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Author for correspondence:
Jessica Purcell
e-mail: jessica.purcell@unil.ch

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Foster carers influence brood pathogen resistance in ants

Jessica Purcell and Michel Chapuisat

Department of Ecology and Evolution, University of Lausanne, Biophore, UNIL-Sorge, 1015 Lausanne, Switzerland

Social organisms face a high risk of epidemics, and respond to this threat by combining efficient individual and collective defences against pathogens. An intriguing and little studied feature of social animals is that individual pathogen resistance may depend not only on genetic or maternal factors, but also on the social environment during development. Here, we used a cross-fostering experiment to investigate whether the pathogen resistance of individual ant workers was shaped by their own colony of origin or by the colony of origin of their carers. The origin of care-giving workers significantly influenced the ability of newly eclosed cross-fostered *Formica selysi* workers to resist the fungal entomopathogen *Beauveria bassiana*. In particular, carers that were more resistant to the fungal entomopathogen reared more resistant workers. This effect occurred in the absence of post-infection social interactions, such as trophallaxis and allogrooming. The colony of origin of eggs significantly influenced the survival of the resulting individuals in both control and pathogen treatments. There was no significant effect of the social organization (i.e. whether colonies contain a single or multiple queens) of the colony of origin of either carers or eggs. Our experiment reveals that social interactions during development play a central role in moulding the resistance of emerging workers.

1. Introduction

Social groups face a particularly great challenge when confronted with pathogens and parasites. The density of individuals, coupled with their often high degree of relatedness, presents an ideal opportunity for pathogens to enter and rapidly spread within a group. In response to this threat, many social organisms are able to mount efficient collective defences that complement their individual immune defences [1,2]. One key problem that social groups face is how to protect naive young from the risk of infection. This can be achieved in several ways. First, inputs from parents can shape the immune resistance of brood through a combination of genetic inheritance of innate defences [3] and parental (=maternal and paternal) effects [4]. Second, extrinsic environmental factors experienced during development, including nourishment, density and social interactions, can further mould the immune resistance of an individual [5–7].

In social insects, workers care for the brood, which could alter the pathogen resistance of brood in multiple ways. In particular, the level of nutrition and hygienic care is likely to influence brood susceptibility to disease. Hygienic care includes mechanical removal of pathogens via allogrooming [8,9] and the deposition of substances that actively repel or kill pathogens [10–12]. The presence of immune-primed workers can also improve brood immune defences in some conditions [13,14]. Some of these mechanisms, such as allogrooming, would primarily function after the colony has been exposed to a pathogen, but others, such as better nutrition, would improve individual resistance even before exposure.

Colony-level traits such as social structure and genetic diversity may also play an important role in shaping individual as well as colony resistance [15,16]. In many social organisms, including bees and ants, colonies of queens that have mated with multiple males tend to exhibit a higher degree of pathogen resistance [17,18]. Colonies headed by multiple queens also have higher genetic diversity, but the impact of queen number on pathogen resistance seems to vary across species [15,19,20]. The mechanisms by which colony genetic diversity improves

pathogen resistance are not yet fully understood. For example, we do not know when genetic diversity contributes to resistance: is it only during infection, or does interacting with individuals with more diverse genotypes already shape the resistance of colony members prior to exposure?

Ants provide ideal systems to investigate the influence of the social environment on offspring traits independently of inherited factors, because the individuals caring for the brood (i.e. workers) typically differ from the ones reproducing (queens and males) [21–23]. Here, our goal is to test whether the identity of the social partners (i.e. carers) encountered during development affects the individual pathogen resistance of newly emerged adults. We used a cross-fostering approach to disentangle the relative influence of care-giving workers from that of factors present in the egg, such as genetic and parental effects.

Our study species, the socially polymorphic ant *Formica selysi* [24], is a natural host of the generalist entomopathogenic fungus *Beauveria bassiana* [25]. We cross-fostered *F. selysi* eggs from both monogynous (=single-queen) and polygynous (=multiple-queen) colonies in groups of foreign carers from each social origin, and investigated whether the ability of the newly eclosed cross-fostered workers to resist *B. bassiana* was influenced by the colony of origin of eggs or the colony of origin of carers. To further investigate the respective influence of inherited factors and carers on pathogen resistance, we tested whether the pathogen resistance of cross-fostered workers correlated with that of adult workers originating from either the same colony as the eggs or the same colony as the carers. Finally, because the genetic diversity of carers may affect the pathogen resistance of brood, we examined whether the social structure (monogynous or polygynous) of the colony of origin of eggs or carers influenced the individual pathogen resistance of the cross-fostered workers. Together, these results will shed light on the intrinsic and social factors shaping pathogen resistance.

