

Post-mortem Changes in Chemical Profile and their Influence on Corpse Removal in Ants

Lise Diez · Laura Moquet · Claire Detrain

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Abstract In order to decrease the risk of pathogen transmission, ants remove corpses from the vicinity of nests, but little is known about the underlying mechanisms. In particular, it is unclear how the odor profile of corpses changes with time since death and how any changes might relate to behavior. We have addressed these questions in the red ant *Myrmica rubra*, where we asked how the time since death determines the ability of workers to discriminate a dead individual from a live one, and whether dead workers are removed in a similar way when they originate from the same or an alien colony. We found that ants could discriminate alien from nestmate corpses up to 2 days after death, since the former continued to elicit aggressive behavior over that period. For dead nestmates, only 15 % of corpses were removed when freshly killed but this rises to 80 % for corpses between 1 to 6 days post mortem. Using gas chromatography, we found that oleic and linoleic acids, which are absent on freshly killed corpses, appeared post mortem and were in higher quantities on those corpses that were ejected from the nest vicinity. When added to fresh corpses, linoleic and oleic acids, alone or blended, enhanced removal to levels observed for corpses of 2–6 days post-mortem. Thus, oleic and linoleic acids appear to be important cues involved in corpse recognition and necrophoresis over a long timeframe, and we advance the hypothesis that these fatty acids in combination with other cues may also trigger other behaviors such as prey retrieval.

Keywords Necrophoresis · Ants · *Myrmica rubra* · Oleic acid · Linoleic acid · Corpse

Introduction

Social insects cooperate in various tasks such as foraging, brood care, or defense against predators. This highly integrated group-living has contributed to their wide ecological success (Wilson 1971). On the other hand, one of the major drawbacks of sociality is the increased risk of pathogen transmission due to high densities of closely-related individuals living in confined and humid nests. Social insects have thus evolved a variety of cooperative defenses against pathogens, which result in a form of “social immunity” (Cremer et al. 2007). Many of these defenses are prophylactic: they act as a first barrier against the intake and spread of pathogens within the colony, and they reduce the need for nestmates to invest in costly activation of their individual immune systems (Schmid-Hempel 2005). The best-known prophylactic defenses used by social insects are the avoidance of infected soil (Drees et al. 1992), the intake of antimicrobial material into the nest such as tree resin in ants (Chapuisat et al. 2007) and propolis in bees (Bankova et al. 2000), the removal of food waste (Hart and Ratnieks 2002; Ribeiro and Navas 2007), and allogrooming among nestmates (Ugelvig et al. 2010), which can be associated with the production of antimicrobial material by the metapleural glands (Vander Meer 2012). Potential sources of infection also can be actively isolated by healthy nestmates. For example, bees remove infected and dead larvae and adults from the hive (Visscher 1983), while termites bury corpses in the nest (Chouvenc and Su 2012; Crosland et al. 1997; Ulyshen and Shelton 2012). Likewise, in most ant species, corpses are removed from the nest (Diez et al. 2011, 2012; Wilson et al. 1958), or in some cases buried inside it (Renucci et al. 2010). Another major way of preventing the spread of pathogens within the colony is the spatial isolation of corpses or diseased individuals that are potential sources of pathogens. In some ant species, moribund ants leave the nest of their own accord before dying (Bos et al. 2012; Heinze and Walter

L. Diez (✉) · L. Moquet · C. Detrain
Unit of Social Ecology, Université Libre de Bruxelles, Brussels,
Belgium
e-mail: lisediez@gmail.com

2010). Nevertheless, workers that die outside the nest may end up quite near the nest entrance, and may need to be transported further from these high-activity areas. Similarly, some corpses carried from inside the nest are deposited near the nest (Diez et al. 2012; Howard and Tschinkel 1976). This reveals an important task for ant workers, *i.e.*, the cleaning and maintenance of nest surroundings.

In the context of corpse removal inside or outside the nest, ant workers have to differentiate between a corpse and a healthy individual. They may also have to discriminate nestmates from other dead insects such as ant competitors or prey. Nestmate recognition is well-known to be based on the detection of non-volatile compounds that are present on the cuticle, such as alkanes, alkenes, (d' Ettore and Lenoir 2009; Lenoir et al. 2009; Martin and Drijfhout 2009) and, in some cases, fatty acids (Franks et al. 1990). Ants perceive these colony-specific, cuticular cues by direct antennal contact or at short distances (Brandstaetter et al. 2008). The following non-exclusive mechanisms are involved in nestmate recognition (d' Ettore and Lenoir 2009). First, there is the “prior association” mechanism, where ants familiarize with nestmates during early stages of life. They are then able to recognize these nestmates as kin, but not other ants with similar profiles. Second, there is phenotype matching, where ants build a neural template from nestmates encountered in early stages of life, and further can recognize never-encountered nestmates as kin by the degree of similarity between the cue-bearer and the template. A third mechanism is the recognition of alleles (Green Beard) that does not require learning, since ants recognize others as nestmates when they share a phenotypic chemical cue resulting from the expression of a common allele. Encountering an alien worker usually triggers aggressive behavior in ants. However, if the alien ant is dead, the threat to the colony does not come from potential aggression or competition, but from potential pathogens on or in the corpse.

