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Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth

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Multiple mating by social insect queens increases the genetic diversity among colony members, thereby reducing intracolony relatedness and lowering the potential inclusive fitness gains of altruistic workers. Increased genetic diversity may be adaptive, however, by reducing the prevalence of disease within a nest. Honeybees, whose queens have the highest levels of multiple mating among social insects, were investigated to determine whether genetic variation helps to prevent chronic infections. I instrumentally inseminated honeybee queens with semen that was either genetically similar (from one male) or genetically diverse (from multiple males), and then inoculated their colonies with spores of *Ascosphaera apis*, a fungal pathogen that kills developing brood. I show that genetically diverse colonies had a lower variance in disease prevalence than genetically similar colonies, which suggests that genetic diversity may benefit colonies by preventing severe infections.

Keywords: disease; genetic diversity; honeybees; polyandry

1. INTRODUCTION

Many females mate with more than one male (polyandry) (Arnqvist & Nilsson 2000), despite the apparent costs of time and predation, because they may benefit by having genetically diverse offspring (Yasui 1998; Jennions & Petrie 2000; Moller & Jennions 2001). Females that mate with many males are rare among social insects (Strassmann 2001), presumably because the female offspring of queens have greater inclusive fitness when they are genetically similar (Hamilton 1964; Crozier & Pamilo 1996; Peters *et al.* 1999; Griffin & West 2002). Nevertheless, increased genetic diversity has been shown to be adaptive in several highly eusocial taxa (Baer & Schmid-Hempel 1999; Cole & Wiernasz 1999; Rosengaus & Traniello 2001). Although many theories have been proposed to explain why polyandry has evolved in social insects (Keller & Reeve 1994; Palmer & Oldroyd 2000; Crozier & Fjerdingstad 2001), evidence to support them has been difficult to obtain.

One proposal for the evolution of polyandry in social insects is that increased genetic diversity lowers the variation in disease prevalence and mortality among members of a colony (Hamilton 1987; Sherman *et al.* 1988, 1998; Schmid-Hempel 1994, 1998; Schmid-Hempel & Crozier 1999). The fathers of the workers, a queen's mates, carry different genes that vary in their resistance to a particular disease, ranging from highly tolerant to highly susceptible. A queen that mates once produces genetically similar workers that all carry the same genes from their father. If, by chance, his genes are highly susceptible to a particular infectious agent, then all of the workers have a high chance of becoming infected and the colony would be impacted severely. A queen that mates multiply, however, produces genetically diverse workers that carry different genes from their respective fathers. By doing this, a queen minimizes the risk that *all* of her worker offspring will be sired by males that carry highly susceptible genes, increasing the probability that the colony, as a whole, will survive. Thus, polyandry yields benefits by reducing the variance in disease prevalence among colonies, not necessarily the average proportion of infected individuals (Sherman *et al.* 1988; Schmid-Hempel & Crozier 1999).

In bumble-bees, Baer & Schmid-Hempel (1999) found that high-diversity colonies, relative to low-diversity colonies, had a lower average disease load and twice the sexual productivity. Baer & Schmid-Hempel (2001) found that the intensity and prevalence of the parasite *Crithidia bombi* decreased with increased colony genetic diversity. *Bombus terrestris* L. queens, however, typically mate only once, evidently because males have evolved a highly efficient mating plug that prohibits additional matings by a queen (Duvoisin *et al.* 1999; Baer *et al.* 2001; Sauter *et al.* 2001). By contrast, honeybees (*Apis mellifera* L.), whose queens mate with an average of 12 males (Tarpy & Nielsen 2002), have among the highest levels of polyandry in all of the social insects (Strassmann 2001). Honeybees are an excellent system in which to investigate the benefits of polyandry because they are hosts to numerous parasites and pathogens (Schmid-Hempel 1998) and can be inseminated instrumentally to vary genetic diversity for experimental purposes (Laidlaw 1977).

Using this technique, I established two groups of honeybee colonies headed by sister queens. To create colonies consisting of genetically similar workers, I instrumentally inseminated queens each with the semen from a single male (drone); each queen's drone came from a different colony. To create colonies consisting of genetically diverse workers, I instrumentally inseminated queens with one drone's-worth of pooled semen from different males, one from each of the same colonies used to inseminate the first group of queens. This mating design, therefore, provides a highly controlled system in which to test the benefits of polyandry.

