Dynamic changes in death cues modulate risks and rewards of corpse management in a social insect

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Summary

1. In social insects, the efficiency of exploitation of resources and colony defence are mediated by chemical communication. In colonies dependent on nutritionally limited resources, corpses represent both a food resource and a risk of pathogens. Because this risk vs. reward changes with time after death, behavioural plasticity in the response to corpses would be advantageous. How social insects regulate this trade-off remains unknown.

2. We conducted quantitative behavioural bioassay and chemical analysis to study corpse management in the eastern subterranean termite, *Reticulitermes flavipes*. We hypothesized that *R. flavipes* responds differently towards corpses with prolonged post-mortem time, and this behavioural plasticity is mediated by the dynamic change in death cues.

3. We show that *R. flavipes* uses a post-mortem signalling system that depends on an early death cue that stimulates corpse retrieval and cannibalism, and late death cues that stimulate recruitment and burial. A blend of 3-octanone and 3-octanol is released immediately after death, with the alcohol (possibly in conjunction with the ketone) playing a clear role as a death cue. This cue enables the colony to recycle nutrients before decomposition occurs and before risk of pathogens increases. The accumulation of late death cues, decomposition products shared by diverse arthropods, elicits a behavioural switch from cannibalism to burial.

4. The ability to detect and respond to the dead is vital to group-living animals. Our study reveals a behavioural shift in corpse management triggered by the interplay of an early death cue and late death cues, which balances risks and rewards associated with corpses. The post-mortem chemical communication highlights the adaptation of a social insect to its ecological niche.

Key-words: cannibalism, chemical communication, eusociality, *Reticulitermes flavipes*, undertaking behaviour

Introduction

The evolution of sociality is integrated with the evolution of chemical communication, which plays a key role in regulating the organization of fundamental behaviours in social colonies (Blum 1974; Hölldobler & Wilson 1990). Social insects rely on various chemical cues to detect environmental features and communicate with colony members in exploitation of food sources (Jarau & Hrncir 2009), as well as colony defence against predators, competitors and disease (Blum 1969; Breed, Guzmán-Novoa & Hunt 2004). The ability to produce and perceive chemical information promotes rewards associated with feeding and minimizes risks via defence, therefore enhances the efficiency of the colony.

Dead individuals are regularly encountered by group-living animals. Corpses can attract feeding by scavengers; however, they pose pathogenic risks and trigger aversion of group members in a wide range of taxa, such as mammals (Prounis & Shields 2013), fish (Hussain *et al.* 2013) and arthropods (Wisenden, Pohlman & Watkin 2001; Nilsson & Bengtsson 2004; Yao *et al.* 2009), including eusocial insects (Howard & Tschinkel 1976; Visscher 1983). While avoidance of corpses is common (Yao *et al.* 2009), undertaking is a colony-level hygienic behaviour that convergently evolved in eusocial species (Sun & Zhou 2013). Honeybees and ants carry dead nestmates and discard them out of the nest to prevent deleterious effects associated with corpses (Wilson, Durlach & Roth 1958; Haskins & Haskins 1974; Howard & Tschinkel 1976; Visscher 1983; Julian & Cahan 1999). Corpse removal in those species is stimulated by post-mortem

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changes of chemical signatures, either through 'increased decomposition death cues' that are primarily fatty acids (Wilson, Durlach & Roth 1958; Haskins & Haskins 1974; Howard & Tschinkel 1976), or 'diminished chemical vital sign' as in the Argentine ant, *Linepithema humile* (Choe, Millar & Rust 2009). Termites that feed on nitrogen-poor lignocellulosic food, on the other hand, could benefit from recycling rather than discarding nutrients from dead nestmates (Moore 1969; Nalepa 1994). Cannibalism of deceased individuals (i.e. necrophagy) frequently occurs in termite colonies (Neoh *et al.* 2012; Sun, Haynes & Zhou 2013). Termites also practice burial behaviour when corpses are in large numbers and infected with pathogenic fungi (Chouvenc & Su 2012), highly decomposed (Chouvenc *et al.* 2012) or from neighbouring congeneric competitors (Sun, Haynes & Zhou 2013). Corpses represent both a source of food and risks of pathogens or intruders for termite colonies, and they are managed through alternative behaviours of cannibalism and burial. Previous attention has focused on burial activity stimulated by fatty acids (Chouvenc *et al.* 2012; Ulyshen & Shelton 2012). However, chemical cues that mediate corpse recycling and regulate the differential undertaking response remain unknown.

Subterranean termites nest under high humidity, and build extensive galleries in soil and decaying wood, predisposing them to a variety of opportunist pathogens (Rosengaus, Traniello & Bulmer 2011). Given that turnover rate of termites in a colony is high (Darlington 1991), encounter of dead nestmates is frequent. Termites are soft-bodied insects that decay quickly in the humid conditions, and the nutritional value presumably drops with time after death. In addition, the presence of corpses in a termite nest poses increasing risk over time. For example, a fungal entomopathogen, *Metarhizium anisopliae*, develops on a termite corpse and produces conidia in 2–3 days (Chouvenc & Su 2012). Our previous study in the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), demonstrated that workers retrieve deceased nestmates within the first 24 h of death (Sun, Haynes & Zhou 2013). In two other subterranean species, *Coptotermes formosanus* and *R. speratus*, workers also cannibalize recently deceased individuals, while they opt to bury the corpses aged for 1–7 days (Neoh *et al.* 2012). Based on these observations, we hypothesized that *R. flavipes* adjusts the undertaking behaviour from corpse recycling to burial as corpse ages. We further hypothesized that the behavioural switch is mediated by the post-mortem change of chemical cues, particularly with an early death cue that facilitates corpse recycling before decay sets in. To test the hypotheses, we examined behavioural response of *R. flavipes* towards nestmate workers with different post-mortem times, investigated the temporal changes in death-related chemicals, and functionally analysed the early and late death cues that modulate corpse management.

Materials and methods

TERMITE COLLECTION AND MAINTENANCE

All *R. flavipes* colonies were collected from Kentucky, USA. Two colonies were collected nearby the Red River Gorge area, Daniel Boone National Forest (Slade). Another colony was collected from the Arboretum, located on the University of Kentucky campus (Lexington). Colonies were obtained during spring and summer using trapping stations filled with dampened cardboard rolls. The rolls were left under wood logs where termite activities were detected. After a week, they were taken to laboratory, and new rolls were placed at the same sites for continuous collection. Once captured termites were extracted from traps, placed in Petri dishes (14·5 cm × 2·0 cm) with moistened unbleached paper towel as food source for 1–2 weeks. After that termites were transferred to round plastic containers (15·0 cm in diameter, 6·5 cm in height). They were provisioned with moistened wood mulch and pinewood blocks. *Reticulitermes* colonies consisted of workers and soldiers upon collection, and they were maintained in complete darkness (L : D = 0 : 24), at 27 ± 1 °C, 80–99% RH. Termites were used for experiments within 6 months of collection from the field.

