

# Chemical basis of nestmate recognition in a defense context in a one-piece nesting termite

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**Abstract** Nestmate recognition is a necessary capacity for the occurrence of discrimination between nestmate and non-nestmate individuals. In one-piece nesting termites, which nest and forage in a single piece of wood, nestmate recognition is poorly studied mainly because the probability of encountering exogenous individuals is low in comparison with separate-piece nesting termites. Previous work described that production of soldiers of *Neotermes chilensis*, a one-piece nesting termite, increased when the risk of invasion of their colony increased, for example when neighboring colonies were present in the same nesting substrate and members of different colonies met when digging galleries. If soldiers are to fulfill their defensive role under these circumstances, they should show nestmate recognition ability; moreover, based on work on other social insects, such nestmate recognition should be based on cuticular compounds (CC). Bioassays were performed in which a soldier of *N. chilensis* was confronted with a nestmate or non-nestmate primary reproductive, pseudergate or another soldier, and in which a soldier was confronted with untreated and with CC-deprived dead primary reproductives. The results showed that soldiers were indeed more aggressive toward non-nestmates than

nestmates for all castes, and that this discrimination was mediated mainly by qualitative (simple matching coefficient) and quantitative (Renkonen index) differences in CC.

**Keywords** Nestmate recognition · Cuticular compounds · Termites · GC–MS · *Neotermes chilensis* · Soldier defense role

## Introduction

Nestmate recognition is a necessary capacity for the occurrence of discrimination between nestmate and non-nestmate individuals (Sherman et al. 1997) and can be inferred from the outcome of the interaction between conspecifics (Smith and Breed 1995) or between an individual confronted with odors from conspecifics (Nehring et al. 2012) from the same or different nests (or colonies); e.g., an organism may be less aggressive toward a nestmate (a colony resident) than toward a non-nestmate (an intruder) (Flores-Prado et al. 2008) or it can exhibit more threat behaviors when confronted with non-nestmate than with nestmate odors (Nehring et al. 2012). An adaptive value of nestmate recognition linked to colony defense in social insects has been postulated (Breed and Bennett 1987), because this ability is related to the success of colonies to defend their resources and to maintain colony viability (Šobotník et al. 2008), especially in species where the likelihood of encounters with individuals from other colonies is high. Thus, the lack of a discrimination system could make a colony vulnerable to invaders of all types (Myles and Nutting 1988). In social insects, chemical communication is the most ancient and widespread mode of communication (Candolin 2003) and is an important determinant of nestmate recognition (Richard and Hunt 2013).

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Termites are a group of eusocial insects that live in colonies formed by hundreds and even millions of individuals (Eggleton 2011). According to their nesting ecology and feeding habits, several classifications of termites have been proposed (Abe 1987; Shellman-Reeve 1997; Donovan et al. 2001; Korb and Hartfelder 2008; Eggleton 2011), all of them including separate-piece nesters which nest and forage in different substrates and one-piece nesters which nest and forage in a single piece of wood.

Termite colonies are composed of individuals belonging to different castes (Korb and Hartfelder 2008). Castes common to all types of termites are: (1) primary reproductives which have reproduction as their sole role, although they feed and take care of the first generation of offspring; (2) true workers or pseudergates in charge of feeding juveniles and other castes and also of extending the nest; while true workers are terminal castes after the first molt in separate-piece nesters, i.e., they are not able to develop into other castes (Korb and Hartfelder 2008; Roisin and Korb 2011), pseudergates are totipotent individuals characteristic of one-piece nesters which remain as such or become alates or soldiers depending on environmental conditions (Roisin and Korb, 2011); (3) alates which develop wings inside the colony, fly away from the colony during a swarming event and, after performing a reproductive behavioral repertoire, may found a new colony and become primary reproductives; and (4) soldiers which are responsible for colony defense.

Competition with other wood- or soil-feeding animals, particularly other termite con- or heterospecific colonies, has been suggested as the biggest threat to colony survival in termites (Šobotník et al. 2010). In separate-piece nesting termites, soldiers defend their colony from intra- and interspecific competitors during foraging and are the first individuals involved in searching a foraging substrate (Traniello and Beshers 1985). In one-piece nesting termites, where non-alate individuals spend all their life inside the nest, soldiers play their defensive role in other contexts. Thorne et al. (2003) showed that when more than one colony of the one-piece nesting termite *Zootermopsis nevadensis* (Termopsidae) share a limited substrate, they perform agonistic behaviors when their galleries meet. Moreover, they showed that invasions of the co-existing colonies are mainly carried out by soldiers and pseudergates. Recently, Aguilera-Olivares et al. (2016) studied nest architecture and caste composition in the one-piece nesting termite *Neotermes chilensis* (Kalotermitidae) and found that in nearly half of the substrates where they live (cylindrical pieces of dry wood ca. 10-cm diameter × 200-cm length) more than one colony (up to 9) co-existed, thus making intercolonial interactions likely. Moreover, they observed that the ratio of soldiers to non-soldiers in a

colony was higher in substrates collected during the pre-swarming period when neighboring colonies were present. Thus, pseudergate differentiation into soldiers seems to increase when the risk of invasion by members of another close-by colony increases.

