

# Queen promiscuity lowers disease within honeybee colonies

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Most species of social insects have singly mated queens, but in some species each queen mates with numerous males to create a colony with a genetically diverse worker force. The adaptive significance of polyandry by social insect queens remains an evolutionary puzzle. Using the honeybee (*Apis mellifera*), we tested the hypothesis that polyandry improves a colony's resistance to disease. We established colonies headed by queens that had been artificially inseminated by either one or 10 drones. Later, we inoculated these colonies with spores of *Paenibacillus larvae*, the bacterium that causes a highly virulent disease of honeybee larvae (American foulbrood). We found that, on average, colonies headed by multiple-drone inseminated queens had markedly lower disease intensity and higher colony strength at the end of the summer relative to colonies headed by single-drone inseminated queens. These findings support the hypothesis that polyandry by social insect queens is an adaptation to counter disease within their colonies.

**Keywords:** disease; genetic diversity; honeybees; polyandry; social insects

## 1. INTRODUCTION

Although queens in most social insect species do not mate with multiple males (Strassmann 2001), polyandry is prominent in certain taxa including yellow jacket wasps (*Vespula*, Ross 1986), leaf-cutter ants (*Atta*, Fjerdingstad *et al.* 1998; *Acromyrmex*, Boomsma *et al.* 1999), army ants (*Eciton*, Denny *et al.* 2004; *Dorylus*, Kronauer *et al.* 2004) and harvester ants (*Pogonomyrmex*, Rheindt *et al.* 2004; Wiernasz *et al.* 2004). The behaviour of mating with multiple males is carried to an extreme in honeybees (*Apis*, Estoup *et al.* 1994; Tarpay & Nielsen 2002). The molecular genotyping of worker bees has revealed effective queen mating frequencies ranging from  $m_e = 10.1$  in *Apis florea* (Palmer & Oldroyd 2001) to  $m_e = 63.0$  in *Apis dorsata* (Wattanachaiyingcharoen *et al.* 2003). Given that polyandry is likely to be costly—for various reasons, including increased predation risk, energetic costs and a reduction of relatedness among colony members—its widespread occurrence begs the question of its benefits.

Numerous hypotheses have been proposed for the selective advantages of the polyandry of social insect queens (reviewed by Crozier & Fjerdingstad 2001). For instance, the resulting increase in intracolony genetic variation could improve the division of labour within a colony (Robinson 1992), lower the genetic load at the sex locus (Page 1980) and reduce the queen–worker conflict over the sex-ratio investment (Ratnieks & Boomsma 1995). Multiple mating might also secure a sufficient number of sperm (Cole 1983). Another hypothesis that has received particular attention in recent years suggests that high genetic diversity within a colony reduces the impacts of parasites (Hamilton 1987; Sherman *et al.* 1988; Schmid-Hempel 1998). This hypothesis assumes that there is heritable variation in susceptibility to parasites; hence, genetically more variable colonies are less likely to

suffer sweeping infections by disease-causing parasites. Genotypic variation for parasite susceptibility is well documented for the honeybee *Apis mellifera* (e.g. Bamrick 1964; Rinderer *et al.* 1975; Palmer & Oldroyd 2003), the bumble-bee *Bombus terrestris* (e.g. Baer & Schmid-Hempel 2003) and the leaf-cutter ant *Acromyrmex echinator* (Hughes & Boomsma 2004).

Although logically compelling, to date, the parasite hypothesis for the evolution of polyandry by social insect queens has received few explicit tests. Studies with the bumble-bee *B. terrestris*, a species in which queens are mostly singly mated, have found that colonies with higher genetic diversity had lower parasite loads and higher production of sexual offspring (Liersch & Schmid-Hempel 1998; Baer & Schmid-Hempel 1999). Studies with the honeybee *A. mellifera*, a species in which queens are always multiply mated, have found that genetically diverse colonies were less likely to contract severe infections of brood diseases than genetically uniform ones (Tarpay 2003; Tarpay & Seeley 2006). However, the honeybee studies involved low-virulence diseases (mainly chalkbrood, caused by the fungus *Ascosphaera apis*) and did not reveal clear fitness differences between the two types of colonies.