UNDERTAKING BIOASSAY WITH DEAD TERMITES

Undertaking responses towards termite corpses with different post-mortem times were tested in two *R. flavipes* colonies with 10 replications for each colony. Nestmate workers were killed by freezing on dry ice for 1 min. Corpses were maintained in covered Petri dishes under the same conditions as their natal colony for 0, 1, 16, 32 and 64 h, respectively. The experimental set-up included a nesting chamber, a round plastic container (15·0 cm in diameter, 6·5 cm in height) containing ~2000 termites (Fig. 1a); and a covered test arena, a 5·5-cm-diameter Petri dish (1·5 cm height) (Fig. 1b) where observations were made. A plastic tube (inner diameter: 0·7 cm; length: 3·0 cm) connected the test arena and the nesting chamber. Colonies were acclimatized in the set-up for 1 month to allow termites to enter the test arena and cover the bottom with faeces and other nest materials. For each trial, an individual corpse was introduced in the vicinity of nest entrance through an entry port (0·5 cm diameter) on the cover of test arena. Behavioural responses by *R. flavipes* in test arena were recorded using a low-light, high-sensitivity video camcorder (Canon VIXIA HF G10, Canon Inc., Tokyo, Japan) under weak visible light for at least 15 min for retrieval behaviour, or for 30 min if burial activity (i.e. depositing soil on or near corpse) was initiated. For burial behaviour, an additional observation was made 1 h after corpse introduction, which always confirmed the first 30-min observation. A piece of plastic sheeting was placed at the bottom of the test arena and replaced after each replication to eliminate chemical residues left by termites. The types of behaviour (retrieval or burial) were documented. Retrieval time, which indicates the duration from the corpse being introduced to the corpse being moved to nesting chamber, was also recorded.

A dish assay was conducted to document if corpses would be cannibalized. Groups of 24 workers with one soldier, added to emulate normal colony caste proportions, were enclosed in individual Petri dishes (5·5 cm in diameter, 1·5 cm in height) (Fig. 1d). The bottom of each dish was scratched to create a coarse surface to facilitate walking behaviour. A piece of moistened unbleached paper towel in semi-circular shape was placed at the bottom, serving as food source and covering half of the bottom. Moistened sand (1 g, water content 15% by weight) was provided as potential burial materials. A hole (0·5 cm in diameter) on the cover, where a nestmate corpse was introduced, was above the section of the dish that was not lined with paper. Groups of
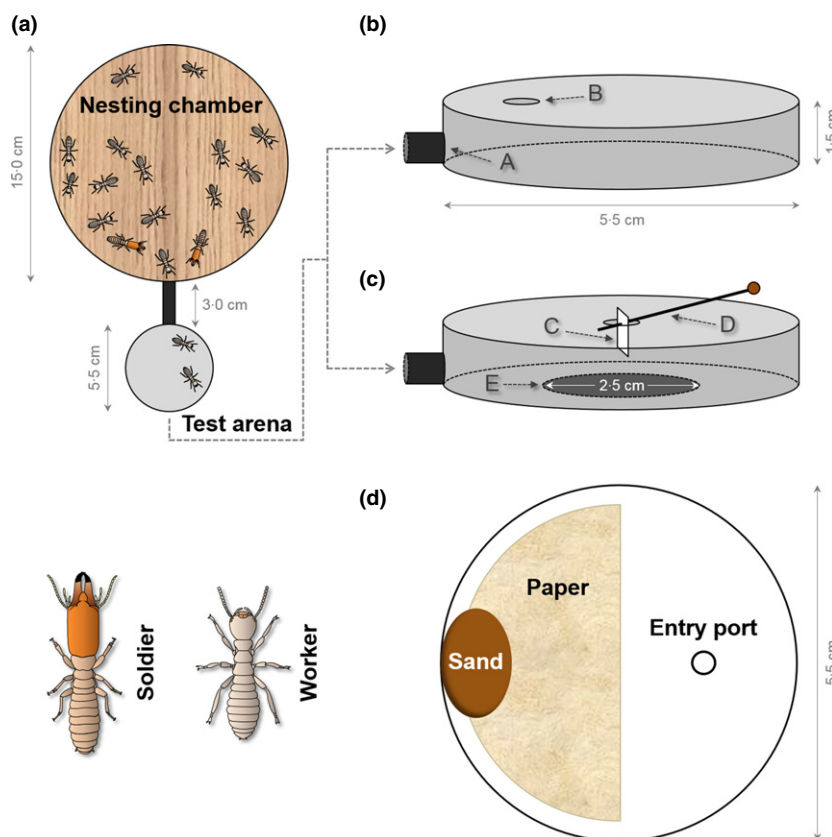
Fig. 1. Schematic drawing of experimental set-ups. (a) Experimental set-up with a nesting chamber and a test arena. (b) Test arena for differential undertaking response. A: nest entrance; B: entry port. (c) Test arena for attraction assay. C: filter paper (0·2 cm × 0·7 cm) with odour source; D: pin holding the filter paper; E: circle (2·5 cm diameter). (d) Experimental set-up for dish assay. In the dish assay, each group of termites consisted of 24 workers and one soldier. Test objects were introduced through the entry port.

termites were maintained in the dishes for 1 day prior to test. Corpses at post-mortem time 0, 1, 16 and 32 h were tested, and each set-up was tested once with one corpse only. Whether corpses were consumed or buried was documented after 24 h. Three replications were made with each post-mortem time for each of two colonies.

ANALYSIS OF DEATH-RELATED CHEMICALS

Chemical analyses were conducted with an Agilent Technologies 6890N gas chromatography (GC) in split-less mode equipped with a DB-5 capillary column (30 m × 0·25 mm × 0·25 μm; Agilent Technologies, Santa Clara, CA, USA), using helium as carrier gas (1·0 mL min^{-1}). Column temperature was raised from 40 °C (2-min hold) at 10 °C min^{-1} to 320 °C (additional 4-min hold). Injection temperature was 280 °C. The GC was coupled with an Agilent Technologies 5975 mass spectrometer (MS). Candidate compounds were identified based on National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health Mass Spectral Library, and further identified by comparison of retention times and mass spectra with synthetic standards (Sigma-Aldrich, St. Louis, MO, USA).

We collected chemicals from live workers and corpses produced by freezing (0, 0·5, 1, 2, 4, 8, 16, 32 and 64 h post-mortem). Corpses at 0 h post-mortem were thawed at room temperature for 5 min before all collection procedures. Volatiles were collected from live workers or corpses with a 100-μm polydimethylsiloxane solid-phase microextraction (SPME) fibre (Sigma-Aldrich). Fifteen workers were placed in a 2-mL glass vial, which was sealed with an aluminium crimp cap with pre-fitted septum (Thermo Fisher Scientific, Waltham, MA, USA). The fibre was extended into the vial for 15 min without contacting termites. After this period, the fibre was introduced into the injection port of the GC and remained there for 1 min, where it was heated to 280 °C. Volatiles identified by SPME were further quantified on corpses with

hexane extraction. Individual workers were submerged in 50 μL of glass-distilled hexane (Thermo Fisher Scientific) (with 10 ng μL^{-1} n-nonadecane as internal standard) for 10 min, and the solution was injected to GC. We made 10 replications of each corpse age for each of two colonies.

To quantify fatty acids from workers, esterification was performed with hexane extracts. At each time point (live, 8, 16, 32 and 64 h post-mortem), a group of six individuals were extracted by 300 μL of distilled hexane (with 10 ng μL^{-1} n-nonadecane as internal standard) for 10 min, when the solution was removed. Two hundred microlitres of BF$_3$-methanol (10%, w/w) was added to react with the solution at 60 °C for 10 min following the manufacturer's instructions (Sigma-Aldrich). Methyl esters as final products were identified using GC/MS and quantified through GC peak areas. Termite extracts were also injected to GC without reaction, and no methyl ester was found. Three replications were made with each of two colonies.

