DINAMARTA VIRGINIO FERREIRA

TERMITE-TERMITE HETEROSPECIFIC COHABITATION MEDIATED BY VIBRATORY CUES

Thesis presented to the Universidade Federal de Viçosa, as part of the Entomology Graduate Program requirements, to obtain the title of *Doctor Scientiae*.

Adviser: Og Francisco Fonseca de Souza

Co-adviser: Leonardo Morais Turchen

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Og Francisco Fonseca de Souza
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À minha família.

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ABSTRACT

FERREIRA, Dinamarta, D.Sc., Universidade Federal de Viçosa, July, 2023. **Termite-termite heterospecific cohabitation mediated by vibratory cues**. Adviser: Og Francisco Fonseca de Souza. Co-adviser: Leonardo Morais Turchen

Distinct organisms can live together in intimate associations, called symbiosis. These heterospecific associations cover the entire spectrum, from parasitism to mutualism. Termite-termite heterospecific cohabitation is an interesting case of symbiosis. Several questions are raised to understand how hosts and inquilines remain living together sharing the same nest. For example, what are the main forces behind this phenomenon? In this context, we focused on two mechanisms: the ability to perceive and distinguish, as well as the process of becoming habituated to vibrations from heterospecific cohabitants. Here, we provide evidence that vibrations emitted by termites are an important communication channel used to perceive heterospecific vibrations. We found that cohabitation is shaped by a combination of inquiline and host behavioural characteristics. The ability to become habituated to heterospecific vibrations is a strong indication that both cohabitants do not perceive each other's vibrations as a threat. Furthermore, the host species does not seek the source of vibration to find the invader, allowing the maintenance of cohabitation. On the other hand, the inquilines can exhibit more distance travelled, independent of presence of vibrations, which would facilitate evasion when meeting the host. We show that the mechanisms linked to vibratory perception and habituation and a set of behavioural adaptations from the host and inquiline termites can facilitate cohabitation.

Keywords: Termites. Cohabitation. Substrate-borne vibration. Habituation.

RESUMO

FERREIRA, Dinamarta, D.Sc., Universidade Federal de Viçosa, julho de 2023. **Coabitação heteroespecífica cupim-cupim mediada por pistas vibratórias**. Orientador: Og Francisco Fonseca de Souza. Coorientador: Leonardo Morais Turchen

Organismos distintos podem viver juntos em associações íntimas, chamadas simbiose. Estas associações heteroespecíficas cobrem todo o espectro, do parasitismo ao mutualismo. A coabitação heteroespecífica cupim-cupim é um caso interessante de simbiose. Diversas questões são levantadas para compreender como hospedeiros e inquilinos permanecem vivendo juntos compartilhando o mesmo ninho. Por exemplo, quais são as principais forças por trás deste fenômeno? Neste contexto, focamos em dois mecanismos: a capacidade de perceber e distinguir, bem como o processo de habituação às vibrações dos coabitantes heteroespecíficos. Aqui, fornecemos evidências de que as vibrações emitidas pelos cupins são um importante canal de comunicação usado para perceber vibrações heteroespecíficas. Descobrimos que a coabitação é moldada por uma combinação de características comportamentais dos inquilinos e dos hospedeiros. A capacidade de se habituar a vibrações heteroespecíficas é uma forte indicação de que ambos os coabitantes não percebem as vibrações um do outro como uma ameaça. Além disso, a espécie hospedeira não busca a fonte de vibração para encontrar o invasor, permitindo a manutenção da coabitação. Por outro lado, os inquilinos podem exibir maior distância percorrida, independente da presença de vibrações, o que facilitaria a evasão no encontro com o hospedeiro. Mostramos que os mecanismos ligados à percepção vibratória e à habituação e um conjunto de adaptações comportamentais do hospedeiro e dos cupins inquilinos podem facilitar a coabitação.

Palavra-chave: Cupins. Coabitação. Vibração transmitida pelo substrato. Habituação.

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SUMMARY

General Introduction

Substrate-borne vibration is a widespread communication channel of insects, playing an essential role in the ecology of these organisms (Virant-Doberlet and Cokl, 2004; Cocroft and Rodríguez, 2005). Vibrations allow the insects to transmit and receive information, facilitating decision-making. After receiving vibrational information, the insect could exploit it in different contexts, including dangerous situations, using vibration to increase its survival (Virant-Doberlet et al., 2019). The detection of vibrations is performed through highly developed sensory organs, such as Johnston's organ in the antennae and the subgenual organ located in the tibia of all six legs, allowing them to perceive and interpret vibrations in the environment (Klowden, 2013). However, the threshold of perception of the vibrations and, consequently, the behavioural responses can depend on the species and kind of vibration received.

In species with higher social interactions, the vibratory signals sent are essential to maintaining self-organisation, as is the case in eusocial colonies (Hunt and Richard, 2013; Leonhardt et al., 2016). Several vibrations are performed to assist social cohesion (social hierarchy, attendance) (Land and Seeley, 2004; Mignini and Lorenzi, 2015) and coordinate group actions (foraging, defence) (Evans et al., 2007; Hager and Kirchner, 2014; Delattre et al., 2015). The colony's defensive behaviour exemplifies the effectiveness of the communication mechanism. When a threat is detected, nestmates use vibratory signals to alert others to the disturbance source and allow them to coordinate their efforts to respond to the threat (Hager and Kirchner, 2013, 2014).

Although social insects rely on substrate-borne vibration as a reliable form of communication, inquilines or parasites (i.e., the guest) can exploit a weakness in it through eavesdropping. Some guest species are able to break down the social insect host's communication channel and use it for their own benefit (Cini et al., 2019). The guest can perceive the host's vibratory cues and may take advantage, such as avoiding encounters (Evans et al., 2009) or mimicking for assistance (Lin et al., 2019). In this case, vibrations mediate the symbiotic interactions.

The obligatory inquilinism in termite nests is a most intricate case of symbiosis. Host and inquiline termites live along sharing the same nest. The investigations presented here use as a model the interaction between the host termite *Constrictotermes cyphergaster* (Silvestri, 1901) (Termitidae: Nasutitermitinae) and its inquiline *Inquilinitermes microcerus* (Silvestri, 1901) (Termitidae: Termitinae).

C. cyphergaster nests are found in some Brazilian regions; the host frequently builds its nest on trees, mainly in the 'Cerrado' biome (Cristaldo et al., 2012), but nests can also be found on rocks in the 'Caatinga' biome (Vasconcellos et al., 2007). The host population consists of thousands of individuals of different castes who contribute to the functioning of the nest (Cunha et al., 2003). In termites, functional roles vary among castes: the royal pair, the king and queen, are responsible for reproduction; alates are future reproductive castes; workers are responsible for foraging, building, repairing, and cleaning the nest; while soldiers improve the exploitation of food resources (do Sacramento et al., 2020), start and defend the foraging trails (Almeida et al., 2016) and defend the nest against intruders (Cristaldo et al., 2016; Oberst et al., 2017). *C. cyphergaster* nests harbour many cohabitants, including *I. microcerus* (Mathews, 1977; Cunha et al., 2003; Cristaldo et al., 2012).

The termites *I. microcerus* are not able to build their nest and become obligatory inquilines in the nests of *C. cyphergaster* (Mathews, 1977). The number of individuals in inquiline colonies is smaller than that of the builder termite, and the colonies are restricted to certain portions of the nest (Cunha et al., 2003), usually close to its core in a mass of dark organic material (Mathews, 1977). *I. microcerus* live inside the host nest; however, they do not assist the host with the defence and repair of the nest (Cristaldo et al., 2016).

The ecological interaction between *I. microcerus* and *C. cyphergaster* is not easily located in the parasitism-mutualism continuum. Furthermore, the research has not yet provided a clear definition of the interaction's costs and benefits. This raises many questions, including: How does cohabitation persist once the inquiline termites never help its host to maintain and defend the nest against intruders? and What other mechanisms might help with the termite-termite cohabitation puzzle? Several mechanisms have already been identified as facilitating termite-termite heterospecific cohabitation. This case of symbiosis seems to remain for some reason, including the morphological, physiological, and behavioural adaptations of both cohabitants.

To thrive inside the host nest, the main strategy of *I. microcerus* seems related to "conflict avoidance", with their host (Florencio et al., 2013). Obvious advantages for *I. microcerus* include the avoidance of building costs and the exploitation of a sheltered, defended and resource-rich environment that is the host nest. Physiological traits such as low concentration of the trail-following pheromone of inquilines, reduce detection by the host (Cristaldo et al., 2014). Simultaneously, inquilines use the host's pheromones to prevent interspecific encounters and escape from the places of greatest risk (Cristaldo et al., 2016). In the event of an encounter, inquilines maintain evasive maneuvers and remain non-aggressive during interactions (Hugo et al., 2020) and use their specialised mandibles for self-defence against attacks from hosts (Clemente et al., 2021). Besides, *I. microcerus* does not overlap its diet with that of their host (Florencio et al., 2013).

Interestingly, the host, when exposed to inquiline chemical cues, does not use them to identify and prevent invasion (Cristaldo et al., 2014, 2016). Another crucial aspect to consider is that, during the host's reproductive period, the colony reduces the defensive caste (soldiers), which can facilitate the entry of inquilines (Rodrigues et al., 2021).

Usually, termites emit vibratory signals for intracolony communication. Termite species use vibrations as a source of information to detect enemies, avoid competitors, prevent overlap of foraging areas, and keep intruders out of their nest (Evans et al., 2009; Hager and Kirchner, 2013; Oberst et al., 2017; Ferreira et al., 2018). Once inside the nest, inquilines and hosts are exposed to cues from each other (Cristaldo et al., 2016), including vibratory one. Could the perception of heterospecific vibration in termites be one of the pieces of this puzzle and help to elucidate the phenomenon of cohabitation in termites? Here we show that vibratory perception might guide the behaviour of both hosts and inquilines. The host can use the vibrations from inquilines to detect them. Meanwhile, the inquiline can employ the vibration to locate the host.

In this thesis, the goal was to unveil if heterospecific vibratory cues might mediate termite-termite cohabitation. The thesis was divided into 2 chapters. Chapter 1 consists of the article “Perceive and distinguish: How do vibration mediate the cohabitation between host and inquiline termites?”, which inspects whether termites are able to perceive and distinguish heterospecific vibratory cues. The remarkable result is that both termites perceive heterospecific vibrations with similar responses. However, do the behavioural responses of both species differ after long exposure to each other’s vibratory cues? The Chapter 2 consists of the article “Cohabitation in termite nests: symbiosis mediated by habituation to heterospecific vibration", which shows if hosts and inquilines differ in their ability to habituate each other. The findings show that cohabiting termites reduce their alertness in response to vibratory cues from one another, indicating that habituation as an important mechanism leading to tolerance and, as a result, termite-termite coexistence.

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Article 1: Perceive and distinguish: How do vibration mediate the cohabitation between host and inquiline termites?

