

## The expression and impact of antifungal grooming in ants

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### Abstract

Parasites can cause extensive damage to animal societies in which many related individuals frequently interact. In response, social animals have evolved diverse individual and collective defences. Here, we measured the expression and efficiency of self-grooming and allo-grooming when workers of the ant *Formica selysi* were contaminated with spores of the fungal entomopathogen *Metarhizium anisopliae*. The amount of self-grooming increased in the presence of fungal spores, which shows that the ants are able to detect the risk of infection. In contrast, the amount of allo-grooming did not depend on fungal contamination. Workers groomed all nestmate workers that were re-introduced into their groups. The amount of allo-grooming towards noncontaminated individuals was higher when the group had been previously exposed to the pathogen. Allo-grooming decreased the number of fungal spores on the surface of contaminated workers, but did not prevent infection in the conditions tested (high dose of spores and late allo-grooming). The rate of disease transmission to groomers and other nestmates was extremely low. The systematic allo-grooming of all individuals returning to the colony, be they contaminated or not, is probably a simple but robust prophylactic defence preventing the spread of fungal diseases in insect societies.

### Introduction

One of the major unresolved questions in evolutionary biology is whether diseases prevent or promote social behaviour. On the one hand, diseases might constrain social evolution (Hamilton, 1987). Social life is associated with a high frequency of contacts among individuals, which is likely to increase the risk of disease transmission (Fefferman *et al.*, 2007; Naug, 2008). Moreover, insect societies usually contain related individuals that remain together in long-lasting nests, providing ideal conditions for the spread of epidemics once the disease has broken into the colony (Hamilton, 1987; Schmid-Hempel, 1998).

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On the other hand, over evolutionary time, social organisms may be able to reduce the impact of disease by evolving collective defences that limit the spread of infection (Cremer *et al.*, 2007; Wilson-Rich *et al.*, 2009; Chapuisat, 2010). In insect societies, many of these defences are behavioural and social and aim at improving the nest hygiene. For example, workers of most species exhibit elaborate waste management behaviours and carry corpses out of their nests (Hart & Ratnieks, 2002; Cremer *et al.*, 2007; Wilson-Rich *et al.*, 2009).

Both solitary and social organisms can groom themselves (self-grooming), but social organisms have the additional advantage of being able to groom each other (allo-grooming). Allo-grooming is an important line of defence in many social animals, because it permits the efficient mechanical removal of parasites such as mites, fleas, nematodes, fungal hyphae or fungal spores from areas that are not easily accessed during self-grooming (Schmid-Hempel, 1998). Allo-grooming is common in mammals, particularly primates, ungulates and some rodents, where it is generally considered to be a form of reciprocity (Mooring *et al.*, 2004; Clutton-Brock,

2009). Allo-grooming is also very widespread in the social insects, where it has been documented in many species of ants, wasps, bees and termites (Rosengaus *et al.*, 1998b; Schmid-Hempel, 1998; Hughes & Boomsma, 2004; Walker & Hughes, 2009).

In addition to the removal of parasites, allo-grooming has important social functions. In mammals, allo-grooming plays a major role in social bonding (Clutton-Brock, 2009; Dunbar, 2010). In the social insects, allo-grooming and trophallaxis (oral exchange of liquid food) result in a transfer of chemical cues among individuals, and this homogenization of the colony odour is important for accurate nestmate discrimination (Boulay *et al.*, 2004). Hence, the social and hygienic roles of allo-grooming are often confounded.

A better understanding of the hygienic function of allo-grooming requires the estimation of its benefits and costs when a group is exposed to parasites. The potential benefits of allo-grooming stem from the removal of parasites, which may increase the survival of the individual being groomed and/or reduce the within-group transmission of parasites if groomers have means to kill the parasites or inactivate infectious particles (Cremer *et al.*, 2007). The costs of allo-grooming include the time and energy spent in grooming, as well as the possibility that the contact with a parasitized individual increases the risk of parasite transmission to the groomers (Rosengaus & Traniello, 1997; Schmid-Hempel, 1998; Cremer *et al.*, 2007).

The expression of allo-grooming is likely to depend on the presence and location of the parasitic threat. Allo-grooming is generally considered to be a conditional response to the presence of dangerous parasites. For example, the frequency of allo-grooming increased in groups of termites and ants that had been experimentally contaminated with *Metarhizium anisopliae* fungal spores (Rosengaus *et al.*, 1998b; Hughes & Boomsma, 2004; Walker & Hughes, 2009). However, allo-grooming might also be a constitutive defence that is systematically applied to individuals that are particularly likely to carry an infection, independent of the actual presence of parasites. Leafcutter ants engage in proactive self-grooming before entering their nests, particularly when the nest chambers contain sensitive fungal crops or brood (Morelos Juárez *et al.*, 2010). Similarly, the systematic allo-grooming of individuals returning to their nest after a foraging trip might be a way to remove undetected microbes before they could be transmitted to nestmates.

The way in which a group responds to parasites may also depend on characteristics of both the parasites and the group. In particular, the amount and efficiency of self- or allo-grooming might vary according to infection dose and infection stage, as removing spores may become increasingly difficult at high doses or when the fungus has had time to attach to the cuticle (Hajek & St. Leger, 1994). Allo-grooming efficiency may also vary with group size. For example, larger groups might be able to

allocate more individuals to allo-grooming and thus remove spores more efficiently than smaller groups (Schmid-Hempel, 1998). On the other hand, smaller groups might have more to lose if one of their members dies or is excluded and might thus put relatively more effort into allo-grooming.

