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Ant queens (Hymenoptera: Formicidae) are attracted to fungal pathogens during the initial stage of colony founding

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Abstract

Ant queens that attempt to disperse and found new colonies independently face high mortality risks. The exposure of queens to soil entomopathogens during claustral colony founding may be particularly harmful, as founding queens lack the protection conferred by mature colonies. Here, we tested the hypotheses that founding queens (I) detect and avoid nest sites that are contaminated by fungal pathogens, and (II) tend to associate with other queens to benefit from social immunity when nest sites are contaminated. Surprisingly, in nest choice assays, young *Formica selysi* BONDROIT, 1918 queens had an initial preference for nest sites contaminated by two common soil entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium brunneum*. Founding queens showed a similar preference for the related but non-entomopathogenic fungus *Fusarium graminearum.* In contrast, founding queens had no significant preference for the more distantly related non-entomopathogenic fungus *Petromyces alliaceus*, nor for heat-killed spores of *B. bassiana.* Finally, founding queens did not increase the rate of queen association in presence of *B. bassiana*. The surprising preference of founding queens for nest sites contaminated by live entomopathogenic fungi suggests that parasites manipulate their hosts or that the presence of specific fungi is a cue associated with suitable nesting sites.

Key words: Colony founding, social immunity, pathogen avoidance, pleometrosis.

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Introduction

Hosts have several lines of defense to resist parasites and pathogens. First, they may avoid contact with the pathogens. If this behavioral defense fails, they may prevent pathogens from entering into their body, and finally stop pathogens from multiplying in their organs, generally by activating the immune system (SCHMID-HEMPEL & EBERT 2003, SIVA-JOTHY & al. 2005). Pathogen avoidance may be particularly cost effective, as it minimizes pathogen-induced damage and avoids the costs of mounting an immune response (SCHULENBURG & al. 2009).

In arthropods, the ability to detect and avoid pathogens varies among species (BAVERSTOCK & al. 2010). For example, mole crickets tunneling through soil avoid generalist fungal entomopathogens (VILLANI & al. 2002, THOMPSON & BRANDENBURG 2005) such as *Beauveria bassiana* and *Metarhizium anisopliae*. In contrast, parasitoid wasps and potato beetles appear unable to detect *B. bassiana*, or do not perceive it as a threat (LORD 2001, KLINGER & al. 2006). The host reaction may vary with conditions and life stage. For example, common flowerbugs avoid *B. bassiana* on leaves but not in soil (MEYLING & PELL 2006), and Japanese beetle larvae keep away from soil contaminated by *M. anisopliae*, whereas adults increase oviposition in presence of the parasite (VILLANI & al. 1994). In a few cases, arthropods were attracted to the fungal pathogens. Specifically, collembolans showed a preference for substrate containing *B. brongnartii*, *B. bassiana* and *M. anisopliae* conidia (DROMPH & VESTERGAARD 2002), and mosquitoes were attracted to spores of *B. bassiana* and *M. anisopliae*, as well as to *B. bassiana* infected caterpillars (GEORGE & al. 2013).

Social insects live in dense groups and often nest in soil, which exposes them to fungal entomopathogens. The ability of termites to detect and avoid *Beauveria bassiana* and *Metarhizium anisopliae* has been well documented (MBURU & al. 2009, RATH 2010, YANAGAWA & al. 2012). Ants are also generally able to detect these fungal pathogens. For example, *Formica selysi* BONDROIT, 1918 workers increased the rate of self-grooming (REBER & al. 2011) when exposed to *Metarhizium brunneum*, and *Lasius neglectus* (VAN LOON, BOOMSMA & ANDRÁSFALVY, 1990) workers increased brood care and sanitary behavior in presence of contaminated workers or brood (UGELVIG & CREMER 2007, TRAGUST & al. 2013). However, in contrast to termites, ants did not seem to avoid the pathogens. Indeed, contaminated individuals were not avoided, and were intensely groomed by nestmates (REBER & al. 2011, KONRAD & al. 2012). Whether ants avoid direct contact with fungal pathogens in other contexts deserves to be further investigated.