Recognition of corpses is also likely to be based on olfactory cues. Two main hypotheses can be formulated. The first is that corpses are recognized due to the disappearance of a signal associated with life. In *Linepithema humile*, iridomyrmecin and dolichodial that are present on live ants disappear within 40 min of death and elicit necrophoresis by nestmates (Choe et al. 2009). The second hypothesis is that recognition and removal of corpses are triggered by the appearance of chemical cues after death. In *Myrmecia vindex*, the presence of oleic acid on cocoons triggers nurses to bury or transport them away from the nest (Haskins and Haskins 1974). Likewise, in *Pogonomyrmex badius* and *Solenopsis saevissima*, oleic acid put on pieces of filter paper seems to initiate their removal to the midden piles (Wilson et al. 1958). However, the same compound also can elicit the opposite behavior by triggering the retrieval of dummies into the nest, especially when the proportion of ants involved in

foraging was high compared to the proportion involved in midden work or nest maintenance (Gordon 1983). There is thus a need for further investigation of the role of oleic acid in ant removal behavior and how quantitative changes of this fatty acid or related compounds on corpses may act upon necrophoric behavior in ants.

In the common red ant *Myrmica rubra*, we first investigated whether ants behave in a similar way toward corpses originating from the same or an alien colony. Second, we tested how the time since death (up to 6 days post mortem) influences the ability of workers to discriminate a dead individual from a live one. Using gas chromatography, we analyzed the chemical profile of removed and non-removed corpses in order to identify compounds that trigger or deter necrophoresis. Finally, as fatty acids seemed to be involved in corpse removal, we quantified changes in the necrophoric behavior of ants faced with freshly-killed corpses to which increasing amounts of oleic and/or linoleic acids were added.

Methods and Materials

Insect Material Colonies of *M. rubra* were excavated from earth banks in a semi-open area on the campus of Gembloux Agro-Bio Tech, Belgium. In the laboratory, ants were kept in plaster nests (Janet type, 150×150×2 mm) connected to a foraging arena (43.5×35 cm) the borders of which were coated with polytetrafluoroethylene (Whitford, Runcorn, UK) to prevent ants from escaping. We connected nest entrances to a corridor (2×7 cm) that channeled the movements of exiting ants. Each nest contained 3 gynes and 500–650 workers, the brood covering 10 to 15 % of the nest area, which is a colony composition commonly found in the field (Elmes 1973). Laboratory conditions were kept at 25±1 °C and 45±5 % RH, with a constant 12:12 h L:D photoperiod. Each nest was provided with a sucrose solution (0.3 M) *ad libitum* and one mealworm, *Tenebrio molitor*, per day as a source of protein and lipid.

Comparison of Behavior Towards Homocolonial or Heterocolonial Corpses We investigated the ability of workers to discriminate between corpses of nestmates and those of alien ants. Therefore, we quantified necrophoretic behavior of ants faced with either hetero- or homocolonial corpses. Moreover, we observed how these behaviors evolved towards corpses at different times since death.

Corpses used in our experiments were ants that were randomly sampled from a colony and killed by freezing for 35 min at –24 °C. Corpses were then left at room temperature (25±1 °C) for 0, 1, 2, 4, or 6 d. Corpses may belong either to the tested colony (nestmates), or to another *M. rubra* colony (alien). The tested corpse then was placed in the corridor connecting the nests, about 1 cm from the nest entrance. For

each condition of time elapsed since death, and for each colonial origin (nestmate or alien) of corpses, we tested 4 corpses in five *M. rubra* colonies ($N=20$).

During the first 5 min following the introduction of a corpse, we counted the number of contacts as well as aggressive behaviors displayed by resident workers towards the corpse. Using these observations, we calculated an “Index of aggression” I that takes into account all observed behaviors weighted according to their level of aggressiveness (*i.e.*, through graded scores ranging from 0 to 3) (Errard and Hefetz 1997). This index of aggression was calculated as $I=(c*0)+(t*1)+(a*2)+(b*3)/(c+t+a+b)$, where c is the number of contacts (no aggression with an associated score of 0: the ant is simply touching the corpse with its antennae), t is the number of threats (weak aggression with a score of 1: the ant faces the corpse with opened mandibles), a is the number of attacks (medium aggression with a score of 2: the ant moves rapidly back and forth towards the corpse), and b is the number of bites (strong aggression with a score of 3: the ant strongly bites the corpse, sometimes curving its abdomen ready to sting).

For each corpse, we also observed whether it was removed far from the nest entrance. Therefore, we recorded the location of the corpse for 1 hr following its introduction. When a corpse was not transported or only relocated a very short distance (≤ 7 cm) within the corridor, it was considered as being non-removed. It was considered as being removed when an ant transported it further than the 7 cm corridor that was connected to the nest entrance.

Changes in Fatty Acids After Death We compared the compounds that were present on removed corpses *vs.* non-removed ones. For each time since death (0, 1, 2, 4, 6 d) and each origin (homocolonial and heterocolonial), we collected all removed and non-removed corpses used in the previous experiment in order to analyze their cuticular profile ($N=40$).

