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Animal Behaviour

Heterospecific detection of host alarm cues by an inquiline termite species (Blattodea: Isoptera: Termitidae)

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Termite inquilines specialize on living in and feeding on a host termite nest. However, the mechanisms allowing survival of two mutually hostile populations confined to a single nest are not understood. Here we report on inquiline termites that respond to their host's alarm cues. Upon detecting these cues, the inquilines do not join in with the host's nest defence, but use this information for their own benefit, to escape danger. Using behavioural bioassays under laboratory conditions, we show that the obligatory inquiline *Inquilinitermes microcerus* (Termitidae: Termitinae) responds both to its own alarm signal and to alarm cues from its host, *Constrictotermes cyphergaster* (Termitidae: Nasutitermitinae). Conversely, this host responded only to its own alarm signal. Despite perceiving their host's alarm cues, inquilines were never observed sharing nest defence with their host in experimentally damaged nests in the field. We argue that this allows inquilines not only to minimize encounters and hence conflict with their hosts, but also to use their host alarm information to escape the host's enemies, which are also likely to be enemies of the inquilines. Our results show a new benefit that inquilines gain from the host, and we discuss the inquiline way-of-life from an evolutionary perspective, as the outcome of constraints and benefits imposed by living in host nests.

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Symbiotic interactions are widespread (Fitter & Garbaye, 1994; Kilner & Langmore, 2011; Kistner, 1979; Savage, 1977; van der Heijden, Bruin, Luckerhoff, van Logtestijn, & Schlaeppi, 2015) and are notable among social insects, as their long-lived colonies and resource-rich nests provide instances of interspecific partnerships covering the whole spectrum from mutualism to parasitism via commensalism (Brandt, Foitzik, Fischer-Blass, & Heize, 2005; Hughes, Pierce, & Boomsma, 2008). Among these associations, nest sharing between hymenopteran hosts and invader species is well documented (Buschinger, 1986; Nash & Boomsma, 2008), being mostly characterized as brood parasitism, i.e. the act of appropriating a colony's workforce to rear one's own young (Kilner & Langmore, 2011; Nash & Boomsma, 2008). Conversely, symbiotic interactions among termite species occupying a single nest (so called 'inquilinism') are less clear. Current evidence is still insufficient to properly frame termite inquilinism within the parasite–mutualist continuum or to understand how inquilines manage to cohabit with their host. Here we approach this latter issue, reporting on an inquiline termite that is able to recognize its termite host's alarm signals. Following this recognition, however, these inquilines do not join the host's efforts for nest defence.

Presumed benefits for inquilines in occupying, rather than building, the nest of another termite should be further enhanced by strategies to reduce costs associated with invasion. Among such strategies the ability to recognize host trail-following cues seems useful to evade detection within the nest (Cristaldo et al., 2014; Wen et al., 2015). Interestingly, this ability to decode the host's chemical cues can also be used by inquilines to offset losses to their host: by deciphering the host's alarm signal, inquilines could join in nest defence, complementing host's defence strategies with their distinct arsenal and tactics, as hypothesized by Coles (1980) and Redford (1984). Such mutualistic behaviour, however, could tend towards commensalism or parasitism if, instead of joining in nest defence efforts, the inquiline uses its decoding ability to detect host communication, fleeing risks signalled by the host. In such cases,

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inquilines are better off when their own chemical cues are not decoded by their hosts. Enhancement of the defence of termite host's nests by non-termite guests has been 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 on interspecific mutual defence have been gathered so far. The ant parasite *Megalomyrmex symmetochus*, for example, uses its alkaloid venom, which is more potent than the biting defence of its host, *Sericomyrmex amabilis*, to enhance host nest defence against agro-predatory ants (Adams et al., 2013).

Here we examine a host–inquiline pair of termite species; the host is *Constrictotermes cyphergaster* Silvestri, 1901 (Termitidae: Nasutitermitinae) and its obligate inquiline is *Inquilinitermes microcerus* Silvestri, 1901 (Termitidae: Termitinae). We focus specifically on the responses given in laboratory assays by both partners to their own and to each other's alarm signals. After that, in the field, we checked for evidence that the inquiline might join in nest defence. We showed that, at least for the system at hand, termite inquilines use host alarm cues for their own benefit, refraining from taking part themselves in nest defence and avoiding risks signalled by the host and/or nest regions crowded by host defenders. We argue that this allows inquilines not only to minimize encounters and hence conflict with their hosts, but also to use their host's alarm information to escape the host's, and potentially their own, enemies.

