependence between nest maturity and invasion by *I. microcerus* remains open to investigation. However, some evidence seems to support this hypothesis, because *Inquilinitermes* sp. have been seen entering *C. cyphergaster* nests through structures built to release their alates (M.A. Bezerra-Gusmão, pers. comm.) and alates are only released from mature nests.

Clearly, all these hypotheses require further testing and we present them as possible explanations to highlight research pathways that might lead to a better understanding of the phenomenon of cohabitation in termite nests. These hypotheses appear to follow naturally from the study reported here, because our focal inquilines and termitophiles did not occupy *C. cyphergaster* nests at random. Instead, nest invasion was apparently dependent on features that make these nests suitable for cohabitation, which may be linked to colony development.

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Chapter 3

Mutual use of trail-following chemical cues by a termite host and its inquiline

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Mutual Use of Trail-Following Chemical Cues by a Termite Host and Its Inquiline

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Abstract

Termite nests are often secondarily inhabited by other termite species (= inquilines) that cohabit with the host. To understand this association, we studied the trail-following behaviour in two Neotropical species, *Constrictotermes cyphergaster* (Termitidae: Nasutitermitinae) and its obligatory inquiline, *Inquilinitermes microcerus* (Termitidae: Termitinae). Using behavioural experiments and chemical analyses, we determined that the trail-following pheromone of *C. cyphergaster* is made of neocembrene and (3Z,6Z,8E)-dodeca-3,6,8-trien-1-ol. Although no specific compound was identified in *I. microcerus*, workers were able to follow the above compounds in behavioural bioassays. Interestingly, in choice tests, *C. cyphergaster* prefers conspecific over heterospecific trails while *I. microcerus* shows the converse behaviour. In no-choice tests with whole body extracts, *C. cyphergaster* showed no preference for, while *I. microcerus* clearly avoided heterospecific trails. This seems to agree with the hypothesis that trail-following pheromones may shape the cohabitation of *C. cyphergaster* and *I. microcerus* and reinforce the idea that their cohabitation is based on conflict-avoiding strategies.

Introduction

A wide variety of species adopt the strategy to live in close association to each other, establishing symbiotic interactions (see e.g. [1–4]). Inquilinism stands among the most specialized forms of symbioses: more than symbiotically interacting in the open space, inquilines cohabit with their host in the nest which these latter have built to house their own relatives [5]. Fundamentally, inquilines are equivalent to better known symbionts, such as gut-inhabiting bacteria, but differ in being naked-eye observable and easily manipulated in lab assays. The ubiquity of inquilinism is impressive and examples include red-headed woodpeckers, cuckoos and cowbirds [6,7], salamanders [8], and especially social insects (see [9–14]). While existing in virtually all known social insects, subtle differences can be observed between Hymenoptera and Isoptera inquilines. In Hymenopterans, inquilines (or "social parasites") live in close contact with their host as brood parasites [15], whereas in termites, inquilines tend to establish themselves apart from their hosts within the nest [16]. In termites the term "inquilinism" is reserved for heterospecific termite-termite cohabitation [14]. Low frequency of direct contact between termite inquilines and their hosts by no means precludes the need to negotiate cohabitation. By occupying a space originally built for

the host's nestmates, feeding on the lining of the nest walls, or stealing stored products [17–20], inquilines most certainly inflict costs to their hosts. Chances of occasional encounters are increased by the fact that cohabitation may take place in volumes as small as 13 litres [14], with inquilines inhabiting the "heart of the hive" and being outnumbered by their host [5]. All this would enhance selective pressures for defensive strategies on the part of the host with consequent development of deceiving strategies on the part of inquilines, establishing arms races likely similar to those observed for cuckoos *versus* hosts in birds and in other social insects [21].

Because termite defence is carried out by blind individuals, it is conceivable to think that both sets of strategies would rely markedly on chemical cues. After all, such cues allow social insects to behave altruistically towards nestmates and discourage the presence of intruders in their society [9]. One of these signals is the trail-following pheromone, which in termites presents surprisingly low chemical diversity, with only 9 active compounds (alcohols, aldehydes, ketone, and hydrocarbons) identified so far [22–24]. This low complexity is further reduced by phylogenetic constraints: in the basal termites (Mastotermitidae, Archotermopsidae, Stolotermitidae), trail-following pheromones are composed of C14 or C18 aldehydes, while in all higher termites, trail pheromones

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comprise C12 alcohols and/or hydrocarbon neocembrene [22,23]. The only exception is *Glossotermes*, member of the phylogenetically transitional family Serritermitidae [25], whose trail-following pheromone has C19 ketone [24]. Single component trail-following pheromones have been identified in all studied termite species except *Prorhinotermes simplex* (Rhinotermitidae), *Amitermes evuncifer* (Termitidae: Termitinae) and several Nasutitermitinae (Termitidae), in which the pheromone always consists of neocembrene and (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol [26–28].

Such a low chemical diversity coupled with high phylogenetic similarity makes trail-following pheromones likely candidates for deceiving strategies on the part of inquilines. After all, manipulation of host-inquiline communication by inquilines would not need new physiologic routes to produce specific compounds.

To the best of our knowledge, no attempt to disentangle host-inquiline communication in termites has been made yet and the mechanisms of cohabitation between them remain enigmatic. Here, we investigated the hypothesis that trail-following pheromones may shape the association between host and inquilines in termites. As a model, we used *Constrictotermes cyphergaster* (Silvestri 1901) and its obligatory inquiline *Inquilitermes microcerus* (Silvestri 1901). *Constrictotermes cyphergaster* (Termitidae: Nasutitermitinae) is a common Neotropical termite species occurring in Brazil, Paraguay, Bolivia, and Northern Argentina [17]. Workers leave the nest in columns and forage at night in the open air under the protection of soldiers [29], and feed predominantly on debris [30] and lichens [19] on the surface of tree barks. *Constrictotermes* spp. nests harbour many organisms but they are not known to commonly house termite inquilines other than *Inquilinitermes* spp. [17,31]. Among these, *I. microcerus* (Termitidae: Termitinae) is known to live exclusively in *C. cyphergaster* nests, in galleries separated from their host's [14], feeding on a highly decomposed diet which may consist of the lining of the nest walls [20]. The colonies are restricted to certain portions of the nest, usually close to its core [32].

To accomplish our aims, we have *(i)* studied the nature of trail-following pheromones in *C. cyphergaster* and *I. microcerus*, and *(ii)* tested for mutual recognition of one another's trails. Gas chromatography coupled with mass spectrometry was used to inspect chemical composition of these pheromones. Additionally, behavioural assays evaluated *(i)* the orientation of workers on conspecific *vs.* heterospecific trails, and *(ii)* the possible use of the host trail-following pheromone by the inquiline (for avoidance or orientation) and the use of the inquiline trail-following pheromone by the host (for detection and elimination of the inquiline).

Materials and Methods

Ethics Statement

A permit for termite collecting was provided by IBAMA to ODS, PFC and JŠ (33094). An export permit was provided by CNPq-Brazil (001347/2012-8) and the import permits were provided by Division of Protection against Harmful Organism-Czech Republic (SRS 032901/2012 and 032904/2012). No specific permits were required for the described studies undertaken in the laboratory with a non-endangered or protected species.

Definitions

We adopt here the same terminology used by [20]: the term "nest" denotes the physical structure built by termites and the term "colony" denotes the assemblage of individuals of a given species, living and cooperating within the nest. "Cohabitation" refers to the simultaneous occurence of colonies of different termite species within a given nest, without implication of reciprocal positive or negative influences.

Study site, Collection and Maintenance

Arboreal nests of *C. cyphergaster* containing colonies of *I. microcerus* were sampled near Sete Lagoas town (19′27°S, 44′14°W; Minas Gerais, Brazil); the site belongs to a vegetational formation physiognomically, but not floristically, similar to savannas ("cerrado"). We collected altogether 13 colonies, of which seven were transported to Viçosa (Minas Gerais, Brazil) in July 2012 and large parts of the six other colonies were transported to Prague in September 2012. The work started in Viçosa, where the colonies were kept in ambient lab conditions ($\pm 26°C$ and low relative air humidity), while in Prague the fragments of colonies were kept inside plastic boxes at temperature $\pm 27°C$ and low relative air humidity. *Constrictotermes cyphergaster* was allowed to forage on large trays where bark covered with algae, mosses and lichens served as food and pieces of cotton soaked with water served as the water source; *I. microcerus* was never observed outside its galleries.

Anatomy of sternal glands

Ten workers of both species, from distinct colonies, were anesthetized on ice and immediately dissected, embedded into Spurr's resin[(TM)] following a well-established protocol, sectioned with a Reichert-Jung Ultracut Microtome[(TM)] and studied with a Carl-Zeiss Amplival[(TM)] optical microscope (for details see [33]). Size of sternal glands was measured by ImageJ software. Additionally, the worker's body size in both species was measured.

Preparation of whole bodies and sternal glands extracts

Whole body extracts (WBE) were prepared from workers (100–400 per sample) submerged in hexane (approximately 10 µl/1 worker) and kept for 24 h at 4°C. After this extraction, a second wash was done with approximately 100 µl of hexane at laboratory temperature, and both washes were merged. Subsequently, the volume of the extract was reduced under the nitrogen flow and the equivalent *per* worker serving as a measure in the bioassays was calculated. Sternal glands extracts (SGE) were prepared from the 4th and 5th sternites dissected from cold-immobilized workers (50–100 glands per sample), submerged into hexane (10 µl/1 gland), extracted for 6 h at 4°C and afterwards merged to a second wash done with 100 µl of hexane at laboratory temperature. After being merged, both extracts (WBE and SGE) were stored at −18°C prior to use. WBE extracts were prepared using host and inquiline workers from three distinct nests (hence, three colonies of hosts and three colonies of their respective inquilines). Likewise, SGE extracts were prepared from another three nests. Each of those colonies have been used only once and extracts originated from them were tested independently as distinct replicates of the bioassays, as described below ("Behavioural experiments" section). Each replicate was comprised of an extract prepared with workers from a single colony.

Pheromone Standards

Synthetic standard of (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (dodecatrienol; RI 1528) was kindly provided by Ullrich Jahn (Institute of Organic Chemistry and Biochemistry, Czech Republic). (1*E*,5*E*,9*E*,12*R*)-1,5,9-Trimethyl-12-(1-methylethenyl)cyclotetradeca-1,5,9-triene (neocembrene; RI 1959) was isolated from tergal glands of *Nasutitermes voeltzkowi* female alates (for details see [34]).

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Chemical analyses

Chemical analyses were performed using comprehensive two-dimensional gas chromatography coupled with mass spectrometric detection (GC×GC/TOF-MS; Pegasus 3D, Leco, St. Joseph, MI, USA). The first dimension column was a non-polar ZB5-MS (30 m, id 0.25 mm, 0.25 μm phase thickness) and the second dimension column was a polar RTX-50 (2 m, id 0.1 mm, 0.1 μm phase). The temperature program for the first column was 50°C (1 min) to 320°C (4 min) at 8°C/min range; the secondary column temperature was set 10°C higher.

Samples were concentrated to approximately 10 μl and then 1 μl was injected in a splitless mode. Injector temperature was 220°C. Helium (flow rate, 1.0 ml/min) was used as a carrier gas. Modulation period was 4 s. TOF-MS detector conditions were as follow: ion source temperature 220°C, detector voltage 1,750 V, filament base voltage −70 V, acquisition rate 100 spectra/s. Redistilled hexane (Merck, for organic trace analysis) was used for extracts and standard solutions.

Behavioral experiments

The following bioassays were performed in open-field on Whatman N° 1 filter paper discs (Ø 15 cm diameter) covered by a large Petri dish, in ambient lab temperature and under dimmed light. Hamilton syringes (10 μl) were used in order to lay down the scent trails (see below) onto the filter paper, in all used concentrations. A worker was deposited in a release chamber (a plastic vial 3 cm in diameter with a 2 mm wide opening) from which it was allowed to walk on the filter paper to follow the scent trail. For each worker tested, a new trail was laid down on a new filter paper. Travelled distances and specific behaviours were recorded for each termite, as described below. Bioassays were carried out independently with both host and inquiline cohabiting workers using extracts made from the respective species and colonies. Each bioassay involved ten workers of both species collected from three colonies drawn randomly from the six available nests.

Intraspecific trail-following. Workers of *C. cyphergaster* and *I. microcerus* were subjected to Y-shape trail-following bioassays (see Fig. 1A), as described in [35] to test their orientation activity to *(i)* their own WBE (in equivalents per cm) or SGE (in equivalents per cm) and *(ii)* standards (neocembrene, dodecatrienol and mixture of neocembrene and dodecatrienol) (concentrations in ng per cm). Equivalent choice tests were also done to compare the orientation activity of SGE and standards in *I. microcerus* workers (see Tab. 1 for an overview of all bioassays).

Interspecific trail-following. Interspecific trail-following bioassays were performed to test *(i)* the orientation on trails made with their own extract (conspecific, CS) *vs.* trails made of the other species (heterospecific, HS) and *(ii)* the possible exploitation of *C. cyphergaster* (host) trail-following pheromone by the *I. microcerus* (inquiline) and *vice versa*. Two types of experiments were performed (see Fig. 1): *(i)* choice test (Y-shape trail-following bioassays) and *(ii)* no-choice test (linear trail-following bioassays), as described below.

In the choice test (see Tab. 2 for an overview of all bioassays), two sets of experiments were done: *(i)* CS trail *vs.* HS trail, and *(ii)* CS trail *vs.* mixed trails (MIX; trail made with both species extracts in an equal proportion, mixed before using). The insects were released at the base of the Y-shape scent trail and the distance travelled was measured while noting the chosen Y arm.

In the no-choice test, two 6-cm trails were laid down from opposite sides of a line. When meeting, such trails would overlap for 2 cm, forming a 10-cm long trail (see Fig. 1B). For each species, two sets of experiments were done: *(i)* CS trail *vs.* HS trail, both
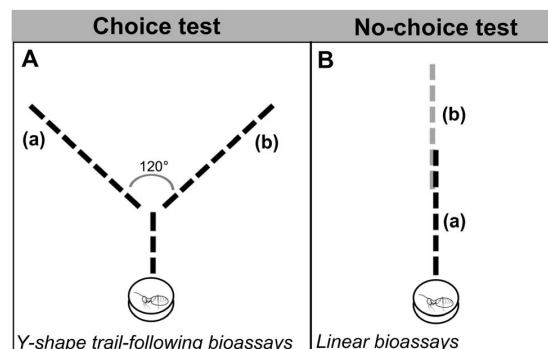
Figure 1. Schematic design of trail-following bioassays: Choice test made with Y-shape trail-following bioassay (A) and no-choice test made with linear bioassay (B). In drawing A, the Y stem was 3 cm long and each one of Y branches was 7 cm long with a 120° angle between them. In drawing B, the trail consisted of two trails of 6 cm long, made of each extract and overlapping for 2 cm in the centre.
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made with WBE and *(ii)* CS trail *vs.* HS trail, both made with SGE (see Tab. 3 for an overview of all bioassays). Termites were released at the end of the 10-cm line, starting from the side where their own species' trail was laid down. The distance travelled on this trail was measured while noting, whether the individual followed the trail, left it, or made U-turns to retreat.

Statistical analyses

All analyses utilized Generalized Linear Models (GLM), choosing error distribution according to the nature of the response variable, as described below. Model simplification was done through contrast analyses with *F* tests, combining treatment levels when it did not cause significant ($P<0.05$) changes in the model, as recommended by [36]. Treatments levels are specified below under description of the respective analysis. All analyses were performed in *R* [37], followed by residual analysis to check the suitability of the error distribution and model fitting. To prevent pseudoreplication, values obtained for each of the 10 workers from a given species and colony were collapsed into a single average value. Because such values come from distinct randomly chosen colonies, they stand as true replicates. Similar procedure was used by [38].

To test whether the compounds identified by GCxGC/TOF-MS could elicit behavioural responses in termites, data from the "Intraspecific trail-following" bioassays were analyzed in two separate models for each species. Both models included "distance followed" by the individuals as response variable with a normal error distribution. One of these models included a categorical independent variable (*x-var*) with two levels: "standard" to represent the respective standard compound being tested and "hexane" to serve as a control. The other model included a categorical independent variable (*x-var*) with two levels: "SGE" to represent the extracts of sternal gland and "WBE" to represent whole body extracts. Each of these models was run independently for each bioassay.

To test whether termites would perceive the heterospecific trail, orienting themselves according to it, two models have been built, for each species independently, regarding the experiment "choice-test" of Section "Interspecific trail-following" of Material and Methods. Both models included as response variable "proportion of workers" opting for a given branch of the Y-shape, thus

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Table 1. Distance followed by *Constrictotermes cyphergaster* and *Inquilinitermes microcerus* workers in Y-shape trail-following bioassays with whole bodies extract (WBE), sternal glands extract (SGE), (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (D) and/or neocembrene (N) (n = 30, degrees of freedom = 3, concentration in body or gland equivalent per cm [Eq/cm]).

