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Evolutionary biology

Genetic diversity confers colony-level benefits due to individual immunity

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Several costs and benefits arise as a consequence of eusociality and group-living. With increasing group size, spread of disease among nest-mates poses selective pressure on both individual immunity and group-level mechanisms of disease resistance (social immunity). Another factor known to influence colony-level expression of disease is intracolony genetic diversity, which in honeybees (*Apis mellifera*) is a direct function of the number of mates of the queen. Colonies headed by queens with higher mating numbers have less variable infections of decreased intensity, though the underlying mechanisms remain unclear. By pathogen-challenging larvae *in vitro*, we decoupled larval immune response from mechanisms of social immunity. Our results show that baseline immunity and degree of immune response do not vary with genetic diversity. However, intracolony variance in antimicrobial peptide production after pathogen challenge decreases with increasing genetic diversity. This reduction in variability of the larval immune response could drive the mitigation of disease observed in genetically diverse colonies.

1. Introduction

For eusociality to evolve, as seen in social insects like honeybees, ants and termites, the fitness benefits (e.g. cooperative brood care) have to outweigh the costs (e.g. potential for increased parasitism). Current genomic analyses suggest that honeybees (*Apis mellifera*) [1], and possibly all Hymenoptera [2], have fewer gene families devoted to immunity than other model insect lineages. Explanations of how social insects are able to combat parasites have thus become all the more relevant. Because honeybees live in societies with one reproductive individual (the queen) and thousands of constantly interacting workers, the dynamics of parasite transmission must also be considered at the group level.

One aspect that influences colony-level disease resistance is polyandry, or multiple mating of the queen. Honeybee queens mate with 12 drones (males) on average (but up to 40+), which creates a high level of intracolony genetic diversity [3]. The adaptive benefit of polyandry has several non-mutually exclusive explanations (reviewed by Palmer & Oldroyd [4] and Smith *et al*. [5]), including mitigated parasitic infestations [6]. Various studies have been able to provide support for this hypothesis across social insects [7–10]. However, little is known concerning the mechanism(s) underlying these patterns. Most studies have focused on the concept that genetically diverse colonies contain patrilines that are more resistant to some strains of pathogens than others and that this may reduce the spread of disease throughout the entire colony [4,11]. Here, we take that idea a step further and explicitly test if the mechanism behind reduced infection intensities seen in genetically diverse colonies could possibly be the result of differences in innate immunity.

Immune responses (both individual and social mechanisms) are likely under different selective pressures at individual and colony levels, so teasing apart the individual versus group pathways of disease mitigation will therefore

elucidate how selection shapes innate and social immunity. Since production of antimicrobial peptides is both heritable [12] and correlated with colony-level resistance to some infections [13], it is possible that in colonies with increased genetic diversity individual larval immune responses can confer colony-level disease resistance. However, studies have shown that there are no differences in physiological immunity at baseline, unchallenged levels among inbred versus outbred individual honeybees [14] or across colonies with relatively high levels of genetic diversity (8–29 patrilines per colony [15]). It still remains unclear, therefore, if individuals from colonies with increased genetic diversity differ in their ability to mount an effective immune response after a pathogen challenge. This is a critically important distinction, given demonstrated colony-level fitness costs for individuals' upregulation in immune response [13], as well as costs at the individual level [16].

The specific goal of this study is to test a prediction of Sherman *et al*.'s hypothesis [6] explaining the evolution of extreme polyandry. Here, we predict that larvae from a genetically diverse colony will, on average, be more responsive to a pathogen challenge than those from a genetically similar colony and that this will result in a reduced variance in immune response with increasing levels of genetic diversity. To do this, we decouple colony-behavioural resistance (social immunity) from individual immunity by rearing larvae *in vitro*. While the ultimate goal is to determine if colony-level disease resistance is a result of this mechanism, this investigation is crucial to evaluate the potential influence of physiological immunity.