2. Material and methods

(a) Study system

We collected *F. selysi* workers and eggs from a well-studied population along the Rhône River between Sierre and Susten in Valais, Switzerland (7°36′30″ E, 46°18′30″ N, altitude 565 m). The focal nests are distributed in a floodplain steppe habitat, in an area of about 1500 × 400 m. This population harbours a mix of monogynous and polygynous colonies. The field colonies are individually marked, and their social structure has been monitored during the past 15 years by regular genotyping of workers at eight polymorphic microsatellite markers; gene flow persists between the two social forms at these loci [24,26]. The two social forms differ from one another in a suite of traits, including queen body size, brood development time, colony size and genetic diversity [27–29]. Workers from monogynous colonies are consistently larger [30], have higher bacterial growth inhibition activity [31] and lower mortality rates than workers from polygynous colonies [15]. Moreover, the survival of workers varies greatly across colonies, both in controls and when exposed to the entomopathogenic fungus *B. bassiana* [32].

(b) Cross-fostering

We collected eggs and workers from 20 monogynous and 20 polygynous field colonies. For each egg-source colony, we introduced 50 eggs into a group of 50 foreign workers of monogynous origin and 50 eggs into a group of 50 foreign workers of polygynous origin (figure 1). Thus, eggs from each colony were reared by one set of workers of monogynous origin and by one set of workers of polygynous origin, in separate experimental groups. We monitored their survival and development until adulthood [29]. We collected all newly emerged cross-fostered workers and placed them with two marked adult workers from their rearing group for 5 days. This allowed cross-fostered workers to receive care until they were fully sclerotized.

After all of the brood completed development, we collected a random subset of eight adult care-giving workers from each of the experimental groups that had successfully reared offspring. In addition, during the brood rearing experiment, we maintained groups of 50 field-collected workers from each colony without brood, and also collected eight of these workers. This sampling scheme allowed us to test the pathogen resistance of cross-fostered workers and adult workers originating from the same colonies as the eggs and carers, respectively (see below). All ants had ad libitum access to water and standard ant food during the entire experiment.

(c) Survival and pathogen resistance

When the cross-fostered workers were 5 days old and fully sclerotized, we deposited 2 µl of either the entomopathogenic fungus *B. bassiana* (10^8 conidia ml^{-1} suspended in 0.05% Tween 20 buffer) or a control solution (0.05% Tween 20 buffer) on their thorax. The *B. bassiana* strain that we used was originally collected from our field site [25; strain B2], and this concentration has caused an intermediate mortality rate in past experiments [25,32,33]. We randomly assigned the first cross-fostered worker emerging from each rearing group to either the *B. bassiana* challenge or control; thereafter, we alternated the treatment applied to each subsequent cross-fostered worker from the same rearing group. This approach ensured that balanced numbers of individuals from the same rearing group were exposed to the pathogen challenge and control, respectively. In total, we had 169 cross-fostered workers in the challenge and 175 cross-fostered workers in the control. These individuals emerged from a total of 44 rearing groups, which produced 7.9 ± 0.9 offspring (mean ± standard error). Thus, some of the rearing groups and hierarchy outlined in figure 1 were lost in the final analysis.

For care-giving workers and adult field-collected workers originating from the same colonies as eggs and carers, we haphazardly collected eight individuals and allocated these to either the *B. bassiana* treatment or the control by shuffling the sampled workers before applying the treatments. If rearing groups from a given field colony failed to rear offspring during the cross-fostering experiment, they were omitted from the pathogen challenge experiment. We measured survival of a total of 584 adult workers; 292 in the pathogen challenge and 292 in the control. These individuals came from 30 field colonies; 18 monogynous and 12 polygynous.

After the application of the solution, individuals were isolated in glass tubes with ad libitum access to standard ant food and water. We monitored survival daily for 14 days; surviving ants were then removed and their heads were measured. Individuals that died during this period were removed, measured and then surface sterilized and maintained in tubes with wet cotton wool for 30 days, in order to determine which corpses produced *B. bassiana* conidia [34].

