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Chemical and vibratory signals used in alarm communication in the termite *Reticulitermes flavipes* (Rhinotermitidae)

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Abstract

Termites have evolved diverse defence strategies to protect themselves against predators, including a complex alarm communication system based on vibroacoustic and/or chemical signals. In reaction to alarm signals, workers and other vulnerable castes flee away while soldiers, the specialized colony defenders, actively move toward the alarm source. In this study, we investigated the nature of alarm communication in the pest *Reticulitermes flavipes*. We found that workers and soldiers of *R. flavipes* respond to various danger stimuli using both vibroacoustic and chemical alarm signals. Among the danger stimuli, the blow of air triggered the strongest response, followed by crushed soldier head and light flash. The crushed soldier heads, which implied the alarm pheromone release, had the longest-lasting effect on the group behaviour, while the responses to other stimuli decreased quickly. We also found evidence of a positive feedback, as the release of alarm pheromones increased the vibratory communication among workers and soldiers. Our study demonstrates that alarm modalities are differentially expressed between castes, and that the response varies according to the nature of stimuli.

Keywords Communication · Defence · Pheromones · Positive feedback · Vibratory behaviour

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Introduction

Alarm communication is common in social animals, and it increases rates of survival (Wyatt 2003; Hunt and Richard 2013). Almost all social insects use alarm communication to coordinate the defensive activities of the entire colony (Leonhardt et al. 2016). These alarm signals are shared through different communication channels, with vibratory and pheromonal communication being the most common (Greenfield 2002; Cocroft and Rodríguez 2005). In termites, as well as their sister group, the subsocial roaches *Cryptocercus* (Seelinger and Seelinger 1983; Connétable et al. 1999; Röhrig et al. 1999; Reinhard and Clément 2002; Hager and Kirchner 2013; Delattre et al. 2015), vibratory and/or chemical signals are produced by disturbed colony members and induce the retreat of workers and other vulnerable castes while attracting soldiers (Reinhard and Clément 2002; Šobotník et al. 2008a, 2010). These mechanisms are essential for termite colony defence, and greatly contribute to their ecological success. For example, tamandua anteaters are specialized predators of *Nasutitermes*, which they find in wood pieces, but they are unable to feed on this termite directly in the nest where the soldier proportion is higher (Lubin and Montgomery 1981).

Many termite species produce substrate-borne vibrations by hitting their heads and/or abdomens on the substrate (Connétable et al. 1999; Röhrig et al. 1999; Hager and Kirchner 2013). All colony members are able to detect vibrations with their subgenual organs, specialized chordotonal vibroreceptors, located on the leg tibiae (Howse 1962, 1965a; Chapman 1998). Such unique alarm communication mechanism may have evolved only once in the common ancestor of termites and *Cryptocercus* (Seelinger and Seelinger 1983), probably after its diet switched from loose substrates to wood, a material through which vibratory signals easily spread. The vibratory alarm signals are generated using several types of oscillatory movements in termites. In this paper, we will follow the terminology introduced by Hill (2014), who recognized two categories of vibrational mechanisms: (1) drumming, commonly used in termites to produce substrate-borne vibrations; (2) tremulation, a body movement performed without any hit on the substrate, used to propagate the alarm selectively to calm nestmates (Kettler and Leuthold 1995; Šobotník et al. 2008b).

Another mean of alarm signalling is the chemical channel. Social insects evolved a rich set of exocrine glands (Billen and Šobotník 2015) producing a wide range of infochemicals. In termites, alarm pheromones are produced either by the frontal or labial glands, which are the defensive glands of soldiers (Šobotník et al. 2008a, 2010; Delattre et al. 2015). Alarm compounds are mostly mono- or sesquiterpenes (see Šobotník et al. 2010 for a review), except in *Mastotermes darwiniensis*, that uses benzoquinone (Delattre et al. 2015).