A corollary of these findings is that soldiers of one-piece nesting termites should exhibit the capacity to discriminate between nestmate and non-nestmate individuals. Moreover, on the basis of work on separate-piece nesting termites, such recognition should be based on the composition of cuticular compounds (CC) (e.g., Clément and Bagnères 1998; Haverty et al. 1999; Kaib et al. 2002, 2004; Uva et al. 2004; Costa-Leonardo et al. 2009; van Zweden and d’Ettorre 2010). In one-piece nesting termites, nestmate recognition has received limited attention, mainly because the encountering probability with exogenous individuals is very low in comparison to separate-piece nesting termites and, as far as we are aware, the chemical signals involved have not been studied. In the present work, we use behavioral bioassays to inquire about the occurrence of nestmate recognition between soldiers and members of other termite castes which may invade nests of *N. chilensis* when co-occurring within the same substrate, and explore the importance of CC in this phenomenon.

## Methods and materials

### Species and study area

*Neotermes chilensis* is endemic to Chile between ca. 26 and 33.5 °S (Ripa and Luppichini 2004). The study areas (Las Chilcas: 32°52’S; 70°52’W and La Dormida: 33°03’S; 71°00’W) are located some 80 km north–northwest of Santiago. The main native hosts of *N. chilensis* at the sites are dry scapes (cylindrical stems of inflorescences, ca. 10 × 200 cm, diameter × length) of *Puya berteroniana* (Bromeliaceae) where it forms colonies containing up to 350 individuals. The populations of *P. berteroniana* studied show patchy spatial distribution in both study areas. Scapes used in the studies contained a single colony and were collected during the pre-swarming period; they were severed from the rest of the plant, surrounded by a mesh and brought to the laboratory in Santiago.

### Behavioral bioassays

To determine the occurrence of nestmate recognition, a soldier was confronted with either a pseudergate, a primary reproductive or another soldier. In a 12-cm diameter Petri dish with filter paper lining its bottom, a focal live soldier was introduced and then confronted with either a live pseudergate ( $N = 7$  nestmate interacting pairs and  $N = 15$

non-nestmate interacting pairs), a live primary reproductive ( $N = 10$  nestmate interacting pairs and  $N = 15$  non-nestmate interacting pairs) or another live soldier ( $N = 8$  nestmate interacting pairs and  $N = 15$  pairs non-nestmate interacting pairs). The bioassay was video-recorded for 15 min with a Sony® Cybershot camera and behaviors were evaluated through playback using the program JWatcher v1.0 (Blumstein et al. 2007).

Following similar studies with termites (Haverty and Thorne 1989; Cornelius and Grace 1994; Bagnères et al. 1991; Jmhasly and Leuthold 1999; Kaib et al. 2004; Šobotník et al. 2008), ants (Roulston et al. 2003), wasps (Lorenzi et al. 1997) and bees (Flores-Prado et al. 2008), behaviors which could be associated to a recognition process, e.g., aggressive (biting, grasping, opening jaws), alarm (drumming) and non-aggressive (antennating, backing up) behaviors were observed. Two additional behaviors were observed which did not seem to derive from a recognition process: standing still and walking; they were not included in the analysis. Table 1 describes the eight discrete behaviors performed by the focal soldier; they were either events (analyzed as the number of times they were observed during each bioassay) or states (analyzed as the total time they were performed during each bioassay).

The different classes of behaviors observed were independently compared in nestmate and non-nestmate interactions using a two-way ANOVA (Siegel and Castellan 1988) with caste (pseudergates, primary reproductives and soldiers) and provenience (nestmates and non-nestmates) as factors. Statistical analyses were performed with the program Statistica 7.0 (StatSoft 2004).