(d) Statistical analyses

We investigated the factors affecting the survival of cross-fostered and adult workers that had either been exposed to *B. bassiana* or kept in control conditions using parametric survival analyses implemented in the survival package of R 3.0.2 [35]. For each survival model, we used the Weibull distribution, which produced the minimum error deviance. We performed statistical tests to address our three hypotheses independently,

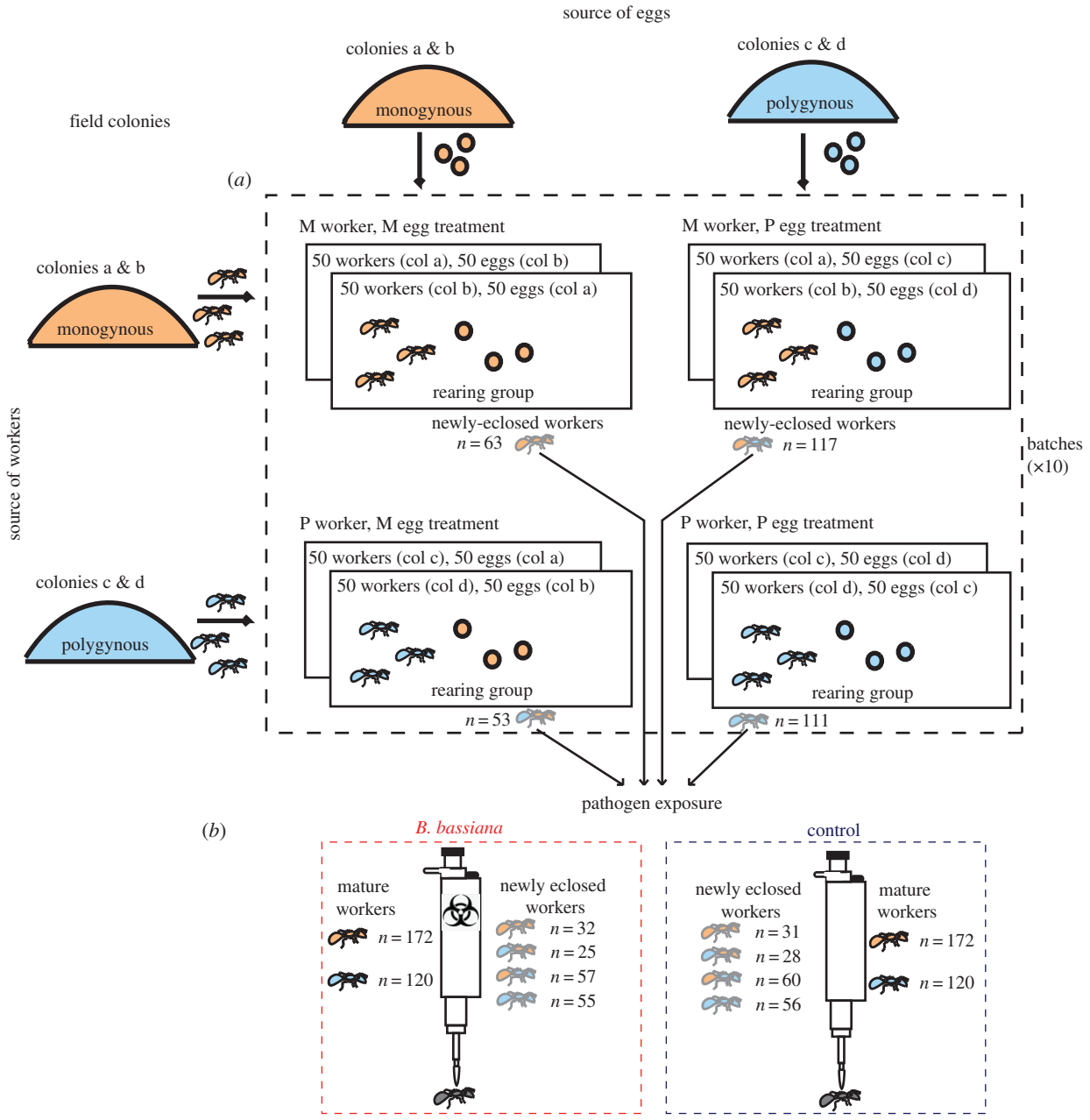

Figure 1. Diagram of cross-fostering experimental design (adapted from [29]). *n* indicates the number of individuals. Newly emerged cross-fostered workers are outlined in grey, whereas field-collected workers are outlined in black. Four unique colonies (labelled a, b, c and d) were used in each of 10 batches, for a total of 40 unique field colonies included in the experiment. In total, there were 80 experimental rearing groups (20 per treatment). The colony of origin refers to the identity of the field colony from which the eggs or workers were collected. The social origin refers to the social structure of the colony of origin (either monogynous or polygynous). (Online version in colour.)

because each contrast was planned as a part of the experimental design. To account for multiple comparisons within the cross-fostered worker survival data, we calculated false discovery rate *q*-values using the qvalue package in R 3.0.2.