In this study, we examined the expression and impact of antifungal grooming in the ant *Formica selysi*. We exposed groups of workers to spores of the fungus *M. anisopliae*. This generalist entomopathogen is common in the habitat of *F. selysi* (A. Reber & M. Chapuisat, unpublished data). In a series of experiments, we examined which factors affected the behavioural response of ants and evaluated the impact of allo-grooming on groomed individuals and grooming individuals. First, we recorded the amount of self-grooming and allo-grooming in groups of workers exposed to various doses of fungal spores deposited in their nest chamber. Second, we examined the allo-grooming rate and its efficiency at removing fungal spores when one contaminated individual was re-introduced into its group, which mimics the situation where a forager returns to the nest. We tested the effect of contamination, time elapsed since contamination and group size. Third, we assessed whether allo-grooming and social contacts increased the survival of ants that had been exposed to a high dose of spores 24 h before being groomed. Finally, we evaluated the risk of disease transmission from contaminated ants to groomers or other group members. Taken together, these experiments shed light on the modalities and impact of grooming as a means of defence against fungal infection in insect societies.

## Materials and methods

### General procedure

We studied grooming in the ant *F. selysi*. This species builds permanent nests in the soil. Workers forage above ground for insect prey and aphid honeydew, which they collect and bring back to the nest (Keller & Zettel, 2001). Hence, like many other ants, *F. selysi* workers are likely to have frequent contact with spores of soil-dwelling entomopathogenic fungi. We sampled *F. selysi* workers from a population located along the river Rhône between Sierre and Susten in the central Valais, Switzerland. In summer 2007, we collected 300 workers from each of 43 single-queen colonies (Chapuisat *et al.*, 2004; Reber *et al.*, 2008). The workers were kept in plastic boxes (13.5 × 15 × 5 cm) lined with fluon, at 25 °C and under a 12-h day/night cycle. The ants had *ad libitum* access to water and a protein-rich jelly food.

We challenged the ants with *M. anisopliae* var. *anisopliae*, an entomopathogenic fungus that is common in the Swiss lowlands (Keller *et al.*, 2003). We used a fungus strain isolated from the soil of our study site and causing high mortality to *F. selysi* workers (Reber *et al.*, 2008;

A. Reber & M. Chapuisat, unpublished data). The fungus was cycled four times in *F. selysi* workers and cultured on a nutritive medium (malt extract agar; BD, Franklin Lake, NJ, USA) at 25 °C for 5–7 days to obtain asexual spores (conidia). The spores were harvested into sterile 0.05% Tween 20 buffer (the nontoxic, highly diluted polysorbate surfactant Tween 20 was used to keep spores in suspension). The concentration of spores was measured with a haemocytometer (Neubauer improved counting chamber). Water and 0.05% Tween 20 buffer were used in controls.

Experimental groups of ants were formed 24 h before the beginning of the experiments and were provided with *ad libitum* food and water. We removed water tubes and food during behavioural observations. The observers were blind with respect to the treatments, because a third person applied the treatments and anonymized the labels at the beginning of the experiments.

Because the effect of allo-grooming on spore removal is likely to depend on the stage of infection and in particular on the degree of attachment of spores, we measured spore removal efficiency when allo-grooming occurred 1 and 24 h after contamination, respectively. This timing was adjusted to the development of the infection in insects: *M. anisopliae* conidia germinate and develop an attachment structure (appressorium) between 12 and 24 h (Hajek & St. Leger, 1994; Moino *et al.*, 2002).

To assess whether the mortality occurring during the experiments was caused by *M. anisopliae*, all corpses were removed from experimental boxes, surface-sterilized (Lacey, 1997) and placed in tubes with wet cotton wool. These tubes were kept at 25 °C and corpses were visually inspected to detect the presence of green stacks of spores that diagnose *M. anisopliae* infection.

### Experiment 1: Grooming after a fungal contamination of the group

We assessed the amount of self-grooming and allo-grooming when group members were jointly exposed to fungal spores. We established 95 experimental groups of 11 workers in Petri dishes (9 cm diameter) lined with flouon. Workers from 19 field colonies were split into five experimental groups per colony, each group being randomly assigned to one of the five following treatments: sterile water, 0.05% Tween 20 buffer, low, medium and high concentrations of *Metarhizium* spores ( $10^4$ ,  $10^6$  and  $10^8$  conidia mL<sup>-1</sup> of 0.05% Tween 20 buffer, respectively). Two hundred microlitres of solution was deposited with a micropipette on a filter paper placed in the centre of the Petri dish.

The behaviour of ants was observed for 30 s at 10, 30 and 70 min after the treatment. We recorded the occurrence of self-grooming (one single ant cleaning herself using her mandibles or legs) and allo-grooming (one ant being cleaned by one or more other ants). We evaluated

the overall effect of treatments on the occurrence of self- and allo-grooming with Kruskal–Wallis tests. When the overall effect of treatments was significant, we further investigated the differences between pairs of treatments with Mann–Whitney tests for independent data.

### Experiment 2: Allo-grooming of contaminated nestmates re-introduced into their group

We observed the behaviour of ants towards contaminated group members that were returned to the group after a period of isolation. We established 40 experimental groups of 30 workers in plastic boxes (13.5 × 15 × 5 cm) lined with flouon. The workers originated from 10 field colonies. In two experimental groups (=replicates) per colony, a worker was re-introduced after having been isolated for 1 h (before conidia had adhered to the cuticle), whereas in the two other experimental groups, a worker was re-introduced after 24 h (after conidia had germinated and adhered to the cuticle). Two workers were removed from each experimental group. For half of the groups (ten groups from the 1-h treatment and ten groups from the 24-h treatment), these two workers were contaminated with fungal spores by depositing on their thorax 1 µL of *M. anisopliae* at a concentration of 10<sup>7</sup> conidia mL<sup>-1</sup> (we used a high dose to ensure that enough conidia would stay on the cuticle, as many are lost during the application process, see results of experiment 3). For the other 20 groups, these two workers were uncontaminated controls that received 1 µL of sterile 0.05% Tween 20 buffer. Each ant removed from the group was isolated in a small Petri dish (3.5 cm diameter) with water and food. One ant was then returned to its group (after 1 or 24 h since contamination), whereas the other one was kept in isolation as a control that could self-groom but was prevented from receiving allo-grooming.

