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Does genetic diversity hinder parasite evolution in social insect colonies?

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Abstract

Polyandry is often difficult to explain because benefits of the behaviour have proved elusive. In social insects, polyandry increases the genetic diversity of workers within a colony and this has been suggested to improve the resistance of the colony to disease. Here we examine the possible impact of host genetic diversity on parasite evolution by carrying out serial passages of a virulent fungal pathogen through leaf-cutting ant workers of known genotypes. Parasite virulence increased over the nine-generation span of the experiment while spore production decreased. The effect of host relatedness upon virulence appeared limited. However, parasites cycled through more genetically diverse hosts were more likely to go extinct during the experiment and parasites cycled through more genetically similar hosts had greater spore production. These results indicate that host genetic diversity may indeed hinder the ability of parasites to adapt while cycling within social insect colonies.

Introduction

Understanding the evolution of multiple mating by females (polyandry) is problematic because the behaviour is probably costly and yet benefits have often been hard to establish (Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000; Simmons, 2001). Most social insect species in the order Hymenoptera (ants, bees and wasps) are more or less monoandrous, but a number of derived genera have evolved high and obligate levels of polyandry (Boomsma & Ratnieks, 1996; Strassmann, 2001). These groups represent interesting cases because some potential material benefits of polyandry are unlikely to apply and because the behaviour is probably particularly costly for the females of most of the species. One leading hypothesis to explain polyandry in social insects is that it results in a more genetically diverse worker population and thereby improves the colony's resistance to parasites (Hamilton, 1987; Sherman *et al.*, 1988).

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There are a number of ways by which having a genetically diverse worker population may improve a colony's resistance to disease. The underlying assumption is that there is genetic variation for resistance to disease, which has been demonstrated in several social insect species (Baer & Schmid-Hempel, 2003; Palmer & Oldroyd, 2003; Hughes & Boomsma, 2004), and is well established in many other animals (Ebert *et al.*, 1998; Little & Ebert, 1999, 2000, 2001; Carius *et al.*, 2001). Variation in resistance may relate either to preventing an infection and/or to reducing within-host parasite growth. Several studies have produced evidence that directly support the hypothesis that genetically diverse colonies may be more resistant to disease (Baer & Schmid-Hempel, 1999, 2001; Hughes & Boomsma, 2004), or may be less prone to extreme rates of infection (Tarpy, 2003). However, to date, none have considered how genetically diverse worker populations impact upon the evolution of a parasite. Social insect colonies can contain thousands or even millions of individuals and can survive for many years. Most parasites, particularly the viruses, bacteria and fungi, have extremely short generation times. Social insect colonies therefore provide the potential, both in terms of number of host individuals and time, for parasites to pass many generations within the worker population. This carries with it the

potential for the parasite to evolve whilst cycling within the colony.

Parasites will normally encounter a range of host genotypes. Assuming these differ in their resistance then a particular parasite genotype will be more or less adapted to the different host genotypes. Consequently, parasite virulence (here defined as the negative effect upon host fitness and including both the probability of infection and the impact of a successful infection) will vary depending upon the specific interaction between host and parasite genotypes. Parasites will evolve towards optimal (for the parasite) levels of virulence for the host genotypes that they interact with. Obtaining optimal virulence will be constrained by having to interact with a range of different host genotypes with different resistance properties. If a parasite is presented with a host population of low genetic diversity, then it will be easier for it to adapt to the restricted range of host genotypes in the population. This generally results in the parasite evolving heightened virulence and transmission characters (Ebert & Hamilton, 1996). The classic example is the evolution of increased virulence in parasites that exploit the monocultures that characterize modern agriculture (Brown, 1994). Very low genetic diversity is something that also characterizes the colonies of most social insects and they therefore represent prime targets for parasites to exploit (Schmid-Hempel, 1998; Boomsma *et al.*, 2005). If colony populations are of higher genetic diversity then this will hinder the evolution of the parasite and may thus reduce the harm that the parasite causes to the colony over successive parasite generations.

Serial passage experiments provide excellent ways of examining how parasites adapt over successive generations. They involve parasites being transferred from one host to another, with the characters of the derived strain then being compared with those of the ancestral strain (Ebert, 2000). This most commonly results in an increase in parasite virulence, sometimes within only a few generations (Ebert, 1998, 2000). The increase in virulence has been suggested to be due to one or more of three reasons (Ebert, 1998, 2000). (1) That the hosts used in serial passage experiments are often genetically similar and therefore easier for the parasite to adapt to than the genetically diverse hosts found in most natural populations. (2) That parasite virulence is normally balanced by the cost of prematurely ending parasite growth by killing the host too quickly, and that this cost of high virulence does not apply to parasites in serial passage experiments because transmission is artificially ensured as part of the experiment. (3) That the artificial transmission that characterizes some serial passage experiments reduces the need for transmission stages and this allows parasites to divert more resources to within-host growth. In a passage experiment using the protozoan parasite *Crithidia bombi*, an increase in virulence was found to occur in colonies of the monoandrous bumblebee *Bombus terrestris* (Schmid-Hempel, 2001). After just three transfers between full-sibling host individuals, the post-selection parasite reduced host condition to a greater degree than did the ancestral strain.

We examined the impact of host genetic diversity on parasite evolution using serial passage experiments with the leaf-cutting ant host *Acromyrmex echinatior* Forel (Hymenoptera: Formicidae: Attini) and the fungal parasite *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) (Deuteromycotina: Hyphomycetes). *A. echinatior* is highly polyandrous, with queens typically mating with around ten males (Sumner *et al.*, 2004). *M. anisopliae* var. *anisopliae* is a virulent, generalist entomopathogen that infects leaf-cutting ants (Alves & Sosa-Gómez, 1983; Humber, 1992; Jaccoud *et al.*, 1999; Hughes *et al.*, 2002; Poulsen *et al.*, 2002; Hughes *et al.*, 2004a,b), as well as many other insects. It is a semelparous, 'obligate killer' (Ebert & Weisser, 1997), producing transmission propagules (spores) shortly after host death. Virulence is expressed during within-host vegetative growth by the direct invasion of host tissues by hyphae, the diversion of host resources to parasite growth and the production of compounds that inhibit the immune response but which are also toxic to the host (Boucias & Pendland, 1998). The within-host interaction of *Metarhizium* parasites is apparently impervious to relatedness, and, unlike certain other parasites (West & Buckling, 2003; Griffin *et al.*, 2004), is characterized by scramble competition rather than cooperation even when within-host relatedness is high (Hughes *et al.*, 2004b). Kermarrec *et al.* (1986) report that a strain of *M. anisopliae* had increased virulence after only 10 passages through leaf-cutting ants of unspecified relatedness.

