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Within- and across-colony effects of hyperpolyandry on immune function and body condition in honey bees (*Apis mellifera*)

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ABSTRACT

Honey bees (*Apis mellifera*) have become a model system for studies on the influence of genetic diversity on disease. Honey bee queens mate with a remarkably high number of males—up to 29 in the current study—from which they produce a colony of genetically diverse daughter workers. Recent evidence suggests a significant benefit of intracolony genetic diversity on disease resistance. Here, we explored the relationship between the level of genetic diversity and multiple physiological mechanisms of cellular and humoral immune defense (encapsulation response and phenoloxidase activity). We also investigated an effect of genetic diversity on a measure of body condition (fat body mass). While we predicted that mean colony phenoloxidase activity, encapsulation response, and fat body mass would show a positive relationship with increased intracolonial genetic diversity, we found no significant relationship between genetic diversity and these immune measures, and found no consistent effect on body condition. These results suggest that high genetic diversity as a result of extreme polyandry may have little bearing on the physiological mechanisms of immune function at naturally occurring mating levels in honey bees.

1. Introduction

Insect societies have long been used for evolutionary study of cooperation and conflict (reviewed by Ratnieks et al., 2006). Hamilton's inequality (Hamilton, 1964) addressed Darwin's concern of sterile workers within the eusocial Hymenoptera and explained how reproductive altruism could be beneficial among related individuals (but see Nowak et al., 2010). Kin selection theory, as applied to eusocial insects, explains that for workers to provide assistance, the benefits of performing a behavior should outweigh the costs after being devalued by the level of relatedness between the actor and the recipient ($rB > C$). While high levels of relatedness seem necessary for this to occur, this is often not observed across colonies within the eusocial Hymenoptera (Crozier and Pamilo, 1996).

Haplodiploidy is exhibited in many social insect systems, including the entire insect order Hymenoptera, whereby males are haploid and females are diploid. Such a genetic system therefore establishes asymmetries in relatedness among individuals within a colony, where daughter workers are related to each other by $r = 0.75$ if they share the same mother and father (supersisters, in the same patriline), but are only related to each other by $r = 0.25$ if they share the same mother and different fathers (half sisters, in different patrilines). Because mean colony relatedness decreases when multiple patrilines are present, increased levels of polyandry (multiple mating by queens) have long been seen as inconsistent with kin selection theory.

While monandry is largely prevalent among the social Hymenoptera (Keller and Reeve, 1994; Strassmann, 2001), polyandry is fairly widespread but unusual, with only a few extreme cases (i.e., hyperpolyandry; Crozier and Fjerdingstad, 2001) exclusive to highly social species. In honey bees (genus *Apis*), queens are known to mate with many males, with colonies consisting of an average of approximately 12 subfamilies depending on the species (Tarpy et al., 2004). Given what we know about kin selection theory and queen–worker conflict, the phenomenon of polyandry in honey bee colonies is therefore puzzling. Mean colony relatedness does not effectively decrease after approximately 10 successful matings; there is an asymptote in this relationship (Rueppell et al., 2008). It has been suggested that the adaptive benefit of polyandry up to this threshold likely originated as a mechanism to avoid the production of sterile, diploid male honey bees (Page, 1980; Ratnieks, 1990; Tarpy and Page, 2002) but that other (non-mutually exclusive) advantages are largely responsible for more extreme levels of hyperpolyandry (Palmer and Oldroyd, 2000; Rueppell et al., 2008).

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Recent data show that hyperpolyandry in honey bees provides a measure of disease resistance to the colony (Palmer and Oldroyd, 2003; Seeley and Tarpy, 2007; Tarpy, 2003; Tarpy and Seeley, 2006). Tarpy (2003) showed that colonies reared from queens inseminated from 24 drones had lower variance in their ability to resist *Ascosphaera apis*, the fungal pathogen that causes chalkbrood disease in honey bee brood, as compared to queens inseminated by only one drone. This observation indicates that multiple matings increase the likelihood that a colony will survive chalkbrood disease, whereas a colony headed by a monogamous queen may be more prone to a sweeping infection. Tarpy and Seeley (2006) followed this study by setting up colonies headed by queens inseminated with either one or 10 drones. They found that colonies with higher genetic diversity had lower intensity of all measured brood diseases (chalkbrood, sacbrood, American foulbrood, and European foulbrood). This observation was confirmed after artificial infection with the bacterium *Paenibacillus larvae*, the causative agent of the highly virulent American foulbrood disease (Seeley and Tarpy, 2007); colonies headed by queens inseminated with sperm from 10 drones had lower disease intensity and higher colony strength (i.e., more brood, heavier, and more populous). In each of these studies, however, only colony-level phenotypes were measured, and thus it is unclear as to what mechanism(s) lead to disease tolerance.