Collected corpses were dropped into 0.1 ml of hexane (SigmaAldrich, $\geq 99\%$) immediately after their removal or 1 hr after their introduction at the nest entrance for non-removed corpses. The corpse was left for 90 min in solvent in order to extract chemical compounds. There were no differences in the quantities of oleic or linoleic acids found on ants using a 90 min ($N=40$) extraction time compared to a shorter extraction time (15 min, $N=20$) often used for hydrocarbon extraction (Wilcoxon rank sum test, oleic acid: $W=7425$, $P=0.241$, linoleic acid: $W=6243$, $P=0.284$).

Samples were analyzed by gas chromatography (GC) on a Thermo Focus DSQ with an AI 3000 autosampler and a flame ionization detector. Aliquots of 1 μ l were introduced by splitless injection into a capillary column (Rtx-5MS, 30 m, 0.25 mm ID, 0.25 μ m film thickness, Restek). Carrier gas was hydrogen (2.1 ml/min), and the injector temperature was 220 °C. The oven temperature was set at 50 °C during the

injection, and then increased to 300 °C at a rate of 20 °C/min. Detector temperature was set at 310 °C. Identification of fatty acids was performed by GC/MS with a Thermo Polaris Q electron ion trap mass spectrometer interfaced with a Thermo Trace GC Ultra GC equipped with a capillary column (DB-5MS, 30 m, 0.25 mm ID, 0.25 μ m film thickness, Thermo-Finnigan). GC conditions were the same as that of GC analyses. In addition, retention times of oleic and linoleic acids were checked on the GC by injecting synthetic standards (Sigma-Aldrich, $\geq 99\%$) interspersed among runs. The amounts of oleic and linoleic acids were quantified by GC using calibration curves drawn by analyzing standards of 16 samples of known concentrations (1 to 25 μ g/ml). We fitted calibration curves of oleic acid (1) and linoleic acid (2) as follows:

$$C_{ol} = 3.80 + (A_{ol} * 6.0 * 10^{-5}) (R^2 = 0.87) \quad (1)$$

$$C_{lin} = 3.25 + (A_{lin} * 5.0 * 10^{-5}) (R^2 = 0.93) \quad (2)$$

with A_{ol} , A_{lin} as the areas (in mV) under peaks, and C_{ol} , C_{lin} as the concentrations (μ g/ml) of oleic and linoleic acids, respectively. Detection limits were 3.80 μ g/ml for oleic acid and 3.25 μ g/ml for linoleic acid. When the concentrations of fatty acids were below these detection limits, we assigned their concentration as equal to zero.

Effect of Oleic and Linoleic Acids on Necrophoric Behavior We aimed to test how increasing quantities of oleic and/or linoleic acid influence corpse removal behavior. Since freshly killed corpses rarely elicit necrophoresis, they were used to test the extent to which the addition of fatty acid extracts on a corpse can trigger its removal. Just after death, freshly killed corpses were placed on filter paper and 0.5 μ l of oleic and/or linoleic acid solution were applied using a microcapillary tube. After letting the hexane evaporate at room temperature for 5 min, the corpse was placed near the nest entrance to test for necrophoresis.

The maximum amounts of fatty acids used were equivalent to those detected on corpses of nestmates that were dead for 2 to 6 d. For this range of time since death, we observed a high removal rate as well as the highest amounts of fatty acids per corpse. The maximum amount of fatty acid put on one corpse was 1.6 μ g for linoleic acid and 1.4 μ g for oleic acid. We prepared stock solutions of oleic, linoleic, and oleic plus linoleic acids in hexane (Sigma-Aldrich, $\geq 99\%$). Preliminary experiments were done to quantify the loss of material following application to a corpse due to contact with the filter paper. The amount of oleic or linoleic acids remaining on a corpse after the application of a known amount of these compounds was determined by GC analysis. We estimated this loss factor and compensated for it by increasing the fatty

acids concentrations by a factor of 1.64. Therefore, stock solutions had concentrations of linoleic and oleic acids of 5.2 and 4.6 $\mu\text{g}/\mu\text{l}$, respectively, and 0.5 μl were applied to freshly killed corpses.

In order to evaluate the necrophoric response threshold of ants towards these fatty acids, we prepared 1/10, 1/100, and 1/1000 dilutions of the stock solutions. We also tested a blended solution of both fatty acids at the highest concentration (corresponding to one corpse equivalent) as well as pure hexane used as a control. For each fatty acid and concentration, as well as for the blend of both fatty acids at maximum concentration, we tested 4 corpses on each of the 5 colonies ($N=20$).

Data Analysis None of the data followed a normal distribution, and all analyses were performed with non-parametrical tests or with generalized linear model (GLM) when it was possible.

Indices of aggression were compared using Kruskal-Wallis tests, followed by Behrens-Fisher Test for *post-hoc* multiple comparisons. The proportion of removed corpses followed a binomial distribution. Then, we used a GLM based on a binomial distribution (link = log) for mean comparisons. Multiple comparisons after the GLM were performed using a Tukey test. The quantities of fatty acids on corpses at different times since death were compared using Kruskal-Wallis tests, followed by Behrens-Fisher Test for *post-hoc* multiple comparisons. The quantities of fatty acids on removed and non-removed corpses were compared using a Wilcoxon rank sum test. Finally, we compared the proportion of removed corpses with increasing quantities of applied fatty acids using Pearson's *chi-squared* test.