We carried out two experiments. The first consisted of six treatments (Fig. 1). Four of these treatments (1a, b, c and d) involved serial passages of a single parasite strain through ants of different levels of genetic diversity. The two main predictions were that the parasite should evolve greater virulence over the course of the experiment, as seen in other serial passage experiments (Ebert, 1998, 2000), and that virulence and/or spore production at the end of the experiment should be negatively correlated with the genetic diversity of the host ants through which the parasite had been passaged. Parasites passaged through genetically similar host ants should thus be able to evolve to a higher virulence than those passaged through more genetically different host ants. The other two treatments in the first experiment (1e and f) were similar, but involved a mixture of two parasite strains (Fig. 1). The prediction here was that the greater diversity of parasites and the addition of within-host competition between parasite strains might stimulate a more rapid adaptation of the parasite than in the single strain treatments. The second experiment used a single parasite strain, but the serial passages involved groups of ants of either low or high diversity (Treatments 2a and b; Fig. 1). This experiment thus attempted to mimic more closely the natural situation in colonies. Competition will

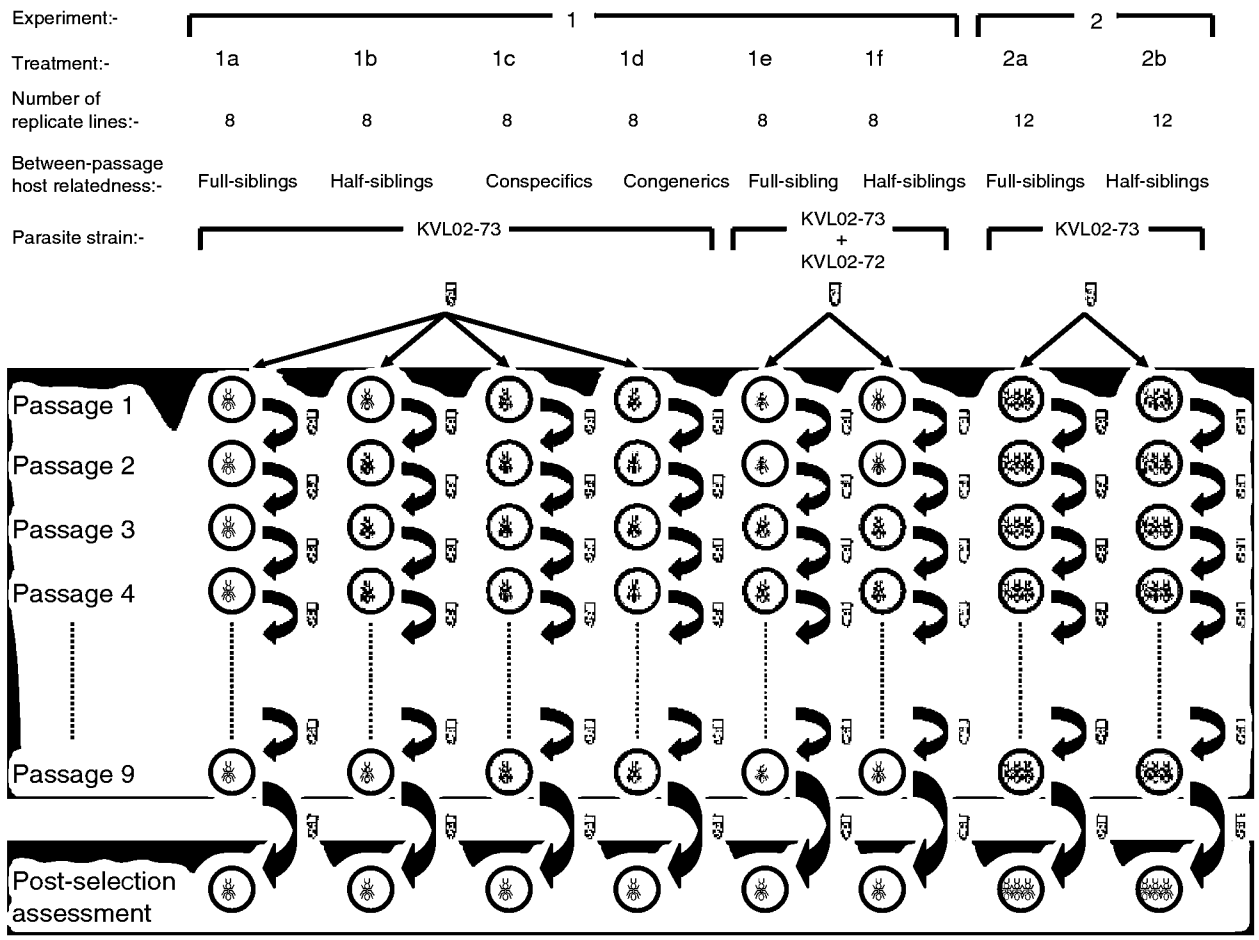

Fig. 1 Experimental design. Nine serial passages were carried out. This was followed by a post-selection assessment in which the derived strains resulting from the ninth passage were compared with the ancestral strain used to start the first passage. The study consisted of eight treatments differing either in the relatedness of hosts used in the different passages, the number of parasite strains used, or whether ants were maintained individually or in groups of three.

be more global in this second experiment and so the derived parasite strains may have lower virulence and greater spore production than the derived strains produced by the first experiment (Frank, 1998). The same logic will also apply within the second experiment. If the two treatments differ in the numbers of ants sporulating and thus being used for subsequent passages, then competition will be more global in the treatment with the greater number of ants sporulating.