Avoiding pathogens may be particularly important for young ant queens attempting to found a new colony independently, without the help of workers. Indeed, in soil-nesting species, generalist fungal entomopathogens appear

to be responsible for a considerable rate of failures during colony founding (BAER & al. 2006). Lone founding queens lack the protection conferred by mature colonies, which may be mediated by allo-grooming (WALKER & HUGHES 2009, REBER & al. 2011), group diversity (HUGHES & BOOMSMA 2004, REBER & al. 2008), sharing of antibiotic substances (FERNÁNDEZ-MARÍN & al. 2006, CHAPUISAT & al. 2007, HAMILTON & al. 2011, TRAGUST & al. 2013) or other forms of social immunity (TRANIELLO & al. 2002, UGELVIG & CREMER 2007, KONRAD & al. 2012). Additionally, founding queens may be more susceptible to pathogens if they found claustrally (without foraging), as deprivation of food has been shown to affect immunity in insects (SIVA-JOTHY & THOMPSON 2002).

Another interesting hypothesis is that ant queens founding new colonies in nest sites that are contaminated by fungal parasites might increase their chances of success by associating with other queens. In line with this hypothesis, workers are more resistant to fungal parasites when they are in groups than when they are alone (HUGHES & al. 2002, JOHNSON 2004). The influence of the presence of parasites on the propensity of queens to associate with other queens during colony founding has not been investigated so far.

Here, we studied the impact of the presence of fungi on the founding behavior of ant queens. The study species, *Formica selysi*, nests in the soil, where it is naturally exposed to fungal entomopathogens (REBER & CHAPUISAT 2012a) such as *Metarhizium brunneum* (formerly *M. anisopliae*, see BISCHOFF & al. 2009) and *Beauveria bassiana*. The frequency of pleometrosis in the field is unknown, but laboratory studies have shown that queens are able to found colonies independently as well as in association with other queens (REBER & al. 2010).

In a series of experiments, we tested whether founding queens detected and showed behavioral resistance to fungal entomopathogens during this crucial and exposed stage of their life-cycle. We first tested if young *Formica selysi* queens avoided nest sites contaminated by *Metarhizium brunneum* and *Beauveria bassiana*. We further examined if queens discriminated nest sites containing non-pathogenic fungi, and whether they distinguished between live and heat-killed *B. bassiana*. Finally, we tested if queens tended to associate with other queens when they had to found colonies in sites contaminated by *B. bassiana*.

Material and Methods

Ants sampling and experimental mating: We collected *Formica selysi* ants (identified using the key in KUTTER 1977) from a well-studied population along the Rhône river between Sierre and Susten in Valais, Switzerland (CHAPUISAT & al. 2004). In summer 2009 and 2010, prior to the nuptial flight, we collected young males, young virgin queens, sexual pupae and workers. We transferred them to the laboratory, where we let the pupae hatch into queens and males. We kept queens and males in separate laboratory colonies, to prevent uncontrolled mating. We supplied the ants with ad libitum water and jelly made of eggs, honey and agar, and maintained them at 25°C with a 12 / 12 h dark / light cycle.

Experimental mating took place outside, in the morning, under direct sunlight. We placed two virgin queens from the same colony with five to ten males from several other colonies in a mating box (REBER & al. 2010). After mating, we kept each young queen in a glass tube with humid cotton wool until the beginning of the experiment.

Experimental nests: The young, freshly mated queens had to found colonies in experimental nests made of glass test tubes (10 cm long and 1.5 cm in diameter). The bottom of each tube was filled with water retained by small cotton wool plugs, and the tubes were wrapped in black paper. Such dark humid tubes constitute good nest sites, mimicking natural holes that are powerful attractants for founding queens (TSCHINKEL 1998). Each experimental nest contained a piece of filter paper (6 × 2 cm) on which we deposited either 500 µl of a solution of fungal spores diluted in 0.05% Tween 20 (fungal treatment), or 500 µl of spore-free 0.05% Tween 20 (control; e.g., CHAPUISAT & al. 2007, REBER & al. 2008). We placed the tubes in arenas (plastic boxes 13.5 cm long × 15 cm wide × 5 cm high) lined with Fluon to prevent queens from escaping.

