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Loss of attraction for social cues leads to fungal-infected *Myrmica rubra* ants withdrawing from the nest

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In social insects, individuals infected by pathogens withdraw from the nest, preventing the spread of diseases among genetically related nestmates and thereby contributing to the 'social immunity' of the colony. Here we investigated the extent to which the isolation of sick ants correlates with changes in their behavioural responses to environmental stimuli that serve as nest-related cues, including light, colony odour and physical presence of nestmates. *Myrmica rubra* ant workers infected by *Metarhizium brunneum* fungus showed a weak but constant attraction to light. By contrast, the progressive withdrawal of moribund workers from the nest appeared to be concomitant with a decline in their attraction towards nestmates or colony odour, which started on the third day after infection. We hypothesized that the fungus impaired the olfactory system of infected ants, preventing them from adequately reacting to chemical blends involved in colony marking and nestmate recognition. Instead of being an active behaviour, the social seclusion of sick ants appears to be the simple outcome of their increasing difficulty in orienting themselves towards nest-related cues. This phenomenon was reinforced by minor positive phototropism and the progressive dysfunction of motor skills as the infection progressed.

Social isolation of moribund individuals that desert their groups has been reported in several species, including vertebrates and invertebrates. This phenomenon has been studied in social insects where withdrawal from the nest appears to be a common response of moribund workers in ants (Bos, Lefèvre, Jensen, & D'Ettorre, 2012; Heinze & Walter, 2010) and bees (Rueppell, Hayworth, & Ross, 2010). In a few striking cases, social withdrawal can be induced by host-specific pathogens in order to complete their life cycle (Hughes et al., 2016; Schmid-Hempel, 1998).

A classic example involves the *Ophiocordyceps unilateralis* fungus, which causes infected ant workers to leave their nest and die in a specific place (generally under leaves) favourable for the growth and dispersal of the fungus (Andersen et al., 2009). However, in most cases, the isolation of dying individuals does not result from a behavioural manipulation by a host-specific parasite. Instead, all moribund ants break off social interactions regardless of whether their reduced life expectancy is due to old age, infection by a generalist pathogen or intoxication following exposure to high CO_2 levels (Heinze & Walter, 2010; Rueppell et al., 2010). Likewise, workers infected by generalist entomopathogenic fungi (such as *Metarhizium anisopliae*, *Metarhizium brunneum* or *Beauveria bassiana*) spontaneously leave their nest for the last hours or days of life without being rejected by colony members (Bos et al., 2012; Leclerc & Detrain, 2016). In these latter cases, withdrawal from the nest by sick workers therefore contributes to the 'social immunity' of the colony (Cremer, Armitage, & Schmid-Hempel, 2007), as it reduces their probability of spreading diseases among genetically related congeners (Boomsma, Schmid-Hempel, & Hughes, 2005; Myers & Rothman, 1995; Schmid-Hempel, 1998). Social seclusion of moribund individuals contributes to a suite of social defences that aim to limit the exposure of groupmates to potential sources of contamination, including, for instance, the avoidance of infected places or congeners (Cremer et al., 2007; Oi & Pereira, 1993, pp. 63–74), allogrooming behaviour (Yanagawa & Shimizu, 2007) waste management (Julian & Cahan, 1999) and necrophoresis, i.e. the removal of dead nestmates (Diez, Le Borgne, Lejeune, & Detrain, 2013; Diez, Lejeune, & Detrain, 2014).

Most studies on the social isolation of diseased workers have focused on the functional and adaptive value of this phenomenon but the underlying mechanisms have not yet been investigated. Thus, the question is raised of whether social withdrawal after a fungal challenge occurs concurrently to changes in the physiology of the infected insect, specifically in its response to environmental stimuli (Moore, 2002; Schmid-Hempel, 1998). For instance, in

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social insects, *Camponotus castaneus* ants, which are mainly nocturnal, become diurnal when infected by the nematode *Rabbium paradoxus* (Poinar, Chabaud, & Bain, 1989), whereas bumblebee workers infected by parasitoid conopid flies actively seek out lower temperatures that delay the development of the parasite (Müller & Schmid-Hempel, 1993). Likewise, the responses of infected individuals to social stimuli can be altered by diseases. For example, the paper wasp *Polistes dominulus* deserts its colony and forms extranidal aggregations with other parasitized wasps when infected by the strepsipteran parasite *Xenos vesparum* (Hughes, Kathirithamby, Turillazzi, & Beani, 2004).

These examples strongly suggest that social isolation shown by moribund workers could be related to changes in their responses to environmental and/or social stimuli. In this study, we investigated whether and how the generalist entomopathogenic fungus *M. brunneum* alters the behavioural responses of sick *Myrmica rubra* ants to environmental stimuli. Specifically, we aimed to link changes in the response of fungus-infected workers to light and/or social stimuli (i.e. colony odour or the presence of congeners) to the dynamics of their withdrawal from the nest, from the first physical contact with spores to the death of the ant host.

METHODS

Maintenance of Ants

We used four colonies of *M. rubra* that contained 200 workers, one queen and 40 larvae of the two first instars. All colonies were collected in Belgium from the localities of Sambreville (50°25′59.62″N, 4°37′22.12″E) and Falisolle (50°25′11.99″N, 4°37′50.41″E) in dead wood and litter in semi-open forests. In the laboratory, each colony was put in a plastic box (47 × 29 cm) with a plaster floor and was settled in a nest consisting of a square glass ceiling (10 × 10 cm) placed 3 mm above the ground and covered with a red filter paper. To prevent ants from escaping, borders were covered with Polyetrafluoroethylene Fluon (Whitford, U.K.). Colonies were fed three times a week with a mealworm, *Tenebrio molitor*, while sucrose solution (0.3 M) was provided ad libitum. Laboratory conditions were kept at 21 ± 1 °C and 50 ± 5% humidity rate, with a constant photoperiod of 12:12 h per day.

