Sci Nat (2016) 103: 70
DOI 10.1007/s00114-016-1394-8

ORIGINAL PAPER

Ants detect but do not discriminate diseased workers within their nest

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Received: 13 May 2016 / Revised: 6 July 2016 / Accepted: 11 July 2016 / Published online: 30 July 2016

Abstract Social insects have evolved an array of individual and social behaviours that limit pathogen entrance and spread within the colony. The detection of ectoparasites or of fungal spores on a nestmate body triggers their removal by allogrooming and appears as a primary component of social prophylaxis. However, in the case of fungal infection, one may wonder whether ant workers are able to detect, discriminate and keep at bay diseased nestmates that have no spores over their cuticle but which constitute a latent sanitary risk due to post-mortem corpse sporulation. Here, we investigate the ability of *Myrmica rubra* workers to detect and discriminate a healthy from a diseased nestmate infected by the entomopathogen *Metarhizium anisopliae*. During dyadic encounters in a neutral location, workers were more aggressive towards isolated sick nestmates on the 3rd post-infection day. However, no such detection or discrimination of fungus-infected nestmates occurred in a social context inside the nest or at the nest entrance. Gatekeepers never actively rejected incoming diseased nestmates that rather spontaneously isolated themselves outside the nest. Our study reveals that ant workers may detect health-dependent cues and that their 'acceptance level' of sick nestmates is tunable depending on the social context. This raises questions about possible trade-offs between a social closure to pathogens and risks of erroneous rejection of healthy nestmates. Social isolation of moribund ants also appears as a widespread prophylactic strategy of social insects allowing them to reduce exposure to pathogens and to spare costs associated with the management of infected individuals.

Communicated by: Alain Dejean

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Keywords Ants · Social immunity · Entomopathogenic fungi · Context-dependence · Nestmate recognition

Introduction

Benefits derived by ants, honeybees, wasps or termites from being eusocial are counterbalanced by increased risks of pathogen transmission. An overlapping of generations as well as a high genetic relatedness between workers make social insects highly susceptible to diseases of which the outbreak is favoured by their life at high densities within the confined space of the ant nest or hive (Myers and Rothman 1995; Schmid-Hempel 1998; McCallum et al. 2001; Boomsma et al. 2005). Therefore, parasites and contagious diseases are recognised as important driving forces in the life history of social species (Tompkins and Begon 1999; Hughes et al. 2016), especially in ground dwelling insects like ants that naturally coexist with several soil entomopathogenic fungi or bacteria (Schmid-Hempel 1998). In ants, there are several lines of defence against sanitary risks that occur at the level of the individuals. First, the cuticle that is covered with antibiotic compounds secreted by metapleural glands provides a physical and chemical barrier against the propagation and penetration of pathogens (Hölldobler and Wilson 1990; Schlüns and Crozier 2009; Yek and Muller 2011). If a parasite nevertheless happens to penetrate the host hemolymph, the immune system of the individual will be stimulated and will produce melanin and microbiocides proteins that facilitate its phagocytosis and its encapsulation (Schmid-Hempel 2005; Siva-Jothy et al. 2005). In addition to these individual responses to threats from pathogens, social insects have evolved cooperative behaviours that result in the avoidance, control or elimination of infectious sources. These behaviours are considered parts of a 'social immunity', since they benefit not only the fitness of

individuals by reducing their investment in innate immunity but also that of the whole colony, by improving group level resistance to pathogens (Cremer et al. 2007; Wilson-Rich et al. 2009; Cotter and Kilner 2010).

The ability of workers to early and efficiently detect the presence of pathogens is a crucial step in social immunity since it is a prerequisite to further trigger the avoidance, removal or cleaning of sources of contamination (Schmid-Hempel 1998; Hughes et al. 2002). In this respect, ectoparasites that are present over the body of adult honeybees or ant workers are usually detected and trigger mutual cleaning—also called allogrooming. This appears as a basic and very effective way for social insects to remove pathogens such as *Antennophorus grandis* mites (Berlese 1903) and also spores of entomopathogenic fungi, such as *Metarhizium anisopliae* (Hughes et al. 2002; Walker and Hughes 2009) and *Beauveria bassiana* (Oi and Pereira 1993) fungi. In the case of ant societies, the increased allogrooming of fungus-infected nestmates (Bos et al. 2012; Walker and Hughes 2009) significantly reduces the pathogen load and the risk of disease outbreak inside the nest (Reber et al. 2011), even though mutual cleaning does not seem to rely on a specific recognition of infectious spores. Instead, it appears as a constitutive line of defence against any external contaminants—biological or chemical—present on the cuticle regardless of the associated sanitary risk (Gratwick 1957; Hlavac 1975; El-Awami and Dent 1995).

In addition to allogrooming, social insects having detected spores may display more specific hygienic behaviours such as the active removal or avoidance of contaminated nestmates. A classic example involves the hygienic behaviour of the honeybee *Apis mellifera* of which workers detect diseased brood, uncap their cells and remove infected larvae from the nest (Boecking et al. 1992; Boecking and Spivak 1999; Spivak and Reuter 2001). Likewise, ant workers are able to selectively remove brood covered with conidia (e.g. *M. anisopliae* conidia in *Cardiocondyla obscurior*: Ugelvig et al. 2010). Marikovsky (1962) also reported the avoidance of *Formica rufa* corpses covered with conidia, which are not removed or consumed, in contrast to non-sporulating cadavers. There is thus ample evidence that ants can detect spores that are present either on the body of living workers just after their contamination or that are produced by sporulating corpses several days post-mortem. In contrast, it is unknown whether workers are able to reduce sanitary risks associated with infected nestmates when there are no external pathogens present on their cuticle. Indeed, between the two critical periods of initial fungal infection of living workers and sporulation of their dead bodies, there is an internal phase of the fungus during which it grows and expands inside the insect body until the death of the infected individual (Hänel 1982). During this internal phase, the infected ants are no longer infectious because all viable spores have penetrated their cuticle (Boucias and Pendland 1998; Gillespie et al. 2000). One may wonder whether ants can detect a diseased individual during this period of 'hidden' infection since they constitute a latent risk of contamination due to the post-mortem sporulation of their corpses. Beyond this ability to anticipate post-mortem sanitary risks of sporulation, this raises more general questions about the ability of ants to detect and react to health-dependent cues.