ATTRACTION ASSAY WITH VOLATILES

Hexane solutions of early- and late-occurring volatiles were made using synthetic chemicals (Sigma-Aldrich) at the concentration equivalent to one corpse at 0 h and 64 h post-mortem, respectively, per μL (Table S1, Supporting Information); and 1 μL of each solution was applied to a strip of filter paper (0·2 cm × 0·7 cm) for test. Hexane was tested as solvent control. To investigate the attractive effect of post-mortem volatiles, the same behavioural set-up consisted of a nesting chamber and a test arena was used, but with a modification on the arena inspired by Reinhard *et al.* (2003) (Fig. 1c). The strip of filter paper with an odour source, held by a #3 insect pin, was introduced to the centre of test arena through a hole on the top. Termites could not make direct contact with the odour source. A circle (2·5 cm diameter) was marked on the floor of the arena directly underneath the odour source. After introduction of each odour source, the

numbers of workers that appeared in the circle were noted every 10 s for the 5-min assay. Treatments using the same colony were separated by at least 1 h.

BURIAL ASSAY WITH PHENOL, INDOLE AND FATTY ACIDS

A dish assay was adopted to test effects of late death cues on burial behaviour (Fig. 1d). Hexane solutions of different compounds were made at the concentration equivalent to one or 10 corpse(s) (64 h post-mortem) per μL, and 1 μL of each solution was applied for each test. The tested quantities were proportional to those extracted with hexane (Table S1). A solution was applied to the surface of a Teflon tube (0·1 cm diameter, 0·4 cm long) of a size to simulate a termite corpse, and hexane was tested as solvent control. After evaporation of solvent (1 min), the tube was dropped through the entry port to the dish. Burial activity was then observed by taking a digital photo after 3 h, and the area covered with sand and faeces was quantified using IMAGEJ 1.48 (National Institutes of Health, Bethesda, MD, USA). The experiment was conducted with 10 replications for each of two colonies, and a new group of termites (24 workers and one soldier) in a clean Petri dish was used each time.

DATA ANALYSES

All statistical analyses were performed using STATISTIX 10 (Analytical Software, Tallahassee, FL, USA). Prior to analysis of variance, retrieval times were log transformed (log (x)), quantities of fatty acids were log transformed (log $(x + 1)$), and the mean numbers of workers per observation in retrieval and burial as well as attraction assay were square root transformed (square root (x)). These transformations yielded values that fit the assumptions of parametric tests. The above data, as well as quantities of volatiles collected by SPME, were analysed by ANOVA with colony, treatment, and interaction between colony and treatment as factors. For mean areas of burial assay, we conducted a Kruskal–Wallis ANOVA, due to non-homogeneity of variance (Levene's Test, $P < 0.05$). All data were pooled from two colonies, but we also analysed each colony separately to demonstrate the patterns were consistent (Figs S1–S5).

Results

UNDERTAKING RESPONSE TOWARDS CORPSES WITH DIFFERENT POST-MORTEM TIMES

Corpses ($\leq$32 h post-mortem) were carried back to the nesting chamber; however, they were buried in the arena if deceased for 64 h (Fig. 2a). Workers walked towards the dead, and retrieval was initiated immediately after antennal contacts (Movie S1). Typically, a worker carried the corpse straight to the entrance, but the retrieval path could be more circuitous. There was no effect of colony on retrieval time (ANOVA; $F_{1,72} = 0.05$, $P = 0.82$), and no interaction between colony and post-mortem time ($F_{3,72} = 0.43$, $P = 0.73$), but post-mortem time category had a significant effect on retrieval time ($F_{3,72} = 3.30$, $P = 0.03$). Workers took a significantly longer time to retrieve 32-h-old corpses than freshly killed corpses (0 h old) (Tukey's HSD all-pairwise comparisons test, $P < 0.05$) (Fig. 2b). Workers walked towards and contacted a 64-h-old corpse in the
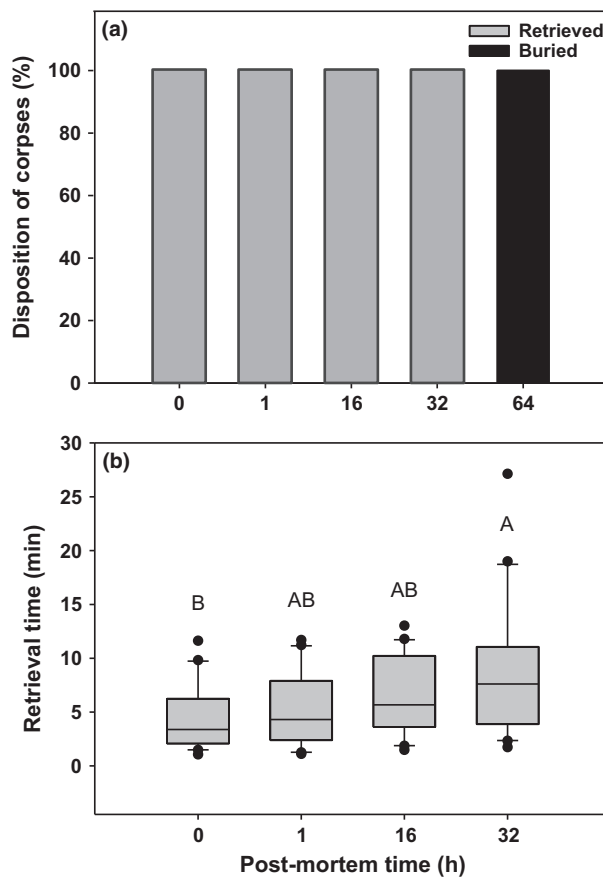

Fig. 2. Undertaking behaviour towards corpses with different post-mortem times. (a) Differential response to corpses ($n = 20$). (b) Corpse retrieval time (mean $\pm$ SEM, $n = 20$). Boxes are bounded by the first and third quartiles, with the band indicating median; whiskers represent the 10th and 90th percentiles; dots outside the whiskers are outliers. Data were pooled from two colonies with 10 replications each colony. Means between groups denoted with the same letter were not significantly different (Tukey's HSD all-pairwise comparisons test, $P > 0.05$).

same manner as a fresh corpse. Then, they picked up soil particles and deposited them on or near the corpse (Movie S2). There were significantly more workers in the test arena during burial than during retrieval; with 8·24 $\pm$ 1·58 and 3·25 $\pm$ 0·70 workers per still video frame taken every 30 s for burial and retrieval, respectively (unpaired t-test: $t_{18} = 2.82$, $P < 0.05$, $n = 10$) (also see Fig. S6a). Burial was observed for 30 min (60 frames) and retrieval for 15 min (30 frames) after corpse introduction. Termites perform alarm behaviour characterized by vigorous vibration of bodies (Crosland & Traniello 1997). Vibration was significantly more frequent in burial than retrieval (mean number of vibrations per minute $\pm$ SEM: 0·27 $\pm$ 0·08 for retrieval during 15 min, and 1·09 $\pm$ 0·26 for burial during 30 min observation period; unpaired t-test: $t_{18} = 3.01$, $P < 0.01$, $n = 10$) (also see Fig. S6b).