To determine if CC were involved in nestmate recognition, experiments were performed with soldiers confronted with primary reproductives, following the description of the preferential post-invasion attack of soldiers toward primary reproductives (Thorne et al. 2003) and taking into account that soldiers are particularly aggressive toward them (Pickens 1934; Thorne and Haverty 1991). Thus, the behaviors of a live soldier were

studied when it was confronted with: (1) a conspecific primary reproductive killed by freezing for 5 min at  $-20\text{ }^{\circ}\text{C}$  ( $N = 15$  nestmates and  $N = 13$  non-nestmates) and (2) a dead primary reproductive which had been immersed in 250  $\mu\text{L}$  of pentane for 10 min to deprive it of its CC ( $N = 20$  nestmates and  $N = 16$  non-nestmates). Data were recorded and behaviors classified as above.

The different classes of behaviors were compared between nestmate and non-nestmate pairs using a two-tailed  $t$  test or a Mann–Whitney  $U$  test when the normality condition of the data was not fulfilled (Siegel and Castellan 1988). Statistical analyses were performed with the program Statistica 7.0 (StatSoft 2004).

Each individual was used only in one bioassay. A total of 93 colonies were used, 53 for the bioassays with live termites and 40 for bioassays with a live soldier and a dead termite. A high number of colonies were used to set up the bioassays due to two limiting factors: only two primary reproductives were present in each colony and smaller colonies tended to have very few soldiers (Aguilera-Olivares et al. 2016). To increase variability in non-nestmate interacting pairs, they were established using colonies in intrapatch, interpatch and intersite combinations in roughly similar proportions; this led to an unbalanced number of replicates between nestmate and non-nestmate treatments. In the nestmate treatments, each interacting pair replicate came from a different colony. In the non-nestmate treatments, for each soldier–caste combination, only one soldier and one opponent were withdrawn from each of the colonies used, and the colonies were selected haphazardly to set up the interacting pair replicates; in other words, no two replicates had soldiers from the same colony or opponents from the same colony. These precautions avoided pseudoreplication.

### Composition of CC and nestmate recognition

The CC of termites used in the behavioral bioassays with live individuals were extracted by immersion in 250  $\mu\text{L}$  of

**Table 1** Behaviors observed in bioassays with *N. chilensis*

Behavior	Description	Event or state
Biting	Bite opponent and perforate its exoskeleton	Event
Grasping	Hold the opponent with the jaws without inflicting apparent damage	Event
Opening jaws	Open the jaws	Event
Drumming	Body vibrations against the substrate	State
Antennating	Touching the opponent with the antennae	State
Backing up	Running away or backing up after contact	State
Standing still	Standing still	State
Walking	Walking along the walls of the arena	State

pentane for 10 min. Five microliters of each extract was combined in a glass vial to produce a master extract which aimed at obtaining a chromatogram containing all possible CC in the population studied. Five microliters of each individual extract and of the master extract was injected into a gas chromatograph coupled with a mass spectrometer (GC–MS, model QP2010 Ultra, Shimadzu, Kyoto, Japan) equipped with an Rtx-5MS crossbond 5 % diphenyl–95 % dimethylpolysiloxane capillary GC column (Restek, Bellefonte, PA, USA: 30 m length, 0.25 mm I.D., 0.25  $\mu\text{m}$  film thickness). The GC was operated in the splitless injection mode. The column temperature was held at 146 °C for 5 min, raised at 3 °C/min to 280 °C, and maintained at that temperature for 5 min. The carrier gas was helium at a flow rate of 0.78 mL/min. The mass spectrometer was used in the electron impact ionization mode (70 eV) with an emission current of 250 mA. The temperatures of the injection port, ion source and interface line were 250, 250 and 280 °C, respectively. The instrument was operated in the scan mode.