Tested species	Extract or standard	Concentration	Distance followed (cm)	*F* value	*P* value
Constrictotermes cyphergaster	WBE	1 Eq/cm	7.5±0.33 **a**	34.6	0.0006
	SGE	10^{-1} Eq/cm	9.3±0.15 **b**		
	SGE	1 Eq/cm	9.4±0.29 **b**		
Inquilinitermes microcerus	WBE	1 Eq/cm	6.3±0.55 **a**	7.01	0.02
	SGE	10^{-1} Eq/cm	8.7±0.43 **b**		
	SGE	1 Eq/cm	8.1±0.30 **b**		
	D	10^{-3} ng/cm	1.5±0.15 **a**	15.92	0.003
	D	10^{-2} ng/cm	5.1±0.58 **b**		
	D	10^{-1} ng/cm	5.3±0.08 **b**		
	D	1 ng/cm	2.5±0.18 **a**		
	N	10^{-4} ng/cm	2.2±0.20 **a**	30.36	< 0.001
	N	10^{-3} ng/cm	2.4±0.10 **a**		
	N	10^{-2} ng/cm	4.9±0.21 **b**		
	N	10^{-1} ng/cm	2.8±0.32 **a**		
	N	1 ng/cm	1.3±0.14 **a**		
	D + N	$10^{-1} + 10^{-2}$ ng/cm	6.0±0.23	159.7	< 0.001
	D + N	$10^{-2} + 10^{-2}$ g/cm	2.9±0.30		

The activity threshold was defined as the minimal concentration eliciting termites to travel over 3 cm on the trail; maximal possible distance was 10 cm (mean ± SE). Hexane was used as a control, and was never followed by termites. Values with the same letters indicate non-significance in Contrast Analyses under Normal distribution.
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requiring binomial error distribution. The first model included a categorical independent variable (x-var) with two levels: "CS" to represent trails made with extracts of conspecifics and "HS" to represent trails made with extracts of heterospecifics. The second model included a categorical independent variable (x-var) with two levels: "CS" to represent trails made with extracts of conspecifics and "MIX" to represent trails made with a mix of extracts of conspecifics and heterospecifics. Each of these models was run independently for each bioassay.

To test whether termites, in perceiving the trail, would be able to exploit it, two models have been built, for each species independently, regarding the experiment "no-choice test" of Section "Interspecific trail-following" of Material and Methods. One of these models included "distance followed" by the

Table 2. Choice test of *Constrictotermes cyphergaster* or *Inquilinitermes microcerus* workers in Y-shape trail-following bioassays with conspecific (CS), heterospecific (HS), or conspecific and heterospecific mixed (MIX) trails made with 10^{-1} sternal glands extract equivalent per cm (n = 30, degrees of freedom = 3).

Tested species	Set of bioassays	*Chi* value	*P* value
Constrictotermes cyphergaster	CS×HS	1.03	<0.001
	CS×MIX	0.70	0.0002
Inquilinitermes microcerus	CS×HS	0.72	<0.001
	CS×MIX	1.03	<0.001

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individuals as response variable, thus calling for the use of normal error distribution. This model included a categorical independent variable (x-var) with two levels: "CS-then-CS" to represent treatments where both sides of the 10-cm line contained trails made with extracts of conspecifics and "CS-then-HS" to represent treatments where one side to the line contained trails made with extracts of conspecifics and the other side contained trails made with extracts of heterospecifics. The other model included "proportion of individuals exhibiting U-turns" relative to the total of tested individuals, as its response variable, thus requiring binomial error distribution. Both models were run independently for each bioassay.

Results

Anatomy of sternal glands

Sternal glands of both species are located on the anterior part of the 5th sternite (Fig. 2). The glands are ovoid in shape and of comparable size (about 80 µm in length and 50 µm in height in both species) but the gland width is slightly larger in *C. cyphergaster* compared to *I. microcerus* (150±7.07 µm *vs.* 120±2.83 µm (mean±SD), respectively) which corresponds to a difference in body sizes: *C. cyphergaster* 4.33±0.41 mm; *I. microcerus* 3.50±0.43 mm (mean±SD).

Chemical analyses

The GC-MS analysis of extracts of *C. cyphergaster* worker sternal glands revealed the presence of dodecatrienol and neocembrene; both retention indices and MS spectra were identical with those of standards. The presence of these two compounds was already identified in *C. cyphergaster* [27]. The detected amounts per

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Table 3. No-choice test of *Constrictotermes cyphergaster* or *Inquilinitermes microcerus* workers in linear trail-following bioassays with conspecific (CS) trail followed by a conspecific trail or a heterospecific (HS) trail made with 10^{-1} whole bodies extract equivalent per cm (WBE) or 10^{-1} sternal glands extract equivalent per cm (SGE) (n = 30, degrees of freedom = 6).

Tested species	Extract	Set of bioassays	Distance followed (cm)	*F* value	*P* value
Constrictotermes	WBE	CS then CS	7.6±0.05	0.04	0.8405
cyphergaster	WBE	CS then HS	7.5±0.03		
	SGE	CS then CS	9.6±0.01	2.94	0.1615
	SGE	CS then HS	9.3±0.02		
Inquilinitermes	WBE	CS then CS	6.4±0.05	**0.70**	**0.0002**
microcerus	WBE	CS then HS	5.3±0.03		
	SGE	CS then CS	8.1±0.02	0.39	0.5631
	SGE	CS then HS	8.7±0.01		

Significant effect is in bold.
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individual were approximately 0.02 ng of dodecatrienol and 1 ng of neocembrene.

In spite of repeated attempts to inject concentrated samples, neither dodecatrienol nor neocembrene or any other known termite trail-following pheromone was detected in WBE or SGE of *I. microcerus* workers.

Intraspecific trail-following bioassays

Trail-following bioassays with WBE and SGE. Both species followed conspecific trails, made either with WBE or SGE. SGE were in both species more efficient in eliciting the trail-following behaviour of workers than WBE (*C. cyphergaster* $P = 0.0006$; *I. microcerus* $P = 0.02$; Tab. 1). There was no significant difference between the two concentrations of SGE used (1 and 10^{-1} gland equivalent/cm) (Tab. 1).

Trail-following activity of *I. microcerus* with standards. *Inquilinitermes microcerus* workers followed dodecatrienol and neocembrene trails. The highest trail-following activity was reached with 10^{-2} and 10^{-1} ng/cm for dodecatrienol ($P < 0.003$; Tab. 1), while only a single concentration of neocembrene (10^{-2} ng/cm) elicited significant trail-following activity ($P < 0.0001$; Tab. 1). The highest overall trail-following activity was observed using a mixture of dodecatrienol and

Figure 2. Worker sternal glands of *Constrictotermes cyphergaster* (A) and *Inquilinitermes microcerus* (B). Scale bars represent 100 µm. Numbers mark particular sternites. Asterisks mark sternal glands. Abbreviations: fb - fat body; g - ganglium.
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neocembrene at concentrations 10^{-1} ng/cm and 10^{-2} ng/cm, respectively ($P = 0.0002$; Tab. 1). Trails made of a mixture of both standards (10^{-1} ng/cm of dodecatrienol and 10^{-2} ng/cm of neocembrene) were significantly more efficient in eliciting the trail-following behaviour of *I. microcerus* workers compared to trails made of each standard alone at the same concentration ($P < 0.02$).

The choice test between trails made with *(i)* the mixture of dodecatrienol (10^{-1} ng/cm) and neocembrene (10^{-2} ng/cm), and *(ii)* trails made with SGE (10^{-1} gland equivalent per 1 cm) resulted in a clear preference of *I. microcerus* workers towards the sternal gland extract ($P < 0.0001$).

Interspecific trail-following bioassays

Choice test. In choice tests, the interspecific trail-following bioassays showed that workers of *C. cyphergaster* significantly prefer the CS trail over the HS trail ($P < 0.0001$, Tab. 2; Fig. 3A), although they prefer significantly the MIX trail over the CS trail ($P < 0.0001$, Tab. 2; Fig. 3B). In contrast, workers of *I. microcerus* always preferred HS trail ($P < 0.0001$, Tab. 2; Fig. 3C) or MIX trail ($P < 0.0001$, Tab. 2; Fig. 3D) over CS trail.

No-choice test. In no-choice tests, workers of *C. cyphergaster* were able to follow the same distance on both CS and HS trails made of WBE ($P = 0.84$, Tab. 3), while *I. microcerus* followed their CS trail for longer distances compared to HS trail ($P = 0.0002$, Tab. 3). When workers of *I. microcerus* reached the HS trail made of WBE, they made a U-turn and retreated back to the release chamber. This behaviour was performed by *I. microcerus* significantly more often than by *C. cyphergaster* ($P < 0.001$; Fig. 4).

However, the same tests performed with SGE instead of WBE resulted in the same distance travelled on the CS and the HS trail for both species studied ($P = 0.16$ for *C. cyphergaster*; $P = 0.56$ for *I. microcerus*; Tab. 3). Moreover, the frequency of U-turn after encountering the trail of the other species did not differ ($P = 0.32$).

Discussion

Trail-following pheromones and their activities

Based on trail-following bioassays and chemical analyses, we confirmed that neocembrene and dodecatrienol are the major compounds of the trail-following pheromone of *C. cyphergaster* [27] and we estimated the quantity of both compounds in the sternal glands of workers to be approximately 1 ng and 0.02 ng, respectively. Despite several attempts to confirm the nature of the trail-following pheromone in *I. microcerus* by measuring sternal
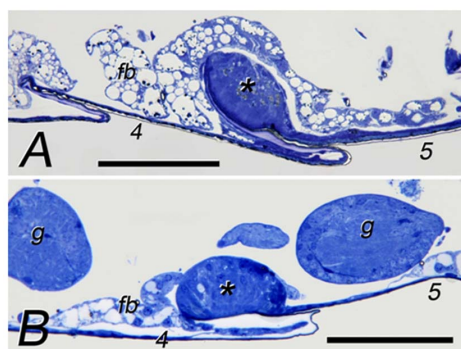
24
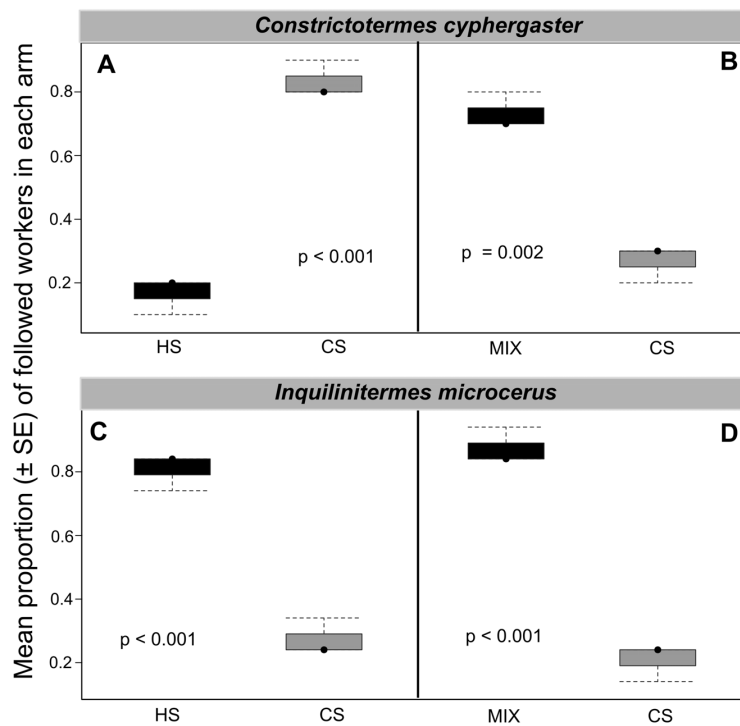
Figure 3. Trail recognition by *Constrictotermes cyphergaster* and its inquiline *Inquilinitermes microcerus*. Each of the four panels depicts a choice test (see Fig. 1A) in which 30 workers (ten from each of three colonies) were exposed to two distinct trails: heterospecific (HS) *versus* conspecfic (CS) in panels A and C; mixed trail (MIX; HS+CS) *versus* conspecific (CS) in panels B and D. In each panel, the vertical axis depicts the mean proportion (± SE) of the number of workers opting for a given arm of the Y-shape. When given the choice between its own trail and that of the other species, both host and inquiline preferred trails of *C. cyphergaster*. If this choice was between own trail and a MIX of both, host and inquiline preferred the MIX trail.f.
doi:10.1371/journal.pone.0085315.g003
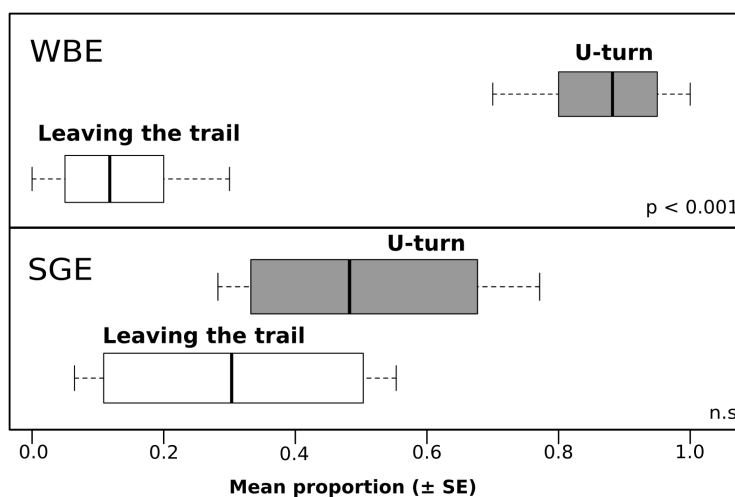
Figure 4. Avoidance of *Constrictotermes cyphergaster* trails by its inquiline *Inquilinitermes microcerus*. Each panel depicts behavioural responses of the inquiline when facing its host trail in a linear bioassay (see Fig. 1B) in which trails were made of whole body extracts (WBE, 10^{-1} whole body equivalent per cm) or sternal gland extracts (SGE, 10^{-1} gland equivalent per cm) of the host. Horizontal axis depicts the mean proportion (± SE) of the number of workers leaving the trail or making U-turns when perceiving the host's trail. *Inquilinitermes microcerus* clearly avoided the WBE host trails making U-turns but do not exhibit such avoidance if the trail was made of SGE.
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glands extracts concentrated up to 200 equivalents in one single injection, the chemical composition could not been confirmed. Theoretically, taking into account the low diversity of trail-following pheromones in termites and their distribution through termite species (for a comprehensive historical account, see [22,23]), three possibilities were expected for the chemical nature of *I. microcerus* trail-following pheromone: *(i)* dodecatrienol alone, *(ii)* mixture of dodecatrienol and neocembrene and less likely but not impossible *(iii)* either of the above plus a minor and unknown compound. Based on our trail-following bioassays, it was clear that dodecatrienol alone is not the trail-following pheromone in *I. microcerus*, since this compound did not elicit termites to follow a long distance on the trail in comparison to a mixture of dodecatrienol and neocembrene (see Tab. 1). It is, thus, likely that when it comes to trail-following pheromones, *I. microcerus* shares the same compounds as *C. cyphergaster*. Minor and unknown compounds may also be present since *I. microcerus* workers clearly preferred their sternal gland extracts over a mixture of dodecatrienol and neocembrene standards in trail-following bioassays ($P < 0.0001$).

Identification of termite trail-following pheromones is known to be difficult due to minute pheromone quantities [39]. More recent techniques like GC-EAD have proven to be useful in such study by highlighting some minor compounds that traditional techniques of chemical analyses could not identify [26]. However, even GC-EAD was not successful in identifying dodecatrienol and neocembrene in *I. microcerus* in part due to extremely short (compared to other termites) lifespan of the antennae isolated from workers. Neocembrene is a non-polar diterpene which is usually easily detected by GCxGC/TOF-MS. The absence of detection of this compound in *I. microcerus* samples is surprising given the results of the behavioural experiments. The amount of neocembrene might be of a few picograms only, which is just below the detection limit. Because of the polarity of dodecatrienol causing a tailing peak, the detection limit of this compound is much higher, between 0.1 and 1 ng/µl, while the expected amount is significantly lower.

Interestingly, trail-following pheromones consisting of both neocembrene and dodecatrienol seem to have evolved several times independently, since they have been identified in all Nasutitermitinae (Termitidae) studied so far (present study, [27]), as well as in the *Amitermes evuncifer* (Termitidae: Termitinae) [28] and in *Prorhinotermes* spp. (Rhinotermitidae) [26]. Alternatively, trail-following pheromones consisting of neocembrene and dodecatrienol may represent synapomorphy of a clade comprising Rhinotermitidae (+ Serritermitidae) + Termitidae [25,40–42], but this hypothesis would require numerous secondary losses of one component (neocembrene) or both components (for distribution of particular compounds in all taxa see [22,23]).

Inquilinitermes microcerus is found only in *C. cyphergaster* nests, but predominantly in parts rarely visited by *C. cyphergaster* (although located in general in the colony centre, representing perhaps the oldest nest parts). Such places seem to represent deposits of waste (hosts faeces and dead bodies) and it is not used by the host colony [17]. *Inquilinitermes microcerus* has never been observed outside the host nest and existing studies indicate that *I. microcerus* workers feed predominantly on the dark grey (abandoned) parts of the nest [17–20]. Thus, although *I. microcerus* is a clear example of an inquiline as defined by [5], it is also similar to 'one-piece' life type termites (e.g. Kalotermitidae). Its ecology may explain the low amount (hundreds of picograms) of trail-following pheromone present in sternal glands of *I. microcerus*: 'one-piece' life type termites as well as obligate inquilines do not need high amounts of trail-following pheromone due to the limited space crossed [35]. The confined

domain of these termites contrasts with the 'separate' or 'central' life type termites, in which workers must use considerable amounts of a trail-following pheromone in order to reach their foraging sites and mark their way back to the nest [43]. Although 'one-piece' life type termites and inquilines are capable of laying trails, the efficiency of trail-following pheromones might not be crucial for colony success since colonies spend their whole life in the same place using it as nest and food source. Thus, the role of the sternal gland secretion in 'one-piece' termites is considered more like a recruitment signal to lead termites to sources of disturbance, rather than an orientation signal [26,44,45]. Nevertheless, it was recently observed that under certain conditions, 'one-piece' termites were able to move from a piece of wood to another and to use connecting runways requiring the utilization of trail-following pheromones as orientation [22,46,47].