2. Material and methods

(a) Establishment of genetically diverse colonies

We established colonies of differing genetic diversity using either artificial insemination or restricted, natural mating of sister queens. Queens mated via either method were maintained in nucleus colonies containing approximately 5000 bees across two apiary locations in Raleigh, NC. We artificially inseminated queens using one, two or 12 drones following previously established protocols [8] to create colonies with below-average genetic diversity. To develop queens mated along a continuum from low to high mating numbers, we reared additional sister queens and placed them as virgins in 3-frame mating nucleus colonies. We monitored the queens daily so that each queen was allowed only one successful mating flight [17]. We collected 48 worker pupae from each colony for genotyping analysis using eight different microsatellites to determine queen mating frequencies, following well-established protocols (see the electronic supplementary material) [3,18].

(b) Larval sampling and infection

We 'grafted' first instar larvae from each colony into 96-well tissue-culture plates containing 100 µl of an artificial larval rearing diet following [19]. Immediately after, we fed 48 larvae on each plate an oral dose of 4 µl of a suspension containing 80 000 *Paenibacillus larvae* spores/µl water mixed from three colony sources. Control larvae were fed 4 µl of water. *P. larvae* is a highly virulent honeybee parasite that causes American foulbrood (AFB) disease in honeybees, and it has been studied extensively in regard to larval immune response (e.g. [4,12,13,20]). We use it as an ecologically relevant model to determine how colony-level genetic diversity influences larval immune response.

Larvae were maintained in a humidified chamber at 34°C for 24 h post-inoculation; preliminary trials determined that at this time point there was a significant immune response and it is prior to any development symptoms. We removed the larvae, washed them with a 10× dilution of PBS and collected them in pools of 12, with 3–10 pools collected per colony (mean number of pools per colony: 6 ± 3; see electronic supplementary material, table S1). We stored samples at −80°C until subsequent analysis.

(c) Assessment of immune response

We extracted RNA from larval pools using a standard TriZol extraction method and synthesized cDNA. We measured transcript levels of the genes encoding the antimicrobial peptides hymenoptaecin and abaecin following well-established protocols using a StepOnePlus Applied Biosystems real-time PCR cycler (see the electronic supplementary material) [21]. *Abaecin* expression correlates with colony resistance to *P. larvae* [13] and *hymenoptaecin* is known to be upregulated in infected larvae [1]. Each gene reaction was normalized with respect to levels of *RPS5*, a well-established housekeeping reference gene. Regression analysis was used to examine if there was a general effect of genetic diversity in immune response across the entire range.

3. Results

(a) Levels of genetic diversity

We determined colony-level genetic diversity by calculating the mating frequency of queens. For naturally mated queens, the number of observed matings (N_o) was 8–30 (13.6 ± 5.5). To account for unequal representation of patrilines—and to have a better depiction of intracolonial genetic diversity—the effective mating frequency (m_e) was calculated and ranged from 4 to 31 (11.1 ± 8.1). Four colonies headed by artificially inseminated queens were also included: one single-drone, two 2-drone and one 12-drone inseminated queens.

(b) Immune response

We determined baseline immune responses of unchallenged larvae from each colony for both *hymenoptaecin* and *abaecin* expression (figure 1). For both antimicrobial peptides, there was no relationship between average relative expression in unchallenged larvae or with the mean relative change in expression owing to pathogen challenge with respect to colony-level genetic diversity. There was a strong upregulation in challenged larvae with respect to unchallenged larvae.

Intracolonial variance among samples in the upregulation of *hymenoptaecin*, but not *abaecin*, in response to AFB challenge significantly decreased with increasing genetic diversity (figure 2; $R^2 = 0.14$, $p = 0.04$).

