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RESEARCH ARTICLE

Higher immunocompetence is associated with higher genetic diversity in feral honey bee colonies (*Apis mellifera*)

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Abstract Honey bees are the most important managed pollinators as they provide key ecosystem services for crop production worldwide. Recent losses of honey bee colonies in North America and Europe have demonstrated a need to develop strategies to improve their health and conserve their populations. Previously, we showed that feral honey bees (colonies that live in the wild without human assistance) exhibit higher levels of immunocompetence than managed colonies in North Carolina (USA). In a first attempt to investigate the underlying mechanisms of this difference in immune response, here we characterize the genetic composition of feral and managed honey bees using microsatellite markers. Our results reveal significant but small genetic differentiation between feral and managed honey bee colonies ($\phi_{CT} = 0.047$, $P = 0.03$) indicating admixture between these two groups. Higher genetic diversity was correlated with higher immune response in feral ($P_{MANOVA} = 0.011$) but not managed bees, despite the fact that the latter group showed significantly higher average genetic diversity ($P_{ANCOVA} < 0.001$). These findings suggest that genetic diversity is positively associated with immunocompetence in feral honey bee colonies, and that the benefits of genetic diversity are obscured in managed bees, perhaps as a result of artificial selection. We hypothesize that high genetic variability provides the raw material upon which natural selection acts and generates adaptive genotypes in unmanaged populations. Feral populations could be useful sources of genetic variation to use in breeding programs that aim to improve honey bee health.

Keywords Microsatellites · Antimicrobial peptides · Defensin · Hymenoptaecin · Management

Introduction

Managed honey bees (*Apis mellifera*), whether in their introduced or native range, are important pollinators in human dominated landscapes. However, even as global demand for crop pollination increases, beekeepers in North America and Europe struggle to maintain robust populations of managed pollinators. The causes of honey bee mortality have drawn widespread attention and research focus (Lee et al. 2015). Undoubtedly, a variety of factors contribute to this decline, but pathogens, *Varroa* mites, poor nutrition and pesticide exposure have been identified as key problems (Potts et al. 2010; Goulson et al. 2015). These threats all intersect at the honey bee immune system, in that poor nutrition and pesticide exposure can cause immunosuppression, which can increase the abundance of pathogens and compromise colony survival (Di Pasquale et al. 2013; Di Prisco et al. 2013; Doublet et al. 2015). Clearly, important interventions in strengthening honey bee

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health need to focus on improving nutrition and reducing pesticide exposure. But such change will likely be slow and as a result, it is also important to understand if mechanisms exist through which beekeepers can improve bee health and thus conserve the pollination services they provide.

High stocking density and aggregation of managed honey bee colonies can increase contact rates among individuals, accelerating pathogen reproductive rate and spread (Seeley and Smith 2015), and potentially favoring the evolution of more virulent forms (Fries and Camazine 2001). In North America, the honey bee population is composed of both managed and feral colonies. Feral colonies were once assumed to have all succumbed to numerous introduced parasites (Kraus and Page 1995; Oldroyd 1999). However, it has more recently been shown that some colonies have survived in certain areas in spite of numerous disease threats (Seeley et al. 2015). This has led several research efforts to identify how non-managed (putatively feral) colonies can survive while managed colonies are having difficulties (Lowe et al. 2011; Appler et al. 2015; Youngsteadt et al. 2015; Magnus and Szalanski 2010). Comparing feral and managed colonies may provide insights into whether and how the biology of persistent natural populations of *A. mellifera* relates to their overall tolerance to disease.

Genetic diversity plays a key role buffering populations against widespread epidemics (King and Lively 2012). In honey bees, multiple studies show that increased genetic diversity at the colony level has direct fitness advantages associated with resistance to pathogens and colony survival (Tarpy 2003; Mattila and Seeley 2007; Tarpy et al. 2013). The mechanisms explaining the association between genetic diversity and pathogen resistance are still unclear. Some evidence shows that individual genotypes differ in their disease resistance, suggesting specific host-pathogen genotype-by-genotype interaction as the most likely mechanism to explain the fitness benefits of higher genetic diversity at the colony level (Evison et al. 2013; Simone-Finstrom et al. 2016). Alternatively, greater genetic diversity may lead to more immune variants at the individual level, and, in doing so, increase overall immunity at the colony level. This relationship has rarely been explored in the literature (Lowe et al. 2011; Evison et al. 2016).

