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ORIGINAL PAPER

Tolerating an infection: an indirect benefit of co-founding queen associations in the ant *Lasius niger*

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Abstract Pathogens exert a strong selection pressure on organisms to evolve effective immune defences. In addition to individual immunity, social organisms can act cooperatively to produce collective defences. In many ant species, queens have the option to found a colony alone or in groups with other, often unrelated, conspecifics. These associations are transient, usually lasting only as long as each queen benefits from the presence of others. In fact, once the first workers emerge, queens fight to the death for dominance. One potential advantage of co-founding may be that queens benefit from collective disease defences, such as mutual grooming, that act against common soil pathogens. We test this hypothesis by exposing single and co-founding queens to a fungal parasite, in order to assess whether queens in co-founding associations have improved survival. Surprisingly, co-foundresses exposed to the entomopathogenic fungus *Metarhizium* did not engage in cooperative disease defences, and consequently, we find no direct benefit of multiple queens on survival. However, an indirect benefit was observed, with parasite-exposed queens producing more brood when they co-founded, than when they were alone. We suggest this is due to a trade-off between reproduction and immunity. Additionally, we report an extraordinary ability of the queens to tolerate an infection for long periods after parasite exposure. Our study suggests that there are no social immunity benefits for co-founding ant queens, but that in parasite-rich environments, the presence of additional queens may nevertheless improve the chances of colony founding success.

Keywords *Lasius niger* · Life-history trade-offs · *Metarhizium* · Allogrooming · Social immunity · Pleometrosis

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Introduction

Sociality brings many benefits, but group living also carries costs, including increases in the risk of parasite transmission, due to a higher within-group contact rate between infected and susceptible individuals (Alexander 1974; Krause and Ruxton 2002; Møller et al. 1993). As such, it is expected that social animals have had to evolve mechanisms in order to cope with an increased parasitic threat (Cremer et al. 2007; Freeland 1976). In addition to defences available to solitary individuals, such as physiological immunity, social animals, such as the social insects, have the added benefit of cooperative disease defences (Altizer et al. 2003; Boomsma et al. 2005; Cremer et al. 2007; Nunn and Altizer 2006, pp. 150–154; Schmid-Hempel 2011, pp. 52–58; Wilson-Rich et al. 2009). In many ways, these defences can be considered analogous to the physiological immune response of an individual organism, and therefore have been termed 'social immunity' (reviewed by Cremer et al. 2007). This additional level of defence can be extremely effective in preventing epidemics, exemplified by the unsuccessful implementation of biological control agents (such as microbial pathogens) against social insect pests (Chouvenc et al. 2011). The social immune defences of the

social insects range from spatio-temporal division of labour (Mersch et al. 2013; Schmid-Hempel 1998, pp. 119–161; Schmid-Hempel and Schmid-Hempel 1993), to active mechanical and chemical neutralisation of parasitic propagules (Graystock and Hughes 2011; Reber et al. 2011; Rosengaus et al. 1998; Tragust et al. 2013; Walker and Hughes 2009; Yek et al. 2012). Social defences such as allogrooming may be particularly crucial during the early stages of colony development, where it is unlikely that spatial and temporal separation of colony members could feasibly be achieved with a few, multitasking workers. Many studies have shown social immunity to be effective in small group sizes, with as few as two individuals, suggesting that social immunity has the potential to function at early stages of colony founding and development (e.g., Hamilton et al. 2011; Hughes et al. 2002; Konrad et al. 2012; Reber et al. 2011; Rosengaus et al. 1998; Tragust et al. 2013; Traniello et al. 2002; Walker and Hughes 2009).

In fact, the most hazardous time for any social insect colony is the founding or incipient phase (Hölldobler and Wilson 1990, pp. 157–163; Schmid-Hempel 1998, p. 12; Wheeler 1910). In a large majority of ant species, new colonies are formed when virgin queens leave the safety of the parental nest to embark upon a mating flight, after which the mated queens must find a suitable location to start a new colony (Hölldobler and Wilson 1990, pp. 157–163). In many cases, queens do this alone and the rate of mortality for founding colonies is exceptionally high, greater than 99 % (Aron et al. 2009). As many social insects construct underground nests, it is likely, given the prevalence of opportunistic soil parasites such as entomopathogenic fungi (Hughes et al. 2004; Keller et al. 2003; Reber and Chapuisat 2012), that parasitic infections occur whilst newly mated queens search for and dig a burrow. For example, in a study of leaf-cutting ant foundresses, 74 % of all mortality appeared to be caused by disease (Fowler et al. 1986), though it is not clear if in this study microbial growth was the cause or consequence of mortality.

Unusually for social animals that are expected to cooperate only with kin, the foundress queens of some ant species can start new colonies with unrelated individuals (but see Nonacs 1990), a mode of colony foundation termed pleometrosis (reviewed by Bernasconi and Strassmann 1999; Bourke and Franks 1995). These pleometrotic queens cooperate to produce the first brood, and, in so doing, gain the benefit of a larger initial worker force, giving them a competitive advantage over single founding queen colonies (Adams and Tschinkel 1995; Aron et al. 2009; Bernasconi and Strassmann 1999; Brown 2000; Nonacs 1990; Sommer and Hölldobler 1995; Tschinkel and Howard 1983; but see Hartke and Rosengaus 2013 for a contrasting view in termites). In addition to such benefits, studies have revealed initial queen survival to be higher for cooperating queens (Adams and Tschinkel 1995; Waloff 1957). The reason for this remains unclear, but it may be that the sharing of costly tasks reduces the strain on each queen, increasing her chances of survival until worker eclosion. Indeed, dissections of haplometrotic (singly founding) ant queens, which died before worker emergence, revealed exhausted fat bodies (Waloff 1957). It has also been suggested that improved survival may be the result of grooming between queens, to remove externally attaching parasites, such as pathogenic fungal conidia (Waloff 1957). This is an intriguing idea given that mutual grooming is a key social immune defence of mature social insect colonies (Cremer et al. 2007; Reber et al. 2011; Rosengaus et al. 1998; Walker and Hughes 2009), and that founding ant queens are vulnerable to cuticle-attaching pathogens (Fowler et al. 1986; Waloff 1957). Attempts to investigate the relationship between disease and number of monogamous pairs, in laboratory studies of a co-founding termite, revealed a negative effect of multiple founding pairs (Hartke 2010). However, in this termite, pleometrosis is rare in the field (~5 % of founding colonies had more than one king, and none had more than one queen; Hartke 2010), and thus it is unclear how these laboratory results may relate to pleometrosis in ants, where co-founding queens occur at much higher levels, and thus pleometrosis is likely to be a much stronger selective pressure on ant queen behaviour (Bernasconi and Strassmann 1999). As of yet, no study has experimentally investigated the combined impact of multiple queens and parasites on the founding of ant colonies.