Materials and methods

Serial passage treatments

The two experiments were carried out simultaneously. The first experiment used single ants maintained in isolation as hosts and consisted of six treatments (Fig. 1). For four treatments the ants were initially treated with a single strain of *M. anisopliae anisopliae* (KVL02-73) and serial passages of the parasite were then carried out through either: (1a) full-siblings, (1b) half-siblings, (1c) nonnestmate conspecifics or (1d) congenerics (*A. octospinosus* also collected from Gamboa, Panama). For the other two treatments the ants were initially treated with the mixed suspension of two strains of *M. anisopliae anisopliae* (KVL02-73 and KVL02-72) and serial passages of the parasite were then through either: (1e) full-siblings, or (1f) half-siblings (Fig. 1). In the second experiment ants were maintained in groups of three, which is sufficient for group-level defences to come into effect (Hughes *et al.*, 2002; Hughes & Boomsma, 2004). Groups were of either: (2a) high or (2b) low genetic diversity, being made up either of ants from three different patrilines or ants from the same patriline (Fig. 1). All of the ants in each group were treated with the suspension of KVL02-73. In the first experiment, two replicate lines were carried out for each of the four experimental colonies, giving a total of eight replicate

lines for each of the six treatments (Fig. 1). In the second experiment, three replicate lines were carried out for each experimental colony, giving a total of 12 replicate lines for each of the two treatments (Fig. 1).

Serial passage procedure

The experiments involved ants from four colonies of *A. echinatior* (Ae33, 48, 112 and 132), which had been collected from Gamboa, Panama and maintained in the laboratory (*ca.* 24 °C and 70% RH) on a diet of bramble leaves and rice grains. The two strains of *M. anisopliae* var. *anisopliae* that were used (KVL02-73 and KVL02-72) had been isolated from the same site in Panama as that from which the ant colonies were collected (Hughes *et al.*, 2004a) and are highly pathogenic to *A. echinatior* (Hughes *et al.*, 2002, 2004b). The isolates were cultured as monospore isolates on Sabouraud dextrose agar. Spore (conidia) suspensions of each isolate were made by flooding recently sporulating culture plates with 0.05% Triton-X solution and scraping off the spores into suspension with a glass rod. The viability of the spores was checked (Lacey & Brooks, 1997) and was >95%. The concentrations of spores in the suspensions were counted using a haemocytometer and both suspensions were diluted to a concentration of 1×10^7 spores mL^{-1}. A mixed suspension was also created containing a total concentration of 1×10^7 spores mL^{-1}, with an equal amount coming from each of the two isolates.

For each passage, 47 small workers (head width: 1.0–1.4 mm) were removed from each of the four colonies. All ants used were collected from the fungus gardens and were of approximately the same cuticular colouration in order to minimize variation in age between individuals. In order to be able to assign ants to treatments based on their genotypes, a single middle leg was carefully removed from each ant. This procedure does not affect the survival of the ants within the time span of the experiments, nor their susceptibility to *M. anisopliae* var. *anisopliae* applied 48 h or more afterwards (Hughes & Boomsma, 2004). DNA was extracted from the ant legs using Chelex beads and amplified at four polymorphic microsatellite loci: Ech1390, Ech3385, Ech4126 and Ech4225 (Ortius-Lechner *et al.*, 2000). The reactions were carried out in 10 μL volumes of 1 μL DNA, 0.5 × reaction buffer, 0.1 mM dNTPs, 0.5 U of Taq polymerase and 0.125, 0.175, 0.175 and 1.0 μM of the Ech1390, Ech3385, Ech4126 and Ech4225 primers, respectively. The DNA was amplified in Hybaid PCR Express Thermal Cyclers, with Ech1390 and Ech4126 being multiplexed and Ech3385 and Ech4225 being amplified individually. A touchdown temperature program was used for Ech1390, Ech3385 and Ech4126. This had an initial denaturing step of 94 °C for 4 min followed by two touchdown sequences of six cycles each (first sequence: 92 °C for 30 s, 65.0–64.0 °C decreasing at 0.2 °C per cycle, and 72 °C for 30 s; second sequence: 92 °C for 45 s, 55.0–52.5 °C decreasing at 0.5 °C per cycle, and 72 °C for 45 s). These were then followed by a sequence of 20 cycles in which the denaturing temperature was 92 °C for 45 s, 52 °C for 30 s, and 72 °C for 45 s. A final elongation step of 72 °C for 60 min completed the amplification process. The programme used to amplify Ech4225 had an initial denaturing step of 94 °C for 2 min, 30 cycles of 92, 52 °C and then 72 °C for 30 s each, and a final elongation step of 72 °C for 59 min. PCR products were run on 5% polyacrylamide gels with an ABI377 automatic sequencer. Allele sizes were scored by comparison with internal size markers and the genotypes of the colony queens and their multiple mates were inferred from the multi-locus offspring genotypes. This then allowed individual workers to be accurately assigned to particular patrilines within colonies.

The 47 ants that were genotyped per colony were assigned based on their patrilines to the different lines within each of the treatments. In Treatments 1a, e and 2a (Fig. 1), each replicate line was started with ants of a different patriline and maintained using ants of the same patriline. In the remaining treatments (Fig. 1), the different lines were started using ants from different patrilines and patrilines were then utilized in a random manner for each subsequent passage, ensuring always that the ants used were from a different patriline to that used in the previous passage (in Treatments 1b and f), or that at least two of the three ants in a group were from patrilines not used in the previous passage (in Treatment 2b).

Quantities of 0.5 μL of the required suspension of fungal spores were applied to the mesosomas of ants using a micropipette, and the ants then maintained in plastic pots (diameter: 2.5 cm; height: 4 cm) with an *ad libitum* supply of water and 10% sucrose water. Two ants were treated and maintained in isolation for each line in the first experiment, while three ants were treated and maintained as a group in the second experiment (Fig. 1). Mortality was monitored on a daily basis with dead ants being removed, surface sterilized (Lacey & Brooks, 1997), and placed in a petri dish with damp filter paper. Each passage lasted 12 days. At the end of this period, all ant cadavers were assessed for the presence of *M. anisopliae* var. *anisopliae* spores using a ranking method. Cadavers were examined under a binocular microscope and given a rank of between 0 (no spores visible) and 10 (surface of cadaver almost completely covered by spores). Following previous work (Hughes *et al.*, 2004b), these ranks were then used to estimate the true numbers of spores present on the cadavers. In the first experiment, a single sporulating cadaver in each replicate line was used to provide spores for the next passage. In the second experiment, all sporulating ants for each replicate line were used. In both cases, the cadavers were placed in eppendorf tubes with 1 mL of 0.05% Triton-X and vortexed for 1 min to remove the spores into suspension. The suspensions were then centrifuged for 2 min to

concentrate the spores into a plug at the bottom of the tubes. The volume of 0.05% Triton-X in the tubes was then adjusted as necessary, based upon the numbers of spores on the cadavers, in order that each suspension had an approximate concentration of 1×10^6 spores mL^{-1}. The spores were then vortexed thoroughly and 0.5 μL quantities of the resuspended spores were used to treat ants in the next passage.