First, we were interested in the relative influence of parental and carer inputs on the resistance of the cross-fostered workers. In order to determine whether the resistance of newly eclosed workers was shaped by genetic or parental effects or by the identity of carers encountered during development, we investigated the survival of cross-fostered workers in relation to (i) their own colony of origin and (ii) the carers' colony of origin. To this end, we performed a survival analysis with the *B. bassiana* challenge, the identity of the colony of origin of eggs, the identity of the colony of origin of carers and the interaction of each with the pathogen challenge as fixed effects. We excluded four experimental groups that produced only one or two cross-fostered workers. The experimental batch (=set of eight rearing groups composed of eggs and workers from two monogynous and two polygynous field colonies) was a random effect in the analysis (figure 1). We report the analysis of deviance for each variable.

To assess whether the pathogen resistance of cross-fostered workers correlated with the one of adult workers originating from either the colony of origin of eggs or the colony of origin of carers, we measured the proportion of adult workers from each field colony that survived in the pathogen challenge and control, respectively. We calculated the Pearson's correlation coefficients between the survival of cross-fostered workers and that of adult workers from (i) the colony of origin of eggs or (ii) the colony of origin of carers (proportions were normalized through logit transformation using the logit function with the

'adjust' option to correct proportions of 0 or 1 to 0.025 and 0.975, respectively, in the 'car' package in R 3.0.2). We excluded two egg colonies of origin for part (i) and five carer colonies of origin for part (ii) owing to small sample sizes. To avoid pseudo-replication, each data point in the correlation analyses corresponds to all adult workers from a given field colony, regardless of the rearing group identity.

Finally, because workers from monogynous and polygynous colonies differ in overall survival [15] and genetic diversity in carers may affect the pathogen resistance of brood, we analysed whether the social origin of the eggs or of the carers influenced the individual pathogen resistance of the cross-fostered workers. We constructed a survival model with *B. bassiana* challenge, social origin of eggs, social origin of carers, brood cohort size at eclosion (which depended upon differential brood mortality during the cross-fostering experiment), individual head size and the interaction term of each characteristic with the pathogen challenge as fixed effects, and rearing group identity as a random effect. To test whether their social origin affects the resistance of adult workers, we first assessed whether the number of brood reared influenced survival or showed a significant interaction with the pathogen challenge. We found no effect of brood rearing ($\chi^2_1 = 1.6$, $p = 0.21$), so we combined data from adult workers that had reared brood (i.e. carers) and those that had been maintained without brood (i.e. original colony workers). We constructed a model with the pathogen challenge, social origin, individual head size and the interaction term of each characteristic with the pathogen challenge as fixed effects, and rearing group as a random effect.

In order to estimate the magnitude of effect of each variable on the expected survival time, we calculated failure time ratios. Failure time ratios that significantly differ from one indicate a factor that either reduces (less than one) or prolongs (greater than one) the life expectancy [36].

3. Results

Exposure to the fungal pathogen *B. bassiana* increased mortality in both cross-fostered and adult workers (cross-fostered workers full model: $\chi^2_{40.4} = 178$, $p < 0.0001$, $q < 0.0001$, pathogen challenge: $\chi^2_1 = 72$, $p < 0.0001$, $q < 0.0001$; adult workers model: $\chi^2_{49.5} = 121$, $p < 0.0001$, pathogen challenge: $\chi^2_1 = 26$, $p < 0.0001$).

The colonies of origin of eggs and carers influenced the survival of cross-fostered workers in different ways. There was a marginally significant interaction between the colony of origin of carers and the pathogen challenge ($\chi^2_{3.25} = 43$, $p = 0.043$, $q = 0.088$), demonstrating that the colony of origin of carers influenced the pathogen resistance of the cross-fostered workers. This effect of the colony of origin of carers was robust to the order of the factors in the model and across model formulations (see the electronic supplementary material, Survival models). By contrast, the effect of the colony of origin of eggs was sensitive to the order of factors in the model. When first in the model, the colony of origin of eggs influenced the overall survival of cross-fostered workers in both control and challenge ($\chi^2_{23} = 53$, $p = 0.0013$, $q = 0.0033$), but the interaction with pathogen challenge was non-significant ($\chi^2_{14.8} = 40$, $p = 0.071$, $q = 0.12$). When second in the model, the colony of origin of eggs had no significant effect on the survival of cross-fostered workers. This result reflects the fact that some degrees of freedom (from the colonies of origin) are shared between the eggs and carers. In total, 24 colonies of origin yielded enough eggs to be included in our analyses, and these were reared by carers from 27 colonies of origin; a total of 12 colonies of origin were represented in both groups. There was substantial variation in the failure time ratios among colonies of origin of both eggs and care-giving workers, indicating a high degree of variation among colonies in overall survival and resistance to *B. bassiana* (see the electronic supplementary material, figure S1 and table S1).