Using foundress queens of the ant *Lasius niger*, we set up incipient laboratory colonies to investigate the effect of queen number on parasite resistance during colony founding, by exposing single and paired foundress queens to the entomopathogenic fungus *Metarhizium pingshaense*. Under laboratory conditions, we observed the behavioural responses of the queens and compared the effect of queen number on resistance to the parasite and queen survival. We expected paired queens to engage in social immune behaviours, such as mutual grooming, to reduce the chance of infection and improve paired queen survival, relative to singly founding queens. Additionally, queen fecundity was measured by counting the number of brood and subsequent workers produced, to reveal the effect of parasite exposure on brood production, in both paired and singly founding queens. We expected paired queens exposed to the pathogen to be able to produce greater quantities of brood, relative to single parasite-exposed queens, as they should exhibit reduced fungal infection and mortality. Thus the chance of paired parasite-exposed queens successfully founding a colony should be improved compared to single parasite-exposed queens.

Methods

Study organisms

L. niger is a common species found throughout the Northern Hemisphere (Wilson 1955), and mature colonies are monogynous with up to 10,000 workers (Sommer and Hölldobler

1995). Alates (winged male and female reproductives) engage in large nuptial flights between July and September, with females mating with one or several males (Aron et al. 2009; Boomsma and Van Der Have 1998; Fjerdingstad et al. 2003), before returning to the ground, removing their wings and digging a nest site. Rates of pleometrosis for the study population are unknown, but based on studies of other *L. niger* populations in Europe, pleometrosis in this species is facultative and has an incidence of approximately 18 %, with the majority of pleometrotic nests containing two co-founding queens (Sommer and Hölldobler 1995). Given the size of the nuptial flights, which are synchronised between many colonies, it is assumed that cooperating queens are also unrelated (Aron et al. 2009; Bernasconi and Strassmann 1999; Boomsma and Leusink 1981). Queen cooperation typically ends with the eclosion of the first workers, triggering brutal queen fighting that results in the death of all but one queen (Aron et al. 2009; Sommer and Hölldobler 1995).

The genus *Metarhizium* is a group of generalist entomopathogenic fungi known to infect many insect species worldwide (Deacon 2006; Roberts and St Leger 2004), including ants (Schmid-Hempel 1998 p 295) and founding queens of our study species (Pull, unpublished data). These fungi can exist in the environment for prolonged periods as pathogenic asexual conidia (the dispersal stage) until they come into contact with the cuticle of a host. Here they first loosely attach, and later germinate, growing into the body to proliferate throughout the insect, eventually leading to host death and then the production of secondary conidia (Deacon 2006). *M. pingshaense* (previously described as *M. anisopliae* var. *anisopliae*, but now recognised as a sister species; Bischoff et al. 2009) is easily cultured in the laboratory, and the conidia can be prepared in a suspension of Triton-X 100 surfactant (to aid wetting), allowing for simple application to a host. We used the strain KVL 02–73 (species confirmed via sequencing of laboratory culture), which was originally isolated from soil surrounding an *Atta cephalotes* leaf-cutting ant nest in Panama (Hughes et al. 2004). We used this exotic strain, which we will henceforth refer to as *Metarhizium*, in our experiment to reduce the impact of co-evolutionary dynamics on host resistance or parasite virulence. However, as *Metarhizium* spp. are ubiquitous generalist pathogens, it is unlikely the use of an exotic strain would affect the queens' ability to detect the presence of the conidia.

Queen collection and pathogen exposure

Mated queens (identifiable by the loss of wings) were collected using pooters (aspirators) after a large nuptial flight in Egham, Surrey, UK, on 8 July 2010, between 18:00 and 20:00. Queens were placed into plastic tubs lined with damp filter paper and with Fluon-coated sides, before being taken to the laboratory. Queens were exposed to either *Metarhizium* conidia solution (conidia suspended in autoclaved 0.05 % Triton-X 100) at a concentration of 1.88×10^8 conidia ml^{-1} (conidia viability was measured as 96 % germination) or autoclaved 0.05 % Triton (control solution), by holding the abdomen gently with soft forceps, and applying 0.5 μl of conidia or control solution to the thorax. The solution was allowed to dry for approximately 30 s, after which the queen was placed into an experimental nest, either singly, or in pairs, with a queen of the same treatment. The conidia solution was vortexed between each treatment and forceps were sterilized in ethanol and flamed to maintain a consistent dose per queen.

Experimental groups

Queens were haphazardly allocated to one of four treatment groups: single queens treated with 0.05 % Triton (single control), single queens treated with fungal conidia (single parasite-exposed), paired queens treated with 0.05 % Triton-X (paired control) or paired queens treated with fungal conidia (paired parasite-exposed). In each group there were 20 replicates, giving a total of 80 colonies and 120 experimental queens. Queens were kept at approximately 21 °C, in 90× 25-mm Petri dishes on a substrate of plaster of Paris, and under a natural light/dark cycle. The plaster was moistened with distilled water weekly, which maintained sufficient humidity levels for fungal germination (queens were also observed drinking this water, and condensation was always present on the dish lids). No food was provided to the queens, as foundresses survive solely on the histolysis of the redundant wing muscles and fat reserves (Hölldobler and Wilson 1990, pp. 157–163; Janet 1907). However, once the first workers eclosed, each colony was subsequently provided with a 50 % honey–water solution and diced beetle larvae (*Tenebrio molitor*), every 2 days.