Here, we present an experimental test of the parasite hypothesis with honeybees, in which we compared colonies with high and low genetic diversity in terms of resistance to a highly virulent bacterial parasite, *Paenibacillus larvae*, causative agent of the disease American foulbrood (AFB), which kills the larvae of honeybees. The spores of this bacterium are transmitted horizontally from colony to colony when an infected colony dies and its spore-laden honey is robbed by bees from neighbouring colonies (Fries & Camazine 2001). Using the apicultural practice of instrumental insemination, we varied 'mating' numbers of queens while controlling for semen volume and therefore sperm number. We established colonies headed by these queens in a common apiary and inoculated them with

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spores of *P. larvae*. We measured each colony's AFB infection twice over the course of one summer, as well as each colony's strength at the end of the summer based on its brood area, adult population and weight change. The experiment was conducted blindly with respect to 'mating' number, in that the queen rearing/insemination phase was conducted by one author (D.R.T.) and the colony inoculation/assay phase was conducted by the other (T.D.S.) and we did not share information until the study's end.

## 2. MATERIAL AND METHODS

### (a) *Queen rearing and inseminations*

One of us (D.R.T.) raised queens following standard protocols (Laidlaw & Page 1997). All were the daughters of a single queen inseminated by a single drone, hence they were full sisters ( $G=0.75$ ). Mature queen cells were placed in small hives containing approximately 1000 adult workers and three combs containing brood, honey and pollen. Each hive's entrance was covered with a screen of queen excluder material through which workers could leave to forage, but (larger) queens could not exit to mate.

Seven days after the queens emerged from their cells (the typical delay between emergence and mating is a week), each queen was captured and instrumentally inseminated with a Schley II insemination apparatus equipped with a Harbo syringe. Each queen was randomly assigned to one of two insemination treatments: single-drone inseminated (SDI) or multiple-drone inseminated (MDI). Drones were collected from 12 unrelated source colonies the day before insemination and stored overnight in separate cages within a large colony. Each SDI queen was inseminated with 1.0  $\mu$ l of semen from a single drone from one of the 12 source colonies; each source colony provided a sperm donor for 2–3 of the 34 SDI queens. Each MDI queen was also inseminated with 1.0  $\mu$ l of semen, but this was a mixture of the semen from 10 drones. The mixed semen was prepared by taking one drone each from 10 of the 12 drone sources, collecting each drone's ejaculate with a Harbo syringe, pooling the semen in a glass vial, and gently mixing it with a metal spatula. We used a different batch of mixed semen for each of the 35 MDI queens. The 10 drones for each MDI queen were chosen randomly from the 12 source colonies, hence the sperm donors came from all 12 colonies. This procedure has been repeatedly shown to produce queens whose offspring are genetically diverse (e.g. Haberl & Moritz 1994).

Each inseminated queen was returned to her hive to begin oviposition. Any queen that did not begin to lay eggs within 5 days following insemination was given a 4 min treatment with CO<sub>2</sub> to stimulate oviposition (seven SDI and five MDI queens). We inspected the brood of each queen following egg laying and removed queens that were producing numerous non-viable or unfertilized eggs. A total of 25 SDI queens and 24 MDI queens survived the insemination procedure and produced high-quality brood. At this point, the two types of queens were designated simply as 'group 1' and 'group 2', so that the remainder of the study was conducted blindly with respect to insemination number.

### (b) *Colony establishment*

On 30 May 2005 (two weeks after the inseminations were completed), the 49 queens were transported northward by 850 km, from D.R.T. in Raleigh, NC to T.D.S. in Ithaca, NY

and the next day each was installed in a small, queenless colony in a single, 10-frame 'Langstroth' (Sammataro & Avitabile 1998) hive. Each colony's hive contained three frames with comb and seven frames without comb (these frames contained beeswax foundation). The three frames of comb contained brood (one frame) and food (two frames); all were partially covered with approximately 1500 worker bees each and taken from 25 hives that were inspected for AFB in April and showed no signs of this disease. The 49 colonies were established 5 m apart in one apiary with the group 1 and group 2 colonies positioned randomly. Every two weeks, the laying pattern of each colony's queen was checked and her presence was verified. Any colony in which the instrumentally inseminated queen was missing (had been superseded) or was laying numerous unfertilized eggs was removed from the study.