METHODS

Study System

The host *C. cyphergaster* is a common Neotropical species occurring in Brazil, Paraguay, Bolivia and northern Argentina (Mathews, 1977). This species is characterized by an unusual lifestyle combining arboreal nests, leaf litter (Moura, Vasconcellos, Araújo, & Bandeira, 2006b) or microepiphyte diet (Bourguignon et al., 2011) and 'open-air' foraging (Moura et al., 2006a). Soldiers use chemical weapons (sensu Prestwich, 1984). The alarm pheromone of *C. cyphergaster* is composed of a mixture of (1S)-α-pinene, myrcene and (E)-β-ocimene, which triggers sophisticated alarm transmission among nestmates (see Cristaldo et al., 2015, for details). Nests of *C. cyphergaster* can house a large number of termitophiles and an obligatory inquiline (*I. microcerus* or *Inquilinitermes fur*). Colonies of *I. microcerus* are known to live apart from the builder, restricting themselves to certain portions of the nest, usually close to its core (Cunha, Costa, Espírito-Santo Filho, Silva, & Brandão, 2003). Aggressive behaviour has been reported to occur between individuals of both colonies in chance meetings (Emerson, 1938). According to Cristaldo, Rosa, Florencio, Marins, and DeSouza (2012), 70% of *C. cyphergaster* nests are inhabited by *I. microcerus* colonies in the Brazilian 'Cerrado' biome. Soldiers of *I. microcerus* use mandibular weapons (sensu Prestwich, 1984). Previous studies have suggested that *I. microcerus* colonies use a 'conflict avoidance' strategy to successfully deal with occupation of termite host nests (see Cristaldo et al., 2014; Florencio et al., 2013).

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 savannah), near the town of Sete Lagoas (19°27′57″S, 44°14′48″W; 800–900 m above sea level), Minas Gerais State, southeastern Brazil. According to Köppen's classification, the study area is subjected to Aw climate ('equatorial with dry winter') (Kottek, Grieser, Beck, Rudolf, & Rubel, 2006).

Behavioural Bioassays: Mutual Response of Cohabiting Species to Alarm Signals

To examine whether cohabiting termite species (hosts and guests) are capable of recognizing and responding to each other's alarm signals, we performed behavioural bioassays in July 2012 as described below. Bioassays were designed to measure the behavioural reaction of termite hosts and their guests when exposed to their own and to each other's alarm signals.

Bioassays were carried out in an experimental arena made from a plastic petri dish (85 mm diameter, 15 mm height) with a slit in the cap and wet filter paper (Whatman No. 1) as substrate (Fig. 1a). Groups of termites from the same colony were transferred into the experimental arenas at least 2 h prior to observation to ensure their acclimation. The number and caste ratio (soldiers:workers) of termite groups used in the bioassays were chosen according to natural caste proportions (1:4.5 for *C. cyphergaster*; 1:8.5 for *I. microcerus*; see Cunha et al., 2003, for details) and to maximize their interaction and survival (Miramontes & Desouza, 1996). After acclimation, we videorecorded the behaviour of the termite group for 2 min before introducing the test stimulus. The test stimulus was loaded on a piece of filter paper (7 × 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 (Fig. 1a). From then on, another video recording was taken for 5 min. Overall, the behaviour of each termite group was videorecorded for 7 min, of which the first 2 min refer to pre-stimulus insertion and the last 5 min refer to post-stimulus insertion (Fig. 1a). Such a procedure is necessary to evaluate the idle activity of groups (see details in Šobotník et al., 2008; Cristaldo et al., 2015). All videos were recorded with a Nikon D300s™.

We carried out the first series of behavioural bioassays to evaluate the alarm reaction to conspecific signals (i.e. stimuli from same species, conspecific trial). We carried out the second series to evaluate the alarm reaction to heterospecific signals (i.e. stimuli from cohabiting species, heterospecific trial): the host was exposed to inquiline signals (Fig. 1b) and vice versa (Fig. 1c).