In certain termite species, the efficiency of alarm signalling is enhanced by positive feedback, a mechanism implying amplification of the signal by newly alerted specimens (Vrkoč et al. 1978; Roisin et al. 1990; Röhrig et al. 1999; Hager and Kirchner 2013; Cristaldo et al. 2015; Delattre et al. 2015). Vibratory positive feedback has been demonstrated in *M. darwiniensis* (Delattre et al. 2015), in several Macrotermitinae (Termitidae; Connétable et al. 1999; Hager and Kirchner 2013; Röhrig et al. 1999), and in *Constrictotermes cyphergaster* (Termitidae: Nasutitermitinae; Cristaldo et al. 2015). Chemical positive feedback relies upon the alarm pheromone present in soldier defensive secretion and is known in *M. darwiniensis* (Delattre et al. 2015) and in several *Nasutitermes* species (Termitidae: Nasutitermitinae) (Roisin et al. 1990; Vrkoč et al. 1978).

While the nature of vibratory or chemical alarm signals is well-known and has been the focus of many studies, the alarm transmission mechanisms have seldom been investigated. Cristaldo et al. (2015) demonstrated that individuals react to alarm pheromone stimulation by vibratory alarm signalling in *C. cyphergaster*, and Delattre et al. (2015) showed that artificial vibratory alarm signals trigger the release of the alarm pheromones in *M. darwiniensis*. In this study, we

carried out a careful examination of alarm communication mechanisms in *Reticulitermes flavipes* (Kollar, 1837) (Rhinotermitidae), including a vibrational and chemical analysis of this behaviour. Since only the basal termites and the most derived termites have been studied, this study aims to improve our understanding of the evolution of alarm communication in termites. *Reticulitermes* is a temperate genus that includes many pest species responsible for billions of dollars of damage annually worldwide (Su and Scheffrahn 2000). We characterized the vibratory and chemical communication in workers and soldiers, and determined their responses to particular danger stimuli, including the alarm pheromones of their colony mates.

Materials and methods

Insect material

All the experiments were carried out on one colony of *Reticulitermes flavipes* collected in Île d'Oléron (France) in 1998, and kept in laboratory stable conditions (26 °C, 90% relative humidity) since then.

Behavioural experiments

All experiments were performed at 26 °C under dimmed red light. We first conducted a behavioural experiment to assess how *R. flavipes* workers and soldiers react to various danger stimuli. To avoid any effect of social deprivation, we formed groups ($N = 12$) of 38 workers and 2 soldiers (natural caste ratio; see Haverty 1977) that were put in 85 mm Petri dishes lined with moist clean Whatman No. 1 filter paper. The groups were left to settle for at least 2 h prior to testing, and between two tests. The stimuli set included: (1) light flash (3 s, 800 lx intensity, 5500–6000 K colour temperature), (2) blow of air, delivered through a fine straw for 3 s, which mimics a breach into the colony, (3) one crushed worker head and (4) one crushed soldier head. Crushed heads were prepared by cutting termites at the level of the prothorax and the heads were crushed on filter paper using a small spatula, allowing the impregnation of the filter paper by the contents of the frontal gland (soldier frontal gland is known to produce alarm pheromone; Reinhard and Clément 2002). We recorded group activity without any stimulation as controls for experiments (1) and (2). For experiments (3) and (4), the controls consisted in the insertion of a blank filter paper. Pieces of filter paper were carefully inserted into the Petri dish through a fine slit in the lid. Each stimulus was tested six times in random order and on randomly chosen groups (3 stimuli per group in random order). Behavioural reactions were recorded using a Canon EOS 6D combined with EF 100 mm f/2.8L Macro IS USM for 3 min before stimulation and 6 min after the stimulation.