In this study, we aimed to determine the influence of colony-level genetic diversity on two standard immune measures—one constitutive (phenoloxidase activity, or PO activity) and one inducible (encapsulation response)—that could be responsible for the recent observations linking genetic diversity to disease resistance. We hypothesized that immune function explains the results we see in genetically diverse versus genetically uniform colonies. With recent advances in our understanding of honey bee immunity (Evans et al., 2006; Wilson-Rich et al., 2008; reviewed in Wilson-Rich et al., 2009), we can test physiological defense mechanisms by exploring the earliest cellular and humoral immune mechanisms that pathogens and parasites encounter once in the honey bee hemocoel. We predict a positive relationship between level of genetic diversity (number of patrilines) and both immune function (phenoloxidase activity and encapsulation response) and body condition (fat body mass). We examined these factors both among colonies (by comparing number of effective patrilines) and within colonies (by comparing among patrilines). Specifically, we predict that as genetic diversity increases, the mean immune function will also increase across colonies, while the variation around the mean will decrease (see Sherman et al., 1988). Within colonies, we predict a patriline effect on immune function and body condition, whereby different sibling groups will vary from each other.

2. Materials and methods

2.1. Specimen collection

1124 honey bees were collected from 22 colonies at the Cummings School of Veterinary Medicine at Tufts University in North Grafton, MA, USA during two non-consecutive years. In 2006, guarding and foraging adult workers were collected from 12 colonies ($N = 266$ individuals, mean ± SD = 22.17 ± 5.18 individuals per colony, range 12–28). In 2009, guarding and foraging adults, as well as brood, were collected from 10 colonies ($N = 858$ individuals total, mean ± SD = 85.80 ± 8.09 individuals per colony, range 66–95 per colony). Brood collection involved mixed samples of larvae and pupae, as the immune function measures used (phenoloxidase activity and encapsulation response) are the same across both developmental stages (Wilson-Rich et al., 2008).

2.2. Immunology and body condition

Phenoloxidase activity (PO), encapsulation response (ER), and fat body mass (FB) were performed following the methods described by Wilson-Rich et al. (2008). Briefly, PO activity (a constitutive measure of immune function with no previous challenge) was measured in samples of bee hemolymph extracted from tested bees. Encapsulation response was quantified after implanting a nylon thread in adult abdomens and by using image analysis software. Finally, fat body mass was measured as the percent change in abdominal weight after an ethyl ether wash. In 2006, 133 adults were assayed for ER and 310 adults for FB. In 2009, 256 individual brood were assayed for PO and 299 adults for FB. Not all individuals collected produced usable data based on limitations of each respective assay, such as inability to collect hemolymph for PO or failure to retrieve the implant for ER. A highly conservative threshold was implemented for PO analyses, whereby the Vmax of enzyme linear phases were used only if $r^2 \geqslant 0.9$. Multiple measures were conducted in an effort to gain more information about the effects of genetic diversity across the dynamic immune system (see Adamo, 2004). We assessed two measures (ER, PO) of immune function and one measure of body condition (FB) for a holistic view of immunocompetence (see Wilson-Rich et al., 2009).

2.3. Genetic analyses

Colony-level genetic diversity and within-colony patrilines were quantified blindly after samples were collected for immune tests. The subfamily of each individual was determined using polymorphic microsatellite genetic analysis (c.f., Delaney et al., 2011; Tarpy et al., 2010). Because of their generally higher mutation rates, microsatellite markers may have large numbers of alleles, which make them particularly suited for paternity analysis (Estoup et al., 1995, 1994). DNA was extracted from all 1124 individuals ($N = 266$ adults from the 2006 collection and $N = 858$ brood from the 2009 collection) using Chelex® 100 (Walsh et al., 1991) and subject to PCR at eight microsatellite loci following Delaney et al. (2011). Paternity was then assigned to each worker following standard methods (Estoup et al., 1994), and the observed mating number and effective paternity frequency was calculated for each queen following Nielsen et al. (2003).

2.4. Statistical analyses

Immune function was compared both across and within colonies based on the number of patrilines, and patriline of origin, respectively. For across colony comparisons, we created a univariate generalized linear model (GLM) to control for any effect of subfamily differences within colonies. The GLM ANOVA incorporated each respective immune measure as discrete dependent variables, the number of subfamilies in each respective colony as the independent variable, and patriline as a covariate nested in each colony. We did not analyze genetics from adults in 2009 (only brood genetics were conducted for the 2009 samples), so these individuals were compared separately from the GLM, and Kruskal–Wallis *H* tests were performed instead. For within-colony comparisons, we first determined whether each data set was normally distributed using Shapiro–Wilk tests. We then performed either ANOVAs or Kruskal–Wallis *H* tests across patrilines followed by post hoc pairwise comparisons, depending upon the normality of the distribution of each respective data set from Levene's tests. Comparisons of variation were conducted also using Levene's tests for unequal variance. Furthermore, we calculated partial eta-squared values (as in Wilson-Rich et al., 2008) to determine the proportion of variance in each immune measure that may be ex-