### (c) *Colony inoculation*

On 14 July 2005, by which time all of the brood and the vast majority of the adults in each colony were the offspring of the experimental queen, there remained 14 group 1 and 14 group 2 colonies. (Note: even after the number of SDI colonies had declined from 25 to 14, fully 10 of the 12 drone-source colonies were still represented among the surviving SDI colonies. Hence, both the SDI and the MDI groups continued to contain a broad representation of the genes in the drone-source colonies.) On the morning of 14 July, each colony was inoculated by T.D.S. with a solution of *P. larvae* spores (10<sup>7</sup> spores per ml). The spore solution was prepared by removing 100 AFB scales (dried larvae killed by *P. larvae*) from a brood comb of a colony that died of AFB the previous summer, crushing the scales in 40 ml of an aqueous saline solution (0.25% NaCl), measuring the spore concentration with a haemocytometer, and then adding saline solution to achieve the desired spore concentration. The inoculation procedure for each colony consisted of removing one frame at a time from each colony's hive, shaking the bees off the frame and into the hive, spraying each side of the frame with 5 ml of the spore solution, returning the frame to the hive, and repeating this for each frame containing comb.

### (d) *Data collection*

T.D.S. inspected each colony twice for AFB disease, on 17 August and 15 September 2005, hence five weeks and nine weeks after the inoculation. He measured the intensity of the disease in each colony by inspecting each frame and (i) counting the number of brood cells with AFB-infected larvae and (ii) categorizing the frame as empty, one-quarter, one-half, three-quarter or full of brood. He summed these data over all 10 frames in each colony's hive and calculated the number of cells of infected larvae per full frame of brood (our measure of 'AFB intensity'). Besides measuring the intensity of AFB in each colony, T.D.S. evaluated each colony's strength on 16–17 September using three measures. First, he measured the size of each colony's brood nest by noting the frames of brood (see above). Second, he measured each colony's weight change between the beginning (1 June 2005) and end (16–17 September 2005) of the experiment. And third, he measured each colony's population by killing the colony with 100 ml of petrol, collecting its adult bees, and weighing both all the bees and a 100-bee sample of these bees. Only after all the data were collected by T.D.S. and reported to D.R.T. on 20 September was it revealed which group of queens was MDI and which was SDI.

**(e) Data analysis**

AFB intensity could not be normalized using standard transformation techniques, thus these data were analysed using non-parametric tests. Comparisons between insemination treatments (SDI versus MDI) for AFB intensity were conducted with Wilcoxon signed-rank tests. O'Brien tests of unequal variance were conducted to distinguish differences in variance between the two groups, as this test is robust against deviations from normality (cf. software text accompanying the JMP statistical package used for the analysis). Comparisons between insemination treatments for change in AFB intensity and for differences in colony strength were conducted with standard *t*-tests. Non-parametric Spearman rank correlations were used to determine the relationships between colony strength and the final measurement of AFB intensity. All tests are one-tailed, with  $\alpha=0.05$  before Bonferroni correction, because the experiment tested the *a priori* prediction of lower and less variable AFB intensity and higher colony strength in colonies with MDI queens than in those with SDI queens.

**3. RESULTS**

We found no difference between the two queen-insemination groups in the probability of infection five weeks after the inoculation with *P. larvae* spores. An AFB infection was found in nine of the 14 SDI colonies and in eight of the 14 MDI colonies. However, we found significant differences between the two queen-insemination groups in the intensity of infection (figure 1). Our first measurements of AFB intensity, made five weeks after inoculation, revealed that the variance in AFB intensity was significantly greater in the colonies headed by SDI queens (O'Brien test of unequal variance,  $F=18.5$ ,  $p=0.0002$ ) but that the mean values of AFB intensity did not differ between the two treatments (Wilcoxon signed-ranks  $\chi^2=2.91$ ,  $p=0.0880$ ). Our second measurements, which were made nine weeks after inoculation, revealed significant differences in both the variances (O'Brien test of unequal variance,  $F=6.27$ ,  $p=0.0189$ ) and the means (Wilcoxon signed-ranks  $\chi^2=5.40$ ,  $p=0.0201$ ) between the two groups, with both the mean and the variance higher for colonies headed by SDI queens. Thus, increased intracolony genetic diversity was associated with reduced likelihood of a colony contracting a severe case of AFB.