We chose to analyse the speed-of-motion of individuals, to assess their reaction to our stimuli. Speed-of-motion increases as workers flee the disturbance source, and soldiers gather next to it (Reinhard and Clément 2002; Šobotník et al. 2008a, 2010). We tracked two randomly chosen workers and both soldiers per group for each record. We estimated the group behaviour 1 min before the stimulus introduction, and 1 and 6 min after (short-term and long-term responses, respectively), using the Mouse-Tracer Software (see Šobotník et al. 2008a). We then computed the variation of speed-of-motion for all the stimuli using the difference between the mean speed before and after the introduction of the stimulus. This method allowed us to normalize the variance between the different termite groups. Variations in speed-of-motion for all danger stimuli were compared to their respective controls.

Chemical analyses

We carried out two experiments to identify the alarm compounds in workers and soldiers of *R. flavipes*. In the first experiment, 20 termites were cut at the level of the prothorax and the anterior parts were successively extracted with 60 µl and 40 µl of hexane. Both extractions took place over two successive nights at 4 °C. Both extracts were merged and one termite equivalent was injected in a 6890N gas chromatograph (Agilent, Santa Clara, CA, USA). The most abundant compounds were identified based on spectral characteristics and published records (summarized in Šobotník et al. 2010). In the second experiment, we crushed heads of five individuals of each caste in a 1.2 mL glass vial with a Pasteur pipette. The headspace extraction of volatiles was carried out using SPME fibre holder for manual sampling equipped with a fused silica fibre coated with 30 µm polydimethylsiloxane (Supelco, Bellefonte, USA). The analytes were desorbed at 220 °C in a split/splitless injector of a 5975B quadrupole mass spectrometer coupled to a gas chromatograph. The separation was achieved on a DB-5 ms capillary column (30 m × 0.25 mm, a film thickness of 0.25 µm, Agilent) at a constant flow mode (1 mL/min) with helium as a carrier gas. The column temperature was held at 40 °C (1 min), gradually increased at 5 °C/min to 200 °C, then gradually increased at 15 °C/min to 320 °C, and held at 320 °C for 3 min. The temperatures of the transfer line, ion source and quadrupole were 280, 230, and 150 °C, respectively. The compounds were ionized at 70 eV electrons.

Vibroacoustic experiments

To decipher the vibratory component of *R. flavipes* behaviour, we formed ten new groups of termites, using the same ratio we used in our behavioural experiments. The aim of this experiment was to determine the interaction between a chemical stimulation and the vibratory component of alarm signalling

in *R. flavipes*. We crushed one worker and one soldier head on different pieces of filter paper (following the same protocol as the behavioural experiment), and used another identical piece of blank filter paper for control. Due to methodological constraints and to avoid the absorption of the termite-produced vibrations, termite groups were placed into a 85 mm Petri dish without filter paper, and moisture was provided by a piece of wet cotton attached to the lid. The bottom of the Petri dish was heavily scratched to allow termites to walk. Our experiments were carried out in an anechoic room at the Czech Technical University in Prague under dimmed red light. All experiments took place on a table hung from the ceiling to avoid any perturbations from the experimenters (see Supplementary video SV1). All experiments were recorded using a SONY DCR-SR72 camera in night-shot mode fixed above the experimental arena. These records were only used to link the behaviour of termite groups to recorded vibrations, and were not used for behavioural analyses.

Vibratory communication was recorded using accelerometers (Brüel and Kjær type 4507 B 005) glued to the bottom of Petri dishes. We analysed the recorded vibratory signals using a Soft dB Tenor recorder (24 bits, sampling frequency 48 kHz) and Matlab software (R 2012a; see ESM 1). Prior to each experiment, we recorded high-resolution videos of both, disturbed and undisturbed groups of termites, to decipher the repertoire of vibrations generated by *R. flavipes* workers and soldiers. We considered recorded frequencies below 15 Hz as low-frequency vibrations, and frequencies above 15 Hz as high-frequency vibrations. These preliminary tests were also used to determine the optimal parameters of frequency filters used for post-processing of vibration records of *R. flavipes* groups in the described environment. This allowed us to reduce the background noise.