Entomopathogenic Fungus

We used *M. brunneum* fungus as the infection factor, a pathogen commonly used in studies about social immunity in ants (Chapuisat, Oppliger, Magliano, & Christe, 2007; Reber & Chapuisat, 2012). We used a commercial strain F52 of the fungus (Met52 EC from Novozymes, Bagsvaerd, Denmark, a commercialized isolate previously identified as *M. anisopliae*) that is produced in the form of rice grains coated with fungal spores. This standard strain allowed us to have a constant pathogenicity and virulence of the pathogen throughout the experiments, in addition to the practical advantages in terms of conservation and use. Furthermore, this generalist entomopathogen, which is known to contaminate and kill more than 200 insect species (Meyling & Eilenberg, 2007), regularly occurs in the natural habitat of *M. rubra* (Groden, 2005). Workers infected by this fungus progressively decrease their nest attendance without being rejected by nestmates. This social withdrawal begins at the third day of infection when the health of individuals begins to deteriorate (Leclerc & Detrain, 2016). Indeed, before the penetration of fungal spores through the cuticle, which takes 48–96 h (Gillespie, Bailey, Cobb, & Vilcinskas, 2000), the contaminated worker, which still bears spores on its cuticle, is infectious for its nestmates but is not yet physiologically impacted by the fungus. Health deterioration starts on the third day of infection after the penetration of spores when the hyphae invade the insect's body (Hänel, 1982). This ultimately leads to the death of the ant, 3–10 days after exposure to the fungus depending on the host species (Khashaveh & Chelav, 2013). Finally, 48–72 h after the host dies, the fungus breaks out of the host's cuticle, sporulates and releases new spores which can infect new hosts (Hänel, 1982).

Infection Protocol

Before starting the behavioural experiments, we had first to contaminate ants with *M. brunneum* spores by using a standardized procedure of infection. We chose to infect foragers since this group of ant workers is likely to be regularly exposed to pathogens when patrolling in their nest surroundings. We randomly sampled a set of workers on the foraging area of each tested colony and put them in groups of five in an Eppendorf (1 ml size) containing a sporulating carcass. The tube was then vortexed at a speed of 2000 rounds/min four times during 10 s. The sporulating carcass was obtained by vortexing a *M. rubra* ant with a rice grain coated with *M. brunneum* spores. After death, the ant was placed over a filter paper soaked with 6 ml distilled water in a closed petri dish and was incubated for 14 days at 25 °C for an optimal germination of conidia.

After having undergone this infection procedure, infected foragers were provided with water and sucrose (0.3 M) ad libitum and were kept separately for 1 h to limit the removal of spores by allogrooming nestmates (Okuno, Tsuji, Sato, & Fujisaki, 2012). A dot of paint (paint marker 751, Edding AG, Ahrensburg, Germany) was put on the abdomen of the ant which was isolated for another 1 h to allow the paint to dry. Finally, tested ants were released in their mother colony until the beginning of the experiments. All experiments were done between 1000 and 1200 hours to prevent any bias of the circadian rhythm on the ant's responses. Paint marking allowed us to discriminate between infected ants and their nestmates and to track them inside their mother colony. The infection protocol was efficient since 84% of infected ants sporulated after their death, the latter being the only ones included in our analysis. Besides infected ants, additional foragers from the same colonies that were used as a control underwent the same infection protocol with the exception that no sporulating carcass was put in the Eppendorf and the dot of paint was of a different colour.

Response to Light

Since a change in phototropism may account for infected ants leaving the nest, we compared how infected ($N = 24$) and healthy ($N = 24$) foragers ($N = 6$ individuals tested per nest and per health condition) responded to light. Ants were tested individually to quantify the response of the ants to a given environmental stimulus, independently of any other factors that may influence their location such as the presence of nearby nestmates. Each tested individual was placed in a set-up consisting of a closed petri dish (55 mm diameter; Fig. 1) of which one half was opaque to create a dark area and the other half was illuminated by a heatless lamp (Philips 20 W) placed 40 cm above the petri dish. Dishes were randomly turned to prevent any experimental bias due to external visual cues that could be perceived by tested ants. After being released at the centre of the petri dish, the individual was filmed during 10 min and then was replaced in its mother colony until the next day. Each ant underwent this test once per day for 5 days to assess how responses to light changed with the progression of the fungal infection. Videos were analysed with Solomon coder software (Andras Péter, solomoncoder.com) to track which half of the set-up the ant used during the test. We then calculated the percentages of time spent by one individual on each half of the set-up. We also recorded the time that an ant spent either on the lit or on

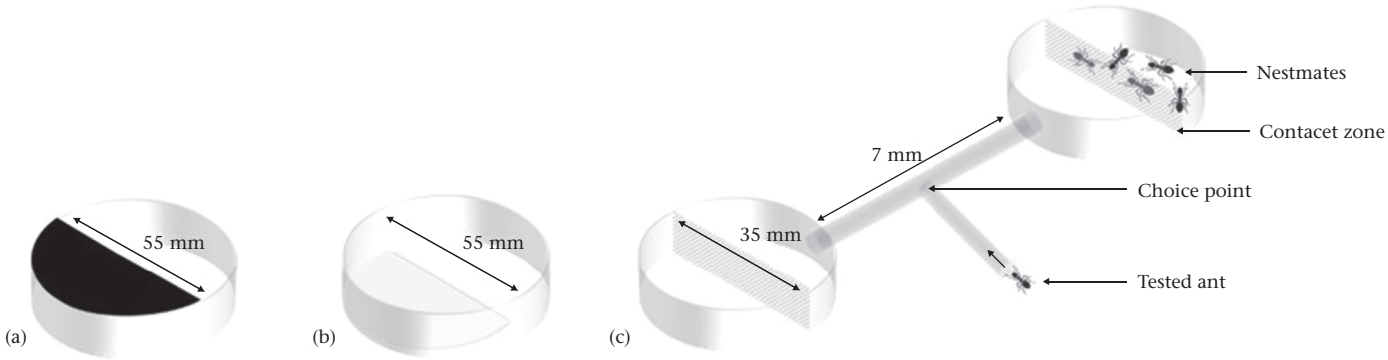

Figure 1. Experimental set-ups for testing attraction to (a) light, (b) colony odour and (c) nestmates.

the dark area. This first 'staying time' allowed us to get, for each ant, the exact time of its departure from the area without this measure being interrupted by the end of the experiment. Only the first staying time that lasted more than 20 s was considered. This allowed us to reduce noise in data collection due to rapid between-area shifts displayed by excited workers that were newly introduced in the set-up.