The two experiments were thus begun with a total of 72 replicate lines of parasite, distributed amongst the eight treatments (Fig. 1). During the passages, three lines had to be discontinued due to there being insufficient ants of the necessary genotype available for infection. There were also 45 'extinction events', in which a line failed to produce any sporulating cadavers after a particular passage. In most treatments, when an extinction event occurred cadavers were utilized from another replicate line within the same treatment and with the most similar history. This was not possible for the three treatments that involved successive passages through the same single host genotype (1a, e and 2a). In these cases the lines were discontinued, except in two cases in Treatment 2a that occurred after the first passage and which were continued using cadavers of the required genotype from Treatment 1a. Thus, of the 72 replicate lines that began the experiments, eight had to be discontinued, two in Treatment 2a had only had eight passages through groups of ants (with the first passage having been through a single ant from Treatment 1a), and a further 24 replicate lines had to be restarted at some point using cadavers from a similar line.

Post-selection assessment of parasite virulence and spore production

Following the completion of nine serial passages, the virulence and spore production of the resulting derived parasite strains were assessed and compared with that of the ancestral strain. A spore suspension of each of the parasite lines was made up at 1×10^6 spores mL^{-1} as described above. A spore suspension of the ancestral KVL02-73 isolate was also made up at 1×10^6 spores mL^{-1}, as was a control solution of 0.05% Triton-X. One hundred and 88 ants from each of the four colonies were genotyped as before and then allocated to treatments based upon their patrilines. The treatments in Experiment 1 were assessed using single ants while those of Experiment 2 were assessed with groups of three ants. For the treatments that had involved passages through full-siblings (1a, e and 2a), the patrilines of ants used in the assessment were the same as had been used during the serial passages. The other treatments had involved passages through a variety of patrilines, and these treatments were assessed using the same patrilines as were used to assess the full-sibling treatments. This was in order to avoid any differences emerging in the assessment as a result of differences in resistance between patrilines. The representation of patrilines was therefore the same across the assessment of the Experiment 1 treatments, and also across the Experiment 2 treatments. Correspondingly, *A. echinatior* workers were used when assessing Treatment 1d and groups of three workers from the same patriline were used when assessing Treatment 2b. Twenty-four ants were treated and maintained in isolation for the assessment of each of the Experiment 1 treatments and 24 groups of three ants for the assessment of each of the Experiment 2 treatments. Due to the variable and restricted number of ants of specific patrilines that were available, different numbers of ants were used from different patrilines. However the number of ants from any particular patriline was matched across treatments. Ants were treated with 0.5 μL quantities of the derived or ancestral spore suspensions or of a 0.05% Triton-X control solution, and their survival was monitored for 14 days. The spore production from the cadavers was ranked 10 days after the end of this period.

Statistical analysis

We examined the occurrence of extinction events in the different treatments of the first experiment using a *G*-test for heterogeneity. This compared whether treatments differed in the same way from the overall occurrence of extinction events. The overall *G* value was also partitioned to give individual *G* values for each treatment that test whether the frequency of extinction events in a particular treatment differed significantly from that occurring overall. We used a standard *G*-test, adjusted using Williams' correction, to compare the occurrence of extinction events in the two treatments in the second experiment.

Four variables were recorded during the nine serial passages: the time of death for treated ants that died, the proportion of treated ants that died, the proportion of ant cadavers that *Metarhizium* sporulated from, and the spore production from any sporulating cadavers ranked as described above. These variables therefore encompassed both the probability of infection and the impact of within-host growth, as well as the efficiency with which the parasite converted host resources into transmission propagules. Repeated measures analyses of variance (RMANOVA) with passage as the repeated measure were used to examine whether any of these variables changed over the course of the passages or differed between the treatments. Values for each parasite line within each treatment were used as replicates, with the times of death and spore ranks of ants within a line being averaged to give a single value per line. Proportions were arcsign transformed prior to analysis. Extinction events (see below) resulted in missing values for one or more of the variables. RMANOVA requires all cells in the analysis to be filled and so missing values were imputed prior to

analysis by maximum likelihood estimation using the expectation maximization (EM) algorithm in SPSS 12.0.1 in order to avoid biasing the analysis (Hill, 1997; Fichman & Cummings, 2003; Strauss *et al.*, 2003). The degrees of freedom were then adjusted down to be conservative. Where Mauchly's assumption of sphericity was violated we used the conservative Greenhouse-Geisser correction (Field, 2000).

To test whether the survival of the ants in the different treatments differed in the post-selection assessment we used RMANOVAS with the parasite lines as replicates and with survival time (days after application) as the repeated measure. For the first experiment (in which single ants were treated), the analysis used the proportional survival of ants treated with each parasite line within each treatment. For the second experiment (in which groups of three ants were treated) the analysis used the proportional survival of ants within a group, averaged for each line. In both cases the analysis was carried out first using all treatments, and was then repeated with the control treatment being excluded. One-way ANOVA were used to test for differences between treatments in the spore production from cadavers using the parasite lines as replicates, with the values for each line being obtained by averaging the spore ranks of sporulating ants treated within that line. *Post hoc* pairwise comparisons of treatments were done using Tukey's HSD test.