The degree to which AFB spread within colonies also differed between the two queen-insemination groups (figure 2). All but four colonies experienced an increase in AFB intensity from measurement 1 to measurement 2 (five and nine weeks post-inoculation, respectively), highlighting the virulence of *P. larvae*. Of the four colonies that experienced a decrease in AFB intensity, three belonged to the MDI treatment. On average, the increase in AFB intensity was significantly higher in colonies with SDI queens relative to those with MDI queens ( $t=2.45$ ,  $p=0.0213$ ), indicating that the disease spread faster within colonies of lower genetic diversity.

When we measured the strength of each experimental colony at the end of the summer (15 weeks after establishment and nine weeks after inoculation), we found that colonies with SDI queens were significantly weaker than those with MDI queens (figure 3), as measured by brood area ( $t=3.85$ ,  $p=0.0003$ ). We also found that adult population ( $t=1.86$ ,  $p=0.0371$ ) and hive weight change ( $t=1.57$ ,  $p=0.0639$ ) were strongly

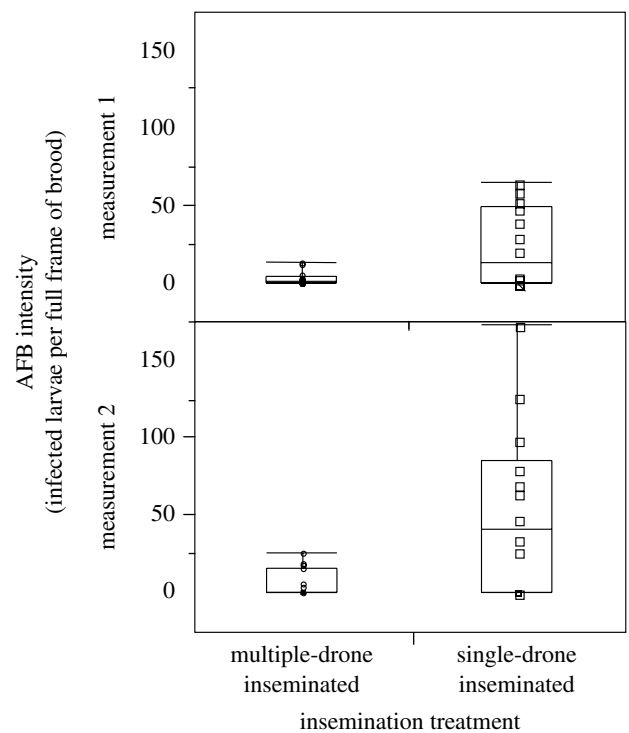


Figure 1. Intensity of AFB disease in honeybee colonies headed by SDI queens and MDI queens. Standard box plots describe the respective distributions of the individual data points. The measurement 1 data were taken five weeks following inoculation and the measurement 2 data were taken another four weeks later.

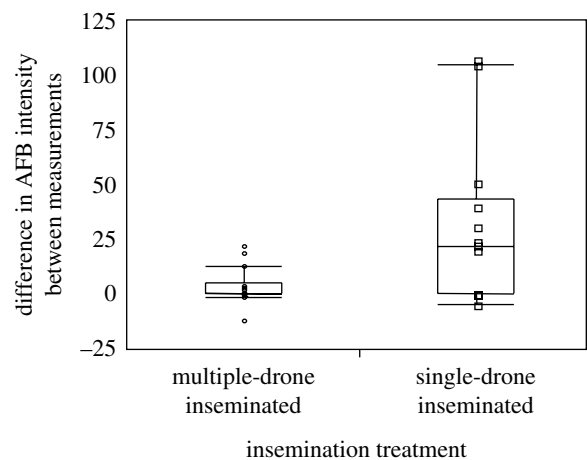


Figure 2. Change in AFB intensity during the study. Measurement 2 value for each colony minus its measurement 1 value is shown. The AFB intensity increased more strongly in SDI colonies than in MDI colonies.

affected by queen-insemination treatment, although the differences between the two groups were not significant statistically after a Bonferroni correction for multiple comparisons ( $\alpha=0.0167$ ). However, we note that this correction is extremely conservative. Probably, all three measures of colony strength would have shown a robust difference if the treatment period had lasted longer than nine weeks, but this was not feasible given seasonal constraints.

Finally, we found that for each of our three measures of colony strength, there was a significant negative correlation with AFB intensity (figure 3; Spearman's  $\rho$  for brood:  $\rho=-0.445$ ,  $p=0.0178$ ; population:  $\rho=-0.733$ ,  $p<0.0001$ ; weight change:  $\rho=-0.740$ ,  $p<0.0001$ ).