1.1 Abstract

Termites use the vibratory communication channel as a source of information to mediate several ecological interactions. Vibrations are used to detect intruders, prevent nest invasions, locate the disturbance source, and avoid encounters with enemies. Termites may use part of these mechanisms to prevent invasions of their nests or avoid encounters with enemies. Despite this, termite nests are invaded by inquiline termites that cohabit with the building species. Once inside the nest, inquilines and hosts are exposed to vibrations from each other. This permanent symbiotic alliance is moulded by several mechanisms—morphological, physiological, and behavioural—that drive both species to live inside the same nest. Could the perception of heterospecific vibration in termites be one of the pieces of this puzzle and help elucidate the phenomenon of cohabitation in termites? Vibratory perception might guide the behaviour of both hosts and inquilines. The host can use the vibrations from inquilines to detect them and prevent nest invasion. Meanwhile, the inquiline can employ the vibration to locate the host and avoid conflicts. Here, we hypothesise that the perception, distinction, and response of termites to heterospecific vibrations compose a mechanism that facilitates obligatory termite-termite cohabitation. We inspected the behavioural responses of the host and inquilines when exposed to each other's playback vibrations, precisely evaluating the duration of alertness and distance travelled by termites. We report that both cohabitating termite species perceive the heterospecific vibration increasing alertness; however, the host species perceives and does not seek the source of vibration. On the other hand, inquilines are generally quite active, walking longer than their host. This should allow them to evade in case of meeting the host. Our results suggest that this heterospecific cohabitation can be facilitated by the unattentive attitude of the host, combined with inquiline activeness.

keywords: cohabit; inquilinism; vibration; avoidance; symbiosis.

1.2 Introduction

Cohabitation can be a challenge! To keep living together, cohabitants must continuously reach compromises to meet each other halfway. This is feasible, albeit with a cost, if cohabitants share the same goal (e.g., conspecific building a nest wall or in cooperative care of young) (West-Eberhard, 1978; Klahn, 1979; Holldobler et al., 2009; Andrade et al., 2016). But if cohabitants consist of individuals from distinct species, goals may be not congruent and compromises not at all achievable (e.g., colony defence) (Cristaldo et al., 2016). Why, then, some species renounce their autonomous lives to bind together in permanent associations? (Estrela et al., 2016) What are the main forces behind this phenomenon?

A somewhat simplistic answer would be that partners should have an advantage in cohabiting. Advantages may include, for instance, shared defence of common areas in the case of mutualistic partners (Higashi and Ito, 1989; Monteiro et al., 2017). As for parasitic ones, heterospecific cohabitation may pose advantages for a single one of the partners (Fuchs and Korb, 2006). But how to explain more intricate cases such as those of heterospecific termite-termite cohabitation, in which partners cannot easily be appointed as parasites, commensals, or even mutualists (Campbell et al., 2016).

In heterospecific termite-termite cohabitation, the inquiline species obtain a sheltered, safe place to rear offspring without the building costs. However, the inquiline never shares tasks with its host, such as defending the nest against intruders. This could help the inquiline to save energy while reducing the risk of injuries (Cristaldo et al., 2016; DeSouza et al., 2016). Furthermore, providing that the inquiline finds food inside the nest (Florencio et al., 2013), it will not need to leave the nest to forage. As a result, the inquiline will not have to deal with environmental challenges outside the nest.

Interestingly, there is no evidence that proves the host benefits from heterospecific cohabitation. This is because inquiline does not help their hosts maintain or defend the nest, as shown above. Furthermore, the host is not able to detect inquilines via chemical cues and prevent the nest from invasions (Cristaldo et al., 2014, 2016). So far, further studies are required to elucidate the costs and benefits of maintaining coexistence for the host.

Cohabitation in termite nests possible due to the mechanisms developed by inquilines for living within their host nest, such as pacifist behaviour to avoid fighting with hosts (Cruz et al., 2018; Hugo et al., 2020), chemical disguise to avoid being found (Cristaldo et al., 2014, 2016), and morphological structures for defence if the encounter is unavoidable (Clemente et al., 2021). Inquilines exploit a window of opportunity during the host's reproductive flights to sneak into the nest, as the allocation of resources for reproduction reduces the colony's defence (Rodrigues et al., 2021).

Once inside the nest, inquilines and hosts are exposed to cues from each other (Cristaldo et al., 2016), part of these cues, resulting from the signals each species use for intracolonial communication. In other systems, such as Hymenoptera's host-parasite associations, invaders are able to invade the host's communication system and use it for their own benefit (Cini et al., 2019). Likewise, inquiline termites do this by using the host's chemical cues to perceive them and avoid encounters (Cristaldo et al., 2014, 2016). Interestingly, the host is exposed to inquiline chemical cues but does not use them to identify and prevent invasion (Cristaldo et al., 2014, 2016). It is plausible to expect a similar situation when it comes to the vibratory signals that cohabiting species would release to their conspecifics. If this vibratory information is detected by cohabiting heterospecifics, they could use it to their own benefit. That is, the inquiline could use the host's vibration to maintain evasive manoeuvres and avoid encounters. Likewise, the host could use the inquilines vibration to locate these intruders in the nest.

In fact, substrate-borne vibrations are a vital communication channel used as a source of information to mediate nestmate interactions and nest defence (Howse, 1964; Bagnères and Hanus, 2015) and can be sent for long-distance (Hager and Kirchner, 2013). Termite species use vibrations as a source of information to detect enemies, avoid competitors, prevent overlap of foraging areas, and keep intruders out of their nest (Evans et al., 2007, 2009; Hager and Kirchner, 2013; Oberst et al., 2017; Ferreira et al., 2018). It is thus plausible to suspect that, cohabitating termites might use vibrations to detect each other. Cohabitation, hence, might be subjected to the ability of cohabitants to perceive vibrations and take appropriate action.

Here, we focus on the cohabiting termite species, the inquiline *Inquilinitermes microcerus* (Silvestri, 1901) (Termitidae: Termitinae) and its host *Constrictotermes cyphergaster* (Silvestri, 1901) (Termitidae: Nasutitermitinae), exploring the perception of heterospecific vibratory signals on both species and whether this perception might facilitate their living together. The host-inquiline termite interaction provides a robust model for symbiosis study, since species share the same nest. Moreover, this interaction exemplifies an obligatory form of symbiosis, as the inquiline termites are incapable of building its own nests and rely on the host nest for survival. Thus, for the inquiline, the perception of the host's vibration could give an advantage and help to avoid encounters. We will test the biological hypothesis that the perception, distinction, and response of termites to heterospecific vibrations compose a mechanism that facilitates obligatory termite-termite cohabitation. We inspect the inquilines and hosts behavioural responses when facing each other's vibratory cues and discuss how these responses can drive this heterospecific cohabitation. We report that both termite species can perceive heterospecific vibratory cues; however, the host species perceives and does not seek the source of vibration to avoid or fight the emitter, suggesting that this behavioral response can facilitate cohabitation.

1.3 Material and methods

1.3.1 Rationale

In order to test our hypothesis, we devised an experiment to inspect the reaction of termites to heterospecific and conspecific vibratory stimuli. To do so, we offered such stimuli to the host and respective inquiline termites in independent assays, recording their behavioural responses for posterior analysis. Four types of assays were carried out, according to the type of stimuli being offered: (i) heterospecific vibrations, (ii) conspecific vibrations, (iii) abiotic vibrations, and (iv) without vibrations. These four assays were replicated 8 times for hosts and 8 times for inquilines. Each replication used a group of termites from a distinct host or inquiline colony, and no termite group was used twice. Assays were devised so that we could be certain whether a given response was actually a consequence of a given stimulus and not a random response. That is, comparing termite responses to the absence of stimulus *versus* their responses to vibrations informed us whether or not the observed behaviour was spontaneously generated, and hence whether or not vibration perception exists. By comparing termite responses to abiotic vibrations *versus* their responses to termite vibrations informed us whether or not termites were able to distinguish between abiotic and biotic vibrations. By comparing termite responses to heterospecific vibrations *versus* their responses to conspecific vibrations informed us whether or not termites can recognise their own species signals. To perform the assays, we confined termites in a low cost arena specially designed for vibratory experiments (the so called "tympanic arena" of Nunes et al., 2018) and played back, onto the arena's floor, a previously recorded vibratory wave. The behavioural responses of the termites were recorded in video for posterior analyses. In the sections below, we give details of all these procedures, materials, and equipments.

1.3.2 Biological model

We use as model species the obligatory inquiline termite *I. microcerus* and its host *C. cyphergaster*, which can be found in the Brazilian Cerrado biome and Caatinga biome (Mathews, 1977). We collected a total of eight termite nests of *C. cyphergaster* near the municipality of Sete Lagoas (19°25'37.9"S 044°10'11.7"W) North of the State of Minas Gerais in the Brazilian Cerrado, in September 2021. All these nest housed colonies of the host and inquiline. The nests were moved to the Department of Entomology of the Federal University of Viçosa. Before the tests, the colonies were acclimatized in a rearing room and kept at 23.8 ± 2.4°C, at relative humidity of 71 ± 6%. All assays were performed within a maximum of six weeks after field collection. On two occasions we extracted fragments of these nests to extract termite individuals for vibratory and behavioural experiments.

1.3.3 Tympanic arenas

We conducted the experiments using a low cost arena especially designed for vibratory assays (the so called "tympanic arena" of Nunes et al., 2018), already tested for the host species. This type of arena consists of a flexible membrane taut by a wooden frame, on top of which a small container holds the termites to be assayed (Fig. 1.1a. By simulating a tympanum, the flexible floor of the arena amplifies the vibrations produced by the termites, easing their detection and record.

As a termite container, we used a plastic Petri dish lid (⌀55 mm) on top of the tympanum. A small hole on the top of the lid allowed readings or served as an air exit, depending on the assay. Another hole on its side allowed air-borne stimuli to be inserted in the container, if needed in that specific assay.

1.3.4 Vibratory assay

In the main assays, termites were stimulated using a mini-shaker to reproduce a vibrations onto the arena's membrane, thereby simulating vibrations from abiotic or biotic sources (see section 1.3.7). Feeding the parameters of these vibrations to the shaker, we instructed it to produce vibrations as close as possible from those actually produced by their actual source (see section 1.3.5 below).

To obtain such parameters, these vibrations were previously recorded from the respective abiotic or biotic source. To obtain the vibrations from the abiotic source, we dropped a styrofoam ball onto the arena's floor. Here, the term "abiotic" does not refer to rain, wind, traffic noise, or similar environmental factors. This vibration was used as an unknown vibratory stimulus for both coexisting termites. To obtain the vibrations from biotic sources (i.e., termites), we confined groups of either host or inquiline termites in the tympanic arena's container, using a blow of air to stimulate them to vibrate. A plastic hose conveyed this blow of air into the container. This hose had one of its end attached to the lateral of the container and the other end attached to a manually operated air pump.

We used a vibrometer (PVD-100; Polytec, Waldbronn, Germany) perpendicularly placed over the tympanum to capture these abiotic and biotic vibratory waves and send them to a recorder (Fig. 1.1 a and b). We focused the vibrometer's laser probe on a piece of reflective tape (1 x 1 mm) pasted onto the arena's floor, in order to enhance the efficiency of the probe. We set the vibrometer's velocity measurement to 100 mm/s (or 25 mm s^{-1} V), with the high-pass filter off and the low-pass filter set at 20 kHz. We used the vibrometer digital output coupled to a digital converter and connected to the microphone input of a high-resolution camera (SONY HDR-CX405TM) to synchronise audio and video recordings.

To collect abiotic vibrations, we recorded the vibratory waves from a styrofoam ball ($\varnothing$= 0.5 mm and weight 1.05 mg) dropped onto the arena's floor (height of 1.5 cm). A small hole was drilled at the center of the container's roof to allow the ball to be dropped onto the arena's floor (Fig. 1.1b). We repeated the assay 10 times. A pilot test was conducted to determine the optimal distance for capturing vibrations from the ball, reducing the noise, and better resembling the vibrations of the termites. After the test, it was found that a distance of 10 cm between the reflective surface (1x1 mm) and the container edge provided the best record. (Fig. 1.1b).