In these experiments, because termite signals overlap, we were unable to analyse individual signals. Therefore, we computed the total amount of energy produced by group vibratory signals after each stimulation. This variable E_{R}, was computed using the following equation:

$$E_{\mathrm{R}} = 10.\log\left[\frac{T_{\mathrm{B}}}{T_{\mathrm{A}}}\frac{\int_0^{T_{\mathrm{A}}}|x_{\mathrm{f}}(t)|^2\mathrm{d}t}{\int_{-T_{\mathrm{B}}}^0|x_{\mathrm{f}}(t)|^2\mathrm{d}t}\right],$$

in which T_{A} is the evaluation period after disturbance (60 s and 360 s, respectively), T_{B} is the evaluation period before disturbance (60 s), and $x_{\mathrm{f}}(t)$ is the filtered acceleration signal (bandpass filter 50–500 Hz) and is a function of time (t).

Statistics

We performed Kruskal–Wallis tests and two-by-two post hoc permutation tests (10,000 permutations) for independent

samples, and we carried out Friedman tests and two-by-two post hoc permutation tests (10,000 permutations) for paired samples. Bonferroni–Holm corrections (Holm 1979) were applied for multiple comparisons among groups. All statistical tests were performed with StatXact software (Cytel Studio, version 9.0.0, 2010).

Results

Behavioural experiments

All groups significantly reacted to all experimental stimuli. We found significant differences among stimulations for both soldiers and workers, both 1 and 6 min after stimulations (short-term *vs.* long-term responses, respectively; Table 1).

Workers were sensitive to the blow of air, the crushed soldier head (CSH) and light flash exposure, but not to the crushed worker head (CWH). Soldiers revealed similar responses, but were also sensitive to the crushed worker head during the first minute after the stimulus introduction (Fig. 1, Fig. S1).

Chemical analyses

Analytical approaches revealed a set of monoterpenes present in soldier extracts and SPME, while no candidate compound was detected in workers, irrespective of method used (Table 2). Extracts of workers and soldiers also contained the cuticular hydrocarbons specific to *R. flavipes* (see, e.g. Bagnères et al. 1990; Vauchot et al. 1998; Perdereau et al. 2010).

Vibroacoustic experiments

In response to the stimuli, soldiers and workers displayed body vibrations that they used to spread alarm within the groups. Using vibroacoustic preliminary tests in conjunction with high-resolution video recordings, we observed that these vibrations were mainly drumming or tremulation. Drumming was produced by abdomen hits against the
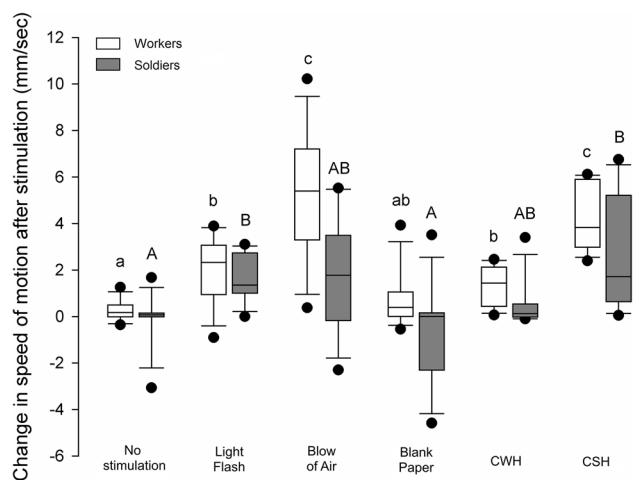

Fig. 1 Change in speed-of-motion of workers (white bars) and soldiers (grey bars) in *R. flavipes* during a 1-min period after exposition to experimental stimuli in comparison to controls. $N = 12$ for each caste and each stimulus. Box plots show the median and 25–75th percentiles. Whiskers show all data excluding outliers outside the 10th and 90th percentiles (circles). Statistical differences are given for $P < 0.05$. *CWH* crushed worker head, *CSH* crushed soldier head

substrate, while tremulation was produced by tactile stimulation of nestmates with the head. In a few cases, soldiers displayed another kind of drumming signal consisting in powerful hits to the ground with their mandibles.

