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Fungus-Farming Termites Selectively Bury Weedy Fungi that Smell Different from Crop Fungi

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Abstract Mutualistic associations such as the fungal farms of insects are prone to parasitism and are consequently vulnerable to attack by weeds and pests. Therefore, efficient farm management requires quick detection of weeds for their elimination. Furthermore, if the available weedicides are non-specific, then the ability of insects to discriminate between crop and weeds becomes essential for targeted application of such compounds. Here, we demonstrate for the first time in fungus-farming insects, that worker castes of the fungus-growing termite *Odontotermes obesus* discriminate between their crop (*Termitomyces*) and the weedy (*Pseudoxylaria*) fungi, even if exposed to only fungal scents. Termites respond to the presence of fungal mycelium or scent alone, by burying the weed with the offered material such as soil or agar, possibly anointing the weed with chemicals in the process. The scent profiles of crop and weedy fungi are distinct and the differences are likely exploited by termites to selectively mount their defences. Sesquiterpene compounds such as aristolene and viridiflorol, which are absent from crop odours, may constitute the "weedy scent". Our results provide a general mechanism of how other fungus-farming insects could avoid indiscriminate application of non-specific fungicides which could lead to poisoning their crops, and have bearing on the stability of the mutualism between termites and their crop fungus in the face of parasitism by weedy fungi.

Keywords Burying behaviour · Fungus-growing termite · Parasite · *Pseudoxylaria* · *Termitomyces* · Volatiles · Symbiosis · Mutualism

Introduction

The parasitism of mutualistic interactions is commonplace (Yu 2001) as occurs in the fig–fig wasp (Borges 2015a), yucca–yucca moth (Althoff 2014) and ant–plant mutualisms (Frederickson 2013), and therefore requires mechanisms to stabilise mutualism in the face of such parasitism (Borges 2015b). Agriculture systems of insects are specialized mutualisms in which insect hosts cultivate their fungal mutualists as crops (Mueller et al. 2005). However, crop monocultures are prone to parasitism by weeds and pests (Sherman et al. 1988; Baer and Schmid-Hempel 1999; Schmid-Hempel and Crozier 1999), which may lead to lowered farming output. Therefore, successful agriculture requires crop protection practices against parasites and diseases which play an important role in farming evolution and ecology. Pertinent to crop protection is the ability of farmers to recognise the presence of disease-causing agents for their successful removal or neutralisation. Thus, agricultural practices should include parasite recognition as an important behavioural task.

Human farmers can visually identify weeds and diseases of their crops, but for automation and large-scale farming purposes, pesticides are frequently employed. Insect farmers such as fungus-farming ants seem to be able to recognise the presence of fungus garden infections (Currie and Stuart 2001), but the mechanism by which they do so is unknown. Fungus-farming beetles are attracted by the volatiles of their own

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symbiotic fungus and even to those of symbiotic fungi of other congeneric beetles, and are not attracted by non-symbiotic fungi when volatiles are offered against blank air (Hulcr et al. 2011), but whether they can discriminate between the volatiles of the two when present together is not known. On the other hand, social insects including termites can recognise the presence of entomopathogens. In response to fungal entomopathogen detection, termites display behaviours such as pathogen alarm behaviour (Rosengaus et al. 1999), walling-off of infected areas of the colony (Milner et al. 1998), allogrooming (Rosengaus et al. 1998; Yanagawa and Shimizu 2007; Yanagawa et al. 2012) and removal of infected termites (Myles 2002). Similarly, when their nest mates die, termites display behaviours directed at cadaver removal either by necrophagy or burial (Myles 2002; Chouvenc et al. 2008, 2012; Chouvenc and Su 2012). Cadaver burial with soil is usually accompanied by deposition of faecal matter and saliva, and such cadavers show little or no sign of microbial growth (Chouvenc et al. 2012; Chouvenc and Su 2012). Therefore, burial seems to be an effective antimicrobial behaviour. Interestingly, the fungus-growing termite *Macrotermes michaelseni*, is repelled by fungal entomopathogens such as *Metarhizium anisopliae* and *Beauveria bassiana*, repellency being mediated by fungal volatile compounds (Mburu et al. 2011, 2013).

Fungus-growing termites are different from other termites in that they have to not only address the challenges posed by entomopathogens but also pathogen attack of their fungal gardens, such as those posed by the weedy fungus *Pseudoxylaria* (Ascomycota), which can overgrow the farms of the mutualistic crop fungus *Termitomyces* (Basidiomycota) if left unchecked (Sands 1969; Batra and Batra 1979; Thomas 1987). *Pseudoxylaria* weeds grow faster than the crop (Visser et al. 2011), are able to grow within the hypercarbic and extremely humid conditions within the termite mound (Katariya 2017), and therefore need to be recognised quickly by the termites to mount an antifungal response, before they overtake the fungus gardens. Additionally, even though a few chemical compounds isolated from the termite–fungus farming system have shown specificity against parasitic fungi (Um et al. 2013; Beemelmanns et al. 2017), most antibiotic-producing bacteria in this system are non-specific and therefore inhibit the crop fungus also (Visser et al. 2012; Kim et al. 2014). Therefore, targeted application of these chemicals has been suggested as a way to circumvent the problem associated with their non-specificity (Boomsma and Aanen 2009; Sen et al. 2009; Poulsen and Currie 2010; Visser et al. 2012). But such targeted application also requires that termites are able to recognise and differentiate between crop and weedy fungi. Research examining such selective action is lacking but important for understanding the ecology of fungus-farm diseases caused by parasitic fungi like *Pseudoxylaria*.