To collect biotic vibrations, we confined groups of either host or inquiline termites in the tympanic arena's container (Fig. 1.1 a). To stimulate them to vibrate, we inserted a blow of air of c.a. 230 cm^3 into the container using an air pump. Here, we used the blow of air to elicit termite vibrations, this vibrations has been described as alarm signalling (Kirchner et al., 1994; Connétable et al., 1999; Röhrig et al., 1999; Hager and Kirchner, 2013). A plastic hose conveyed this blow of air into the container. This hose had one of its ends attached to the lateral of the container and the other end attached to a manually operated air pump. An opening on the container's roof created an air exit. We inserted the air blow twice inside the container, within an interval of 60s; the total video recording time was 120 seconds (Fig. 1.1a). Proportions of workers: soldiers confined were 12:3 for hosts and 15:1 for inquilines. This proportion ensures an absolute density of 0.12 (area/area)which maximizes the interactions between individuals (Miramontes and DeSouza, 1996). Once placed in the container, the subjects were given at least 1 h to acclimatize under 24 ± 2.3°C temperature, 79.7 ± 4.6% relative humidity, and artificial light before the assays. A pilot test was conducted to determine the optimal position to point the laser probe onto the arena's floor, to capture termite vibratory signals. Thus in this assay, we used a distance of four centimetres between the reflective film and the container edge to provide the best record.

We carried out all assays on a vibration isolating table (63-500 Series Micro-g; TMC, Peabody, MA, USA) to decrease external interference (i.e., background noise).

After recording the vibrations, we extracted from these recordings the parameters to instruct the mini-shaker (type 4810; Brüel & Kjaer) to reproduce each biotic or abiotic wave in the main assays. All vibratory parameters are presented at Table 1.1.

Before proceeding to the main assays, however, we validated this procedure inspecting whether the shaker (Fig. 1.1c) would faithfully reproduce the waves as compared to their original source (see section 1.3.5).

1.3.5 Programming the mini-shaker

After obtaining and analysing the abiotic and biotic vibrations (see section 1.3.4), we extracted their specific describing parameters from the recordings. These parameters are essential to instruct the mini-shaker to replicate the artificial waves from biotic or abiotic source during the main assays. We extracted the vibratory parameters based on sound analysis, as indicated by Sueur et al. (2018).

We developed unique protocols for each vibration, obeying its respective parameters. Each protocol was individually uploaded to the mini-shaking, and in sequence, we observed whether the vibratory waves generated followed all parameters of the protocol. The parameters used are presented at Table 1.1.

Table 1.1: List of parameters, its unit of measurement and respective description.

Parameter	unit	Description
Beat	un	A single touch on the substrate
Burst	un	Sequence of beats in a lump
Duration of a burst	s	The interval between the start and end of each lump of beats
Number of beats in a burst	un	The number of times a touch is detected on the substrate in each burst
Pause between beats	s	The interval between one beat and another, inside a burst
Interval between bursts	s	The interval between the end of a burst and the start of another
Maximum amplitude in a burst	(mm/s)	Highest value reached by a waveform
Dominant frequency in a burst	Hz	The number of cycles per second of the waveform with the highest amplitude inside a burst

Specifically, for styrofoam ball vibration, we create the protocol to simulate unknown stimuli to termites. The temporal parameters and the number of beats were different from those emitted by termites. However, we adjusted the amplitude to not exceed the normal amplitude perceived and emitted by termites.

1.3.6 Validating the mini-shaker vibrations

The following procedure was done to verify whether or not the mini-shaker was faitfully reproducing the vibrations to be used in the assays. We compared vibratory waves recorded from abiotic (styrofoam ball) and biotic (termites) sources (section 1.3.4) with their respective playback produced by the mini-shaker onto the arena's floor.

A small hole on the top of the container allowed the laser probe to pass and touch the arena's floor (Fig. 1.1c). To generate the artificial vibration, the appropriate parameters (section 1.3.5) were uploaded to an Arduino Uno board (RS Components, Wädenswil, Switzerland) equipped with an ATmega328p microprocessor (Atmel, San Jose, CA, USA) as a wave generator. The signal sent by Arduino was received by a power amplifier (type 2718; Brüel & Kjaer, Nærum, Denmark) and transmitted to the mini-shaker (type 4810; Brüel & Kjaer). The tip of mini-shaker touched the floor of the tympanic arena softly, 10 cm away from the container edge. The artificial vibration generated by the mini-shaker was thus captured by the laser probe and recorded for eventual analysis.

In order to validate the protocols (section 1.3.5) and the vibrations produced by the mini-shaker, the vibratory data were subjected to Generalized Linear Modelling (GLM) using as full model

$$\text{y} \sim \text{vibration}$$

where “vibration" is a categorical x-variable with two levels: “playback", referring to the vibrations produced by the mini-shaker; and “recorded", referring to the abiotic or biotic vibrations, that is, the vibrations recorded from the styrofoam ball or from termites. Modelling was run independently to compare vibrations produced by: (i) mini-shaker versus hosts; (ii) mini-shaker versus inquilines; and (iii) mini-shaker versus styrofoam ball.

For each of the above comparisons several full models have been built, each of them using one of the following y-variables: (i) duration of a burst; (ii) number of beats in a burst; (iii) pause between beats; (iv) interval between bursts; (v) maximum amplitude in a burst; (vi) dominant frequency in a burst. Full description of each of these y-vars is given at Table 1.1.

We use the Poisson errors for y-vars expressed as counts and Gaussian for the others. When detected overdispersion, the Poisson model was corrected using Quasipoisson distribution (Crawley, 2012).

No differences were detected between the recorded and playback vibrations in all cases. This allowed us to proceed using the mini-shaker as the source of vibrations in the main assays.

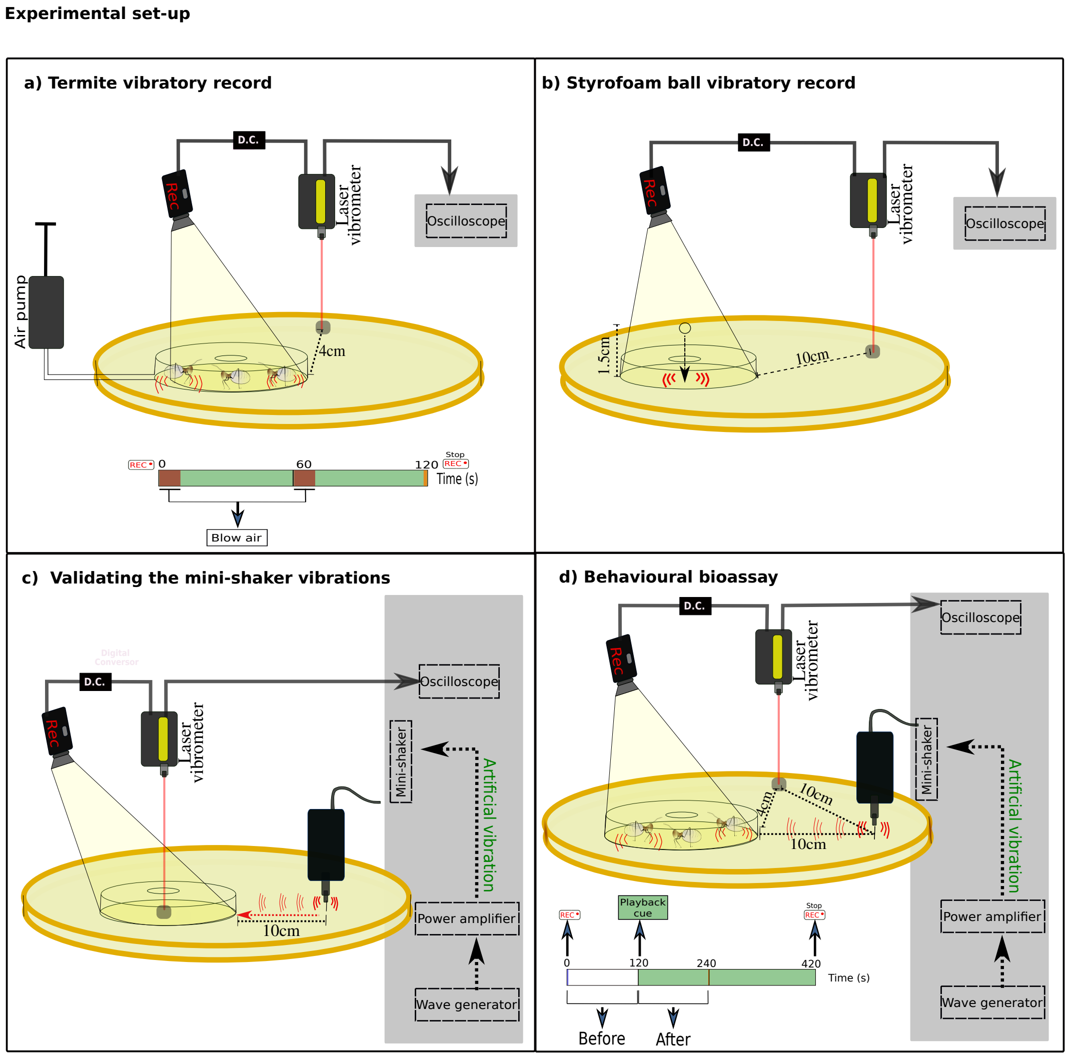

Figure 1.1: Schematic illustrations of the experimental set-up. **a)** Recording vibrations of termites; **b)** Recording vibrations of styrofoam ball; **c)** Validation of vibrations playback by mini-shaker; and **d)** Termite behavioural bioassay. D.C.: Digital conversor.

1.3.7 Behavioural bioassays

Once the mini-shaker was validated (section 1.3.5), we proceeded with behavioural assays, to verify if termites perceive and distinguish vibrations from distinct sources (abiotic and biotic). To do so, we exposed the host and inquilines to mini-shaker vibrations that mimicked vibration from distinct sources (abiotic and biotic), video recording their reactions. These recordings were fed into Boris software (Friard and Gamba, 2016), to build a full ethogram of the termite reactions at each assay.

Four types of assays were carried out, according to the type of stimuli being offered: (i) heterospecific vibrations, (ii) conspecific vibrations, (iii) abiotic vibrations, and (iv) without vibration. Four groups of termites were extracted from the same colony to use one group per assay type. In doing so, a termite group was assayed only once, while that given colony was exposed to all the four types of assays. We used eight distinct colonies for each of the species under scrutiny, giving hence 32 true replicates (4 termite groups x 8 colonies) for host termite and 32 true replicates for inquiline termite species.

Previously to start the bioassay, termites were given two hours to acclimate, under artificial lighting conditions. The proportion of workers: soldiers confined in each arena was identical to that used in the previous trial (see section 1.3.4).

Each vibrational protocol (section 1.3.5) was uploaded to an Arduino Uno board and sent to the mini-shaker for playback onto the tympanic arena's floor (Fig. 1.1 d). The mini-shaker was positioned 10 cm away from the container edge, as dictated by a previous pilot test. Each assay was recorded on video and audio. The total time of the bioassay lasted 420 seconds, and from zero to 120 seconds there was no stimulus; from 120 seconds on, the stimulus was inserted continuously until the end of the bioassay. Specifically, to test the current hypothesis, we use the record of 240 seconds, divided into 120 seconds before and 120 seconds after the start of the vibratory stimulus (Fig. 1.1 d). The assay without vibratory stimulus is the termites' basic behavioural reaction in an experimental condition, which means there is no external stimulus (i.e.absence of playback), thus the mini-shaker was turned off and released no vibratory waves.