It is known that an epigeous structure initially houses the colony of *C. cyphergaster* while an arboreal nest is built only after the population exceeds a certain number [31]. The back and forth movement of workers from the initial nest to the new one under construction clearly involves the use of a trail-pheromone. It has also been showed that *I. microcerus* preferably occupies *C. cyphergaster* arboreal nests of rather larger size ($\geq$13 L.; [14]) but the mechanisms of the *C. cyphergaster* nest location by *I. microcerus* remain mysterious. It seems probable that the invasion of the inquiline starts by penetration of an *I. microcerus* dealate pair which uses visual cues at long distance to identify a suitable nest, but also chemical cues (including trail-following pheromone) perceived at short distance. The identical chemical nature of the trail-following pheromone of both species may help imagoes of *I. microcerus* to find and enter the nest of their hosts. Trail following by cohabitants to migrate between nests was already observed in lycaenid caterpillars and their host ants (see [48]). Some staphylinid termitophiles are also able to follow trails laid by their termite hosts, hypothetically not only to locate their resource (the termites), but also to locate their few conspecifics [49].

Interspecific trail following: How does trail following shape the relationships between *C. cyphergater* and *I. microcerus*?

This study showed that *I. microcerus* workers always preferred the trails made of *C. cyphergaster* extracts (Fig. 3C) or the MIX trails (Fig. 3D) rather than trails made of their own extracts. Conversely, *C. cyphergaster* workers prefer trails made by their own extracts (Fig. 3A) or by both extracts (Fig. 3B) compared to trails of *I. microcerus* extracts. These results may be due to the quantity of pheromone being higher in *C. cyphergaster* than in *I. microcerus*. These behavioural results might be linked to the size of the sternal gland being larger in *C. cyphergaster* than in *I. microcerus*. We cannot exclude that minor compounds present in the sternal gland secretion of *C. cyphergaster* may explain these trail preferences even though we did not detect any relevant compounds.

The U-turn behaviour performed by *I. microcerus* workers after detecting WBE of the host (Fig. 4) indicates perception and active avoidance of the host smell, probably due to presence of repellent compound(s) secreted by *C. cyphergaster* workers for their own protection during foraging in the open air. This hypothetical compound is obviously not present in the sternal gland secretion, and may originate either from the enlarged mandibular glands of workers [50] or from the faeces of *C. cyphergaster* workers, which are known to be laid onto their trails [51,52]. Moreover, powerful repellents are expected in open-air foraging termites to minimize predation upon them. Although soldiers play the prime role in defence and their high numbers (44% during the dry season and 33% during the wet season – see [29]) explain *Constrictotermes*

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success, we may also expect the appreciable effect of repellents used to avoid predation. Chemical repellence has been already reported as effective against mammalian termite predators [53].

The strategy used by the inquiline is to build its nest inside the host nest with no connection between the two nests thereby minimizing probable conflicts. Due to its comparable body size, the host could probably penetrate inquiline gallery system and kill all inhabitants based on its numerical dominance, if the inquiline would not able to detect gaps in the nest by using the trail-following pheromone of the host. *Inquilinitermes microcerus* can rely on the chemical cues of *C. cyphergaster* to inhabit its nest without risk of confrontation. Another possibility could be the use by *I. microcerus*, of *C. cyphergaster* trail-following pheromone as an indication of opportunities or threats. A low concentration of the trail-following pheromone due to a breach that needs to be sealed by the inquiline may be considered as a low level threat and may trigger investigation for *I. microcerus*. In contrast, a high concentration encountered when an important breach is created into the nest or when the inquiline digs into chambers with relatively fresh and potentially infectious corpses, may induce a quick retreat in *I. microcerus*. This could explain the different results between whole body and sternal gland extracts in Fig. 4.

In short, our results seem consistent with the hypothesis that trail-following pheromones may shape the cohabitation of *C. cyphergaster* and its guest *I. microcerus*. This is the first study evaluating chemical communication between two closely associated termite species. It seems evident that the inquiline is able to use the host's chemical cues to evade detection within the nest. While strictly in line with previous findings that cohabitation by this same pair of species is eased by the use of distinct diets [14], our results reinforce the idea that inquilinism by *I. microcerus* is based on conflict-avoidance strategies.

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Author Contributions

Conceived and designed the experiments: PFC ODS JS DSD. Performed the experiments: PFC ODS JK JS DSD. Analyzed the data: PFC ODS ERL JS DSD. Contributed reagents/materials/analysis tools: JS DSD ODS ERL. Wrote the paper: PFC ODS JK KK JS DSD. Chemical analyses: AJ. Anatomical study: KK JS.

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Chapter 4

How do you say 'SOS'? Alarm transmission in advanced termite *Constrictorermes cyphergaster* (Blattodea: Termitoidea: Termitidae: Nasutitermitinae)

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Abstract

Alarm signalling represents a keystone of social communication in all social insects. Although there are two ways of alarm signalling in termites, mechanical (e.g. vibration) and chemical (e.g. pheromone), these are not exclusive but rather complementary. This work represents the very first attempt to understand the functional significance of both kinds of signals in a termite species. Here, extensive testing led to identification of the alarm pheromone made of abundant monoterpenes $(1S)$-α-pinene and myrcene, along with minor compound, monoterpene (E)-β-ocimene. The vibratory alarm signals consist in series of beats produced predominantly by soldiers in response to various endangering stimuli. Exposing termite groups to various mixtures containing the alarm pheromone (smashed soldier heads, frontal gland extracts, mixture of all monoterpenes, the alarm pheromone mixture made of standards) resulted in significantly increased intensity of vibratory alarm communication. Lower doses (2 soldier equivalents) provoked higher numbers of vibratory signals compared to higher (6 soldier equivalents), which rather induced long-term running of all termites without stops necessary to perform vibratory behaviour. Surprisingly, even worker smashed heads increased significantly the number of vibratory movements, showing that not only frontal gland secretion is perceived and answered by colony members. Our results show that there obviously are different levels of alarm signals in termites, and lower excitement is shared with nest-mates rather using vibratory signals, while the alarm pheromone is released during more endangering situations.

Introduction

Negative interactions, such as predation and competition, have long been considered the major ecological factors structuring biotic communities (Shurin & Allen, 2001; Chese et al., 2002). To avoid costs imposed by such interactions, and consequently to increase the reproductive success, a wide range of defensive strategies evolved in all organisms. Apart of direct defence, mechanical (biting, kicking, fleeing) or chemical (release of toxic or repellent compounds), indirect defence consists in emission of highly-specific vocalizations and/or chemical substances (referred as alarming, alarm communication, alarm pheromone, alarm call etc.) informing relatives about imminent threats (Klump & Shalter, 1984). Although there is alarm communication in manifold animals (see for example Reed, 1969; Hews, 1988; Macedonia, 1990; Hollèn & Radford, 2009; Vander-Meer et al., 2010), this defensive behaviour is best pronounced in social insects (Blum, 1985; Verheggen et al., 2010).

In termites, the alarm communication is known since as early as 1779 (Konig *ex* Snyder, 1956). It can be transmitted by mechanical and/or chemical means, both leading to a variety of defensive behaviours, such as rapid running, attraction to the source, dispersal of colony members and/or aggressive postures (for review see Prestwich, 1984; Šobotník et al., 2010c). The mechanical alarm signalling, transmitted by substrate-borne vibrations, is present in all termite species and castes studied so far (see Howse, 1962, 1964b, 1965; Stuart, 1963, 1988; Kirchner et al., 1994; Connétable et al., 1998, 1999; Röhrig et al., 1999; Hertel et al., 2011). These vibratory movements are produced by drumming the head or abdomen against floor or ceiling of wood, nest or gallery (Howse, 1962; Stuart, 1963), and are perceived by specialized chordotonal receptor called subgenual organ attached to the cuticle of proximal tibia (Howse, 1964b,a; Chapman, 1998). While the mechanical alarm signalling is in primitive termites such as *Zootermopsis*, *Kalotermes* and *Coptotermes* restricted to a single or few termites close to the disturbance place (Howse, 1965; Stuart, 1988; Kirchner et al., 1994; Hertel et al., 2011), advanced termites (Termitidae) exploit

positive feedback reaction in order to spread the signal throughout the nest (Stuart, 1988; Connétable et al., 1998, 1999; Röhrig et al., 1999).

Chemical signals, i.e. alarm pheromones, are always produced by the frontal gland, a defensive organ without any equivalent among other insects (Noirot, 1969). All known alarm pheromones are components of the soldier frontal gland secretion and are released by fighting or excited termite soldiers (Šobotník et al., 2010c). Although the presence of chemical alarm communication is known to occur in numerous representatives of Rhinotermitidae and Termitidae (see Prestwich, 1984; Šobotník et al., 2010c), the exact compounds responsible for the role were identified only in *Prorhinotermes canalifrons* (Rhinotermitidae; Šobotník et al. (2008b)), three *Nasutitermes* (Vrkoč et al., 1978; Lindström et al., 1990; Roisin et al., 1990) and in a single *Velocitermes* species (Valterová et al., 1988). Positive feedback also occurs in chemical alarm signalling (see Vrkoč et al., 1978).

The Neotropical genus *Constrictotermes* (Termitidae: Nasutitermitinae) contains seven described species (Mathews, 1977; Krishna et al., 2013), with *Constrictotermes cyphergaster* Silvestri1901 being a common species in the savannah formations ('Cerrado') in Brazil, Paraguay, Bolivia and northern Argentina (Mathews, 1977). *Constrictotermes* is, along with several oriental genera, characterised by an unusual life-style combining arboreal nests and micro-epiphyte or leaf-litter diet (Moura et al., 2006a; Bourguignon et al., 2011). The food is harvested usually in the night by worker columns protected by numerous soldiers along the paths (Moura et al., 2006b). Their nests play an import role in the "Cerrado" diversity due to housing high numbers of termitophiles and obligatory inquilines of genus *Inquilinitermes* (Cristaldo et al., 2012; Florencio et al., 2013).

This work represents, up to our best knowledge, the first attempt to understand the relations between mechanical and chemical means of communication in a termite species.

Material & Methods

Ethics statements

Termite collections in Brazil were carried with permission of IBAMA (33094) to ODS, PFC and JŠ. Material transport was in agreement with Brazilian and Czech laws; export permits from Brazil was provided by CNPq-Brazil (001347/2012-8) and the import permits by Division of Protection against Harmful Organism of the Czech Republic (SRS 032901/2012 and 032904/2012).

Termite material

Experiments were conducted on 17 colonies of *C. cyphergaster* collected in July and September 2012 near Sete Lagoas town (S 27°19', W 14°44'; Minas Gerais, Brazil). Colonies collected in July 2012 were transported to Viçosa (Minas Gerais, Brazil), and kept in ambient laboratory conditions. Considerable parts of others colonies, collected in September 2012, were transported to Prague, and kept inside plastic boxes at temperature $\pm$ 27 ^{o}C and low relative air humidity.

First experiments were done in Viçosa in order to find out the presence/absence of alarm communication in *C. cyphergaster*. The second part, done in Prague, included the chemical composition of frontal gland secretion, the alarm responses to particular standards and the mechanical alarm communication (see below).

Structural work: Optical and electron microscopy

Ten *C. cyphergaster* soldiers were anesthetized on ice and fixed according to protocols described in Šobotník et al. (2010b,a). Semithin sections (0.5 μm) were cut using Reichert-Jung Ultracut Microtome™ and stained with methylene blue for optical microscopy. Sections were studied using a Carl-Zeiss Amplival™ optical microscope combined with Canon EOS 500D™ digital camera. For transmission electron microscopy, ultrathin sections (60–80 μm) were stained using the protocol developed by Reynolds (1963) and observed using Jeol 1011™ transmission electron microscope.

Observation of alarm behaviour

In order to observe the alarm behaviour in natural conditions, 10 arboreal nests of *C. cyphergaster* were subjected to experimental disturbance by piercing a hole using a pocket knife (approx. 5 cm deep) through the nest wall in the middle section of the nest. We scored the number of termites, soldiers and workers, appearing at the damaged point, and we also observed and noted down their behaviour.

Subsequently, the alarm behaviour was studied in laboratory conditions. Prior to experimentation, we compared the alarm responses at the red light and under dimmed daylight. Although the behavioural responses were always more pronounced under red light, we had to switch the experimentation to dimmed daylight in part of experiments (see "Chemical communication" section below), because the low-intensity red light often disallowed to distinguish between workers and soldiers, which are in *C. cyphergaster* quite similar in terms of body size, shape and coloration (see Mathews (1977), but we needed to treat them separately (see below).

Sample preparation

Frontal Gland Extracts (FGE): Soldiers were anesthetized on ice and dissected into head and rest of body. Heads with nasus cut off were placed into hexane (10 μL per head), and extracted for least for 24 h at 4 ^{o}C followed by another washing with 5-10 μL of hexane per head. Final samples were stored at -18 ^{o}C and used for chemical analyses and bioassays. According to the known volume of extract, we recounted one soldier frontal gland equivalent used as a measure in the bioassays.

Standards: Camphene as well as both enantiomers of α-pinene, β-pinene and limonene were purchased from Sigma-Aldrich; myrcene and (E)-β-ocimene originated from our laboratory store. Mixture of monoterpenes (MM) was prepared from standards after quantification of particular monoterpenes in the mixture using (1S)-α-pinene as an external standard.

All samples were extracted in hexane purchased from Merck and redistilled prior to use.

Chemical analyses

Chemical analyses have been carried out by gas chromatography coupled with mass spectrometric detection (quadrupole DSQ II, Thermo Scientific). The nonpolar ZB-5MS column (30 m, id 0.25 mm, 0.25 μm phase thickness) was used. Temperature program was 50 ^{o}C to 120 ^{o}C at 8 ^{o}C/min rate and then to 320 ^{o}C at 15 ^{o}C/min rate. 1 μm of the extracts (representing approximately 1/10 of termite equivalent) was injected in a splitless mode. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. Identification of particular compounds was based on a comparison of their retention indices and fragmentation patterns with MS library (NIST MS Search 2.0) and the published data (Adams, 2007). The final confirmation and quantification was done with available standards. The retention indices were calculated from the retention times of n-alkanes (nC10–nC30).

To distinguish between enantiomers of detected monoterpenes we used GC-FID (HP 6850 Series) with chiral column HP-CHIRAL-20B (30 m, id 0.25 mm, 0.25 μm phase thickness). Temperature program was from 40 ^{o}C (1 min) to 60 ^{o}C (13 min) at the rate of 50 ^{o}C/min then to 110 ^{o}C (12 min) at 10 ^{o}C/min and finally at the same rate to 150 ^{o}C (50 min). Hydrogen was used as a carrier gas. We compared retention times of commercial standards with compounds from extracts.

Experimental design and parameters measured

Experimental arenas were made from plastic Petri dish (ø 85 mm) with a slit in the cap and wet filter paper (Whatman N° 1) as substratum (see Roisin et al., 1990; Šobotník et al., 2008b). Groups of 5 soldiers and 20 workers were transferred from the colonies into experimental arenas at least 2 h prior to observation to ensure their acclimatization after manipulation. The number of termites and caste ratio was chosen according to natural caste proportions (see Cunha et al., 2003, S:W=1:4.5) and to maximize their interactions and survival (see Miramontes & DeSouza, 1996). The tested stimulus (see below) was loaded on a piece of filter paper (7 x 3 mm) and introduced immediately into the experimental arena through a slit in the dish cap. The filter paper was hung out of reach of termites by a pin bridge over the slit. Each group was tested once for one stimulus and once for the control with at least

2 h of quiet between the experiments. Recording lasted for 7 minutes, of which 2 were before the stimulus insertion and 5 after; this setting is necessary to evaluate idle activity of a group, and it was used in all types of experiments.

Stimuli tested in the experiments (chemical and mechanical) were: (1) untreated paper (UP) (blank control), (2) pure hexane (Hex) (solvent control), (3) smashed worker heads (SWH) (smashed control), (4) smashed soldier heads (SSH), (5) soldiers' frontal gland extracts (FGE), (6) mixture of monoterpenes in natural ratio observed in soldier head extracts prepared from available standards (MM) and (7) the alarm pheromone (AP) in ratio observed in soldier head extracts [(1S)-α-pinene:myrcene:(E)-β-ocimene approximately 100:88:1; for details see Table 4.2]. An overview of stimuli with respective quantity (amount and gland equivalent) used in the experiments is shown in Table 4.1.

Table 4.1: Overview of stimuli tested: Stimuli, type, amount used (frontal gland equivalent) and number of repetitions per stimuli in the chemical experiments (CE) and in the mechanical experiments (ME).

Stimuli	Type	Amount (Glands/Eq.)	CE	ME
Untreated paper	Blank control	-	6	6
Hexane	Solvent control	2 or 6 Glands/Eq*	18	24
Smashed workers head	Smashed head control	2 heads	6	8
Smashed soldiers head	Natural Stimulus	2 heads	6	8
Soldier's frontal gland extracts	Natural Stimulus	2 Glands/Eq	6	8
Monoterpenes mixture	Synthetic Stimulus	6 Glands/Eq	6	8
Most active compounds	Synthetic Stimulus	6 Glands/Eq	6	8

* The amount of hexane used in the experiments varied with the treatment, but it was always the same as the amount of the soldier's frontal gland extracts (2 Glands/Eq. vs. 6 Glands/Eq.).