We observed the behaviour of group members for 10 min after the re-introduction of the contaminated or control worker. We recorded the number of individuals that allo-groomed the re-introduced ant (=number of allo-groomers) and the total time during which the re-introduced ant was allo-groomed by one or more ants (=duration of allo-grooming).

Forty-eight hours after the first round of behavioural observations, the experiment was repeated, but the treatments were reversed, so that groups that had first received a contaminated worker received a control one, and vice versa. Hence, each experimental group received one contaminated and one control ant, in randomized order. Two groups had to be removed from the analysis because we lost sight of the re-introduced ant during the observation.

The effect of the fungal treatment, round of observation (first or second ant being re-introduced) and time since contamination on the number of allo-groomers and duration of allo-grooming were tested with three-way ANOVA. We used a mixed model with three fixed factors,

the fungal treatment (contaminated or control), round of observation and time elapsed between contamination and re-introduction (1 or 24 h), and a random factor, the field colony from which the workers originated. Nonsignificant terms were sequentially removed, using likelihood ratio tests. In addition, we used a Pearson's correlation test to examine whether the number of allo-groomers was correlated with the duration of allo-grooming.

We performed two additional control experiments. First, we assessed whether the 0.05% Tween 20 buffer elicited grooming. Second, we evaluated whether the amount of self-grooming before and after re-introduction differed between control and contaminated ants (see Appendix S1).

### Experiment 3: Effect of allo-grooming on spore removal

To estimate the impact of allo-grooming on spore removal, we compared the number of spores remaining on contaminated workers that had been re-introduced in their groups or kept in isolation (Hughes *et al.*, 2002). Immediately after the 10-min period of grooming and observation, groomed and isolated ants were placed individually in microcentrifuge tubes filled with 500  $\mu\text{L}$  of 0.05% Tween 20. The tubes were vortexed for 1 min to re-suspend all remaining spores. The Tween solution was completely evaporated by placing the tubes in a speedvac for 6 h and the spores were re-suspended in 30  $\mu\text{L}$  of 0.05% Tween 20. For each sample, we took three independent measures of the concentration of conidia with a Neubauer haemocytometer and calculated the total number of spores remaining on the surface of the worker from the average of these three measures.

The effects of allo-grooming and time since contamination on the number of spores remaining on the surface of the contaminated ant were tested with two-way ANOVA. The number of spores was  $\sin(x + 360)$  transformed to ensure normality and homoscedasticity. We used a mixed model with two fixed factors, the social treatment (allo-groomed or kept in isolation) and time elapsed between contamination and re-introduction (1 or 24 h), as well as a random factor, the field colony from which the workers originated. In addition, we used a Kendall's rank correlation test to examine whether the number of spores remaining on contaminated ants was correlated with the duration of allo-grooming.

To detect whether the fungal disease had been transmitted from contaminated ants to groomers or other group members, we monitored the mortality in the experimental groups until 7 days after the end of the second re-introduction and checked whether the corpses produced *Metarhizium* spores.

### Experiment 4: Effect of group size on spore removal

We also examined whether spore removal by allo-grooming increased with group size. For this, we

re-introduced contaminated ants in small (three workers) and large (12 workers) groups, respectively. We used workers originating from 29 field colonies to form 29 experimental groups of five workers and 29 experimental groups of 14 workers. The workers were marked with spots of paint to distinguish contaminated and control ants from other group members. The groups were kept in Petri dishes (9 cm diameter) lined with fluon. Two ants were removed from each experimental group. We contaminated these two ants by depositing on their thorax 1  $\mu\text{L}$  of *M. anisopliae* at a concentration of  $10^8$  conidia  $\text{mL}^{-1}$  (only a small proportion of the spores are still on the ant cuticle following the 24 h in isolation, see Results). We kept each contaminated ant isolated for 24 h in a small Petri dish (3.5 cm diameter) with water and food. One of these contaminated workers was then returned to its group for 1 h, where it was allo-groomed. The other contaminated worker was kept in isolation.

Immediately after this 1-h period of grooming or isolation, we measured the number of spores remaining on the body of contaminated workers as described earlier. We used a two-way ANOVA to test the effect of social treatment (allo-groomed or kept in isolation) and group size on the number of spores remaining on workers. The number of spores was log-transformed to ensure normality and homoscedasticity. As described previously, we also recorded mortality and corpse sporulation to detect whether there had been any transmission of the fungal disease.

### Experiment 5: Effect of allo-grooming on the survival of contaminated ants

To examine the impact of allo-grooming and social contacts on the survival of contaminated ants, we compared the survival of ants that had been kept in isolation, had social contacts but no allo-grooming, or were allo-groomed for 2.5 h beginning 24 h after exposure to a high dose of spores. We formed 132 experimental groups of 16 workers. The workers originated from 33 field colonies. Two experimental groups (=replicates) per colony received contaminated ants, whereas the two other experimental groups received control ants. The workers were marked with spots of paint to distinguish contaminated or control ants from other group members. The groups were kept in Petri dishes (9 cm diameter) lined with fluon.