Impact of pathogens on nest choice: We staged nest choice assays to investigate (I) if queens avoided founding colonies in sites contaminated by fungal entomopathogens (II) if the presence of non-entomopathogenic fungi also affected their nest choice. In these tests, the queens had to choose between a nest containing fungal spores and an identical but spore-free control nest. We tested the choice of queens with respect to the presence of *Beauveria bassiana* and *Metarhizium brunneum*, two fungal entomopathogens that are common in the site where we sampled the ants (REBER & CHAPUISAT 2012a), *Fusarium graminearum*, a pathogen of plants belonging to the same order as *B. bassiana* and *M. brunneum* (the Hypocreales), and *Petromyces alliaceus*, a non-entomopathogenic, phylogenetically more distant fungus belonging to another class (the Eurotiomycetes). We did not detect *F. graminearum* in the site where we sampled the ants, whereas *P. alliaceus* was very common (REBER & CHAPUISAT 2012a). These four species of fungi belong to the subdivision Pezizomycotina in the Ascomycota.

We tested 80, 62, 40, and 40 queens for *Beauveria bassiana*, *Metarhizium brunneum*, *Fusarium graminearum*, and *Petromyces alliaceus*, respectively, depending on the number of queens available at the time of the experiment. We used fungal solutions at 8.9×10^7, 3.5×10^7, 7.8×10^5 and 1.5×10^7 spores / ml, respectively. We selected these concentrations to account for the marked size differences between the spores of the four fungal species, in particular the larger spores of *F. graminearum*. These concentrations also tend to compensate for the marginally higher lethality of *M. brunneum*, as compared to *B. bassiana* (REBER & CHAPUISAT 2012a).

We introduced one queen in the middle of each arena. We recorded the position of each queen first shortly after introduction and then on a daily basis over a period of 13 days (nine days for *Beauveria bassiana*). We analyzed the initial choice of queens, which was given by the first nest in which we found them. We also examined in which nests the live queens were on the last day. When a queen died, we surface sterilized its corpse with 14% bleach and kept it in an Eppendorf tube with wet cotton wool to check if it died from infection and produced fungal spores (REBER & al. 2011).

To get insight into temporal variation in the position of queens, we analyzed (I) the number of days spent in the in-

itial nest, (II) the proportion of queens that visited another nest and (III) the total number of nest switches made by the queens, relative to the duration of the experiment. We examined if these measures of queen movements depended on the fungus used in the assay, on whether the first nest chosen was inoculated by a fungus or not, or on an interaction between these two factors. We only report significant results for these analyses.

In a follow-up experiment, we examined whether founding queens discriminated between live and dead spores of *Beauveria bassiana*. We tested 120 queens, which had to choose between three nests sites, one inoculated with live spores (9.2×10^7 spores / ml), one with heat-killed spores (9.3×10^7 spores / ml) and one with control solution. We monitored the position of the queens over a period of 13 days. For the heat-killed treatment, we autoclaved the fungus solution at 121°C for 20 minutes. With a microscope, we checked that the sterilization process had not affected the external structure of the spores. We also confirmed that the heat-killed spores did not grow on malt extract agar nutritive medium.

Impact of pathogen on queen association: To test if the presence of *Beauveria bassiana* spores influenced the propensity of queens to associate with other queens during colony founding, we placed two queens in an arena containing a single nest site, which was either contaminated by spores of *B. bassiana* (6.9×10^7 spores / ml) or contained the usual control solution. We tested 152 pairs of queens that we associated at random. Half of the pairs had access to a contaminated nest site, and the other half to a control nest site.

We monitored the position of the queens over a period of six days, recording if they were in or out of the nests. We estimated the initial frequency of queen association as the proportion of nests that contained two live queens on the second day of the experiment. At this time, 96% of the nests contained at least one queen. We analyzed the data with a general linear model including the type of nest (contaminated vs. uncontaminated) and whether the queens in a pair originated from the same or from different field colonies as explanatory variables. All statistical analyses were performed in R 3.0.2 (R CORE TEAM 2013). In all tests where an initial and final measure were analyzed, we adjusted the *p*-values with a Bonferroni correction to account for multiple comparisons.