We tested whether fungus-growing termites can discriminate between the crop fungus *Termitomyces* and the weedy fungus *Pseudoxylaria*. For this, we used *Odontotermes obesus* (subfamily: Macrotermitinae), a commonly available fungus-growing termite (Katariya et al. 2017; Zachariah et al. 2017) as our model system. We examined termite ability to respond differentially to different fungal mycelia as well as only fungal scents in two-choice assays. We hypothesised that scent profiles of the crop and weedy fungi should be different for the blind termites to distinguish between them. Therefore, we also collected and identified fungal scents from different isolates of both fungal genera.

Methods and Materials

Termite Collection For all assays, fresh termites were collected during the day (10 am–2 pm) from two different nests (I16 & I20) of the fungus-growing termite *O. obesus*, present in the Indian Institute of Science campus (Bangalore, India). Different termite castes (minor workers, major workers, and soldiers) were kept separately in glass petri-plates containing moist tissue papers to decrease mortality owing to desiccation.

***Termitomyces versus Pseudoxylaria* and Either Fungus *versus* Blank Choice Assays** *Termitomyces* and *Pseudoxylaria* fungi isolated from *O. obesus* nests were cultured on potato dextrose agar (PDA) at 30 °C. For the assays, plugs punched from 7 to 12 day-old *Termitomyces* cultures growing as spread plates and 3–5 day-old *Pseudoxylaria* plates point inoculated in the centre were used. In cell culture dishes (Greiner CELLSTAR, 35 × 10 mm, with vents), plugs of *Termitomyces* (from the spread plate) and *Pseudoxylaria* (from an actively growing culture edge) were placed on the agar surface, culture facing up, equidistant from the centre (centre of each plug 8 mm away from the centre of the dish; Fig. 1a). Termites were offered their native *Termitomyces* isolates (i.e. fungi isolated from their own nests). The *Pseudoxylaria* isolate used belonged to a common genotype found across termite nests (the most common genotype of the most prevalent OTU) (Katariya et al. 2017). For the choice between fungus and blank, the blank was a PDA plug from a plate incubated at 30 °C for 5–7 days without any fungal culture. Each dish contained three termites of one of the worker castes (minor or major worker) and incubated in the dark at room temperature (RT) for different durations, viz. 15, 30, 45 and 60 min. The same termites were never re-tested. Assays were conducted from 10 am till 7 pm, i.e. during the day. Termites utilised the agar to cut small pieces which we term "boluses" and deposited them over and near the fungal plugs. All the boluses in a circle of diameter 8.8 mm around the plugs, where most of the deposition was concentrated, were collected and weighed. In cases, where only few boluses were