For smashed head treatments, workers and soldiers were anesthetized on ice and then dissected into head and rest of body. Heads were smashed by the tweezers pressure against the piece of filter paper. FGE and MM were obtained as described in the 'Sample preparation' subsection. The AP was obtained from preliminary tests with standards of all monoterpenes found in the *C. cyphergaster* frontal gland secretion, alone and in combinations with others (see Tab. 4.4 for details). The

compounds that triggered higher speed of termite motion were selected as AP and used in the main experiments (see "Identification of alarm pheromone" in Results section). A Hamilton microsyringe (10 μL) was used to load the Hex, FGE, MM and AP onto the test filter paper.

Chemical Communication Testing

All experiments were performed in laboratory at ± 27 ^{o}C under dimmed daylight. The behaviour of termites groups was recorded with either Nikon D300s or Canon EOS 500D. To check the behavioural response of termites to chemical alarm (different stimulus), two parameters were evaluated: *(i)* the speed of termite motion according to caste, and *(ii)* the total number of vibratory movements during the experiment. The speed of motion was measured in one soldier and one worker randomly selected at the beginning of each experiment, and analyzed using Mouse-Tracer software (for details see Šobotník et al., 2008b). The difference in speed of motion before and after a stimulus insertion was used as a measure of the alarm activity. Overall number of vibratory movements was counted without consideration of the caste performing it in order to get data comparable to mechanical communication results (see below). The total number was recounted per minute and divided by the total number of individuals (n=25) in the experimental arenas, and used for subsequent statistical analyses.

Mechanical Communication Testing

Experiments were performed in the anechoic room at the Faculty of Electrical Engineering (Czech Technical University in Prague, Czech Republic) under red light and on the table hung from the ceiling. This set up was necessary for elimination of undesired vibrations originated either from background noise or vibration of the floor. We used plastic Petri dishes with coarsened bottom allowing easy walking to termites, because filter paper effectively absorbed the vibrations elicited by termites. The high sensitivity accelerometers (Brüel & Kjær type 4507 B 007, nominal sensitivity 100 mW/ms^{-2}) were fixed to the centre of the Petri dish bottom. The signals from accelerometer were recorded by Soft dB Tenor recorder (24 bit, sampling frequency 48 kHz).

Termites groups were exposed to different stimulus and their responses were analyzed using Matlab software. The vibratory signals were transformed to the frequency domain by Short-Time Fourier Transformation. The analysis of correspondence between video record and vibratory signals proved that the first structural mode of the Petri dish is excited exclusively by the vibratory movements of a termite, and thus only the first structural mode of Petri dish was used for the evaluations. The threshold (magenta dash line in Fig. 4.4A) proportional to power of the vibration signal was computed. The series with one burst above the threshold and at least two other bursts were recounted per minute and divided by the total number of individuals (n=25) in the experimental arenas, and used for subsequent statistical analyses. We have also combined video- and vibratory signals recording to see possible differences in drumming behaviour of workers and soldiers (n=3).

Statistical analyses

To check the effect of stimulus (*x-var1*) and caste (*x-var2*) on the speed of termite motion (*y-var*), the data were subjected to linear-mixed effects modelling with '*stimulus*', '*caste*' and their first-order interaction as fixed factors, and '*caste*' nested within '*nest identity*' as the random factor (Crawley, 2007; Pinheiro & Bates, 2000). The use of this statistical approach was necessary because workers and soldiers, i.e. the categories forming the variable '*caste*' (*x-var2*), belong to the same nest and hence are not independent from each other. Likelihood ratio tests compared full models to the null model composed only by random effects. Model simplification was done through Contrast analyses with F tests, removing non-signicant (P>0.05) terms sequentially, starting from the most complex one and then lumping treatment levels together within retained categorical variables.

To inspect the effect of stimulus (*x-var1*) on the vibratory movements (*y-var*), either in the chemical or mechanical communication testing, the data were subjected to Generalized Linear Modelling (GLM) under normal errors. Model simplification was performed by contrast analyses as described above.

All analyses were performed in R (R Development Core Team, 2012), followed by residual analysis to check the suitable of the error distribution and model fitting. Linear-mixed effects modelling used *lme* function from R package *nlme* (Pinheiro

et al., 2011).

Results

Appearance of the frontal gland

Frontal gland is in soldiers of *C. cyphergaster* (Fig. 4.1) developed as a large sac filling considerable volume of the cephalic capsule, especially dorsal, posterior and lateral parts. The ventral, posterior and lateral parts of the frontal gland sac are formed by the secretory epithelium, which dorsally and anteriorly changes into non-secretory epithelium of the reservoir and evacuating channel ending at the tip of nasus. Whole frontal gland is surrounded with a thick basement membrane, formed by many laminae of loose material in the anterior parts, whose number decreased posteriorwards.

Secretory epithelium of the frontal gland varies in thickness between 5 and 25 μm (in general thicker in the centre, somewhat thinner marginally) and is formed by uniform columnar cells (class 1 according to Noirot & Quennedey, 1974). The epithelium is lined with a highly modified cuticle, whose basal part (0.5-1 μm thick) is electron-lucent and compact, medial part (about 2 μm thick) is made of fibrous material (heterogeneous appearance) and apical continuous epicuticle made of single layer about 20 um thick; basal and medial parts are usually separated by a space filled with flakes of electron-dense secretion. Apical plasma membrane forms dense microvilli up to 5 μm long. Approximately apical halves of cell membranes are connected by intercelular junctions, with a single zonula adherens being followed by a long septate junction. Nuclei are highly irregular, up to 8 μm in the largest dimension, located in the basal portions of the cells. Secretory organelles include rich tubular smooth endoplasmic reticulum (ER) located predominantly in the apical half of the secretory cells, and rarer rough ER intermingled with smooth ER. Secretory vesicles (up to 1.5 μm in diameter) are located predominantly basally, and are filled with electron-dense material. Although their release from secretory cells was not observed, the flakes of electron-dense secretion located between the secretory cells and the overlaying cuticle strongly resemble the content of these vesicles.

Mitochondria are very populous and located predominantly in the cell bases.

Secretory cells class 3 (according to Noirot & Quennedey, 1974) surround the evacuating channel of the frontal gland and expel their products into it. They are located within the connective tissue (basement membrane formed by multiple layers) surrounding whole frontal gland, but are equipped with own basement membrane of slightly different appearance. The cells are relatively small (about 15 μm in the largest dimension), with relatively large irregular nuclei (up to 8 μm in the largest dimension). End apparatus is lined with short (about 1 μm) and dense microvilli, among which the porous receiving canal is inserted. Secretory organelles comprise rough ER and numerous Golgi bodies. Secretory vesicles are of two principal types: *(i)* small electron-dense vesicles were never observed to be released at the cell apex, but rather seemed to dissolve into *(ii)* larger lucent vesicles (up to 2.5 μm in diameter). Canal cells are small and simple, without many organelles, but with a small nucleus showing extensive chromatin aggregates.
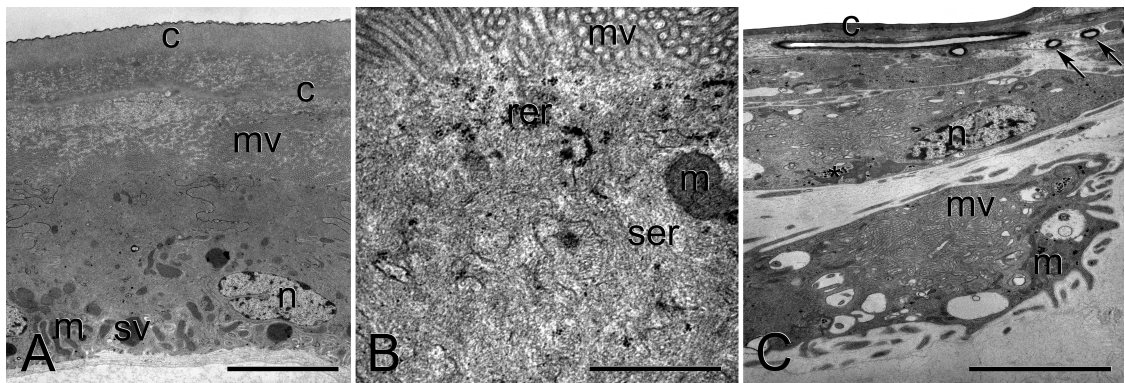
Figure 4.1: Ultrastructure of the frontal gland of *C. cyphergaster* soldier. Class 1 secretory cells (A); note the gap between two layers of cuticle filled with heterogeneous secretion. Detailed view on the class 1 secretory cell apex (B). Class 3 secretory cells (C); asterisk marks glycogen rosettes, arrows mark conducting canals. Scale bars represent 5 μm (A and C) or 1 μm (B). Abbreviations: c, cuticle; m, mitochondria; mv, microvilli; n, nucleus; rer, trough endoplasmic reticulum; ser, smooth endoplasmic reticulum; sv, secretory vesicle.

Alarm communication in natural conditions

Constrictotermes cyphergaster showed a quick alarm response to experimental disturbance in the nest wall. The time of the first individuals arrived to the disturbed point ranged from 5 to 60 seconds with an average of 31±1.8 sec. (men ±SD). In most of sampled nests (8 out of 10), soldiers were the first arriving caste followed usually only by other soldiers. However, in a few nests the soldiers were followed by workers; workers anyway always arrived in low quantity (maximum 2 workers [0.3±0.06] compared to 78 soldiers [26.6±2.44]). When occurred at the nest surface, soldiers showed typical alarm behaviour, like running around the leak and searching for the enemy, the zig-zag walking and scanning the space with antennae, vibratory signalling and alerting nestmates when encountered.

Analytical Chemistry

GC-MS analysis revealed the presence of 7 monoterpenes, 2 sesquiterpenes and 1 diterpene in the frontal gland secretion of *C. cyphergaster* soldiers. Concerning the alarm properties of the secretion, we focused on the most volatile compounds from the mixture, i.e. monoterpenes and their enantiomeric properties. The results are shown in Tab. 4.2 and Fig. 4.2.

Table 4.2: Monoterpenes found in *Constrictotermes cyphergaster* frontal gland secretion with respective retention index (RI), quantity (%) and amount per gland. Compounds in bold represent the alarm pheromone.

Compound	RI	Quantity (%)	Amount per gland (ng)
(1S)-α-pinene	944	43.7	1000
Camphene	956	11.6	280
(1S)-β-pinene	983	6.4	150
Myrcene	992	37.0	880
R-limonene	1033	0.5	12
(*Z*)-β-ocimene	1039	0.3	6
(*E*)-β-ocimene	1049	0.5	12
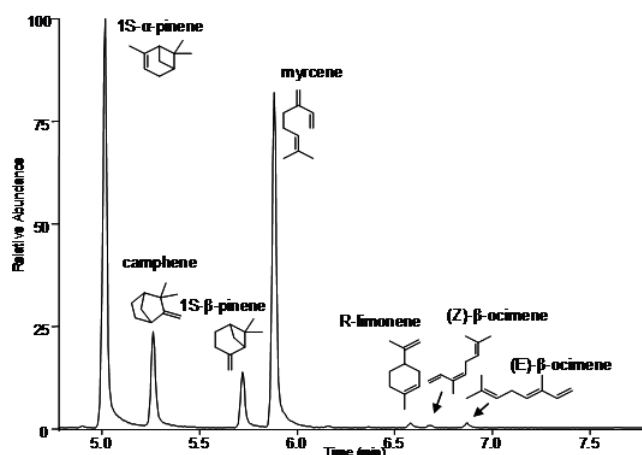
Figure 4.2: Detail of GC-MS chromatogram showing all monoterpenes detected in the frontal gland secretion of *Constrictotermes cyphergaster* soldiers.

Identification of the alarm pheromone

Testing all detected monoterpene enantiomers and their combinations (see Supplementary Material (Tab. 4.7) for more details) led to a single combination, which significantly (P< 0.0001) increased the speed of termite motion in comparison with UP and Hex. The mixture of *(1S)*-α-pinene, *(E)*-β-ocimene and myrcene in natural ratio thus represents the alarm pheromone of *C. cyphergaster*, with the effect fully comparable to SSH and FGE. Both tested signal strengths (2 or 6 equivalents) induced the alarm behaviour, and responses obtained using 6 equivalents were adequately stronger in terms of speed of motion (see Fig. 4.7), and were used in subsequent experiments.

Alarm behaviour

Chemical communication testing

The speed of motion of *C. cyphergaster* was strongly affected by different stimuli, with soldiers moving always significantly faster compared to workers (likelihood ratio= 151.051, P< 0.0001; see Fig. 4.3A). Significantly positive alarm responses were triggered by SSH, FGE, MM and AP, arranged in ascending order (P< 0.0001). The responses are clearly dose-dependent, as evidenced by significantly (P< 0.03) higher level of response to 6 equivalents compared to 2 equivalents (see Fig. 4.7).

The number of vibratory movements scored from video records was affected by the stimuli nature ($F_{6,33}$= 2.43, P= 0.04, Fig. 4.3B). SSH induced the highest numbers of vibratory movements, and SWH, FGE, MM, AP elicit significantly higher number of vibratory behaviours compared to UP and Hex (P< 0.04), although no significant differences among SWH, FGE, MM, AP were detected.

(A)

(B)
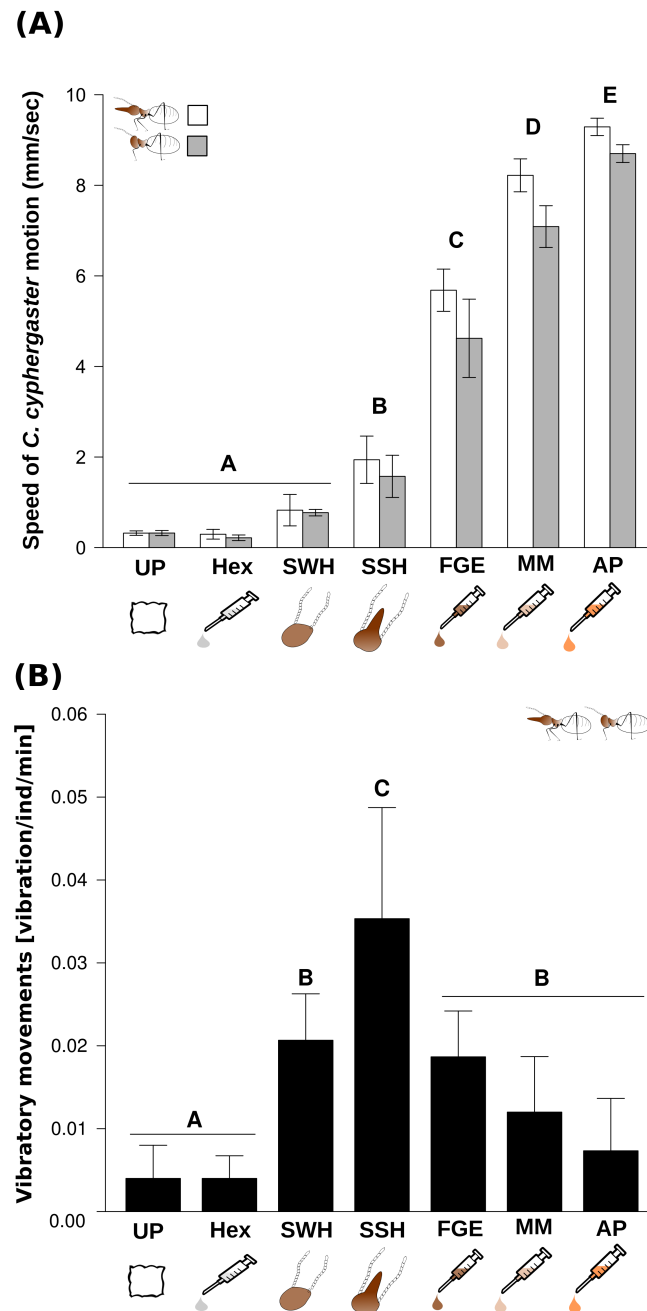
Figure 4.3: Behavioural responses of *Constrictotermes cyphergaster* workers and soldiers during chemical bioassays. **A.** Average (±S.E.) of speed of motion in soldiers and workers according to type of stimulus. **B.** Average number (±S.E.) of vibratory movements per minute in soldiers and workers combined according to type of stimulus. The columns marked with different letters are significantly different. Stimuli: UP - untreated paper; Hex - hexane; SWH - smashed workers head; SSH - smashed soldiers head; FGE - soldier's frontal gland extract; MM - mixture of all monoterpenes; AP - alarm pheromone.

Mechanical communication testing

Our direct observation revealed existence of two basic means of vibratory communication, drumming and shaking (vibratory movement during which the substrate either is or is not repeatedly hit by termite body), and only the drumming can be noted by vibratory recording. Drumming behaviour may consist in solitary beats, which are more often combined into bursts of several beats (see Fig. 4.4C). The number of beats per burst ranged from 2 to 7 (mean: 3.2±1.18 in soldiers and 2.6±1.09 in workers). A typical drumming series (see Fig. 4.4B) includes several bursts followed by several more singular beats. Table 4.3 shows a comparison between characteristics of vibratory movements during bioassays in *C. cyphergaster* soldiers and workers.