Recently, we found that feral honey bees show higher levels of immunocompetence than do managed colonies (Youngsteadt et al. 2015). However, in that study we did not identify a mechanistic basis for the differences in immune gene expression between feral and managed colonies. Here we characterize the genetic composition of these colonies to investigate the role of genetic diversity in the underlying difference of immune function found in managed and feral colonies of *A. mellifera*. Specifically, we explore three possible links between the genetic diversity and composition of these coloniesóas measured using neutral genetic markers that do not, themselves, confer fitness effectsóand their immune function. First, feral and managed honey bees could be genetically differentiated, and thus show differences in their levels of immune function. Second, higher immune function in feral honey bees could be the result of higher overall genetic diversity in this group of colonies. Last, we explicitly test whether immune gene expression is correlated with within-colony (not population level) genetic diversity. We assessed colony level genetic diversity using microsatellite markers in feral and managed honey bee colonies. Our results provide evidence for the beneficial effects of maintaining high levels of genetic diversity in feral honey bees, and that these colonies may exhibit novel genetic variation that could be used in breeding programs towards improving honey bee resistance to diseases.

Materials and methods

Sampling

We sampled 35 honey bee colonies (14 feral and 21 managed) from a 5000 km^2 area around the city of Raleigh, NC (USA). Managed hives belonged to hobbyist beekeepers, and they were non-migratory and established in the area for at least 1 year prior to sampling (see Youngsteadt et al. 2015 for details). Because of the large variation in management practices among beekeepers, we could not ascertain or verify the longevity of each queen but only the colony, and as such we did not know if the queens were purchased commercially from an outside population or raised and mated locally. Feral colonies were naturally established, at least a year before our sampling, in cavities such as tree trunks and buildings, and were not manipulated by humans in any way. We collected a group of foragers from the entrance of each colony to assess genetic diversity and conduct the immune assays. Because feral colonies were often located within inaccessible cavities, we were unable to sample workers from within colonies, and we therefore consistently collected only foragers from both managed and feral colonies. We used an average of 23 (min = 9; max = 48) and 13 (min = 7; max = 30) individuals per colony for the genetic analysis and immune assays, respectively.

Immunocompetence

We focused on three antimicrobial peptides (AMPs)óabaecin, defensin-1, and hymenoptaecinót hat were differentially expressed in feral and managed colonies in our previous study (Youngsteadt et al. 2015). As described in that study, we quantified AMP transcripts using qRT-PCR from RNA extracted from pooled abdomens of at least nine individuals after a lipopolysaccharide (LPS) immune

challenge. Briey , we extracted RNA using the TRIzol method (Life Technologies) with some modifications to the manufacturerís protocol: the centrifugation step for phase separation and RNA precipitation was at 16,260 g; the centrifugation step for the RNA wash was at 6532 g; and the RNA pellet was washed twice before final suspension. We then prepared cDNA using RNA template, Superscript II reverse transcriptase (Invitrogen), and a set of random 7-mer primers (S3 Table in Youngsteadt et al. 2015). We performed qRT-PCR reactions using the Power SYBR Green Master Mix (Applied Biosystems) in reactions with a final volume of 10 µL using the following thermal protocol: 95 C for 10 min; 40 cycles of 94 C for 20 s, 60 C for 30 s, 72 C for 1 min, and 78 C for 20 s.; and one cycle of 95 C for 15 s, 60 C for 1 min, and 95 C for 15 s. We ran all reactions in duplicate and calculated their average difference in threshold cycle values between each target and β-actin (ΔCT) in the StepOnePlus software (see Materials and methods in Youngsteadt et al. 2015 for details). We used the ΔCT values to compute the abundance of each AMP relative to its own rarest detected transcript using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). We then compared AMP transcription levels between feral and managed bees using one-way ANOVAs.