Three focal ants were removed from each experimental group. For 66 groups, these three workers were contaminated with fungus by depositing on their thorax 1  $\mu\text{L}$  of *M. anisopliae* at a concentration of  $10^8$  conidia  $\text{mL}^{-1}$ , as in experiment 4. For the other 66 groups, the three workers received 1  $\mu\text{L}$  of sterile 0.05% Tween 20 control buffer. Each contaminated or control worker was kept isolated for 24 h in a small Petri dish (3.5 cm diameter) with water and food. Two workers were then returned to their group. One of the two could be allo-groomed by the

other group members. The other was placed in a small Petri dish covered by mesh, allowing social contacts such as antennal touching or trophallaxis, but preventing allo-grooming. The third worker was kept in isolation as a control that could self-groom but was deprived of allo-grooming and social contacts. After 2.5 h, the two re-introduced ants were removed from their experimental groups and kept in isolation. Over the next 10 days, we monitored the mortality of the focal ants and checked whether their corpses produced *Metarhizium* spores.

We compared the survival of treated and control workers in a parametric survival analysis, using the function *survreg* of the program R (R Development Core Team, 2006). We built a model with two fixed factors, the fungal treatment (contaminated or control) and social treatment (isolated, having contacts through mesh, or allo-groomed). We included the experimental group nested in the colony of origin as a random factor using the frailty argument for the *survreg* function (Therneu et al., 2003). The interaction between fixed factors was tested using a likelihood chi-square ratio test. We used a Weibull distribution, which produced the minimum error deviance. All statistical analyses were performed in R version 2.8.1 (R Development Core Team, 2006).

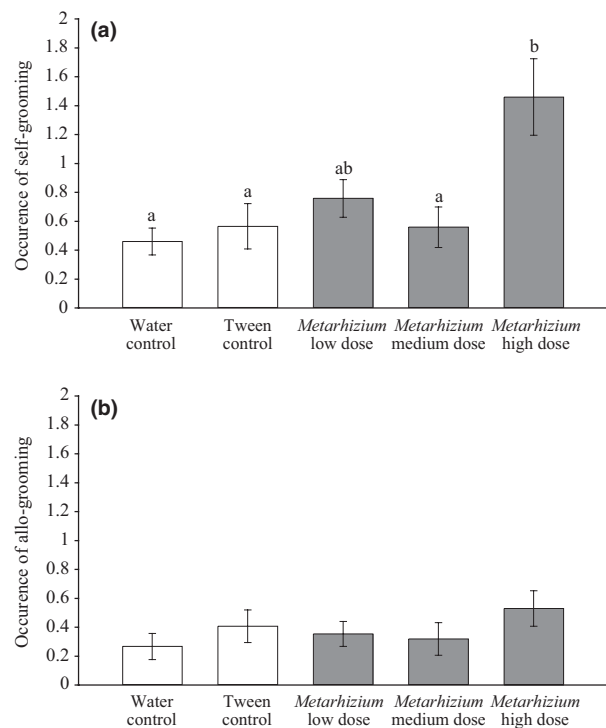
## Results

### Experiment 1: Grooming after a fungal contamination of the group

Overall, the amount of self-grooming rose significantly when groups of ants were jointly exposed to increasing doses of *Metarhizium* spores placed on filter paper in the group (Fig. 1a;  $H_{4,90} = 11.9$ ,  $P = 0.02$ ). The amount of self-grooming was significantly higher in the presence of high dose of fungal spores than in the presence of Tween control ( $U = 95.5$ ,  $n_1 = n_2 = 19$ ,  $P = 0.01$ ), water control ( $U = 275$ ,  $n_1 = n_2 = 19$ ,  $P = 0.005$ ) or medium dose of spores ( $U = 251$ ,  $n_1 = n_2 = 19$ ,  $P = 0.01$ ; Fig. 1a), even after a sequential Bonferroni-adjustment of the significance threshold. In contrast, there was no significant effect of treatment on the amount of allo-grooming in this experiment (Fig. 1b;  $H_{4,90} = 3.6$ ,  $P = 0.5$ ).

### Experiment 2: Allo-grooming of contaminated nestmates re-introduced into their group

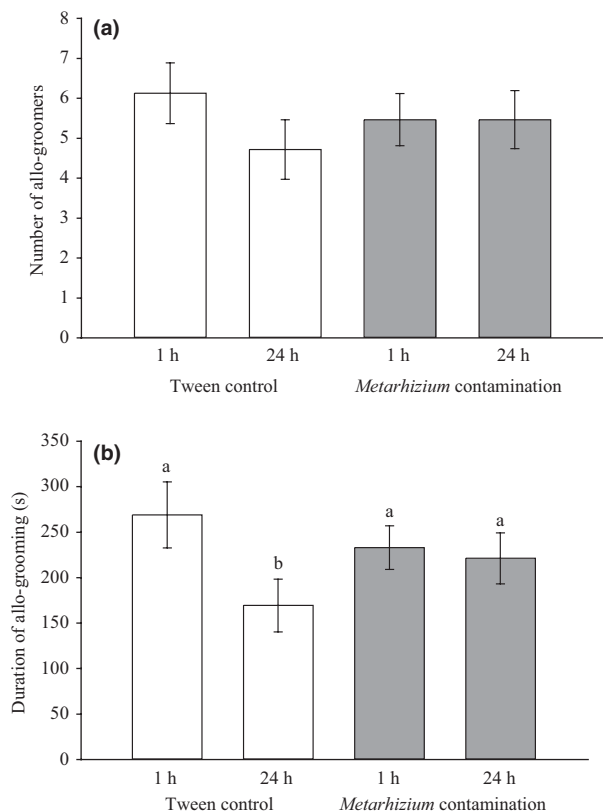
When an ant was returned to its group after a short (1 h) or long (24 h) period of isolation, it was groomed intensively by an average of five ants and for more than one-third of the total observation time (Fig. 2). All re-introduced ants were groomed, independently of whether they had been contaminated by fungus or not. Indeed, exposing the ant to fungal spores before re-introduction had no significant effect on the number of groomers (Fig. 2a;  $F_{1,65} = 0.02$ ,  $P = 0.9$ ) nor on the duration of allo-grooming (Fig. 2b;  $F_{1,65} = 0.1$ ,  $P = 0.7$ ).