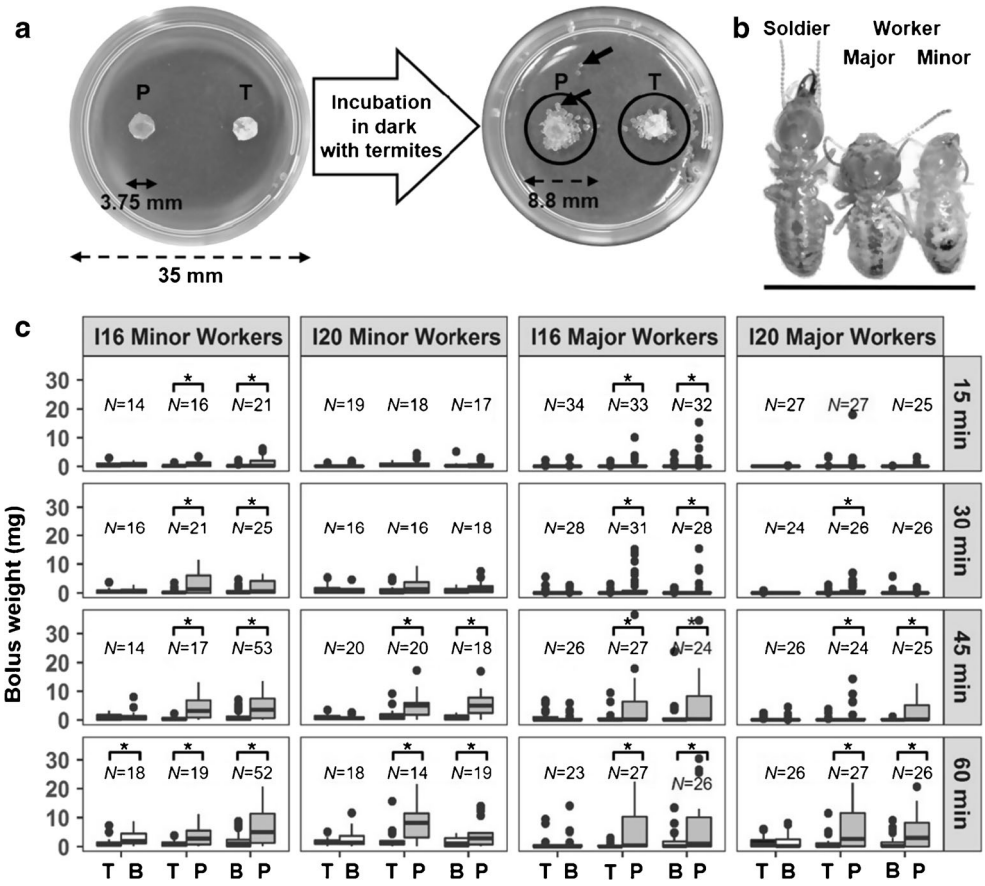

Fig. 1 Differential agar bolus deposition by worker termites in two choice assays (*Termitomyces* versus *Pseudoxylaria* or either fungus versus a control). (**a**) Left: Assay set up. Right: Agar bolus deposition by termites on fungal plugs. All boluses (indicated by arrows) in a circle of diameter 8.8 mm were collected and weighed. (**b**) Different castes of the fungus-growing termite *Odontotermes obesus* (scale bar = 5 mm). (**c**) Box plots representing mass of agar boluses (mg) deposited after 15, 30, 45 and 60 min on plugs of *Termitomyces* (T), *Pseudoxylaria* (P) and control or Blank (B) by minor and major workers from two different nests I16 and I20 of *O. obesus*. Horizontal lines inside boxes represent medians. Filled circles represent outliers. *N* = sample sizes. * = significant difference ($P < 0.05$) in pairwise comparisons using Wilcoxon signed rank tests

deposited, the weights of deposited boluses were estimated from the weight of a known number of boluses (Fig. S1 and supplementary methods in Supplementary Material). Even though in such circular and visually symmetrical arenas with unrestricted movement, the possibility of any directional bias is unlikely as compared to a Y-tube or T-maze, we still took precautions to avoid any inadvertent biases. For example, we haphazardly introduced termites into the dish, such that the first encounter of a termite with a particular plug was on any side (i.e. right or left). Similarly, dishes were incubated on a table in such a way that the position of plugs (inside the dish) was random with respect to the corners of the table. Also, since the incubation was in the dark, there was no possibility of bias due to directionality of light cues. All experiments were carried out from June to September (2013) except for minor workers of the I16 nest which were performed from June to July (2015). This was because of the high mortality, very low activity and subsequent unavailability of I16 minor workers during the summer of 2013 resulting in very small sample sizes.

Sustained Response in *Termitomyces versus Pseudoxylaria* Choice Assay To examine if the behavioural response to crop and weed fungi was sustained even after 60 min, we compared bolus deposition for the choice between *Termitomyces* and *Pseudoxylaria* at the end of 1, 2 and 3 hr. Apart from the worker caste, we also used soldiers for this particular assay. These experiments were carried out from June to July (2013).

***Termitomyces versus Pseudoxylaria* Choice Assay with Soil** To validate our experiments with a natural substrate, we used autoclaved garden soil (mesh size = 75 μ) (amount equivalent to ~3 mL) in sterile cell culture dishes in place of the agar. Autoclaved MilliQ water (1.65 mL) was sprinkled to wet the soil and two discs (diam. = 8.8 mm) of sterile aluminium foil were placed equidistant from the centre of the dish (centre of the disc 8 mm away from the centre of the dish). *Termitomyces* and *Pseudoxylaria* plugs were placed over the aluminium foil discs and incubated with termites. All the soil boluses deposited on the discs were weighed at the end of the experiment, i.e. 3 hr. These experiments were carried out in March (2016).

***Termitomyces versus Pseudoxylaria* Volatile Choice Assays** These assays were conducted in sterile 6-well cell culture plates (Nest Biotech, flat bottom with low evaporation). Around 0.5 mL of agar (2%, autoclaved) was poured in each well and two PCR caps (Axygen, flat 0.2 mL PCR strip caps, thin wall) were fixed in an inverted position during agar solidification, equidistant from the centre (centre of the cap

8 mm away from the centre of the dish), such that they form cups for the fungal plugs (Fig. 2a). After solidification, fungal plugs of both *Termitomyces* and *Pseudoxylaria* were placed inside these PCR caps (facing up). Around 1 mL more agar was added to the wells to fill them to the height of the PCR caps. PCR caps were then covered with 6.4 mm diameter discs (Merck Millipore, Durapore GVWP04700, 0.22 μ) concealing the fungal plugs inside. All the boluses in a circle of diameter 8.8 mm were collected and weighed. These experiments were carried out from May to June (2015).

***Termitomyces* and *Pseudoxylaria* Volatile Collection** Fungal plugs were obtained as described above for behavioural experiments and either 50–60 plugs of *Pseudoxylaria* or 250–280 plugs of *Termitomyces* were placed in a single glass petri-plate (diam. = 5 cm). Five conditioned silicone (also known as polydimethysiloxane or PDMS) tubes (ST) were introduced in the petri-plate, cordoned off with a strip of aluminium foil so that the STs were not in direct physical contact with the fungal plugs. The plate was sealed with parafilm and incubated at RT in the dark. After 3 hr of incubation, all STs were removed carefully with the help of forceps and stored together in a clean 2 mL glass vial at RT in the dark until use. There were eight isolates for both *Pseudoxylaria* and *Termitomyces* isolated from different *O. obesus* nests (I16, I19, I20, I21, I38, O57, O63, O65, O77, O90, O92, O96, G4 and G5) present in Bangalore (India) (Katariya et al. 2017). For each isolate we had two replicates of volatile collection (except for the P96 *Pseudoxylaria* isolate for which we had three replicates). These experiments were carried out from August to November (2016).