All means are given with standard deviations. All tests are two-tailed with alpha set at 0.05. We used R 2.12.2 (<http://www.r-project.org>) for all statistical analyses, modeling and artwork.

Results

Comparison of Behavior Towards Homocolonial or Heterocolonial Corpses Overall, 87.6 % of the observed aggressive behaviors were bites, and 9.5 % were attacks. Nestmate corpses were not treated like alien corpses, especially during the first days after death (Fig. 1). On average, the index of aggression for nestmate corpses was 0.27 ± 0.30 ($N=100$) and was not influenced by the time elapsed since death (Kruskal-Wallis test, $KW=7.69$, $df=4$, $P=0.104$). The behavior of ants was quite different when contacting a corpse originating from another colony. The index of aggression towards alien corpses up to 2 d post-mortem was 0.50 ± 0.35 ($N=60$), and this was higher than towards nestmate corpses

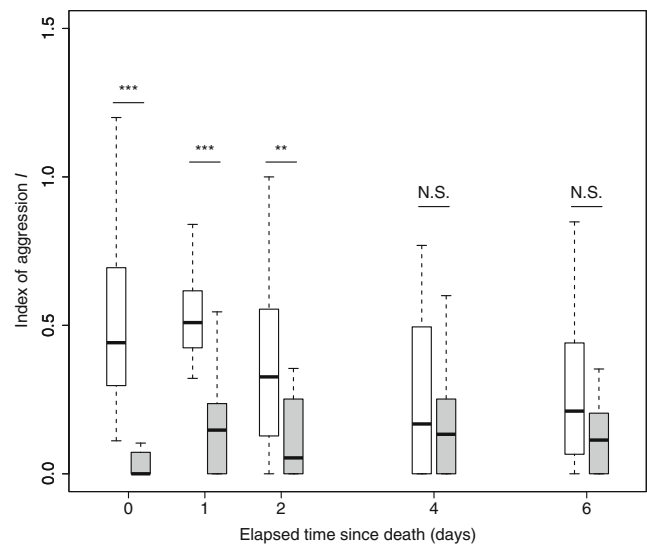


Fig. 1 Index of aggression towards corpses at different times since death. *White*: alien corpses ($N=20$). *Grey*: nestmate corpses ($N=20$). Above each pair of boxplots, indices of aggression were compared between homocolonial and heterocolonial corpses for different times since death using Wilcoxon rank sum tests. (N.S. : $P>0.05$, ** : $P<0.01$, *** : $P<0.001$)

(Wilcoxon rank sum test, $P<0.01$). Such aggressive behavior towards alien corpses decreased with time (Kruskal-Wallis test, $KW=17.95$, $df=4$, $P=0.001$): the index of aggression was no longer different from that observed for nestmate corpses at 4 d post-mortem (0.27 ± 0.28 , $N=20$) (Wilcoxon rank sum test, $P>0.05$). These results indicate that workers discriminated between alien and nestmate corpses only for 2 d after death.

In addition to changes in aggressiveness, we investigated whether the removal of corpses was influenced by their hetero- or homocolonial origin as well as by the time elapsed after death (Fig. 2). First, we tested if some colonies systematically removed more corpses than others, but we found no significant influence of colony on the proportion of removed corpses (GLM, $z_{4,199}=-1.66$, $P=0.090$). The interaction between corpse origin and the time since death had a significant influence on the proportion of removed corpses (GLM, $z_{9,199}=2.45$, $P=0.014$). Therefore, we tested separately the influence of these two factors. There was no significant influence of the time since death on the removal rate of alien corpses that were always quickly removed at rates higher than 70 % (GLM, $z_{4,199}=0.93$, $P=0.353$). On the other hand, for nestmate corpses, the time since death influenced the proportion of removed corpses (GLM, $z_{4,199}=3.67$, $P<0.001$). While freshly killed corpses were weakly removed (10 % removal), the removal rate began to significantly increase one-day after death, and thereafter reached values higher than 80 % removal rate (Tukey test, $z_{1,199}>4.039$, $P<0.001$). As regards the influence of heterocoloniality, alien corpses were more frequently removed than nestmate ones only for freshly-killed

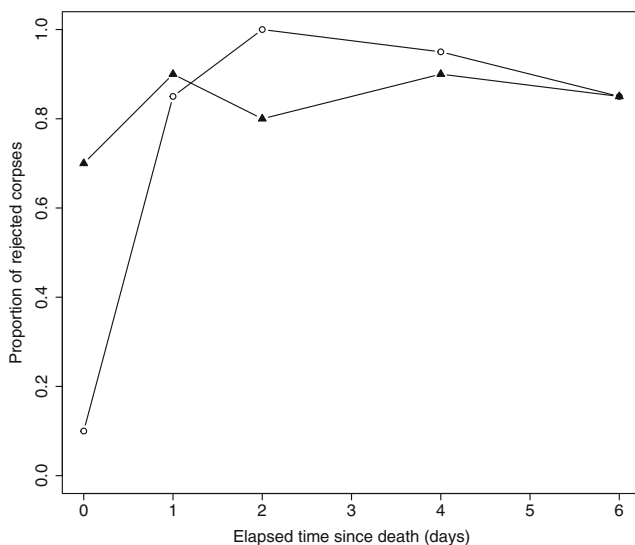


Fig. 2 Proportion of removed corpses as a function of time since death. *White circles*: nestmate corpses ($N=20$); *black triangles*: Alien corpses ($N=20$)

ants (GLM, $z_{1,199}=-3.41, P<0.001$). For corpses dead for one to six days, there was no difference in the removal behavior of ants with hetero- or homocolonial origin (GLM, $z_{1,199}<0.617, P>0.537$).