We found that drumming and tremulation were used in combination by workers and soldiers, forming thus complex vibrations. These vibrations occurred in two kinds of bursts (series of hits). The first kind of burst was at a high frequency of 31 ± 4 Hz, while the second kind of burst occurred at a low frequency of 7.4 ± 1.3 Hz. High and low-frequency vibrations were often performed together, the high-frequency bursts preceding the low-frequency bursts (Fig. 2, video SV1). This could be defined as the typical pattern of vibratory behaviour in *R. flavipes*. Occasionally, the high- and low-frequency bursts were performed separately after disturbance. A third kind of drumming burst was specifically displayed by soldiers,

Table 1 Differences in speed-of-motion between all stimulations after stimulus introduction for workers and soldiers in *R. flavipes*

	Workers	Soldiers
Short-term response (1 min)	$H_5 = 46$ $P < 0.001$	$H_5 = 39.61$ $P < 0.001$
Long-term response (6 min)	$H_5 = 46.01$ $P < 0.001$	$H_5 = 27.24$ $P < 0.001$

Kruskal–Wallis for multiple comparisons statistic values (H_5) and P values are provided for both castes

Table 2 Chemical compounds identified by GC–MS from *Reticulitermes flavipes* worker and soldier samples (hexane extracts or headspace SPME)

Extracts		SPME	
Workers	Soldiers	Workers	Soldiers
CHC	α-Pinene	Ø	α-Pinene
	β-Pinene		β-Pinene
	Limonene		Limonene
	CHC		Unknown compound

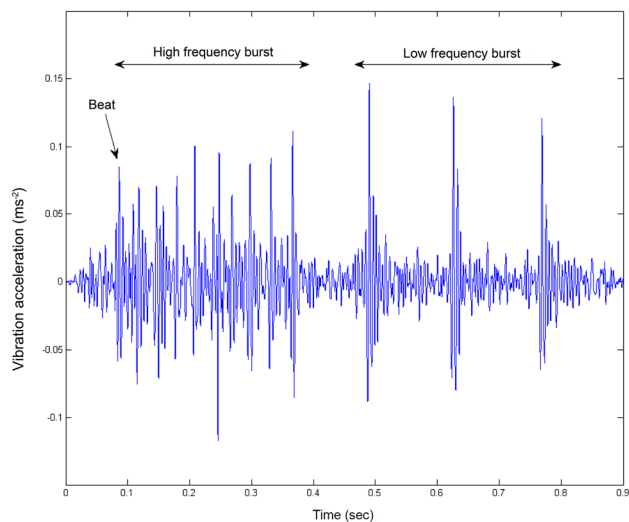Ø no volatile compound detected, *CHC* cuticular hydrocarbons

Fig. 2 Example of vibratory behaviour showing the typical structure of the vibratory movements of workers and soldiers of *Reticulitermes flavipes*. The complex vibrations occurred at two distinct frequencies, described as high- (31 Hz) and low- (7.4 Hz) frequency bursts

Fig. 3 Energy ratio difference in vibrations recorded in groups ($N = 10$) before and after the introduction of the stimulus. Box plots show the median and 25th–75th percentiles. Whiskers show all data excluding outliers outside the 10th and 90th percentiles (circles). Statistical differences are shown for $*P < 0.05$ and $**P < 0.01$. *CO* control blank paper, *CWH* crushed worker head sample, *CSH* crushed soldier head sample

Table 3 Differences in vibratory behaviour (permutation tests for paired samples) recorded in termite groups ($N = 10$) before and after the introduction of the stimulus