Results

Impact of pathogens on nest choice: Surprisingly, founding queens showed a strong initial preference for nests containing fungal pathogens. When given the choice between a nest contaminated with *Beauveria bassiana* and a spore-free control nest, 75% of the 72 queens that entered a nest were first found in the contaminated one (Fig. 1a; exact binomial test: $p < 0.0001$; all p-values are two-tailed; eight queens died without entering any nest). In the tests with *Metarhizium brunneum*, 67.7% of the 62 queens were first found in the nest containing the fungal pathogen (Fig. 1a; exact binomial test: $p = 0.014$).

Founding queens also showed a strong initial preference for nests containing the non-entomopathogenic but phylogenetically close fungus *Fusarium graminearum*: 80% of the 40 queens chose the nest containing the fungus (Fig. 1; exact binomial test: $p = 0.0004$). The preference was weaker and not statistically significant when we tested the non-entomopathogenic but phylogenetically more distant fungus *Petromyces alliaceus*: 65% of the 40 queens chose the nest containing the fungus (Fig. 1a; exact binomial test: $p = 0.16$).

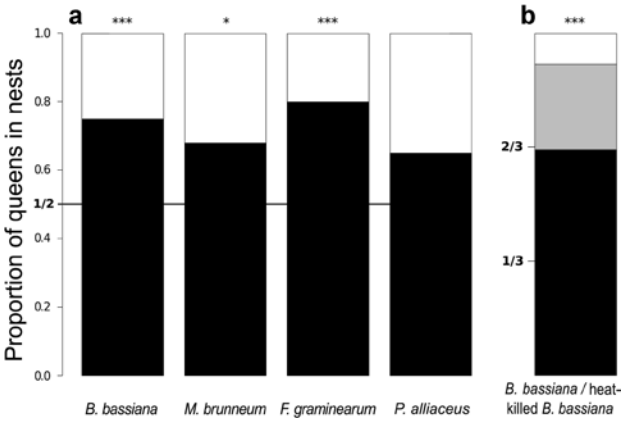

Fig. 1: a) Proportion of queens that were first found in nests containing the fungi *Beauveria bassiana*, *Metarhizium brunneum*, *Fusarium graminearum*, and *Petromyces alliaceus* (black bars), as compared to control nests (white bars). b) Proportion of queens that were first found in nests containing live *B. bassiana* (black bar), heat-killed *B. bassiana* (grey bar) and control (white bar). Horizontal bars indicate "no choice", at 1/2 for a) and 1/3 and 2/3 for b). Asterisks signal significant deviations from 1/2 for a) and 1/3 for b) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The number of days spent by the queens in the initial nest depended on the fungus ($\chi^2 = 10$, $df = 3$, $p = 0.018$; it was higher in assays involving *Fusarium graminearum*). On average and across all fungi tested, the queens that had initially chosen a fungus-inoculated nest stayed for longer in it than queens that had initially chosen a spore-free nest (fungus vs. spore-free: 3.7 ± 2.6 days vs. 2.4 ± 1.7 days, respectively; $\chi^2 = 9.3$, $df = 1$, $p = 0.002$), and made fewer relative nest switches thereafter (fungus vs. spore-free: 0.18 ± 0.12 vs. 0.22 ± 0.13; $\chi^2 = 5.1$, $df = 1$, $p = 0.024$). For the proportion of queens that switched nests, there was a significant interaction between the fungus species and the type of nest initially chosen ($\chi^2 = 12.5$, $df = 3$, $p = 0.006$). This is because, in contrast to the pattern observed with the other three fungi, queens that had initially chosen a nest inoculated with *Petromyces alliaceus* were more likely to visit another nest than the queens that had initially chosen a spore-free nest.

Overall, the mortality of queens depended on the fungi they were exposed to (binomial test, entomopathogens vs. non-entomopathogens: $\chi^2 = 12.2$, $df = 1$, $p = 0.0005$). A larger proportion of queens died in treatments where one of the two nest sites was contaminated by an entomopathogenic fungus than by a non-entomopathogenic fungus (27.5% and 11.3% for *Beauveria bassiana* and *Metarhizium brunneum*, respectively, versus 2.5% and 0% for *Fusarium graminearum* and *Petromyces alliaceus*, respectively). Eight out of the 22 queens that died during the test involving *B. bassiana* produced the typical white spores of this pathogen. None of the queens that died during the test involving *M. brunneum* sporulated with this pathogen. However, two queens that died after the end of the experi-

ment produced *M. brunneum* spores, which confirms that this fungus was infectious.