Changes in Fatty Acids After Death We quantified changes in the chemical profiles of the cuticles of corpses following death, which may induce corpse removal. Using GC and GC/MS analyses, we identified two compounds that appeared on the ant cuticle after death as linoleic and oleic acids (Fig. 3). Changes in the amount of these compounds were quantified and averaged per corpse as a function of time since death (Fig. 4). The amount of linoleic and oleic acid increased with time post-mortem (Kruskal-Wallis test, linoleic acid: $KW=61, 0, df=4, P<0.001$; oleic acid: $KW=71, 0, df=4, P<0.001$). We never detected linoleic or oleic acid on freshly killed corpses (detection threshold $0.35 \mu\text{g/corpse}$). One day after death, the average quantity of fatty acids was $0.252 \pm 0.305 \mu\text{g/corpse}$ ($N=40$) for linoleic acid, and $0.320 \pm 0.364 \mu\text{g/corpse}$ ($N=40$) for oleic acid. These amounts were higher than on freshly killed corpses (Behrens-Fisher test, linoleic acid: $t_{2,39}=5.6, P<0.001$; oleic acid: $t_{2,39}=5.6, P<0.001$). From day 2 to day 6 after death, the quantity of linoleic acid continued to increase and reached $0.530 \pm 0.427 \mu\text{g/corpse}$ ($N=120$). Likewise, the quantity of oleic acid increased up to $0.826 \pm 0.602 \mu\text{g/corpse}$ ($N=120$). The quantities of fatty acids on corpses of 2 to 6 days since death were not different from one another (Behrens-Fisher Test, linoleic acid: $t_{2,39}<1.7, P>0.257$; oleic acid: $t_{2,39}<1.0, P>0.681$) but they were higher than the quantities detected on fresh corpses or on corpses of 1 day after death (Behrens-Fisher-Test, linoleic acid: $t_{2,39}>3.4, P<0.01$; oleic acid: $t_{2,39}>3.7, P<0.001$).

Effect of Oleic and Linoleic Acids on Necrophoric Behavior Out of the 100 nestmate corpses tested, the ants removed a total of 75. Nestmate corpses that were not removed, whatever their age, had an average combined quantity of linoleic and oleic acids of $0.33 \pm 0.71 \mu\text{g/corpse}$ ($N=25$) (Fig. 5). The combined amount of these two fatty acids was higher on corpses that were removed ($2.23 \pm 5.51 \mu\text{g/corpse}$, $N=25$) (Wilcoxon rank sum test, $W=1442, N=20, P<0.001$). Corpses that had more than $2.5 \mu\text{g}$ of linoleic plus oleic acid on their cuticle were always removed by nestmates, indicating