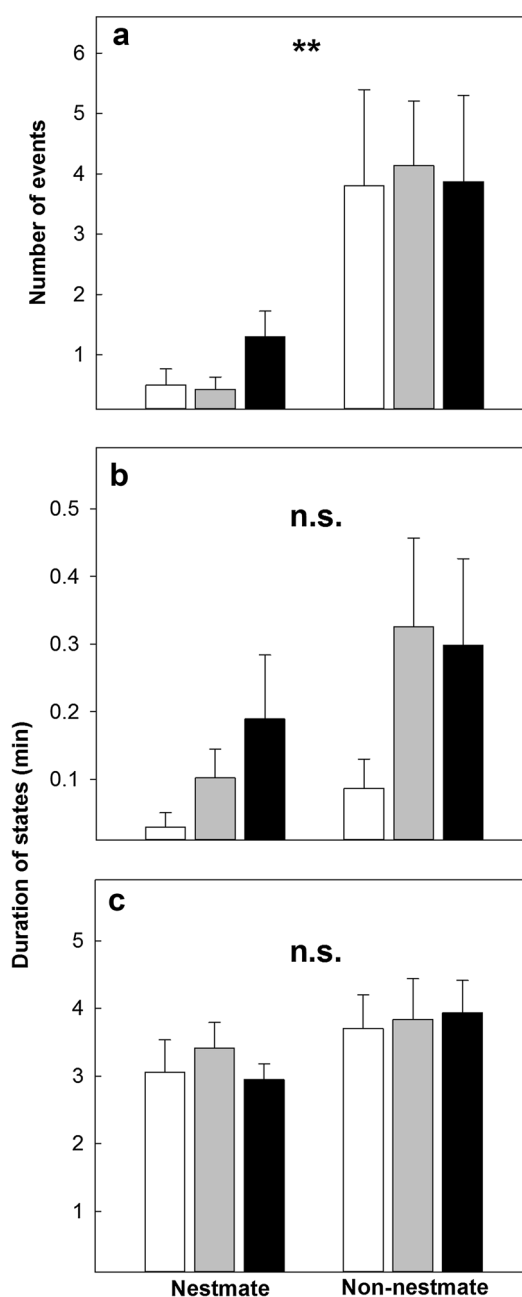
Chromatographic peaks were identified by comparison of their mass spectrum and retention index with those of authentic standards—when available—or with data from the literature (El-Sayed 2014). To determine the double bond position in alkenes, dimethyl disulfide (DMDS) derivatization was used following Attygalle (1998).

The individual chromatographic profiles were compared between nestmate and non-nestmate pairs using the simple matching coefficient (Krebs 1989), calculated as the number of positive and negative matches (in terms of the presence or absence of a given compound) between interacting pairs divided by 31, the total number of peaks detected. Thus, the simple matching coefficient for each comparison varied from 0 to 1. Chromatographic profiles were also compared using the Renkonen index based on quantitative compositions (Krebs 1989) and calculated as the sum of the smaller percentage composition of each compound over all compounds in the mixture. Thus, Renkonen index varied from 0 to 100 %. Simple matching coefficients and Renkonen indices for nestmate and non-nestmate pairs were compared using a two-tailed *t* test.

## Results

### Behavioral bioassays

When a focal soldier was confronted with a live individual, the number of aggressive events (Fig. 1a) was higher in non-nestmate than in nestmate interactions ( $F_{1,69} = 8.42$ ;  $P = 0.005$ ), but did not differ between castes or in the interaction between both factors ( $F_{2,69} = 0.058$ ,  $P = 0.943$  and  $F_{2,69} = 0.095$ ,  $P = 0.910$ , respectively). There were



**Fig. 1** Bioassays (mean  $\pm$  standard error) between the following interacting pairs: soldier–soldier (white box), soldier–pseudergate (gray box) and soldier–primary reproductive (black box). **a** Aggressive events, **b** alarm states and **c** non-aggressive states. Non-significant differences were found between castes. Double asterisk significant difference between nestmates and non-nestmates ( $P < 0.01$ ); n.s. non-significant differences between nestmates and non-nestmates ( $P > 0.05$ )

no differences in the time spent in alarm behavior between non-nestmates and nestmates, between castes, and in the interaction between both factors ( $F_{1,69} = 2.064$ ,  $P = 0.156$ ;  $F_{2,69} = 1.646$ ,  $P = 0.201$ ;  $F_{2,69} = 0.276$ ,  $P = 0.760$ , respectively) (Fig. 1b). There were no

differences in the time spent in non-aggressive behaviors between non-nestmates and nestmates, between castes, and in the interaction between both factors ( $F_{1,69} = 2.356$ ,  $P = 0.130$ ;  $F_{2,69} = 0.103$ ,  $P = 0.902$ ;  $F_{2,69} = 0.138$ ,  $P = 0.872$ , respectively) (Fig. 1c).

When the focal soldier was confronted with a dead primary reproductive, the number of aggressive events was higher in non-nestmate than nestmate interactions ( $U_{13,15} = 32$ ;  $P = 0.003$ ; Fig. 2a); however, there were no differences between nestmate and non-nestmate interactions in the time spent in alarm (Fig. 2b) and non-aggressive behaviors (Fig. 2c) ( $U_{13,15} = 78$ ,  $P = 0.370$ ;  $U_{13,15} = 72$ ,  $P = 0.249$ , respectively). When the dead reproductive was deprived of its CC by immersion in pentane, there were no differences between nestmate or non-nestmate interactions in the number of aggressive behaviors (Fig. 2a) and in the times spent in alarm (Fig. 2b) and in non-aggressive (Fig. 2c) behaviors ( $U_{16,20} = 150.5$ ,  $P = 0.772$ ;  $U_{16,20} = 147$ ,  $P = 0.680$ ;  $t_{34} = -1.173$ ;  $P = 0.249$ ;  $U_{16,20} = 173.0$ ,  $P = 0.849$ , respectively).