Attraction towards Colony Odour

Healthy ants are known to be attracted by their colony odour which favours their aggregation (Depickère, Fresneau, & Deneubourg, 2004). We tested whether withdrawal of infected individuals from the nest could be related to an alteration in their attraction to colony odour. Healthy ($N = 24$) or infected ants ($N = 24$) that were evenly sampled from four nests were each put in a separate petri dish (Fig. 1) with a filter paper cut in half (55 mm diameter) on the base. Half of the filter paper was devoid of chemical marks while the other half was previously put in the mother colony of the tested ant for 24 h to be chemically marked by the ants' footprints (Devigne & Detrain, 2002; Lenoir, Depickère, Devers, Christidès, & Detrain, 2009). The two pieces of the filter paper were randomly placed in the dish to avoid a possible experimental bias. For 5 days after the infection procedure, each ant was filmed during a 10 min session per day with the purpose of comparing how attraction to area marking changed for infected workers and their healthy counterparts. Solomon coder software was used for coding the location of individuals over time. As described above, we also calculated the first staying time of tested ants on either the unmarked area or the area chemically marked by the colony.

Attraction towards Healthy Nestmates

We conducted tests in a T-maze set-up to investigate a possible change in the social attraction of infected ants towards nestmates (Fig. 1). Forty-eight focal ants ($N = 24$ healthy and $N = 24$ infected workers) were sampled from four nests ($N = 6$ per nest and per condition). Twenty-four hours after being infected, the tested ant was introduced in the set-up consisting of a T-shaped plastic tube (7 mm diameter and 7 cm long) with branches leading to two closed petri dishes (35 mm diameter; Fig. 1). Each petri dish was divided in half by a 'contact zone', consisting of a metallic grid that allowed antennal contacts between the tested ant and nestmates but prevented individuals from moving to the other side. Before each test, five workers belonging to the same colony as the tested ant were randomly put into the most remote half of one of the petri dishes while the other dish was left empty. After being put in the T-shaped plastic tube, the tested individual was filmed for 10 min and then replaced in its mother colony. Each tested ant underwent this test once per day for 5 days. The T-maze set-up allowed us to assess a possible remote perception of volatile compounds emitted by nestmates by observing the direction taken by ants at the choice point, defined as the intersection of the T-shape (Fig. 1). We also noted the first visited dish and measured the latency to reach it. Finally, the tested ants' search for physical contacts with nestmates was assessed by using Solomon coder software to calculate the ratio of the time the tested individual had spent in the contact zone close to nestmates over the total time spent in the corresponding petri dish. As a control, we also calculated the relative time they spent in the contact zone of the empty dish. To get a reference value for the social attraction/avoidance of *M. rubra* ants towards non-nestmates, six healthy individuals were taken from the four *M. rubra* nests ($N = 24$) and were tested in the presence of five workers originating from a foreign colony during a 1-day session.

Statistical Analysis

Except when otherwise specified, data were analysed with Statistica version 10 (Statsoft, Tulsa, OK, U.S.A.). Nonparametric tests with a significance level of alpha = 0.05 were used since no data met the normality criteria. With regard to phototropism and colony odour attraction, general linear mixed-effect models (GLM) were used to investigate the effect of treatment, colony and time on the time spent by tested ants on the lit or the marked area. For all GLM analyses, colony and treatment were treated as categorical variables, whereas time was considered as a continuous variable. Moreover, time and treatment were specified as fixed effects, colony as a random effect and ant identity as a nested random factor within colony to account for the repeated measurements performed on tested ants (Pinheiro & Bates, 2000). Full models included treatment, colony and time as explanatory variables, and all relevant second-order interactions. When a significant interaction effect occurred, a Friedman test was applied to test for temporal changes within each treatment, while daily comparisons between healthy and infected tested ants were made by using Tukey post hoc tests. We assessed for how long ants remained on one side of the set-up. For the first staying times on one side of the set-up, we drew survival curves that represented the percentage of tested ants that were still present after a given amount of time had elapsed since they reached this side. Survival curves of staying times were then compared for both healthy and infected ants using log-rank (Mantel–Cox) tests. In addition, these percentages of tested ants remaining on an area were ln-transformed and fitted by a linear regression whose slope provides an estimate of the probability of an ant leaving this area per unit of time. We estimated the

slope parameters using a linear least-square method and linearization was considered as satisfying when the best fitting of transformed data showed a coefficient of determination above 0.90. This condition was met only in experiments measuring ants' response towards colony odour, for which we compared slopes of linearized distributions with GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, U.S.A.). The software first shared the value of the slope to fit one line to all data sets and then fitted individual slopes to each data set. The *F* test compared the fits of these models and reported a *P* value (two-tailed) testing the null hypothesis that the slopes are all identical.

For attraction towards nestmates, the direction taken by ants at the choice point was analysed for each day and each condition using binomial tests with a theoretical probability of 0.5. We compared for each day the proportions of healthy and sick ants that made U-turns in the set-up by using chi-square tests. Moreover, to rule out any side-effect on U-turn behaviours, we used McNemar chi-square tests to compare for each day of each condition the proportions of ants turning back when heading towards the dish containing congeners or towards the empty dish.

Furthermore, we used a GLM analysis to assess the effect of treatment, colony and time on the latency of an ant to reach one dish as well as on the ratios of the total time spent by the tested ant on the contact zone over the total time spent in the corresponding dish. When an effect was significant, post hoc comparisons between healthy and infected tested ants were made using a Tukey test. Finally, ratios of time spent on the contact zone with nestmates were compared to those spent on the contact zone of the empty dish for each health condition and each experimental day by using a Wilcoxon matched-pairs test.

Ethical Note

No licences or permits were required for this research. Ant colonies were collected with care in the field and maintained in nearly natural conditions in the laboratory. Ants were provided with suitable nesting sites, food and water thus minimizing any adverse impact on their welfare. After the experiments, fungal-infected ants were removed from their foraging area to protect colonies from disease spread and were killed by freezing. The rest of the colony was kept in the laboratory and reared until their natural death.