By the end of the experiment, fewer queens remained in the nests, and the initial preference for contaminated nests was much reduced or non-existent. In the test with *Beauveria bassiana*, 63.5% of the 52 live queens that were still in a nest at the end of the experiment were in the contaminated one (exact binomial test: $p = 0.14$), while in the test with *Metarhizium brunneum* this was the case of 50.9% of the 55 queens (exact binomial test: $p = 1$). In the test with *Fusarium graminearum*, 55.3% of the 38 queens in a nest occupied the one containing spores (exact binomial test: $p = 1$), while in the test with *Petromyces alliaceus* significantly more queens were found in the control nest (71.9% of the 39 queens found in a nest; exact binomial test: $p = 0.019$).

This change in the position of queens over time was not entirely explained by queen mortality: at the end of the experiment, we detected no significant preference for contaminated nests when we included dead queens in the analysis (exact binomial tests: *Beauveria bassiana*: $p = 0.098$, *M. anisopliae*: $p = 0.79$, *Fusarium graminearum*: $p = 0.63$, *Petromyces alliaceus*: $p = 0.009$).

When given a choice between nest sites inoculated with live *Beauveria bassiana*, heat-killed *B. bassiana* or a control, the queens significantly preferred the nest with the live entomopathogen (66%, 25%, 9% were found in each type of nest, respectively: Fig. 1b; $\chi^2 = 58.3$, $df = 2$, $p < 0.0001$; $n = 116$ queens, as four queens died without entering any nest site). By the end of the experiment, a marginally higher proportion of the queens remained in the nest with the live fungus (out of the 101 queens that were still in a nest at the end of the experiment, 44%, 27% and 29% were in a nest with live *B. bassiana*, heat-killed *B. bassiana* and control, respectively; $\chi^2 = 5.8$, $df = 2$, $p = 0.11$).

Impact of pathogen on queen association: When two queens had access to a single nest during colony founding, the presence of the fungal pathogen *Beauveria bassiana* in the nest had no significant influence on the initial propensity of queens to associate. On the second day of the experiment, 57.9% of the 76 contaminated nests contained two live queens, as opposed to 50% of the 76 control nests ($\chi^2 = 0.95$, $df = 1$; $p = 0.33$). Whether the queens originated from the same or from different field colonies had no significant influence on their initial propensity to associate ($\chi^2 = 0.005$, $df = 1$; $p = 0.95$). On the last day of the experiment, the presence of *B. bassiana* still had no significant impact on the frequency of queen association: 22.4% of the contaminated nests contained two live queens, as opposed to 25% of the control nests ($\chi^2 = 0.15$, $df = 1$, $p = 1$).

Discussion

Ant queens founding new colonies independently are under high risk of dying from exhaustion (CAMARGO & al. 2011), desiccation (MANKOWSKI & MORRELL 2011), competition (ADAMS & TSCHINKEL 1995), predation (NICKERSON & al. 1975) or infection by parasites (BAER & al. 2006). As the presence of fungal pathogens in the soil jeopardizes the survival of the queen and her first brood, we expected that queens would avoid settling in nests contaminated by entomopathogenic fungi. In sharp contrast to this expectation, in nest choice assays *Formica selysi* queens showed a strong and significant initial preference for nests contaminated by the common generalist fungal entomopathogens *Beauveria bassiana* and *Metarhizium brunneum*, as opposed to spore free control nests. This preference of ant queens for entering contaminated nest sites is surprising, because *B. bassiana* caused significant mortality to the queens in this experiment, and both *B. bassiana* and *M. brunneum* killed workers in other experiments (CHAPUISAT & al. 2007, REBER & al. 2008, PURCELL & al. 2012). Moreover, the two pathogens are common in the natural habitat of this ant species (REBER & CHAPUISAT 2012a).