Microsatellite genotyping

We extracted DNA from hind legs of forager bees using 150 µL of 5% Chelex solution and 5 µL of Proteinase K (10 mg/mL). We incubated the mix at 55 C for 1 h, 99 C for 15 min, 37 C for 1 min, and 99 C for 15 min. To store the DNA, we transferred the supernatant into a new tube until PCR analyses. We genotyped eight microsatellite markers: A113, A24, A88, Ap43, Ap81, B124, ApJC2, and A76 (Supplementary Table S1) using the protocol described in Tarpy et al. (2010). We conducted multiplex PCR reactions in 2 sets (Plex 1: A24, A88, B124, ApJC2; Plex 2: A113, Ap43, Ap81, A76) using annealing temperatures of 55 and 54 C for Plex 1 and Plex 2, respectively. Allele sizes of PCR products were estimated on an ABI 3730 DNA Analyzer (Applied Biosystems) at the Genomic Sciences Laboratory of North Carolina State University, USA. We used the software Genemapper 4.0 (ABI) for allele scoring.

Data analysis

We used the Chakraborty (1992) and Brookfield 1 (1996) equations to estimate null allele frequencies in MicroChecker v.2.2.3 (Van Oosterhout et al. 2004). We also removed drifters (worker bees from other colonies) from our dataset after identifying individuals that did not share one of the maternal alleles with the putatively inferred queen. To test whether feral and managed colonies were genetically differentiated, we randomly selected one individual from each colony that showed no missing data. We tested HardyW einberg equilibrium (HWE) for each locus in the feral and managed populations separately using the function hw .testí in the R package *pegas* (Paradis 2010). We tested for population differentiation using three G-statistics (Neiís G_{ST}, Hedrickís G_{ST}, Jostís D_{est}) with the function di ff_statsí in the package *mmod* (Winter 2012). These G-statistics differ on how estimation of population structure is corrected by the varying levels of variability in different sets of genetic markers (Meirmans and Hedrick 2011). We also performed an analysis of molecular variance (AMOVA) to test for population genetic differentiation between feral and managed bees using colonies as the sampling unit. For the AMOVA analysis, we used the function poppr .amovaí of the R package *poppr* (Kamvar et al. 2014). To further investigate genetic differentiation, we used the discriminant analysis of principal components (DAPC) implemented in the package *adegenet* for R (Jombart et al. 2010). This multivariate model-free approach does not assume HWE to cluster individuals based on prior population information. Allowing for e xibility on violations to HWE was important because genetically related individuals from the same colony were included in the analysis, which may lead to deflits in the proportion of heterozygous. For the DAPC analysis, we determined if there was genetic differentiation between colonies and/or populations based on the ability to reassign individuals to their colonies of origin (K = 35), or to the f eral *vs* managedí group (K = 2). We determined the number of principal components with the function xv alDapcí after running 1000 replicates on a training set using 90% of the data. We used the number of principal components associated with the highest number of successful reassignments and the lowest root mean square error. For the AMOVA and DAPC analyses, we removed individuals with more than 50% missing data from the dataset with the function missingnoí in *poppr* (Kamvar et al. 2014).

We characterized the levels of genetic diversity of each colony by estimating expected heterozygosity (a unitless parameter that summarizes allele frequenciesóa lso known as Neiís unbiased gene diversityóH_{exp}), and rarefied allelic richness (number of alleles per locus corrected by the smallest sample size, A_R) using *poppr* and *hierfstat* (Goudet 2005; Kamvar et al. 2014). These metrics were used to quantify genetic diversity and provide unbiased estimates of genetic variability from small and unequal samples sizes (Kalinowski 2004; Nei 1978). To compare the levels of genetic diversity between feral and managed colonies, we used an analysis of covariance (ANCOVA) using mean H_{exp} and A_R per locus per population as a covariate to control for variability in microsatellite markers (Boff et al. 2014). We

used a multivariate analysis of variance (MANOVA) to test the hypothesis that higher genetic diversity was correlated with higher levels of AMP transcription in feral and managed colonies. We calculated a multivariate F using Wilksí λ, after log-transforming the data to meet the assumption of normality, and scaling the data to equalize the contribution of all three AMPs to the analysis regardless of their overall abundance. For visualization purposes, we built linear correlations between genetic diversity (H_{exp} and A_R) and each AMP separately.