The present study investigates whether *Myrmica rubra* ants are able to determine the health status of living nestmates having been exposed to the entomopathogenic fungus *M. anisopliae*. First, we assess whether ant workers are able to discriminate an infected nestmate by carrying out daily dyadic encounters starting from its initial exposure to fungal spores until its death. Then, within the less artificial environment of the ant nest, we track the time evolution of the type and rate of social interactions that are displayed towards a nestmate before and after its infection by the fungus. Finally, we compare patterns of interactions with infected workers at the nest entrance since it is a key place for checking and preventing nest intrusion not only by foreign ants but also by diseased nestmates (Honey bees: Couvillon and Ratnieks 2008; ants: Hölldobler and Wilson 1990). A better knowledge on how *M. rubra* ants assess the health status of fungus-infected nestmates even in the absence of spores will highlight their ability to anticipate sanitary risks and to accordingly adjust their prophylactic behaviours and social interactions.

Material and methods

Maintenance of ants and contamination protocol

Four queenless colonies of 200 *M. rubra* workers and 40 larvae of the first two instars were used for each experiment. In the laboratory, each colony was housed in a plastic box (47 × 29 cm) with a plaster floor. Borders were coated with polytetrafluoroethylene Fluon (Whitford, UK) to prevent ants from escaping. Square glass plates of 10 cm wide, placed 3 mm above the ground and covered with a red filter were used as nests (total nest area of 49 cm^2 (7 × 7 cm), see Fig. 2). Colonies were fed with a mealworm (*Tenebrio molitor*) three times per week while water and sucrose solution (0.3 M) were provided ad libitum. Laboratory conditions were kept at 21 ± 1 °C and (50 ± 5 % humidity rate, with a constant photoperiod of 12 h/day.

All tested ants were taken out from colonies of equal size, kept within the same nest type and were fed with the same food type for more than one month in order to standardise environment-related cues that may influence the cuticular profile of workers. Likewise, all colonies were orphaned since queens would make the cuticular profile more variable, more dynamic and thereby more likely to blur health-related changes of the cuticular profile. We also carried out preliminary experiments that compared how ants discriminate between five nestmates and five alien workers

in four queenright and four queenless colonies ($N = 20$ individuals tested for each condition). Workers in queenless colonies were found to be as aggressive towards alien ants as workers from queenright colonies (91 and 89 % of the first ten interactions consisted in bites in queenless and queenright colonies, respectively. Mann-Whitney test: $U = 0.14, N1 = 20, N2 = 20, P = 0.93$). Workers in both queenless and queenright colonies never show any agonistic behaviours towards nestmates. Thus, orphaning of *M. rubra* colonies did not seem to alter the discrimination and recognition abilities of workers, as it is the case for several other ant species (Ichinose 1991; Lahav et al. 1998).

The infection vector was the *M. anisopliae* fungus that is commonly used in studies relating to social immunity in ants (Hughes and Boomsma 2004; Chapuisat et al. 2007; Reber and Chapuisat 2012), bees (Butt et al. 1998) and termites (Calleri et al. 2006). We used a commercial strain of Metarhizium fungus (Strain F52 from Novozymes), that allows us, beyond the practical advantages in terms of conservation and use, to have a constant pathogenicity and virulence of the pathogen throughout the experiments. This generalist entomopathogenic fungus is known to infect more than 200 insect species (Osborne and Landa 1992; Shah and Pell 2003; Meyling and Eilenberg 2007), requires direct contact to be transmitted to the host and occurs in the natural habitat of *M. rubra* ant species (Groden 2005; Rodrigues et al. 2005; Yan 2005, but see, Evans et al. 2010). Before starting the behavioural experiments, we had to infect a set of ant workers that were randomly sampled out of the foraging area of each tested colony. We chose to infect foragers because they are the most exposed to pathogens under natural conditions when they explore the outer environment or exploit food resources. Groups of five healthy foragers underwent the following standardised infection protocol. Once taken out from the foraging area of their colony, they were put together in an Eppendorf (1 ml) with one sporulating corpse of a nestmate. This sporulating corpse was obtained by previously placing a *M. anisopliae*-infected dead ant over a filter paper soaked with 6 ml distilled water in a closed Petri dish and by incubating it at a temperature of 25 °C for 14 days. Finally, the Eppendorf that contained the five nestmates and the sporulating corpse was then vortexed four times during 10 s at a speed of 2000 rounds/min (Fig. 1).

After having undergone this infection protocol, each of the five foragers was kept separately in an individual box (18 × 3 cm) for 1 h and was provided with water and sucrose (0.3 M) *ad libitum,* before being returned to its mother colony. This 1-h isolation aimed at increasing the success of infection by limiting the removal of fungal spores via allogrooming (Okuno et al. 2012; Zhukovskaya et al. 2013). As a control group, five additional ants that were sampled from the same colony underwent the same protocol, excepting that no sporulating corpse was put in the Eppendorf. When the experiment required the marking of infected or control individuals, a dot of paint (Edding paint marker 751, Edding AG, Ahrensburg, Germany) was deposited on the ant abdomen. Marked individuals were isolated for one extra-hour to allow paint to dry, before being returned to their mother colony. Finally, data were only taken into account if the infected status of the ant was later confirmed by a post-mortem sporulation of the fungus over its corpse. In total, 81.3 % of the ants infected with *M. anisopliae* spores died from the infection.

Dyadic encounters

We investigated whether *M. rubra* workers specifically recognise a fungus-infected nestmate by measuring daily changes in their interactions during dyadic encounters. For each focal ant, three dyadic encounters were carried out per day for 5 days, the first set of encounters being launched two hours after the infection procedure. Only tested ants (here called focal ants) that survive for at least 5 days were taken into account. As a result, 31 healthy and 30 infected ants were tested in total with eight individuals per condition being drawn out of each colony (excepting one healthy ant missing for the 4th nest and one infected ants that died before the end of the experiment in the 3rd and 4th nests). In addition, five individuals taken from four alien nests ($N = 20$) were tested during a 1-day session to get a reference value of *M. rubra* aggression level towards non-nestmates. Every day, the focal ant that was either healthy (control) or infected by *M. anisopliae*, was faced successively with three different 'checking' nestmates during dyadic encounters. Checking and focal ants were marked with a dot of different colours for identification. Five minutes before the encounter, the checking ant was individually placed in a circular arena of 9 cm diameter. The focal ant was introduced in this arena but was allowed to calm down by keeping it isolated within a Fluon-coated cylinder (1.5 cm diameter) for 1 min. The cylinder was then removed and all pairwise interactions with the checking nestmate were measured for 2 min, starting from the first contact. By following exactly the same protocol, we then transferred the focal ant into a second and finally a third arena, each time in the presence of a new checking ant. The three tests were done successively with the focal ant being allowed to calm down for 1 min inside the cylinder between each pairwise interaction. Between successive daily sets of three dyadic encounters, the focal ant was isolated in a separate box (18 × 3 cm) for resting and feeding. Behavioural observations allowed us to calculate indices of aggression (IA) inspired by Bos et al. (2012) (see also Errard and Heffetz 1997), as follows:

$$I = \frac{0 \cdot c + 1 \cdot t + 2 \cdot a + 3 \cdot b}{c + t + a + b}$$

c The number of contacts (no aggression with a score of 0): the checking ant touches the 'focal' ant with its antennae.