Behavioural observations

Descriptive behavioural observations were made by randomly selecting five nests per treatment group at the beginning of the 2010 experiment. These queens were observed for 5 min each immediately after exposure, daily for the following week, and then twice a week for a further 3 weeks, with all self-grooming (including any metapleural gland or acidopore grooming), allogrooming (mutual grooming of one ant by another) and trophallaxis (the sharing of crop contents between two ants) behaviours during the observation period being recorded (as described in Wilson 1971, pp. 281–295). Additionally, 112 queens were collected after a mating flight on 23 July 2012 and set up into the four treatments as above, but exposed to a higher dosage of conidia (2 μl of conidia solution at a concentration of 5×10^8 conidia ml^{-1}) in order to elicit a greater antiseptic grooming response from the queens. Additionally, the nests of these queens were covered with red plastic, to

avoid light exposure affecting queen behaviour (given that they usually nest in darkness). Scan sample observations were made, observing all queens for approximately 2 s, three times a day for 2 days, starting 10 h after parasite exposure (due to time constraints immediately after exposure). Again, all self-grooming, allogrooming and trophallaxis behaviours were recorded. In addition, a further 36 queens were collected and set up as above, after a nuptial flight on the 25 July 2012. Queens were exposed to the same dosage and the same behaviours were recorded via scan sampling, three times a day for 2 days, but starting immediately after parasite exposure. Again, the dishes of these queens were covered with red plastic. In these latter experiments queens were only observed for the first 2 days, as it is during this period that grooming is most effective, when conidia are yet to fully adhere to the cuticle (Konrad et al. 2012). Both groups of 2012 queens were only used for behavioural observations and were not included in any other aspect of the study. In all observations, behaviours were recorded by naked eye, as *L. niger* queens are large and their movements slow. Although we focus on grooming and trophallaxis, it should be noted we also observed for other behaviours that might have occurred (e.g., aggressive interactions, direct formic acid spraying and antennation).

Brood production

The experiment was ended after 140 days and a brood count was made of all offspring (eggs, larvae, pupae and workers) produced by each colony, using a light microscope and counter. Based on previous work, it is assumed the queens in the paired queen nests both contributed towards brood production (Aron et al. 2009).

Cadaver treatment

Petri dishes were inspected daily for queen mortalities. Cadavers were removed from the nest and surface sterilised using standard procedures (Lacey and Brooks 1997). Briefly, cadavers were dipped in 70 % ethanol, washed in distilled water and placed into 0.05 % sodium hypochlorite (NaClO) for 60 s. The cadavers were then washed three times in distilled water, before being placed onto sterile damp filter paper in a Petri dish. Surface sterilisation prevents the growth of microbes on the cadaver cuticle that might compete with or obscure *Metarhizium* growth. The cadavers were checked daily for external hyphal growth and *Metarhizium* sporulation.

Data analysis

A Cox proportional hazards model was used to analyse queen survival, including queen treatment, queen number and their interaction as predictors, and queen longevity as the response. As paired queen survival is non-independent, Petri dish number was included as a random factor, using a frailty function (Mills 2011, pp. 164–178). We tested the assumptions of this model (i.e., proportional hazards, influential observations and model adequacy), which revealed the effect of treatment to be non-proportional. As such, we included treatment in the model as a time-dependent covariate (Mills 2011, pp. 151–155), which both fulfilled the assumption of proportional hazards and improved the overall fit of the model. To conduct pairwise comparisons, the Peto and Peto modification of the Gehan–Wilcoxon test (equivalent to the Wilcoxon–Breslow test; both place more weight on early deaths (Mills 2011, pp. 79–83), such as those caused by *Metarhizium* at the beginning of this study) was used for formal comparisons between the survival curves of the different queen groups. Subsequent p values were corrected using the Holm–Bonferroni (H-B) method. A two-way ANOVA was used to compare the total amount of brood produced by a queen or queens in a nest, after ensuring that the data satisfied the assumptions of the test (i.e., Cook's distance, dfbetas, dffits, leverage, equality of variances and the distribution of residuals were also checked), and a Tukey's HSD test was used to carry out post-hoc comparisons. Similarly, a two-way ANOVA with a Tukey's HSD test was used to compare the total number of workers produced by a queen or queens in a nest, but we excluded from this analysis those queens that died before day 54, the first day on which a worker eclosed in the experiment, to prevent them confounding the results. Additionally, we used a second Cox proportional hazards model to examine the proportion of colonies with eclosed workers over time, including queen number, treatment and their interaction as variables, and, again, we excluded those queens who did not survive until day 54. This model fulfilled all assumptions, and subsequent pairwise comparisons, using the log-rank test (that places equal weights on events; Mills 2011, pp. 79–83), were used to compare the cumulative event curves. Again, p values were corrected using the Holm–Bonferroni method. We also analysed the number of queen-right colonies at the end of the experiment (i.e., those whose queen/s survived until 140 days), using a Generalized Linear Model (GLM) with binomial error structure (as colonies were either queen-right or not) and log link function, ensuring the model conformed to assumptions (i.e., Cook's distance, dffits, leverage, equality of variances). Treatment, queen number and their interaction were included as fixed factors. The overall significance of the model, and the effects of the fixed factors, were tested by comparing to nested null and reduced models, respectively, using likelihood ratio tests. Reordering of factor levels was carried out to obtain pairwise post-hoc comparisons, and resulting p values were corrected using the Holm-Bonferroni method. To explore the potentially

confounding effects of queen mortality and queen fecundity, we carried out a second set of analyses, where the number of brood and workers of only surviving queen-right colonies, were analysed. The results of these analyses were not qualitatively different to the analyses described above, and hence we provide them as supplementary information (Online Resource 1). Lastly, the observational data from the 2012 experiments were combined over the 2 days of observations (separately for each experiment) and the total counts of all behaviours were analysed, using chi-square tests and Fisher's exact tests, when observations were too small. All statistical analyses were carried out using R version 2.15.2 (R Core Team 2012), the package 'Survival' for the time-to-event analyses (Therneau 2013) and the package 'gmodels' for chi-square and Fisher's tests (Warnes et al. 2012).