**Fig. 1** Expression of (a) self-grooming and (b) allo-grooming when group members were jointly exposed to *Metarhizium anisopliae* spores. The groups were exposed to water, Tween buffer, low, medium or high dose of *Metarhizium* deposited on a filter paper, respectively. Mean ( $\pm$ SE) occurrence of (a) self-grooming and (b) allo-grooming in groups of 11 individuals, averaged over three observation scans of 30 s per group. Different letters indicate treatments that differed significantly from one another.

The time elapsed between contamination and re-introduction had no significant effect on the number of allo-groomers (Fig. 2a;  $F_{1,65} = 0.94$ ,  $P = 0.3$ ) but had a marginally significant effect on allo-grooming duration (Fig. 2b;  $F_{1,65} = 3.92$ ,  $P = 0.05$ ), which was significantly shorter for control ants re-introduced after 24 h ( $F_{1,27} = 5.6$ ,  $P = 0.02$ ). The number of allo-groomers and allo-grooming duration were positively correlated ( $r = 0.7$ , d.f. = 76,  $P < 0.0001$ ).

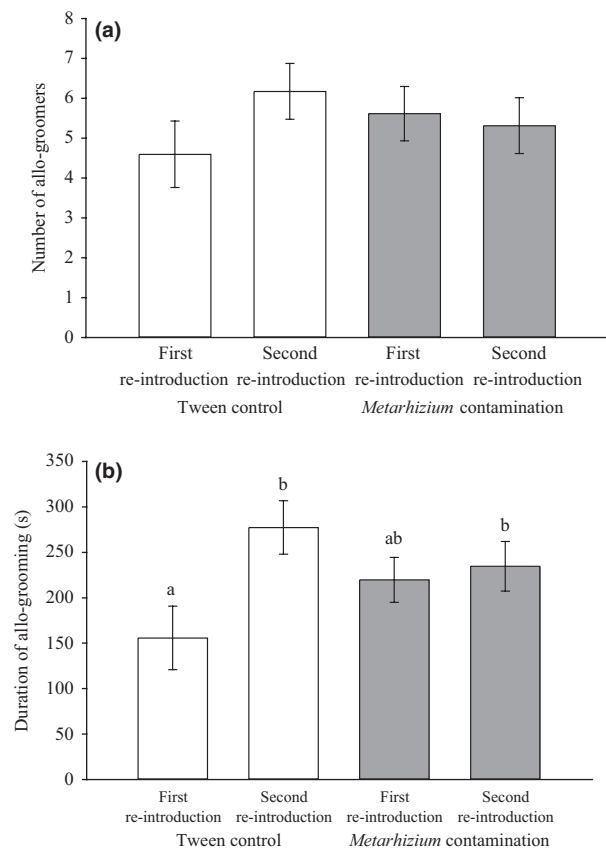
The first and second ants re-introduced were groomed by a similar number of ants (Fig. 3a;  $F_{1,65} = 0.9$ ,  $P = 0.3$ ), but overall the second ants being re-introduced were allo-groomed for a significantly longer time than the first ones (Fig. 3b;  $F_{1,62} = 6.9$ ,  $P = 0.01$ ). There was a significant interaction between the round of observation (first or second ant being re-introduced) and the fungal treatment on the duration of allo-grooming (Fig. 3b;  $F_{1,62} = 4.1$ ,  $P = 0.05$ ). The control ants were groomed for a significantly longer time when they were re-introduced following the previous re-introduction of a contaminated ant than when they were the first ones to be re-introduced (Tween control, first vs. second re-introduction:  $t_{38} = 3.1$ ,



**Fig. 2** Expression of allo-grooming when a nestmate contaminated with *Metarhizium anisopliae* spores was re-introduced in its group. The recipient group had 28 ants and allo-grooming was measured over a period of 10 min. (a) Mean ( $\pm$ SE) number of ants allo-grooming the re-introduced ant. (b) Mean ( $\pm$ SE) duration of allo-grooming. The expression of allo-grooming is given in function of the contamination of the re-introduced ants (control or *Metarhizium*-contaminated) and time elapsed between contamination and re-introduction (1 or 24 h). Different letters indicate treatments that differed significantly from one another.

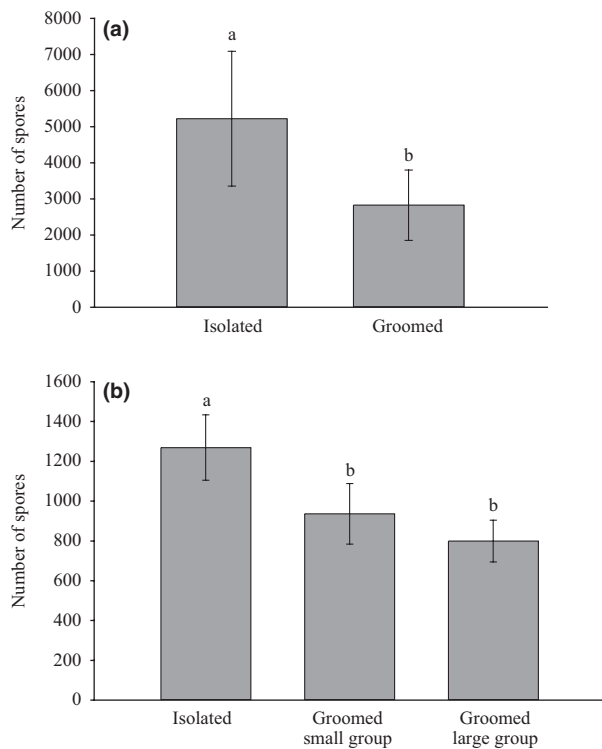
$P = 0.002$ ). In contrast, the contaminated ants were not groomed for longer when they were re-introduced after a control ant than when they were the first ones to be re-introduced (*Metarhizium*-contaminated, first vs. second re-introduction:  $t_{38} = 0.4$   $P = 0.7$ ). This suggests that the reaction towards control and contaminated ants differs in subtle ways and that the grooming of noncontaminated ants increases when the group had been previously exposed to the pathogen.