Stimuli tested were those known to provide some 'alarm signal' in termites and their respective controls: (1) untreated paper (blank control); (2) hexane (solvent control); (3) crushed worker's head ('crushed head treatment' control); (4) crushed soldier's head ('crushed head treatment'); (5) frontal gland extract. For the crushed head treatments (soldier, control), we anaesthetized each individual on ice, removed its head (Fig. 1a) and immediately crushed the head by pressing it against the test filter paper with tweezers. Hexane was purchased from Merck and redistilled prior to use. To prepare frontal gland extract, we anaesthetized soldiers on ice and removed the heads. We then cut open each head and placed it in hexane (10 µl per head) for 24 h at 4 °C, followed by a second washing of hexane (10 µl per head). Final samples were stored at −18 °C and used in the bioassays. A Hamilton microsyringe (10 µl) was used to load hexane and frontal gland extract onto the test filter paper, using a volume twice that used to extract a single head. All stimuli tested were prepared separately for host and inquiline species. For each of the stimuli tested (blank control, hexane, crushed worker's head, crushed solder's head and frontal gland extract), six repetitions were recorded using individuals from different colonies ($N = 6$), totalling 30 repetitions for each conspecific and heterospecific bioassay in host and guest species.

Typical alarm reactions in termites involve behavioural changes such as (1) substrate-borne vibrations (shaking and/or drumming;
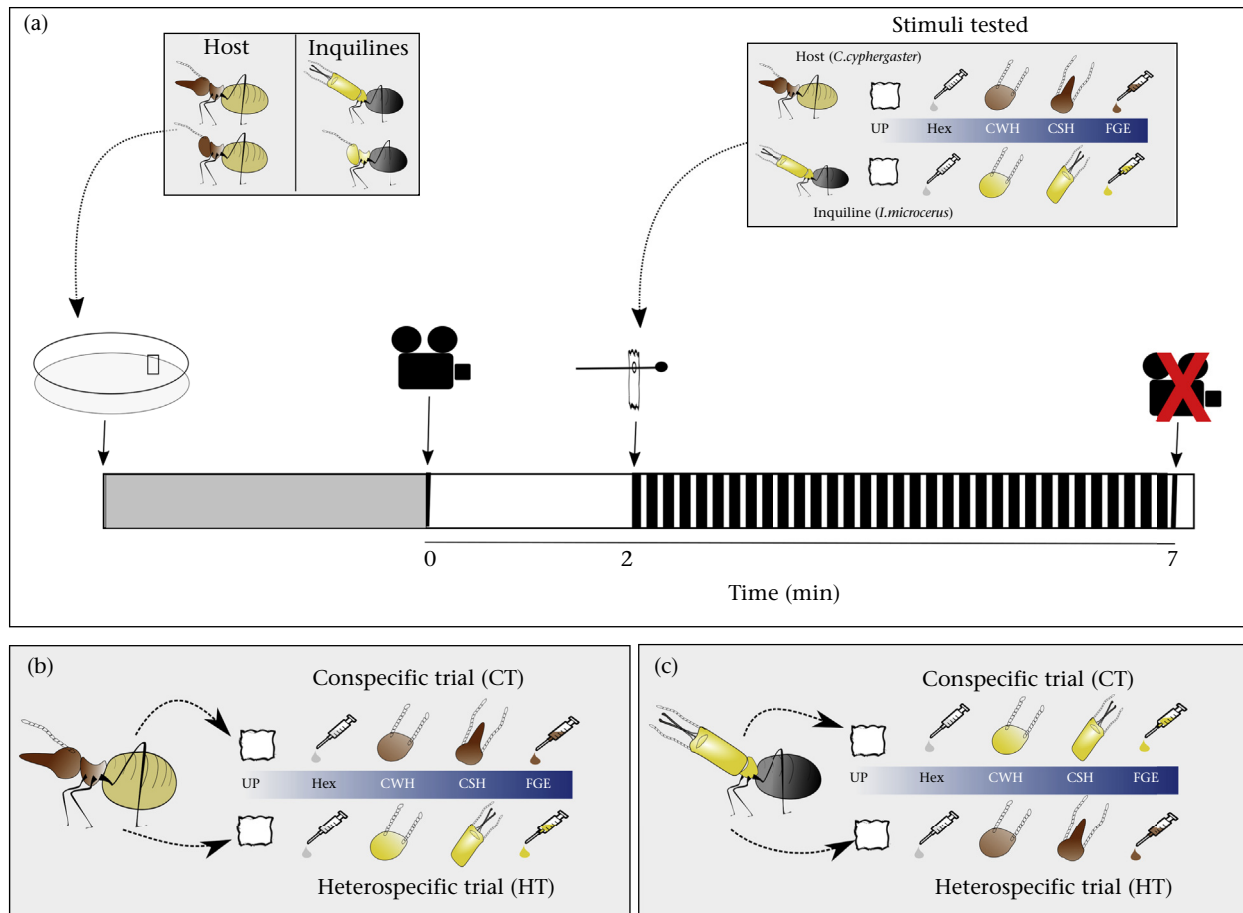
Figure 1. Schematic diagram showing the experimental set-up used for behavioural assays of the host *Constrictotermes cyphergaster* and its inquiline *Inquilinitermes microcerus*. Groups of workers and soldiers of hosts and of inquilines occupying the same nest were released separately in the experimental arena and allowed to acclimate for at least 2 h. The behaviour of each termite group was videorecorded for 7 min, of which the first 2 min refer to pre-stimulus insertion and the last 5 min refer to post-stimulus insertion. CT = conspecific; HT = heterospecific. Stimuli were: untreated filter paper (UP), or filter paper with hexane (Hex), crushed worker's head (CWH), crushed soldier's head (SSH), or frontal gland extracts from soldiers' heads (FGE).