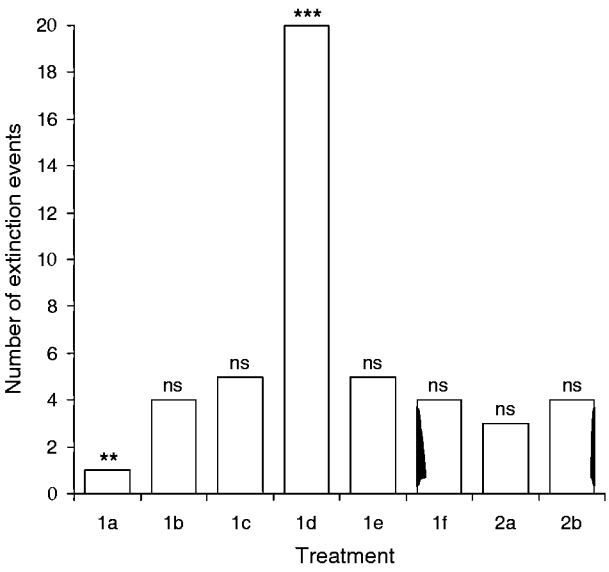

Fig. 2 Number of extinction events (a line failing to produce any sporulating cadavers in a particular passage) across treatments during the nine-passage course of the experiment. See Fig. 1 for details of treatments. The significance levels are shown for *G* tests comparing the frequency of extinction events in each treatment with the overall frequency (* $P < 0.01$, ** $P < 0.001$, n.s. = $p > 0.05$).

Results

Extinction events during the serial passages

In the first experiment, extinction events were rare in most treatments but were significantly less frequent in Treatment 1a and significantly more frequent in Treatment 1d ($G_{\text{Het},5} = 33.4$, d.f. = 5, $P < 0.001$; Fig. 2). In Treatment 1d, there were significant differences between passages in the occurrence of the extinction events (Fisher's exact test, $P < 0.05$). Half of the events occurred on passages 2 and 4 (6 and 4 events, respectively), three events on passage 8, and only one or two events during the other passages. Passages 2, 4 and the other even numbered passages were through the congeneric *A. octospinosus* workers. Significantly more extinction events thus occurred during passages through *A. octospinosus* than during those through *A. echinatior* ($G_{\text{adj}} = 9.66$, d.f. = 1, $P < 0.01$). The frequency of extinction events did not differ between the treatments in the second experiment ($G_{\text{adj}} = 0.138$, d.f. = 1, n.s.).

Changes in parasite traits during the serial passages

Four parasite traits were recorded over the course of the nine passages: time of host death, proportion of hosts dying, proportion of cadavers sporulating and spore production of sporulating cadavers. In the first experiment, both the time of death and the ranked spore production of sporulating cadavers decreased significantly over the course of the nine passages (time of death: $F_{5,191} = 18.2$, $P < 0.0001$; spore production: $F_{7,219} = 18.6$, $P < 0.0001$; Fig. 3). This decrease was similar in all of the treatments in this experiment (time of death: $F_{26,191} = 1.19$, n.s.; spore production: $F_{32,219} = 0.861$, n.s.). The main effect of treatment was significant for time of death, with ants in Treatment 1d taking slightly longer to die (time of death: $F_{5,42} = 7.52$, $P < 0.0001$; Fig. 3). The spore production of cadavers did not differ significantly between treatments overall, although it did appear somewhat lower in Treatment 1d (spore rank: $F_{5,42} = 0.859$, n.s.; Fig. 3). Both the proportion mortality and the proportions of cadavers sporulating also changed significantly over the passages, but here the changes differed between treatments (proportion mortality: $F_{25,210} = 1.91$, $P < 0.01$; proportion sporulating: $F_{30,224} = 1.8$, $P < 0.01$). No overall pattern is discernible, but in both cases Treatment 1d was associated with the lowest proportions, particularly on passages 2 and 4 (Fig. 3).

In the second experiment there was no effect of treatment on the way any of the traits changed over the course of the passages (time of death: $F_{5,115} = 0.417$, n.s.; proportion mortality: $F_{4,87} = 0.511$, n.s.; proportion sporulating: $F_{5,98} = 0.327$, n.s.; spore rank of cadavers: $F_{4,80} = 0.458$, n.s.). There were also no overall differences between the treatments (time of death: $F_{1,22} = 1.5$, n.s.; proportion mortality: $F_{1,22} = 0.029$, n.s.; proportion