In the dish assay, we introduced 0- to 64-h-old corpses to groups of termites enclosed in single Petri dishes. With a single corpse introduced to each dish, workers consumed

0- to 32-h-old corpses (100%, $n = 6$ each post-mortem time: 0, 1, 16 and 32 h; see Fig. S7 for cannibalism), whereas they buried corpses deceased for 64 h (100%, $n = 6$).

POST-MORTEM CHANGES IN CHEMICAL PROFILES

Immediately after death, workers emitted two volatiles, 3-octanone and 3-octanol (Fig. 3a). These two volatiles reached maximum quantities at 0·5 h and 1 h post-mortem, respectively, rapidly decreased in 4 h and reached the lowest level by 64 h post-mortem. There was a significant difference between post-mortem time categories (ANOVA; 3-octanone: $F_{9,180} = 118·42$, $P < 0·01$; 3-octanol: $F_{9,180} = 108·15$, $P < 0·01$) and a colony effect (3-octanone: $F_{1,180} = 11·26$, $P < 0·01$; 3-octanol: $F_{1,180} = 5·62$, $P = 0·02$), but there was no interaction between colony and post-mortem time (3-octanone: $F_{9,180} = 1·38$, $P = 0·20$; 3-octanol: $F_{9,180} = 0·99$, $P = 0·45$). In contrast, two different volatiles, phenol and indole, were produced by corpses with longer post-mortem times (Fig. 3b). Phenol was detected at 8 h post-mortem, and then increased in quantity and became most abundant 64 h after death (ANOVA; post-mortem time: $F_{9,180} = 9·01$, $P < 0·01$; colony:

$F_{1,180} = 7·56$, $P < 0·01$; there was an interaction between colony and post-mortem time: $F_{9,180} = 5·76$, $P < 0·01$). Similarly, indole was detected at 16 h through 64 h post-mortem (ANOVA; post-mortem time: $F_{9,180} = 14·88$, $P < 0·01$; colony: $F_{1,180} = 0·25$, $P = 0·62$; there was an interaction between colony and post-mortem time: $F_{9,180} = 0·53$, $P = 0·85$). 3-Octanone and 3-octanol (freshly dead), and phenol and indole (highly decayed) were the only volatiles consistently associated with deceased workers. No volatile was detected from live workers.

Six fatty acids found ranged from C14 to C18, including myristic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid and stearic acid. All six compounds were detected from live workers, but they gradually built up after death (Fig. 4). There was a significant increase in fatty acids with time after death (ANOVA; myristic acid: $F_{4,20} = 5·06$, $P < 0·01$; palmitoleic acid: $F_{4,20} = 18·18$, $P < 0·01$; palmitic acid: $F_{4,20} = 20·19$, $P < 0·01$; linoleic acid: $F_{4,20} = 37·54$, $P < 0·01$; oleic acid: $F_{4,20} = 20·76$, $P < 0·01$; stearic acid: $F_{4,20} = 14·73$, $P < 0·01$), but there was no effect of colony on quantity (myristic acid: $F_{1,20} = 0·03$, $P = 0·87$; palmitoleic acid: $F_{1,20} = 0·28$, $P = 0·60$; palmitic acid: $F_{1,20} = 0·15$, $P = 0·70$; linoleic acid: $F_{1,20} = 0·15$, $P = 0·70$; oleic acid: $F_{1,20} = 0·01$, $P = 0·91$; stearic acid: $F_{1,20} = 0·05$, $P = 0·83$), and no interaction between colony and postmortem time (myristic acid: $F_{4,20} = 0·30$, $P = 0·87$; palmitoleic acid: $F_{4,20} = 0·60$, $P = 0·67$; palmitic acid: $F_{4,20} = 0·77$, $P = 0·56$; linoleic acid: $F_{4,20} = 0·93$, $P = 0·46$; oleic acid: $F_{4,20} = 0·86$, $P = 0·51$; stearic acid: $F_{4,20} = 0·57$, $P = 0·69$).

FUNCTIONAL ANALYSIS OF DEATH-RELATED CHEMICALS

Attractant effect of post-mortem volatiles

When 3-octanone, 3-octanol, or a blend of the two early-occurring volatiles equivalent to one freshly killed corpse
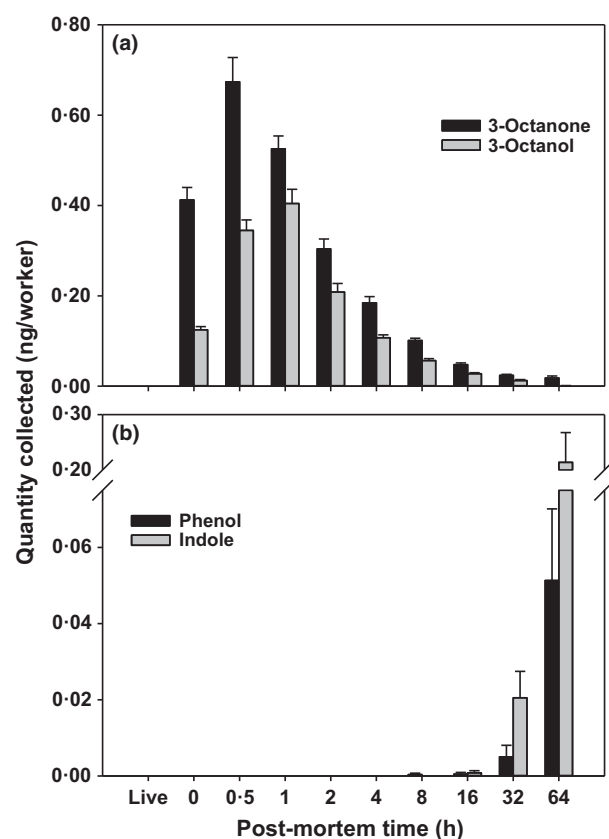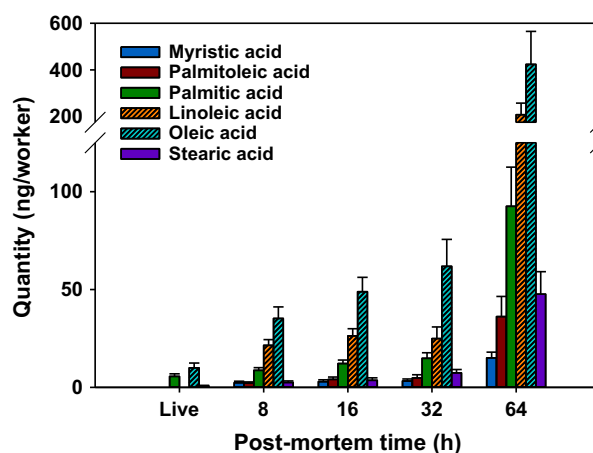

Fig. 3. Temporal profiles of death-related chemicals on *R. flavipes* workers. (a) and (b) show early-occurring volatiles, 3-octanone and 3-octanol, and late-occurring volatiles, phenol and indole, respectively. Quantity collected by SPME fibre was presented (mean ± SEM, $n = 20$). (SPME does not accurately reflect the emission rate from corpses.) Data were pooled from two colonies with 10 replications each colony.

Fig. 4. Fatty acids collected from *R. flavipes* workers after death. Quantity extracted with hexane was presented (mean ± SEM, $n = 6$). Data were pooled from two colonies with three replications each colony.