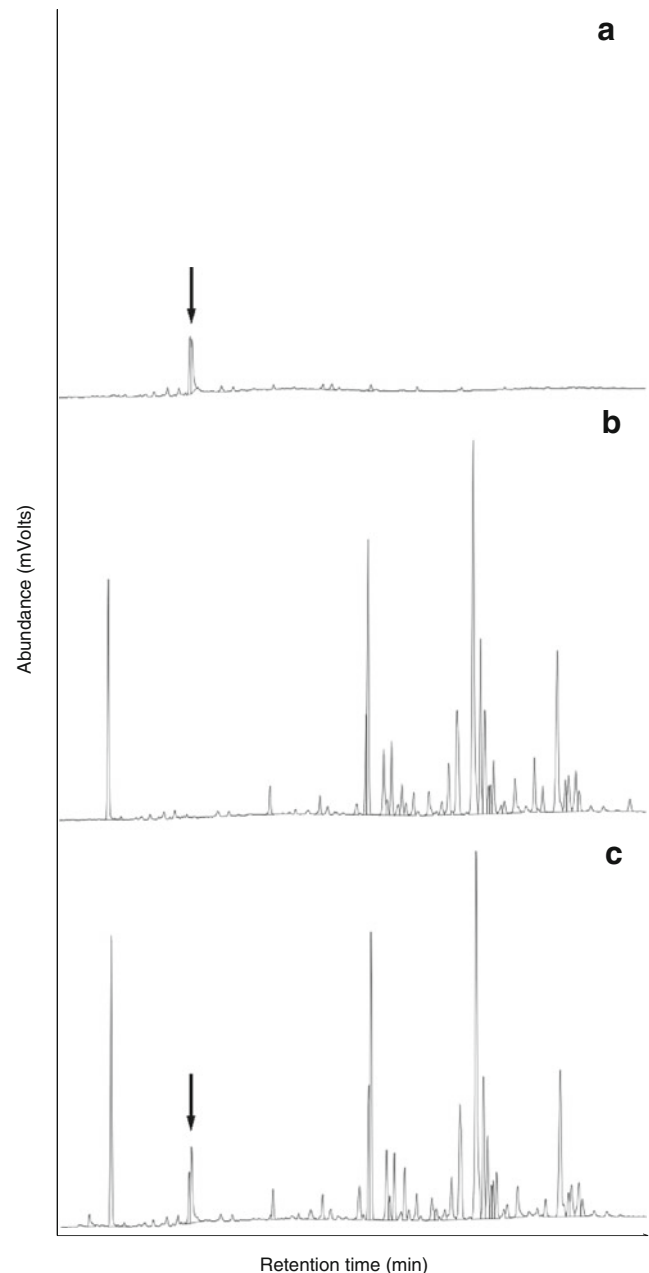


Fig. 3 Gas chromatograms. **a** Oleic and linoleic acid standards. **b** Freshly killed *Myrmica rubra* corpse. **c** *M. rubra* corpse 2 d after death. *Black arrows* indicate oleic and linoleic acids

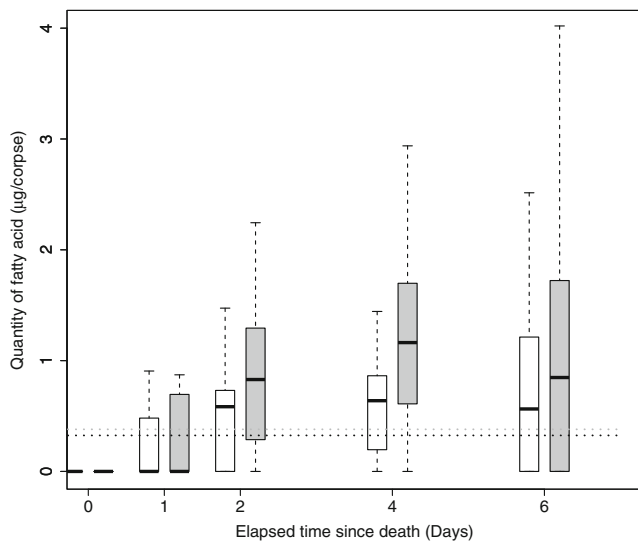


Fig. 4 Quantity of fatty acids found on corpses as a function of the time elapsed since death. *White*: linoleic acid ($N=40$). *Grey*: oleic acid ($N=40$). *Black dash line*: detection limit of linoleic acid. *Grey dashed line*: detection limit of oleic acid

that these two fatty acids may play an important role in corpse removal.

To demonstrate the role of linoleic and/or oleic acid in necrophoresis, we added extracts of these fatty acids to freshly killed corpses and quantified resulting changes in the rate of removal (Fig. 6). First, the addition of hexane solvent on corpses had neither attractive nor repulsive effects on ants: the number of contacts towards corpses (32.8 ± 37.0 , $N=20$) was similar to that towards freshly killed corpses without any addition of solvent (35.8 ± 35.9 , $N=20$) (Wilcoxon rank sum test, $W=1003$, $N=20$, $P=0.279$). Control corpses (on which

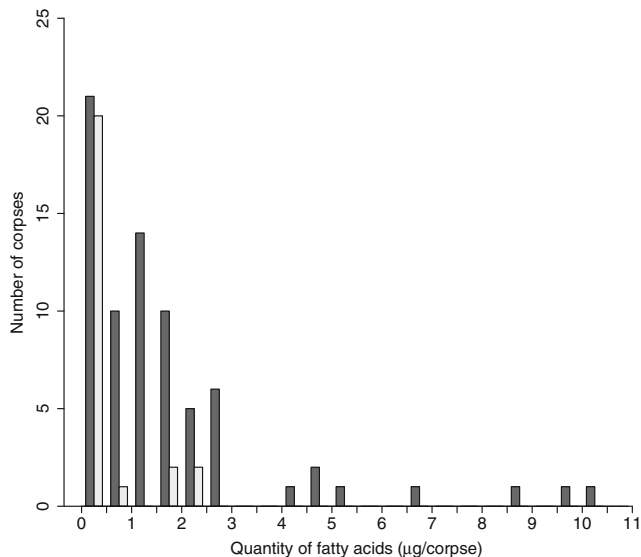


Fig. 5 Frequency distribution of nestmate corpses as a function of quantity of fatty acids (oleic plus linoleic acid) detected on their body. *Light grey*: Non-removed corpses ($N=25$). *Dark grey*: Removed corpses ($N=75$)