see details in Cristaldo et al., 2015; Delattre et al., 2015) and (2) escape from a source of disturbance (Cristaldo et al., 2015; Delattre et al., 2015; Šobotník, Jirosová, & Hanus, 2010). To check the alarm response in the bioassays, we measured two parameters as indicative of alarm reaction in termites: (1) body-shaking movements and (2) running speed. Both parameters were measured independently for each stimulus in each conspecific or heterospecific bioassay. To do so, the respective video was loaded and visually inspected in Mouse-Tracer software (for details see Šobotník et al., 2008). To take account of body-shaking movements, we pressed a predefined key on the keyboard each time a termite was seen vibrating in the experimental arena. From each video, we then obtained the sum of all vibrational events. Final counts, used in subsequent statistical analysis, refer to the difference in the number of vibratory events before and after a stimulus insertion. We determined the termites' running speed by positioning the cursor on an arbitrarily selected individual, which was followed on the computer screen. The cursor position was initially expressed in pixels/s and subsequently converted into mm/s. For each recording, termite speed was initially measured in one soldier and one worker and subsequently averaged among both. Final running speed (mm/s) used in subsequent statistical analysis was estimated by the difference in the running speed before and after a stimulus insertion. To minimize observer bias, all collection and analysis of data was carried out blind with respect to the treatment used.

Nest Defence in Natural Conditions

To determine which species were involved in the host nest defence, we subjected arboreal nests of *C. cyphergaster* cohabited by *I. microcerus* ($N = 36$; different from those used in the behavioural laboratory assays) to experimental physical disturbance in the field. Disturbance was made to a point located halfway between the nest's base and the top, using the stainless-steel blade of a pocket knife to jab the nest on its exterior wall, as described in DeSouza et al. (2016). After 40 s had elapsed, we scored the total number and identity of termite soldiers and workers appearing at the damaged point (i.e. host, inquilines, or host plus inquilines). Nests were subsequently broken apart to confirm the presence of *I. microcerus*.

Statistical Analyses

To test the effects of stimuli (blank control, hexane, crushed worker's head, crushed solider's head and frontal gland extract), source of stimuli (conspecific or heterospecific) and their first-order interaction on the number of body-shaking movements and running speed, we analysed the data in independent models for each species, using analysis of deviance (ANODEV) for generalized linear models (GLM). Body-shaking movements were analysed under Poisson error distribution with log link, corrected for

overdispersion with quasi-Poisson function. Running speed was analysed under normal error distribution with identity link. Analysis were conducted independently for each species in order to avoid pseudoreplication, since host and inquilines belong to the same nest and hence are not independent from each other. Model simplification, when necessary, was conducted by extracting explanatory terms from the initial model and evaluating the subsequent change in deviance, as recommended by Crawley (2007). We assessed differences among treatments by contrast analysis, lumping treatment levels as long as this did not cause significant ($P < 0.05$) changes in models as described in Crawley (2007, page 368).

Analyses were performed in R (R Development Core Team, 2009) followed by residual analysis to verify the suitability of error distribution and model fitting.