(1×) was introduced, workers swept their antennae in the air, moved towards the odour source, and lifted their heads and antennae underneath the odour source for a short time before moving away. More workers moved towards 3-octanol and the blend than the solvent control (Fig. 5a; Tukey's HSD all-pairwise comparisons test, $P < 0.05$). The response to 3-octanone was not significantly different from controls and did not improve the response to the blend. There was a significant effect of odour source on number of workers attracted (ANOVA; $F_{3,72} = 7.36$, $P < 0.01$), as well as a colony effect ($F_{1,72} = 7.76$, $P < 0.01$), but there was no interaction between odour source and colony ($F_{3,72} = 0.21$, $P = 0.89$).

The late-occurring volatiles, phenol, indole or a blend, attracted significantly more workers compared to solvent control (Fig. 5b; ANOVA, $F_{3,72} = 14.33$, $P < 0.01$; Tukey's HSD all-pairwise comparisons test, $P < 0.05$). There was a colony effect ($F_{1,72} = 5.77$, $P = 0.02$), but there was no interaction between odour source and colony ($F_{3,72} = 0.98$, $P = 0.41$).

Burial activity induced by late death cues

Teflon tubes were treated with a blend of all late-accumulating chemicals (six fatty acids, phenol and indole; indicated as FAPI) and a hexane extract of 64-h-old corpses at two different quantities, equivalent to one corpse (1×) or 10 corpses (10×) (Table S1). The higher dose stimulated more burial behaviour (Kruskal–Wallis one-way nonparametric ANOVA, $H_4 = 45.20$, $P < 0.01$). At 1×, there was no significant burial activity triggered by FAPI or hexane extract of 64-h-old corpses in comparison with control (Dunn's all-pairwise comparisons test, $P > 0.05$). However, at 10×, both FAPI and the corpse extract induced significantly more burial behaviour (Dunn's all-pairwise comparisons test, $P < 0.05$) (Fig. 6a). Therefore, the dose of 10× was used in subsequent experiments.

The roles of volatiles and fatty acids in burial activity were tested by exposing workers to tubes treated with solvent (hexane), a blend of late-occurring volatiles (phenol and indole, indicated as PI), a blend of six fatty acids

identified (FA), or the FAPI blend. Both FA and FAPI triggered significant burial activity (Kruskal–Wallis one-way nonparametric ANOVA, $H_3 = 26.65$, $P < 0.01$; Dunn's all-pairwise comparisons test, $P < 0.05$), but workers did not deposit significantly more sand on PI-treated tubes compared to the solvent control (Dunn's all-pairwise comparisons test, $P > 0.05$) (Fig. 6b).

Burial activity was stimulated by oleic acid alone, a blend of linoleic acid and oleic acid, a blend of all six fatty acids, as well as an extract of 64-h-old corpses (Kruskal–Wallis one-way nonparametric ANOVA, $H_6 = 81.83$, $P < 0.01$; followed by Dunn's all-pairwise comparisons test, $P < 0.05$). There was no significant difference between these four treatments (Dunn's all-pairwise comparisons test, $P > 0.05$) (Fig. 6c). The results suggested that oleic acid, the most abundant fatty acid from dead workers, was the most active in stimulating burial behaviour, followed by linoleic acid. The remaining four fatty acids did not appear to trigger undertaking behaviour.

Discussion

BENEFITS OF CANNIBALISM

In adaptive cannibalism, the consumer benefits by acquiring nutrients from the victim or corpse. Gregarious locusts that consumed conspecifics showed improved survival and migratory abilities (Simpson *et al.* 2006). Sexual cannibalism promotes female fecundity in mantids (Barry, Holwell & Herberstein 2008). In subterranean termites, cannibalism of corpses is an important mechanism for nitrogen conservation (Hungate 1941). Wood is rich in carbon but poor in nitrogen, with a carbon: nitrogen ratio ranging from 75 : 1 to 247 : 1. In comparison, C : N ratio in termite bodies varies between 4 : 1 and 7 : 1 (Tayasu *et al.* 1997). Unlike wood-feeding termites, in the litter-feeding higher termites, *Microcerotermes crassus* and *Globitermes sulphureus*, workers forage for highly decomposed plant materials with higher nitrogen content, and corpse cannibalism rarely occurs (Neoh *et al.* 2012). These observations suggest the
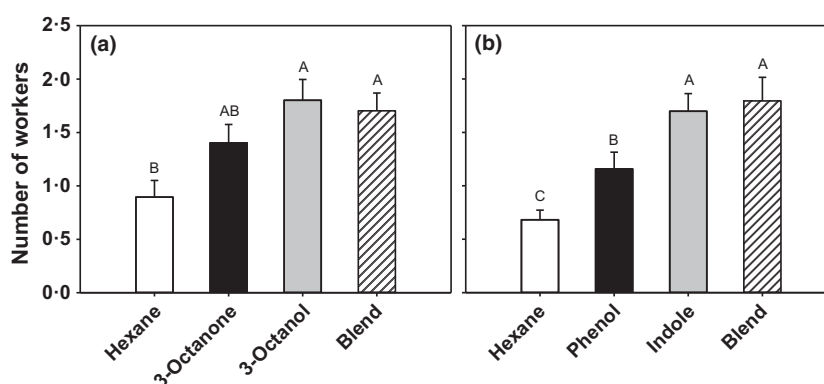

Fig. 5. Attractive effect of post-mortem volatiles. Here, it presented the number of workers that moved underneath odour source per observation in treatments with early-occurring (a) and late-occurring (b) (mean ± SEM, $n = 20$). Data were pooled from two colonies with 10 replications each colony. Means between groups labelled with the same letter were not significantly different (Tukey's HSD all-pairwise comparisons test, $P > 0.05$).
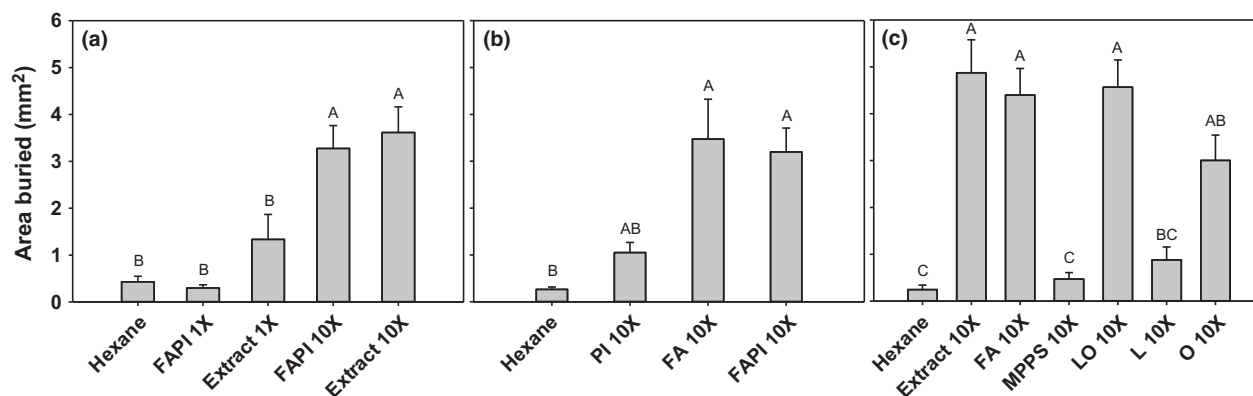
Fig. 6. Burial activity induced by late death cues. (a) Effect of two doses on burial behaviour. (b) Effect of volatiles and fatty acids. (c) Effect of different groups of fatty acids. Areas buried with sand (and faeces) within 3 h were presented as mean ± SEM (*n* = 20). Data were pooled from two colonies with 10 replications each colony. Groups denoted with the same letter were not significantly different (Dunn's all-pairwise comparisons test, *P* > 0·05). '1×' and '10×' indicate tested quantity equivalent to one or 10 corpse(s) at 64 h post-mortem, respectively. Extract: hexane extract of corpse(s) at 64 h old; FAPI: blend of all six fatty acids, phenol and indole; PI: blend of phenol and indole; FA: blend of six fatty acids; LO: blend of linoleic acid and oleic acid; MPPS: blend of the other four fatty acids (myristic acid, palmitoleic acid, palmitic acid and stearic acid); L: linoleic acid; O: oleic acid.