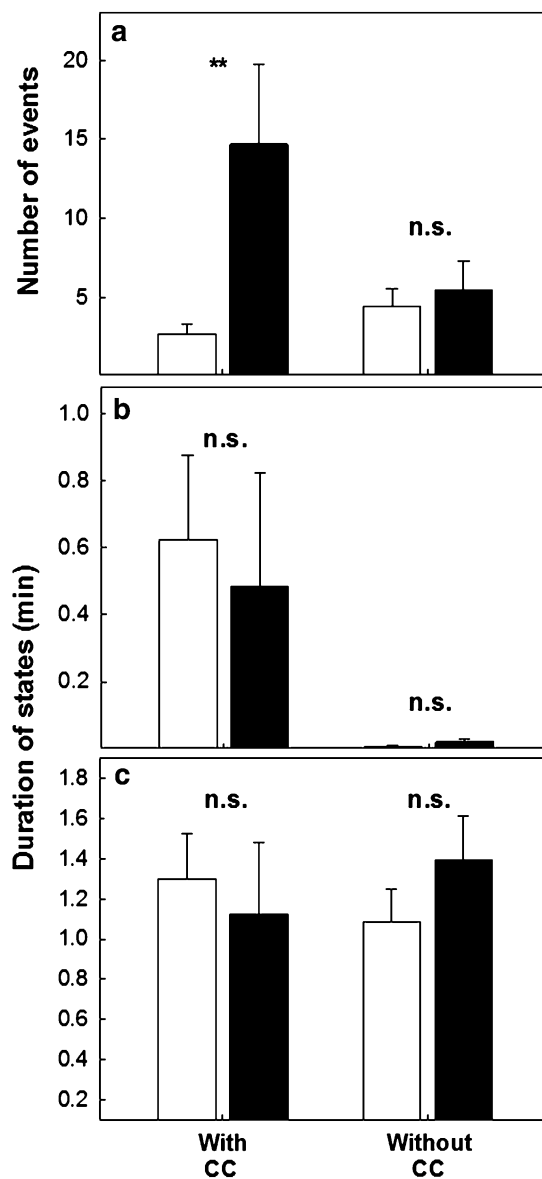
### Cuticular compounds

Thirty-one peaks were detected in the master chromatogram (Table 2; Fig. 3), 27 of which could be identified. Identified compounds were all hydrocarbons: 8 *n*-alkanes (25.8 %), 17 methylalkanes (54.8 %) and 2 *n*-alkenes (6.4 %). The mass spectra of unidentified compounds (13 %) are given in Appendix.

Given that caste was not a significant factor in behavioral bioassays, CC profiles were compared between nestmate and non-nestmate pairs, independently of caste; significant differences were found in the simple matching coefficient ( $t_{36} = 3.019$ ;  $P = 0.004$ ) and the quantitative Renkonen index ( $t_{36} = 2.724$ ;  $P = 0.009$ ) between nestmate and non-nestmate pairs (Fig. 4).

### Discussion

Among social insects, nestmate recognition has been widely demonstrated (Smith and Breed 1995) on the basis of the outcome of the interaction between individuals from the same or different nests, whereby individuals attack non-nestmates more often than nestmates, and nestmates exhibit little mutual aggression (Michener and Smith 1987; Gamboa 2004). It has also been inferred from the outcome of the interaction between an individual confronted with odors from conspecifics from the same or different nests (or colonies), whereby individuals exhibit more threat behaviors when confronted with non-nestmate than with nestmate odors (Nehring et al. 2012). In our study, *N.*