All footage was taken using SONY HDR-CX405TM digital video camera set to record 30 frames per second at Full HD (1920 × 1080 60p) saved in .MTS file in Advanced Video Coding High Definition (AVCHD) format, with a sample rate of 48 kHz, with 16-bit resolution.

1.3.8 Compiling the behavioural data

Later on, with the video recordings were visually inspected using BORIS Software (Friard and Gamba, 2016), in order to produce a full inventory of the behaviours exhibited by termites. Prior to video observation, a list of behaviours was previously defined.

To ensure that each individual was only observed once, a print-screen in Inkscape software [version 0.92.5] (Inkscape Project, 2020) obtained at time 0 in each video file, at which time all termites were marked and named, and the same identity of each individual was inserted in Boris.

Inside Boris we linked each behaviour to a key on the keyboard. Each time that specific behaviour occurs, the key is engaged, meaning the behaviour starts. When the behaviour stopped, the key was revoked. We perform this procedure on all behaviours that occur in sequence. Thus, we observed the behaviour performed by each termite individually throughout the entire video for a total of 240 seconds.

Finally, the behaviours observed in all bioassay generate a complete inventory of the behaviours exhibited by termites. After collecting the data, we conducted a statistical analysis and interpreted the results.

In addition, we fed the video recordings to Ethoflow software (Bernardes et al., 2021), to track movement patterns of each of the individuals confined in the arena along the whole assay. A total of more than 800 individuals were tracked once every 1/30 s, along with 240 s, totaling c.a. 5 million datapoints.

1.4 Statistical analyses

The data collected from assays were analyzed using the R-software [version 4.0.2] R Core Team (2021) run in the RStudio interface [version 2021.09.2+382] RStudio Team (2021).

1.4.1 Analysing the behavioural data

Behavioural analyses aimed to verify whether cohabitant termites would be able to perceive each other's vibratory cues. To do so, we tested whether these termites showed distinct alert and walking responses to the vibrations the mini-shaker produced mimicking (i) heterospecific vibrations, (ii) conspecific vibrations, and (iii) abiotic vibrations. The responses to these "treatments" were compared to the responses to (iv) without vibration as a controlling factor to separate spontaneous vibrations from those triggered by the treatments.

In order to make sure that the analysed responses were due exclusively to the factor under scrutiny, we subtracted the responses recorded before the application of the stimuli from those recorded after it. That is to say, we subtracted the responses recorded in the first 120 s of the assay from those responses recorded after 120 s until the end of the assay (240 s). The same procedure was done for the assay without vibrations. In doing so, we obtained a "net value" for all responses, this net value is used a the y-var in all cases detailed below.

Statistical modelling used as a full model

$$\text{y} \sim \text{stimuli*sp + nest}$$

where "stimulus" is a categorical x-var, with four levels (i.e., the "treatments" described above), "species" is a categorical x-var with two levels ("host" or "inquiline") and "nest" is a categorical x-var with eight levels, each level being one of the eight nest identifiers. This last x-var entered the model as a blocking factor.

Two full models have been created, each one holding a y-var that corresponds to a metric describing two types of behavioural response: alertness and displacement.

Alertness corresponds to any behaviour denoting inspection or alarm, that is: (i) performing antennation on nestmates or on the arena's surface; (ii) banging the head or the abdomen on the arena's floor, i.e., "drumming"; (iii) meandering around the arena as if exploring it and (iv) remaining stationary but moving antenna and head while pointing them upward.

To the displacements were considered the total distance travelled within the arena along the assay.

The y-var describing the duration was the time elapsed between the onset and the cessation of an alert response, summed up across all occurrences of that response along the assay. Because arenas held distinct numbers of individuals (15 host or 16 inquiline individuals), this value was divided by 15 or 16, giving the timespan of these responses *per capita*. The net value of duration of alert responses was calculated, as described above.

The y-var describing the distance walked that is the total distance walked by each termite individual, summed up across all individuals in the arena. This value was divided by the number of individuals in the arena, giving the average distance walked *per capita* along the assay. The net value of distance was calculated as above.

Statistical modelling proceeded using Generalized Linear Models (GLM), under Gaussian error distribution. Model simplification was done through contrast analyses with F tests, extracting explanatory terms or combining treatment levels when it did not cause significant ($P<0.05$) changes in the model, as recommended by Crawley (2012). Residual analyses were performed to verify error distribution and model suitability, including tests for overdispersion.

1.5 Results

1.5.1 Characterization of recorded and playback vibration

As stimulated by the air blow, termites performed a vertical oscilatory movement (VOM, *sensu* Howse, 1964) beating their head or abdomen against the substrate. This is commonly known as "drumming". Workers and soldiers exhibit it, but in *I. microcerus* and *C. cyphergaster* the workers perform drumming more frequently than soldiers do.

Termites drumming occurs in bursts of activity interspersed by periods of rest. Each burst is composed of a sequence of beats on the arena's floor. These bursts and beats were recorded by the vibrometer as a vibratory sequence composed of lumps of peaks (Figs. 1.2a and 1.3b) in which each lump is a burst and each peak is a beat. Such a vibratory profile is mathematically characterized by the parameters showed in Tables 1.2, 1.3 and 1.4. At this table, we present not only the recorded vibratory profiles (that is, for host and inquiline termites as well as the styrofoam ball) but also their respective playback vibratory profiles as produced by the mini-shaker. Graphic representation of each pair of recorded and playback vibrations is presented in the

Figures 1.2, 1.3 and 1.4.

After conducting statistical analyses, we validated the mini-shaker vibrations, and we found that the termite's playback vibrations (from hosts and inquilines) were similar to the recorded vibrations, as no differences were detected as presented in Tables 1.2 and 1.3. For the styrofoam ball, we adjusted the amplitude value to ensure it did not exceed the typical amplitude perceived and produced by termites (see Table 1.4).

Table 1.2: Parameters characterizing the recorded vibrations from host termite *C. cyphergaster* and from its playback counterpart (produced by the mini-shaker). Column names ending in "rec" refer to the vibratory parameter recorded directly from the termite in contact with the arena's floor. "host rec" = recorded vibration of the host; "host playb" = the playback vibration produced by the mini-shaker to mimick the host recorded vibration; "stats" = the result of the comparison between the recorded and the playback parameter. Parameter names are self evident.

Parameter		values (mean ± se)		
name	unit	host rec	host playb	stats
Duration of a burst	s	1.41 ± 0.09	1.55 ± 0.001	$F_{1;18}$=2.09 ; *P*= 0.16
Number of beats in a burst	un	5.6 ± 0.3	6.0	$F_{1;18}$= 2.53; *P*=0.12
Interval between beats	s	0.12 ± 0.009	0.13 ± 0.001	$F_{1;18}$=2.10; *P*=0.16
Maximum amplitude in a burst	(mm/s)	0.55 ± 0.11	0.58 ± 0.009	$F_{1;18}$=0.06 ; *P*=0.80
Dominant frequency in a burst	Hz	243.8 ± 40.5	264.52 ± 0.068	$F_{1;18}$=0.24; *P*=0.62

Table 1.3: Parameters characterizing the recorded vibrations from inquiline termite *I. microcerus* and from its playback counterpart (produced by the mini-shaker). Column names ending in "rec" refer to the vibratory parameter recorded directly from the termite in contact with the arena's floor. "inquiline rec" = recorded vibration of the inquiline; "inquiline playb" = the playback vibration produced by the mini-shaker to mimick the inquiline recorded vibration; "stats" = the result of the comparison between the recorded and the playback parameter. Parameter names are self evident.

Parameter		values (mean ± se)		
name	unit	inquiline rec	inquiline playb	stats
Duration of a burst	s	1.67 ± 0.19	1.54 ± 0.016	$F_{1;18}$= 0.458; P= 0.50
Number of beats in a burst	un	5 ± 0.42	5.0	$F_{1;18}$=0 ; P=1
Interval between beats	s	0.11 ± 0.01	0.13 ± 0.009	$F_{1;18}$= 1.15 ; P=0.29
Maximum amplitude in a burst	(mm/s)	0.38 ± 0.05	0.44 ± 0.02	$F_{1;18}$= 0.84; P=0.369
Dominant frequency in a burst	Hz	227.79 ± 24.9	222.64 ± 1.53	$F_{1;18}$= 0.043; P=0.83

Table 1.4: Parameters characterizing the recorded vibrations from styrofoam ball and from its playback counterpart (produced by the mini-shaker). Column names ending in "rec" refer to the vibratory parameter recorded directly from styrofoam ball dropping onto the arena's floor. "ball rec" = recorded vibration of the ball; "ball playb" = the playback vibration produced by the mini-shaker to mimick the ball recorded vibration; "stats" = the result of the comparison between the recorded and the playback parameter. Parameter names are self evident.

Parameter		values (mean ± se)		
name	unit	ball rec	ball playb	stats
Duration of a burst	s	0.30 ± 0.01	4.2 ± 0.009	-
Number of beats in a burst	un	1	3	-
Interval between beats	s	NA	1.87 ± 0.005	-
Maximum amplitude in a burst	(mm/s)	2.9 ± 0.0062	0.54 ± 0.005	$F_{1;18}$=55410; P<0.05
Dominant frequency in a burst	Hz	162.68 ± 0.37	162.56 ± 0.08	$F_{1;18}$= 0.1008; P=0.75

Constrictotermes cyphergaster

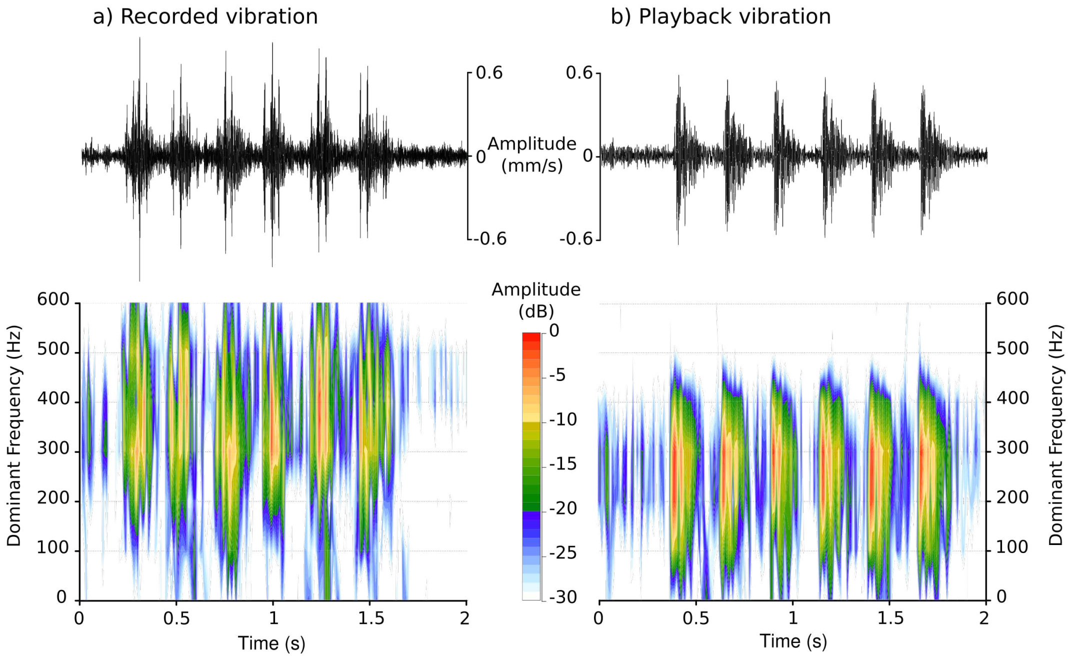

Figure 1.2: Oscillogram and spectrogram of **a)** recorded vibration and **b)** playback vibration of host termite *C. cyphergaster*, recorded with a laser vibrometer. The oscillograms indicate the amplitude (mm/s), and the spectrograms represent the frequency (Hz) through time; the respective power spectra based on the amplitude dB is indicated by the heat scale