RESULTS

Response to Alarm Signals

The mean number of body-shaking movements performed by the host species *C. cyphergaster* groups were significantly affected by stimuli and source of stimuli (ANODEV: $F_{2,34} = 12.23$; $P = 0.0001$; Table 1) but not their interaction. In the conspecific treatment, body-shaking movements attained maximum values for termites exposed to crushed soldier's head. Values significantly lower than these (but equivalent among themselves) were observed for termites subjected to crushed worker's head and frontal gland extract. Termites subjected to controls (blank control and hexane) presented the lowest values for body-shaking movements (ANODEV: $P = 0.004$; Fig. 2a). Meanwhile, body-shaking movements did not differ between types of stimuli in the heterospecific treatment (ANODEV: $P = 0.279$; Fig. 2b). Surprisingly, the inquiline *I. microcerus* never displayed vibratory movements, either in the conspecific treatment or in the heterospecific treatment assays, possibly indicating the absence of such behaviour in this species.

The running speed of *C. cyphergaster* was significantly affected by the interaction of stimuli and source of stimuli (ANODEV: $F_{2,30} = 137.45$, $P < 0.001$; Table 1). In the conspecific treatment, increased speed was observed in individuals exposed to crushed worker's head, crushed solider's head and frontal gland extract, in
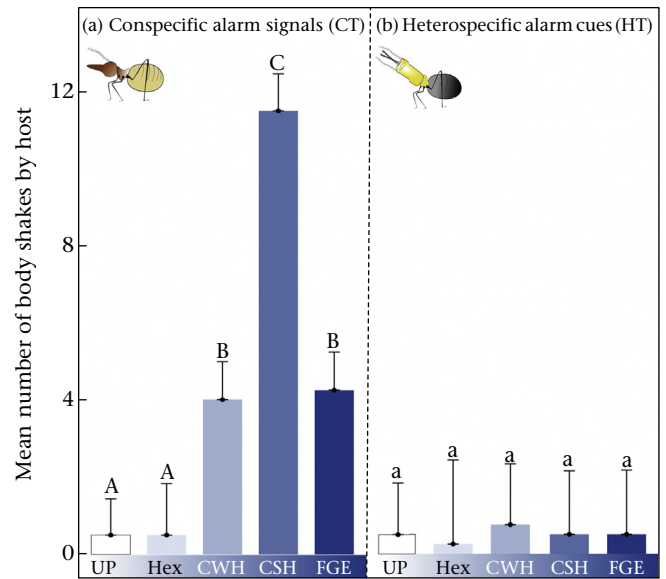

Figure 2. Mean ± SE number of body-shaking movements by the host *Constrictotermes cyphergaster* in response to (a) conspecific and (b) heterospecific (inquiline) alarm cues. See Fig. 1 for abbreviations of stimuli.

ascending order, as compared to the controls (blank control and hexane) (ANODEV: $P < 0.001$; Fig. 3a). In contrast, the running speed of *C. cyphergaster* was not significantly affected by the stimuli from heterospecifics (i.e. guests) (ANODEV: $P = 0.5104$; Fig. 3b, Supplementary Video S1). In the conspecific assays, the speed of *C. cyphergaster* was significantly greater than that in the heterospecific assays (ANODEV: $P < 0.0001$; Table 1, Fig. 3).

In *I. microcerus*, running speed was also significantly affected by the interaction of stimuli and source of stimuli (ANODEV: $F_{2,30} = 85.65$, $P < 0.001$; Table 1). In both conspecific and heterospecific assays, increased speed was observed in individuals subjected to crushed worker's head, crushed soldier's head and frontal gland extract, in ascending order, as compared to the controls

Table 1
Effects of stimuli and source of stimuli (conspecific versus heterospecific cues) on body-shaking behaviour and running speed of the host (*C. cyphergaster*) and running speed of inquilines (*I. microcerus*)

	df	Deviance	Resid. df	Resid. dev.	F	P(>F)
Host (*C. cyphergaster*)						
Body-shaking vibrations						
Null model			39	201.55		
Stimuli (a)	4	69.38	35	132.16	9.77	<0.001
Source of stimuli (b)	1	65.44	34	66.72	36.86	<0.001
a:b	4	8.13	30	58.59	1.14	0.3544
Running speed						
Null model			39	111.68		
Stimuli (a)	4	45.485	35	66.20	128.82	<0.001
Source of stimuli (b)	1	23.409	34	42.79	265.19	<0.001
a:b	4	40.145	30	2.64	113.70	<0.001
Inquiline (*I. microcerus*)						
Running speed						
Null model			39	43.90		
Stimuli (a)	4	36.836	35	7.07	167.98	<0.001
Source of stimuli (b)	1	3.238	34	3.83	59.05	<0.001
a:b	4	2.190	30	1.64	9.98	<0.001