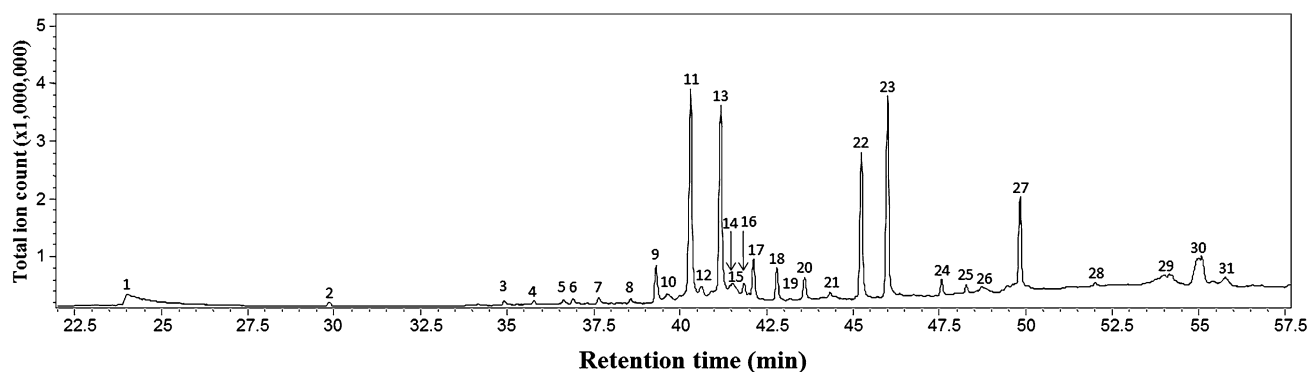
**Fig. 2** Bioassays (mean  $\pm$  standard error) between a soldier confronting a primary reproductive killed by freezing with its CC and a soldier confronting a primary reproductive killed by freezing without its CC. Interacting pairs were nestmates (white box) and non-nestmates (black box). **a** Aggressive events, **b** alarm states and **c** non-aggressive states. Double asterisk significant difference ( $P < 0.01$ ); n.s. non-significant differences ( $P > 0.05$ )

*chilensis* soldiers showed significantly more aggressive behaviors toward non-nestmates than toward nestmates, independently of the caste of the individual confronting the soldier, thus showing nestmate recognition. This process has been reported in many separate-piece nesting termites (e.g., Traniello 1981; Adams 1991; Takahashi and Gassa 1995; Jmhasly and Leuthold 1999; de Camargo-Dietrich and Costa-Leonardo 2000; Kirchner and Minkley 2003; Kaib et al. 2004; Marins and DeSouza 2008; Šobotník et al.

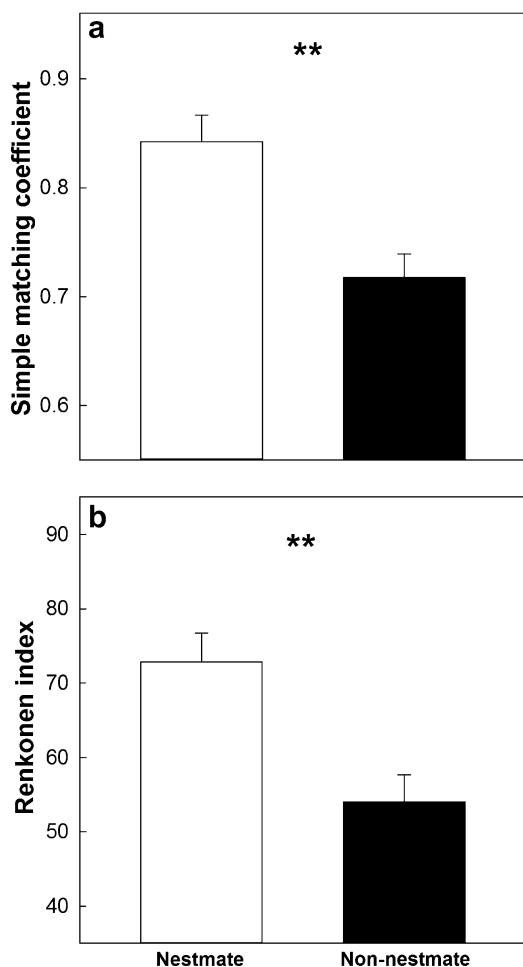
**Table 2** Compounds detected in cuticular extracts of *N. chilensis*