The number of individual bursts (drumming series) per minute was affected by the different types of stimuli ($F_{6,39}$= 2.52 , P = 0.03; Fig. 4.5). SSH, FGE, MM and AP increased the number of bursts per minute compared to UP, Hex and SWH (P= 0.01). On the other hand, the total number of vibratory movements (beats) per individual was not affected by the different types of stimulus (P= 0.45).
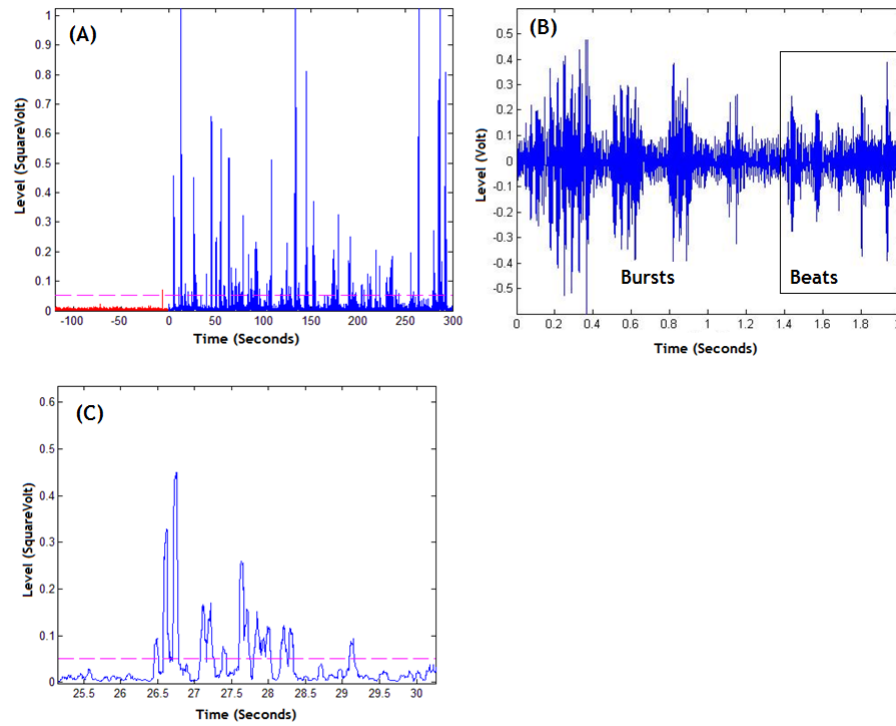
Figure 4.4: Physical characteristics of vibratory communication in the mechanical experiments. **A.** Response to AP (alarm pheromone). The threshold (magenta dash line) proportional to power of the vibration signal. Red line represents the activity of the group before insertion of treated paper and blue line represents the activity after insertion of treated paper. **B.** Time domain record of one of the typical drumming series. Note four bursts followed by five more beats. **C.** Detail of a single burst made of 4 beats.

Table 4.3: Comparison between characteristics of vibratory movements in *Constrictotermes cyphergaster* soldiers and workers during mechanical bioassays (mean±SD).

	Soldiers	Workers
Number of series	3.2 ±1.04	2.0 ±1.02
Average number of bursts in series	6.0 ±2.62	7.8 ±4.03
Average duration between bursts (sec.)	0.236 ±0.02	0.218 ±3.06
Average duration between beats (sec.)	0.060 ±0.02	0.064 ±0.08
Average duration of bursts (sec.)	0.167 ±2.61	0.119 ±2.56
Average number of beats per burst	3.2 ±1.18	2.6 ±1.09
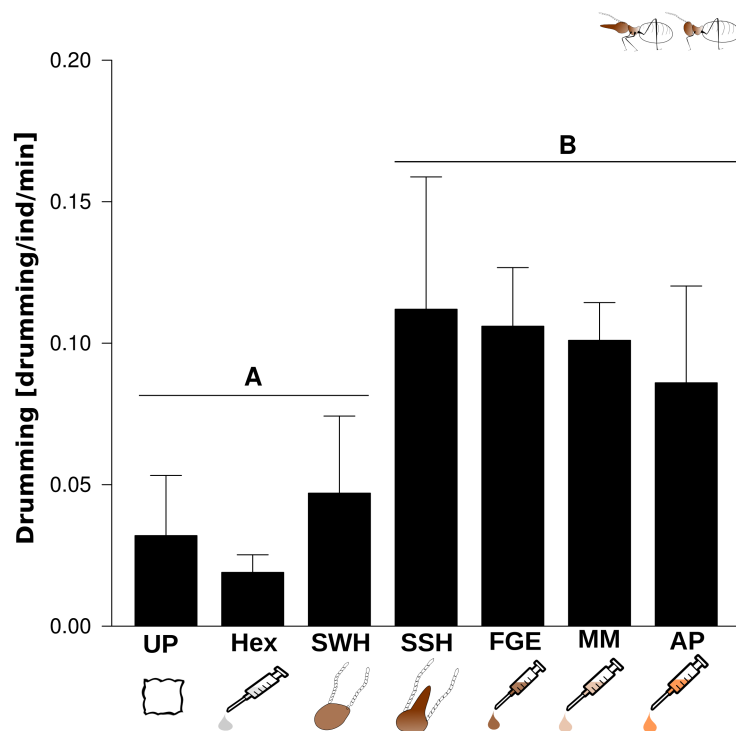
Figure 4.5: Average number (±S.E.) of vibratory movements per minute in mechanical behavioural bioassays according to type of stimulus. The columns marked with different letters are significantly different. Stimuli: UP - untreated paper; Hex - hexane; SWH - smashed workers head; SSH - smashed soldiers head; FGE - soldier's frontal gland extract; MM - mixture of all monoterpenes; AP - alarm pheromone.

Discussion

Nasutitermitinae represent one of the most advanced and successful termite lineages (Inward et al., 2007; Legendre et al., 2008; Engel et al., 2009; Legendre et al., 2013), as evidenced by their cosmotropical distribution, orientation on diverse diet from sound wood or microepiphytes to highly decomposed plant materials and also by the efficient defence strategies relying upon numerous small soldiers exploiting exclusively the chemical warfare. Likewise all other Nasutitermitinae species, *C. cyphergaster* soldiers eject from the nasus tip strings of defensive secretion entangling and incapacitating small enemies, and inducing long-term scratching and cleansing behaviour in larger ones (Redford, 1984a). At the same time, the frontal gland secretion clearly alerts calm nestmates, in which it induces a profound change in behaviour towards typical alarm responses. Alarm responses were described in detail also in other termite species, and consist in increased of speed of motion of soldiers and workers, their gathering at the alarm source or hiding from it (Roisin et al., 1990; Reinhard & Clément, 2002; Reinhard et al., 2003; Šobotník et al., 2008b), and these activities are usually accompanied by vibratory movements (see Kirchner et al., 1994; Connétable et al., 1998, 1999; Röhrig et al., 1999; Reinhard & Clément, 2002; Reinhard et al., 2003; Hertel et al., 2011). All above-mentioned behavioural changes were observed in *C. cyphergaster*, providing a clear evidence of alarm response transmitted by both chemical (Fig. 4.3A and 4.3B) and mechanical (Fig. 4.4 and 4.5) signals.

Frontal gland secretion of Nasutitermitinae soldiers is made of sticky diterpenes dissolved in monoterpene mixture (for reviews see Prestwich, 1984; Šobotník et al., 2010c). While the function of irritating agents can be a commonplace for all monoterpenes (Deligne et al., 1981; Baker & Walmsley, 1982; Prestwich, 1984), the alarm pheromone function was proved only in few monoterpenes produced by *Nasutitermes* (Vrkoč et al., 1978; Lindström et al., 1990; Roisin et al., 1990) and *Velocitermes* (Valterová et al., 1988) soldiers. α-pinene is the most common compound showing

the alarm pheromone function, occurring alone in *N. princeps* (Roisin et al., 1990) or in mixture in *N. ripertii* (Vrkoč et al., 1978), *Velocitermes velox* (Valterová et al., 1988) and *C. cyphergaster* (*present study*). Surprisingly, in our case this compound alone shows no activity, but is essential for the blend function (see Tab. 4.4). Other two components responsible for the chemical alarm signal in *C. cyphergaster* are (*E*)-β-ocimene and myrcene, both matched to pheromone function for the first time in termites. While *(1S)*-α-pinene and myrcene represent dominant components of *C. cyphergaster* frontal gland secretion, (*E*)-β-ocimene represents only a minor component, which is anyway needed for the alarm function of the secretion. (*E*)-β-ocimene is known to act as aggregation pheromone in beetle *Alphitobius diaperinus* (Barlet et al., 2009) and is also present in the venom gland secretions of several ants and wasps (Keegans et al., 1993; Dani et al., 1998). Myrcene is common compound produced by termite frontal glands (for review see Šobotník et al., 2010c, Appendix A), but is also known as sex pheromone in shield bugs (Pentatomidae: Acanthosomatinae; Staddon, 1990) and some jewel beetles (Buprestidae: *Agriotes*; Yatsynin et al., 1996). All other monoterpenes identified in *C. cyphergaster* frontal gland secretion are known to occur in other Nasutitermitinae, except for (*E*)-β-ocimene and (*Z*)-β-ocimene (see Tab. 4.2), found only in *Syntermes* (Syntermitinae) (Baker et al., 1981). Different classes of compounds (alcohols, aromatic compounds, ketone) were identified by Azevedo et al. (2006), in addition to monoterpenes partially overlapping with our results. Such differences (distinct chemotypes) are repeatedly reported in termites, especially due to geographic distance (see e.g. Goh et al., 1984; Prestwich, 1984; Valterová et al., 1988; Krasulová et al., 2012), and evolutionary history (Quintana et al., 2003; Perdereau et al., 2010). Larger molecules were out from the scope of our work, but several sesquiterpenes and a diterpene were previously identified in *C. cyphergaster* soldier frontal gland secretion as well (see Baker et al., 1984; Azevedo et al., 2006).

Frontal gland, the sole source of alarm pheromones, is in *C. cyphergaster* developed modally (see Holmgren, 1909; Noirot, 1969; Santos & Costa-Leonardo, 2006). It seems that the shape and organization of the frontal gland belongs to definitional feature of Nasutitermitinae soldiers, along with whole anatomy of the head. The fundamental characters of frontal gland thus comprise *(i)* pear-shaped gland

reservoir connected to fontanelle by an excretory duct; *(ii)* posterior and ventral parts formed by class 1 cells only; *(iii)* non-secretory epithelium pierced bearing numerous class 3 secretory cells. On the other hand, our observation excludes the existence of specific muscle emptying the frontal gland reservoir reported by Santos & Costa-Leonardo (2006). The muscles adjoining the frontal gland reservoir are indeed mandibular muscles, but these are stretched between posterior (occipital part) head and mandibular tendon, similarly to soldiers of all other termites. The major difference in head structure in soldier of Nasutitermitinae compared to e.g. Rhinotermitidae is that the posterior head is in nasute completely filled with the reservoir, but predominantly with mandibular muscles in biting soldiers. This observation suggests specific function of the mandibular muscles in nasutes, and forming the direct pressure upon reservoir as suggested by Holmgren (1909) is actually not excluded. At the same time, termite soldiers were observed to bite without expelling the frontal gland secretion as well as releasing and imbibing the secretion from and back to frontal gland reservoir without mandible opening and closing (Šobotník, *pers. observ.*). Although it seems impossible to get a proof of the exact mechanism of frontal gland secretion release, we would not exclude the possibility that the needed pressure comes from contraction of abdominal intersegmental muscles as suggested by Šobotník et al. (2010a) for Rhinotermitidae imagoes. If this is true, then the tentorial-fontanellar muscle would prevent fontanelle from being plugged by the frontal gland epithelium.

Vibratory alarm communication occurs in all termite species studied so far (see Howse, 1962, 1964b, 1965; Stuart, 1963, 1988; Kirchner et al., 1994; Connétable et al., 1998, 1999; Röhrig et al., 1999; Reinhard & Clément, 2002; Hertel et al., 2011), as well as in *Cryptocercus* (Bell et al., 2007), sister-group of all termites (Inward et al., 2007; Cameron et al., 2012). Although other cockroaches are able to perceive the substrate-borne vibration, the communication through drumming is evolutionary novelty of *Cryptocercus*-Termitoidea (*sensu* Lo et al., 2007) clade. Our results show that vibratory communication is used also in meanings different from alarm, as only the number of bursts significantly increased according to type of stimulus (*P= 0.01*; Fig. 4.5), while the total number of beats did not (*P= 0.45*). These results may indicate that undisturbed termites share their calmness with others using singular

beats and the disturbance causes clumping these beats into bursts with alarm meaning. Bursts of beats are indeed known to bear alarm function (Kirchner et al., 1994; Connétable et al., 1998, 1999; Röhrig et al., 1999; Reinhard & Clément, 2002; Hertel et al., 2011), but as the total number of beats *per* experiment was never scored before, our results cannot be compared with any other reports. However, the use of vibratory communication for other purposes was already described in termites, e.g. in tactile interactions between two individuals (Sbrenna et al., 1992; Leis et al., 1994), to determine the wood size and the quality of food source (Inta et al., 2007; Evans et al., 2009a) and also to distinguish own species from others in order to avoid competition (Evans et al., 2009b). Future studies comparing the alarm responses (including vibratory movements) to different stimuli (light, air, alarm pheromone, intruder) are anyway needed.

The most surprising finding was the significant increase in number of vibratory movements in response to smashed heads treatments in chemical bioassays. SSH enhanced the number of the vibratory movements more than alarm pheromone in the chemical bioassays, moreover, SWH (control in fact) provoked similar frequency of vibratory movements as that observed after the alarm pheromone exposure (see Fig. 4.3B), but haven't increased the speed of motion (see Fig. 4.6; more details also present in Fig. 4.9 and 4.10). At the same time, number of bursts (drumming series) has not increased after SWH exposure in mechanical bioassays (see Fig. 4.5), i.e. only the frequency of shaking but not of drumming increased. These results point at two interesting phenomena: *(i)* some compounds present in head of both worker and soldier are perceived by other colony members, which respond to them by vibratory behaviour but not by increased speed of motion, or *(ii)* there are different levels of excitement, and these are evidenced by vigorousness of vibratory behaviour, of which only the most intense can be detected by vibratory sensors, while the less intense serve only for contact perception. In fact, our results showed that alarm behaviour includes more components, which are mediated by different means and are displayed by different intensities probably according to disturbance nature and intensity ('dose-dependent effect'; see Supplementary Material - Fig. 4.8). The explanation may consist in *(i)* presence of repellent compound(s) occurring in the *C. cyphergaster* heads or *(ii)* presence of frontal gland in *C. cyphergaster* workers. The repellent

compound(s) were postulated according to results of trail-following behaviour in *C. cyphergaster* and *Inquilinitermes microcerus* (see Cristaldo et al., 2012), and can originate from enlarged mandibular glands, described in detail by Costa-Leonardo & Shields (1990). Frontal gland is known to occur in workers of many soldierless Apicotermitinae (see Šobotník et al., 2010a), and have been observed also in workers of several other species since then (Šobotník, *pers. observ.*), future studies must focus in the function of frontal glands in workers.
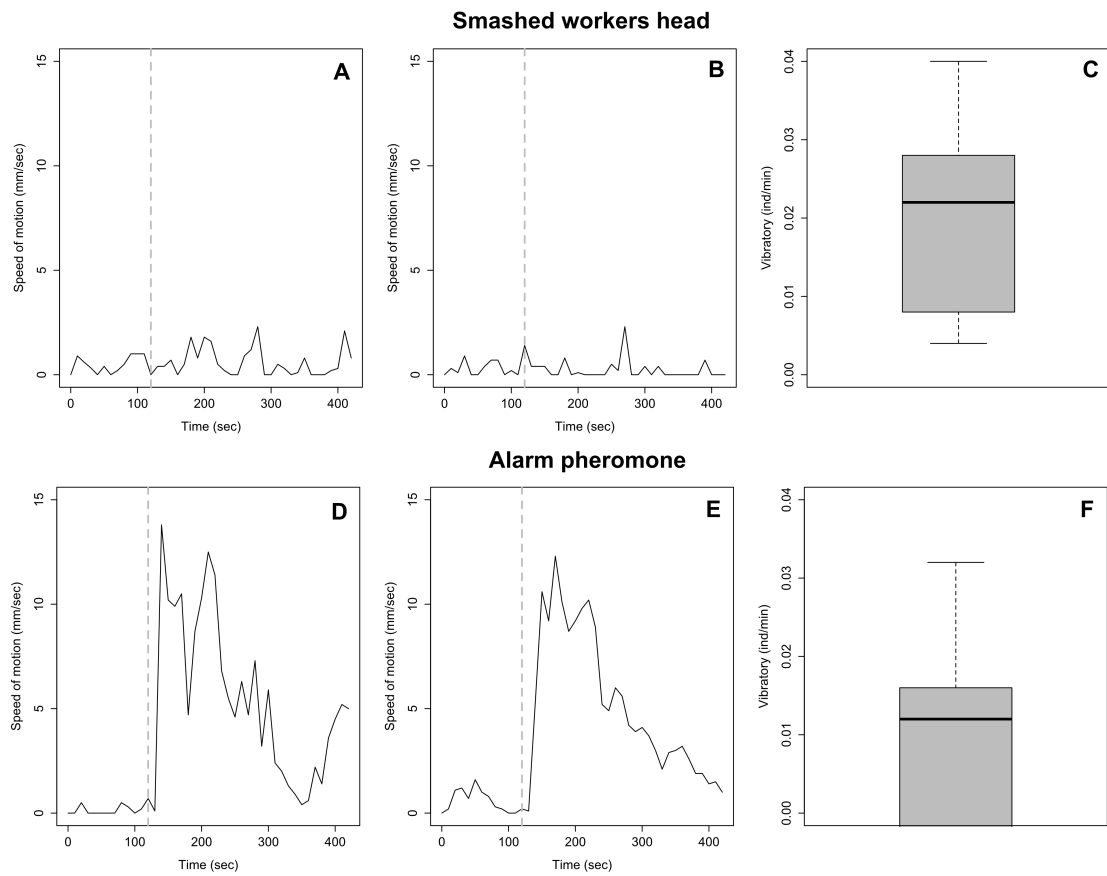
Figure 4.6: Dynamic of alarm behaviour in SWH (**A, B, C**) and AP (**D, E, F**). The typical dynamics of speed of motion of soldiers (**A, D**) and workers (**B, E**) in the course of the experiment, and the number of vibratory movements counted from all recordings (**C, F**). The curves represent measured distances travelled by termites second by second.