RESULTS

Response to Light

There was no interaction effect between the treatment and the change over time of the ants' response to light (GLM: Treatment*-Time interaction: $F_{1,227} = 0.38$, $P = 0.84$). The percentage of time an individual spent on the lit area did not change over time from the first to the last experimental day (GLM: Time effect: $F_{4,223} = 0.59$, $P = 0.67$). However, infected ants were consistently more present over the lit area than healthy ones during the experiment (GLM: Treatment effect: $F_{1,223} = 10.56$, $P = 0.001$; Fig. 2).

For healthy ants, the survival curves of their first staying times were similar when they stayed in the lit or in the dark areas of the set-up (Table 1). For instance, the presence of illumination in one area did not influence the median staying time of healthy ants on the first day of the experiment (Fig. 3a, Table 1) or on the fifth final day of the experiment (Fig. 3b, Table 1). Only on day 2 were staying times of healthy ants slightly longer over the lit area (Table 1).

In contrast, on the first day following infection, the median staying time of infected ants was twice as high in the lit area than the dark area, even though the associated survival curves did not differ significantly (Fig. 3c, Table 1). The phototropism of infected ants was confirmed by comparing survival curves for the following days. Indeed, the staying times of infected individuals were significantly longer in the lit area than the dark area from the second day onwards (Table 1). For example, on the last day of the experiment, the median staying times was 335 s in the lit area compared to just 135 s in the dark area (Fig. 3d).

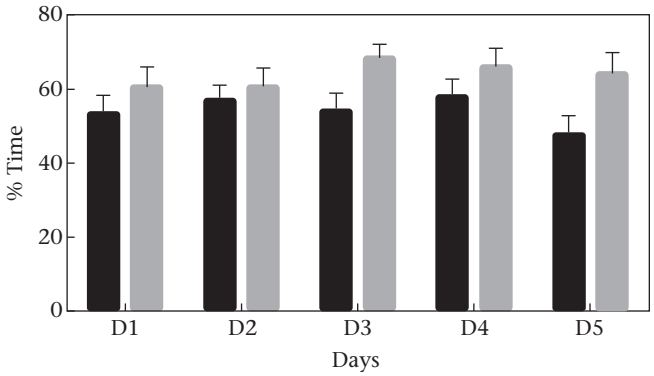

Figure 2. Change in presence of ants over the lit area as a function of days elapsed since the infection procedure. Change in presence (mean + SE) is given by the percentage of time spent by a worker on the lit side of the set-up during a 10 min session. Black bars: healthy individuals ($N = 24$); grey bars: infected individuals ($N = 24$).

Attraction towards Colony Odour

The percentage of time spent in the area marked by colony odour changed differently over time for healthy and infected ants (GLM: Time*Treatment interaction: $F_{4,227} = 3.82$, $P = 0.002$; Fig. 4). Indeed, while healthy ants spent around 60% of the time in the marked area for each experimental day (Friedman tests for repeated measures: $\chi^2 = 1.95$, $P = 0.74$), the percentage of time that infected ants spent in the marked area tended to decline over time, although in a nonsignificant way (Friedman tests for repeated measures: $\chi^2 = 7.5$, $P = 0.11$; Fig. 4). Daily comparisons of the rate of presence according to health status showed that infected ants spent significantly less time in the marked area than healthy ants from the third day of the experiment onwards (GLM: Tukey post hoc tests: day 3: $P = 0.02$; day 4: $P = 0.008$; day 5: $P < 0.001$; Fig. 4).

The survival curves of first staying times showed that healthy ants stayed significantly longer in the marked area than the unmarked side of the set-up for all experimental days, except the first day (Fig. 5a and b, Table 2). On the fifth (final) day, the median first staying time spent in the marked area was almost twice that in the unmarked area (Fig. 5b, Table 2). The chemical marking of the substrate did not influence the resting behaviour of infected ants throughout the experiment (Fig. 5c and d). Indeed, the survival curves drawn from their first staying times did not differ when ants were in the marked or the unmarked areas of the set-up (Table 2). For example, the median times of the first stay in the marked area on the first (180 s) and fifth (175 s) days were very similar to those recorded for the unmarked area (162.5 s and 205 s on day 1 and day 5, respectively; Fig. 5c and d, Table 2).

Ln-transformed data of the survival curves were satisfactorily linearized, with an R^2 coefficient of determination higher than 0.9 for each experimental condition and each experimental day (Table 2). This indicates that the probability of an ant leaving an area per unit of time was constant over time and could be assessed by the slope of linearized survival curves. The comparison of slope values (Table 2) confirmed that healthy *M. rubra* ants were more likely to stay close to their colony odour for all experimental days (except day 1). For instance, the probability of a healthy ant leaving

Table 1
First staying times of ants on the lit or on the dark area as a function of days elapsed since the infection procedure

		Healthy ants (N=24)				Infected ants (N=24)			
		Lit area	Dark area	χ^2	Log-rank test (P)	Lit area	Dark area	χ^2	Log-rank test (P)
Day 1	N	13	11			13	11		
	Median (s)	155	135	0.09	0.76	170	90	2.01	0.16
Day 2	N	12	12			15	9		
	Median (s)	135	112.5	4.16	0.04*	165	85	10.29	<0.001
Day 3	N	14	10			12	12		
	Median (s)	240	200	0.03	0.85	192.5	95	7.72	0.006
Day 4	N	11	13			15	9		
	Median (s)	185	175	0.65	0.42	230	115	3.86	0.04
Day 5	N	9	15			13	11		
	Median (s)	115	150	0.05	0.81	335	135	4.56	0.03

Staying times are given as median values (s). N is the sample size. Based on staying times, the survival curves of ants on each side of the set-up were drawn and then compared using a log-rank test. Log-rank statistics (χ^2) and the related P value are summarized in the table.