4. Discussion

The current narrative for behavioural versus physiological immunity among the social insects is that there may be trade-offs between the two [1,2], though it is likely that many disease resistance traits are also complementary or additive. Our results, using the bacterial agent of American foulbrood disease as a model, suggest that larval immunocompetence alone can be a strong driver in the reduction of disease. Given that increasing levels of genetic diversity reduced intracolonial variance of larval antimicrobial peptide production, we provide clear evidence for a mechanism that

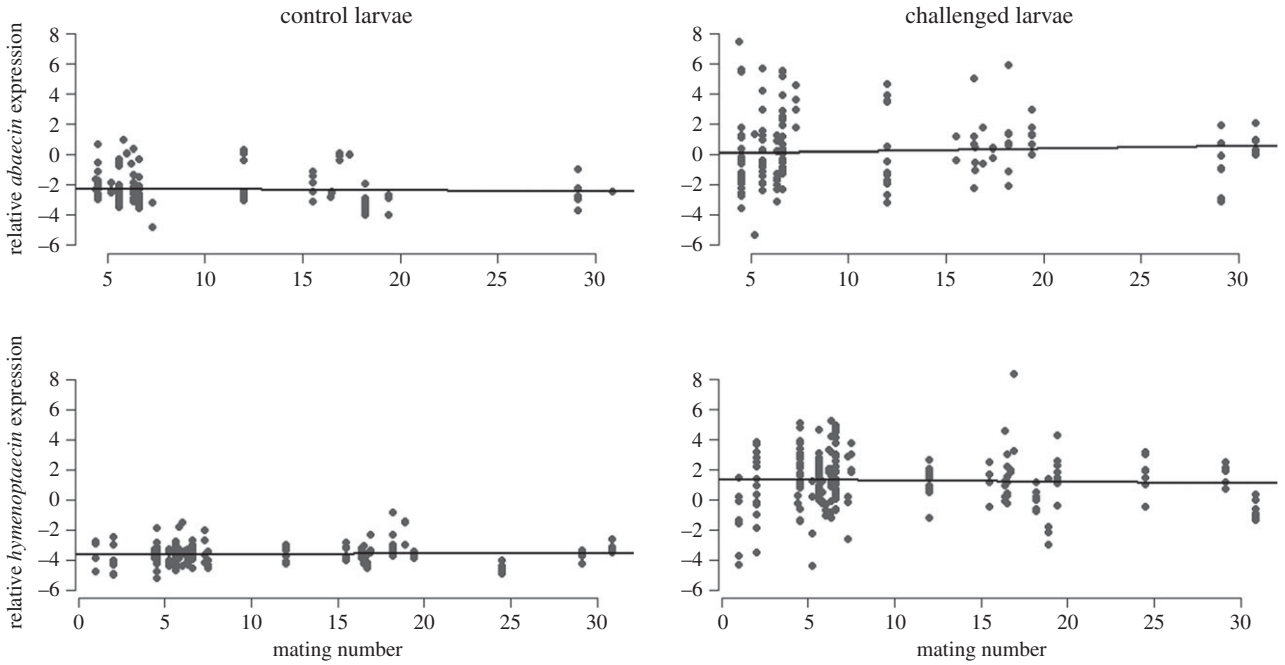

Figure 1. Relative expression of *abaecin* and *hymenoptaecin* in unchallenged (control) and challenged larvae from colonies ranging in level of genetic diversity. There was no significant relationship for relative expression and mating frequency for either gene based on linear regressions (*hymenoptaecin*—unchallenged: $R^2 = 0.002$, $p = 0.48$; challenged: $R^2 = 0.009$, $p = 0.16$; *abaecin*—unchallenged: $R^2 = 0.002$, $p = 0.61$; challenged: $R^2 = 0.003$, $p = 0.49$).