Founding queens showed a similar preference for *Fusarium graminearum*, a plant pathogen belonging to the order Hypocreales, which also contains *Beauveria bassiana* and *Metarhizium brunneum*. In contrast, founding queens had no significant initial preference for the more distantly related non-entomopathogenic fungus *Petromyces alliaceus*. There was a trend, however, and the sample size was lower, so that a general initial preference for fungi can't be excluded. Finally, the queens showed a much stronger preference for live than for heat-killed spores of *B. bassiana*. Together, these results indicate that queens are attracted to live fungi belonging to the order Hypocreales, which are parasites of plants, invertebrates, or even other fungi (SPATAFORA & al. 2007).

The unexpected preference of founding queens for nest sites containing live entomopathogenic fungi may be explained in several ways. First, the fungal pathogens may manipulate their hosts, luring them with odor cues in order to increase infection probability. *Beauveria* and *Metarhizium* attract collembolans (DROMPH & VESTERGAARD 2002), as well as mosquitoes (GEORGE & al. 2013). The hypothesis that *Beauveria* and *Metarhizium* manipulate uninfected insect hosts deserves further investigation (GEORGE & al. 2013). Records of pathogens attracting their hosts are indeed rare, as hosts are under strong selection to resist manipulation and avoid virulent pathogens (POULIN & al. 1994).

Second, the presence of fungi may be a cue associated with suitable nesting sites, or may provide some direct or indirect benefits to the queens. For example, the presence of fungi may indicate favorable ecological conditions, for example humid, humus-rich soil. The queens might theoretically feed on fungi, but this seems unlikely given the lack of records of such behavior (SANDERS 1964, AYRE 1967, cited by CANNON & FELL 2002).

Finally, contact of the queen with a pathogen might improve the defense of her offspring against the same pathogen. This process, known as "trans-generational immune priming", has been observed in diverse invertebrate taxa, such as bumble-bees (MORET & SCHMID-HEMPEL 2001, SADD & al. 2005), *Daphnia* (see LITTLE & al. 2003), moths (TIDBURY & al. 2011) and beetles (ROTH & al. 2010). The potential occurrence of trans-generational immune priming deserves to be investigated. It would however be surprising, given that trans-generational immune priming has not been reported in ants so far, and that we found no evidence of individual immune priming in workers (REBER & CHAPUISAT 2012b) or queens (D. Gálvez & M. Chapuisat, unpubl.) of *Formica selysi*.

Some queens died during the course of the experiment, and some moved between nests, so that in the assays involving pathogenic fungi the queens tended to be equally present in inoculated and spore-free nests by the end of the experiment. Queen movement did not appear to be

prompted by the perception of queens that they were, or could be, infected. We found no significant difference between queens that initially entered contaminated nests and queens that initially entered spore-free nests for the number of days spent in the initial nest or the probability to abandon it. Only queens that initially entered *Petromyces alliaceus*-inoculated nests were more likely to leave, suggesting that they were repelled by this non-entomopathogenic fungus. Indeed, in the assay involving *P. alliaceus*, most of the queens were in spore-free nests at the end of the experiment. A recording of queen movements in real time over a longer period, until the queens begin to lay eggs, would be useful to examine how initial nest choice, nest switching and mortality jointly determine the final settlement and success of the queens.

We also tested if the propensity of queens to associate with other queens during colony founding increased when nest sites were contaminated by *Beauveria bassiana*. Indeed, by joining others, queens might benefit from allogrooming or from other forms of social immunity (CREMER & al. 2007, REBER & al. 2011). However, founding queens did not increase the rate of queen association in presence of *B. bassiana*, which indicates that pleometrosis is not a conditional response to benefit from social immunity when the risk of infection is high.

Overall, our results indicate unexpected patterns in the colony founding behavior of ant queens in presence of fungi, including entomopathogens. Indeed, the queens did not avoid the fungal pathogens and one of the non-entomopathogens tested – to the contrary, they showed an initial preference for spore-inoculated nest sites. This surprising and potentially fatal attraction might result from parasite manipulation, or may be associated with correlated factors that are normally beneficial to the queens.

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