Our results show that the levels of alarm signalling are dose-dependent, allowing probably handling different disturbances with differing intensity of responses. For example, AP in 2 head equivalent provoked a lower speed of motion (3.95±0.33) but a high number of vibratory movements (0.022±0.004), while 6 equivalents induced

a high speed of motion (8.99±0.16) and lower numbers of vibratory movements (0.010±0.0005) (see Supplementary Material – Fig. 4.8). In other words, the increase in the speed of motion caused decrease in the number of vibratory movements and *vice-versa*, and these responses might be adaptive in natural conditions, as low level disturbance can be fixed by recruitment of more soldiers, while the escape and hide might be the best response to a large disturbance. Regardless of the exact mechanism creating such behavioural pattern, these findings may help to understand the defensive mechanisms in their complexity, but are also important for future studies, which must take into account stimulus intensity.

The evolutionary trend in the vibratory alarm propagation consists in increasing complexity of the signal, starting from bursts of unstable number of beats in the most primitive termite (e.g. *Zootermopsis*, Archotermopsidae; Stuart, 1988), through stabilization of beat number per burst (*Coptotermes*, Rhinotermitidae; Hertel et al., 2011) to existence of positive feedback reported in several Macrotermitinae (Termitidae; Connétable et al., 1998, 1999; Röhrig et al., 1999)). *Constrictotermes cyphergaster* seems to be rather an exception from this role, being a member of the most advanced subfamily Nasutitermitinae, one of few termites able of foraging in the open during the daylight, as a proof of the efficacy of its defensive strategies, but at the same time showing rather unsophisticated pattern of vibratory communication with unstable number of beats per burst. In spite of a simple pattern of vibratory communication, the positive feedback is in *C. cyphergaster* clearly displayed at least by vibratory alarm response to alarm pheromones. *Nasutitermes* is known to respond to alarm pheromone by release of further pheromone (see Vrkoč et al., 1978), so the presence of positive feedback in the alarm behaviour seems to be a feature specific to the most advanced termites belonging to Termitidae (see also Connétable et al., 1998, 1999).

To conclude, termites are eusocial cockroaches (Inward et al., 2007; Cameron et al., 2012), and their complex social life made them use more sophisticated means of communication compared to solitary relatives. Concerning the alarm function, termites and *Cryptocercus* evolved alarming through body vibrations, and only advanced termites (Rhinotermitidae and Termitidae) rely also upon alarm pheromones. The present study demonstrated the dual function of the alarm signalling system

in *C. cyphergaster.* In addition to vibratory communication known in all termites, the soldier frontal gland secretion contains the alarm pheromone, which is the mixture of abundant [(1S)-α-pinene, myrcene] and rare [(E)-β-ocimene] monoterpenes, all contributing to the desired function. The three-component alarm pheromone is reported for the first time in termites, and may provide necessary specificity to alarm signal even in the open air, where *C. cyphergaster* is foraging for food. The behavioural changes due to chemical alarm signalling include the increase of walking speed and number of vibratory movements in both soldiers and workers. The responses are clearly dose-dependent, as lower doses (represented by 2 soldier equivalents) enhance more the vibratory communication while higher doses (represented by 6 soldier equivalents) affect stronger the walking activity.

Acknowledgements

SUPLEMENTARY MATERIALS

Identification of Alarm signal

Methods

All monoterpenes found in *Constrictotermes cyphergaster* soldier frontal gland secretion (see Tab. 4.2) were tested alone or in combination of two or three compounds in order to identify the composition of the alarm pheromone. The significant increase in the speed of termites motion in comparison with UP and Hex was the only measure in the initial phases of testing. Different concentrations of the signals (2 *vs.* 6 equivalents according to quantification made on natural extracts) were tested later in order to find the optimal setting for testing synthetic compounds.

Results

Of all compounds tested, only the combination of *(1S)*-α-pinene, *(E)*-β-ocimene and myrcene increased the speed of termites motion significantly in comparison with UP and Hex ($P < 0.0001$; see Tab. 4.4) and represent thus the alarm pheromone. The pheromone induced responses comparable to SSH and FGE ($P < 0.0001$). Both signal strengths (2 and 6 equivalents) were able to provoke the alarm behaviour, and responses obtained using 6 equivalents were adequately stronger (see Fig. 4.7; Tab. 4.4) and therefore the higher concentration was used in majority of experiments.

Table 4.4: Overview of synthetic compounds tested in order to identify the activity of particular compounds produced by the frontal gland of *C. cyphergaster* soldier. Tested standards are indicated together with respective signal strengths (gland equivalents), number of repetitions (n) and mean ±s.e. of speed of motion in soldiers and workers. Untreated paper and hexane were used as control. Significant (P < 0.0001) effects are in bold.

	Standards tested	Eq	n	Speed of termites motion (mean ±s.e.)	
				Soldiers	**Workers**
Alone	Hexane	2 Eq	10	0.19±0.02	0.12±0.08
		6 Eq	10	0.40±0.12	0.16±0.05
	α-pinene	2 Eq	4	0.56±0.12	0.32±0.02
		6 Eq	8	0.86±0.23	0.46±0.16
	β-ocimene	2 Eq	6	0.44±0.10	0.30±0.21
		6 Eq	8	0.47±0.10	0.21±0.17
	Myrcene	2 Eq	4	0.33±0.04	0.12±0.02
		6 Eq	4	0.42±0.10	0.31±0.09
	Limonene	2 Eq	4	0.40±0.03	0.13±0.01
		6 Eq	4	0.48±0.03	0.35±0.02
	Camphene	2 Eq	4	0.22±0.09	0.18±0.03
		6 Eq	4	0.32±0.07	0.15±0.01
	Hexane	2 Eq	10	0.21±0.09	0.10±0.02
		6 Eq	10	0.40±0.10	0.36±0.03
	α-pinene and β-ocimene	2 Eq	8	0.60±0.12	0.32±0.09
		6 Eq	8	0.80±0.10	0.48±0.05
	α-pinene and Limonene	2 Eq	6	0.32±0.04	0.45±0.20
		6 Eq	6	0.33±0.18	0.13±0.06
	α-pinene, β-pinene and Camphene	2 Eq	8	0.23±0.08	0.42±0.07
		6 Eq	4	0.43±0.03	0.20±0.10
	α-pinene, β-pinene and β-ocimene	2 Eq	4	0.10±0.01	0.19±0.08
		6 Eq	4	0.58±0.10	0.33±0.02
	α-pinene, β-ocimene and Camphene	2 Eq	4	0.23±0.05	0.64±0.14
		6 Eq	4	0.30±0.07	0.10±0.04
	α-pinene, β-ocimene and Myrcene	**2 Eq**	**6**	**4.56±0.59**	**3.35±0.31**
		6 Eq	**6**	**9.29±0.18**	**8.70±0.19**
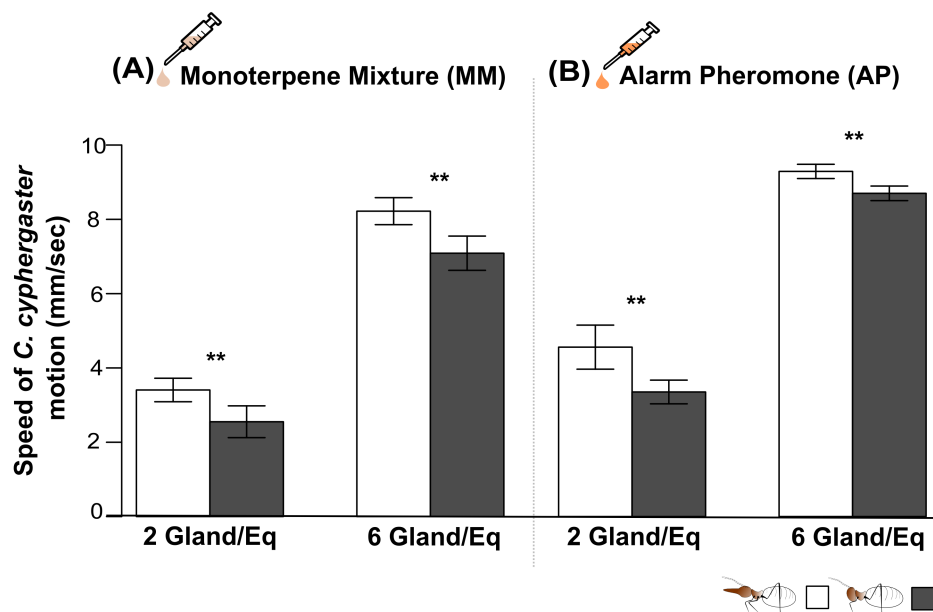
Figure 4.7: Effect of synthetic compounds on the speed of termite motion. **A.** Monoterpenes mixture; **B.** The alarm pheromone mixture. Note that although both signal strengths show significant effect, 6 equivalent treatment produces higher responses.
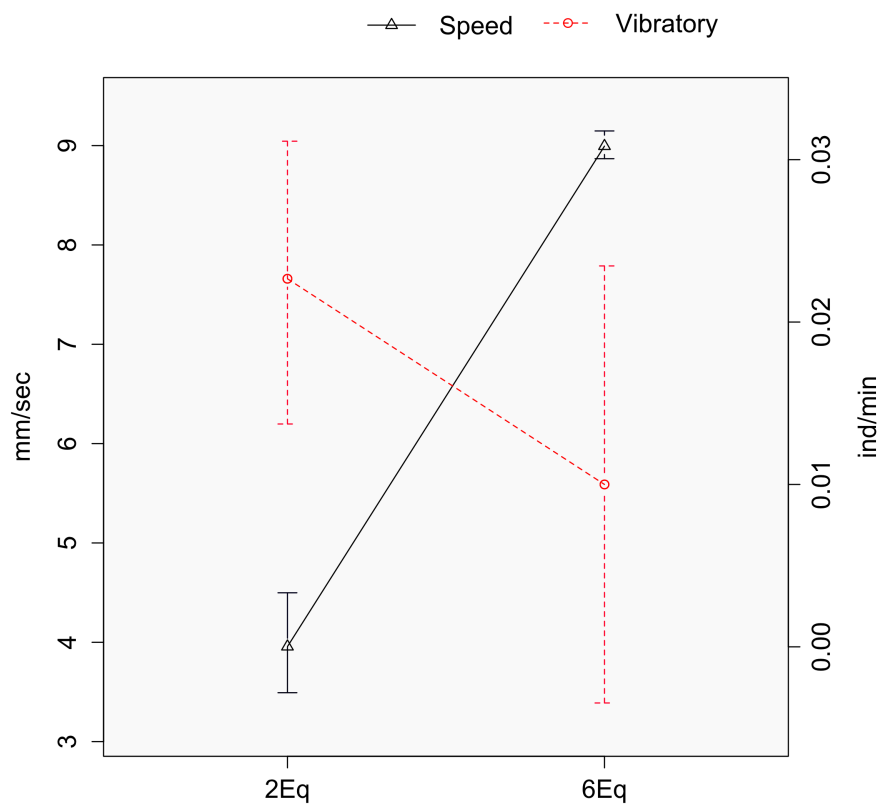
Figure 4.8: Dose-dependent effects of *C. cyphergaster* alarm pheromone in chemical alarm experiments. Note that 2 equivalents induced a lower speed of motion (3.95±0.33) and higher number of vibratory movements (0.022±0.004), while 6 equivalents provoked high speed of motion (8.99±0.16) and lower number of vibratory movements (0.010±0.0005).
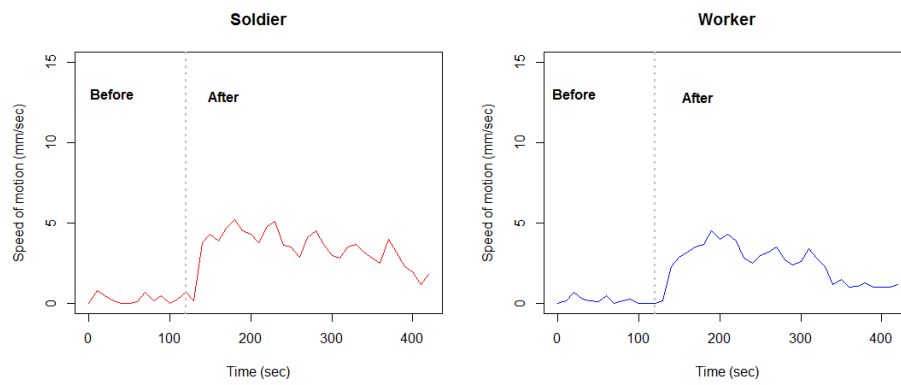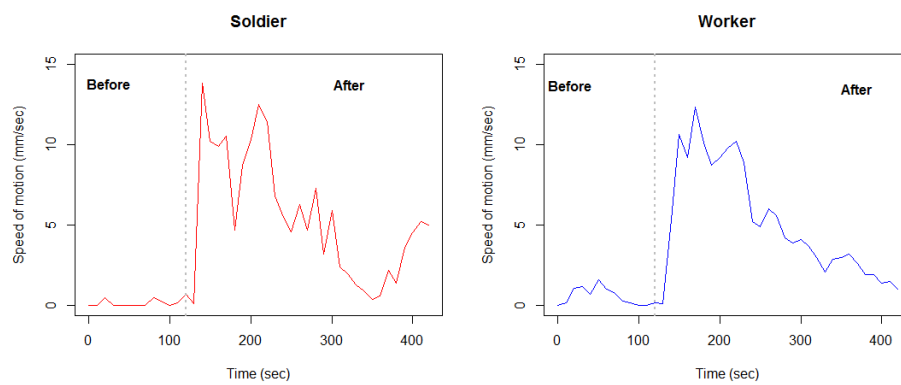
Figure 4.9: Dynamics of walking speed in *C. cyphergaster* soldiers and workers exposed to alarm pheromone in 2 (upper) and 6 (bottom) gland equivalents. Dashed line indicates the insertion of stimuli.
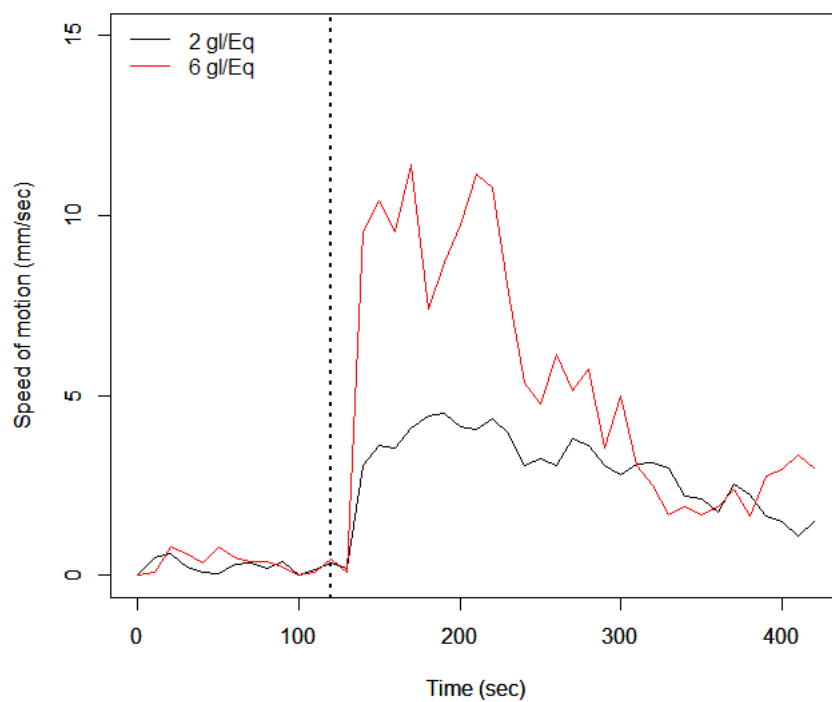
Figure 4.10: Dynamic of walking speed in *C. cyphergaster* exposed to alarm pheromone in 2 (red curve) and 6 (black curve) gland equivalents. Dashed line indicates the insertion of stimuli.

Foreign language skill by guest? Eavesdropping of host alarm signal by inquiline termite species (Blattodea: Termitoidea: Isoptera)

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Abstract

The coexistence of two or more termite species in a single nest is frequently observed in nature. However, how do termite cohabiting species manage such cohabitation remains open to investigation. Here, we investigated whether distinct termite species cohabiting the same nest could eavesdrop each other's alarm cues. Using behavioural bioassays under laboratory conditions, we show that the obligatory inquiline *Inquilinitermes microcerus* responds not only to its own alarm signal but also alarm cues from its host. Conversely their host *Constrictotermes cyphergaster* is able to perceive only its own alarm signal. Inquilines were never observed helping the defense of experimentally damaged nests in the field, despite perceiving their host alarm. Therefore, it seems plausible to infer that termite inquilines eavesdrop their host alarm in their own benefit, e.g., to evade danger signalled by the host or, at least, to avoid nest regions crowded by defenders at a given time.