The contribution of carers towards the pathogen resistance of newly eclosed workers was confirmed by the relationships between the survival of cross-fostered workers and adult workers. On the one hand, the survival of cross-fostered workers was significantly correlated with the survival of adult workers from the colony of origin of eggs in the control (figure 2*a*; Pearson's correlation $r_{16} = 0.50$, $p = 0.034$), but there was no significant relationship between the two in the pathogen challenge (figure 2*b*; $r_{16} = 0.057$, $p = 0.82$). On the other hand, there was a positive correlation between the survival of cross-fostered workers and the survival of adult workers from the colony of origin of carers in the challenge (figure 2*d*; $r_{21} = 0.46$, $p = 0.025$), but no relationship between the two in the control (figure 2*c*; $r_{21} = 0.29$, $p = 0.19$). These results further suggest that the colony of origin of eggs influences the baseline survival of cross-fostered workers, whereas the colony of origin of carers influences the ability of cross-fostered workers to resist the fungal pathogen. Across all colonies, adult workers tended to survive infection in higher proportions than cross-fostered workers (see the electronic supplementary material, Odds ratio).

The head size of cross-fostered workers was significantly associated with their overall survival ($\chi^2_1 = 90.5$, $p < 0.0001$, $q < 0.0001$; interaction with pathogen challenge: $\chi^2_{2.7} = 0.04$, $p = 0.99$, $q = 0.72$); the time ratio suggests that larger workers are much more likely to survive for the duration of our experiment than smaller ones (see the electronic supplementary material, table S1). By contrast, the social origin of eggs, social origin of carers and brood cohort size had no significant effect on survival (figure 3*a*; social origin of eggs: $\chi^2_1 = 0.22$, $p = 0.64$; interaction with pathogen challenge: $\chi^2_{1.3} = 0.085$, $p = 0.86$; social origin of carers: $\chi^2_1 = 1.88$, $p = 0.17$; interaction with pathogen challenge: $\chi^2_{1.4} = 3.53$, $p = 0.1$; brood cohort size: $\chi^2_1 = 0.91$, $p = 0.34$; interaction with pathogen challenge: $\chi^2_{1.1} = 0.39$, $p = 0.58$; $q > 0.15$ for all comparisons). None of these factors had significant interactions with the pathogen challenge, suggesting that they do not have a strong influence on the ability of cross-fostered workers to resist the fungal pathogen. Out of the 85 cross-fostered workers that died in the pathogen challenge, 80% of the corpses exhibited *B. bassiana* conidia, whereas none of the 17 workers that died in the control exhibited conidia.

In line with the results from cross-fostered workers, the head size of adult workers influenced their overall survival, but did not alter their ability to resist the pathogen ($\chi^2_1 = 103.1$, $p < 0.0001$; interaction with pathogen challenge: $\chi^2_1 = 0.70$, $p = 0.28$). Although workers from monogynous colonies were significantly larger than those from polygynous colonies (two-sample *t*-test $t_{543} = 12.9$, $p < 0.0001$; see also [30]), there was no significant effect of the social origin on overall survival or pathogen resistance (figure 3*b*; social origin: $\chi^2_1 = 1.6$, $p = 0.2$; interaction with pathogen challenge: $\chi^2_{1.3} = 0.58$, $p = 0.55$). Of the 84 adult workers that died in the pathogen challenge, 65% of the corpses exhibited *B. bassiana* conidia, whereas none of the 37 workers that died in the control exhibited conidia.