Results

Queen survival

As expected, no deaths in either control group were attributable to *Metarhizium*. However, the fungus caused 100 % of the mortalities in the single parasite-exposed queens, and all mortalities in the paired parasite-exposed queens that occurred before the start of dominance fights in the paired queens. Queen–queen fighting after worker eclosion appeared to cause the majority of the remaining mortalities for the latter group, but 31 % of queens who died during the queen reduction phase also produced *Metarhizium* growth, and so the true cause of death for these queens is uncertain. These latent fungal infections were present in both the single and paired parasite-exposed treatments, occurring 57–110 days after parasite exposure. The application of *Metarhizium* conidia had a significant impact on the survival of queens, but the hazard of *Metarhizium* death decreased over time, as expected based on the biology of the fungus (Deacon 2006) (Fig. 1a; interaction of treatment and time: hazard ratio [HR]=0.01, χ^2=15.02, $p<0.01$). Further, paired queens also experienced greater mortality, with a hazard risk 1.5 times greater than that of single queens (HR=1.53, χ^2=7.23, p=0.01). Additionally, there was a marginally non-significant interaction between queen treatment and number (HR=0.85, χ^2=3.71, p=0.05). When pairwise comparisons were made, we found that the survival of single parasite-exposed queens (65 % of which survived) was almost significantly lower than that of single control queens (90 % of which survived) (Fig. 1a; post-hoc comparisons with H-B correction, p=0.05). In contrast to expectations, there was no positive effect of queen number on survival when exposed to *Metarhizium*, with single parasite-exposed queens exhibiting similar levels of survival to paired parasite-exposed queens (30 % of which survived) (post-hoc comparisons with H-B correction, p=0.14). Moreover, pleometrosis had a negative effect on survival, with paired control queens exhibiting increased mortality compared to single control queens (post-hoc comparisons with H-B correction, $p<0.01$). Despite the same number of queens dying, there was a slight but significant difference between the survival curves of paired control and paired parasite-exposed queens (post-hoc comparisons with H-B correction, p=0.048), with paired

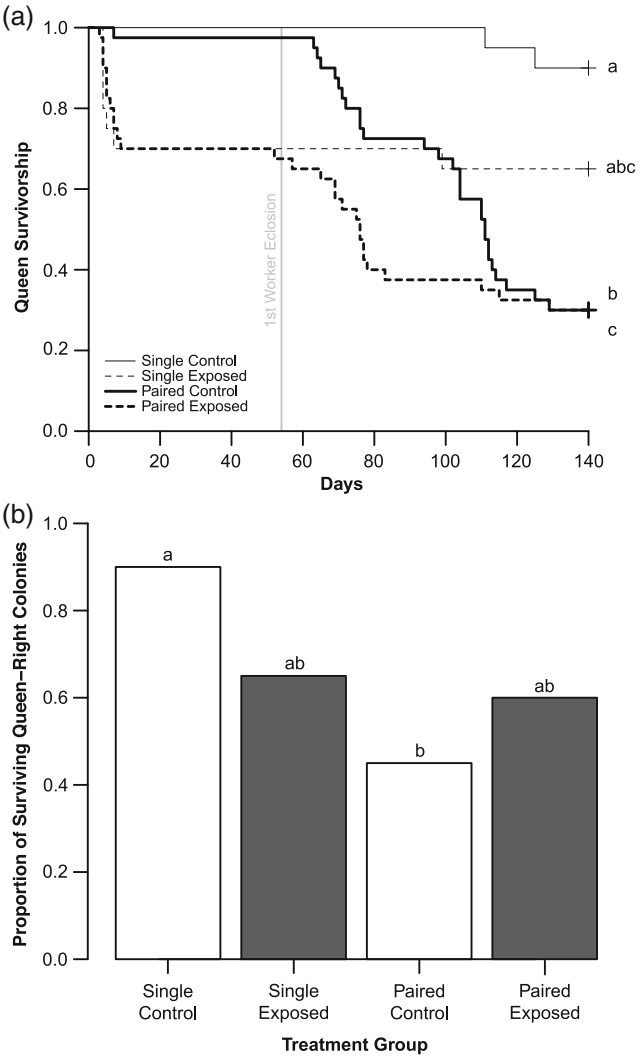

Fig. 1 **a** The cumulative survival of *L. niger* queens that were kept either singly or in pairs and either exposed at the beginning of the experiment to the fungal pathogen *Metarhizium* or treated with a control solution (n=20 single or 20 paired queens; *crosshairs* represent censored cases). Queens were kept under standardised laboratory conditions and food was provided only after worker eclosion (after day 55). *Letters* denote those survival curves that differ significantly from one another ($p<0.05$) in pairwise post-hoc tests. **b** The proportion of queen-right, viable *L. niger* ant colonies present 140 days after exposure to either *Metarhizium* (*grey bars*) or a control solution (*white bars*), with queens kept either singly or in pairs (n=20 nests per group). *Letters* denote the experimental groups that differ significantly from one another ($p<0.05$) in post-hoc analysis

control queens having an improved survival over paired parasite-exposed queens.