Chemical Analysis by GC-MS Thermal desorption (TD)-GC-MS analysis was performed on a Unity^2 TD unit (Markes International) connected to a quadrupole GC-MS (Agilent HP GC model 6890 N, MS model 5973 N). All 5 STs were placed in 89 mm steel TD tubes (Markes International) for desorption (5 μL of thymol (0.4 μg/ml) was injected on to the TD tube surface as an internal standard). Samples were desorbed under a stream of helium for 5 min at 200 °C. All substances desorbed from the STs were cryo-focused at −10 °C onto a cold trap (Graphitized Carbon, Marks International). After desorption, the cold trap was heated to 200 °C within 10 s, and analytes were injected onto a HP-5MS column (30 m long, 0.25 mm i.d., 0.25 μm film thickness; Agilent) with helium as the carrier gas at a constant linear velocity of 50 cm s^{-1}. The TD-GC interface was held at 120 °C. The GC oven gradients for the analysis were started with initial temperature of 50 °C for 3 min, then ramped to 80 °C at 3 °C min^{-1}, then to 90 °C at 2.9 °C min^{-1}, next to 120 °C at 2.8 °C min^{-1}, and finally to 160 °C min at 2.7 °C

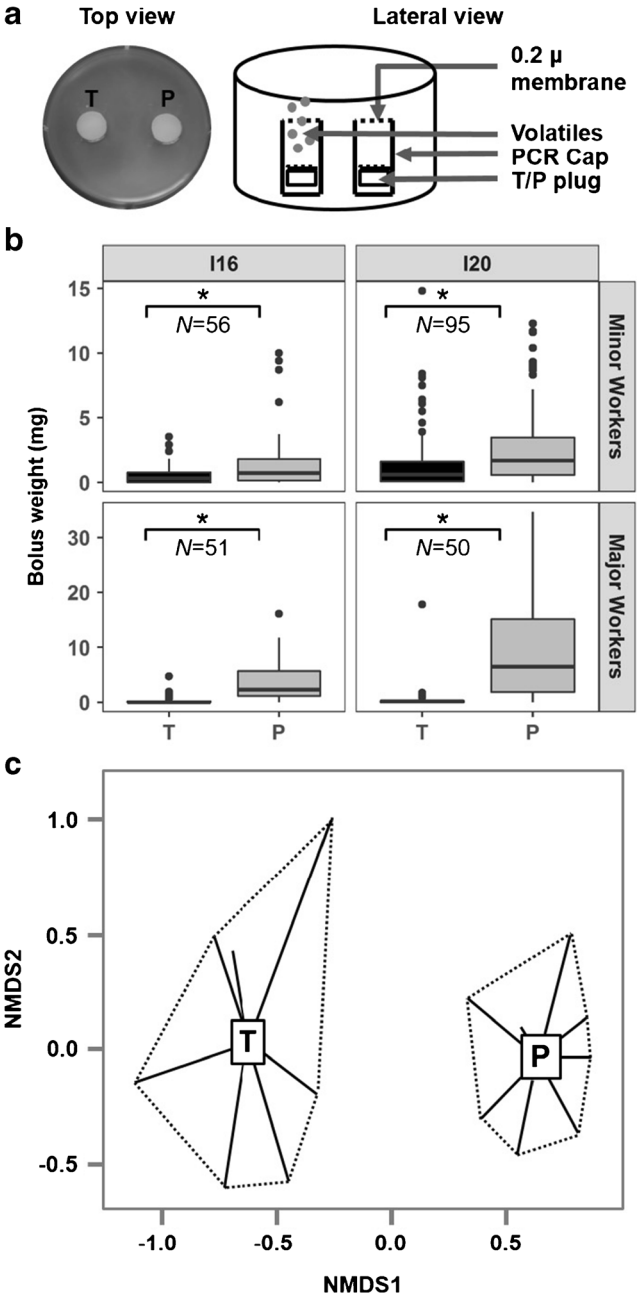

Fig. 2 Differential agar bolus deposition in volatile choice assays (*Termitomyces* versus *Pseudoxylaria*) and distinct volatile profiles of crop and weedy fungi. (**a**) Left: Top view of assay set up showing a single well of a sterile 6-well cell culture plate. Right: Schematic of lateral view of the well. (**b**) Box plots representing mass of agar boluses (mg) deposited after 3 hr on discs placed over *Termitomyces* (T) and *Pseudoxylaria* (P) plugs by minor and major workers of two different nests I20 and I16 of the fungus-growing termite *O. obesus*. Horizontal lines inside boxes represent medians. Filled circles represent outliers. *N* = sample sizes. * = significant difference ($P < 0.05$) in pairwise comparisons using Wilcoxon signed rank tests. (**c**) Non-metric multidimensional scaling (NMDS) ordination of volatile compounds of the two fungi *Termitomyces* (T) and *Pseudoxylaria* (P) ($N = 8$ isolates each), based on Bray-Curtis distance, rotated by principal component analysis

min^{-1} and held for 10 min. Electron impact (EI) spectra were recorded at 70 eV in scan mode from 38 to 300 m/z using a scan speed of 2000 Da s^{-1}. The transfer line was held at 280 °C and the ion source at 230 °C.

Data Processing and Statistical Analysis All statistical analyses were performed in Rstudio 1.0.136 (RStudio Team 2016), user interface for R 3.3.2 (R Core Team 2016). Wilcoxon signed rank tests were used to test for statistical difference (at $\alpha = 0.05$) between masses of bolus deposited on different plugs in each choice offered, i.e. pairwise comparisons between plugs in each dish. Data were pooled from experiments conducted on different days and plotted using ggplot2 ver 2.2.1 (Wickham 2009). Dishes in which termite did not form boluses were not included in the analysis. However, dishes in which termites made boluses but did not deposit them on plugs/discs were recorded as zero deposition.