2. MATERIAL AND METHODS

(a) *Inseminations*

All of the experimental queens were daughters of a singly inseminated queen, and thus were 'super sisters' (full sisters) to each other (coefficient of relatedness, $G = 0.75$). I chose 24 colonies as the male (drone) sources, all of which were unrelated to the maternal source and to each other. I obtained sexually mature drones by capturing them at their hive entrances as they returned from unsuccessful mating flights. I instrumentally inseminated (Laidlaw 1977) each of 24 queens with *ca*. 1.0 µl of semen from a single drone from a different source to create a group of queens with genetically similar worker offspring ($G = 0.75$). I then collected the semen from a second drone from each source and mixed their semen loads. I inseminated another 24 queens with 1.0 µl aliquots of the pooled semen to create a group of queens with genetically diverse worker offspring ($G \approx 0.274$). Thus, the sources of genetic variation were the same for both groups, but this variation was primarily *among* colonies in the genetically similar group whereas it was primarily *within* colonies in the genetically diverse group.

(b) *Colony establishment*

I introduced the inseminated queens to separate colonies that were free of disease, similar in their worker populations, and standardized for their quantities of brood and food stores. I blindly placed the colonies within two isolated apiaries to minimize their competition for forage and provided the colonies with standard disease preventions for the parasitic mite *Varroa destructor* and bacterial infections. At night, I recorded the initial hive weights to the nearest 0.1 kg using a platform scale. As the colonies increased their populations, I provided additional hive bodies with frames of wax foundation to permit colony expansion.

(c) *Data collection*

Besides manipulating genetic diversity, I measured two other heritable traits that influence the phenotypes of honeybee colonies. First, I measured the viability of worker brood in each colony because it is an important factor in colony growth and survival (Woyke 1980; Tarpy & Page 2001). Brood viability is largely a consequence of the removal of inviable, diploid male larvae as a result of the honeybee's single locus sex determination system (Cook & Crozier 1995) and may explain why honeybee queens mate multiply (Page 1980). I estimated the viability of worker brood within each colony by counting the number of occupied versus unoccupied brood cells in five latitudinal transects of contiguous capped brood. Note that this measure is not brood viability as a consequence of the sex locus *per se*, rather it is a measure of 'effective' brood viability (Woyke 1984).

Second, I measured the level of hygienic behaviour (uncapping brood cells and removing dead brood) in each colony because it is a well-known mechanism of resistance to brood diseases (Rothenbuhler 1964; Spivak & Downey 1998). I estimated the percent hygienic behaviour of each colony using the pierced brood assay (Spivak & Downey 1998) of up to 70 capped brood cells. The proportion of these cells that were uncapped and empty after 24 h was my measure of percentage hygienic behaviour. I performed this assay only after most colony members, particularly the middle-aged bees that perform this behaviour, were offspring of the experimental queens. Although these two behaviours are independent from each other (i.e. hygienic bees are not necessary for diploid male removal), they are both likely to be affected by changes in intracolony genetic diversity.

I permitted the queens to oviposit for six weeks after they were introduced to their colonies so that most of each colony's members were offspring of the experimental queens. I then inoculated the colonies with spores of *Ascosphaera apis*, the fungal pathogen that causes chalkbrood disease, which I obtained from a single colony. I ground up the spores, added them to a mixture of pollen and 40% sucrose solution, and fed each colony 170 g of the inoculum (Gilliam *et al.* 1988). On the 5th and 15th days after inoculation, I counted the number of chalkbrood 'mummies', the unequivocal sign of a diseased individual, within the brood combs of each colony. I also estimated the total brood nest area of each colony (± 86 cm^2) so that I could calculate a standard measure of disease prevalence. I re-inoculated the colonies three weeks after the first inoculation using spores that I obtained from a different diseased colony, and I estimated the prevalence of disease following the same procedure.

I euthanazed the colonies on the 15th week of the experiment to obtain precise population measurements. I weighed all adult nest-mates to the nearest 0.028 kg, then a sub-sample of 100 workers to the nearest 0.01 g, and estimated the number of individuals within each colony. I also determined the final weight of each colony's hive, taking into account any equipment added during the experiment, to estimate the change in colony mass over the course of the 15-week experiment. Several colonies were removed throughout the experiment, either because the queens were not laying eggs within one week of their inseminations, were superseded or accidentally killed during the experiment, or had depleted their sperm stores before the end of the summer. Thus, the final sample sizes were 18 in the genetically similar group and eight in the genetically diverse group.