Number of queen-right colonies at the end of the experiment

Overall, the model comparing the number of surviving queen-right colonies at the end of the experiment was significant (Fig. 1b; likelihood ratio test comparing full and null model: $\chi^2=10.24$, $df=3$, $p=0.02$). The interaction between queen number and treatment was significant (likelihood ratio test comparing full and reduced model: $\chi^2=4.42$, $df=1$, $p=0.04$), suggesting a compounding effect of both on the number of surviving queen-right colonies. The number of single control, single-exposed and paired-exposed queen-right colonies surviving to the end of the study did not significantly differ from one another (Fig. 1b; post-hoc comparisons with H-B correction, all $p>0.05$). Additionally, the numbers of single-exposed and paired-exposed queen-right colonies, compared to paired control queen-right colonies, were not significantly different (post-hoc comparisons with H-B correction, all $p>0.05$). However, the number of queen-right single control queen colonies was significantly higher than the number of paired control queen colonies (18 single control vs. 9 paired control queen colonies; post-hoc comparisons with H-B correction, $p<0.05$).

Brood production

Our experiment revealed a negative effect of *Metarhizium* exposure on the total amount of brood produced by queens (Fig. 2a; two-way ANOVA, $F_{1,\ 76}=22.7$, $p<0.01$) and an effect of queen number (two-way ANOVA, $F_{1,\ 76}=6.20$, $p=0.02$). There was also a marginally non-significant interaction between queen number and treatment (two-way ANOVA, $F_{1,\ 76}=3.8$, $p=0.06$). Post-hoc comparisons revealed that single parasite-exposed queens had a greatly reduced number of brood compared to all other queen treatments (Fig. 2a; all Tukey's HSD $p<0.05$). However, we found no difference between the numbers of brood produced by paired parasite-exposed queens and the control groups (single control queens: Tukey's HSD, $p=0.38$; paired control queens: Tukey's HSD, $p=0.20$). Surprisingly, there was no difference in the number of brood produced between single control and paired control queens (Tukey's HSD, $p=0.98$).

Worker production

Queen number (Fig. 2b; two-way ANOVA, $F_{1,\ 69}=10.27$, $p=0.01$) and *Metarhizium* exposure (two-way ANOVA, $F_{1,\ 69}=6.73$, $p=0.02$) had an impact on the number of workers produced by queens. However, the interaction was not significant (two-way ANOVA, $F_{1,\ 69}=0.73$, $p=0.40$). Single parasite-exposed queens showed a significant reduction in the number of workers produced compared to paired-parasite-exposed queens (Fig. 2b; Tukey's HSD, $p=0.03$) and paired control queens (Tukey's HSD, $p<0.01$). There was, however, no significant reduction in worker production between single parasite-exposed queens and single control queens, with the latter being intermediate between single-exposed queens and both paired queen groups, to which single control queens also did not differ (all Tukey's HSD $p>0.05$). Lastly, paired parasite-exposed and paired control queens did not differ in the number of workers they produced (Tukey's HSD, $p=0.54$).

Cumulative proportion of colonies with eclosed workers

Exposure to *Metarhizium* had a negative effect on the cumulative proportion of colonies with eclosed workers relative to control groups (Fig. 2c; HR=0.25, $z=-2.98$, $p=0.01$), whereas queen number had no impact (HR=1.30, $z=0.80$, $p=0.43$). The interaction of the two was also non-significant (HR=2.79, $z=1.76$, $p=0.07$). As expected, there were no differences in the cumulative proportion of colonies with workers, between single control queens, paired control queens and paired parasite-exposed queens (Fig. 2c; all post-hoc comparisons with H-B correction, $p<0.05$). However, more colonies in all three groups produced workers than the colonies of single parasite-exposed queens, indicating a strongly negative impact of *Metarhizium* exposure on the ability of these single queens to raise workers (Fig. 2c; post-hoc comparisons with H-B correction, all $p<0.01$).

Behavioural observations

In the 2010 study, we found that the ants were very inactive and few interactions were recorded. Immediately after exposure, queens in 98 % of paired queen nests exhibited self-grooming behaviours. Allogrooming was only observed in one paired parasite-exposed queen nest, where one queen cleaned the other twice. However, for all queens, grooming behaviours were reduced in subsequent observations, with allogrooming observed only once more (in a paired parasite-exposed queen nest). Self-grooming occurred occasionally, 1–2 times per nest per observational session. Neutral interactions, i.e., antennating, were witnessed multiple times throughout the observatory period. Aggressive interactions were non-existent during the observational sessions. However, once workers emerged and queens began to fight for dominancy, aggressive behaviours were very common and lasted long periods, until one or both of the queens were killed. These behaviours included biting (to the extent of removing limbs), and also the spraying of formic acid into the exoskeleton joints of the competing queens. In the first of the 2012 experiments, where behaviours were recorded for queens for 2 days after exposure (excluding the first 10 h immediately