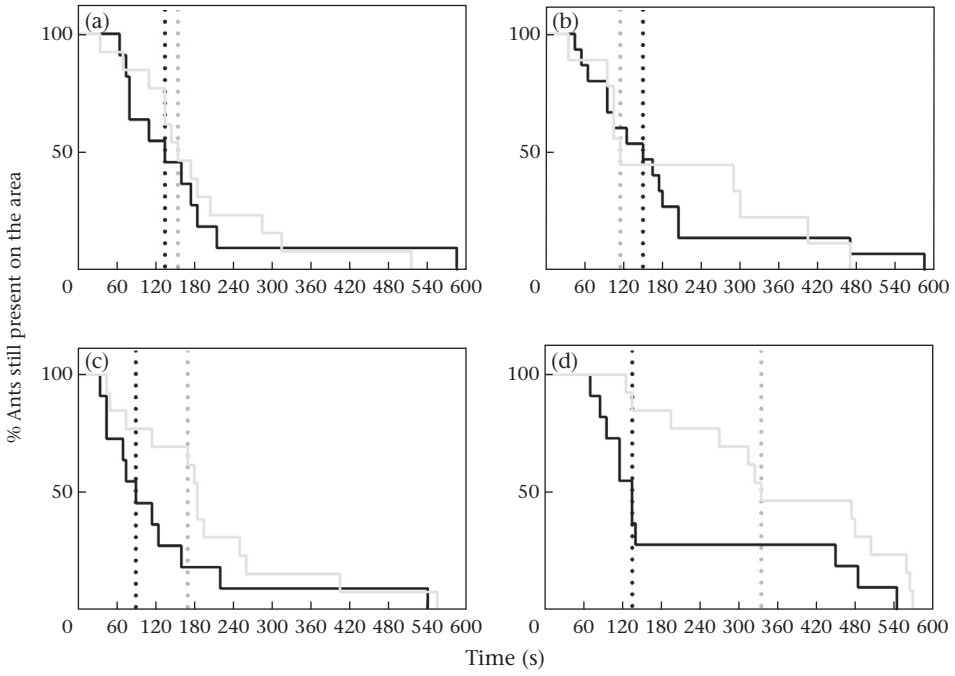

Figure 3. Survival curves drawn from the first staying times observed for (a, b) healthy ($N = 24$) and (c, d) infected ants ($N = 24$) on the lit (grey line) and dark areas (black line) of the set-up. Each curve shows the percentage of ants still present on one area for a given staying time. These curves were drawn for (a, c) the first day and (b, d) the fifth day that followed the infection procedure. The vertical lines indicate the median times at which half of the ants had left the dark area (black dashed line) or the lit area (grey dashed line).

the marked area per unit of time was about twice (probability of 0.007 at day 2) to seven times (probability of 0.005 at day 4) lower than the probability of leaving the unmarked area (probability of 0.016 and 0.031 at day 2 and day 4, respectively; Table 2). In contrast, once infected, ants were as likely to stay in the marked or unmarked areas. The slopes representing the probabilities of quitting the unmarked area over time were similar to those describing probabilities of leaving the marked area, whatever the experimental day (Table 2).

Attraction towards Healthy Nestmates

Once ants were engaged in the T-maze set-up and had reached the choice point, the latency of infected ants to enter a petri dish changed differently from that of healthy nestmates (GLM: Time∗Treatment interaction: $F_{4,227} = 5.51$, $P < 0.001$; Fig. 6a). While the latency of healthy individuals was constant over time (Friedman test for repeated measures: $\chi^2 = 6.46$, $P = 0.17$), there was an increasing latency for infected ants which moved more slowly with time, most probably because their health was deteriorating (Friedman test for repeated measures: $\chi^2 = 23.78$, $P < 0.001$; Fig. 6a). On the last experimental day, infected workers required, on average, more than 3 min to reach one dish, with their latency being 11-fold longer than that of healthy ants (GLM: Tukey post hoc test: $P < 0.001$; Fig. 6a). At the choice point, both infected and healthy ants were as likely to orient over one branch of the T-maze set-up, whatever the experimental day (binomial tests: all P values > 0.05). Apart from one tested ant on the fourth day, all the healthy ants consistently reached the petri dish towards which they initially oriented at the choice point (chi-square tests: all P values > 0.05; Fig. 6b). By contrast, infected ants were more likely to change their initial direction. Such U-turns were observed from the third day of infection and were significantly more frequent on the fifth day (Fig. 6b). Indeed, significantly fewer infected ants maintained their choice (66.6%) than healthy nestmates (100%) on the last experimental day (chi-square test: $\chi^2 = 14.02$, $P < 0.001$; Fig. 6b). Interestingly, we found no side-effect of nestmates' presence on U-turn behaviour, with this behaviour appearing to occur

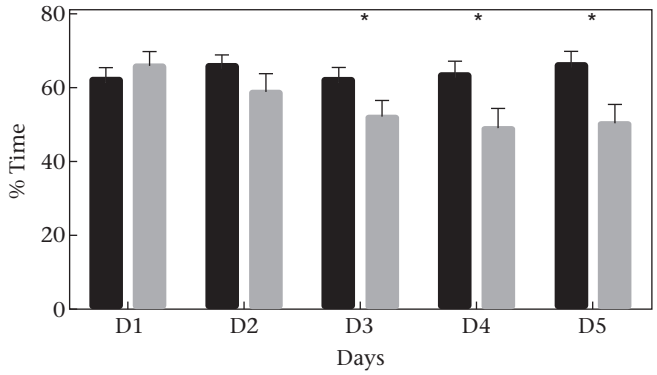

Figure 4. Change in presence of ants over the area marked by their colony odour as a function of days elapsed since the infection procedure. Change in presence (mean + SE) is given by the percentage of time spent by a worker on the marked side of the set-up during a 10 min session. Black bars: healthy individuals ($N = 24$); grey bars: infected individuals ($N = 24$). Pairwise comparisons were made using a Tukey post hoc test ($^*P < 0.05$).

randomly, regardless of the initial direction in which ants were heading at the choice point (McNemar chi-square test: day 4: $\chi^2 = 1.13$, $P = 0.29$; day 5: $\chi^2 = 2.29$, $P = 0.13$).