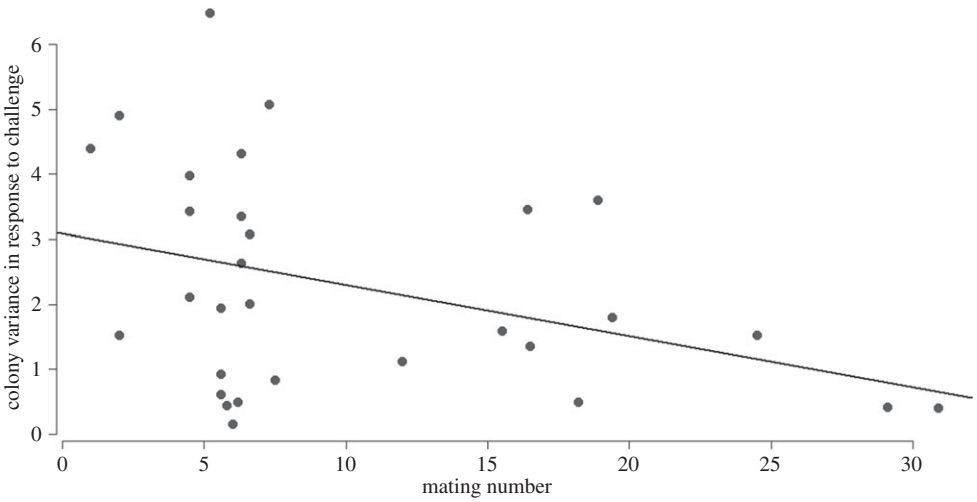

Figure 2. Intracolony variance in *hymenoptaecin* expression after challenge with American foulbrood decreases as genetic diversity increases, with each point indicating the variance among samples from a single colony ($R^2 = 0.14$, $p = 0.04$).

may explain previous studies documenting the same phenomenon for disease expression at the colony level [4,8,20]. Since immune expression of larvae in challenged colonies has been shown to be associated with subsequent reduction in levels of disease at the colony level [13], there is other empirical support for this as the mechanism.

Also noteworthy is that we only found a difference relating to the immune response of challenged larvae across the gradient of genetic diversity and that there were no differences in the baseline immune production in unchallenged larvae. Since a high constitutive production of antimicrobial peptides can have costs for both individuals and colonies [13,16], selection may favour inducible expression rather than constitutive expression. A recent study has in fact shown that constitutive expression does not seem to correlate with subsequent ability to fight pathogen exposure [22], which supports the findings presented here. The mechanism explaining intracolonial variance of immune expression could be explained by differing response thresholds, whereby some larvae are able to respond more quickly or more strongly to pathogens and thus show higher levels of immune gene expression after a challenge. The fact that intracolonial variance decreases with increasing genetic diversity could also be, in part, explained by the initial larval environment. While we removed the colony environment from this experiment as much as possible, larvae were still present in the colonies as eggs and newly hatched first instars. Previous research has shown that genetic diversity increases colony productivity and overall health, which could possibly result in healthier larvae upon hatching, which in turn results in a more stable response in larvae from these colonies.

While we show that physiological immune responses correlate to previous findings regarding colony-level phenotypic differences, subsequent *in vivo* studies need to confirm that

larval immune response can sufficiently explain individual and colony resistance to disease. It is still likely, however, that in an uncontrolled hive setting individual physiological immunity and group-level social immunity behaviours work in concert [23]. Future work will address the magnitudes of effects of individual and social immune traits in unselected colonies and those selected for increased social immunity responses.

Our findings support the idea that variance-based selection—whereby intercolony variance is reduced by multiple mating—may explain the evolution of polyandry in social insects [24]. Furthermore, based on previous theoretical and empirical data concerning the threshold mating frequency on the polyandry continuum of honeybees [24,25], our data are consistent with an effective paternity frequency of approximately 7 maximizing the effects of polyandry in this system. This is significantly lower than the average observed mating frequency for *A. mellifera* and lower than what is typically tested when attempting to determine if intracolonial genetic diversity influences behaviour and colony fitness [15,18]. It seems reasonable, therefore, that selection for hyperpolyandry in social insects has acted on queens to achieve a threshold mating frequency above which the benefits have diminishing fitness consequences.

Data accessibility. Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.ct92j.

Authors' contributions. M.S.-F. and M.W. collected all data, M.S.-F. and D.R.T. completed experimental design, data analysis and interpretation, and all authors contributed to and approved the final manuscript.

Competing interests. The authors declare no competing interests.

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