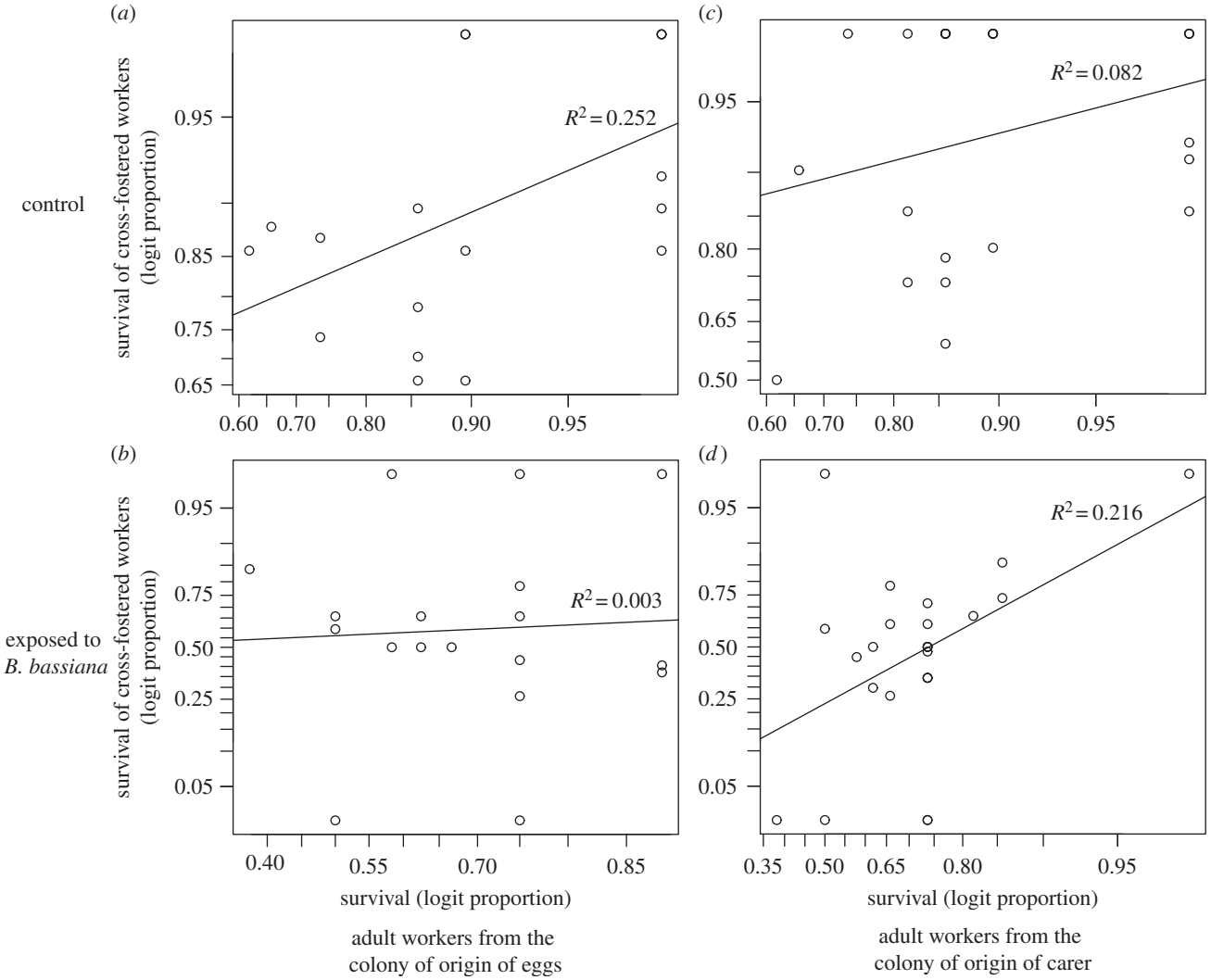

Figure 2. Correlations between the survival of cross-fostered workers and either adults from the colony of origin of eggs (*a*,*b*) or adults from the colony of origin of care-giving workers (*c*,*d*), in the controls (*a*,*c*) and when exposed to *B. bassiana* in the pathogen challenge (*b*,*d*). The baseline survival of cross-fostered workers in the control is significantly correlated with the baseline survival of adult workers from the same colony of origin (*a*), but there is no relationship between the two in the pathogen challenge (*b*). By contrast, the survival of cross-fostered workers is significantly correlated with the survival of adult workers from the colony of origin of carers in the challenge (*d*), but there is no relationship between the two in the control (*c*).

4. Discussion

Social insects have developed an impressive arsenal of collective defences to combat their pathogens and parasites, but the degree to which social interactions during development influence individual resistance remains virtually unexplored. Using a cross-fostering approach, we show that the overall viability of newly eclosed, cross-fostered *F. selysi* workers is influenced by factors that are already present in eggs. By contrast, a significant amount of the variation in the ability of cross-fostered workers to resist the entomopathogenic fungus *B. bassiana* depends on the identity of the care-giving workers. Interestingly, this variation in individual pathogen resistance persists when the cross-fostered workers are isolated from their carers, and therefore cannot be attributed to remedial behaviours, such as allogrooming and trophallaxis.