Keywords: communication; inquilinism; alarm calls; eavesdropping; Isoptera; social information

Introduction

Symbiotic interactions are ubiquitous in nature and include species from all of five kingdoms (Douglas, 1994). Typical examples include the bacteria in digestive system of mammals (Savage, 1977), flowering plants and their pollinators (Sakai, 2002) and guests species living along with the builder in nests of birds (Kilner & Langmore, 2011) and social insects (Kistner, 1979). Although widespread through organisms, it is among social insects that this kind of interaction is arguably most striking, as their long-lived colonies provide a whole ecosystem for thousands of invertebrate and vertebrate species exhibiting all hues of symbiotic partnerships (see e.g. Brandt et al., 2005; Hughes et al., 2008).

Guests in nests of social insects are often other social species belonging to the same group of nest builder (see Wilson, 1971), which result in many characteristics important for co-evolutionary dynamics (Brandt et al., 2005). In the majority of the cases, such interactions involve complex adaptive and counter-adaptive mechanisms, establishing "arms races" between host and guests species (see Brandt et al., 2005; Kilner & Langmore, 2011). Social parasites in Hymenoptera, for instance, employ multiple strategies to overcome barriers to colony invasion, evolving morpho-physiological and behavioural strategies to confront, confound, or avoid completely their host (for a review see Nash & Boomsma, 2008). Hymenopteran hosts, on their turn, evolve counteracting strategies to detect and face the invader (Kilner & Langmore, 2011).

Differently from current understanding regarding hymenopterans, our comprehension of the mechanisms behind symbiotic interactions among termite species cohabiting a single nest remain open to investigation, only recently being studied under functional (as opposed to pattern-finding) perspective (e.g. Cristaldo et al., 2012; Darlington, 2012; Florencio et al., 2013; Cristaldo et al., 2014). Inquiline termites form a particular group of guests because, in constrast to social parasites in Hymenoptera, they seem to not exploit the brood care behaviour or the entire social

system of their host. The absence of it by no means implies the absence of costs. From the host's perspective, the presence of inquiline species can be costly once they seem to feed on stored products and/or on the lining of the nest walls (Noirot, 1970; Mathews, 1977; Bourguignon et al., 2011; Florencio et al., 2013). At the very least, costs could be triggered when inquilines use a space originally built for the host's nestmates.

To deal with such conflicts, a plausible hypothesis would sustain that inquiline selection favours the adoption of strategies to minimise costs to the host, which inquilines could achieve (*i*) inflicting low loss or (*ii*) offsetting losses with an associated benefit. Inquiline termite species may use conflict-avoidance strategies and thereby pose milder loss to their host through *(i)* the establishment of colonies apart from their hosts within the nest (Grassé, 1986; Cunha et al., 2003), *(ii)* differentiation in resource use (*diet segregation*; Florencio *et al.*, 2013) and/or *(iii)* the use of the host trail-following cues to evade detection within the nest (Cristaldo et al., 2014). However, it seems also plausible that termite host species derive a benefit from the association with their inquilines. According to Coles (1980) and Redford (1984b), the presence of multiple termite species with different defensive strategies in the same nest can enhance the defense of termitaria. If it happens, the interaction between host and inquilines could be classified as mutualistic, in which the host provides shelter to its inquilines and these, in turn, enhance nest defense. Non-termite guests of termites enhancing the defense of host nest was already observed in *Amitermes laurensis* (Termitidae: Termitinae) and their termitophile ants (Higashi & Ito, 1989). As for termite-termite associations, to the best of our knowledge, no direct experimental data has been gathered so far.

In order to join host's armed forces thereby helping nest defense, inquilines must inform and get informed about potential threats. To do so, inquiline species must decode their host alarm and, likewise, the host must decode inquilines' alerts. Conversely, sneaky perceiving the host alarm cues can be also a strategy used by parasites (Nash & Boomsma, 2008) as a means of intercepting host communication, avoiding risks signalled by the host and/or nest regions crowded by host defenders. In such case, inquilines are better off when hosts can not perceive their cues.

Here we provide evidence, at least for the system at hand, that termite inquilines

use host alarm cues in their own benefit, avoiding risks signalled by the host and/or nest regions crowded by host defenders. By doing so, inquilines can easily avoid two potential risks: to be killed by host predators or disturbed by host defenders. We arrived at this conclusion using behavioural bioassays under laboratory conditions, where we analysed the response of the host and its inquiline to its own and to each other's alarm signals. After that, in the field, we observed the species involved in the defense of experimentally damaged nests, checking for evidence of joining nest defense on the part of the inquiline. Field and lab assays have been conducted using the termite host *Constrictotermes cyphergaster* Silvestri, 1901 (Termitidae: Nasutitermitinae) and its obligatory inquiline *Inquilinitermes microcerus* Silvestri, 1901 (Termitidae: Termitinae).

Material & Methods

Terms Definition

The term 'nest' denotes the physical structure built by termites and 'colony' denotes the assemblage of individuals of a given species living and cooperating within the nest. 'Cohabitation' and 'coexistence' are used as synonyms and refer to the simultaneous occurrence of colonies of different termite species within a given nest, without implication of reciprocal positive or negative influences. 'Inquiline' and 'guest' are used as synonyms and refer to heterospecific termite-termite cohabitation. All these definitions were already used by other authors in the termite-termite cohabitation studies (see Cristaldo et al., 2012; Florencio et al., 2013; Cristaldo et al., 2014).

System

Constrictotermes cyphergaster is a common Neotropical species occurring in Brazil, Paraguay, Bolivia and Northern Argentina (Mathews, 1977; Krishna et al., 2013). This species is characterised by an unusual life-style combining arboreal nests, leaf-litter (Moura et al., 2006a) or micro-epiphyte diet (Bourguignon et al., 2011) and 'open-air' foraging with soldiers as guard (Moura et al., 2006b). Workers as well soldiers are monomorphic and all males (Moura et al., 2011). Alarm pheromone is composed by a mixture of three monoterpenes: $(1S)$-α-pinene, myrcene and (E)-β-ocimene, which trigger a multimodal alarm transmission among nestmates (Cristaldo et al., prep). Their nests play an import role in the 'Cerrado' diversity due to housing high numbers of termitophiles and an obligatory inquiline *Inquilinitermes microcerus* (Cunha et al., 2003; Cristaldo et al., 2012; Florencio et al., 2013; Cristaldo et al., 2014). *Inquilinitermes microcerus* is known to live in galleries separated from the builder's (Mathews, 1977). Colonies are restricted to certain portions of the nest, usually close to its core (Cunha et al., 2003).

Study site

Arboreal nests of *C. cyphergaster* inhabited with colonies of *I. microcerus* were sampled in the Brazilian 'Cerrado', an environment physiognomically but not floristically similar to a savannah, near Sete Lagoas town (19' 27° S, 44' 14° W; altitude 800-900 m above sea level), Minas Gerais State, South-eastern Brazil. In Köppen's classification, the study area has an *Aw* climate (equatorial with dry winter) (Kottek et al., 2006). The native biota is considered fire-dependent or at least fire-tolerant (see Coutinho, 1990; DeSouza et al., 2003).

Mutual response to alarm signals

In order to check whether termite cohabiting species (host and guest) are able to recognize and respond the alarm signal from heterospecific, behavioural bioassays were done in July 2012 as described bellow. Bioassays aimed to measure the 'alarm' reaction in termite host and their guest when exposed to to its own (Coespecific - CS) and to each other's (Heterospecific - HS) alarm signals. The measurement of alarm reaction in CS was used as our 'true control' in order to have a comparison between response found in HS treatments.

Behavioural bioassays set up

Bioassays were carried out in an experimental arena made from plastic Petri dish (⌀ 85 mm) with a slit in the cap and moisture filter paper (Whatman N° 1) as substratum. Groups of termite from the same colony were transferred into the experimental arenas at least 2 hours prior to observation ensuring their acclimatization. After acclimatization, the behaviour of termite group was video recorded for two minutes before introduce the tested stimulus. The tested stimulus (see below in 'Stimuli tested' subsection) was loaded on a piece of filter paper (7 x 3 mm) and immediately introduced into the arena through the slit in the dish cap. It was hung out of reach of termites by a pin bridge over the slit. From then on, another video recorded was done for five minutes. At all, the behaviour of termite group was video recorded for seven minutes, of which the first two minutes refers to before stimulus insertion and five minutes refers to after stimulus insertion. Such procedure is nec-

essary to evaluate idle activity of groups and it is a well-established methodology to study alarm behaviour (see e.g. Šobotník et al., 2008a). All videos were recorded with a Nikon™ D300s.

The first series of behavioural bioassay was carried out to evaluate the alarm reaction to CS signals, i.e. stimuli from same species tested. The second series was carried out to evaluate the alarm reaction to HS signals, i.e. stimuli from cohabiting species (host was exposed to inquiline signals and *vice-versa*).

Stimuli tested and their preparation

Stimuli tested were those that it is known to provide some 'alarm signal' in termites (see for details Deligne et al., 1981; Prestwich, 1984; Šobotník et al., 2010c) and their respectively controls: (*i*) untreated paper (UP) (blank control), (*ii*) smashed workers head (SWH; smashed treatment control), (*iii*) smashed soldiers head (SSH), (*iv*) hexane (Hex; solvent control) and (*v*) frontal gland extract (FGE). An overview of stimuli with respective description, type and quantity used in the experiments is shown in Tab. 1.

For smashed head treatments, workers and soldiers were anaesthetized on ice and dissected into head and rest of body. Heads were immediately smashed by the tweezers pressure against the test filter paper. Hex used was purchased from Merck and redistilled prior to use. FGE was prepared using soldiers anaesthetized on ice and then dissected into head and rest of body. Heads with a cut off were placed into the hexane (10 μL per head), and extracted for 24 h at 4°C followed by a second washing with 10 μL of hexane per head. Final samples were stored at -18°C and used in the bioassays. According to the known volume of extract, we recounted the amount for two soldiers frontal gland equivalent which was used in bioassays. A Hamilton microsyringer (10 μL) was used to load Hex and FGE onto the test filter paper.

For each one of stimuli tested (UP, SWH, SSH, Hex and FGE), it was performed four repetitions with individuals from different colonies, totalling 20 repetition for CS bioassays and 20 repetition for HS bioassays in each species.

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Table 5.1: Overview of stimuli tested with description, type and quantity (amount) used in the bioassays. All stimuli described were prepared from conspecific and heterospecific species.

Stimuli	Abreviation	Description	Type	Amount used
Untreated paper	UP	Filter paper without stimulus	blank control	–
Smashed workers head	SWH	Workers head mashed by the tweezers pressure against the filter paper	smashed control	2 *heads*
Smashed soldiers head	SSH	Soldiers head mashed by the tweezers pressure against the filter paper	smashed treatment	2 *heads*
Hexane	Hex	Pure hexane load with micro-syringe onto filter paper	solvent control	2 *glands/Eq.*
Frontal gland extract	FGE	Extract of soldiers frontal gland load with micro-syringe onto filter paper	natural treatment	2 *glands/Eq.*

Termites group

The number and caste ratio of termites groups used in the bioassays were chosen according to natural caste proportion (S:W= 1:4.5 for *C. cyphergaster* and 1:8.5 for *I. microcerus*; see Cunha et al., 2003) and to maximize their interaction and survival (see Miramontes & DeSouza, 1996; DeSouza et al., 2001). For *C. cyphergaster* (host), the group was composed by five soldiers and 20 workers and for *I. microcerus* (guest), two soldiers and 20 workers composed a group.

Measurement of alarm response

In termites, typical alarm reaction involves behavioural changes such as (*i*) substrate-borne vibrations and (*ii*) escape away of a disturbance source (for review see Prestwich, 1984; Šobotník et al., 2010c; Hunt & Richard, 2013). To check the alarm response in the bioassays, two parameters were taken as indicative of alarm reaction: (*i*) vibratory movements and (*ii*) speed of termites motion. Both parameters were measured independently in each one of CS and HS stimuli, using Mouse-Tracer software.

Vibratory movements was obtained pressing a specific key for each vibratory movement displayed by all termites individuals in the experimental arena. For each record, the sum of all vibrational events was measured. Final vibratory events was obtained with difference in the mean of vibratory events (vibratory events *per* time) before and after a stimulus insertion, and used for subsequent statistical analyses.

Speed of termite motion was obtained positioning the cursor on the selected individual, which was followed on the computer screen. The cursor position *per* second was initially expressed in pixels and subsequently converted into millimetres. For each record, the speed of motion was initially measured in one soldier and one worker and subsequently converted in an average. The selection of termites to track in all experiments was designed to include all possible termite statuses at the beginning of the experiment as describe in Šobotník et al. (2008a). Final speed of motion in *mm/sec* was obtained with difference in the speed of motion before and after a stimulus insertion, and used for subsequent statistical analyses.

Nest defense in natural conditions

In order to check which cohabiting species are involved in the host nest defense, 36 arboreal nests of *C. cyphergaster* were subjected to experimental disturbance in July 2008 ($n = 16$) and July 2012 ($n = 20$).

Experimental disturbances were done in field by piercing a hole through the nest wall in the middle section of the nest using a pocket knife (approx. 5 cm deep), as described in Cristaldo (2010). We scored the number of termites, soldiers and workers, appearing at the damaged point and we also noted down the species (host or inquilines).

Statistical analyses

In order to check whether termite cohabiting species would perceive the HS alarm signal, data were analysed using Generalized Linear Models (GLM), choosing the error distribuition according the response variable as describe bellow. All analyses were performed in R (R Development Core Team, 2012), followed by residual analysis to verify the suitability of error distribution and model fitting.

In order to check if stimuli affect the number of vibratory events, three statistical models have been built, for each species independently. All models included 'vibratory events' as response variable ($y - var$) under Poisson distribution. One of the models included as explanatory variable ($x - var$) the type of stimuli (UP, SWH, Hex, SSH and FGE) in the CS bioassays. The second model also included as explanatory variable ($x - var$) the type of stimuli, but now in the HS bioassays. The third model included as explanatory variable ($x - var$) the source of stimuli, *i.e.* from CS or HS species. Model simplification, in the case of the first two models, was done through Contrast Analysis with $Chi - squared$ tests, lumping treatment levels (*i.e.stimuli*) together as long as this lumping did not cause significant (P < 0.05) changes in the model.

In order to check if stimuli affect the speed of termite motion, three statistical models have been built, for each species independently. All models included 'speed of motion' in mm/sec as response variable ($y - var$) under Normal distribution. One of the models included as explanatory variable ($x - var$) the type of stimuli (UP,

SWH, Hex, SSH and FGE) in the CS bioassays. The second model also included as explanatory variable ($x - var$) the type of stimuli, but now in the HS bioassays. The third model included as explanatory variable ($x - var$) the source of stimuli, *i.e.* from CS or HS species. Model simplification, in the case of the first two models, was done through Contrast Analysis with F tests as described above.

Results

Mutual response to alarm signals

Vibratory events

The total number of vibratory events displayed in *C. cyphergaster* was significantly affected by stimuli from CS ($F_{[4,15]}$= 7.066; p < 0.002). The highest number of vibratory events was observed in SSH stimulus followed by SWH and FGE compared to UP and Hex ($F_{[2,17]}$= 16.081; p < 0.001; Tab. 5.2). Otherwise, the total number of vibratory events displayed was not affected by stimuli from HS (χ^2= 15.589; d.f.= 20; p= 0.902; Tab. 5.2). In CS stimuli the total number of vibratory events displayed in *C. cyphergaster* was significantly higher compared to HS stimuli ($F_{[1,38]}$= 65.441; p < 0.001).

Surprisingly *I. microcerus* never displayed vibratory movements behaviour, neither in CS or HS stimuli, which can indicate the absence of such behaviour in *I. microcerus*.

Table 5.2: Minimum, maximum and mean ($\pm$SE) values of vibratory events displayed by *Constrictotermes cyphergaster* in the behavioural bioassays, for both CS and HS stimuli. *Inquilinitermes microcerus* never displayed such behaviour.

Type	Stimuli	Minimum	Maximum	Mean$\pm$SE	P-values
CS	UP	0	2	0.50$\pm$0.50 **a**	< **0.001**
	Hex	0	2	0.50$\pm$0.50 **a**	
	SWH	1	8	4.00$\pm$1.58 **b**	
	SSH	2	20	11.50$\pm$4.42 **c**	
	FGE	3	5	4.25$\pm$0.47 **b**	
HS	UP	0	1	0.50$\pm$0.50	0.902
	Hex	0	1	0.25$\pm$0.25	
	SWH	0	2	0.75$\pm$0.47	
	SSH	0	1	0.50$\pm$0.28	
	FGE	0	1	0.50$\pm$0.29	

Speed of termite motion

The speed of *C. cyphergaster* motion was significantly affected by different stimuli from CS ($F_{[4,15]}$= 145.97; $p < 0.001$; Fig. 5.1). Increase of speed was observed in SWH, SSH and FGE, in ascending order, compared with UP and Hex ($F_{[3,16]}$= 207.39; $p < 0.001$). Unlike, the speed of *C. cyphergaster* motion was not affected by the stimuli from HS (guest) ($F_{[4,15]}$= 0.8591; p= 0.5104; Fig. 5.1). In CS stimuli the speed of *C. cyphergaster* was significantly higher compared to HS stimuli ($F_{[1,38]}$= 10.077; p= 0.002; Fig. 5.1).