Data were analysed using generalized linear models (GLM) under quasi-Poisson (body-shaking vibration) and normal (running speed) error. See Methods for details.
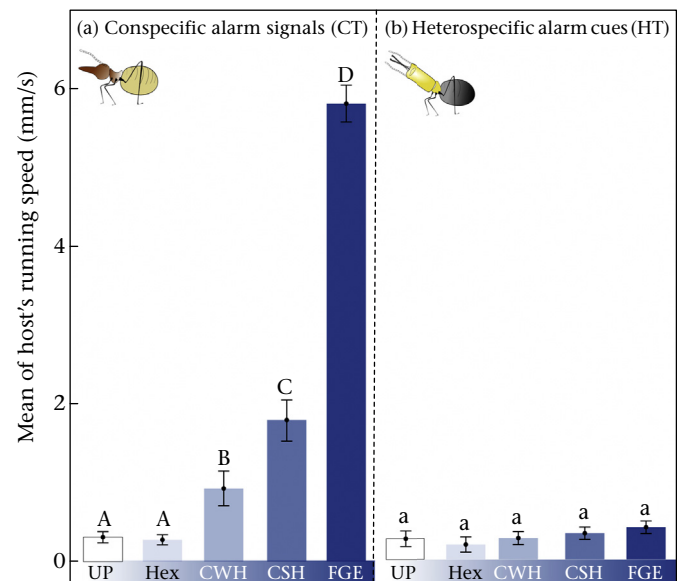

Figure 3. Mean ± SE running speed of the host *Constrictotermes cyphergaster* in response to (a) conspecific and (b) heterospecific (inquiline) alarm cues. See Fig. 1 for abbreviations of stimuli.
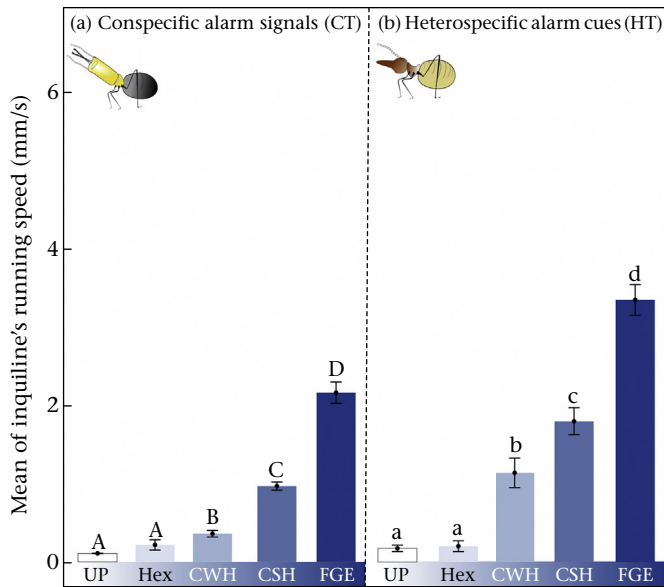
Figure 4. Mean ± SE running speed of obligatory inquiline *Inquilinitermes microcerus* to (a) conspecific and (b) heterospecific (host) alarm cues. See Fig. 1 for abbreviations of stimuli.

(blank control and hexane) (ANODEV: conspecific: $P < 0.001$; Fig. 4a; heterospecific: $P < 0.001$; Fig. 4b). In the heterospecific assays, the speed of *I. microcerus* was significantly higher compared to that in the conspecific trials (ANODEV: $P < 0.0001$; Table 1, Fig. 4). This seems to support the idea that *I. microcerus* are capable of perceiving their own alarm as clearly as they perceive the alarm signal of their host (see Supplementary Video S1).

Host Nest Defence in the Field

Colonies of *I. microcerus* were present in all 36 *C. cyphergaster* nests sampled. Disturbance in the nest wall of *C. cyphergaster* nests cohabited by colonies of *I. microcerus* provoked quick alarm responses from *C. cyphergaster* individuals (mean ± SE = 11.33 ± 2.14) but not from *I. microcerus*, whose soldiers or workers were never observed at the disturbed point in any of the 36 assayed nests (Fig. 5a,b). An overall summary of host and inquiline individuals at disturbed spots per assayed nest is shown in Fig. 5c.