Plots of increase in bolus deposition on different plugs (*Termitomyces*, *Pseudoxylaria* and blank) with time showing adjusted means (Fig. S2) were drawn using phia package version 0.2–1 in R. Adjusted means (least square means) are predicted values from a multiple regression equation and thus adjusted for the imbalances arising as a result of interacting variables (here plug identity and time).

Fungal Volatile Analysis Compound identification was based on retention times, calibration with known alkanes and the National Institute of Standards and Technology (NIST) library of mass fragmentation spectra. Area under the chromatogram peak of each compound was used to calculate the proportional abundance of each volatile organic compound (VOC). For the volatile data analysis, out of the two replicates per isolate, we used the replicate which had the greater number of compounds for further analysis to capture as many compounds as possible while keeping the data independent of each other. Since the replicates of some isolates (two isolates of both *Pseudoxylaria* and *Termitomyces*) had the same number of compounds, we created all possible combinations of isolates ($N = 16$ datasets) such that each dataset had eight isolates of both fungi with each isolate represented by only one replicate. We randomly selected one dataset as a representative (Table S1) for the rest of the analysis and confirmed our results with the remaining datasets. First, in order to compare patterns of scent composition between crop and weedy fungi, we performed a non-metric multidimensional scaling (NMDS) using the function metaMDS in the package Vegan (version 2.4–2) with VOC proportions. Prior to the analysis, data were first square transformed and then standardised by a Wisconsin double standardization using the function 'wisconsin' in Vegan. We used Bray-Curtis distances for the NMDS analysis. The null hypothesis of no difference in patterns of scent composition between fungi was tested with a permutational multivariate analysis of variance (PERMANOVA) using the function 'adonis' in Vegan. Prior to analysis, homoscedasticity was confirmed using the 'betadisper' function in Vegan. Random Forests (RF) analysis of the VOC proportion data was used to identify the key compounds that can explain the dissimilarities in the volatile profiles of the fungi and thus can also be used as predictors of genus identity (Ranganathan and Borges 2010). We used 100 bootstrap iterations for this analysis with the package varSelRF (version 0.7–5).

Results

Behavioural Response of Worker Termites with Access to Crop and Weedy Fungi (and Blank) in Two-Choice Assays We found that termites deposited a greater number of boluses on *Pseudoxylaria* than *Termitomyces* fungal plugs (Fig. 1c); this difference was significant at 15 min for both minor and major workers of nest I16 and remained so till 60 min. But for nest I20, the difference was evident only at 30 and 45 min for minor and major workers respectively and remained so till the end of the experiment. In a choice between *Pseudoxylaria* and a blank plug, termites again deposited a greater mass of boluses on the *Pseudoxylaria* plug (Fig. 1c), with a significant difference at 15 min for both minor and major workers of nest I16 but for nest I20 only at 45 min which remained significant till 60 min. For the choice between *Termitomyces* and a blank plug, the amount of deposition was never found to be significantly different (except for one comparison where deposition was greater on the blank plug than on *Termitomyces*), even at the end of 60 min for both types of workers of both nests (Fig. 1c). We also found that with increase in time, the overall bolus deposition on plugs also increased but the overall amount of deposition was far more on *Pseudoxylaria* as compared to *Termitomyces* or blank plugs for both minor and major workers (Fig. S2).

Sustained Behavioural Response of Worker Termites to Crop and Weedy Fungi We found that minor and major workers favoured bolus deposition on *Pseudoxylaria* plugs (Fig. 3). This was evident for nest I20 for all time points. However, for nest I16 this was true only for major workers. Additionally, the total bolus deposition was lower for minor workers than major workers. We also tested the soldier caste and found that soldiers did not make (let alone deposit) boluses even at the end of 24 hr (Fig. S3).