After entering the dish containing congeners, the ratio of time spent by healthy and infected ants close to nestmates changed in a different way from that of their infected counterparts (GLM: Time * Treatment interaction: $F_{4,237} = 6.34$, $P < 0.001$; Fig. 7a). Indeed, healthy ants spent around half of their time contacting nestmates through the grid for each experimental day (Friedman test for repeated measures: $\chi^2 = 5.63$, $P = 0.23$; Fig. 7a), whereas the attraction of infected individuals towards nestmates decreased as infection progressed (Friedman test for repeated measures: $\chi^2 = 24.5$, $P < 0.001$; Fig. 7a). As a result, infected ants spent significantly less time close to nestmates than their healthy counterparts from the third day of the experiment onwards (GLM: Tukey post hoc Tests: day 3: $P = 0.02$; day 4: $P = 0.003$; day 5: $P < 0.001$; Fig. 7a). Both healthy and infected ants spent a small amount of time close to the grid of the empty dish, with this behaviour remaining consistent through time (GLM: Time * Treatment interaction: $F_{4,237} = 2.37$, $P = 0.15$; Fig. 7b).

Finally, healthy ants stayed significantly longer on the contact zone when perceiving nestmates than on the grid of the empty dish for all experimental days, except day 1 (Wilcoxon matched-pairs test for day 1: $T = 114$, $P = 0.19$; day 2: $T = 52$, $P = 0.03$; day 3: $T = 63$, $P = 0.01$; day 4: $T = 65$, $P = 0.009$; day 5: $T = 51$, $P = 0.002$; Fig 7a and b). By contrast, the attraction of infected individuals towards nestmates changed as infection progressed. On the first day, infected ants were attracted to congeners, staying significantly longer on the contact zone close to nestmates than on the empty grid (Wilcoxon matched-pairs test for day 1: $T = 48$, $P = 0.002$; Fig 7a and b). Subsequently, the percentage of time spent close to nestmates decreased significantly and no longer differed from the time spent on the grid of the empty dish (Wilcoxon matched-pairs test for day 2, 3, 4, 5: all P values > 0.05). On the last day of the experiment, the relative time that infected ants spent in contact with congeners (12.6%) was as low as the time spent on the contact zone of the empty petri dish (16.4%; Fig. 7a and b) or the time spent by healthy workers in contact with alien ants (5.5%; Mann–Whitney tests: $U = 273$, $P = 0.45$; Fig. 7a).

DISCUSSION

When ants are near death, they isolate themselves from congeners by staying longer outside the nest (Heinze & Walter, 2010; Leclerc & Detrain, 2016). Here, we have shown that this progressive shift in location was not related to a temporal change in the behavioural response of fungus-infected individuals to light, which they may use as a cue to differentiate between the nest interior and

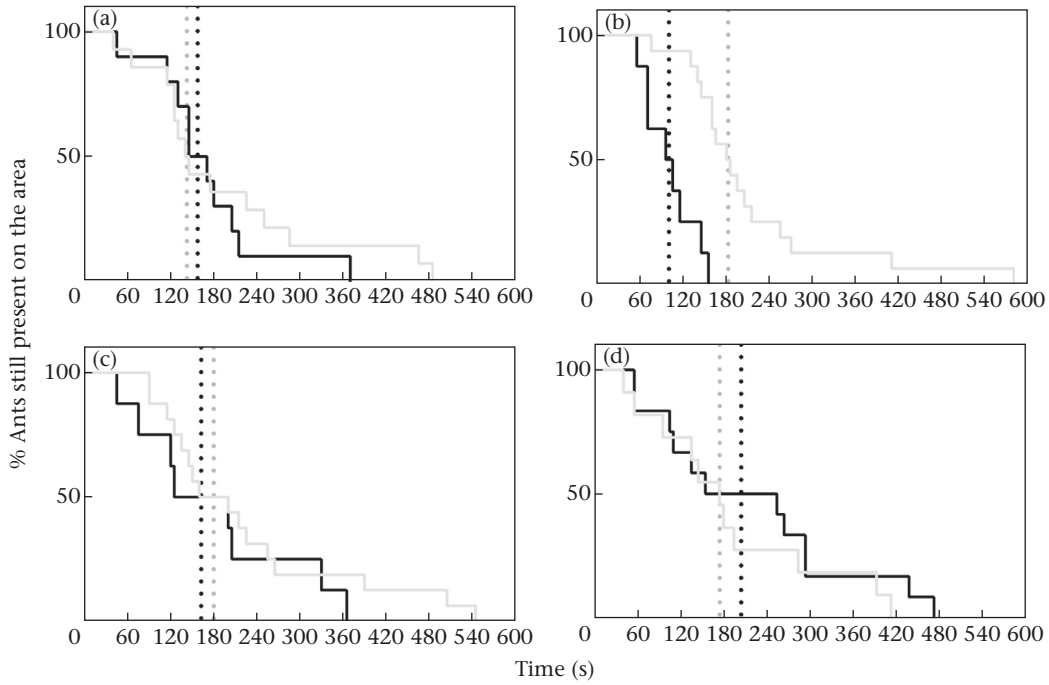

Figure 5. Survival curves drawn from the first staying times observed for (a, b) healthy ($N = 24$) and (c, d) infected ants ($N = 24$) on the unmarked area (black line) or the area marked by the colony odour (grey line). Each curve shows the percentage of ants still present on one area for a given staying time. These curves were drawn for (a, c) the first day and (b, d) the fifth day that followed the infection procedure. The vertical lines indicate the median times at which half of the ants had left the unmarked area (black dashed line) or the marked area (grey dashed line).