adaptive value of 'trophic' corpses is associated with feeding habit of the species. Termites share a common ancestor with cockroaches (Inward, Beccaloni & Eggleton 2007). Cockroaches, which are phytophagous or opportunistically omnivorous, are faced with the same dilemma of nitrogen acquisition, and they cannibalize live conspecifics (Nalepa 1994).

Death of sterile individuals in social colonies is frequent. In mature colonies of a mound-building termite, *Macrotermes michaelseni*, non-reproductive populations remain constant with a turnover rate of 1·4% per day (estimated corpse production of 18 076 per day in a colony with 1 280 895 workers and soldiers, see table 3 in Darlington 1991). If a similar turnover rate occurs in *R. flavipes*, which consists of 0·2–5·0 million termites per colony (Su, Ban & Scheffrahn 1993), 2800–70 000 dead individuals are expected each day. Loss of nutrients is inevitable if corpses were not recycled. Cannibalism of deceased individuals before decomposition occurs, therefore, is important for nitrogen conservation. Other nutrients and resources including carbohydrates, lipids, and potentially, endosymbionts, will also be recovered. In addition, cannibalism may reduce the chance of pathogenic attack by destroying their source. Also, antimicrobial components in termite saliva and guts inhibit the spread of pathogens (Lamberty *et al.* 2001; Chouvenc, Su & Robert 2009).

BEHAVIOURAL SWITCH FROM CANNIBALISM TO BURIAL

Reticulitermes flavipes altered the undertaking behaviour from cannibalism to burial when corpses decomposed for 64 h. The observations are consistent with previous findings that *C. formosanus* and *R. speratus* only fed on newly dead nestmates (Neoh *et al.* 2012). In our study, there was a substantial accumulation of fatty acids extracted from corpses by 64 h, indicating the progression of decomposition. The level of fatty acids might play an important role in predicting pathogenic risk. In the red imported ant, *Solenopsis invicta*, infection with fungus *Metarhizium anisopliae* accelerates corpse decomposition and promotes corpse removal by workers (Qiu *et al.* 2015). The level of fatty acids also dictates the speed of corpse removal in the European fire ant *Myrmica rubra*, in which decayed dead nestmates with a higher fatty acid titre were removed more quickly (Diez, Moquet & Detrain 2013). Corpse removal maintains colony hygiene and enhances colony fitness in ants (Diez, Lejeune & Detrain 2014). In termites that do not forage outside of the nesting system, physically isolating corpses through burial is a parallel hygienic practice. The burial material utilized by workers consists of faeces and chewed food mixed with saliva, both of which have antifungal properties (Rosengaus, Guldin & Traniello 1998; Chouvenc & Su 2012). The behavioural switch from feeding on corpses to colony defence against pathogens suggests a balance between rewards and risks.

THE INTERPLAY OF EARLY AND LATE DEATH CUES

In *R. flavipes*, 3-octanone and 3-octanol were released synchronously with death, allowing for immediate recruitment of workers to consume the corpses, with 3-octanol playing a clear role. The rapid release of and response to the early death cue is an adaptation to conserve nutrients, energy and labour (see Fig. 7 for summary). Based on the fact that 3-octanone and 3-octonal are released immediately after death and peak within 1 h, we suspect that they are likely produced and stored by live workers. It is not known whether termites use 3-octanone and 3-octanol in communication functions other than death cue, but interestingly,

the two compounds are also found in four congeneric species (*R. santonensis*, *R. lucifugus*, *R. grassei* and *R. banyulensis*) (Reinhard *et al.* 2003) and ants. In several ant genera including *Manica* (Fales *et al.* 1972), *Crematogaster* (Crewe, Blum & Collingwood 1972) and *Camponotus* (Duffield & Blum 1975), either one or both of these volatiles function as alarm pheromone; and in *Myrmica*, 3-octanol was reported to attract workers (Cammaerts *et al.* 1985; Cammaerts & Mori 1987). The association of 3-octanone and 3-octanol with multiple social insects is intriguing and awaits further exploration in social, phylogenetic and ecological contexts.

Fatty acids stimulate burial behaviour in *R. flavipes* (this study), and a distantly related termite *Pseudacanthotermes spiniger* (Chouvenc *et al.* 2012), suggesting that fatty acid-mediated burial may be widespread in termites. The ability to recognize a 'fatty acid death cue' is advantageous to diverse organisms as these chemicals may indicate proximity to a threat posed by either predators or disease (Yao *et al.* 2009). Fatty acids from dead individuals, primarily oleic acid and linoleic acid, elicit avoidance behaviour in many gregarious arthropods including amphipods (Wisenden, Pohlman & Watkin 2001), springtails (Nilsson & Bengtsson 2004) and social caterpillars (Yao *et al.* 2009). In eusocial insects, fatty acid-induced undertaking behaviour occurs in Hymenoptera (Wilson, Durlach & Roth 1958; Haskins & Haskins 1974; Howard & Tschinkel 1976; Diez, Moquet & Detrain 2013) and Isoptera (termites), being a part of social repertoire to eliminate corpses. Fatty acids identified in this study have low volatility and initiate burial activity by workers upon direct contact. Phenol and indole are late death cues that direct workers to the corpses. Thus, burial involves an integration of volatiles that attract workers and fatty acids that stimulate burial. Phenol and indole are degradation products of proteins (Spoelstra 1977). These two
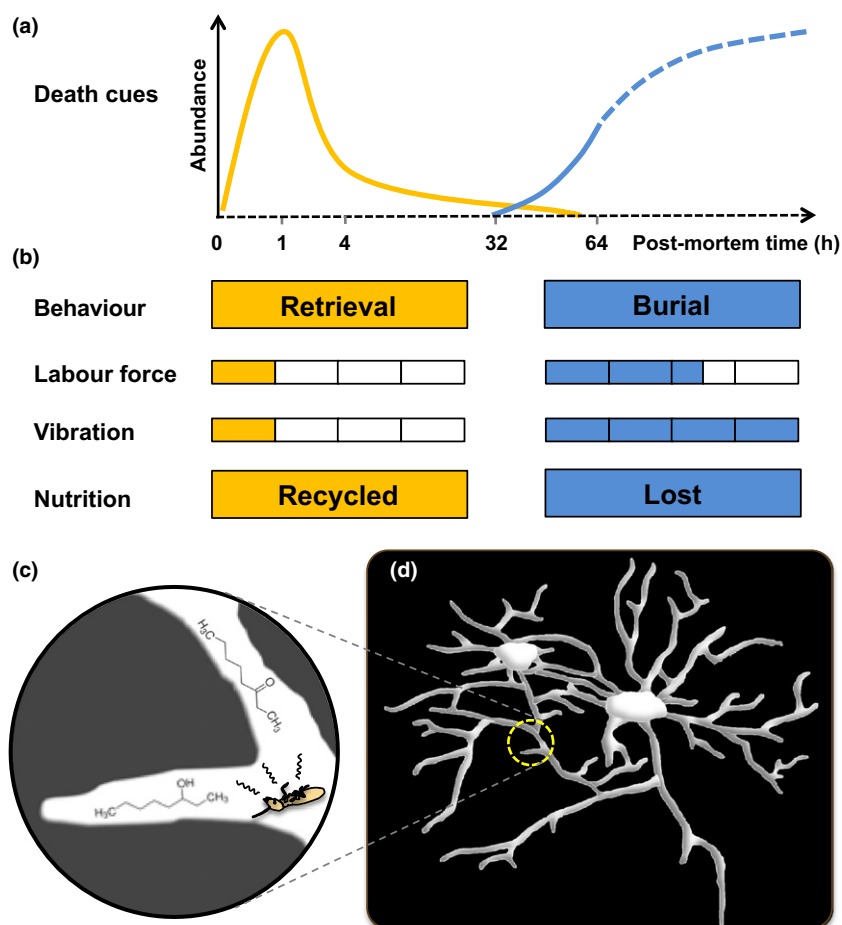