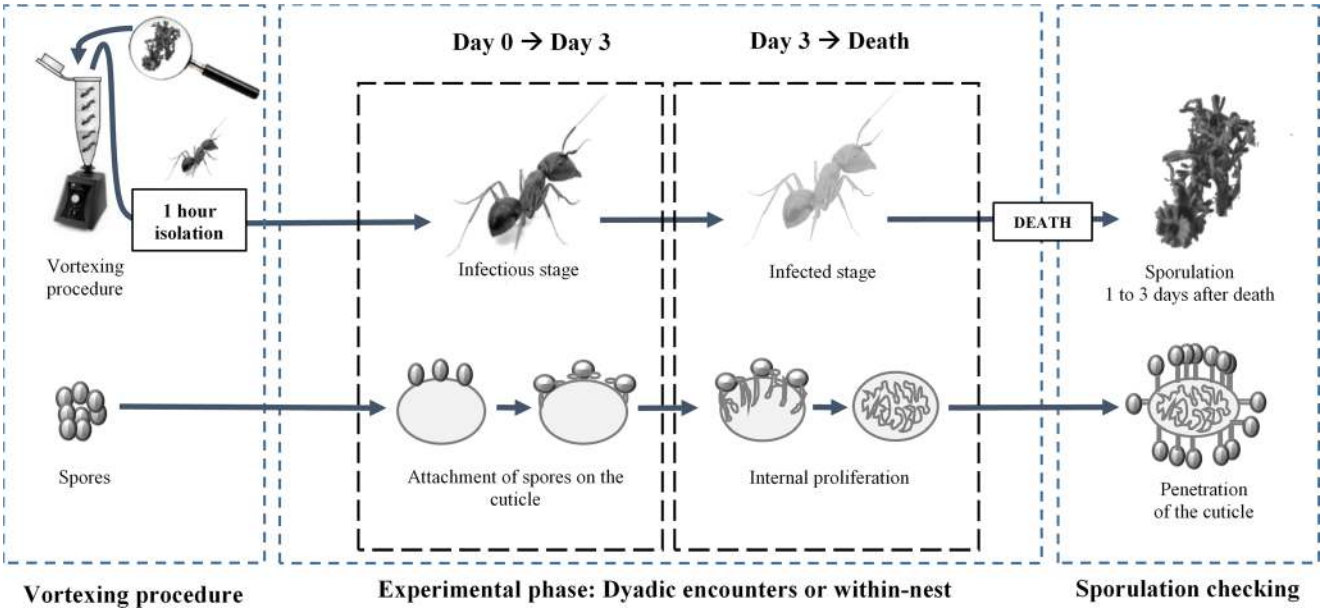

Fig. 1 Vortexing procedure and infection steps of the focal ants. Eppendorf containing five foragers and a sporulating corpse was vortexed for the infection procedure of the focal ants. Only five foragers without a sporulating corpse were used for the control condition

t The number of threats (low aggression score of 1): the checking ant opens its mandibles in front of the focal ant.

a The number of attacks (medium aggression score of 2): the checking ant made rapid movements back and forth when facing the focal ant.

b The number of bites (strong aggression score of 3): the checking ant strongly bites the focal ant, sometimes curving its abdomen and attempting to sting.

IA Values were averaged over the three checking nestmates in order to take into account possible differences in the genuine level of aggressiveness of ant workers. Daily changes in interactions and aggressiveness level during dyadic encounters were compared for healthy and fungus-infected focal ants in order to evidence whether and when workers may detect a worsening of the health condition of their conspecifics.

Interactions inside the nest

Since a conspecific may be tolerated in one recognition context but avoided or rejected in another (Starks et al. 1998; Tanner and Adler 2009) depending on the presence of congeners (Buczkowski and Silverman 2005; Downs and Ratnieks 2000), the level of familiarity or the value of resources at stake (Gamboa et al. 1991; Starks et al. 1998; Buczkowski and Silverman 2005), we also studied interactions of *M. rubra* workers with infected individuals within the social context of their nest. Focal ants first underwent the vortexing procedure without a sporulating corpse in order to assess the impact of this experimental procedure on behaviour of the ants (Fig. 1, $N = 22$ with six individuals being sampled out from four nests (excepting one ant missing for the 3rd and 4th nests that died before the end of the experiment)). They were then individually marked by using coloured dots and were all replaced in their mother colony after an isolation period of 1 h. Two hours after their nest reintroduction, we localised each marked ant and we recorded the first ten behaviours displayed by nestmates that contacted this focal ant. These behaviours consisted either in aggressive displays directed against the focal ant such as threat, attack and bite or in non-agonistic interactions such as antennal contact, allogrooming (as a 'donor' or as a 'receiver') and trophallaxis. In addition, the number of self-grooming events was counted for 10 min per day for each focal individual. These observations were carried out every day for 12 days, alternatively on morning and afternoon sessions. On the 6th day, all marked individuals were removed from their nest and again underwent the vortexing procedure but this time with a sporulating corpse in order to be fungus infected. After an isolation period of 2 h, they were observed for six extra-days, in the same way as described above.

Overall, each focal ant was individually followed for 12 days with a total of 120 behavioural data, that were compared before (days 1 to 6) and after the ant was infected by *M. anisopliae* fungus (days 7 to 12). In addition, we recorded the number of marked focal individuals that were seen at least once inside the nest by observing their location during 1 h each day (based on two 30-min sessions in the morning and in the evening). This allowed us to see whether their presence within the nest or in the foraging area changed with their closeness to death.

Interactions at nest entrance

We observed how workers react to the nest entry of nestmates that were infected or not ($N = 47$ healthy and 27 infected focal ants sampled out from four nests). All focal individuals first underwent the vortexing procedure, with or without a sporulating corpse for the infected and the healthy condition, respectively. After being isolated for 1 h, all individuals were marked with a dot of paint and then waited for one more hour before being replaced in their mother colony. All ants were tested 72 h later (3 days post-contamination) when most fungal spores had pierced the cuticle (Boucias and Pendland 1998; Gillespie et al. 2000; Arthurs and Thomas 2001) and when discrimination and rejection by nest guards is likely to occur as suggested by the lowered nest frequentation of diseased ants reported in several ant species (Heinze and Walter 2010; Bos et al. 2012). The experimental setup consisted of a small half-circular arena of 3.5 cm diameter that was centred in front of the nest entrance, making the nest the only way out (Fig. 2). Once the focal ant had reached the nest, it was prevented from leaving it by blocking the entrance with a plastic plug. The nest entrance area was then filmed until the focal ant moved deeper into the nest for at least 10 s. We recorded the time spent into the nest entrance area as well as all the behaviours displayed by nestmates towards the focal ant. Videos were analysed with Solomon Coder software (copyright András Péter), which calculated durations and frequencies of the following events: antennal contacts, allogrooming (as a donor or as a receiver), trophallaxies, threats, attacks, bites and selfgrooming.