In *I. microcerus*, the speed of motion was significantly affected by different stimuli from CS ($F_{[4,15]}$= 130.49; $p < 0.001$; Fig. 5.1) and also by stimuli from HS ($F_{[4,15]}$= 78.625; $p < 0.001$; Fig. 5.1). In both, CS and HS stimuli, the increase of speed was observed in SWH, SSH and FGE, in ascending order, compared to UP and Hex ($F_{[3,16]}$= 173.03; $p < 0.001$ to CS stimuli and $F_{[3,16]}$= 111.69; $p < 0.001$ to HS stimuli). There was no differences between the speed of *I. microcerus* in CS and HS stimuli ($F_{[1,38]}$= 3.025; $p = 0.09$; Fig. 5.2). This seems to support the idea that *I. microcerus* are able to perceive their own alarm as well as the alarm signal of their host.
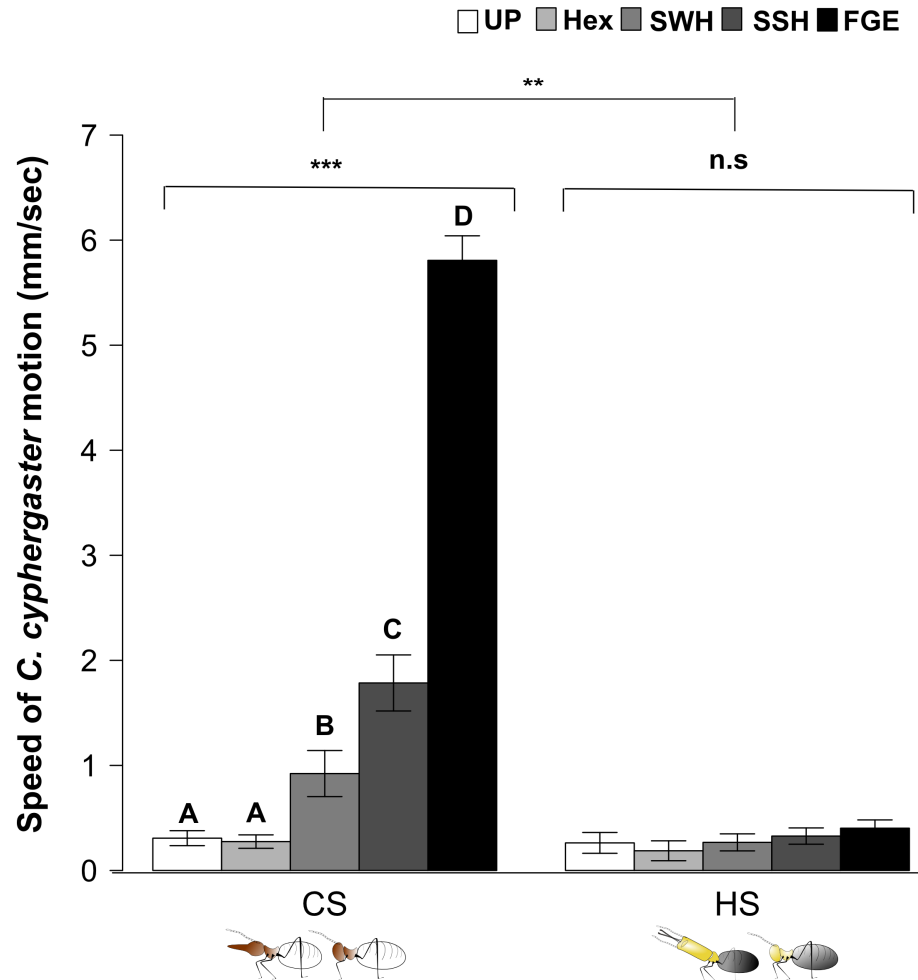
Figure 5.1: Alarm recognition of host *Constrictotermes cyphergaster* to conspecific (CS) and heterospecific (HS - its guest) signals. The vertical $axis$ decipts the speed of *C. cyphergaster* motion (mean±SE) exposed to different stimuli (bars) from CS and HS during behavioural bioassays. Significance indicated as ***p < 0.001; **p < 0.01; n.s. not significant: p> 0.05. *Stimuli:* UP: untreated paper; Hex: Hexane; SWH: smashed workers head; SSH: smashed soldiers head; FGE: soldier's frontal gland extract.
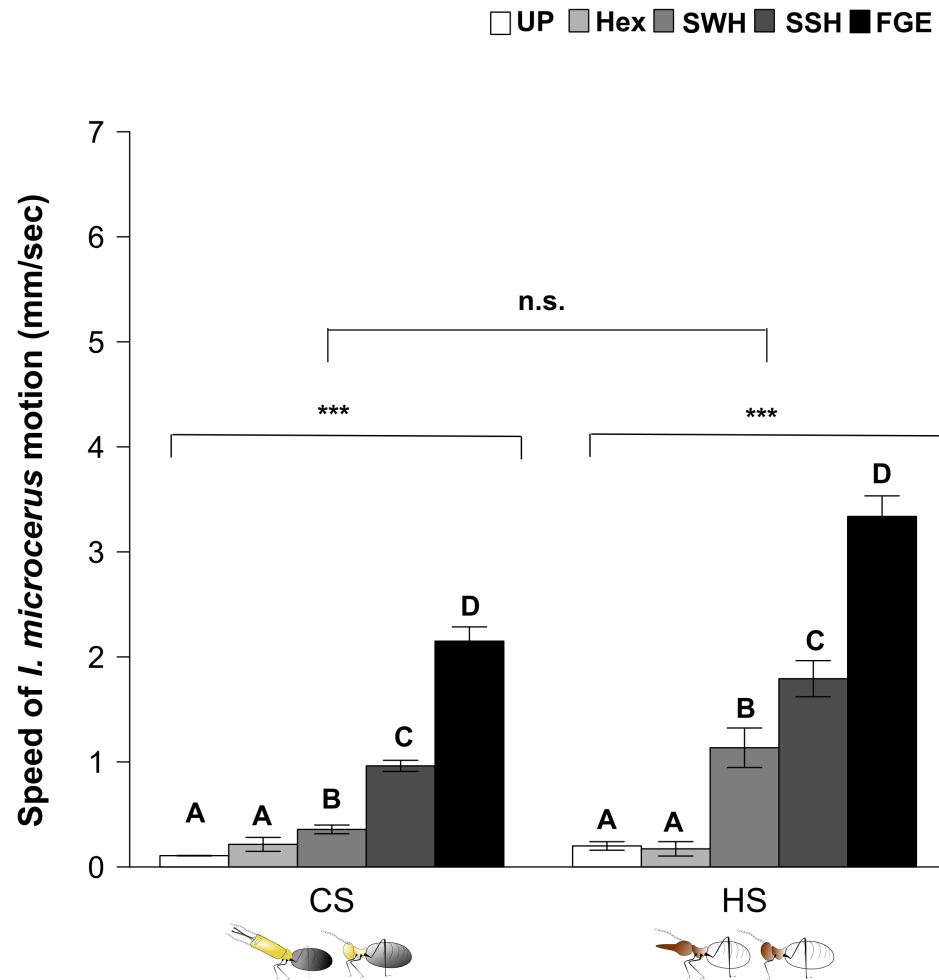
Figure 5.2: Alarm recognition of guest *Inquilinitermes microcerus* to conspecific (CS) and heterospecific (HS - its host) signals. The vertical $axis$ decipts the speed of *I. microcerus* motion (mean±SE) exposed to different stimuli (bars) from CS and HS during behavioural bioassays. Significance indicated as ***p < 0.001; n.s. not significant: p> 0.05. *Stimuli:* UP: untreated paper; Hex: Hexane; SWH: smashed workers head; SSH: smashed soldiers head; FGE: soldier's frontal gland extract.

Defense of nest cohabited in natural conditions

Disturbances in the nest wall of *C. cyphergaster* nests inhabited with colonies of *I. microcerus* showed a quickly alarm response, which were always performed by individuals of *C. cyphergaster* colony (Tab. 5.3). In the most of sampled nest, *C. cyphergaster* soldiers were the first caste arriving followed usually by other soldiers. In few nests, *C. cyphergaster* workers were also observed but always in low quantity (maximum of two workers). Neither soldiers or workers of *I. microcerus* were observed at disturbed point in all sampled nest. Overall summary of individuals at disturbed point per nest sampled was shown in Fig. SM01.

Table 5.3: Mean ($\pm$SE) of *C. cyphergaster* and *I. microcerus* individuals at disturbed point in the wall of *C. cyphergaster* nests inhabited with colonies of *I. microcerus* in natural conditions. Experiments performed in 2008 ($n = 16$) and 2012 ($n = 20$) in a Brazilian 'cerrado' (Sete Lagoas-MG).

	Individuals at disturbed point	
Species on defense	2008	2012
C. cyphergaster	4.0$\pm$0.97	17.2$\pm$3.25
I. microcerus	0.0$\pm$0.0	0.0$\pm$0.0

Discussion

Our results have shown that the the ability to recognize alarm signals by the studied termite hosts and their inquilines was asymmetric: *C. cyphergaster* did not recognize the alarm cues of their inquilines *I. microcerus* (see Fig. 5.1), but *I. microcerus* recognize alerts from both, their hosts as well as their conspecifics (see Fig. 5.2). In recognizing alerts from the host they were able to respond to it, increasing their walking speed (Fig. 5.2). In addition, *I. microcerus* were never observed acting in defense of the host nest (see Tab 5.3). It seems therefore plausible to conclude that these inquilines are 'eavesdropping' (*sensu* Matessi et al., 2008) their hosts' alarm signals, establishing a form of 'informational parasitism' (see Nuechterlein, 1981), in which they use the host signals to evade danger.

The ability to detect and use visual, acoustic, olfactory and other heterospecific signals is frequently observed among organisms (Matessi et al., 2008). It is often used by predators and parasites to locate their prey (Zuk & Kolluru, 1998) but it has been already recorded among species from the same trophic level, such as mixed-groups of lemurs or of primates (see e.g. Seyfarth & Cheney, 1990; Zuberbuhler, 2000; Seiler et al., 2013). Because these latter have shared or similar predators, the ability to eavesdrop the alarm signals emitted by heterospecifics can provide considerable life saving information. It follows that such an ability must be even more evident among species coexisting in confinement and hence exposed to the same threats, such as wood feeding termites confined in the same log or host and inquilines of social insects cohabiting the same nest. In termites confined in wood, eavesdropping of superior competitors by their weaker counterparts has been already documented (Evans et al., 2009b): vulnerable dry wood species *Cryptotermes secundus* are able to detect vibrational cues of their superior competitor *Coptotermes acinaciformis*, avoiding places in which this species is present and thus decreasing risks of confrontation.

Two theoretical scenarios seem plausible for the specific case of eavesdropping

by hosts and inquilines: on the one hand, alerts can be mutually shared so that both species could join defense forces or, at least, flee more efficiently. Conversely, if only one of the cohabitant species is able to glean information from heterospecifics, it could profit from this extended watching in a somewhat parasitic way.

This last case seems to be appliable to the results here obtained for *C. cyphergaster* and its inquiline *I. microcerus*. Despite perceiving and responding to their host's alarm cues in lab bioassays (Fig. 5.2), *I. microcerus* have never been recorded to join defense by attending events of simulated breaking and entering in nests, carried out in numerous (c.a. 36) field assays carefully kept apart in space and time (Tab. 5.3). While such a behaviour does not convey evident benefits to the host, on the part of the inquilines it seems of double value: to inform about imminent threats suffered by their cohabitants (and themselves by extension) and to reveal the proximity to nest regions crowded by alerted host defenders, eager to evict any invader. This sneaky behaviour is reinforced in the inquilines by the use of alarm chemical compounds to which their host is unable to react (Fig. 5.1). Most remarkably, inquiline was never observed performing vibrational events; a typical alarm behaviour observed among all termite studied so far (see Šobotník et al., 2010c). That is, inquilines truly perform eavesdropping as they *listen without the speaker's knowledge*, according the definition of dictionary Oxford.

It seems compelling to conclude that evolution has shaped hiding behaviours in *I. microcerus*, possibly favouring diminished cohabitation costs, as proposed by Florencio et al. (2013) and further inspected by Cristaldo et al. (2014), arguably allowing to divert investments to reproduction, as hinted by Cunha et al. (2003). This latter possibility, while still open to investigation, finds support on the fact that *I. microcerus* colonies have been consistently observed to produce proportionately more alates than soldiers while their *C. cyphergaster* cohabiting host colonies produced proportionately more soldiers than alates in the same nest and season: in a preliminary full survey of the whole population in five cohabited nests, we found, on average, 2.14±3.66 alates to every soldier in the inquiline colonies and 0.21±0.38 alates to every soldier in the host colonies.
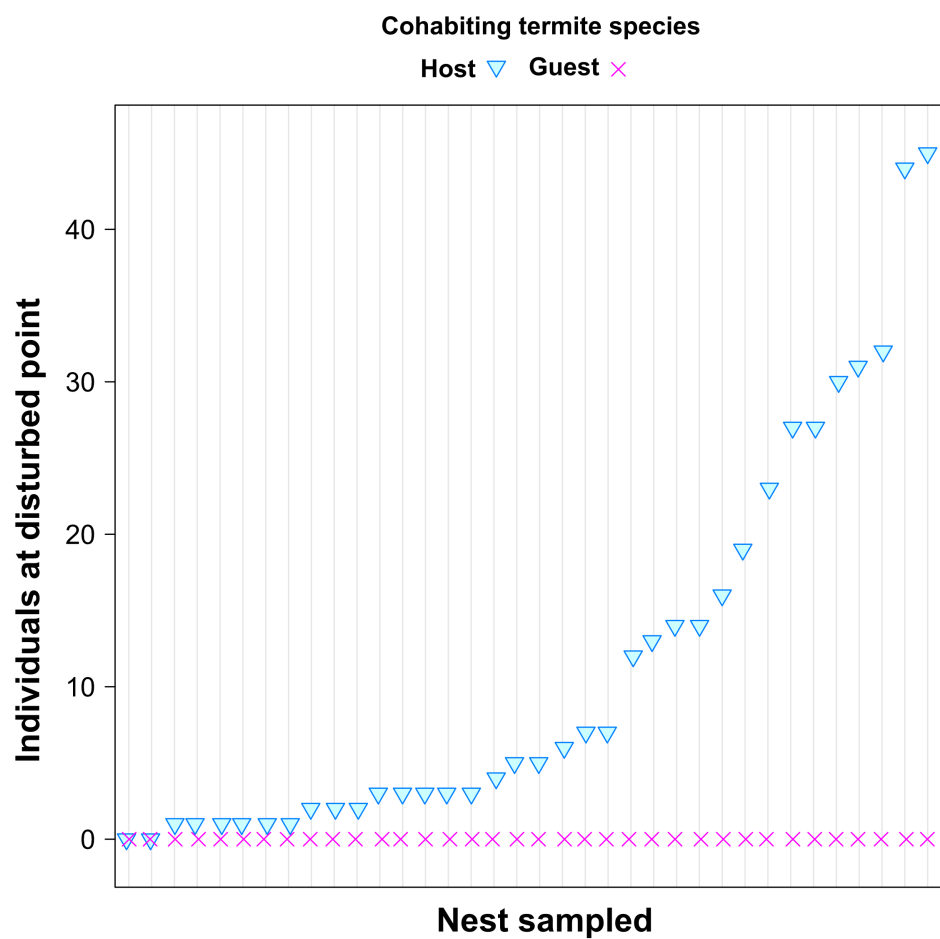
Even if Cunha et al.'s hypothesis is eventually proven unsustained, a clear picture still seems to be emerging for the inquilinism by *I. microcerus* on *C. cyphergaster*: we

found no evidence for mutualism in the form of combined nest defense, as proposed by Coles (1980) and Redford (1984b) for inquilines possessing armoury distinct from their host (as it is the case here). Rather, our data fits very well with previous results which suggest that cohabitation in this case is eased by the inquilines actively avoiding encounters and hence conflicts with their host. That is, *I. microcerus* not only avoid diet overlaps with their host (Florencio et al., 2013) but also decode their host trail (Cristaldo et al., 2014) and alarm (this work) chemical signals, most likely as means to avoid confrontation arising from overlaps in space.

Acknowledgements

Supplementary Material

Figure SM01. Total number of *Constrictotermes cyphergaster* (triangle) and *Inquilinitermes microcerus* (asterisk) individuals at disturbed point in the wall of *C. cyphergaster* nests inhabited with colonies of *I. microcerus*. Each vertical line in grey refers a one nest sampled in 2008 and 2012 in a Brazilian $'cerrado'$. For details see Material & Methods.

Chapter 6

General Conclusions

The results generated by the present thesis showed that, at least for the system at hand, the colonization of termite nest by obligatory inquiline is linked with its host's colony development, which seems to indicate that inquiline needs its host colony active and mature to live in the nest (Chapter 2; Cristaldo et al., 2012). To live in the nest along with its host colony, inquiline species adopt the 'conflict-avoidance' strategy to establish the coexistence over ecological and evolutionary time. Such 'conflict-avoidance' strategy occurs through chemical insignificance in trail-following (Chapter 3; Cristaldo et al., 2014) and alarm (Chapter 5) pheromones by inquiline species. In addition, it was possible to provide evidence that inquiline species gains benefit of defense by eavesdropping its host alarm cues (Chapter 5). Beyond these insights on termite-termite obligatory association, we provided important findings to termite biology itself, including for instance: (i) the first thorough analysis of a termite alarm showing that the advanced termite *C. cyphergaster* integrates chemical and mechanical signals to escalate calmness into alert and then into alarm and also that distinct intensities of the same chemical signal elicits different responses, evidencing semantic properties in the alarm communication of this species (Chapter 4) and (ii) important biological features of the poorly studied inquiline *I. microcerus*.

All in all, the findings generated by this thesis adds a new dimension not only to termitology, but also to other important fields of science. I hope that the presented results are considered an important contribution to our efforts to understand these unresolved association between termite species in a single nests and that could generate new hypothesis to future works.

Chapter 7

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