Inquilinitermes microcerus

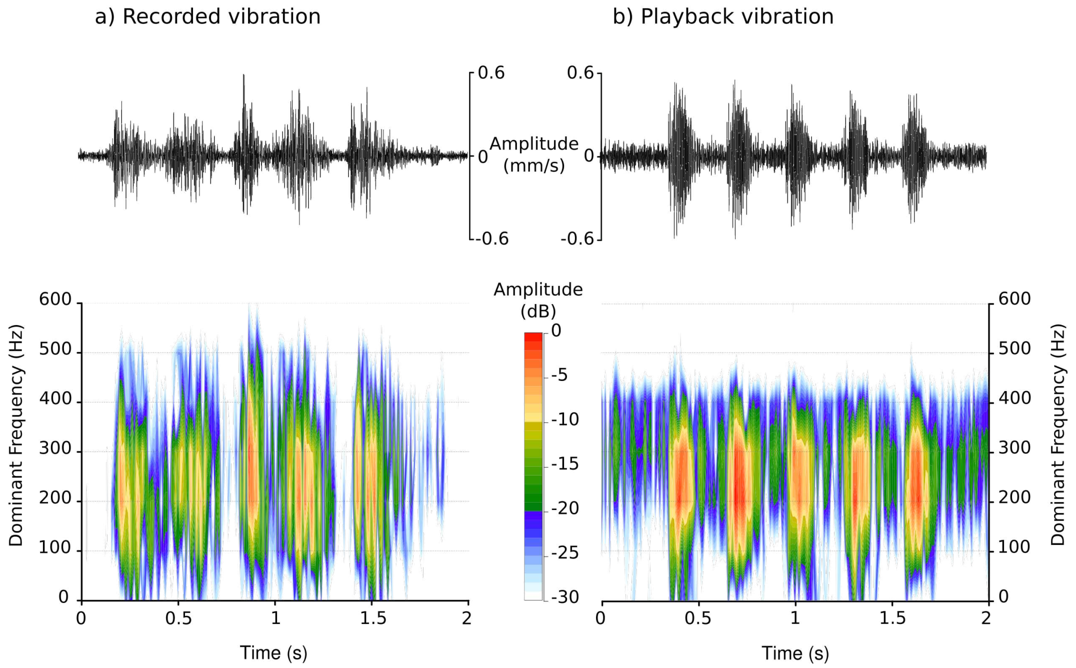

Figure 1.3: Oscillogram and spectrogram of **a)** recorded vibration and **b)** playback vibration of inquiline termite *I. microcerus*, recorded with a laser vibrometer. The oscillograms indicate the amplitude (mm/s), and the spectrograms represent the frequency (Hz) through time; the respective power spectra based on the amplitude dB is indicated by the heat scale

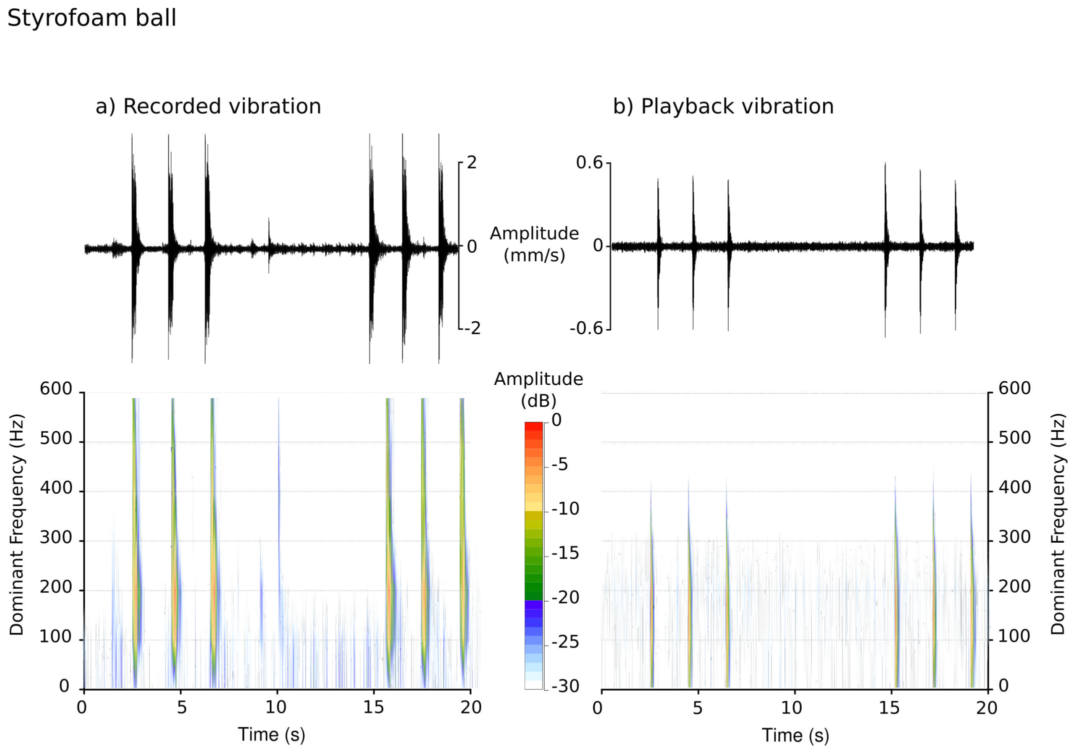

Figure 1.4: Oscillogram and spectrogram of **a)** recorded vibration and **b)** playback vibration of styrofoam ball, recorded with a laser vibrometer. The oscillograms indicate the amplitude (mm/s), and the spectrograms represent the frequency (Hz) through time; the respective power spectra based on the amplitude dB is indicated by the heat scale

1.5.2 Termites behavioural responses

Our findings reveal that both termite species *C. cyphergaster* and *I. microcerus* adjust equally their behaviour in the presence of vibration, as evidenced by an increase in the duration of alert behaviour, indicating perception. However, the termite responses are vibration-dependent; termites only increase their net duration of alertness when stimulated by unfamiliar vibrations (heterospecific and abiotic) ($F_{1,55}$ = 11.8776, P<0.001) (Fig. 1.5). In contrast, termites exposed to none and own vibration had a shorter net duration of alertness. Additionally, the results indicate that termites are able to distinguish conspecific vibratory signals from other types of vibratory cues by differing in responses to their net duration of alertness.

Below, we show the all models and contrasts analysis used to inspect the effects of stimuli on net duration of alertness in *C. cyphergaster* and *I. microcerus* (Table 1.5).

Table 1.5: Models and contrasts used to inspect the effects of stimuli (`st`), species identity (`sp`) and nest of origin (`nt`; blocking factor) on the net duration of alertness *per capta* (y-var). `st` is a categorical x-var with levels: `none`: no stimuli applied; `abiot`: abiotic vibrations; `consp`: conspecific vibrations; `heterosp`: heterospecific vibrations. In the model description below, factors within parentheses are amalgamated and a colon (:) means an interaction between variables.
m1: $y \sim st[none, abiotic, conspec, heterosp] + sp + nt + st : sp$
m2: $y \sim st[none, abiotic, conspec, heterosp] + nt$
m3: $y \sim st[(none, conspec), abiotic, heterosp] + nt$
m4: $y \sim st[(none, conspec, abiotic), heterosp] + nt$
m5: $y \sim st[(none, conspec),(abiotic, heterosp)] + nt$

Contrasts	Res. d.f.	Res. dev.	df	Dev.	F	P
m2 × m1	49	4319.6	4	82.853	0.235	0.9173
m3 × m2	53	4402.5	1	84.445	1.0166	0.3179
m4 × m3	54	4486.9	1	634.57	7.6371	0.0078**
m5 × m3	54	4486.9	1	0.15326	0.0018	0.9659

** P < 0.01

We verified that the net distance travelled *per capta* does not depend on the stimulus applied to termites, neither on the interaction between stimuli and species. However, cohabitant termites exhibit different patterns of net distance travelled ($F_{1,60}$ = 4.1148, P=0.04). The net distance travelled *per capta* in *I. microcerus* (9.33 cm) was greater than *C. cyphergaster* (-25.85 cm) (Fig. 1.6).

1.6 Discussion

Our results show that cohabitating termites use substrate-borne vibrations as a source of information and are able to perceive and distinguish vibrations. The combination of the host *C. cyphergaster* and its inquiline *I. microcerus* behaviours in response

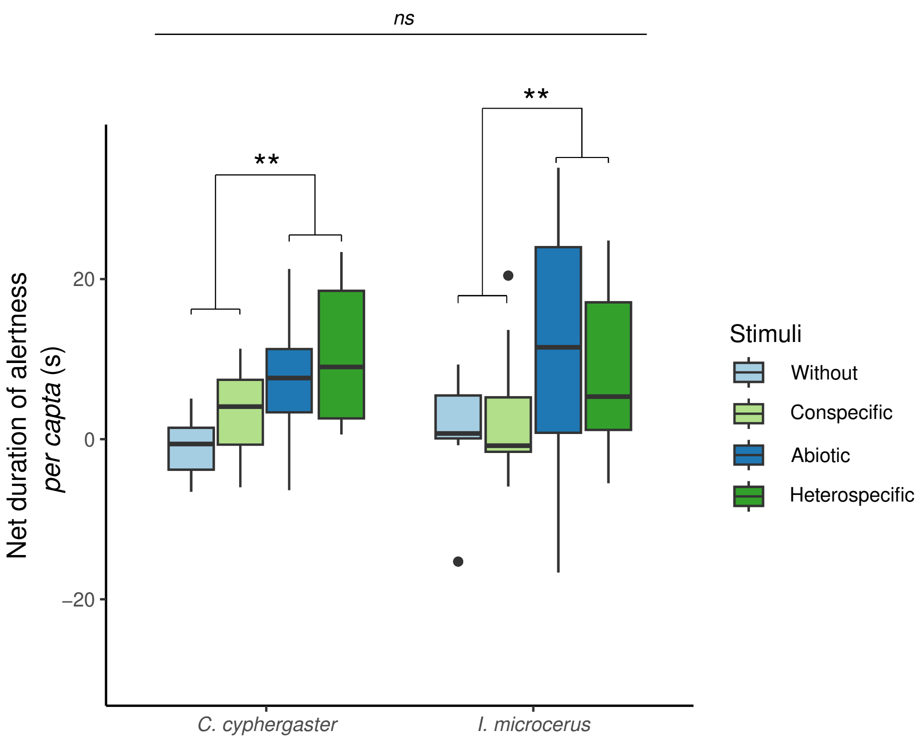

Figure 1.5: Boxplot of net duration of alert response *per capta* of the termite host *C. cyphergaster* and its inquiline *I. microcerus*. “Net distance” refers to subtracting the responses recorded before the stimulus application from those recorded after it. Boxes of different color refers to different stimuli: without vibration (sky blue bar), abiotic (navy blue bar), conspecific vibratory cue (light green bar) and heterospecific vibratory cue (dark green bar). The boxes display median and interquartile range. Outliers are displayed in the plot. Species exhibit similar behavior, but the respond differ between stimuli ($P<0.05$). None and conspecific stimuli differ from abiotic and heterospecific stimuli.

to heterospecific vibration suggests that vibratory perception can be one of the mechanisms that facilitate termite-termite cohabitation.