Results

Immunocompetence

As previously reported by Youngsteadt et al. (2015), feral colonies showed signiﬁ antly higher levels of expression for abaecin ($F=8.694$, df = 1, $P=0.006$), defensin ($F=9$, df = 1, $P=0.005$), and hymenoptaecin ($F=6.649$, df = 1, $P=0.015$) than did managed colonies (Supplementary Figure S1). On average, bees from feral colonies expressed twice as many transcripts as did managed colonies (Youngsteadt et al. 2015 and Supplementary Figure S1).

Genetic differentiation and genetic diversity

We genotyped 783 individuals with an average of 21.8 (SD 7.96) workers per colony. Loci ApJC2 and A76 were not included in the analyses due to unreliable allele scoring. The number of alleles per locus of the ﬁa l set of six microsatellites varied between 8 and 18, and the expected heterozygosity per locus from 0.59 to 0.79 (Supplementary Table S1). No null alleles were detected in the six microsatellite loci (Supplementary Table S2). The Chi square statistic for HWE indicated that that all loci were at equilibrium in the managed and feral populations (Supplementary Table S3). Overall estimates of genetic differentiation were 0.024 (95% CI 0.00780.045) for Neiís G_{ST}, 0.142 (95% CI 0.04910. 2479) for Hedrickís G_{ST}, and 0.1003 (95% CI 0.03340.1795) for Jostís D_{est} based on average heterozygosity.

We partitioned genetic variation between three levels of organization: individuals, colonies, and populations (feral *vs* managed). The majority of variation, 75%, was found between individuals within colonies ($\phi_{IS} = 0.248$, $P=0.01$, Table 1). Differences between colonies accounted for 20% of the variation ($\phi_{SC} = 0.211$, $P=0.01$). Finally, feral and managed populations were signiﬁ antly differentiated, even though their differences accounted for only 5% of the overall genetic variation we detected ($\phi_{CT} = 0.047$, $P=0.03$). We assigned individuals to the feral or managed populations with 81% success rate (based on DAPC; Fig. 1),

Table 1 Results of analysis of molecular variance (AMOVA) testing for differentiation between feral and managed, and among colonies in *Apis mellifera*

	df	σ^2	% variance	ϕ-statistic	P-value
Between feral and managed	1	0.026	4.69	0.047	0.02
Between colonies within feral or managed	34	0.111	20.07	0.211	0.01
Within colonies (error)	619	0.415	75.23	0.248	0.01

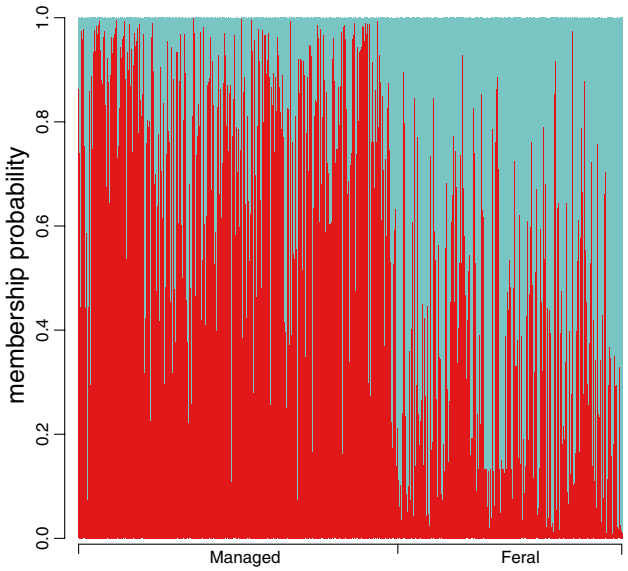

Fig. 1 Membership probabilities from the discriminant analysis of principal components (DAPC). Each *vertical line* represents one individual from managed (*left* panel) and feral (*right* panel) colonies; the *colored portions* of *each line* represent the proportion of each individualís genotypes that belong to the managed (*red*) or feral (*blue*) population. Thus, a completely *blue line* represents an individual whose genetic composition was unambiguously that of a feral bee. (Color figure online)

suggesting population differentiation between these groups of colonies. Assignment-success rate of individuals into their colonies of origin was lower (65%), but this function was higher in managed (89%) than feral (69%) colonies. Managed colonies showed higher levels of genetic diversity (H_{exp}) (ANCOVA; $F=47.756$, df = 1, $P<0.001$) and allelic richness (A_R) (ANCOVA; $F=102.698$, df = 1, $P<0.001$) than feral bees (Fig. 2).