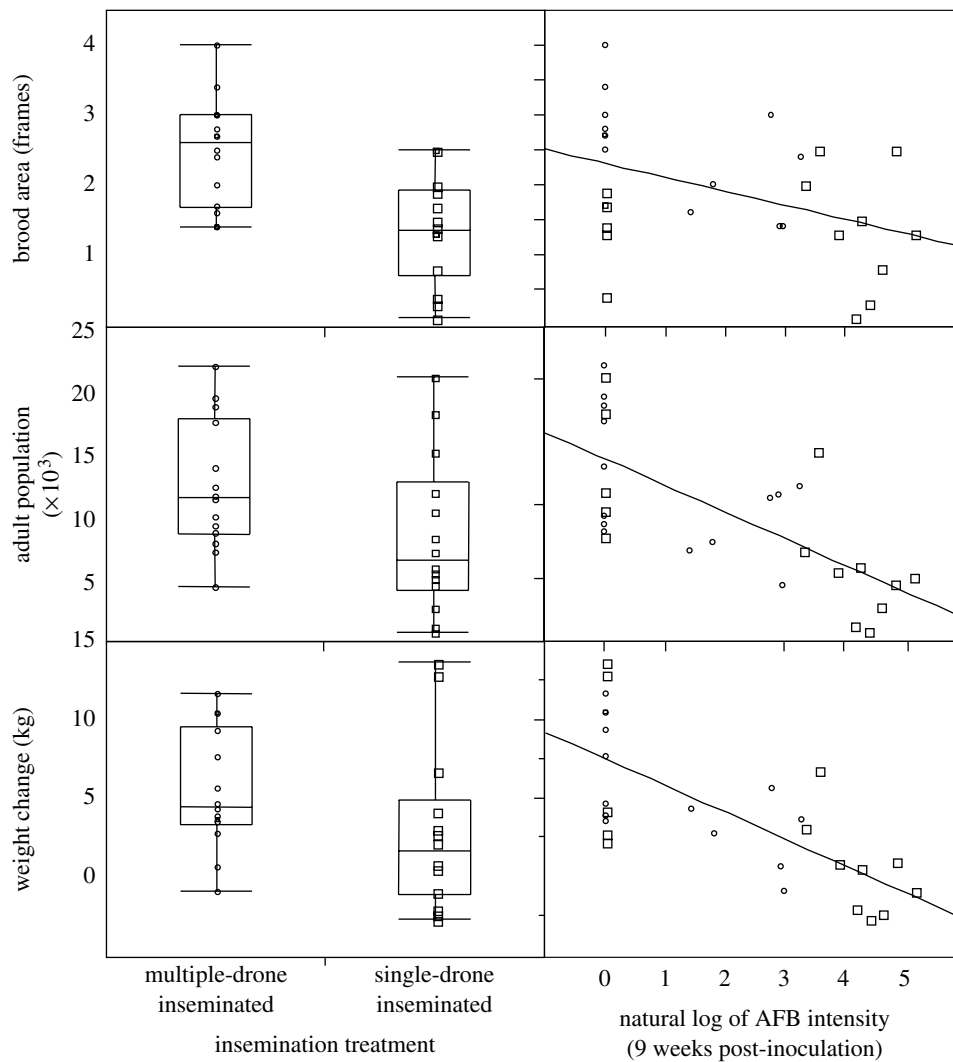


Figure 3. Three measures of colony strength at the end of the summer: brood area, adult population and colony weight change. All were higher, on average, for colonies with MDI queens compared with those with SDI queens. Moreover, each measure of colony strength was negatively correlated with the intensity of AFB at the end of the study; lines in the right-hand plots are least-squares fits to the transformed data.