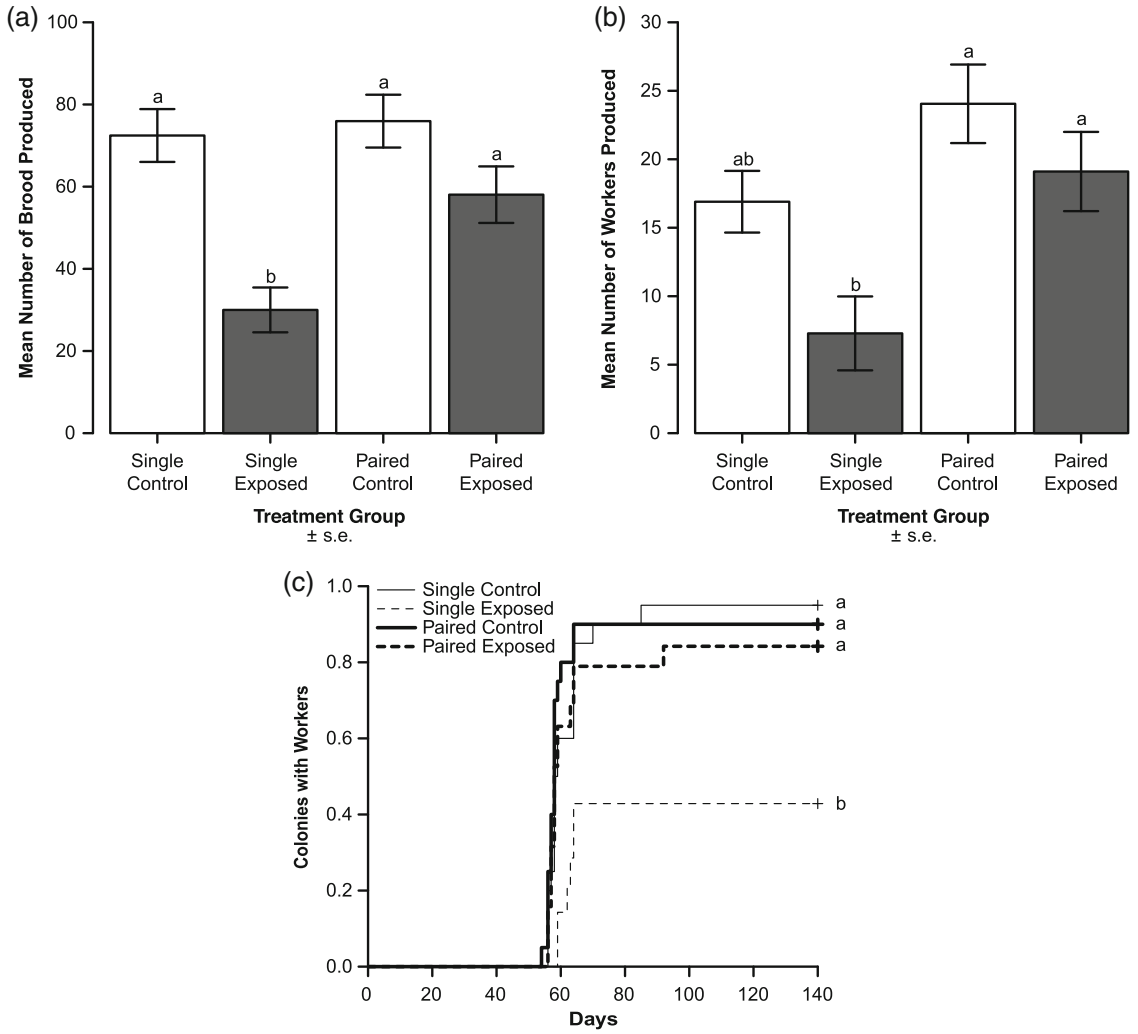

Fig. 2 The mean (±SE) number of **a** total brood items (*n* nests=20 per group) and **b** workers (*n* nests=single control: 20, single exposed: 14, paired control: 20, paired exposed: 19), recorded for single or paired *L. niger* ant queens exposed to either the fungal pathogen *Metarhizium* (*grey bars*) or a control solution (*white bars*), at the respective time of queen or last queen death (in paired queen nests) or 140 days after exposure, for all censored cases. Queens that died before the age of being able to produce workers (taken as day 54, the first worker eclosion in the study) are excluded in **b**. *Letters* denote mean values that differ significantly from one another ($p<0.05$) in Tukey HSD post-hoc comparisons of two-way ANOVAs. **c** The cumulative proportion of colonies with eclosed workers, founded by either single or paired *L. niger* queens exposed to *Metarhizium*, or a control solution at the start of the study (*crosshairs* represent censored cases; *n* nests=single control: 20, single exposed: 14, paired control: 20, paired exposed: 19). Again, queens that died before the age of being able to produce workers (taken as day 54, the first worker eclosion in the study) are excluded. *Letters* denote cumulative event curves that differ significantly from one another ($p<0.05$) in pairwise post-hoc comparisons

after parasite exposure), we found that there was a difference in self-grooming between single control and single-exposed queens (Fig. 3a; $\chi^2=5.32$, $df=1$, $p=0.02$), with more single-exposed queens self-grooming (3 % of single control vs. 9 % of single-exposed observations). The same result was seen for paired queens (Fig. 3a; $\chi^2=19.4$, $df=1$, $p=0.01$), with exposed paired queens self-grooming more than paired control queens (1 % of paired control vs. 7 % of paired-exposed observations). However, there was no significant increase in allogrooming between paired control and paired-exposed queens (Fig. 3a; 0 % of paired control vs. 2 % of paired-exposed observations; Fisher's exact test, $p=0.25$). In the second 2012 experiment, where queens were observed immediately after exposure for 2 days, we found the same general pattern as above. There is an increase in self-grooming between single control and single parasite-exposed queens (Fig. 3b; 13 % of single control vs. 31 % single-exposed observations; $\chi^2=5.36$, $df=1$, $p=0.02$) but no increase in self-grooming between paired control and paired-exposed queens (Fig. 3b; 24 % of paired control vs. 35 % of paired-exposed observations; $\chi^2=3$, $df=1$, $p=0.09$). Again, we found no significant increase in allogrooming between paired control and paired-exposed queens (Fig. 3b; 6 % paired control vs. 2 % paired-exposed queens; Fisher's exact test, $p=0.30$). No other social