Statistical analyses

Data were analysed with Statistica version 10 (Hammer et al. 2001). For all data, non-parametric tests with a significance level of alpha = 0.05 were used. As advocated by Oron and Hoff (2006) when data set needs a tie correction and parametric assumption is violated, we used permutation Kruskall-Wallis tests to analyse nested effects in our hierarchical design. We checked the homogeneity of colonies' responses by carrying out Kruskal-Wallis permutation tests that compared observed data distributions with randomised data distributions ($N = 1000$). Concerning dyadic encounters, colonies displayed similar levels of aggression towards infected congeners (permutation Kruskal-Wallis tests: Day 1: $H = 0.59$, $P = 0.89$; Day 3: $H = 3.27$, $P = 0.35$; Day 5: $H = 0.33$, $P = 0.95$). Likewise, no between-colony differences were observed towards healthy nestmates (permutation Kruskal-Wallis tests: Day 1: $H = 2.11$, $P = 0.5$; Day 3: $H = 0.99$, $P = 0.80$; Day 5: $H = 3.42$, $P = 0.33$). Concerning behaviours made within the nest, all P values were higher than 0.05 for each day and for all types of social interactions (antennal contacts, allogrooming, trophallaxis and self-grooming behaviour). Having checked for this between-colony homogeneity, we pooled behavioural responses for further statistical analyses.

Concerning the analysis of dyadic encounters, the temporal evolution of behaviour's frequencies as well as of indices of aggression were assessed using Friedman tests for repeated measures. The test compares the absolute value of the differences for all paired values with a critical value that is determined using a normal approximation with suitable adjustment of alpha to take the multiple comparisons into account. For each experimental day, the number of antennations as well as the aggression indices were compared towards healthy versus infected focal ants by using Mann-Whitney tests. Furthermore, the percentages of focal ants involved at least once in each agonistic behaviour were compared between infected and healthy individuals by using Chi-square tests.

Concerning ants' behaviours inside the nest, the temporal evolution of the percentages of antennal contacts as well as of social interactions with the focal ant were assessed using Friedman test for repeated measures. We used Cochran test for binary data to analyse how the percentages of focal ants involved in a specific social interaction evolved through time. The impact of infection was assessed by comparing for each observation day, with Chi-square tests, the percentages of healthy versus infected focal ants involved in trophallaxis, mutual grooming or self-grooming. As regards the location of focal ants, we evaluated with Cochran tests whether the level of nest attendance by marked individuals changed through time. For each experimental day, comparisons between percentages of healthy and infected focal ants seen inside the nest were done using Chi-square tests.

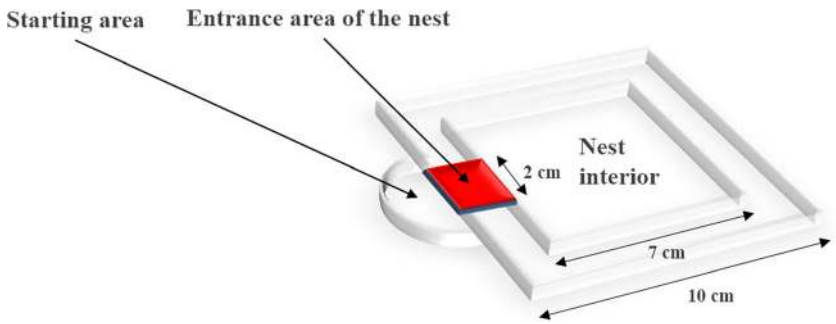

Fig. 2 Schematic setup of the experiment. Camera recorded all behaviours occurring in the entrance area of the nest (2 × 1.5 cm) until the 'focal' individual moved deeper into the nest

As for the nest entrance, the staying duration as well as the time spent performing a given behaviour were compared between healthy and infected ants by Mann-Whitney tests. Pairwise comparisons of percentages of healthy versus infected focal ants being involved in a given behaviour were done using Chi-square tests.

Results

Dyadic encounters

The total number of interactions of checking ants with infected nestmates increased over time (Friedman test for repeated measures: $\chi^2 = 31.77$, $N = 30$, $P < 0.001$). The same trend was observed with healthy ants but was slighter and not significant (Friedman test for repeated measures: $\chi^2 = 9.87$, $N = 31$, $P = 0.08$, Fig. 3). Fungus-infected ants were also more involved in interactions with checking congeners than healthy ones on the fifth day after the vortexing procedure (Mann-Whitney test: $U = 327$, $N1 = 31$, $N2 = 30$, $P = 0.047$). This evolution was not due to changes in the number of antennal contacts by checking individuals that did not differ through time (Friedman tests for repeated measures: $\chi^2 = 3.73$, $N = 30$, $P = 0.59$ and $\chi^2 = 3.11$, $N = 31$, $P = 0.68$ for the healthy and infected ants, respectively).

In contrast, the level of aggressiveness (IA) significantly increased over successive daily dyadic encounters both when the focal ant was healthy and was fungus-infected (Friedman test for repeated measures: $\chi^2 = 47.69$, $N = 31$, $P < 0.001$ for healthy ants; $\chi^2 = 64.29$, $N = 30$, $P < 0.001$ for infected ones (Fig. 4a)). The highest IA values were observed on the fifth day of dyadic encounters (healthy individuals: mean IA ± SD = 0.16 ± 0.03, $N = 31$; infected individuals: mean IA ± SD = 0.26 ± 0.04, $N = 30$) but remained quite low