Cohabitating termite species can perceive and distinguish vibratory stimuli, but the behavioural response does not differ between species. Termites exhibit alertness for a longer time when exposed to unfamiliar vibratory cues (either heterospecific or abiotic) than in response to a conspecific stimulus or without a vibratory stimulus. Although both cohabitating termites perceive unfamiliar vibrations, they do not distinguish the vibration coming from the other (i.e., heterospecific) from abiotic vibrations. The hosts may use the response to unfamiliar vibratory cues as a reliable source of information to detect treats (e.g., predators on the nest walls). A similar ability was recorded for other termite species (Hager and Kirchner, 2014). Once inside their host’s nest, inquilines are also exposed to these stimuli and make use of them for the same goal, detect treats (e.g., host presence). The increase in alertness happens with the perception of unfamiliar vibrations, and this may indicate to us that these vibrations can help prevent encounters between cohabitants or aid in the search for enemies.

The substrate vibrations are able to convey the information through galleries

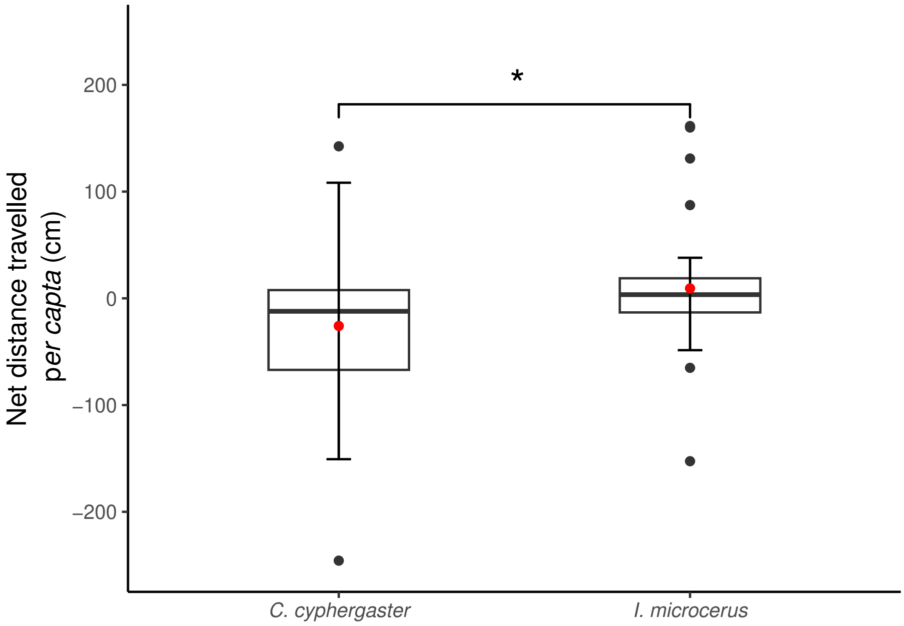

Figure 1.6: Boxplot of distance travelled *per capta* of *C. cyphergaster* and *I. microcerus*. "Net distance" refers to the subtracting of the responses recorded before the application of the stimuli from those recorded after it. The boxes display median and interquartile range. Outliers are displayed in the plot. The red dots represent the mean ± *se* of the distance travelled in each specie. The species differ statistically *($P<0.05$).

over long distances (Röhrig et al., 1999; Hager and Kirchner, 2013). However, this information can be intercepted by unintended receivers (Peake, 2005; Evans et al., 2009; Virant-Doberlet et al., 2019) and, therefore, be used as a source of information to guide their behaviours (Virant-Doberlet and Cokl, 2004; Cini et al., 2019). Termites exposed to vibratory cues from competitors uses the information to avoid encounters (Evans et al., 2009) and ascertain the direction of the disturbance's source (Hu et al., 2003). Here we show a new perspective on the use of vibratory cues by termites: cohabitants increase alertness when perceiving heterospecific vibrations. Heterospecific cue perception between termite cohabitants has already been shown for the chemical communication channel, but in this case, only inquilines are able to sense host cues (Cristaldo et al., 2016). Here we show that both cohabitant species can eavesdrop on each other's vibratory cues.

However, despite perceiving heterospecific vibrations, hosts and inquilines do not change their walking pattern, perhaps to reduce the energy cost with constant searches, waiting for signal reinforcement or complementary information (Partan and Marler, 1999). Here, we show evidence that the inquiline walking pattern can keep it out of harm's way. The net distance travelled *per capita* for termites confined in an arena did not depended on the stimulus applied but differed between species. The individuals of *C. cyphergaster* were less attentive throughout the experiment, whereas *I. microcerus* kept patrolling the arena throughout the whole experimental timeframe. These contrasting walking patterns between host and guest species may lead to cohabitation. While *C. cyphergaster* only performs short walks, *I. microcerus* travels far longer distances (regardless of any cue). This allows *I. microcerus* to explore large areas of the host nest, making it easier to find safe and suitable places for its own colony.

Further, the difficulty in distinguishing heterospecific from abiotic vibrations may explain why termite hosts do not seek the vibrations source when exposed to them. Since heterospecific vibrations can be confused with abiotic vibrations, inquilines remain unnoticed, ensuring their access to privileged areas of the nest, as cited above. If this dynamic arose from an intrinsic limitation of termites ability to distinguish between vibrations different from the conspecific one, from an evolutionary perspective, it conferred a clear advantage to the invading termite, enabling cohabitation.

To maintain cohabitation, the inquilines have a set of adaptations to avoid contact and conflict with the host. Additionally, if needed mandibular morphological traits of inquilines are used for self-defence against attacks from hosts (Clemente et al., 2021). Physiological traits such as low concentrations of the trail-following pheromone reduce detection by the host (Cristaldo et al., 2014). Inquiline's behavioural adaptations support cohabitation, including evasive manoeuvres to avoid encounters, being non-aggressive during interactions (Hugo et al., 2020), and a not overlapping diet with their host (Florencio et al., 2013). Inquilines use the host's chemical alarm cues for their own benefit to anticipate escape from the places of greatest risk (Cristaldo et al., 2016). Furthermore, they are never found parts of the nest that have been recently damaged, where host soldiers are bound to gather (DeSouza et al., 2016; Cristaldo et al., 2016).

Combining the multitude of studies on obligatory inquilinism in termites, the current scenario remains incomplete in terms of uncovering the host's strategies to avoid invasion by inquiline species. The host does not use chemical communication channels to detect the inquilines and prevent nest invasion (Cristaldo et al., 2016). Furthermore, there is a trade-off between defence and reproduction in host termites, making the colony vulnerable to invasions when it invests in reproduction (Rodrigues et al., 2021). In this context, we offer further insights into the persistence of termite coexistence. Hosts do not use inquiline vibrations as an information source to prevent them from staying.

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Article 2: Cohabitation in termite nests: symbiosis mediated by habituation to heterospecific vibration

2.1 Abstract

Habituation is the reduction in the response to a stimulus after continuous exposure to it. Termite nests are frequently invaded by other termite species, called inquilines, that cohabit along with the building species, and, once inside the nest, inquilines and their hosts are exposed continuously to cues from each other, including vibrations. Here we test the hypothesis that heterospecific termite-termite cohabitation may be mediated by hosts and inquilines habituating to each other vibratory cues. We continually exposed both termite species to each other's vibrations and analysed the group's alert behaviour. Our results show that cohabitating termites habituate equally to vibratory cues from one another by reducing alertness behaviour. In doing so, they seem to fail to recognise each other's vibratory cues as dangerous. It seems plausible to state, then, that habituation may lead to mutual tolerance between hosts and guests, thereby easing termite-termite coexistence.

keywords: coexistence; inquilinism; vibration; cohabit; learn.

2.2 Introduction

Habituation is a type of non-associative learning that occurs when an animal that is repeatedly exposed to a specific stimulus reduces its behavioural reaction to the stimulus (McDiarmid et al., 2019). This reduction in reaction might depend on the stimulus threat level (Langen et al., 2000; Dissegna et al., 2021); low-risk cues may generate quickly habituation than high-risk cues. In animals, it is the most basic form of learning (Thompson and Spencer, 1966; Peeke, 1984). Initially, a stimulus elicits a behavioural reaction. However, over time, if the stimulus becomes insignificant (e.g., if it does not convey danger), the animal can return to its relaxed state (Gray, 2005; Bullock et al., 2009) and transfer energy to other actions crucial to survival (Roberts, 2014; Ginsburg and Jablonka, 2021). In contrast, if the stimulus conveys information about a possible risk, an animal may ensure its survival by not becoming habituated (Dehaudt et al., 2019). Therefore, habituation is context-dependent (Dissegna et al., 2021) and enables the animal to learn about a stimulus and then reject it when it is irrelevant.

Habituation may be the primary mechanism that ensures the coexistence between organisms of the same or different species (Langen et al., 2000; Grangier et al., 2007). Sharing the same place at first can involve conflict and competition. The outlanders need to learn about the environment and its stimuli (i.e., biotic and abiotic), as well as ecological interactions, to react appropriately, reducing negative interactions (Rice and Silverman, 2013; Hugo et al., 2020). On the other hand, the disrupted organism can initially try to avoid invasion, but if it fails, it must deal with the intruder's environmental disturbances and alter its behaviour to minimise stress (Santicchia et al., 2022). Thus, learning about new stimuli and how to respond to them may lead to coexistence.

Termite nests can be invaded by several other organisms (Kistner, 1969; Costa et al., 2009; Marins et al., 2016), including other termite species that cohabit along with the building species. These later are called "inquilines" in termitological literature. Inquiline species settle in a host nest, avoiding building costs and exploiting an environment that is sheltered and full of resources (Florencio et al., 2013). Once inside the nest, inquilines and their hosts are exposed to cues from each other (Cristaldo et al., 2016). Host-guest coexistence is then possible if each species learns how to respond in a suitable manner to the new stimulus that is provided by these cues. Inquilines tend to respond to host cues, avoiding conflict (Cristaldo et al., 2014; Hugo et al., 2020), and that should reduce the stress imposed to the hosts. A low level of stress would not justify a strong investment in defence on the part of the hosts. It is then plausible to expect hosts to get habituated to their inquilines, which ultimately would enable coexistence.