Stimulation	Short-term response (1 min)	Long-term response (6 min)
CO	−0.959 $P = 0.359$	−0.5501 $P = 0.5977$
CWH	−2.067 $P = 0.021$	−2.279 $P = 0.014$
CSH	−1.908 $P = 0.006$	−1.574 $P = 0.057$

CO control blank paper, *CWH* crushed worker head, *CSH* crushed soldier head

Table 4 Comparison between vibrational responses to crushed termite heads ($N = 10$) (permutation tests for paired samples)

Stimulation comparison	Short-term response (1 min)	Long-term response (6 min)
CO vs. CWH	−1.839 $P = 0.063$	−2.378 $P = 0.027$
CO vs. CSH	−1.841 $P = 0.047$	−1.579 $P = 0.088$

CO control blank paper, *CWH* crushed worker head, *CSH* crushed soldier head

Discussion

Alarm communication is an important component of the defensive strategies of many gregarious, colonial and social animals, and is used to coordinate defensive activities. In *Reticulitermes flavipes*, alarm signals are spread via tremulations, substrate-borne vibrations and alarm pheromones. Irrespective of the communication channel, two modes of alarm transmission can be distinguished in natural situations: general alarm responses follow strong disturbance and affect large termite groups, while subtler specific alarm communication involves a few specimens reacting to low-level disturbance, such as the encounter of alien individual into the gallery system (Howse 1965b; Stuart 1963, 1988).

Here we studied the general alarm responses of *R. flavipes*, and clearly showed that potential dangers, represented

and consisted of repeated hits to the substrate with the mandibles, at an average frequency of 26 ± 2 Hz. Unfortunately, this particular behaviour occurred too scarcely (only 40 series recorded in all experiments combined) to be analysed in our vibroacoustic work.

Reticulitermes flavipes groups reacted to the alarm pheromone by producing vibroacoustic signals (Fig. S2). The intensity of these signals was nearly identical 1 and 6 min after the stimulation, with the exception of the response to CSH, which was lower after 6 min (Table 3; Fig. 3). The responses to CSH were stronger in the first minute after the stimulus introduction, while CWH stimulation triggered a long-lasting effect increasing with time during the timeframe of the experiment (Table 4).
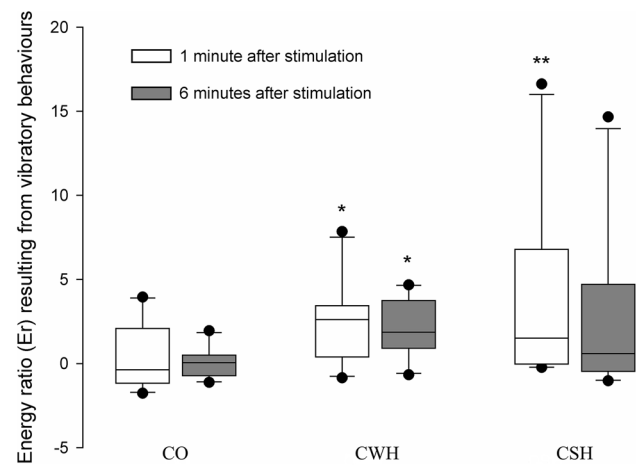
by air current, light flash, or crushed nestmate heads, are treated with differing types of alarm responses. The responses to air currents and crushed soldier heads were the most pronounced, and triggered immediate increase of speed-of-motion and quantity of vibroacoustic signals in workers and soldiers. These behavioural changes were abrupt and often lasted over 6 min.

The observed vibroacoustic signals were made of complex vibratory movements, combining drumming and tremulations. They were produced by workers and soldiers repeatedly hitting their abdomen on the substrate. These examples of vibroacoustic communication combine two kinds of bursts differing in beat frequencies, as described in *Coptotermes gestroi*, another species of Rhinotermitidae (see Hertel et al. 2011). A duration-dependent effect could be perceived and CSH samples elicited the strongest reaction during the first minute after the stimulation.