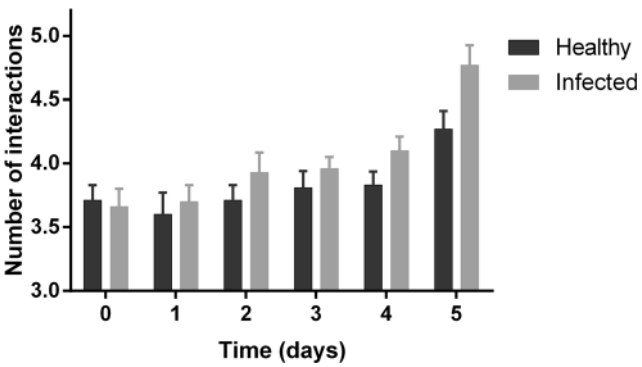

Fig. 3 Daily changes in the number (mean ± SD) of interactions performed during dyadic encounters with either an infected or healthy nestmates. Each focal individual was daily involved into three dyadic encounters of which we averaged the numbers of interactions per individual. Days correspond to time elapsed since the vortexing procedure. *Black bars*, healthy individuals ($N = 31$); *white bars*, infected individuals ($N = 30$)

compared with agonistic behaviours directed towards alien workers (mean IA ± SD = 0.96 ± 0.095, $N = 20$. Mann-Whitney tests: foreign vs. healthy: $U = 24$, $P < 0.001$; foreign vs. infected: $U = 46$, $P < .001$). Indeed, agonistic displays against nestmates mainly consisted in threats (72.9 % of all the observed agonistic behaviours, $N = 277$) while attacks or bites were observed only occasionally and mostly from the fourth day onwards (Fig. 4c, d). As regards the impact of health status of the focal ant, the level of aggressiveness towards fungus-infected nestmate was significantly higher than towards healthy nestmates on their 3th and 4th days of infection (Mann-Whitney tests: Day 3: $U = 316.5$, $N1 = 31$, $N2 = 30$, $P = 0.032$; Day 4: $U = 313.5$, $P = 0.029$ (Fig. 4a)). This was mostly due to diseased nestmates being more frequently threatened than healthy workers, even though this difference was not significant (Chi-square test: Day 3: $\chi^2 = 3.8$, $N1 = 31$, $N2 = 30$, $P = 0.051$; Day 4: $\chi^2 = 3.7$, $P = 0.056$ (Fig. 4b)). Likewise, one can notice a nearly significant difference on the fifth day, where 29.03 % of healthy and 53.3 % of infected focal ants were attacked or bitten at least once by a checking ant (Chi-square test: $\chi^2 = 3.72$, $N1 = 31$, $N2 = 30$, $P = 0.054$ (Fig. 4c, d)).

Interactions inside the nest

Regardless of their health status, all focal individuals were contacted by nestmates at least once for each session of observation. No agonistic behaviours, even mild ones such as threats were observed before or after the focal ants were exposed to fungal spores. Antennal contacts accounted for 94.2 % (SD = ±0.7, $N = 132$ (1 session of observation × 22 focal ants × 6 days)) and 93.9 % (SD = ±0.8, $N = 132$) of all the observed interactions that were performed by focal individuals before and after being infected, respectively (Fig. 5). On average, these percentages of antennations did not significantly change through time during the first experimental phase when the focal ant was not yet infected (Friedman test: $\chi^2 = 8.09$, $N = 22$, $P = 0.15$) as well as after being exposed to fungal spores (Friedman test: $\chi^2 = 10.14$, $N = 22$, $P = 0.07$ (Fig. 5)). Likewise, the percentages of social behaviour did not change through time and remained scarce both before and after exposure to the fungus (Fig. 5).

A closer look into social behaviours showed that the percentage of ants involved at least once in a trophallaxis did not vary over time (Cochran test for binary data: healthy ants: $Q = 4.62$, $N = 22$, $P = 0.46$; infected ants: $Q = 9.51$, $N = 22$, $P = 0.09$) and did not differ according to the health status of the focal ants (Chi-square tests: all P values above the significance level of alpha (0.05) whatever the day (Fig. 6a)).

In the same way, the percentages of individuals involved at least once in mutual grooming—either as a donor or a receiver—did not change significantly over time when the focal ants were healthy (as a donor: Cochran test for binary data:

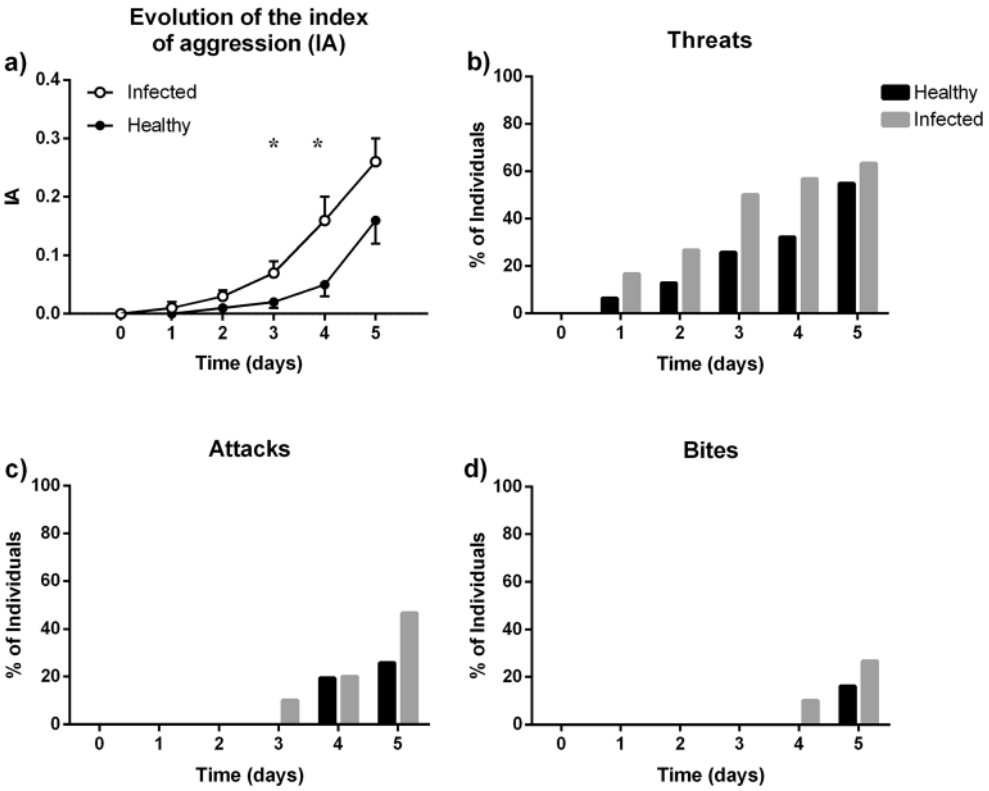

Fig. 4 Dyadic encounters. **a** Daily evolution of the index of aggression (*IA*; mean ± SD) towards either an infected or a healthy nestmate. Each focal individual was daily involved in three dyadic encounters of which we averaged IA values. *Black circles*, healthy individuals ($N = 31$); *white circles*, infected individuals ($N = 30$). Pairwise comparisons were made using Mann-Whitney test (N.S., $P > 0.05$; *$P < 0.05$). **b**–**d** Percentages of individuals who received at least once a threat (**b**), an attack (**c**) or a bite (**d**) during dyadic encounters. Days correspond to time elapsed since vortexing procedure. *Black bars*, healthy individuals ($N = 31$); *grey bars*, infected individuals ($N = 30$)