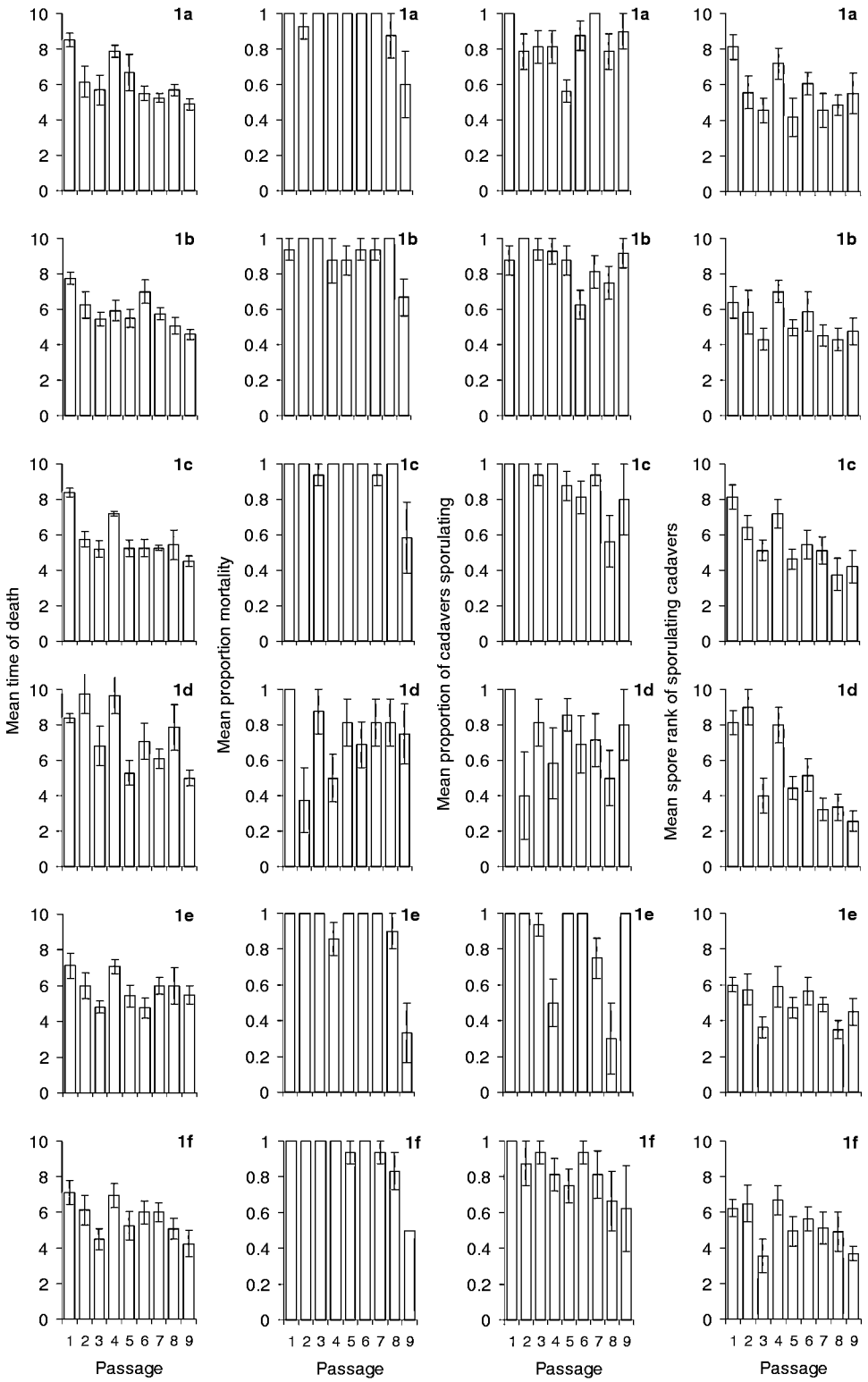

Fig. 3 Differences between treatments in the first experiment (passaging through single ants) during the nine passage course of the experiment in, from left to right, mean (±SE) time of death of individuals that died, mean (±SE) proportion mortality of individuals in a line, mean (±SE) proportion of cadavers in a line that sporulated and mean (±SE) rank of spores produced by sporulating cadavers in a line. See Fig. 1 for details of treatments.

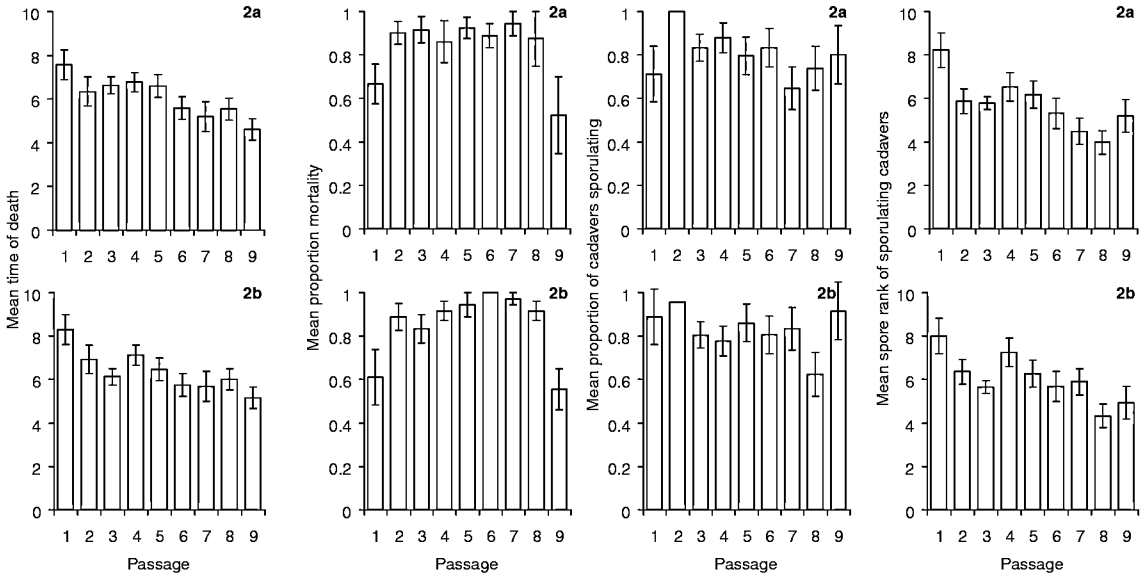

Fig. 4 Differences between treatments in the second experiment (passaging through groups of three ants) during the nine passage course of the experiment in, from left to right, mean (±SE) time of death of individuals that died, mean (±SE) proportion mortality of individuals in a line, mean (±SE) proportion of cadavers in a line that sporulated and mean (±SE) rank of spores produced by sporulating cadavers in a line. See Fig. 1 for details of treatments.

sporulating: $F_{1,22} = 0.005$, n.s.; spore rank of cadavers: $F_{1,2} = 2.99$, $P = 0.098$). However, as in the first experiment both time of death and the spore rank of sporulating cadavers decreased significantly over the course of the nine passages (time of death: $F_{5,115} = 9.85$, $P < 0.0001$; spore rank: $F_{5,80} = 5.96$, $P = 0.0005$; Fig. 4). The proportion mortality and the proportion of cadavers sporulating also changed significantly over the passages but not in a consistent manner (proportion mortality: $F_{4,87} = 10.4$, $P < 0.0001$; proportion sporulating: $F_{5,98} = 3.72$, $P < 0.01$; Fig. 4).

Post-selection assessment of virulence and spore production

In both experiments the survival of ants was significantly lower when treated with the derived or ancestral strains of *Metarhizium* than with the control solution (first experiment: $F_{21,159} = 2.37$, $P < 0.01$, Fig. 5a; second experiment: $F_{6,128} = 7.37$, $P < 0.0001$, Fig. 5b). In the first experiment, the survival of ants exposed to the ancestral strain or to the derived strains of Treatment 1d was approximately double that of ants exposed to some of the other derived strains (overall effect size after 14 days $f = 0.28$ (Cohen, 1988); Fig. 5a), while in the second experiment the survival of ants treated with the derived strains was also lower than of those treated with the ancestral strain ($f = 0.22$; Fig. 5b). However, in neither experiment were these differences significant (first experiment: $F_{18,137} = 1.04$, n.s.; second experiment: $F_{4,80} = 0.409$, n.s.).