DISCUSSION

Our results have shown that the response to alarm signals by the studied termite hosts and their inquilines was asymmetric (see Supplementary Video S1): *C. cyphergaster* did not respond to the alarm cues of their inquilines *I. microcerus* (Fig. 3), but *I. microcerus* responded to the alarm cues from both the hosts and their conspecifics (Fig. 4). Although inquilines were able to respond to the alarm cues from their conspecific and hosts, they were never observed acting in defence of the host nest (Fig. 5). It is therefore possible that the inquilines detection of their hosts' alarm cues serves as a form of 'public information' (Danchin, Giraldeau, Valone, & Wagner, 2004; Valone, 2007), in which host signals are used to obtain information about threats and allow danger to be evaded.

The ability to detect and use heterospecific visual, acoustic, olfactory and other cues is frequently observed among organisms (Matessi, Matos, & Debelsteen, 2008). It is often used by predators and parasites to locate their prey or host (Vandermoten, Francis, Haubruge, & Leal, 2011; Zuk & Kolluru, 1998), but it has also been

recorded among species from the same trophic level, such as mixed-groups of frogs (Phelps, Rand, & Ryan, 2007), lemurs (Seiler, Schwitzer, Gamba, & Holderied, 2013), primates (Seyfarth & Cheney, 1990; Zuberbühler, 2000) and birds (Fallow & Magrath, 2010; Haff & Magrath, 2013; Leavesley & Magrath, 2005; Leniaud, Dedeine, Pichon, Dupont, & Bagnères, 2009; Magrath, Pitcher, & Gardner, 2009; Magrath et al., 2016). Because the groups share the same or similar predators, the ability to detect alarm calls produced by heterospecifics can provide valuable 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 social insect hosts and inquilines occupying the same nest. In fact, guests of ants conduct most of the nest defence against agropredator ants on behalf of their host ants (Adams et al., 2013).

In termites confined in wood, heterospecific detection of cues from superior competitors by their weaker counterparts has been previously documented (Evans et al., 2009): vulnerable dry wood species *Cryptotermes secundus* (Kalotermitidae) are able to detect vibrational cues of their superior competitor *Coptotermes acinaciformis* (Rhinotermitidae), avoiding places in which this species is present and thus decreasing risks of confrontation. Heterospecific detection of chemical signals has been reported to be a common strategy to exploit food resources in ants (by social parasites or mutualists; Menzel & Blüthgen, 2010; Menzel, Pokorny, Blüthgen, & Schmitt, 2010; Powell, Del-Claro, Feitosa, & Brandão, 2014), in stingless bees (Nieh, Barreto, Contrera, & Imperatriz−Fonseca, 2004) and also in termites (by neighbouring colonies) (Cristaldo et al., 2016). We are aware of no other examples of heterospecific detection of chemical alarm signals in social insects however.

Two theoretical scenarios seem plausible for the specific case of heterospecific detection of alarm signals by hosts and inquilines in termites: on the one hand, alerts can be mutually shared so that both species could join forces for defence, or at least increase efficiency in defence. Conversely, if only one of the cohabitant species is able to glean information from heterospecifics, it could profit from the other's vigilance in a commensal fashion. This last case seems to be applicable to the results here obtained for *C. cyphergaster* and its inquiline *I. microcerus*. Despite perceiving and responding to their host's alarm cues in laboratory bioassays (Fig. 4), *I. microcerus* have never been recorded to join defence by attending events of simulated breaking and entering in nests, carried out in numerous (i.e. 36) field assays (Fig. 5). While such behaviour does not confer clear 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, which might act to evict an invader and also reduce costs of producing their own signal. This sneaky behaviour is reinforced in the inquilines by the use of alarm chemical compounds to which their host does not react (Fig. 3b). Most remarkably, inquilines were never observed performing vibrational movements, a typical alarm behaviour observed among all termites studied so far (see Šobotník et al., 2010). That is, inquilines detect the host's cues without the host's knowledge. Interestingly, more than simply detecting their host's alarm cues, inquilines are able to perceive distinct levels in the alarm signals of their host (for details see Cristaldo et al., 2015): running speed of *I. microcerus* was significantly different according to the stimulus from their host (Fig. 4b).