Overall, ants that were treated with 0.05% Tween 20 buffer, with or without *Metarhizium* spores, were groomed by about five nestmates for 4 min, on average, whereas ants that had received a drop of sterile water or the dry treatment were allo-groomed by one or two nestmates for about 1 min, on average (Figs 2 and S1). Hence, the application of Tween 20 buffer, with or without spores, elicited a significantly longer and more vigorous allo-grooming interaction than the dry or water



**Fig. 3** Expression of allo-grooming towards the first and second nestmate re-introduced in their group (the two ants were re-introduced with an interval of 48 h). For each group, the first and second nestmates re-introduced in the group had alternative treatments (control or contaminated, respectively). The recipient group had 28 ants and allo-grooming was measured over a period of 10 min. (a) Mean ( $\pm$ SE) number of ants allo-grooming the re-introduced ant. (b) Mean ( $\pm$ SE) duration of allo-grooming. The expression of allo-grooming is given in function of the contamination of the re-introduced ants (control or *Metarhizium*-contaminated). Different letters indicate treatments that differed significantly from one another.

only treatments (Fig. S1; grooming duration:  $F_{5,49} = 5.17$ ,  $P = 0.0007$ ; number of groomers:  $F_{5,49} = 5.62$ ,  $P = 0.0004$ ). However, the proportion of time that nestmates spent allo-grooming re-introduced ants that had only been exposed to the water or dry treatments was significantly higher than the background level of allo-grooming among untreated ants, even when the re-introduced ants had been separated for only 1 h (Fig. S1; background level of allo-grooming was zero for all ten groups observed; *Bonferroni-corrected proportion tests* ( $\alpha = 0.025$ ): water treatment vs. background  $\chi^2 = 601.8$ , d.f. = 1,  $P < 0.0001$ ; dry treatment vs. background  $\chi^2 = 620.6$ , d.f. = 1,  $P < 0.0001$ ). This suggests that the ants systematically allo-groom all ants returning to the



**Fig. 4** Effect of allo-grooming and group size on spore removal. (a) Mean ( $\pm$ SE) number of spores remaining on the body of *Metarhizium*-contaminated ants that were kept in isolation or re-introduced in their group (28 ants) for 10 min. (b) Mean ( $\pm$ SE) number of spores remaining on the body of *Metarhizium*-contaminated ants that were kept in isolation, re-introduced in small groups (three ants), or re-introduced in large groups (12 ants) for 1 h. Different letters indicate treatments that differed significantly from one another.

group, independent of the treatment. In the re-introduction experiments, the amount of self-grooming did not vary significantly across treatments, neither when the individuals were observed in isolation (Fig. S1;  $F_{5,79} = 0.77$ ,  $P = 0.57$ ) nor when they were observed after returning to their group ( $F_{5,31} = 1.36$ ,  $P = 0.27$ ).

### Experiment 3: Effect of allo-grooming on spore removal

Contaminated ants re-introduced into their experimental group, where they could be allo-groomed by 28 other ants for up to 10 min, had significantly fewer fungal spores than ants kept in isolation, which could only self-groom (Fig. 4a;  $F_{1,65} = 6.44$ ,  $P = 0.01$ ). Hence, this short period of allo-grooming resulted in a 46% decrease in the number of spores remaining on the body of contaminated ants, relative to ants that could only self-groom. The number of spores remaining on the ants was negatively correlated with the duration of allo-grooming ( $t_{38} = -0.2$ ,  $P = 0.05$ ).

The time elapsed between contamination and re-introduction had no significant effect on the number of spores remaining on the ants ( $F_{1,65} = 0.2$ ,  $P = 0.6$ ). Moreover, the efficiency of allo-grooming was similar after a short and long period of isolation, as there was no interaction between the social treatment (allo-groomed or kept in isolation) and the time elapsed between contamination and re-introduction ( $F_{1,65} = 1.26$ ,  $P = 0.2$ ).

There was very limited transmission of disease from experimentally contaminated individuals to groomers or other group members. In total, 137 of the 1120 group members that potentially had been in contact with one contaminated nestmate died within 1 week of the second re-introduction, but only one of these 137 corpses produced *Metarhizium* spores.

### Experiment 4: Effect of group size on spore removal

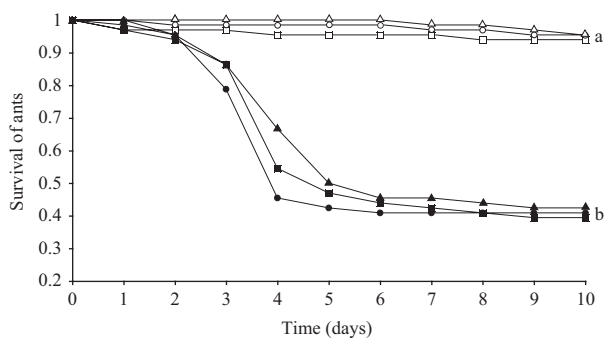
There were significantly fewer fungal spores on contaminated ants that had been re-introduced in groups of three or twelve ants for 1 h than on contaminated ants that were kept in isolation (Fig. 4b;  $F_{1,116} = 4.42$ ,  $P = 0.04$ ). However, group size had no significant effect on the number of spores remaining after grooming (Fig. 4b;  $F_{1,116} = 0.1$ ,  $P = 0.7$ ), and there was no significant interaction between social treatment (allo-groomed or kept in isolation) and group size (Fig. 4b;  $F_{1,116} = 0.0004$ ,  $P = 0.9$ ).