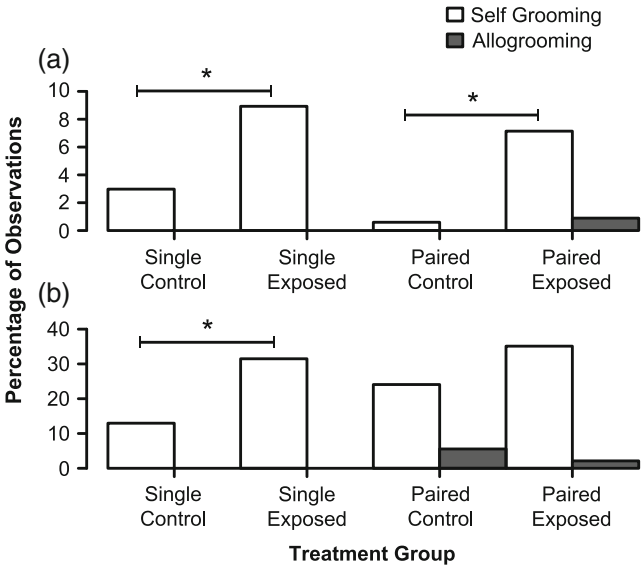

Fig. 3 The scan sample observations from the second set of queen experiments (year 2012). Queens were either single or paired queens and exposed to the pathogen *Metarhizium* or a control solution. Self-grooming was recorded for all queens (*white bars*: possible for all queens) or allogrooming recorded for paired queens (*grey bars*: paired queens only). In **a** observations were conducted for 2 days, starting 10 h after parasite exposure ($n=28$ single or 28 paired queens), whereas in **b** observations were conducted for 2 days including the period immediately after exposure ($n=9$ single or 9 paired queens). *Lines* denote experimental groups that differ from one another ($p<0.05$) in either chi-square or Fisher's exact tests

immunity behaviours were observed (e.g., antiseptic formic acid spraying).

Discussion

Our experiments show that co-founding *L. niger* queens do not engage in cooperative social immune behaviours, and thus pathogen-defence is not a direct benefit of pleometrosis. Specifically, we found no difference in survival between single or paired parasite-exposed queens to their respective control groups. A similar result was observed by Hartke (2010) in the termite *Nasutitermes corniger*, although she found that pathogen-imposed mortality actually increased with group size, perhaps due to within-group transmission of the pathogen. However, we did find a strong negative effect of *Metarhizium* exposure on both brood production and the cumulative proportion of nests with workers in single parasite-exposed queens. The size of this positive effect of co-foundress associations in the face of a parasite-challenge is particularly remarkable given that it was achieved by groups of just two gynes. Paired parasite-exposed queens were able to produce similar numbers of brood, relative to the control groups, and in addition, the proportion of paired parasite-exposed queen nests with workers did not differ from the control groups. This result also stands in contrast to previous studies in termites, where no effect of the fungal pathogen *M. anisoplae* on brood production was found, irrespective of founding group size (Hartke 2010). A final remarkable result of our study was the apparent ability of queens to suppress viable *Metarhizium* infections for several weeks to months after exposure. This is not only suggestive of a surprisingly strong immune system in ant queens, but also, given the reduction in fecundity of single parasite-exposed queens, a potential trade-off between the immune response and reproduction, during the most hazardous time of a queen ant's life.

Paired queen ants in both experimental treatments demonstrated some level of association (e.g., joint brood piles, initial lack of aggression towards conspecifics), however, there was a distinct lack of mutual grooming, which is a frequent behaviour in groups of workers (Wilson 1971, pp. 281–295). The almost complete absence of allogrooming suggests that co-founding queens do not engage in cooperative anti-parasite defences during colony foundation, which is surprising, given that allogrooming is an adaptive first line of defence for social insect workers encountering nestmates exposed to fungal parasites (Okuno et al. 2011; Reber et al. 2011; Rosengaus et al. 1998; Tragust et al. 2013; Ugelvig et al. 2010; Ugelvig and Cremer 2007; Walker and Hughes 2009). There may be several reasons for this. Typically, only one queen survives to become the reproducing individual in the nest (Sommer and Hölldobler 1995). Therefore, all associated queens can be viewed as competitors, with pleometrosis a temporary association, only lasting as long as each queen benefits from it (Bernasconi and Strassmann 1999; Holman et al. 2010; Rissing et al. 1989). Sommer and Hölldobler (1995) demonstrated that workers preferentially feed the most fertile queen, which influenced the outcome of queen fights. As queens have limited resources, they should therefore invest these into reproduction (ovary development, brood feeding, etc.), both to compete with rival colonies and perhaps improve their chances of becoming the future reproductive, rather than engaging in energy-consuming behaviours that benefit future rivals. Another possible explanation is that queens may not rely heavily on behavioural disease defences, due to a potentially enhanced immune system possessed during the colony-founding phase. Indeed, up-regulation of the immune system after mating has been demonstrated in the founding queens of *Atta* leaf-cutting ants (Baer et al. 2006), *Formica* wood ants (Castella et al. 2009) and both the founding queens and kings of dampwood termites (Calleri et al. 2007). Leaf-cutting ant queens also have larger antibiotic metapleural glands for their body size, compared to workers (Hughes et al. 2010) and bumblebee gynes appear to be more resistant to trypanosome parasites than their worker sisters (Ulrich et al. 2011). This hypothesis may also be supported by the observation that queens were able to survive many weeks with viable *Metahrizum* infections, which is suggestive of a robust

immune response. It is therefore possible that queens in an association do not engage in social immune behaviours, due to a shift from a dependency on social immunity to a dependency on the individual immune system. While it is possible that the arbitrary pairing of co-founding queens in this study (as opposed to allowing queens to actively choose a conspecific to found with) may also explain to some degree the lack of social immune behaviours recorded, this seems unlikely because pleometrosis appears to be a random choice in this species, and is not influenced by queen weight or size (Aron et al. 2009; Sommer and Hölldobler 1995).