$Q = 1.08$, $N = 22$, $P = 0.96$; as a receiver: Cochran test for binary data: $Q = 7.45$, $N = 22$, $P = 0.19$ (Fig. 6b, c)) or fungus infected (as a donor: Cochran test for binary data: $Q = 3.21$, $N = 22$, $P = 0.67$; as a receiver: Cochran test for binary data: $Q = 9.38$, $N = 22$, $P = 0.09$ (Fig. 6b, c)). The percentages of self-grooming ants decreased over time both during the healthy phase (Cochran test for binary data: $Q = 11.79$, $N = 22$, $P = 0.038$) and the second phase following fungal infection (Cochran test for binary data: $Q = 14.35$, $N = 22$, $P = 0.014$ (Fig. 6d)). One can notice higher —although not significant— percentages of infected individuals being groomed or performing self-grooming on the third day after exposure to fungus (Chi-square test: $\chi^2 = 3.48$, $N = 22$, $P = 0.062$ for mutual grooming (Fig. 6c) and $\chi^2 = 3.27$, $N = 22$, $P = .07$ for self-grooming (Fig. 6d)).

With respect to the location of individuals, the level of nest frequentation by focal ants was similar during the first phase of the experiment (Cochran test for binary data: $Q = 2.80$, $N = 22$, $P = 0.73$). However, once infected by the fungus, we observed a strong decrease in the number of diseased ants seen inside the nest (Cochran test for binary data: $Q = 43.25$, $N = 22$, $P < 0.001$ (Fig. 7)). Rates of nest attendance by healthy and infected individuals significantly differed from the third day onwards (Chi-square tests: Day 3: $\chi^2 = 7.38$, $N = 22$, $P = 0.007$; Day 4: $\chi^2 = 11.6$, $P < 0.001$; Day 5: $\chi^2 = 19.25$, $P < 0.001$ (Fig. 7)).

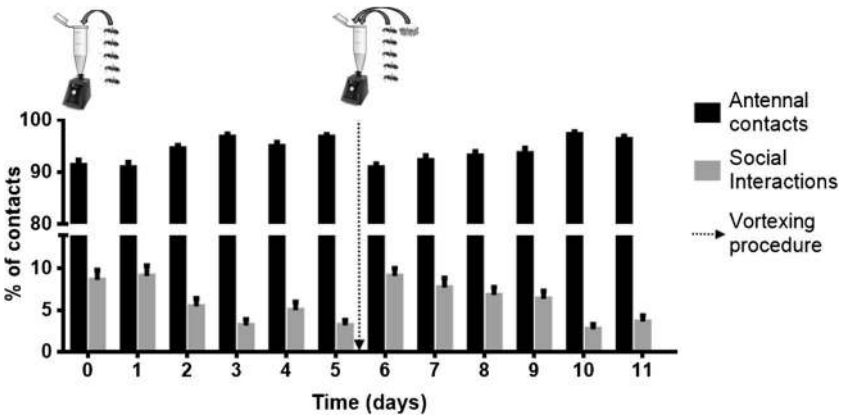

Fig. 5 Interactions inside the nest. Percentages (mean ± SD) of antennal contacts (*black bars*) and social behaviours —here allogrooming + trophallaxis— (*grey bars*) among all interactions observed during one daily session ($N = 22$ focal ants). *Arrows* indicate the time of the two vortexing procedure (1st as control and 2nd with infected corpse)

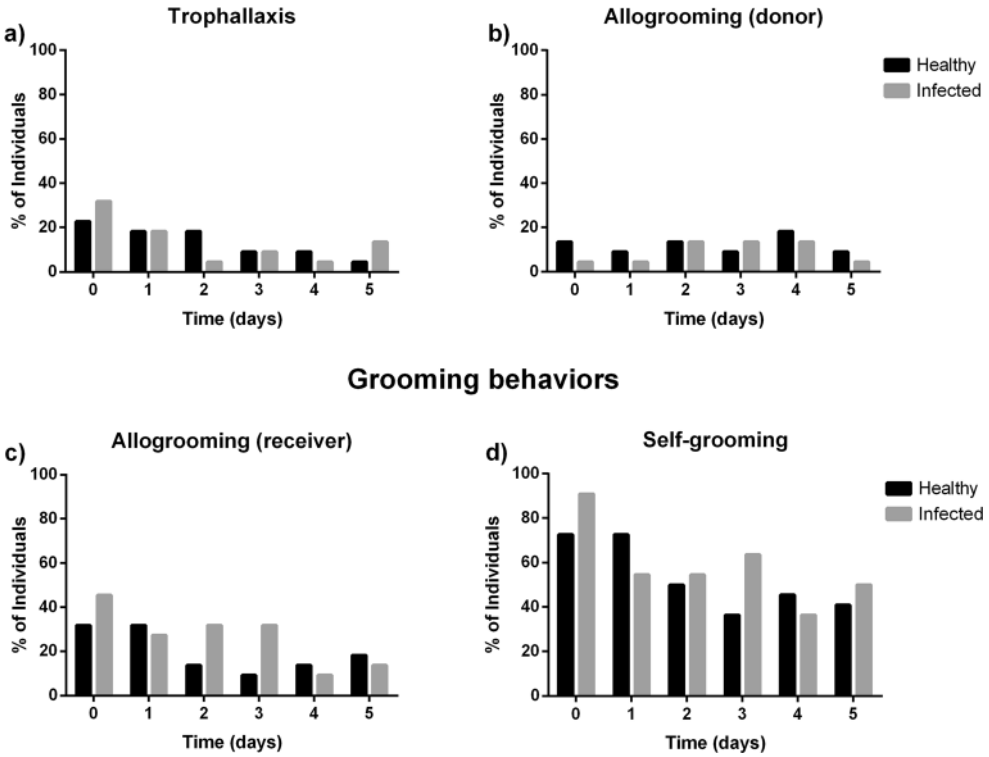

Fig. 6 Interactions inside the nest. **a**–**c** Percentages of individuals who were involved at least once in a trophallaxis (**a**) or an allogrooming: as a donor (**b**) or as a receiver (**c**). **d** Percentages of individuals who performed at least one self-grooming behaviour within a 10-min session. Days correspond to time elapsed since vortexing procedure. *Black bars*, healthy individuals (before being infected. $N = 22$); *grey bars*, diseased individuals (after being infected. $N = 22$)