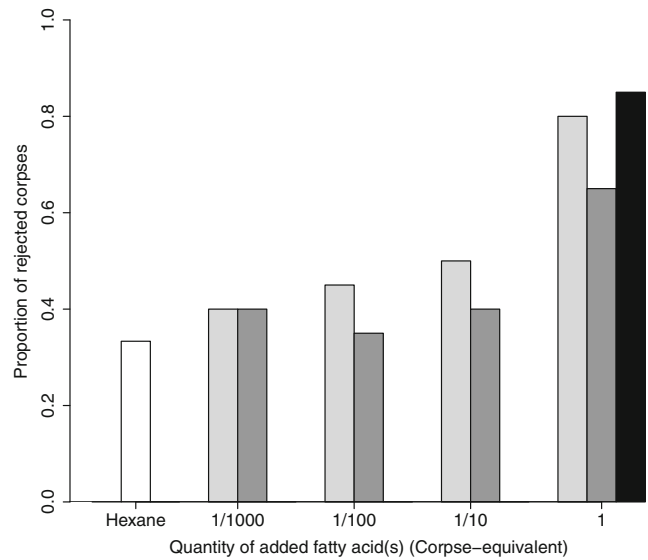


Fig. 6 Proportion of removed corpses as a function of the quantity of fatty acid(s) added on the corpse. One corpse-equivalent is equal to $1.6 \mu\text{g}$ for linoleic acid and $1.4 \mu\text{g}$ for oleic acid ($N=20$). *White*: control (hexane), *Light grey*: Linoleic acid, *Dark grey*: Oleic acid, *Black*: Linoleic + oleic acids

hexane was added) induced more removal events (33 %) than freshly killed corpses (16 %) (Pearson's *chi-squared* Test, $X^2=4.3$, $N=20$, $P=0.038$). However, the removal rate of freshly killed corpses became even higher when we added the same quantity of fatty acids as the mean amount found on a corpse of 2 to 6 days after death (linoleic acid: $1.6 \mu\text{g}$; oleic acid: $1.4 \mu\text{g}$). This induced a removal rate of 80 % with added linoleic acid, 65 % with added oleic acid and 85 % with both fatty acids added. All these rates of corpse removal were higher than for the control (corpse with added hexane: 33 %) (Pearson's *chi-squared* Test, Linoleic acid: $X^2=8.7$, $N=20$, $P=0.003$; Oleic acid: $X^2=4.8$, $N=20$, $P=0.041$; both fatty acids: $X^2=10.9$, $N=20$, $P<0.001$). We also tested the effect of smaller amounts of fatty acids that were equivalent to 1/10, 1/100, or 1/1000 of maximal quantities found per corpse. The addition of these small amounts on a corpse did not increase its removal rate relative to control corpses (Pearson's *chi-squared* Test, $N=20$, $P>0.05$).

Discussion

During their lives, social insects constantly use chemical cues for recognition, namely discriminating nestmates from strangers (d' Ettore and Lenoir 2009). After death, changes in these chemical cues are expected to lead to the recognition of corpses, and to the adaptation of worker behavior towards corpses. Short-term changes in corpse odors are assumed to trigger corpse removal from the nest. In *L. humile*, a change in the cuticular profile of corpses within 40 min of death induces corpse removal (Choe et al. 2009). Indeed, dolichodial and

iridomyrmecin that are produced and stored in the pygidial gland disappear from an ant's cuticle, suggesting that these compounds act as a "life signal" for nestmates. In *Temnothorax lichtensteini*, fresh corpses are buried mainly in the nest, whereas older (16 hr) corpses are mainly transported to a new location inside or outside the nest (Renucci et al. 2010). These different behaviors suggest that chemical cues associated with death have evolved and differ according to the time since death. The cause of death can also influence corpse removal behavior, probably by influencing the odors released from the corpse. In *Solenopsis invicta*, ants killed by *Beauveria bassiana* are removed more quickly than freeze-killed ants (Fan et al. 2012).

Short-term changes in the chemical profiles of dead ants are important cues that will trigger the removal of corpses from the nest. While some workers deposit corpses near their nest entrance (Diez et al. 2012; Howard and Tschinkel 1976) and some ants even die outside the nest (Bos et al. 2012; Heinze and Walter 2010), the ability of workers to recognize corpses will affect how ants clean the surroundings of the nest entrance over the longer-term. In *M. rubra*, we have shown long-term after-death changes in the chemical profile of non-volatile compounds such as fatty acids. Amounts of both oleic and linoleic acids are below the detection level on freshly-killed corpses, which were rarely removed. Nevertheless small amounts of these compounds are certainly present in live and freshly killed ants, as about 0.2 μg total of fatty acids was found in *M. rubra* heads (Brian 1973). Oleic and linoleic acids quantities increase at the same rate as the likelihood of removal, *i.e.*, increasing within the 24 hr post mortem and then being stable up to 6 d after death. The timing of the increase of fatty acid quantities in *M. rubra* is of the same order of magnitude as for other ant species. In *Atta Mexicana* and *Formica japonica*, oleic acid appears, respectively, within 10 and 48 hr of death (Akino and Yamaoka 1996; López-Riquelme et al. 2006). Oleic acid probably results from the enzymatic hydrolysis of triglycerides in the corpse (Akino and Yamaoka 1996) and, as well as linoleic acid, it is a common component of lipids in insect fat bodies (Gilby 1965). Those fatty acids that are released after death probably reach the cuticle surface through pore canals and epicuticular channels, which are the usual pathways for lipids to reach the body surface (Lockey 1988).