For the first experiment, the cadavers of ants treated with either the ancestral strain or the strains derived from Treatment 1a produced significantly more spores than the cadavers of ants treated with the strains derived from Treatments 1c and d ($F_{6,39} = 4.06$, $P < 0.01$; $f = 0.77$; Fig. 6a). The mean spore ranks also differed between the derived strains alone ($F_{5,31} = 5.122$, $P < 0.01$). While the difference in spore production between Treatment 1a and b was not significant, the effect size was still very large ($f = 0.62$). These differences were not due to individuals in the different treatments having had different lengths of time to produce spores because there was no relationship between spore production and the length of time that had passed between death and the assessment of spore production ($F_{1,37} = 0.0001$, n.s.). In the second experiment, spore production did not differ between ants treated with the ancestral strain and with the derived strains ($F_{2,24} = 0.144$, n.s.) (Fig. 6b).

Discussion

Parasite evolution over the serial passages

The first prediction of the experiment was that parasite virulence would be increased by the serial passages, as is observed in most such studies (Kotiw *et al.*, 1995; Ebert, 1998,2000; Schmid-Hempel, 2001; Cooper *et al.*, 2002; McClelland *et al.*, 2004). In both experiments, the time to host death decreased significantly in all treatments during the nine passages. Although no statistically

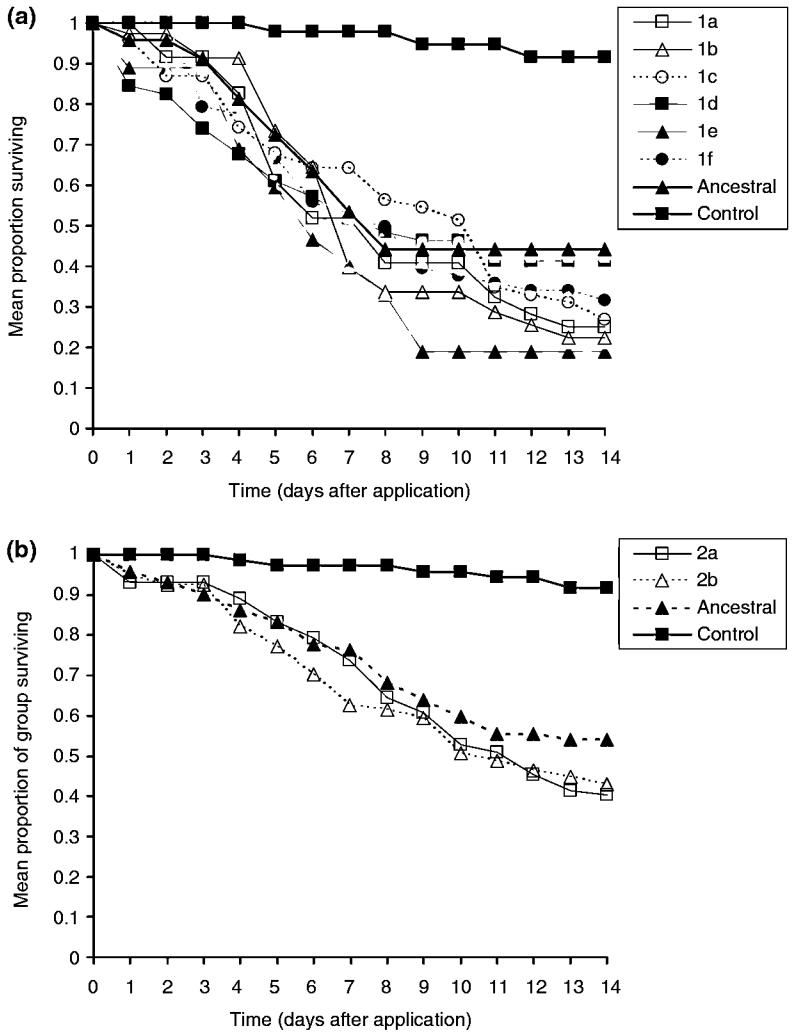

Fig. 5 Virulence of derived and ancestral strains of the *Metarhizium* parasite in the post-selection assessment, as measured by host ant survival, for (a) Experiment 1, which used single ants, and (b) Experiment 2, which used groups of three ants. All ants were treated with 0.5 μL of a 1×10^6 spores mL^{-1} suspension or with a control solution of 0.05% Triton-X. See Fig. 1 for details of treatments. Error bars removed for clarity.

significant differences in virulence were detected between the derived and ancestral strains in the post-selection assessment, in both experiments the virulence of the derived strains was greater than that of the ancestral strain and the effect size was moderate. It therefore appears that parasite virulence did increase, but that the increase was only moderate and the post-selection assessment lacked the statistical power to detect it. The lack of a greater change in virulence may represent a genuine lack of adaptation, possibly because the virulence of the *Metarhizium* strains was already near the maximum attainable, or could be due to only nine passages having been carried out. While *Metarhizium* and other entomopathogenic fungi have been reported to show changes in virulence and spore production within approximately this number of passages (Schaerffenberg, 1964; Kermarrec *et al.*, 1986; Hayden *et al.*, 1992; Shapiro-Ilan *et al.*, 2002; Quesada-Moraga & Vey, 2003; Vandenberg & Cantone, 2004), it is fewer than used in most serial passage experiments (Ebert, 1998, 2000).

The spore production of cadavers also decreased over the course of the nine passages in all treatments and in the post-selection assessment most of the derived strains from the first experiment had reduced spore production compared to the ancestral strain. Similar reductions in the production of transmission stages have been seen in other serial passage experiments (Kotiw *et al.*, 1995; Cooper *et al.*, 2002; Lázaro *et al.*, 2003). In some cases this may be due to a bottleneck effect caused by a limited number of transmission stages being used in each passage. However the number used in this study (approximately 500 spores per host individual) is more than often used in serial passage experiments (Ebert, 1998, 2000) and was probably sufficient to prevent this. There are two more likely explanations for the decrease in spore production associated with the passages. First, that the selection pressure on the parasites to produce a maximal number of spores was relaxed because controlled doses of spores were used during the passages. They might thus have been selected to divert resources