CSHs were not the only stimuli which could trigger vibrations, CWH revealed significant effect as well, as evidenced by the walking activity of soldiers and vibratory activity of both soldiers and workers. Our results concur with previous observations on *C. cyphergaster* (Termitidae, Nasutitermitinae) (Cristaldo et al. 2015), in which the CWH also provoke marked behavioural responses in soldiers. *C. cyphergaster* workers possess enlarged mandibular glands with defensive function (Costa-Leonardo and Shields 1990), which are possibly the source of chemicals responsible for the change in behaviour. In contrast, no gland with defensive role is known in *R. flavipes* workers, and the source of the excitement remains unknown.

Soldiers of *R. flavipes* also used their head and mandibles to perform powerful hits to the substrate, which produced a strong substrate-borne vibratory drumming-like signal, much stronger than the abdominal drumming. The same way of drumming has also been observed in Archotermopsidae (Kirchner et al. 1994), Rhinotermitidae (Hertel et al. 2011) and Macrotermitinae (Termitidae) (Connétable et al. 1999; Hager and Kirchner 2013; Kettler and Leuthold 1995; Röhrig et al. 1999). The strongest signals are probably used to warn nestmates and recruit soldiers from deeper inside the colony.

Our chemical analysis showed high quantity of α- and β-pinene, and limonene produced by the frontal gland of *R. flavipes* soldiers. These compounds form the alarm pheromone in *R. flavipes*, as it has been previously suggested by several authors (Bagnères et al. 1990; Parton et al. 1981; Reinhard et al. 2003), although not all compounds might be necessary for the function, as they were never tested separately. The alarm pheromones involve various compounds classes, such as monoterpenes (also in *R. flavipes*), sesquiterpenes (for review see Šobotník et al. 2010) or quinones (Delattre et al. 2015), and are known to be released from the soldier defensive glands, namely the labial glands in

Mastotermitidae and the frontal gland in Rhinotermitidae and Termitidae (Delattre et al. 2015; Kaib 1990; Kriston et al. 1977; Pasteels and Bordereau 1998; Reinhard and Clément 2002; Roisin et al. 1990; Šobotník et al. 2008a; Vrkoč et al. 1978).

Until recently, alarm positive feedback was only demonstrated either for vibroacoustic (Connétable et al. 1999; Delattre et al. 2015; Hager and Kirchner 2013; Röhrig et al. 1999) or chemical signals (Roisin et al. 1990; Vrkoč et al. 1978). Integrative studies appeared only recently, and vibroacoustic feedback to chemical alarm has been shown only in the basal *Mastotermes* (Mastotermitidae; Delattre et al. 2015) and the derived *Constrictotermes* (Termitidae: Nasutitermitinae; Cristaldo et al. 2015). In this study, we found the third example of vibroacoustic feedback to chemical alarm, as workers' and soldiers' speed-of-motion increased after exposure to head volatiles. Moreover, as showed in Connétable et al. (1999), it is likely that termites spread the alarm further using complex vibratory communication based on tremulations and drumming, contributing to the general state of colonial defensive activity (i.e. running away from the threat).

Our work is the first exhaustive study on the alarm communication strategies in a species of Rhinotermitidae. Both workers and soldiers of *R. flavipes* reacted to all stimulations (light flash, air currents and crushed nestmate heads) with various degrees of excitement and displaying different vibratory movements. These observations show specialized alarm communication strategy based on complementary modalities, which could trigger an efficient response according to the nature and intensity of endangering stimulus. Moreover, *R. flavipes* is a pest species in Western USA (Evans 2011; Evans et al. 2013) and has been introduced to several places around the world (Bagnères et al. 1990; Smith et al. 2006; Evans et al. 2013), where it became invasive (originally described as *R. santonensis*, and later synonymized with *R. flavipes* by Austin et al. 2005). The dominance of *R. flavipes* over *R. grassei*, which has already been observed in the field (Perdereau et al. 2011), might, at least partially, be explained by its sophisticated alarm communication strategy.

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