Fig. 7. Graphic summary of the energy output and nutritional balance associated with differential undertaking responses. (a) An early death cue (orange line) peaks within 1 h and rapidly decreases over 4 h. In contrast, late death cues (blue line) start to increase after 32 h. (b) Retrieval behaviour involves a small labour force and only low levels of vibrational signalling; thus, energy costs of this behaviour are low. Burial behaviour involves 2·5 times more workers than retrieval, and higher frequency of vibrational signalling (4× compared to retrieval). Retrieval behaviour results in low energy costs, and it allows the colony to recycle nutrients through cannibalism. Burial is not only energetically costly, but the nutrients contained in the corpse are lost. The timely release of the early death cue, consisting of 3-octanone and 3-octanol (c), allows workers to locate the dead within a short period of time (<4 h) inside the dark and complex nesting matrix (d) to maximize the colony fitness by practicing retrieval instead of burial behaviour, as retrieval minimizes the loss of nutritional resources while preventing pathogen propagation, and limits the energy output associated with the undertaking behaviour (b).

compounds are comparable to cadaverine and putrescine in vertebrates, which have been shown to stimulate burial in rats (Pinel, Gorzalka & Ladak 1981) and avoidance in fish (Hussain *et al.* 2013).

Conclusions

We found that a dynamic post-mortem signalling system mediates a behavioural switch from corpse recycling to burial. The differential response balances nutritional rewards and pathogenic risks associated with dead colony members. The use of early death cue may occur in other termite species with similar nesting ecology and feeding habits, where cannibalism is the most efficient strategy for managing corpses. We suggest that synchronous release of 3-octanone and 3-octanol with death and the corresponding behavioural and sensory response are adaptations that promote colony fitness. According to the 'disposable soma' theory, altruistic workers are 'somatic' and disposable (Kirkwood 1987; Rueppell & Kirkman 2005). Upon death, deceased workers no longer support the colony through labour; instead, they eventually become a pathogenic risk. The programmed release of an early death cue allows workers to continue their colony support immediately after death.

Authors' contributions

QS, KFH and XZ conceived and designed the experiments; QS conducted the experiments; QS and KFH analysed the data; KFH and XZ contributed reagents/materials/analysis tools; QS, KFH and XZ wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Data accessibility

The data sets supporting the conclusions of this article are available in the Supporting Information (Data S1).

References

Barry, K.L., Holwell, G.I. & Herberstein, M.E. (2008) Female praying mantids use sexual cannibalism as a foraging strategy to increase fecundity. *Behavioral Ecology*, **19**, 710–715.

Blum, M.S. (1969) Alarm pheromones. *Annual Review of Entomology*, **14**, 57–80.

Blum, M. (1974) Pheromonal bases of social manifestations in insects. *Pheromones* (ed. M. Birch), pp. 190–199. North-Holland Publishing, Amsterdam, the Netherlands.

Breed, M.D., Guzmán-Novoa, E. & Hunt, G.J. (2004) Defensive behavior of honey bees: organization, genetics, and comparisons with other bees. *Annual Review of Entomology*, **49**, 271–298.

Cammaerts, M.C. & Mori, K. (1987) Behavioural activity of pure chiral 3-octanol for the ants *Myrmica scabrinodis* Nyl. and *Myrmica rubra* L. *Physiological Entomology*, **12**, 381–385.

Cammaerts, M.C., Attygalle, A.B., Evershed, R.P. & Morgan, E.D. (1985) The pheromonal activity of chiral 3-octanol for *Myrmica* ants. *Physiological Entomology*, **10**, 33–36.

Choe, D.-H., Millar, J.G. & Rust, M.K. (2009) Chemical signals associated with life inhibit necrophoresis in Argentine ants. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 8251–8255.

Chouvenc, T. & Su, N.-Y. (2012) When subterranean termites challenge the rules of fungal epizootics. *PLoS One*, **7**, e34484.

Chouvenc, T., Su, N.-Y. & Robert, A. (2009) Inhibition of *Metarhizium anisopliae* in the alimentary tract of the eastern subterranean termite *Reticulitermes flavipes*. *Journal of Invertebrate Pathology*, **101**, 130–136.

Chouvenc, T., Robert, A., Sémon, E. & Bordereau, C. (2012) Burial behaviour by dealates of the termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) induced by chemical signals from termite corpses. *Insectes Sociaux*, **59**, 119–125.

Crewe, R.M., Blum, M.S. & Collingwood, C.A. (1972) Comparative analysis of alarm pheromones in the ant genus *Crematogaster*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **43**, 703–716.

Crosland, M.W.J. & Traniello, J.F.A. (1997) Behavioral plasticity in division of labor in the lower termite *Reticulitermes fukienensis*. *Naturwissenschaften*, **84**, 208–211.

Darlington, J.P.E.C. (1991) Turnover in the populations within mature nests of the termite *Macrotermes michaelseni* in Kenya. *Insectes Sociaux*, **38**, 251–262.

Diez, L., Lejeune, P. & Detrain, C. (2014) Keep the nest clean: survival advantages of corpse removal in ants. *Biology Letters*, **10**, 20140306.

Diez, L., Moquet, L. & Detrain, C. (2013) Post-mortem changes in chemical profile and their influence on corpse removal in ants. *Journal of Chemical Ecology*, **39**, 1424–1432.

Duffield, R.M. & Blum, M.S. (1975) Identification, role and systematic significance of 3-octanone in the carpenter ant, *Camponotus schaefferi* Whr. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **51**, 281–282.

Fales, H.M., Blum, M.S., Crewe, R.M. & Brand, J.M. (1972) Alarm pheromones in the genus *Manica* derived from the mandibular gland. *Journal of Insect Physiology*, **18**, 1077–1088.

Haskins, C.P. & Haskins, E.F. (1974) Notes on necrophoric behavior in the archaic ant *Myrmecia vindex* (Formicidae: Myrmeciinae). *Psyche*, **81**, 258–267.