Table 2
First staying times of ants on the marked and unmarked areas as a function of days elapsed since the infection procedure

		Healthy ants (N=24)					Infected ants (N=24)				
		Marked area	Unmarked area	Test	χ^2/F	P	Marked area	Unmarked area	Test	χ^2/F	P
Day 1	N	14	10				16	8			
	Median (s)	142.5	157.5	Log-rank	0.31	0.58	180	162.5	Log-rank	0.77	0.38
	Slope$\times10^{-1}$ (R^2)	−0.06 (0.95)	−0.08 (0.91)	F test	2.27	0.15	−0.05 (0.97)	−0.06 (0.95)	F test	0.45	0.51
Day 2	N	15	9				13	11			
	Median (s)	175	75	Log-rank	11.29	<0.001	175	140	Log-rank	1.61	0.20
	Slope$\times10^{-1}$ (R^2)	−0.07 (0.95)	−0.16 (0.95)	F test	35.89	<0.001	−0.05 (0.95)	−0.07 (0.92)	F test	1.59	0.22
Day 3	N	16	8				14	10			
	Median (s)	175	90	Log-rank	15.47	<0.001	155	100	Log-rank	0.005	0.94
	Slope$\times10^{-1}$ (R^2)	−0.04 (0.93)	−0.24 (0.93)	F test	44.09	<0.001	−0.06 (0.97)	−0.06 (0.91)	F test	0.19	0.67
Day 4	N	15	9				13	11			
	Median (s)	210	115	Log-rank	4.63	0.031	115	155	Log-rank	1.03	0.31
	Slope$\times10^{-1}$ (R^2)	−0.04 (0.91)	−0.31 (0.97)	F test	48.66	<0.001	−0.06 (0.98)	−0.04 (0.91)	F test	2.01	0.17
Day 5	N	16	8				11	12			
	Median (s)	182.5	100	Log-rank	15.9	<0.001	175	205	Log-rank	0.67	0.67
	Slope$\times10^{-1}$ (R^2)	−0.06 (0.91)	−0.18 (0.94)	F test	27.33	<0.001	−0.05 (0.95)	−0.05 (0.92)	F test	1.04	0.32

Staying times are given as median values (s). N is the sample size. Based on staying times, the survival curves of ants on each side of the set-up were drawn and then compared using a log-rank test. Log-rank statistics (χ^2) and the related P value are summarized in the table. Ln-transformed data of survival curves were best fitted with a regression line the slopes and the R^2 value of which are presented in the table. The opposite of slope value gives the probability of an ant leaving the area. The slopes of linear fittings were compared between ants staying on the marked and on the unmarked area by using F tests the statistics and related P values of which are given in the table.

external environment. By contrast, the social isolation of infected ants occurred concurrently with a pronounced decline in attraction towards social cues: namely, the level of colony area marking and the physical presence of nestmates. This decrease in social attraction was observed from the third day after infection, when the health of the ant starts deteriorating (Gillespie et al., 2000; Hänel, 1982).

Light is known to regulate the exit of foragers from the nest (Frisch & Aschoff, 1987; Moore & Rankin, 1993), by acting on circadian rhythms in most eusocial species (Kasper, Reeson, Mackay, & Austin, 2008). Here, we found that infected *M. rubra* ants stayed significantly longer in the lit than in the dark area of the set-up from the second day of the experiment onwards. Fungus-contaminated ants may use this slightly positive phototropism to

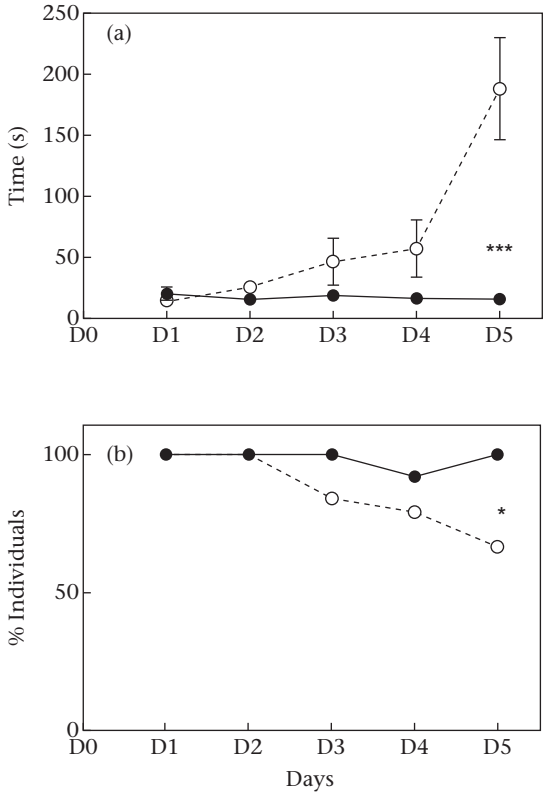

Figure 6. (a) Latency (mean ± SE) to enter the first visited petri dish and (b) percentages of individuals that maintained their initial heading direction as a function of days elapsed since the infection procedure. Filled circles: healthy individuals (N = 24); open circles: infected individuals (N = 24). Pairwise comparisons were made using (a) a Tukey post hoc test or (b) a chi-square test (*P < 0.05; ***P < 0.001).

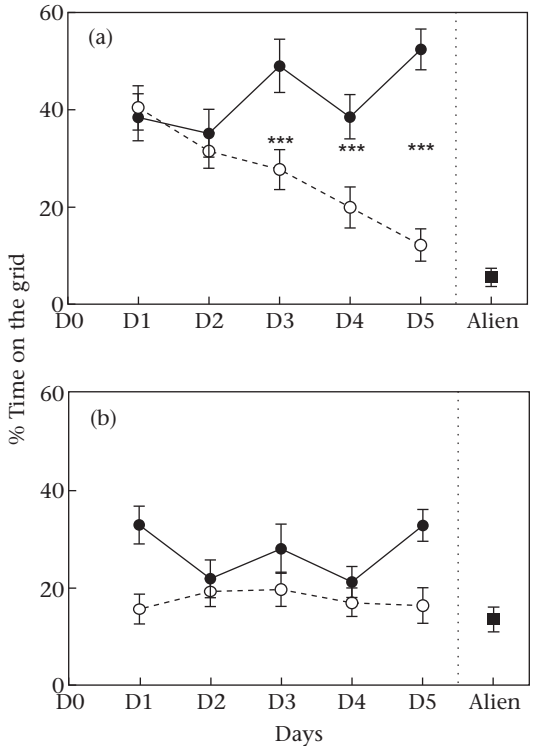

Figure 7. Percentages of time (mean ± SE) spent by tested individuals on (a) the contact zone of the dish occupied by nestmates or (b) the empty dish, as a function of the time elapsed since the infection procedure. Filled circles: healthy individuals (N = 24); open circles: infected individuals (N = 24). Alien (filled squares): attraction towards alien *M. rubra* ants was measured by the percentage of time (mean ± SE) spent by healthy ants (N = 24) in contact with workers from another colony. Pairwise comparisons were made using a Tukey post hoc test (***P < 0.001).

search for ultraviolet light to kill spores (Braga, Rangel, Fernandes, Flint, & Roberts, 2015; Rotem, Wooding, & Aylor, 1985) and/or may benefit from heat to stimulate innate immunity (Blanford & Thomas, 2001). However, the fitness gain of standing under light for infected ants may be counterbalanced by the enhancing effect of heat on the development of the fungus within the insect's body (Blanford, Thomas, & Kedwards, 1999). Regardless of the potential sanitizing effects of ultraviolet light and/or heat, light does not appear to be the main stimulus leading to the progressive isolation of infected workers outside their nest. Indeed, their slightly higher attraction to light was constant over time, differing from the dynamics of social seclusion that abruptly increased from the third day after infection until death.