It seems compelling to conclude that evolution has shaped hiding behaviours in *I. microcerus*, possibly favouring diminished cohabitation costs, arguably allowing them to divert investments to reproduction, as hinted by Cunha et al. (2003). A clear picture seems to be emerging for the inquilinism by *I. microcerus* on
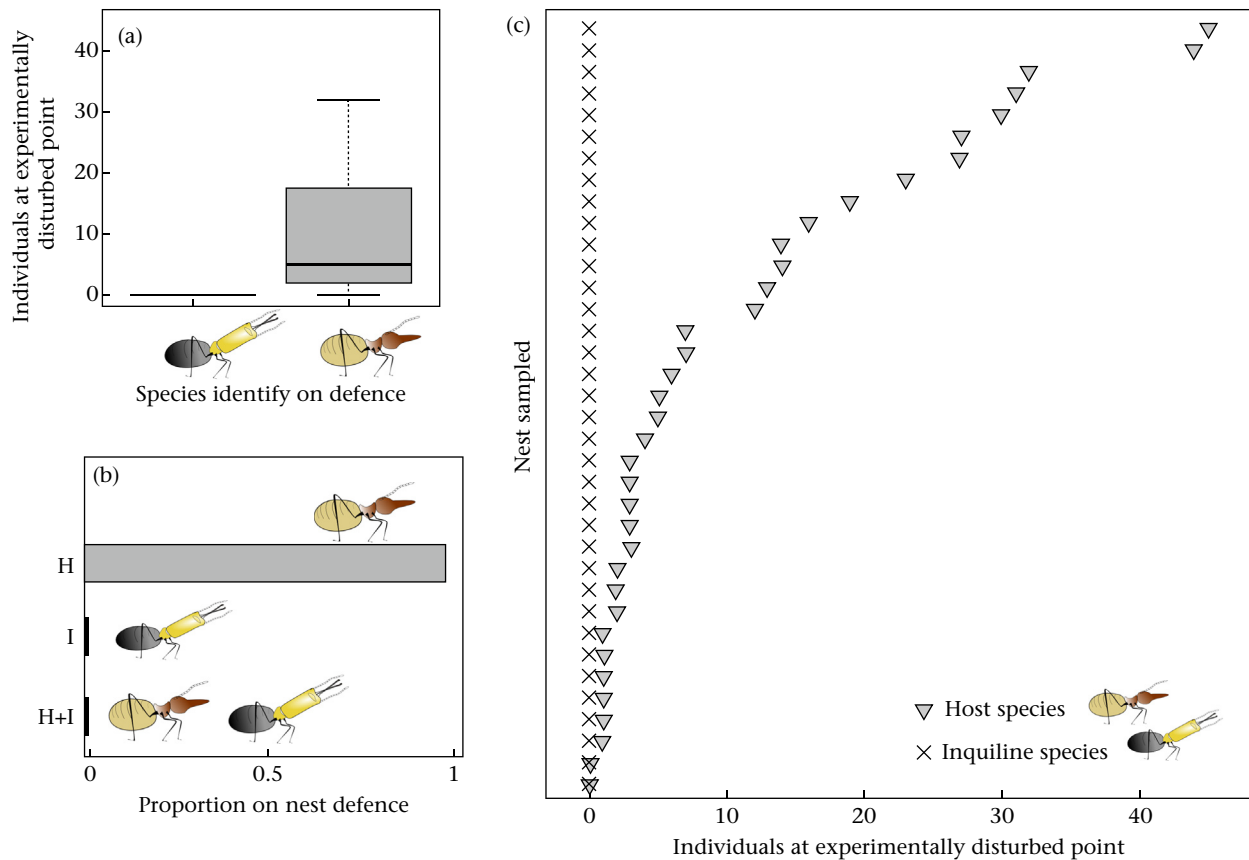
Figure 5. Defence of *Constrictotermes cyphergaster* nests (host) in natural (field) conditions. (a) Mean ± SE number of host and inquiline individuals at experimentally disturbed point. (b) Proportion of hosts (H), inquilines (I) or hosts plus inquilines (H+I) involved in host nest defence. (c) Overall summary of host and inquiline individuals present at experimentally disturbed points per assayed nest.

C. cyphergaster: we found no evidence for mutualism in the form of combined nest defence, as proposed initially by Coles (1980) and Redford (1984) for termite inquilines possessing weaponry distinct from their host (as is the case here). Rather, our data connect 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 and avoid their host trail (Cristaldo et al., 2014) and alarm (this study) cues, most likely as means to avoid confrontation arising from overlaps in space. The results shown here provide new insights into the nature of inquilinism in termites while providing better groundwork for models of cohabitation in social insect colonies. To the best of our knowledge, no previous study on inquilinism in social insects has shown inquilines capable of detecting their host's alarm cues.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2016.07.025.

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