Two lines of evidence support the conclusion that the identity and individual characteristics of carers play a large role in shaping the pathogen resistance of the brood they rear. First, the colony of origin of carers influenced the ability of the cross-fostered workers to resist the fungal pathogen. Specifically, particular groups of carers had a differential influence on the survival of cross-fostered workers exposed to B. *bassiana* when compared with controls. This effect of carers was evidenced by the significant interaction between the colony of origin of carers and the pathogen challenge, a result that was highly robust to the model choice (see the electronic supplementary material). By contrast, the colony of origin of eggs was significantly associated with the overall survival of the resulting brood, but the interaction with the pathogen challenge was not significant. Moreover, this result was sensitive to the order of the fixed factors in the statistical model, suggesting that the origin of carers and factors present in the egg jointly influence the baseline survival of cross-fostered brood. Second, the pathogen resistance of cross-fostered workers was positively correlated with that of adult workers from the colony of origin of their carers, which indicates that more resistant workers tend to rear brood that will also be more resistant to the same pathogen. There was no such correlation in control conditions. By contrast, the survival of cross-fostered workers was correlated with the survival of adults from the colony of origin of eggs in control conditions, but not when exposed to *B. bassiana*. Together, these results confirm that the origin of eggs influences the baseline survival, whereas the origin of carers influences the pathogen resistance of the cross-fostered brood.

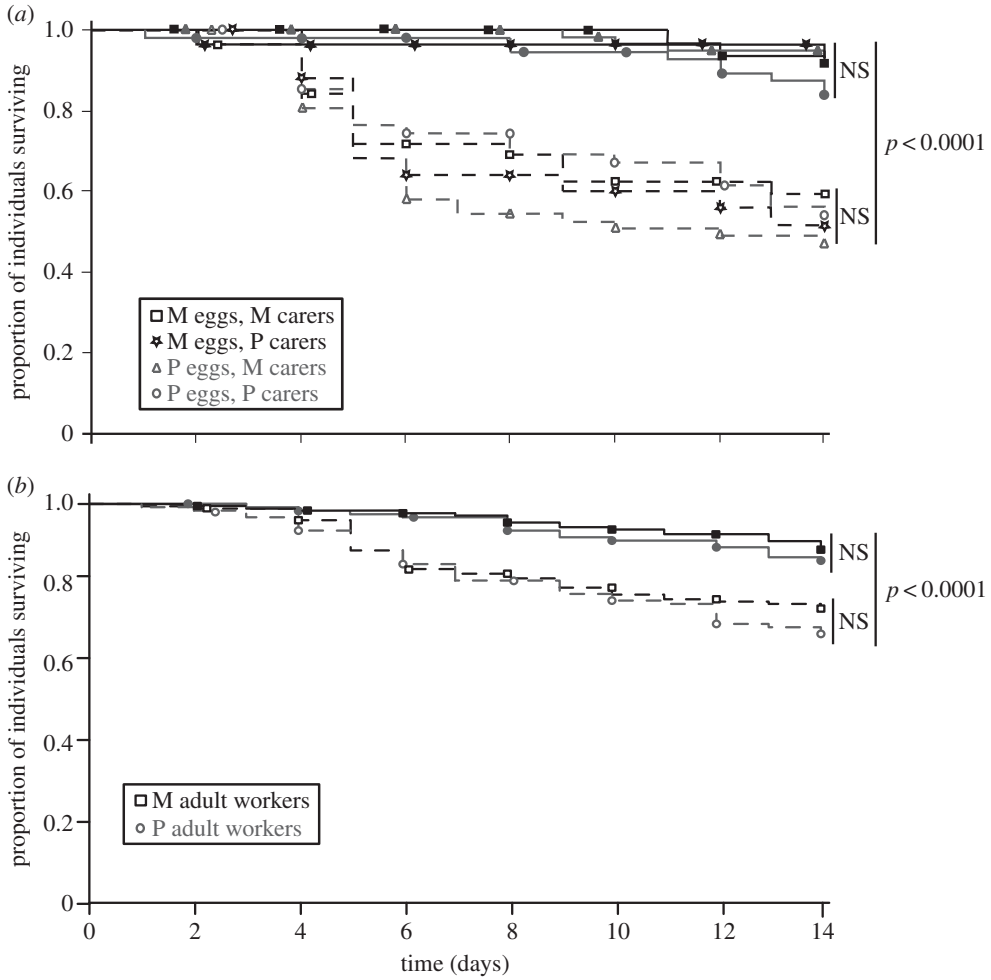

Figure 3. Kaplan–Meier survival curves showing the proportion of cross-fostered, (*a*) newly eclosed workers and (*b*) adult workers surviving in controls (solid lines) and when exposed to *B. bassiana* (dashed lines). In both cases, individuals exposed to the pathogen had increased mortality relative to controls, but there were no significant survival differences associated with social origin.