We detected no disease transmission from the contaminated ant to other group members. Only 16 of the 435 group members that potentially had been in contact with one contaminated nestmate died during the following week, and none of these 16 corpses produced *Metarhizium* spores.

### Experiment 5: Effect of allo-grooming on the survival of contaminated ants

The ants experimentally contaminated with *Metarhizium* spores suffered from a significantly higher mortality than control ants (Fig. 5; mortality after 10 days was 59% and 5% for contaminated and control ants, respectively;  $\chi^2 = 166.5$ , d.f. = 1,  $P < 0.0001$ ). The elevated mortality in contaminated ants was almost entirely caused by *Metarhizium* infection, as 90% of their corpses produced *Metarhizium* spores. In contrast, none of the corpses from control ants produced *Metarhizium* spores.

Allo-grooming did not protect the contaminated ants from the *Metarhizium* infection, at least under the limited conditions tested. Indeed, the survival of contaminated ants did not differ significantly when they could be allo-groomed, had social contact through mesh that prevented allo-grooming or were kept in isolation (Fig. 5;  $\chi^2 = 0.4$ , d.f. = 2,  $P = 0.8$ ). Moreover, there was no interaction between the fungal treatment (contaminated or control) and social treatment (allo-groomed, having



**Fig. 5** Effect of allo-grooming and social contacts on the survival of ants exposed to *Metarhizium*. Control ants (exposed to Tween buffer, open symbols) or *Metarhizium*-contaminated ants (closed symbols) were re-introduced in their group where they could be allo-groomed (circles), re-introduced in their group where they could have social contacts through mesh but no allo-grooming (triangles) or maintained in isolation where they could only self-groom (squares). After 2.5 h, the focal ants were kept in isolation and their survival (proportion of ants alive) was monitored for 10 days. Different letters indicate treatments that differed significantly from one another.

contacts through mesh, or isolated;  $\chi^2 = 0.7$ , d.f. = 2.4,  $P = 0.8$ ).

## Discussion

By cooperating, social animals can increase their resistance to environmental stressors, predators or parasites (Andersson, 1984; Hughes *et al.*, 2002; Andras *et al.*, 2007; Cremer *et al.*, 2007). Allo-grooming permits the removal of infectious particles from the body of other individuals, which is likely to increase the collective resistance to parasites at a potential cost to groomers (Schmid-Hempel, 1998; Hughes *et al.*, 2002; Cremer *et al.*, 2007; Clutton-Brock, 2009). Because allo-grooming combines social and hygienic functions, it is important to examine when and where it is expressed, and what are its benefits and costs in terms of disease resistance.

We found that the behavioural response of *F. selysi* ant workers to the presence of *M. anisopliae* fungal spores depended on the context and dose. When a social group was jointly exposed to a high dose of spores deposited in their nest chamber, the frequency of self-grooming increased significantly. This shows that the ants were able to detect the presence of spores, which is in line with previous studies on ants and termites (Jaccoud *et al.*, 1999; Ugelvig & Cremer, 2007; Mburu *et al.*, 2009; Ugelvig *et al.*, 2010). However, and somewhat surprisingly, this joint exposure of group-mates to spores did not result in a significant increase in the frequency of allo-grooming. It is possible that the presence of spores on filter paper did not result in a high degree of contami-

nation of the ants' body, even if it did cause significant mortality in previous experiments (Chapuisat *et al.*, 2007; Reber *et al.*, 2008).

Most individuals that were re-introduced into their group after a short or long period of isolation were immediately and intensively groomed by group-mates, independent of whether or not they had been contaminated by fungal spores. The intensity of allo-grooming was increased by the use of the surfactant Tween, when compared to the water or dry treatments. This response likely results from the ability of the surfactant to wet the hydrophobic cuticle of the ant (Rostas & Blassmann, 2009). Hence, the grooming response appears to be increased if the ants carry an unusual chemical substance adhering to their cuticle and potentially altering their odour. Such a modulation may serve both a hygienic function and the social function of re-homogenizing the colony odour (Boulay *et al.*, 2004).

Overall, these results suggest that allo-grooming is a proactive and prophylactic response directed towards all individuals returning to the nest, independent of the presence of fungal spores but modulated by chemical alterations. Individuals returning from exterior trips are likely to carry dangerous infectious propagules that may contaminate the nest and brood. The systematic and modulated cleaning of individuals returning to the nest by other individuals might thus serve to maintain the nest hygiene. In line with this hypothesis, leaf-cutting ant workers engage in intensive self-grooming upon entering nest chambers that contain brood or crops (Morelos Juárez *et al.*, 2010).

The allo-grooming of uncontaminated ants lasted for longer when the re-introduction occurred after a first re-introduction of a contaminated ant. This suggests that the ants further modulate their behaviour in response to the risk of infection, which they can evaluate from previous contacts with contaminated individuals. A similar result was observed in *Acromyrmex echinator*, where a second exposure to *Metarhizium* elicited a higher level of allo-grooming (Walker & Hughes, 2009). This also indicates that *F. selysi* workers are able to sense the presence of fungal spores or infection and may adjust their reaction accordingly at a later stage, even if they do not immediately increase the amount of allo-grooming towards contaminated individuals.