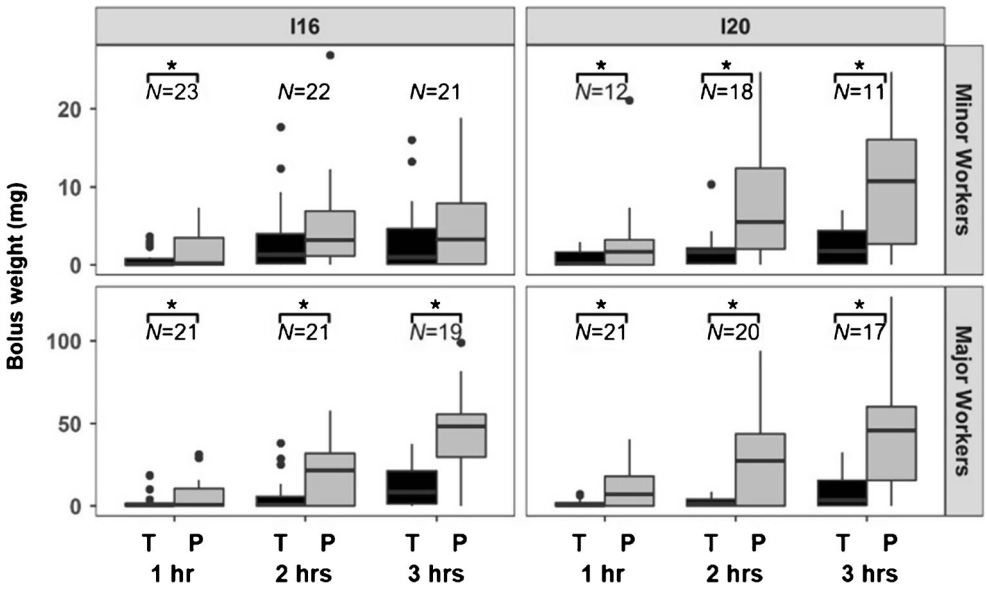

Fig. 3 Sustained differential agar bolus deposition by worker termites in *Termitomyces* versus *Pseudoxylaria* choice assays. Box plots representing mass of agar boluses (mg) deposited after 1, 2 and 3 hr on plugs of *Termitomyces* (T) and *Pseudoxylaria* (P) by termites (minor and major workers) from two different nests I16 and I20 of the fungus-growing termite, *O. obesus*. Horizontal lines inside boxes represent medians. Filled circles represent outliers. *N* = sample sizes. * = significant difference ($P < 0.05$) in pairwise comparisons using Wilcoxon signed rank tests

Sustained Behavioural Response of Worker Termites to Crop and Weedy Fungi with a Natural Substrate (Soil) Both minor and major workers successfully made boluses with soil which is a natural substrate for termites and deposited a greater mass of boluses on *Pseudoxylaria* plugs as compared to *Termitomyces* plugs at the end of 3 hr (Fig. 4).

Behavioural Response of Termites to Volatiles of Crop and Weedy Fungi When physical access to the plugs of *Termitomyces* and *Pseudoxylaria* was blocked, i.e. termites had access only to volatiles released from the fungal plugs, we found that both minor and major workers (of both nests I16 and I20) deposited significantly more boluses near *Pseudoxylaria* as compared to *Termitomyces* at the end of 3 hr (Fig. 2b).

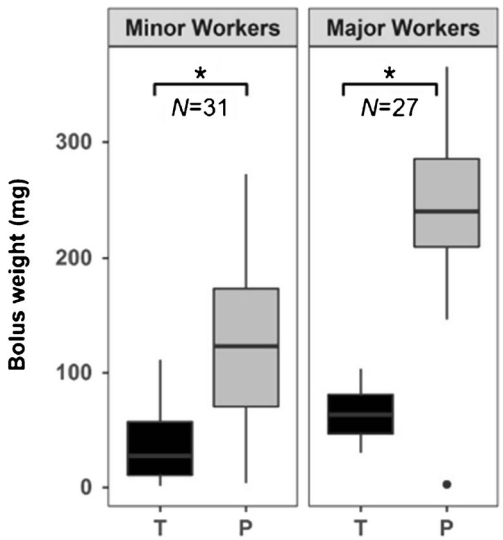

Fig. 4 Soil bolus deposition by worker termites in *Termitomyces* versus *Pseudoxylaria* choice assays. Box plots representing mass of soil boluses (mg) deposited after 3 hr on plugs of *Termitomyces* (T) and *Pseudoxylaria* (P) by termites (minor workers and major workers) of nest I16 of the fungus-growing termite, *O. obesus*. Horizontal lines inside boxes represent medians. Filled circles represent outliers. *N* = sample sizes. * = significant difference ($P < 0.05$) in pairwise comparisons using Wilcoxon signed rank tests

Volatile Profiles of Crop and Weedy Fungi A randomly selected dataset of *Termitomyces* and *Pseudoxylaria* fungi yielded 28 and 24 VOCs respectively; some compounds were common to both; there were 41 unique compounds across fungi (Table S1). When we compared the scent profiles of both fungi, we found that they differed considerably (Fig. 2c) and isolates of the same fungal genus grouped together based on NMDS ordination. PERMANOVA analysis confirmed that the volatile profiles of the isolates varied significantly between the two fungal genera (pseudo $F_{1,15} = 4.49$, $P = 0.001$). We found similar results for all the remaining 15 datasets (data not shown). Random Forests analysis revealed that the combination of aristolene, pogostol and viridiflorol best explained the difference between the fungal genera at 61% model frequency (Fig. S4). Among the remaining 15 datasets, the combination of aristolene and viridiflorol was found to have very high model frequency (94–100%) in eight of the datasets (Fig. S4).

Discussion

We report for the first time that the worker castes of fungus-growing termites differentiate between their crop fungus *Termitomyces* and the weedy fungus *Pseudoxylaria*. Using a novel assay that exploited the hard-wired material-handling behaviour of termites, we found that both minor and major workers utilised agar and a natural substrate like soil to form boluses and deposited them to a greater extent on the weed

compared to the crop, effectively burying the weed. These results show that fungus-farming termites not only have the behavioural capacity to distinguish between ecologically relevant fungi, but also demonstrate a mechanism of how antifungal chemicals could be selectively applied on the weedy fungi. These fungi produce characteristic scents and the emitted volatiles alone are sufficient for the termites to discriminate between them. This elucidates the mechanistic basis of the burial behaviour response of termites and unravels the sensory ecology of the host–mutualist–parasite tripartite interaction. This also demonstrates how insect farmers may recognise and thus limit the spread of garden infection, thereby shaping the disease ecology of fungus farms.