The relationship between the pathogen resistance of cross-fostered workers and adult workers from the colony of origin of their carers can be explained by several non-mutually exclusive mechanisms. First, adults that are in better condition or have large energetic reserves might rear stronger workers. If this were the case, however, we would also expect a positive correlation between the survival of cross-fostered workers and adult workers in control conditions. Second, care-giving workers could actively share immune compounds and anti-pathogenic substances with brood during development, for example by trophallaxis [37] or allogrooming [12,38]. Carers may also transmit their microbiota to brood, which can affect pathogen resistance [39,40].

The significant effect of carers on the pathogen resistance of cross-fostered workers reflected substantial variation in resistance among colonies from which carers originated. Inter-colony variation may emerge from differences in the innate immunity of colony members or differences in their microbiota. Alternatively, some colonies may have previously encountered this pathogen prior to collection, which could result in a more effective response upon secondary exposure [9,41,42; but see 33]. Further work is needed to elucidate the mechanisms underlying the large variation in pathogen resistance among colonies and the non-genetic transmission of resistance to the developing brood. Whatever the mechanisms, our results indicate that the individual immune resistance of social insects is shaped by early social interactions with carers.

In our study, the social origin (i.e. whether the field colony was monogynous or polygynous) of brood and carers had no significant effect on the survival of cross-fostered workers in control conditions, nor when they were exposed to *B. bassiana*. The survival and pathogen resistance of adult workers similarly did not depend on their social origin. Thus, the genetic diversity of the colony of origin of brood and carers had no strong influence on the individual pathogen resistance of cross-fostered workers challenged in isolation. It should be noted that, by isolating workers from one another immediately after the challenge, we focus on the effect of past social interactions and individual immunity on pathogen resistance, and so might miss effects of group genetic diversity owing to variation in therapeutic behaviour, such as pathogen detection and grooming [8,15,19].

In line with previous studies, larger workers had higher survival in both control conditions and when exposed to the pathogen [43], and there was no significant difference associated with social origin in the size of cross-fostered workers [29]. The field-collected adult workers of monogynous origin were significantly larger than those of polygynous origin [30], but this size difference was not sufficient to result in differential survival between workers of monogynous and polygynous

origin. The brood cohort size did not influence the survival of cross-fostered workers or of care-giving workers in control conditions or when exposed to the pathogen. This result fits with our previous finding that the worker-to-brood ratio during development did not influence the ability of newly emerged workers to resist infection [32]. It also suggests that brood rearing activities are neither compromising nor enhancing the future survival and immunity of care-giving workers in the conditions tested.

More broadly, our study contributes to ongoing research on the relative contribution of the genetic origin of individuals versus their experience during development on emergent phenotypes. We show that carers play an important role in shaping an adult phenotype with strong implications for survival. The influence of the rearing environment on adult phenotypes is in line with recent studies demonstrating epigenetic control of many phenotypic traits, including disease susceptibility, in humans [44]. Our findings also raise general questions about the mechanisms through which carers affect phenotypes. There is abundant evidence in many systems that nutrition and hygiene during development influence an array of different phenotypic traits [45]. Other mechanisms, such as the transfer of hormones, enzymes and gut microbiota, and indirect genetic effects mediated through carers are only now being discovered and await future exploration [40,46].

Overall, the major result of our experiment is that the colony of origin of the carers affects the ability of cross-fostered workers to resist a fungal infection, whereas the colony of origin of the eggs shapes their baseline survival. The mechanism underlying this pattern may be parallel to mammalian transfers of antibodies through milk, which can be transmitted from the mother [47] or from another carer through allosuckling [48]. In insects, mothers can transfer to eggs hormones and other factors that may improve their offsprings' fitness and survival, as do birds, reptiles and fishes [49]. In addition, extended care of offspring beyond the egg stage allows for post-hatching transfers, either from the mother or from other social partners. In our experiment, the origin of the carers appears to be critically important to the pathogen resistance of the cross-fostered ants. Thus, social interactions with specific partners during development play a central role in shaping the pathogen resistance of naive offspring.

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