Allo-grooming was an efficient way to remove fungal spores from the body of group-mates. In experiment 3, the contaminated ants that were kept in isolation and could only self-groom had on average 5200 spores still present on their cuticle. Contaminated ants that could be allo-groomed for 10 min, in addition to any previous self-grooming, had about half this number of fungal spores remaining on their body. It is likely that allo-grooming permitted the removal of spores from body parts that were beyond the reach of self-grooming individuals. Re-introduced ants were also usually groomed by several individuals, which further increased the efficiency of



spore removal. Indeed, the total duration of allo-grooming was positively correlated with the number of allogroomers and negatively correlated with the number of conidia remaining on the surface of the groomed ant. Similarly, leaf-cutting ants and *Coptotermes formosanus* termites maintained in groups had fewer spores remaining on their body than individuals maintained in isolation (Hughes *et al.*, 2002; Yanagawa & Shimizu, 2007).

The efficiency of allo-grooming did not depend on the size of the group in which the contaminated ants were re-introduced. Moreover, when re-introduced into large groups, only a small fraction of the workers (four to six on average) groomed the contaminated ant. It thus seems possible that the number of ants that are able to efficiently groom a single individual at the same time is limited by space constraints or by some self-regulating mechanism of task allocation. The efficiency of allo-grooming at removing spores also did not depend on the time elapsed between contamination and re-introduction. This might be attributed to a weak adherence of this fungus, as suggested by the fact that *M. anisopliae* conidia bond less effectively to the insect cuticle than conidia from other entomopathogenic fungi (Yanagawa *et al.*, 2008).

In our experiments, the grooming of contaminated ants did not protect them from the fungal infection. The mortality caused by the fungal parasite was very high, as nearly 60% of the contaminated ants died from a *Metarhizium* infection. The survival of contaminated ants was not higher when they could be allo-groomed than when they only had social contact through a mesh preventing allo-grooming or were kept in complete social isolation. This result contrasts with several experiments that had shown that being in a group increased the survival of termites or ants challenged with *M. anisopliae* (Rosengaus *et al.*, 1998b; Hughes *et al.*, 2002; Traniello *et al.*, 2002). A major difference was that in our experiment, the contaminated individuals had only a relatively short contact with nestmates (up to 2.5 h) and were not maintained in the group until they died. Hence, the initial dose of spores might have been too high to permit their efficient removal by nestmates in this limited period of allo-grooming. Moreover, the ants were re-introduced 24 h after contamination, which is likely to reduce the effect of allo-grooming on ant survival if the conidia have already germinated and adhered to the cuticle. More generally, the curative effect of being in a group might require prolonged social interactions involving grooming, feeding or transfers of antibiotics.

The fungus strain that we used was virulent, as shown by the high mortality that it caused to experimentally contaminated ants. However, despite the high dose applied, the rate of disease transmission from contaminated ants to groomers and other group-mates was very low. In the course of our experiments, more than 1500 ants were exposed to a contaminated nestmate and only one of them died and produced *Metarhizium* spores. Low

rates of transmission of *Metarhizium* have already been documented in leaf-cutting ants, *Cardiocondyla* ants and *Zootermopsis* termites (Rosengaus *et al.*, 1998b; Hughes *et al.*, 2002; Ugelvig *et al.*, 2010). These low rates of disease transmission suggest that social insects are able to inactivate the spores that they remove. In several other ant and termite species, workers are indeed able to neutralize fungal spores by segregating them under nest material, destroying them mechanically by a compression in an infra-buccal pocket and/or exposing them to UV sunlight or antibiotics (Febvay *et al.*, 1984; Rosengaus *et al.*, 1998a; Jaccoud *et al.*, 1999; Hughes *et al.*, 2002; Poulsen *et al.*, 2002; Fernandez-Marin *et al.*, 2006; Little *et al.*, 2006; Cremer *et al.*, 2007). Whatever the mechanism, our results suggest that the removal of *Metarhizium* spores by allo-grooming is a low risk activity for the groomers.

In summary, *F. selysi* workers returning to their group after a period of isolation were systematically allo-groomed by nestmates, independent of the presence of *Metarhizium* spores on their bodies. The intensity of the response to returning ants appears to be increased in response to a substance coating the cuticle of the returning ant, or if the group has been previously exposed to a pathogen carried by a returning ant. A short period of allo-grooming permitted the removal of a large fraction of fungal spores from the bodies of heavily contaminated workers. The allo-groomers had little risk of becoming infected, but allo-grooming did not seem to protect the contaminated ants, at least under the set of conditions tested, which involved a high initial dose of spores and one late bout of allo-grooming. The systematic allo-grooming of returning foragers thus seems to be a constitutive prophylactic behaviour that protects the group against the entrance of infectious propagules, rather than a behaviour elicited by the presence of the parasites and aimed at protecting already heavily contaminated or infected individuals.

Overall, worker response to contaminated nestmates seems highly variable in social insects, ranging from social avoidance (Marikovski, 1964; Hart & Ratnieks, 2001; Ballari *et al.*, 2007; Heinze & Walter, 2010) to intensive allo-grooming (Rosengaus *et al.*, 1998b; Hughes & Boomsma, 2004; Walker & Hughes, 2009). The allo-grooming response may depend on the ecological context, including the identity of the parasite as well as the social and spatial organization of the host colony. For example, allo-grooming may be targeted at individuals that are potentially at risk of bringing infectious particles into the colony (this study) or be directed towards the most sensitive group members, such as brood (Ugelvig & Cremer, 2007). Allo-grooming may also provide protection beyond the removal of infectious propagules if the exposure of naïve individuals to nonlethal doses of a parasite increases their future immunity (Traniello *et al.*, 2002; Ugelvig & Cremer, 2007).

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Additional control experiments.

**Figure S1** This figure presents an additional control experiment to complement the experiment presented in Fig. 2 of the main text.

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