(d) *Analysis*

I transformed the average number of mummies per brood frame over the four measurements by natural $\log(x + 1)$ so that the data were distributed normally (Sokal & Rohlf 1995). Similarly, the percent brood viability and hygienic behaviour were $\arcsin(x)$ transformed. I then analysed the data with forward stepwise-regression models, using apiary, initial colony population, percentage brood viability and percentage hygienic behaviour as the independent variables (Sokal & Rohlf 1995). Any variable that was significant at the $\alpha = 0.10$ level was included in a multiple-regression analysis from which I generated the residuals. I then performed a Bartlett's test for unequal variances to determine a difference in variance between the two treatments (Sokal & Rohlf 1995). To compare the means of the two groups, I performed a simple *t*-test for those variables with equal variances and a Welch's ANOVA for those with unequal variances. All statistics are reported with one-tailed probabilities, unless otherwise noted, since there is a directional expectation towards genetically diverse colonies.

3. RESULTS

As expected, genetic diversity had a significant effect on brood viability and hygienic behaviour. The mean brood viability was the same for the two treatments (Welch's ANOVA, $F_{1,28} = 0.08$, $p = 0.392$), but the variances were significantly different (Bartlett's test of unequal variances, $p = 0.019$; figure 1*a*). There was also a significant difference in the variance in percentage hygienic behaviour

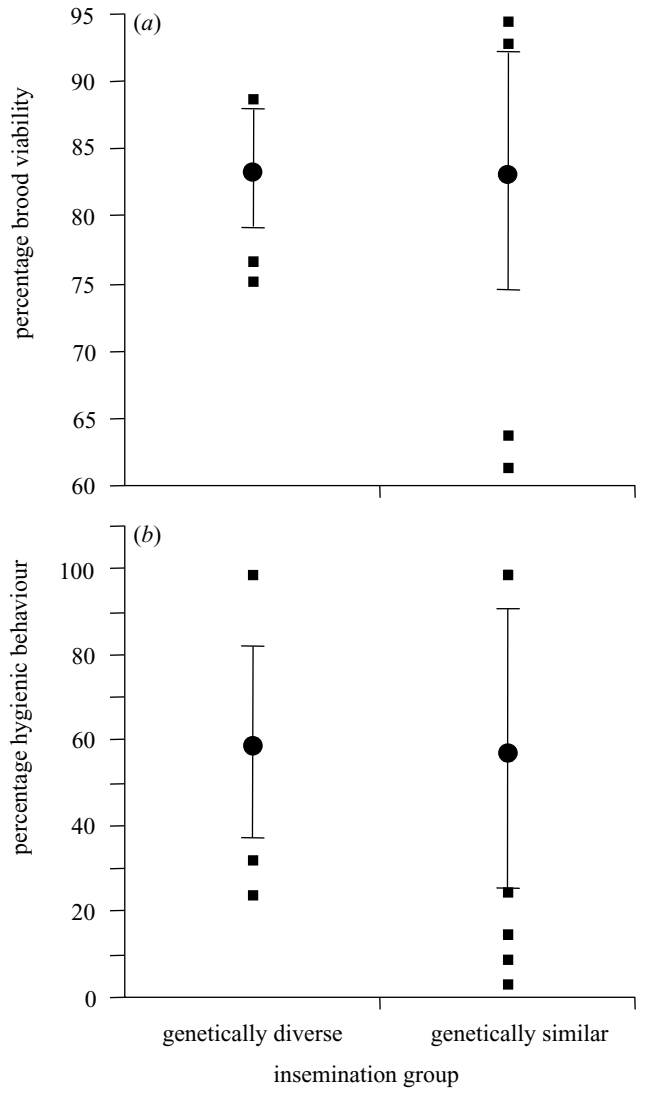

Figure 1. The effects of genetic diversity on brood viability and hygienic behaviour. As expected, increased genetic diversity as a result of polyandry significantly reduced the variances in the proportion of non-viable brood (*a*) and the percent hygienic behaviour (*b*). The axes are given as percentages, but the analyses used arcsine-transformed values. The mean ± s.d. is given for each group, and the data points greater than one s.d. are provided for a sense of range in the measures.

between the two groups, but not their means ($F_{1,24} = 1.39$, $p = 0.125$, Bartlett's test, $p = 0.009$; figure 1*b*). Moreover, hygienic behaviour was associated with a greater disease recovery (calculated as the change in disease prevalence between the two measurements) in diseased colonies for the first inoculation ($r^2 = 0.400$, two-tailed $p = 0.004$) but not for the second inoculation ($r^2 < 0.001$, two-tailed $p = 0.998$).