No.	RI	Compound	Identification method
1	1919	Unidentified 1	–
2	2117	Unidentified 2	–
3	2300	Tricosane	ST, RI, MS
4	2329	11-Methyltricosane	RI, MS
5	2364	2-Methyltricosane	RI, MS
6	2373	3-Methyltricosane	RI, MS
7	2400	Tetracosane	ST, RI, MS
8	2433	9-Methyltetracosane	RI, MS
9	2463	2-Methyltetracosane	RI, MS
10	2470	3-Methyltetracosane	RI, MS
11	2500	Pentacosane	ST, RI, MS
12	2517	Unidentified 3	–
13	2535	11-Methylpentacosane	RI, MS
14	2548	7-Methylpentacosane	RI, MS
15	2553	5-Methylpentacosane	RI, MS
16	2562	2-Methylpentacosane	RI, MS
17	2573	3-Methylpentacosane	RI, MS
18	2599	Hexacosane	ST, RI, MS
19	2614	Unidentified 4	–
20	2632	11-Methylhexacosane	RI, MS
21	2662	2-Methylhexacosane	RI, MS
22	2699	Heptacosane	ST, RI, MS
23	2732	11-Methylheptacosane	RI, MS
24	2798	Octacosane	ST, RI, MS
25	2828	13-Methyloctacosane	RI, MS
26	2899	Nonacosane	ST, RI, MS
27	2964	2-Methylnonacosane	RI, MS
28	2998	Triacontane	ST, RI, MS
29	3093	1-Hentriacontene	RI, MS, CD
30	3129	13-Methylhentriacontane	RI, MS
31	3184	7-Dotriacontene	RI, MS, CD

Identification was achieved by comparison of their mass spectra with those of standards (ST), by comparison of their mass spectra (MS) and their retention indices (RI) with those available in the literature, and by chemical derivatization (CD)

**Fig. 3** Master chromatogram of *Neoterme chilensis*. Numbers above the peaks correspond to the number of compounds in Table 2





**Fig. 4** Comparison of simple matching coefficient (**a**) and Renkonen index (**b**) between nestmates and non-nestmates using combinations of soldiers with all other castes. *Double asterisk* significant differences ( $P < 0.01$ )

2008), where workers and soldiers from different colonies may encounter each other during foraging tasks. On the other hand, in one-piece nesting termites, nestmate recognition has been reported only for colonies of two *Zootermopsis* species sharing the nesting substrate, when their galleries met (Haverty and Thorne 1989; Thorne et al. 2003).

The drumming behavior has been described as an alarm signal produced by soldiers when they are disturbed (Kirchner et al. 1994; Connétable et al. 1999, Hager and Kirchner 2013; Hunt and Richard 2013). The main function of alarm signals is to warn nestmates of potential threats, thereby eliciting in nestmates behaviors such as escape, interruption of feeding, recruitment toward the signal and/or drumming by other soldiers (Rosengaus et al. 1999; Inta et al. 2009). Connétable et al. (1999) showed that a group effect occurs when a soldier is disturbed; thus, isolated soldiers produce lower levels of alarm signals than soldiers accompanied by nestmates. Our results showed that the

focal soldier performed alarm behaviors for very short times and differences when the focal soldier was confronted with a nestmate or a non-nestmate was not significant; this is likely due to the requirement of a social context for the signals to be continuously produced or to a lack of specificity of alarm signals. Non-aggressive behaviors consisted of antennating and backing up behaviors; we are unsure on how to interpret the lack of significant difference between nestmates and non-nestmates.

When soldiers were confronted with a dead primary reproductive, more aggressive behaviors were performed toward non-nestmate than nestmate individuals, an effect that disappeared when CC had been extracted from the carcasses. This shows that nestmate recognition was mediated, at least partly, by CC, as has been shown earlier for many species of separate-piece nesting termites (e.g., Cornelius and Grace 1994; Clément and Bagnères 1998; Haverty et al. 1999; Kaib et al. 2002, 2004; Uva et al. 2004; Costa-Leonardo et al. 2009; van Zweden and d’Ettorre 2010).

Finally, the composition of CC was found to be constituted predominantly of hydrocarbons (at least 87 %), i.e., methyl-alkanes (54.8 %), followed by *n*-alkanes (25.8 %) and only two alkenes (6.4 %); these results are in agreement with those found in other termite species (Howard et al. 1982; Howard and Blomquist 2005; van Zweden and d’Ettorre 2010). We were unable to identify 13 % of the CC, which could well be hydrocarbons. Analyses of chemical profiles revealed higher similarity between nestmates than between non-nestmates; both genetic relatedness as well as the possibility of exchanging CC through contact within the colony may be the basis of similarity between nestmates. These results, jointly with the bioassay results, are in agreement with studies demonstrating correlations between differences in colony-specific CC profiles and aggressive behaviors displayed by individuals from different colonies (Takahashi and Gassa 1995; Kaib et al. 2004; Howard and Blomquist 2005; Dronnet et al. 2006; van Zweden and d’Ettorre 2010).