This behaviour of depositing boluses closely replicates the commonly observed, natural behaviour of termites in which worker termites deposit soil on any foreign material introduced into their nest mound (Batra and Batra 1966; L. Katariya, pers. obs.) or on infected and dead nest mates (Myles 2002; Yanagawa et al. 2011; Chouvenc et al. 2012; Chouvenc and Su 2012). Worker termites, in a choice between a *Termitomyces* isolate from their own nest and an isolate of *Pseudoxylaria* that is common across termite nests, deposited more boluses on the latter. This shows that worker termites have the ability to discriminate between crop and weedy fungi.

To determine whether the behaviour of differentially burying *Pseudoxylaria* plugs is only context dependent, i.e. expressed only in a situation when the crop fungus is also present, we offered termites a choice between either *Termitomyces* or *Pseudoxylaria* versus a blank plug in similar two-choice assays. Here also, termites deposited a greater mass of boluses on *Pseudoxylaria* than the blank. This means that greater bolus deposition on *Pseudoxylaria* occurs even in the absence of *Termitomyces*. Hence, presence of crop fungi in the vicinity is not required for the termites to respond with such burial behaviour towards weedy fungi. For the choice between *Termitomyces* and blank plugs, the amount of deposition was never significantly different (except for one comparison) (Fig. 1c) and was extremely low (Fig. S2). These results indicate that termites treat a blank stimulus similar to *Termitomyces* with respect to bolus deposition within the duration of the experiment. Also, termites seem to avoid burying crop fungi (and blank plugs) excessively unlike the response to the weedy fungi.

Whereas the earlier experiments indicate that the behavioural response to crop and weedy fungi is quick, with onset as soon as 15 min, we also found that this behaviour is sustained for long durations. This sustained behavioural response seems to be aimed at burying the weedy fungi completely. Additionally, the total bolus deposition was lower for minor workers than major workers. This may be because minor workers make smaller boluses than major workers (Fig. S1). However, inside the termite mound both castes may work together to bury the parasites. Since soldiers did not make (let alone deposit) boluses (Fig. S3), this indicates that soldiers are not involved in the burial response towards crop parasites. This is similar to the social immunity defences exhibited by ants where only worker castes are known to display behaviours such as allogrooming (Walker and Hughes 2009). Also, both minor and major workers successfully formed boluses with soil and deposited a greater mass of boluses on *Pseudoxylaria* compared to *Termitomyces*. This substantiates our earlier results with agar indicating that this burying behaviour is not a laboratory artefact and suggests that termites utilise the soil present in their nest to deal with the infected parts of their farms.

The differences in the time course of the burial responses between nests and castes could be a reflection of differing levels of termite activity. That termites deposit boluses even on the blank and on *Termitomyces* is interesting, though the deposition is lower on the crop fungus than blank or the weed. One possible reason for this lower deposition on *Termitomyces* could be the negative effect of bolus deposition on growth since termites deposit faeces and saliva during the burying process (Myles 2002; Chouvenc et al. 2012; Chouvenc and Su 2012). Termite faeces and saliva have antimicrobial activity including antifungal peptides and have been implicated in reducing the microbial load of buried termite cadavers (Rosengaus et al. 1998; Lamberty et al. 2001; Chouvenc et al. 2012; Chouvenc and Su 2012). This may also be why even where we found a significant difference in deposition between *Termitomyces* and blank plugs, deposition was lower on *Termitomyces* (Fig. 1c). We therefore propose that bolus deposition is a behavioural defence of fungus-growing termites against parasitic fungi such as *Pseudoxylaria* and is likely how termites anoint parasitic fungi with the non-specific fungicides isolated from this system (Visser et al. 2012; Kim et al. 2014) and avoid poisoning their crop. Even if there are specific fungicides against the parasites (Um et al. 2013; Beemelmanns et al. 2017), it would benefit termites to apply them only on selected areas since production of secondary metabolites is costly (Vining 1990); this cost may be ultimately incurred by the termite hosts of the antibiotic-producing bacteria, if the fungicides are of microbial provenance. Therefore, this study shows for the first time how insect farmers may selectively apply chemical defences against the parasites of their gardens. Batra and Batra (1966) reported that *Odontotermes* sp. workers plastered a petri dish containing *Cunninghamella* fungus with moist soil, when placed inside the nest, leading to fungus killing. They also reported that workers plastered the sprouting [*Pseudo*]*xylaria*-like fungus with soil moistened with their saliva which reduced its growth. This further supports the hypothesis that termites may use bolus deposition as an antimicrobial application mechanism against parasitic fungi. Additionally, fungus-growing termites may have co-opted the bolus deposition behaviour as a

behavioural defence against fungal farm parasites from a pre-existing behaviour repertoire against entomopathogenic fungi that is likely present in many social insects including lower termites (Myles 2002; Chouvenc and Su 2012).

While fungus-growing ants show grooming and weeding as behavioural defences directed towards the presence of fungal weeds and parasites in their gardens (Currie and Stuart 2001) and also differentiate between different strains of the crop fungus (Bot et al. 2001; Viana et al. 2001) and mutualistic *Pseudonocardia* bacteria (Zhang et al. 2007), the mechanisms by which such discrimination occurs is unknown. However, chemicals such as VOCs produced by fungi and bacteria may provide a mechanistic basis for the specificity of these responses (Biedermann and Kaltenpoth 2014). This seems to be true for the fungus-farming termites as they display a differential burying response even when offered fungal scent alone. In some of our assays, physical access to the fungal plugs was blocked and termites had access only to the volatiles released from the fungal plugs which could pass through membrane discs. Here also, termites exhibited the same enhanced burial response towards the weedy fungus showing that fungal volatiles are sufficient to initiate the burying behaviour.