Indeed, termites can habituate to several biological and non-biological stimuli when repeatedly exposed to them. Light (Stuart, 1963), fungal conidia (Myles, 2002), fungal semiochemicals (Grace, 1989), air puffs (Hertel et al., 2011) and vibratory disturbance (Hu et al., 2003) have been all recorded as stimuli to which termites are prone to habituate to. In the case of vibratory disturbance, becoming habituated can be most dramatic because these stimuli are relevant to perceive and avoid enemies (Evans et al., 2009; Virant-Doberlet et al., 2022). This study will investigate the habituation mechanism to heterospecific vibrations between cohabitant termites, the host *Constrictotermes cyphergaster* (Silvestri, 1901) (Termitidae: Nasutitermitinae), and its obligatory inquiline *Inquilinitermes microcerus* (Silvestri, 1901) (Termitidae: Termitinae). We will test the hypothesis that termite cohabitation may be mediated by hosts and inquilines habituating to each other. We inspected whether groups of inquilines and hosts, when exposed to each other's vibrations, decrease their alertness over time. Our results show that cohabitating termites habituate equally to vibratory cues from one another by reducing alertness behaviour. In sequence, we discuss how these responses can drive this heterospecific cohabitation.

2.3 Material and methods

2.3.1 Rationale

In order to test our hypothesis that habituation mediates coexistence between hosts and inquilines, we inspected the dynamics over time of the responses of these termites to each other's vibrations. Our hypothesis would be rejected if termite responses do not fade over time when they are under continuous stimulation by heterospecific vibratory cues.

To test this hypothesis, we offered a given stimulus to host and inquiline termites in independent assays, recording their behavioural responses for posterior analysis. Stimuli have been offered continuously along 300 seconds.

Assays were designed to verify whether a given fading response was actually an indication of habituation rather than a mere consequence of termites getting tired. That is, if termite reactions in the absence of stimuli faded in a similar manner as they faded under vibratory stimuli, this fading would be, most likely, a consequence of weariness.

Once termite weariness was ruled out, we ran additional assays to disentangle habituation to unspecified abiotic stimuli from habituation to specific biotic stimuli, such as conspecific or heterospecific vibratory stimuli. To do so, termite responses to abiotic vibrations were compared to their responses to biotic vibrations. Additionally, termite responses to conspecific vibrations were compared to their responses to heterospecific vibrations.

2.3.2 Biological model

All assays were carried out using a termite host *C. cyphergaster* and its termite inquiline, *I. microcerus*. *C. cyphergaster* nests are common in Brazilian savannah ("Cerrado") and semi-arid environments ("Caatinga") (Mathews, 1977), and are the most abundant nest building in these environments (Vasconcellos et al., 2007). The host species build epigeal and/or arboreal nests. The nest's defense is carried out by soldiers caste, who have chemical weapons against intruders (Prestwich, 1984), and uses vibratory cues to indicate to nestmates about dangers (Cristaldo et al., 2015). Colonies of *C. cyphergaster* can house a range of termitophiles and obligatory inquiline species, including *I. microcerus* (Mathews, 1977; Cunha et al., 2003; Costa et al., 2019). To maintain inside the host nest *I. microcerus* use a "conflict avoidance" strategy to successfully deal with the occupation of termite host nests (Cristaldo et al., 2014; Florencio et al., 2013; Hugo et al., 2020).

2.3.3 Tympanic arenas

We conducted the experiments using a low cost arena especially designed for vibratory assays (the so called "tympanic arena" of Nunes et al., 2018). This type of arena consists of a flexible membrane taut by a wooden frame, on top of which a small container holds the termites to be assayed (Fig. 2.1). By simulating a tympanum, the flexible floor of the arena amplifies the vibrations produced by the termites, easing their detection and record. As a termite container, we used a plastic Petri dish lid (⌀55 mm) on top of the tympanum.

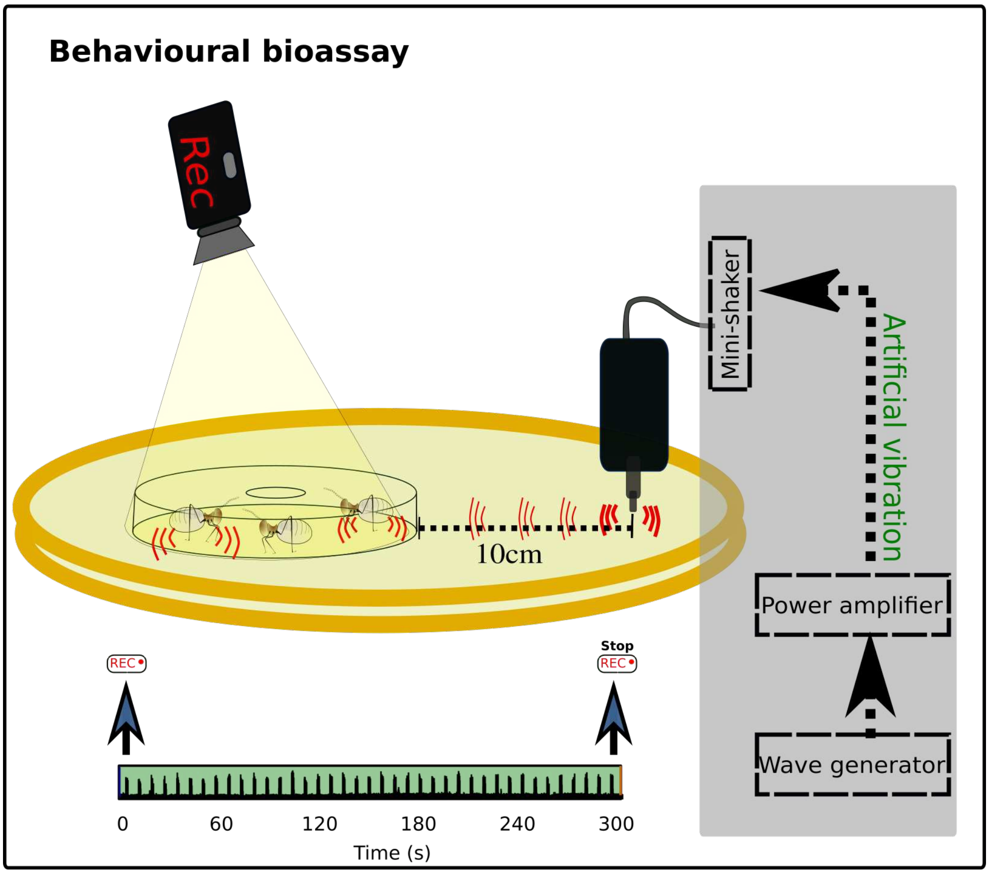

Figure 2.1: Schematic illustrations of the experimental set-up to record behavioural responses of termites exposed continually to artificial vibratory stimuli.

2.3.4 Behavioural bioassays

The following assays were done to verify whether or not the cohabitating termites are able to be habituated to vibrations. We continually exposed termite groups to vibration from distinct sources (abiotic and biotic), video recording their reactions. These recordings were fed into Boris software (Friard and Gamba, 2016) to build a full ethogram of the termite reactions at each assay.

Four types of assays were carried out, according to the type of stimuli being offered: (i) heterospecific vibrations, (ii) conspecific vibrations, (iii) abiotic vibrations, and (iv) without vibration. Four groups of termites were extracted from the same colony to use one group per assay type. In doing so, a termite group was assayed only once, while that given colony was exposed to all four types of assays. We used eight distinct colonies for each of the species under scrutiny, giving hence 32 true replicates (4 termite groups x 8 colonies) for host termite species and 32 true replicates for inquiline termite species. In each termite group, the proportion of workers: soldiers confined in each arena was identical to that used in the previous experiment (see Chpt. 1, section 1.3.4). After confining termites in the container, they were given at least two hours to acclimate, under artificial lighting conditions.

We used recorded vibrations from termite groups of both species separately create the playback vibrations. Each vibrational protocol was uploaded to an Arduino Uno board and sent to a mini-shaker for playback onto the tympanic arena's floor (see Chpt. 1 , Fig. 1.1 d). The mini-shaker was positioned 10 cm away from the container edge (Fig. 2.1), as dictated by a previous pilot test. Each assay was recorded on video and audio. The bioassay lasted for 420 seconds. No stimulus was applied from zero to 120 seconds; from 120 seconds on, the vibratory stimulus was inserted continuously until the end of the bioassay. Specifically, to test the current hypothesis, we use the last 300 seconds after the start of the vibratory stimulus (Fig. 1.1 d). The assay without vibratory stimulus is the termites' basic behavioural reaction in an experimental condition, which means there is no external stimulus (i.e.absence of playback), thus the mini-shaker was turned off and released no vibratory waves.

All footage was taken using SONY HDR-CX405TM digital video camera set to record 30 frames per second at Full HD (1920 × 1080 60p) saved in .MTS file in Advanced Video Coding High Definition (AVCHD) format, with a sample rate of 48 kHz, with 16-bit resolution.

2.3.5 Compiling the behavioural data

To unveil whether the cohabiting termites were able to habituate to vibratory stimuli, we inspected the progression of alert behaviours along the entire running time of the assay. To do so, video recordings of each assay were visually inspected using Boris software (Friard and Gamba, 2016), in order to produce a complete inventory of the alert behaviours exhibited by termites.

Inside Boris, we linked each behaviour to a key on the keyboard, as well as each termite. Thereby, each termite in the arena was followed along the whole assay (total time = 300 s) and recorded all behaviour performed. Each time that specific behaviour occurred, the key was engaged, recording hence the onset of the behaviour. When the behaviour stopped, the key was revoked. This allowed us to produce a full list of all behaviours performed by all individuals in the arena, along with the starting and ending times of each of these behaviours for each of these individuals. The behaviours were then classified into "alert behaviours" and "non-alert behaviours" for further analysis.

This list (or 'ethogram') was summarised into a dataframe where we computed the number of individuals exhibiting a given behaviour at every second of the assay. This dataframe is used here to test our hypothesis.

2.3.6 Statistical Analysis

Statistical analysis verified whether or not the termites reduced their alert behaviour, along the bioassay consequently indicating habituation to vibratory stimuli.

This reduction (or increment) in alert behaviours was quantified individually for each arena, calculating the slope of the curve:

$$\text{sum.alert} = \text{int} + \text{sl} \;.\; \text{time}$$

where 'sum.alert' is the total number of individuals exhibiting any alert behaviour, 'int' is the intercept, 'sl' is the slope and 'time' is the moment along the timespan 0 - 300 s of the assay. In doing so, we could characterize the whole dynamics of that arena by a single value (the slope). This circumvented temporal pseudo-replication which our analysis would incur if it disregarded the fact that we took several readings from the same the arena along 300 s.

Alert behaviours correspond to any behaviour denoting inspection or alarm, that is: (i) performing antennation on nestmates or on the arena's surface; (ii) banging the head or the abdomen on the arena's floor, i.e., "drumming"; (iii) meandering around the arena as if exploring it and (iv) remaining stationary but moving antenna and head while pointing them upward.

After calculating the slopes for each arena we could compare them between treatments using the model

$$\text{slope} \sim \text{stimulus} + \text{spp} + \text{stimulus:spp} + \text{nest}$$

where 'stimulus' is a categorical variable representing the treatments applied to the arena's floor. The four treatments were (i) heterospecific vibrations, (ii) conspecific vibrations, and (iii) abiotic vibrations. A fourth level ('without') was the control, that is, absence of playback.

The term 'spp' represents the species (host or inquiline) and 'stimulus:spp' is the interaction term. The variable 'nest' is a blocking factor to account for the fact that from each one of the nests we extracted four groups of individuals of a given species (host or inquiline) to be allocated in four arenas, each of these arenas being used to test a single treatment.

Statistical analyses were carried out under R [version 4.0.2] R Core Team (2021) run in the RStudio interface [version 2021.09.2+382 RStudio Team (2021).