In *N. chilensis*, the ratio of soldiers to non-soldiers was shown to increase inside the colony when the risk of invasion was greater, for example when two or more colonies develop inside the same piece of wood (Aguilera-Olivares et al. 2016). In this situation, soldiers must possess the ability to discriminate nestmates from non-nestmates and to display more aggressive behaviors toward the latter to avoid the infiltration of their colony. In this work, soldiers of *N. chilensis* were shown to discriminate between nestmates and non-nestmates, on the basis of the outcome of the aggressive behaviors displayed by bioassayed individuals, independently of the caste confronted. Additionally, recognition was demonstrated to be

mediated, at least in part, by CC. These findings contribute to better understanding the role of soldiers in a one-piece nesting termite.

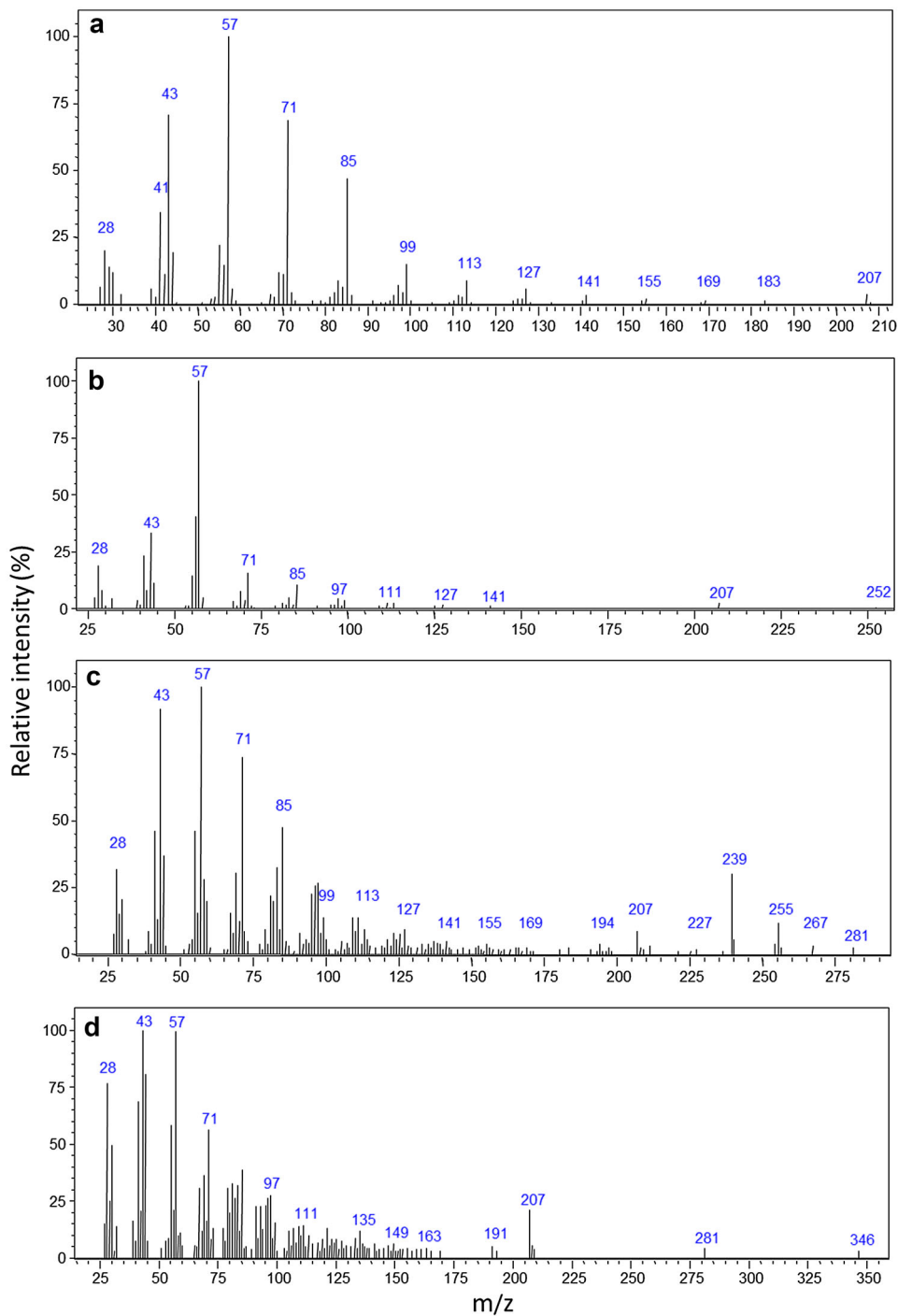
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## Appendix

See Fig. 5.

**Fig. 5** Mass spectra for unidentified cuticular compounds from individuals of *N. chilensis*, as shown in Table 2. Retention indexes were: **a** 1919, **b** 2117, **c** 2517 and **d** 2614





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