The ability of termites to respond to volatile compounds alone with a burial response is particularly interesting because the interior of the termite mound is a dark environment. Since these termites lack functional eyes, they are expected to have heightened chemoreception as these results demonstrate, i.e. single fungal plugs of diameter less than a centimetre elicit not just burying behaviour but differential action between fungi. Similarly, in fungus-growing ants, the proactive self-grooming behaviour which helps to prevent garden contamination, is stimulated by the presence of the fungal crop that was hypothesised to be sensed through crop volatiles (Morelos-Juárez et al. 2010). Fungus-growing beetles also seem to use volatiles for fungal recognition as they are attracted towards the scent of crop fungi in an olfactometer assay but are repelled by antagonistic *Trichoderma* sp. (Hulcr et al. 2011). However, unlike volatiles of entomopathogenic fungi which usually lead to aversion behaviour in termites (Hussain et al. 2010; Yanagawa et al. 2012), including fungus-growing termites (Mburu et al. 2009), we found a burial response of workers towards the weedy *Pseudoxylaria*. This could be because leaving a newly sprouting farm parasite unattended by absconding will negatively impact the fungus farm. Alternatively, it is also possible that in our particular assay, since the termites are enclosed in a dish, they have no opportunity to leave the arena, and therefore respond with burying behaviour. However, even inside the stable confines of the nest, it would be non-adaptive for termites to show aversion behaviour to *Pseudoxylaria*; on the contrary it is expected that they may respond to its presence by weeding and/or consuming it (for sterilisation in the gut) apart from burying the infected area. Also, even though we have used a small arena for these behavioural experiments compared to the large dimensions of the fungus comb and nest, within a mound there are millions of termites constantly patrolling their farms and, once a threat is recognised, additional termites can be quickly recruited to the source with the help of "pathogen alarm behaviour" or pheromones (Roisin et al. 1990; Rosengaus et al. 1999; Gerstner et al. 2011).

Termites may be able to deposit boluses differentially on *Termitomyces* and *Pseudoxylaria* plugs due to differences in the volatiles released by the two fungi. When we compared the scent profiles of the fungi, we found that they differ considerably. This difference in volatile profiles may facilitate the differential response of the termites in agar and soil-based assays. The combination of sesquiterpene compounds such as aristolene and viridiflorol may constitute the "weedy scent" to identify the parasitic fungus as these volatiles are absent from the scent of *Termitomyces* but present in *Pseudoxylaria*. Fungi are known to produce volatile compounds that can attract insects (Spiteller 2015), e.g. the sesquiterpene alcohol chokol K produced by the endophytic fungus *Epichloe* attracts "pollinator" *Botanophila* flies (Schiestl et al. 2006) and (3R)-1-octen-3-ol attracts wood-living beetles (Fäldt et al. 1999), thus contributing to the distribution of fungal spores (Spiteller 2015). However, in the present case, termites may be utilising volatile by-products of *Pseudoxylaria* metabolism as parasite recognition cues. While it is also possible that the interacting mycelium of *Termitomyces* and *Pseudoxylaria* could alert the termites to garden infection (Visser et al. 2011) by producing volatiles as a result of this interaction, in our assays termites were easily able to identify and respond to the presence of the parasite in the absence of the crop fungus even though the offered biomass of *Pseudoxylaria* was miniscule (just 0.1 cm^2 plug compared to the humungous surface of a fungus comb (Duringer et al. 2007)). In an actual termite fungus farm, any *Pseudoxylaria* infection may generate volatiles that are very distinct from the background odour emitted by the huge mass of crop fungi and possibly detectable by workers constantly tending the farm. Such detection even by few termites in any part of the farm may lead to recruitment of additional workers leading to growth suppression of parasites by the selective and local application of antifungal defences and ultimately removal of the infected piece from the healthy parts of the farm.

It will be interesting to investigate whether fungus-growing termites can show a similar differential response between different *Pseudoxylaria* isolates that may differ in volatile profiles (Table S1), a phenomenon similar to the relationship between virulence and repellency of entomopathogenic fungi exhibited by termites (Mburu et al. 2009; Yanagawa et al. 2012). Additionally, since *Termitomyces* isolates also have

qualitative differences in their volatile profiles (Table S1), it will be valuable to investigate whether termites can utilise these differences to discriminate between native (nest) and non-native mutualistic fungi. This may help answer important questions pertaining to partner-choice mechanisms (Aanen et al. 2009). Finally, it will be important to examine the presence of antifungal compounds in the bolus depositions which may explain why termites preferably deposit more agar and soil on the weedy fungus.

In summary, our results show that the worker castes of fungus-growing termites can differentiate between their mutualistic crop fungus *Termitomyces* and the weedy fungus *Pseudoxylaria*. In soil and agar-based assays, both minor and major workers utilised the substrate to form boluses and deposited them to a greater extent on *Pseudoxylaria*, effectively burying the fungal parasite. These fungi produce volatiles which alone are sufficient for the termites to discriminate between them. These results provide the first test of the ability of insect farmers to distinguish between crop and weedy fungi along with a mechanism for how this discrimination may result in selective application of non-specific weedicides. These results advance our knowledge of the parasite ecology of fungus farms. Moreover, these results also underscore the important role of behaviour in the stability of such ancient mutualisms (Nobre et al. 2011; Roberts et al. 2016).

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