Interactions at the nest entrance

On the 3rd day of infection, we looked for changes in the ways ants interact at the nest entrance area. The mean time spent into the nest entrance before moving deeper inside did not differ between healthy individuals and infected ones (Mann-Whitney test: $U = 524$, $N1 = 47$, $N2 = 27$, $P = 0.18$), as well their mean number of interactions (Mann-Whitney test: $U = 610.5$, $N1 = 47$, $N2 = 27$, $P = 0.79$, Table 1). Within the nest entrance, the percentage of focal ants that were antennated at least once as well as the duration of these antennal contacts did not differ according to the health condition of the focal ant. (Chi-square test: $\chi^2 = 0.21$, $N1 = 47$, $N2 = 27$, $P = 0.65$ and Mann-Whitney test: $U = 481$, $N1 = 42$, $N2 = 25$, $P = 0.57$ for comparison of percentages and contact durations respectively, Table 1).

In the same way, neither the percentages of individuals that received at least one allogrooming (Chi-square tests: $\chi^2 = 0.06$, $N1 = 47$, $N2 = 27$, $P = 0.80$, Table 1) nor the times being allogroomed (Mann-Whitney test: $U = 9.5$, $N1 = 6$, $N2 = 4$, $P = 0.68$, Table 1) differed between healthy and infected focal ants. Finally, the percentages of individuals grooming themselves (Chi-square test: $\chi^2 = 0.71$, $N1 = 47$, $N2 = 27$, $P = 0.40$, Table 1) as well as the duration of self-cleaning did not differ between healthy ants and their fungus-infected nestmates (Mann-Whitney test: $U = 7$, $N1 = 4$, $N2 = 4$, $P = 0.89$, Table 1). Focal ants never received trophallaxis nor agonistic behaviours, even mild ones, from nestmates.

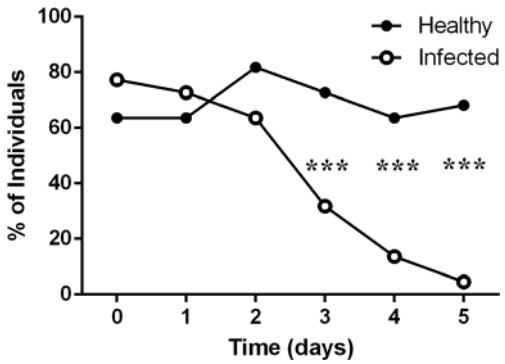

Fig. 7 Rate of nest attendance. Percentages of individuals seen inside the nest at least once during daily sessions of observation. Days correspond to time elapsed since vortexing procedure. *Black circles*, healthy individuals (before being infected; $N = 22$); *white circles*, diseased individuals (after being infected; $N = 22$). Pairwise comparisons were made using Chi-square test (*N.S.*, $P > 0.05$; ***$P < 0.001$)

Discussion

This study shows that, even in the absence of spores over the cuticle, diseased *M. rubra* workers were quickly discriminated during dyadic encounters by being more challenged than their healthy counterparts on the 3rd and 4th days of fungal infection. However, no such discrimination occurred within the social context of the nest. Furthermore, ants located at the nest

Table 1 Interactions at the nest entrance

	Healthy	Infected	Statistical test
Mean number of total interactions	4.9 + 0.9, $N = 47$	4.7 + 0.9, $N = 27$	M-W test: $U = 610.5$, $P = 0.79$
Mean time spend in the entrance area	60 s + 10.4, $N = 47$	69.6 s + 16.1, $N = 27$	M-W test: $U = 524$, $P = 0.18$
Antennal contacts			
% of individuals	89.5 %, $N = 47$	92.5 %, $N = 27$	Chi^2 test: $\chi^2 = 0.21$, $P = 0.65$
Mean time (±SD)	27.9 s + 8.1, $N = 42$	39.9 s + 15.4, $N = 25$	M-W test: $U = 481$, $P = 0.57$
Allogrooming (as receiver)			
% of individuals	12.5 %, $N = 47$`	14.8 %, $N = 27$	Chi^2 test: $\chi^2 = 0.06$, $P = 0.80$
Mean time (±SD)	22.3 s + 7.9, $n = 6$	13.2 s + 1.7, $N = 4$	M-W test: $U = 9.5$, $P = 0.68$
Self-grooming			
% of individuals	8.3 %, $N = 47$	14.8 %, $N = 27$	Chi^2 test: $\chi^2 = 0.71$, $P = 0.40$
Mean time (±SD)	103.1 s + 29.3, $N = 4$	97.9 s + 17.8, $N = 4$	M-W test: $U = 7$, $P = 0.89$

This table gives the time spent (mean ± SD) and number (mean ± SD) of interactions performed at the nest entrance according to the health status of tested individuals. It also reported the percentages of individuals involved at least once in a given behaviour as well as the duration of this behaviour. The average and SD time values are given in seconds. All individuals were tested 72 h after vortexing procedure (with or without a contaminated corpse)

entrance that were commonly interacting with incoming nestmates did not actively reject diseased individuals.

In a neutral context of dyadic encounters, the level of aggressiveness towards healthy or fungus-infected nestmates progressively increased. Such agonistic behaviours are considered as reliable indicators of impaired nestmate recognition (Liu et al. 1998; Boulay et al. 2000; Lenoir et al. 2001) when dissimilarity between cuticular profiles exceeds a given threshold (Jutsum et al. 1979; Lenoir et al. 1999; Dalecky et al. 2007). These agonistic behaviours did not seem to be a response to a cuticular profile collapse resulting from the orphaning of ant colonies, since, in this case, aggression would have occurred from the first dyadic encounters. Instead, most of the triggered agonistic behaviours towards isolated nestmates were mild ones such as short-lasting threats and their occurrence increases over time what suggests a progressive drift of the cuticular profile of focal ants from the chemical template of their colony (Stuart and Herbers 2000; Boulay et al. 2004; Schmidt et al. 2010). This progressive alteration of the cuticular profile most probably results from the experimental social isolation of the focal ant as also reported in several studies (Boulay and Lenoir 2001; Lenoir et al. 2001).

It is of great interest that the aggression level grows more steeply over successive dyadic encounters with diseased nestmates. Indeed, on the 3rd and 4th days of infection, a significantly higher rate of agonistic behaviours were directed towards sick nestmates, although viable spores had all penetrated the insect body at this stage of infection (Boucias and Pendland 1998; Gillespie et al. 2000; Arthurs and Thomas 2001). This higher aggressiveness occurred when infected ants began to be physiologically impacted by the entomopathogenic fungus of which the hyphal bodies evade the host immune response (Hänel 1982; Gillespie et al. 2000; Hung and Boucias 1992; Kurtti and Keyhani 2008; Bidochka et al. 2010).