#### 4. DISCUSSION

The main aim of this investigation was to conduct a rigorous test of the hypothesis that increased genetic variation within a social insect colony, resulting from multiple mating by the colony's queen, increases the colony's resistance to parasites. Our findings strongly support this hypothesis. In a blind comparison between colonies headed by SDI queens versus colonies headed by MDI queens, we found that the MDI colonies were lower than the SDI colonies in both the mean level of AFB infection and the variance in the level of AFB infection, nine weeks after inoculating each colony with spores of *P. larvae*. In principle, having a genetically diverse worker population may improve a colony's resistance to disease either by preventing an infection or by reducing within-colony parasite growth, or both. In actuality, the MDI and the SDI colonies in this study were equally vulnerable to becoming infected, but they differed markedly in their vulnerability to extreme levels of infection. Only the SDI colonies showed phenotypes with extremely low resistance to AFB infection. This finding of differential within-colony parasite growth, but not of differential resistance to infection, corroborates earlier experimental studies with bumble-bees (Baer & Schmid-Hempel 1999), honeybees

(Tarpay 2003; Tarpay & Seeley 2006) and leaf-cutter ants (Hughes & Boomsma 2004), and it supports the suggestion of Baalen & Beekman (2006) that increased genetic heterogeneity within a colony is unlikely to decrease the frequency of parasite infections. There is also the possibility that having greater genetic diversity within a social insect colony hinders the ability of parasites to adapt while cycling within a colony (Hughes & Boomsma 2006), but the present study does not evaluate this possible additional link between genetic diversity and disease resistance.

Besides finding differences in disease loads between the SDI and the MDI colonies, we found differences between the two types of colonies in colony-state variables that affect colony fitness: brood population, adult population and weight gain. We believe that the MDI colonies possessed larger populations (brood and adult) and higher weight gains than the SDI colonies, because the MDI colonies had far lower levels of the fatal brood disease AFB. This belief is supported by the fact that each of our three measures of colony strength was strongly and negatively correlated with the intensity of AFB infection. However, we cannot exclude the possibility that the larger populations and higher weight gains of the MDI colonies

arose, at least in part, because intracolony genetic diversity promoted task efficiency as well as disease resistance. The task-efficiency hypothesis proposes that increased genetic diversity allows a more complete expression of a genetically based caste system, leading to a more efficient worker force. Specifically, if the response thresholds for performing tasks are genetically determined, then genetically more diverse colonies may possess a higher level of individual specialization and thus may have greater task efficiency (Beshers & Fewell 2001; Page & Erber 2002). Heritable differences among workers in the probability of performing various tasks have been documented in many social insects (e.g. Robinson & Page 1988; Page *et al.* 1995; Julian & Cahan 1999; reviewed by Robinson 1992). In addition, genetically more diverse colonies of honeybees are reported to be slightly better than less diverse ones at maintaining a stable temperature in their brood nests (Jones *et al.* 2004). However, other empirical studies report little or no impact of genetic diversity on task efficiency (Rosset *et al.* 2005) and studies of polyandry using simulation models suggest that increased genetic diversity to enhance task efficiency is unlikely to be the mechanism promoting the evolution of polyandry (Brown & Schmid-Hempel 2003).

Besides elucidating the specific role of disease in the evolution of polyandry in honeybees, our findings highlight the general need for more theoretical work on the relationship between genetic diversity and disease resistance in social insect colonies. The model in the original theoretical study of this topic (Sherman *et al.* 1988) assumes that whether or not a worker (larva or adult) in an infected colony survives depends only on whether or not she bears an allele for disease resistance (see Evans & Pettis 2005). Consequently, multiple mating reduces the variance, but does not raise the mean, of the proportion of a colony's workers that survive an infection. However, it may be that resistant workers can provide resistance to others without resistance (Rosengaus *et al.* 2000). For example, adult workers with a strong tendency to remove AFB-infected larvae probably provide protection to other AFB-susceptible larvae in the hive (Spivak & Reuter 2001). Under these circumstances, multiple mating can raise the mean as well as reduce the variance of the proportion of a colony's worker brood that survive an infection, particularly as the infection spreads within the colony. This is indeed the pattern that we found in the present study.

Overall, we conclude that when a honeybee colony is infected by the virulent disease AFB, increased genetic diversity among the workers will reduce disease intensity and will enhance colony strength. If further studies with other diseases confirm this result, then it will be clear that the parasite hypothesis explains, at least in part, why highly polyandrous honeybee queens are favoured by natural selection.

We thank Jennifer Keller and Joshua Summers for their assistance with the fieldwork during the inseminations, and Drs Randy Worobo and Melissa Mundo for their advice on the microbiological methods. We also thank Christina Grozinger, Kern Reeve, Paul Sherman, Ed Vargo and Kirk Visscher for their helpful comments and suggestions on the manuscript. This study was funded by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant no. 2003-35302-13387.

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