Our work confirms that linoleic and oleic acids are important chemical cues involved in necrophoresis, and addition of linoleic and oleic acids to a fresh corpse increases necrophoresis by *M. rubra* workers. Similarly in *Pogonomyrmex barbatus*, *A. mexicana* and *M. vindex*, the addition of pure oleic acid on filter paper or on a cocoon induces its transport to waste piles (Haskins and Haskins 1974; López-Riquelme et al. 2006; Wilson et al. 1958). In the termite *Reticulitermes virginicus*, workers show higher burial behaviors towards dummies treated with oleic acid (Ulyshen and Shelton 2012). On the other hand,

for alates of *Pseudacanthotermes spiniger* termites, fatty acids can trigger burial behavior providing they are combined with indole and phenols (Chouvenc et al. 2012). Furthermore, in honeybees, fatty acids including oleic and linoleic acids seem to be involved in hygienic behavior against the parasitic mite *Varroa destructor*: honeybees from *Varroa*-resistant hives show higher responses to these mites' fatty acids than bees coming from mite-susceptible colonies (Martin et al. 2002).

The role of oleic and linoleic acids as the chemical triggers of necrophoresis in social insects could have evolved from a general avoidance behavior elicited by these fatty acids. For instance, in the context of exploration in *Crematogaster scutellaris*, *Formica cunicularia*, and *Lasius* sp., oleic and linoleic acids have a repellent effect at a dose of 2.5 μg , which is the same as the dose triggering 100 % removal in our experiments. This repellent effect seems to be specifically induced by these two acids since other saturated fatty acids (lauric, myristic, palmitic, and stearic) have no repellent effect on these species, even at higher doses (Dani et al. 1996). Likewise, in gregarious species, oleic and linoleic acid also act as a repellent: cockroaches and isopodes avoid shelters covered with these fatty acids, as well as shelters covered with dead conspecific extracts (Rollo et al. 1994).

While fatty acids are involved in corpse removal, it is surprising that the same compounds can also trigger opposite behaviors such as retrieval into the nest. In many social species, oleic and linoleic acids trigger the removal of diseased or dead conspecifics outside the nest, while oleic acid can also elicit the retrieval of dummies into the nest, as in *P. badius* (Gordon 1983). One can hypothesize that it is the association of fatty acids with cuticular hydrocarbons, together with the context, that determines ant behavior. Fatty acids are widely present on the cuticle of social insects (Cavill et al. 1970; Liebig et al. 2000). (*e.g.*, *Apis mellifera*, *Harpegnathos saltator*, *Myrmecia gulosa*) as well as non-social insects that are potential prey for ants (*e.g.* *T. molitor*, *Melanoplus bivittatus*, *Leptinotarsa decemlineata*) (Lockey 1988). In a context of foraging, fatty acids associated with the odor of an insect prey could then elicit retrieval to the nest. Likewise, oleic acid is found in seeds and elaiosomes, and has been identified as a main cue triggering seed retrieval by ants (Brew et al. 1989; Greene et al. 2013; Marshall et al. 1979; Pfeiffer et al. 2010; Skidmore and Heithaus 1988). On the other hand, in the context of waste management inside the nest, fatty acids on corpses that are associated with colonial odors as well as on inedible cuticular parts of prey would trigger a centrifugal movement, *i.e.*, their removal outside the nest.

Moreover, the chemical context—the presence of homocolonial or heterocolonial odors—also influences ants' behavior towards corpses, as it influences behavior towards live ants (d' Ettore and Lenoir 2009). In the field, *M. rubra* ants from other colonies occasionally can walk around a nest

entrance and can be met during competition for resources (Garnas et al. 2007). If workers encounter these alien ants, they will probably attack the stranger and may kill it. Therefore, alien corpses may be found near the nest, and have to be transported away for hygienic reasons. In our study, fatty acids associated with colonial odor elicited necrophoresis and low aggressiveness towards dead nestmates. On the other hand, alien corpses—which bear another cuticular profile of hydrocarbons as well as fatty acids after 24 hr—elicited both a high rate of removal and numerous aggressive behaviors. This shows the importance of both colonial odor and fatty acids in the discrimination of corpses and further decision-making. After more than 4 d post-mortem, aggressive behaviors decreased to the level seen with nestmate corpses, indicating that the discrimination of alien corpses may be more difficult to achieve after this time. After death, alien ants are not a threat by themselves, but as nestmate corpses they can carry pathogens. The adaptive response towards an alien corpse is to carry it away rather than to attack and bite it. Thus, fatty acids may act as a cue that elicits the most appropriate behavior towards alien dead corpses.

This work has shown the role played by fatty acids in the removal of corpses far from the nest vicinity. As these fatty acids appear on *M. rubra* corpses only after one day since death, it will be worth investigating whether—beside non-volatile fatty acids—the profile of volatile odors undergoes changes within the few hours following death and also acts upon corpse removal. Furthermore, since it is imperative for the colony to remove sources of infection quickly, one may wonder whether differences in chemical cues on the cuticle of diseased workers can be used by ants to discriminate infected corpses on the basis of their contaminating potential and to elicit specific behaviors such as burial or accelerated necrophoresis (Choe et al. 2009; Renucci et al. 2010). It also could be interesting to investigate whether the association of fatty acids with other compounds in preys could trigger different behaviors such as retrieval to the nest, consumption and removal.

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