Statistical modelling proceeded using Generalized Linear Models (GLM), under Gaussian error distribution. Model simplification was done through contrast analyses with F tests, extracting explanatory terms or combining treatment levels when it did not cause significant ($P > 0.05$) changes in the model, as recommended by (Crawley, 2012). Residual analyses were performed to verify error distribution and model suitability, including tests for overdispersion.

2.4 Results

2.4.1 Termites behavioural reaction

Habituation to vibratory stimuli occurred in cohabitating termite species. However, the number of hosts and inquilines reducing the alertness behaviour differed among vibratory stimuli (Fig. 2.2). Termite individuals from both species did not become habituated to conspecific vibratory stimuli but become habituated to unfamiliar (heterospecific and abiotic) stimuli.

In average, in the beginning of all assays there were 1.3 alert termites. The number of termites exhibting alertness in the beginning of the assay is not affected by the species identity ($F_{1,62} = 2.819$, P=0.1) or by the type of stimulus ($F_{3,59} = 1.13$, P=0.344).

When continuously exposed to conspecific signals, the number of alerted termites did not change along the whole assay. This scenario is not significantly different from that of the assay in which termites were not exposed to any stimulus at all ($F = 1.07$, P=0.3) but it differs from the scenario in which termites where subjected to abiotic and heterospecific stimuli ($F_{1,62} = 10.468$, P=0.001).

In its turn, when continuously exposed to heterospecific vibratory cues, the number of alerted termites reduced along the whole assay. This scenario is not significantly different from that of the assay in which termites were exposed to an abiotic stimulus (Fig. 2.2) ($F = 0.85$, P=0.59).

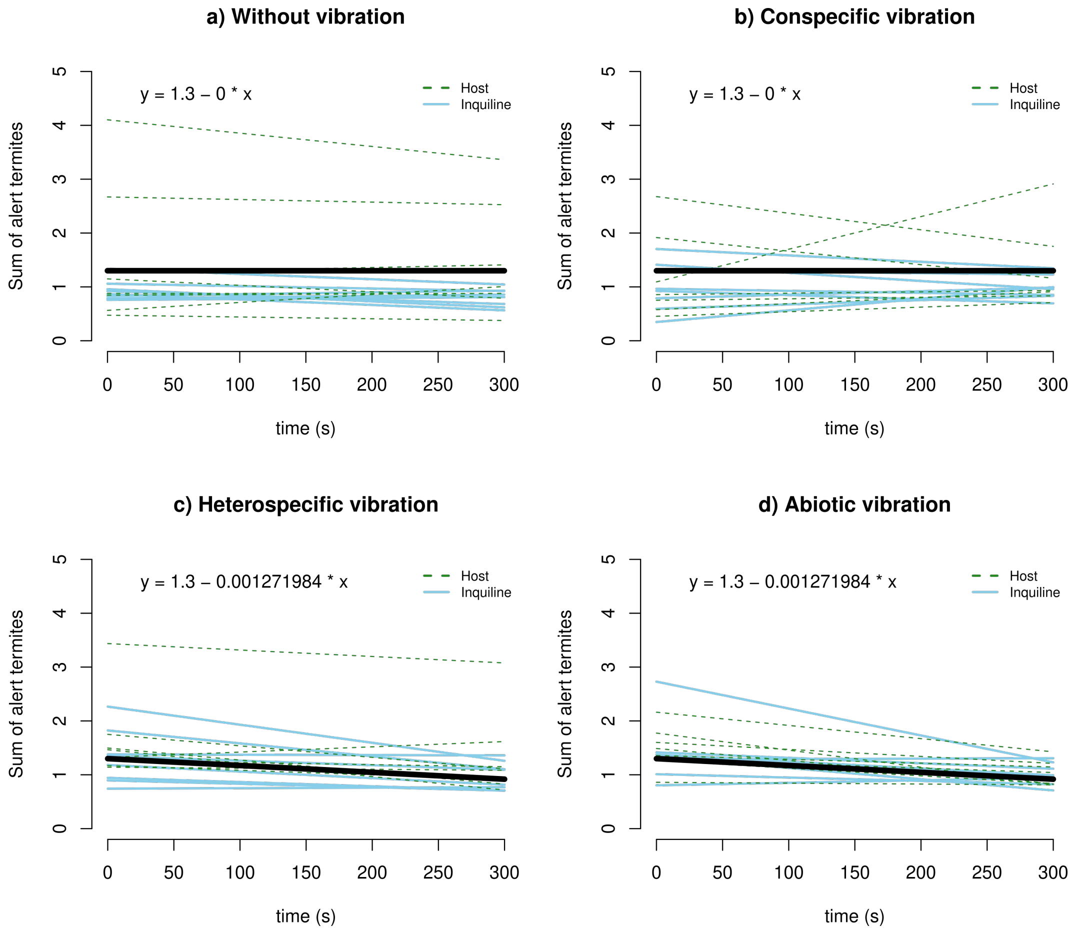

Figure 2.2: The alertness reaction of cohabitating termites to continuous exposition to artificial vibratory stimuli. Frames represent the the number of termites which reacted to: a) no stimulus; b) conspecific stimulus; c) heterospecific stimulus; and d) abiotic stimulus. *C. cyphergaster* are represented by curves in *green* colour. The data from *I. microcerus* are represented by lines and dots in *sky blue* colour. Each of the green and blue lines represent a given termite nest. The black line is the curve estimated at final model.

2.5 Discussion

Substrate-borne vibrations are important mediators of ecological interaction. However, the reliability of the signal is dependent on the ability of the receiver to perceive, decode, and benefit from information (Virant-Doberlet et al., 2019; Searcy and Nowicki, 2006). Our findings show that cohabiting termites become habituated to substrate vibrations, as indicated by the negative slope of behavioural alert response curves. Specifically, we gathered evidence that the coexistence of different termite species in a single nest (that is, "heterospecific termite-termite cohabitation") may be mediated by the ability of the host and inquiline to become habituated to each other's vibratory cues. This ability would, ultimately, prevent cohabitants from wasting energy reacting to an unharmful. heterospecific.

Termite groups, when exposed to abiotic and heterospecific vibrations, reduced the alertness along the time. The reduction of behavioural reactions to each other's stimuli can lead to tolerance and facilitate heterospecific sharing of the same habitat (Rice and Silverman, 2013). The reduction in alertness in the presence of a heterospecific vibration is indicative that cohabitant termites do not recognise each other as a danger.

Similar waning responses occur with species that cohabit the same habitat (as opposed to cohabit the same nest), living in close range, the so called "dear enemies" (Temeles, 1994). Species which behave as "dear enemies" are those that tend to be friendlier to neighbouring heterospecifics than to those living farther apart. In such cases, there is comparatively low aggressiveness and alert behaviours in heterospecific encounters between neighbours. Such a strategy will only make sense if the neighbour is unharmful. After all, in other ecological interactions becoming habituated can be mortal, especially in cases of the cues from predators (Oberst et al., 2017), heterospecific competitors (Evans et al., 2009), and other abiotic potentially harmful vibratory disturbances (Hu et al., 2003). Once the cohabitating species here studied live apart inside the nest and do not compete for food (Florencio et al., 2013), the vibratory cues would not indicate harm, quickly becoming irrelevant.

It is known that in a termite nest, there are invasions by a vast array of organisms (Costa et al., 2009), which arrive at the place in search for shelter and food (Monteiro et al., 2017). The host and the inquilines must be continuously aware of all these possible new inhabitants as potential predators or competitors, bringing imminent danger. This might explain why we observed alertness to not wane under conspecific vibration and in the absence of other vibrations. The slope of the curve is close to zero, indicating constancy in the alert behavior. Conspecific vibrations are reliable and must not be ignored (Virant-Doberlet et al., 2019), for it may convey information about danger. Likewise, the absence of vibrations is a scenario biologically rare, hence calling for alertness. Remaining alert to both situations seems to be the best strategy, as it will ensure the acquisition of information about the source of the stimulus (Biedenweg et al., 2011) and, if necessary, making a decision. This strategy has already been observed for organisms from other groups that remain alert, ensuring the survival of individuals, especially the most vulnerable (Hu et al., 2003; Dehaudt et al., 2019). This sounds particularly relevant for *I. microcerus* inquilines, as their colonies normally present low defensive potential due to low number of individuals, among which soldiers appear in low proportion (Cunha et al., 2003). Thus, not becoming habituated to conspecific vibrations or to the absence of vibrations can be an important strategy to guarantee the colony's security and survival.

Once exposed to inquiline vibrations, hosts become habituated and do not seek the source of the disturbance (Chpt. 1), which facilitates cohabitation. These results indicate that the vibratory communication channel alone is not used by hosts for immediate decision-making. It is possible that they wait for quick reinforcement through a chemical signal (Cristaldo et al., 2016) to trigger behavioural change, which, if not occurring, indicates that there was not a strong threat to the stability of the colony and the individuals can return to their relaxed state.

Here, we show that termites become habituated to heterospecific vibrations and are able to adjust their alertness to each vibration. Habituation is crucial to facilitate the peaceful cohabitation of organisms of different species, reducing their stress response. The final balance between habituation and cohabitation appears closely related and helps explain why different termite species remain cohabiting.

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General Conclusion

Obligate cohabitation in termite nests has been intensely studied to unveil how those species maintain cohabitation since the interaction is not easily linked to parasitism-mutualism. Furthermore, the research had not yet provided a clear definition of the interaction's costs and benefits. Inquiline termites remain within the host's nest for reasons beyond temporary and immediate benefits. In contrast, the advantage of maintaining cohabitation appears unknown for the host species. This permanent symbiotic alliance is moulded by several mechanisms as morphological, physiological, and behavioural from both cohabitants. Each of these forms a piece of the puzzle that helps unravel the enigma of cohabitation.

One of the open questions is: Could the detection of heterospecific vibration in termites be one of the pieces of this puzzle and help elucidate the phenomenon of cohabitation in termites? Vibratory cues emitted by heterospecifics might guide the behaviour of both hosts and inquilines. The host can use the vibrations from inquilines to detect them and prevent nest invasion. Meanwhile, the inquiline can employ the vibration to locate the host and avoid conflicts.

Here, we can include perception, distinction, and habituation to heterospecific cues as important mechanisms to maintain cohabitation. Termites of both species are able to perceive heterospecific vibrations, increasing their alertness; and distinguish of the conspecific vibrations. This difference in behavioural responses shows that termites are able to distinguish their own signals from true risks, behaving accordingly. Further, both cohabitants become habituate to heterospecific by reducing the alertness after long exposure to vibrations. Indicating that initially to exposure termites react to heterospecific vibrations, however, return to its relaxed state, probably after a time the cue does not exhibit risk, thus the animal can transfer energy to other cues, which are crucial to survival.

All this evidence shows how important vibrations are for termites to perceive the environment and detect threats. In addition, there is a strong indication that the recognition of nestmates and non-nestmates can occur through vibrations. Nevertheless, the occurrence of the distinction between conspecific and heterospecific vibrations does not seem to be an issue for maintaining cohabitation. Despite distinguishing the vibrations, the host does not seek the heterospecific vibration source. Thus, even the inquiline vibrating inside the nest for intraspecific communication does not pose challenges, ensuring cohabitation. Our results suggest that the ability to perceive, distinguish, and habituate to vibratory cues and the appropriate behavioural attitude from both the host and the inquiline are key mechanisms in the success of cohabitation.