As the parasite and pathogen model predicts (Sherman *et al.* 1988; Schmid-Hempel & Crozier 1999), the variation in disease prevalence was much lower in the genetically diverse group than in the genetically similar group ($F_{1,24} = 2.21$, $p = 0.077$, Bartlett's test, $p < 0.0001$; figure 2*a*) even with brood viability taken into account statistically ($F_{1,24} = 1.92$, $p = 0.090$, Bartlett's test, $p = 0.002$; see § 2). This result demonstrates how raising a colony's genetic diversity can lower the probability of a large proportion of a colony contracting an infection. There was no significant difference in the change in colony mass between the two groups (figure 2*b*), even with a significant effect of apiary ($t_{24} = 0.44$, $p = 0.332$, Bartlett's test, $p = 0.204$). Finally, the two groups had statistically equivalent average colony populations at the end of the summer

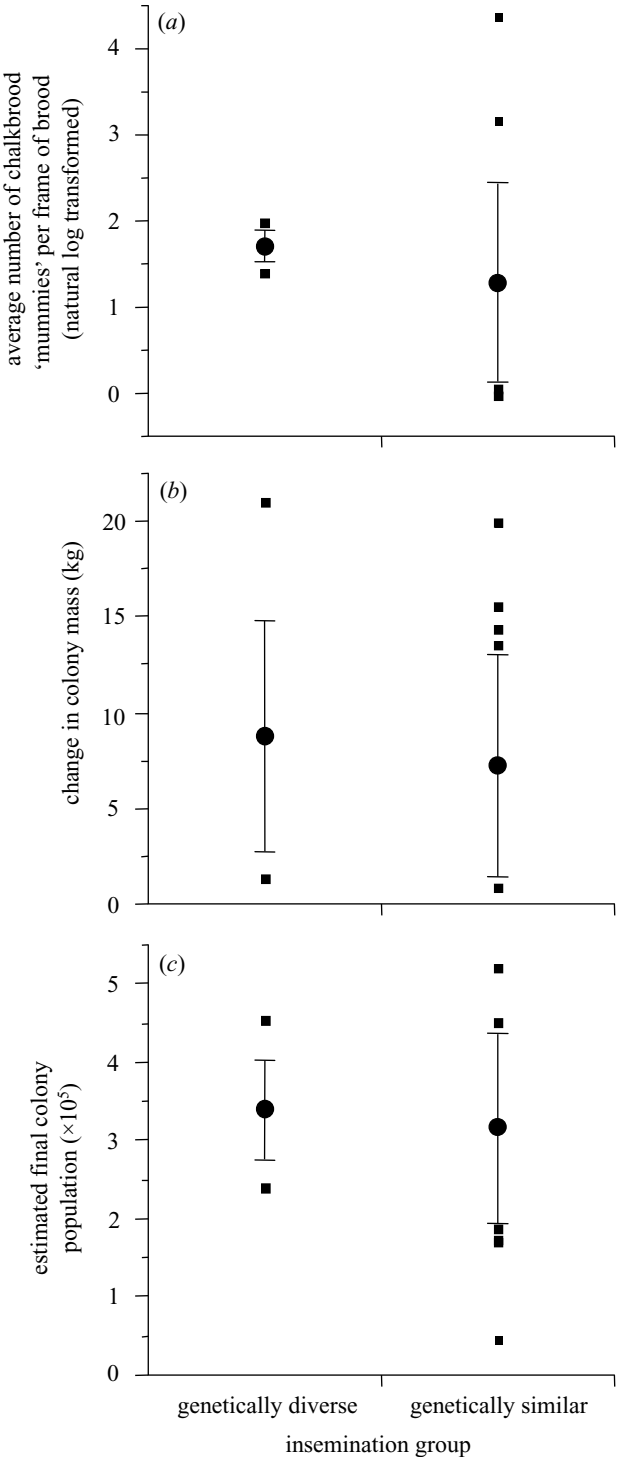

Figure 2. The effects of genetic diversity on disease prevalence and colony fitness measures. Increased genetic diversity significantly decreased the variance in the proportion of infected individuals within the colonies (*a*). The variance in colony mass was not significantly affected by genetic diversity (*b*), but it was for final colony population (*c*). Data points are explained in figure 1.