Hölldobler, B. & Wilson, E.O. (1990) *The Ants*. Harvard University Press, Cambridge, MA, USA.

Howard, D.F. & Tschinkel, W.R. (1976) Aspects of necrophoric behavior in the red imported fire ant, *Solenopsis invicta*. *Behaviour*, **56**, 157–178.

Hungate, R.E. (1941) Experiments on the nitrogen economy of termites. *Annals of the Entomological Society of America*, **34**, 467–489.

Hussain, A., Saraiva, L.R., Ferrero, D.M., Ahuja, G., Krishna, V.S., Liberles, S.D. *et al.* (2013) High-affinity olfactory receptor for the death-associated odor cadaverine. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 19579–19584.

Inward, D., Beccaloni, G. & Eggleton, P. (2007) Death of an order: a comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. *Biology Letters*, **3**, 331–335.

Jarau, S. & Hrncir, M. (2009) *Food Exploitation by Social Insects: Ecological, Behavioral, and Theoretical Approaches.* CRC Press, Boca Raton, FL, USA.

Julian, G.E. & Cahan, S. (1999) Undertaking specialization in the desert leaf-cutter ant *Acromyrmex versicolor*. *Animal Behaviour*, **58**, 437–442.

Kirkwood, T.B.L. (1987) Immortality of the germ-line versus disposability of the soma. *Evolution of Longevity in Animals: a Comparative Approach* (eds A. Woodhead & K. Thompson), pp. 209–218. Plenum Press, New York, NY, USA.

Lamberty, M., Zachary, D., Lanot, R., Bordereau, C., Robert, A., Hoffmann, J.A. *et al.* (2001) Constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. *Journal of Biological Chemistry*, **276**, 4085–4092.

Moore, B.P. (1969) Biochemical studies in termites. *Biology of Termites* (eds K. Krishna & F.M. Weesner), pp. 407–432. Academic Press, Cambridge, UK.

Nalepa, C.A. (1994) Nourishment and the origin of termite eusociality. *Nourishment and Evolution in Insect Societies* (eds J.H. Hunt & C.A. Nalepa), pp. 57–104. Westview Press, Boulder, CO, USA.

Neoh, K.-B., Yeap, B.-K., Tsunoda, K., Yoshimura, T. & Lee, C.-Y. (2012) Do termites avoid carcasses? Behavioral responses depend on the nature of the carcasses. *PLoS One*, **7**, e36375.

Nilsson, E. & Bengtsson, G. (2004) Endogenous free fatty acids repel and attract Collembola. *Journal of Chemical Ecology*, **30**, 1431–1443.

Pinel, J.P., Gorzalka, B.B. & Ladak, F. (1981) Cadaverine and putrescine initiate the burial of dead conspecifics by rats. *Physiology & Behavior*, **27**, 819–824.

Prounis, G.S. & Shields, W.M. (2013) Necrophobic behavior in small mammals. *Behavioural Processes*, **94**, 41–44.

Qiu, H., Lu, L., Shi, Q., Tu, C., Lin, T. & He, Y. (2015) Differential necrophoric behaviour of the ant *Solenopsis invicta* towards fungal-infected corpses of workers and pupae. *Bulletin of Entomological Research*, **105**, 607–614.

Reinhard, J., Quintana, A., Sreng, L. & Clement, J.-L. (2003) Chemical signals inducing attraction and alarm in European *Reticulitermes* termites (Isoptera, Rhinotermitidae). *Sociobiology*, **42**, 675–692.

Rosengaus, R., Guldin, M. & Traniello, J.A. (1998) Inhibitory effect of termite fecal pellets on fungal spore germination. *Journal of Chemical Ecology*, **24**, 1697–1706.

Rosengaus, R., Traniello, J.A. & Bulmer, M. (2011) Ecology, behavior and evolution of disease resistance in termites. *Biology of Termites: a Modern Synthesis* (eds D.E. Bignell, Y. Roisin & N. Lo), pp. 165–191. Springer, Dordrecht, the Netherlands.

Rueppell, O. & Kirkman, R.W. (2005) Extraordinary starvation resistance in *Temnothorax rugatulus* (Hymenoptera, Formicidae) colonies: demography and adaptive behavior. *Insectes Sociaux*, **52**, 282–290.

Simpson, S.J., Sword, G.A., Lorch, P.D. & Couzin, I.D. (2006) Cannibal crickets on a forced march for protein and salt. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 4152–4156.

Spoelstra, S.F. (1977) Simple phenols and indoles in anaerobically stored piggery wastes. *Journal of the Science of Food and Agriculture*, **28**, 415–423.

Su, N.-Y., Ban, P.M. & Scheffrahn, R.H. (1993) Foraging populations and territories of the eastern subterranean termite (Isoptera: Rhinotermitidae) in southeastern Florida. *Environmental Entomology*, **22**, 1113–1117.

Sun, Q., Haynes, K.F. & Zhou, X. (2013) Differential undertaking response of a lower termite to congeneric and conspecific corpses. *Scientific Reports*, **3**, 1650.

Sun, Q. & Zhou, X. (2013) Corpse management in social insects. *International Journal of Biological Sciences*, **9**, 313–321.

Tayasu, I., Abe, T., Eggleton, P. & Bignell, D.E. (1997) Nitrogen and carbon isotope ratios in termites: an indicator of trophic habit along the gradient from wood-feeding to soil-feeding. *Ecological Entomology*, **22**, 343–351.

Ulyshen, M. & Shelton, T. (2012) Evidence of cue synergism in termite corpse response behavior. *Naturwissenschaften*, **99**, 89–93.

Visscher, P.K. (1983) The honey bee way of death: necrophoric behaviour in *Apis mellifera* colonies. *Animal Behaviour*, **31**, 1070–1076.

Wilson, E.O., Durlach, N.I. & Roth, L.M. (1958) Chemical releaser of necrophoric behavior in ants. *Psyche*, **65**, 108–114.

Wisenden, B., Pohlman, S. & Watkin, E. (2001) Avoidance of conspecific injury-released chemical cues by free-ranging *Gammarus lacustris* (Crustacea: Amphipoda). *Journal of Chemical Ecology*, **27**, 1249–1258.

Yao, M., Rosenfeld, J., Attridge, S., Sidhu, S., Aksenov, V. & Rollo, C.D. (2009) The ancient chemistry of avoiding risks of predation and disease. *Evolutionary Biology*, **36**, 267–281.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Corpse retrieval time in two colonies of *Reticulitermes flavipes*.

Fig. S2. Temporal change of death-related volatiles collected from *Reticulitermes flavipes* workers in two colonies.

Fig. S3. Post-mortem accumulation of fatty acids on *Reticulitermes flavipes* workers from two colonies.

Fig. S4. Attractive effect of post-mortem volatiles tested in two colonies.

Fig. S5. Burial activity of *Reticulitermes flavipes* toward death-related chemicals tested in two colonies.

Fig. S6. Comparison of energy cost between retrieval and burial.

Fig. S7. Cannibalism of freshly killed corpses by *Reticulitermes flavipes* in dish assay.

Table S1. Quantities of compounds tested in undertaking bioassay.

Movie S1. Video clip showing corpse retrieval behaviour by *Reticulitermes flavipes*.

Movie S2. Video clip showing burial behaviour by *Reticulitermes flavipes*.

Data S1. Supporting data.