The spatial distribution of workers is known to be shaped by their response to direct cues, such as direct interactions with nestmates (Depickère et al., 2004), and to indirect cues, such as chemical marking the intensity of which is directly related to the ants' density (Detrain & Deneubourg, 2008; Devigne & Detrain, 2002; Lenoir et al., 2009). Our results show that the attraction of ants towards both direct and indirect social cues was strongly altered by fungal infection. Indeed, infected ants showed a gradual decline in attraction to nestmates or to their colony odour. Infected ants even completely lost any preference for social cues on the last day of the experiment, just before their death. Such altered responses to social cues following infection have already been recorded in several insect species. For instance, the microsporidian parasite *Paranosema locustae* prevents locusts from forming aggregations and induces gregarious individuals to shift back to a solitary behaviour (Shi et al., 2014). Likewise, virus-infected aphids are no longer attracted by conspecifics' odour (Ban, Ahmed, Ninkovic, Delp, & Glinwood, 2008). We never observed aggressive displays by congeners confirming that the social seclusion of infected individuals did not result from rejection (Leclerc & Detrain, 2016). Likewise, we did not observe any active avoidance of nestmates by infected ants since they never avoided heading towards congeners either at the choice point of the set-up or at the grid interface. Furthermore, the time spent by infected ants near nestmates became similar to that on the empty side but remained around twice that spent by healthy ants in the presence of alien ants. This supports the idea that social seclusion of infected individuals was not the result of deliberate avoidance of social interactions with nestmates, but was rather the outcome of dysfunctional perceptive and motor systems as infection progressed.

Social attraction in fungus-infected *M. rubra* workers declines at day 3 after infection, when the fungal hyphae proliferate within the host's body and damage its tissues (Hänel, 1982). At this step of the infection process, *M. rubra* workers showed an increasing latency to explore the T-shape set-up due to impaired mobility of legs (personal observations). Furthermore, a slight paralysis of the antennae prevents individuals from correctly orienting themselves (Gewecke, 1974), which might explain the frequent U-turns displayed by moribund individuals. Fungal infection should also have impaired the olfactory system of ants, preventing them from fully perceiving chemical compounds in the environment as reported for bacteria- infected fruit flies that failed to respond to food olfactory cues (Peng, Nielsen, Cunningham, & McGraw, 2008). Likewise, mosquitoes infected by *B. bassiana* or *M. brunneum* fungi show a decline in the electrophysiological sensitivity of olfactory sensilla to CO_2, which impairs their host-seeking efficiency (George et al., 2011). Failure to perceive chemical compounds might also explain why infected ants were never seen collecting food or standing close to a food source (Bos et al., 2012; Heinze & Walter, 2010).

Thus, the isolation of infected ants is probably due to their impaired olfactory abilities to (1) perceive low concentrations of certain compounds, such as chemical markings, (2) decode complex chemical blends, such as the cuticular hydrocarbon profile used to mark the colony's home range, or (3) recognize nestmates. Further investigation is required on the extent to which moribund workers display altered responses to pheromones that are less complex or that are emitted in larger quantities, such as the secretions of mandibular glands, which serve as alarm pheromones and as an attractant for *M. rubra* workers (Cammaerts-Tricot, 1973). This information would provide evidence on a possible 'hierarchy' in the loss of olfactory abilities of diseased insects as a function of the complexity, concentration and functional value of the pheromone being perceived.

Besides, one may wonder whether moribund ants behave as old 'healthy' workers which also exhibit social seclusion a few hours or days before death (Heinze & Walter, 2010). In this way, it has been shown that an infection may accelerate the ageing process (e.g. HIV in humans, Ances et al., 2010), which strongly correlates with a decrease in behavioural and physiological performance in several organisms, ranging from flies (Grotewiel, Martin, Bhandari, & Cook-Wiens, 2005) to humans (Burke & Barnes, 2006). Specifically, eusocial species have developed a way to regulate ageing that maintains individual performance throughout the life span of workers, which continue learning, responding to stimuli or performing complex tasks until the last days of their life (Giraldo et al., 2016; Rueppell, Christine, Mulcrone, & Groves, 2007; but see ; Remolina, Hafez, Robinson, & Hughes, 2007). Further research should identify to what extent the social seclusion of infected workers results from an alteration of the same metabolic and sensorimotor pathways as those affected in the ageing process, but with much faster dynamics of deterioration.

Finally, from an evolutionary perspective, the impact on pathogen fitness of withdrawal from the nest of infected individuals is difficult to estimate for generalist parasites such as *Metarhizium* fungus. Indeed, withdrawal from the nest can be detrimental by reducing propagation of the fungus among nestmates but can also be beneficial by facilitating its dispersal and prompting transmission to new insect hosts.

For the ant host, withdrawal from the nest appears to be an efficient prophylactic strategy since, by dying outside the nest, infected ants limit the risk of spreading disease among nestmates through their sporulating carcass. Overall, social isolation of any moribund ants is likely to have been selected in social insects to minimize the sanitary risks of carcass management, to reduce time-consuming hygienic tasks and to save the costs incurred in setting up complex regulatory systems for the recognition and withdrawal of nestmates with reduced life expectancy.

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AUTHOR CONTRIBUTIONS

JBL and CD designed the experiments. JBL conducted the experiments and analysed the data. All authors contributed equally to drafting and writing the article.

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