Does genetic diversity correlate with immunocompetence?

Overall, AMP transcription levels were significantly correlated with heterozygosity and marginally

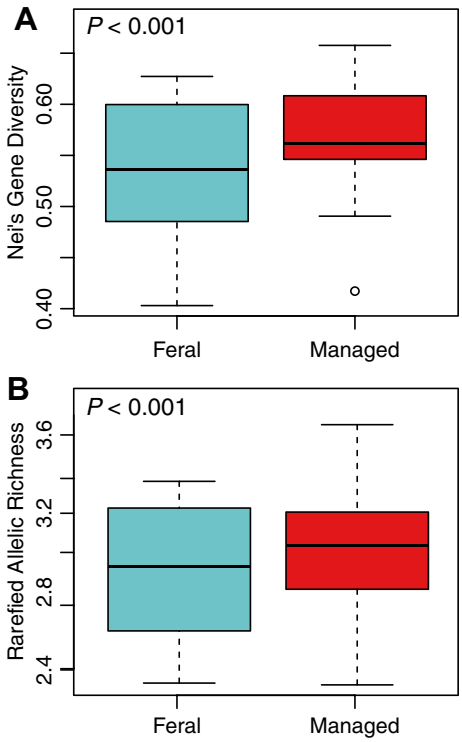

Fig. 2 *Boxplots* showing differences in levels of genetic diversity between feral (*blue*) and managed (*red*) colonies. Genetic diversity is expressed as: **a** Neiís unbiased gene diversity (H_{exp}), and **b** Rarefied allelic diversity (A_R). Significant values are the result of ANCOVA using mean number of alleles per population as a covariate. (Color figure online)

Table 2 Results of the multivariate analysis of variance analysis (MANOVA) testing for the effect of gene diversity (H_{exp}) and allelic richness (A_R) on the transcription levels of antimicrobial peptides in feral and managed colonies of *Apis mellifera*

	Abaecin	Defensin	Hymenoptaecin
Feral			
H_{exp}	0.371 (0.555)	**22.258 (<0.001)**	**5.199 (0.044)**
A_R	0.394 (0.542)	**9.359 (0.011)**	4.068 (0.068)
Managed			
H_{exp}	2.643 (0.128)	0.0675 (0.426)	0.17 (0.687)
A_R	2.687 (0.125)	3.234 (0.095)	1.102 (0.313)

Values denote F and P values (in parenthesis)

Statistically significant associations are indicated in bold

correlated with allele richness in feral bees (MANOVA; H_{exp}: $F_{3,9} = 10.554$, $P=0.003$; A_R: $F_{3,10} = 8131$, $P=0.076$). However, AMP transcription was not correlated with genetic diversity in managed bees (H_{exp}: $F_{3,11} = 0.8$, $P=0.519$; A_R: $F_{3,11} = 2643$, $P=0.128$) (Table 2). Specifically, we found that defensin and hymenopteacin expression increased with genetic diversity at the colony level in feral bees (H_{exp}: adjusted $R^2_{DEF}=0.639$, adjusted $R^2_{HYM}=0.259$; A_R: adjusted $R^2_{DEF}=0.411$, adjusted $R^2_{HYM}=0.204$) but not managed honey bee colonies (H_{exp}: adjusted $R^2_{DEF} = -0.024$, adjusted $R^2_{HYM} = -0.063$; A_R: adjusted $R^2_{DEF}=0.138$, adjusted $R^2_{HYM} = -0.007$; Fig. 3).

Discussion

Our results reveal three main findings about the genetic composition of feral and managed honey bee colonies and its relationship with immunocompetence. First, we found feral and managed colonies to show low genetic differentiation in the sampled area (Raleigh, NC, USA). Second, managed colonies have significantly higher levels of genetic diversity than do feral colonies. Last, we found a significant positive correlation between genetic diversity and the levels of AMP expression in feral but not in managed colonies. Collectively, we interpret these results as evidence for the importance of natural selection maintaining genetic diversity for high immunocompetence in unmanaged *A. mellifera* colonies.