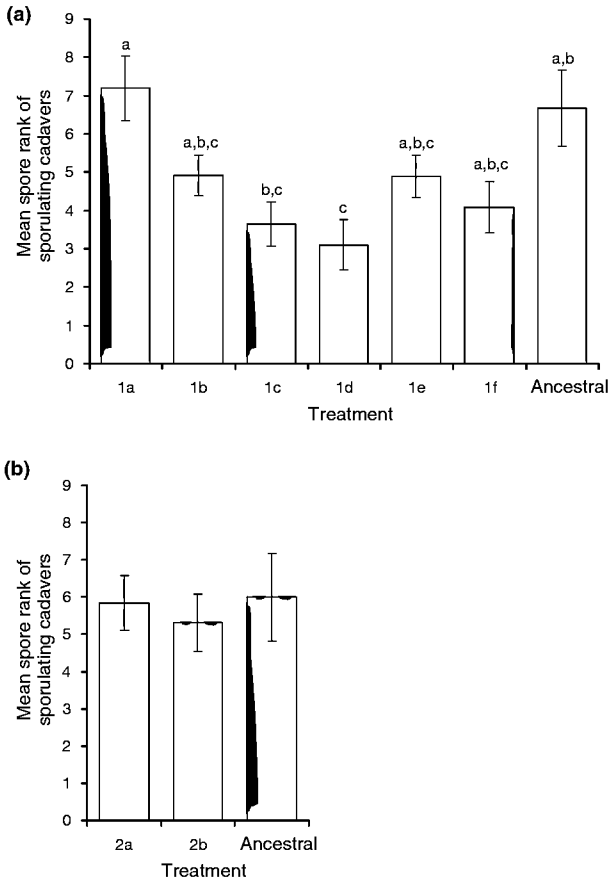

Fig. 6 Spore production of derived and ancestral strains of the *Metarhizium* parasite in the post-selection assessment for (a) Experiment 1, which used single ants and (b) Experiment 2, which used groups of three ants. See Fig. 1 for details of treatments. In (a), different letters signify pairwise treatment comparisons that differed significantly at $P < 0.05$ using Tukey's HSD test.

from spore production into other traits. Second, that the short duration of the passages forced the parasite to switch from vegetative within-host growth to spore production earlier than allowed an optimum production of spores. Each passage was restricted to 12 days. This is less time than these strains of *Metarhizium* sometimes take to kill and sporulate from *Acromyrmex* ants at the dosage used, and certainly less than required for maximum sporulation (Hughes *et al.*, 2002, 2004b). It seems likely that these two selection pressures combined to cause the decreased spore production of the derived strains. Both may be realistic. Social insects, including leaf-cutting ants, are extremely fastidious about removing dead or dying individuals from the colony (Schmid-Hempel, 1998; Boomsma *et al.*, 2005). A rapid sporulation time is thus virtually a prerequisite for a parasite such as *Metarhizium* to survive in a social insect colony.

Parasite diversity and the scale of competition

There appeared to be little or no effect of parasite diversity on virulence, but spore production was somewhat lower in the mixed parasite treatments (1e and f) than in the equivalent single strain treatments (1a and b) in the post-selection assessment. Although no formal comparison of the two experiments was possible, there was no evidence of the strains in the second experiment (where competition was more global) being less virulent or producing more spores than the strains in the first experiment (where competition was more local), nor of virulence or spore production differing between Treatments 2a and b. The lack of evidence for effects of parasite diversity or the scale of competition on the evolution of the *Metarhizium* strains may indicate that the within-host dynamics of *Metarhizium* are relatively impervious to relatedness, as has been suggested before (Hughes *et al.*, 2004b), or that the effects were too subtle to be detected with the experimental design used.

Effect of host genetic diversity on parasite evolution

Several aspects of the results indicate that the genetic diversity of hosts through which the parasite was passaged did impact upon parasite fitness. Treatments differed in virulence during the serial passages and, although not significant, the effect size in the post-selection assessment was moderate. Both during the passages and in the post-selection assessment, parasites passaged through the most genetically dissimilar hosts appeared to have the lowest virulence. The effect of treatment on spore production in the post-selection assessment was stronger, with strains passaged through full-sibling hosts having greater spore production than the other strains. In addition, extinction events were rarest when the parasite was passaged through full-sibling hosts and were far more common when the parasite was passaged between hosts of different species.

The effect of host genetic diversity was clearest between the most extreme treatments (full-siblings and congenerics; Treatments 1a and d), being exemplified by the relatively high frequency of extinction events in Treatment 1d. The initial passages through the ants of the congeneric host *A. octospinosus* resulted in the parasite taking longer to kill the host, and, at least partly as a consequence, being less likely to kill it and to sporulate within the 12 day time limit of the passage. The difference was sufficient to result in a major increase in the frequency of extinction events. In addition to the clear differences relating to the congeneric treatment, extinction events were particularly rare in Treatment 1a, the greatest reduction in spore production in the post-selection assessment occurred between the full-sibling and half-sibling treatments (1a and b) and the effect size of this difference was large. Thus even the half-sibling level of host diversity appeared to have a negative effect on parasite fitness.

Transmitting between more genetically similar hosts therefore benefited the evolution of the parasite over this nine-generation experiment. Host genetic diversity is generally predicted to hinder parasite adaptation (Brown, 1994; Ebert & Hamilton, 1996; Ebert, 1998, 2000; but see McClelland *et al.*, 2004) and the current results provide further support for this. While the effect was clearest between full-siblings and congenerics, it was also large between full-siblings and half-siblings which is the level of genetic diversity that results from polyandry. It therefore seems likely that the increased genetic diversity of workers that polyandry produces may inhibit the adaptation of a parasite within the colony and thus improve the probability of colony survival and reproduction. Although it has been suggested that genetic diversity may increase the range of parasites that can infect a colony (Boomsma & Ratnieks, 1996; van Baalen & Beekman, unpublished data), previous work has shown that polyandry can benefit social insect colonies because high genetic diversity colonies may be more resistant to disease or may express a less extreme resistance phenotype (Baer & Schmid-Hempel, 1999, 2001; Tarpy, 2003; Hughes & Boomsma, 2004). Parasite evolution has previously been little considered and the current study provides some evidence to suggest that high genetic diversity may also make colonies harder for parasites to adapt to. Although the experiment involved relatively few generations and was limited to four colonies, it did provide interesting insights. Serial passage experiments have proved powerful tools for investigating the evolution of parasites (Ebert, 1998, 2000) and further work should utilize them to investigate the impact of genetic diversity in social insect colonies on parasite evolution.

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