Therefore, one may assume that fungus growth inside the insect body cavity has modified the body odour of infected ants, possibly through an activation of the immune system as demonstrated for cuticular compounds in honeybees (Salvy et al. 2001; Richard et al. 2008; Evans and Spivak 2010). This would support existing evidence for a close dependency between the body odour of ant workers and their internal physiological state. For example, *Linepithema humile* workers become aggressive towards nestmates reared on new insect preys (Liang and Silverman 2000) due to diet-induced differences in their cuticular profile. Similarly, the removal of dead ants outside the nest is triggered by post-mortem changes of cuticular profile such as by the release of fatty acids (linoleic and oleic) (in *Pogonomyrmex badius*: Wilson et al. 1958; in *Myrmecia vindex*: Haskins and Haskins 1974; in *Lasius niger*: Diez et al. 2013) or by the loss of life signals (dolichodial and iridomyrmecin in *L. humile*: Choe et al. 2009). Further GC_MS analyses are needed to investigate whether the detection and discrimination of illness in *M. rubra* ants results from changes in the cuticular chemical profile and/or from the presence of new disease-specific compounds over the body of infected individuals. Until now, only Bos et al. (2012) have reported a nearly significant difference between cuticular profiles of healthy and sick *Camponotus aethiops* congeners on their third day of infection.

Quite unexpectedly, our results do not support the hypothesis that discrimination and aggression towards sick nestmates would be enhanced inside the nest in order to reinforce prophylaxis by setting diseased congeners apart. By contrast, we found out that individuals behaved similarly with congeners, regardless of their health status and displayed no form of aggression. Only a higher number of allo- and self-grooming behaviours occurred during the first 2 days for both healthy and infected ants. This confirms that body cleaning is a basic,

ubiquitous and essential prophylactic measure for any individual that entering the nest, which allows not only to update the colonial visa but also to limit risks of pathogen spread through the colony (Reber et al. 2011). Frequent allogrooming persisted longer —for two extra-days— in the case of fungus-exposed individuals, as also observed for *Camponotus aethiops* ants (Bos et al. 2012). This enhanced allogrooming benefits to contaminated ants through the removal of spores attached on their cuticle but also to healthy nestmates that may gain resistance against pathogens (Ugelvig and Cremer 2007; Walker and Hughes 2009). Such an 'immune priming' phenomenon mediated by allogrooming however remains controversial (Hauton and Smith 2007; Gonzalez-Tokman et al. 2010; Reber and Chapuisat 2012).

Finally, we paid special attention to discriminatory behaviours performed at the nest entrance since it is the key location at which gatekeepers check the cuticular profile of any entering ants and may reject those that do not match with the colonial template. When infected individuals entered the nest, gatekeepers behaved in a similar way as for healthy nestmates and displayed no sign of aggression. This does not necessarily imply that the gatekeepers were unable to discriminate healthy from infected nestmates but simply that they did not react to perceived differences by exhibiting aggression (Steiner et al. 2007; Tsuji 2010). Indeed, in order to reduce errors such as nestmate rejection, guards should accept a certain level of dissimilarity between the colony template and the cuticular profile of incoming ants (as it takes place for foragers of which body odours is altered during their foraging journey; Wagner et al. 1998; Greene and Gordon 2003).

In the case of fungus-infected *M. rubra* workers, one may assume that body odour changes were under the maximum chemical dissimilarity that a gatekeeper would tolerate without rejecting the entering individual. This 'acceptance' of dissimilarity is expected to be tunable namely depending on the level of polygyny (Reeve 1989). Indeed, the only known case of active rejection of diseased individuals is found in honeybees affected by the Deformed Wing Virus (DWV) (Baracchi et al. 2012) of which the monogyny makes the colonial visa probably less complex and thereby results in stricter recognition processes of diseased nestmates. On the other hand, several polygynous ant species of which the high genetic diversity results in a high complexity of their colonial visa were more tolerant to dissimilarities between nestmates' chemical profiles (Tsutsui et al. 2003; Fürst et al. 2011). In the same way, the high-level polygyny of *M. rubra* colonies (Elmes 1973; Seifert 2007) could explain why workers and more specifically gatekeepers show no aggressive reactions even when meeting fungus-infected nestmates with a potentially altered body odour.

From a sanitary perspective, the absence of active rejection of infected nestmates is probably not damaging to *M. rubra* colonies due to the spontaneous decrease of nest frequentation by diseased individuals. This spatial shift from the nest interior to the outside began on the third day of infection, when the health status of workers started to deteriorate. This spontaneous isolation that was also found in fungus-infected *Temnothorax* ants (Heinze and Walter 2010), *Camponotus aethiops* (Bos et al. 2012) as well as in honey bees *A. mellifera* (Rueppell et al. 2010) appears as a simple way for the infected individual to increase its inclusive fitness, by minimising contacts with related individuals and by preventing the pathogen to potentially devastate the colony (Cremer and Sixt 2009; Wilson-Rich et al. 2009). Beside striking cases of behavioural manipulation of the host by the parasite (Hughes et al. 2016), seclusion of infected individuals outside their nest thus appears as a widespread behaviour that was selected for in social insects when benefits of preventing disease outbreaks in the nest outweigh the potential cost of losing workers (Rueppell et al. 2010).

It is now clear that parasite pressure has shaped many life traits and behaviours of social insects to prevent epidemics within their colony (Hughes 2005; Martin et al. 2011). In the case of fungal infection in *M. rubra* ant colonies, the pressure for an accurate discrimination and an early detection of contaminated nesmates seems to be loose or unnecessary, maybe due to the fact that spontaneous isolation of diseased individuals constitutes a first, simple but effective mechanism of social immunity. Further comparative studies about the detection and acceptance levels of diseased ants are needed to better understand how ant colonies balance the benefits of increased hygiene with the time/energy costs associated to the management of infected workers or with the risks of erroneous rejection of nestmates.

Acknowledgments We thank the anonymous referees for their comments and Julien Hendrickx for helping to collect ants. This study was funded by a Ph.D. grant to Mr. Leclerc from FRIA (Fonds pour la Recherche dans l'Industrie et dans l'Agriculture) and by a research credit (CDR J.0092.16) from FRS-FNRS (Fonds de la Recherche Scientifique). C.D. is Research Director from the Belgian National Fund for Scientific Research (FNRS).

Compliance with ethical standards We declare that the experiments comply with the current laws of Belgium.

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