($F_{1,24} = 0.34$, $p = 0.282$), but had significantly different variances (Bartlett's test, $p = 0.038$; figure 2*c*).

Some of the observed treatment effect on colony population (see figure 2*c*) can be attributed to the viability of worker brood. Brood viability had a significant effect on final colony population ($r^2 = 0.284$, two-tailed $p = 0.006$) and is affected by increased genetic diversity as a result of multiple mating (see above). But even when the effects of brood viability and initial population are taken into account statistically (see § 2), the effect of genetic diversity on the variance in worker population is still strong, albeit not significant statistically ($t_{23} = 0.26$, $p = 0.400$, Bartlett's test, $p = 0.054$). However, chalkbrood is a benign pathogen relative to most other brood diseases (Schmid-Hempel 1998). Indeed, the highest infection rate of these colonies was 151 infected individuals per frame of brood, or *ca*. 2.3% of the brood, which may have had a minimal impact on colony population. Nevertheless, the relationship between disease prevalence and population suggests that the chalkbrood infections had a negative cumulative effect on colony population ($r^2 = 0.174$, two-tailed $p = 0.038$).

4. DISCUSSION

Polyandry may be considered a 'bet-hedging' reproductive strategy (Yasui 2001; but see Hopper 1999), because females can effectively reduce the error in assessing mate genetic quality by mating with multiple males. Such strategies are based on the principle of reducing the variance in fitness over time (Gillespie 1976; Real 1980; Hopper 1999) and this study demonstrates that polyandry lowers the variance in several important parameters that impact colony fitness in honeybees. Rather than providing an increase in average population or a decrease in total disease prevalence, polyandry appears to reduce the variance in these measures. Hence, polyandry may prevail in a population because monandrous queens produce colonies that are more likely to fail and thus are selectively disfavoured.

Reducing the variance among colony phenotypes is a common principle of the 'genetic diversity' hypotheses for the evolution of polyandry in social insects (see Palmer & Oldroyd 2000 for review; Crozier & Fjerdingstad 2001). Increased genetic diversity affects the division of labour within colonies (Beshers & Fewell 2001) by creating a worker force that is collectively more 'average' (Page *et al.* 1995). This effect on worker tasks is particularly pronounced for behaviours that are strongly influenced by genotype and have a significant impact on colony phenotype, such as hygienic behaviour (Rothenbuhler 1964; Spivak & Downey 1998). Increased genetic diversity also lowers the variance in brood viability as a consequence of homozygosity at the sex locus (Cook & Crozier 1995; this study), thus reducing the chance that a queen produces an excess number of non-viable, diploid males (Page 1980; Tarpy & Page 2001). Low brood viability negatively impacts several colony variables, such as population and food stores (Woyke 1980, 1981; Tarpy & Page 2002) and recently has been shown to lower colony winter survival (Tarpy & Page 2002).

There is genotypic variation in the resistance to many, if not most, of the diseases that infect honeybee colonies. Different genotypes have been shown to be differentially infected with chalkbrood (Gilliam *et al.* 1988; this study), the parasitic mites *Varroa destructor* (Guzman *et al.* 1996; Harbo & Hoopingarner 1997) and *Acarapis woodi* (Gary & Page 1987; Danka & Villa 1996; Guzman *et al.* 1998), *Nosema apis* (Woyciechowski *et al.* 1994), and American foulbrood (Rothenbuhler & Thompson 1956; Bamrick 1964). This study demonstrates that multiple mating by a queen can minimize the probability that her colony contracts a severe chalkbrood infection, which in turn promotes colony growth and, presumably, increases fitness. It is plausible that the same principle holds for other honeybee diseases, making parasites and pathogens significant selective agents for increased intracolony genetic diversity.

It is unclear whether polyandry evolved in honeybees in response to parasites and pathogens, or if reducing the variance in disease prevalence is an inevitable consequence of multiple mating. It is clear, however, that increased genetic diversity within colonies provides them with several benefits, and thus should be viewed as a trait with pluralistic consequences. Future work should determine the impact of other parasites and pathogens and the relative fitness benefits of these multiple mechanisms.

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