We saw two clear phases of queen mortality: the first caused largely by *Metarhizium* between 4–26 days after fungal exposure, and a second that appears to be primarily driven by queen fighting between 58 and 118 days after exposure (the queen reduction phase, during which, consistent with Reber et al. (2010), both queens could be killed). However, extraordinarily, queens from both the single and paired parasite-exposed queen groups died, and produced *Metarhizium* growth, outside of the initial fungal-death phase. One single parasite-exposed queen died 99 days after exposure, and five paired parasite-exposed queens that died between 57 and 110 days, during the queen reduction phase, all produced *Metarhizium*. It is not clear in the latter case if these infections were the cause of mortality, or if the queens were instead killed during dominancy fights. In either case, latent fungal growth suggests queen ants in this study were able to suppress viable *Metarhizium* infections for a number of weeks to several months after exposure. Low-level, non-lethal infections of *Metarhizium* have been demonstrated to manifest in workers of *L. neglectus* under laboratory conditions, and lead to the stimulation of the ants' immune system and subsequent parasite resistance (Konrad et al. 2012). However, it is not known in what capacity low-level infections exist, e.g., whether the fungus enters a 'dormancy' phase, waiting until the immune system is weakened due to stress, or if the fungus is prevented from growing through continual suppression by the immune system. It is also unknown whether these low level infections can be eventually cleared from the ant's body. However, in bumblebees, workers infected with bacteria can clear low-level infections of a gut parasite (Sadd and Schmid-Hempel 2006). In our study, we did not test directly for the presence of the fungus in the bodies of the queens, but all queen cadavers were surface-sterilised and observed for fungal outgrowth (Fig. 4). It is unlikely that queens became 're-exposed' to the fungus, via conidia that inadvertently ended up on surface of the substrate, as conidia groomed off the queens themselves would be neutralised through the use of formic acid and/or the infrabuccal pocket (Graystock and Hughes 2011; Oi and Pereira 1993; Tragust et al. 2013), and initial application of conidia outside of the nest will have prevented the transfer of viable conidia to the nest substrate. This is supported by the lack of infections in

Fig. 4 The cadaver of a *L. niger* queen supporting extensive *Metarhizium pingshaensae* fruiting bodies. *Metarhizium* spp. are general entomopathogens whose fungal conidia attach to the cuticle of an insect. Here the conidia can germinate and penetrate the body of the host, producing hyphae that then deplete the insect's resources and breakdown tissue, eventually resulting in host death (Deacon 2006)

nanitic workers, which might be expected if viable conidia were present in the nest. Given that nuptial flights are synchronised between local colonies (Boomsma and Leusink 1981), variation in queen age or cuticle sclerotization cannot explain this temporal variation in *Metarhizium* mortality, and as all nests were constructed and maintained equally, variation in conditions is also an unlikely explanation. Therefore, it seems probable that these surprising infections resulted from successful conidia germination during the initial exposure of the parasite, and were present in the body of the queen for the duration of the experiment. That single parasite-exposed queens produced reduced numbers of brood relative to paired parasite-exposed queens and control groups also supports this idea, providing evidence of a trade-off between reproduction and immunity.

Mounting an immune defence is costly and can cause a reduction in other components of the host's fitness, e.g., lower fecundity, slower growth, reduced competitiveness (reviewed by Schmid-Hempel 2011, pp. 105–123). More specifically, many empirical studies show that immunity can be traded off against reproduction/fecundity (e.g., in sheep: Festa-Bianchet 1989; birds: Gustafsson et al. 1994; in damselflies: Siva-Jothy et al. 1998; in *Drosophila*: Fellowes et al. 1998; in mosquitos: Yan et al. 1997; Ahmed et al. 2002; in aphids: Gwynn et al. 2005; in crickets: Copeland and Fedorka 2012; in moths: Boots and Begon 1993; termites: Calleri et al. 2005; Calleri et al. 2007). As a closed system, the queens in this study had no option but to reallocate resources away from egg laying/brood-feeding, and perhaps into the immune suppression of parasite growth instead. The resulting underdeveloped colonies of the single parasite-exposed queens would have had a

low chance of survival under natural conditions, where competition between other incipient and mature colonies is high (Bernasconi and Strassmann 1999; Sommer and Hölldobler 1995). As we did not weigh the queens in this study, we cannot make conclusions about the effect of individual variation in queen resources, which might affect their ability to withstand fungal infections. However, it seems likely queens with larger reserves would have a greater capacity to survive, whilst still producing enough brood. Indeed, when two parasite-exposed queens are present, similar numbers of brood and workers are produced, relative to both single and paired control queens, suggesting that by engaging in cooperative brood production and care, less strain is placed on the reserves of each individual queen. Interestingly, in laboratory studies of the termite *N. corniger* there was no direct impact of fungal treatment on offspring production (Hartke 2010). This was suggested to be due to a lack of a trade-off between reproduction and immunity in larger groups (Hartke 2010), although the low sample size due to pathogen imposed mortality may also explain the lack of observed treatment effects. One potential implication of the reproduction–immune trade-off is that, if ant queens can tolerate the parasite until the eclosion of workers, then it may be possible for them to completely clear the infection once they are no longer resource limited. Indeed, several queens from this study that were exposed to the parasite were still alive 3 years later (Pull, personal observation). Furthermore, the numbers of surviving queen-right single-exposed and paired-exposed queen colonies did not differ (as opposed to the controls), suggesting that in parasite-rich environments there may be no disadvantage of pleometrosis, and so when the chances of acquiring an infection are high, co-founding may be beneficial.

Our study is the first to test if cooperating ant queens participate in social immune defences, demonstrating that in fact pleometrotic *L. niger* queens do not engage in the well-documented collective immune responses seen in the workers of many social insects (Cremer et al. 2007; Hughes et al. 2002; Reber et al. 2011; Rosengaus et al. 1998; Yanagawa and Shimizu 2007). However, an indirect benefit of pleometrosis was observed in the fecundity of queens, with paired parasite-exposed queens maintaining competitively high numbers of brood and more colonies with eclosed workers. Also, the number of surviving incipient experimental colonies was not different between single and paired parasite-exposed queens. Consequently, in the parasite-rich environments in which ant queens found colonies (Hughes et al. 2004; Reber and Chapuisat 2012), cooperating with additional queens may improve the chance of successful colony foundation, through ensuring enough brood are raised. Additionally, this study suggests the presence of extremely robust immune systems in ant queens, with queens apparently able to suppress, but not clear, parasitic fungal infections for prolonged periods. Further studies of the dynamics of immunocompetence in social insect queens will be important for understanding the repercussions of life-history trade-offs, made during the founding stage, on colony organisation and fitness.

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