We show that feral and managed colonies share most of their genetic variability but are nonetheless genetically differentiated ($\phi_{CT} = 0.018$, $P=0.02$). In North America, feral colonies are much rarer than they were historically due to population declines triggered by the introduction of the mite *Varroa destructor* in the mid-1980s (Kraus and Page 1995). Most feral colonies were therefore thought to be recent escapes from managed bees, and thus represent a subset of the lineages present in managed populations. But new evidence suggests that at least some highly isolated populations of feral bees have persisted and are almost entirely genetically distinct from local managed populations (Seeley et al. 2015). Where feral bee populations represent persistent populations and where they are more recent escapees is as of yet not fully resolved. In our study region, the signifi ant but low genetic differentiation between feral and managed bees is expected if feral bees derive primarily from recent swarms.

In line with the hypothesis that the feral bees in our study region are of recent origin, we found higher genetic diversity in managed than feral honey bee colonies. Even though the fne ss benefis of high genetic diversity at the colony level are well-established in honey bees (Tarpy 2003; Mattila and Seeley 2007), the ways in which these benefits bear out among feral bees at the population level are less clear (but see below). In our system, the relative amount of neutral genetic variation between feral and managed populations does not correlate with the observed difference in immune response. Feral bees had a stronger immune response, even though they were less diverse. However, our fidings indicate that the increased genetic diversity of managed *A. mellifera* may not bear fitness benefits that

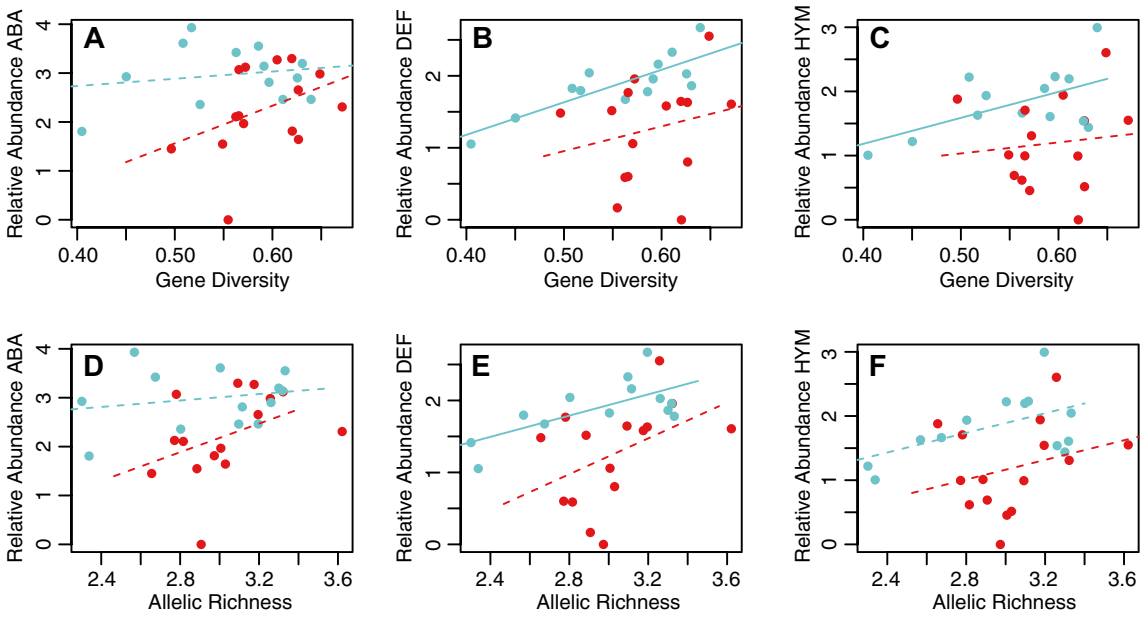

Fig. 3 Linear relationship between genetic diversity and immunocompetence in colonies of *Apis mellifera*. Genetic diversity is expressed as **a c** gene diversity (H_{exp}), and **d f** rarefied allelic richness (A_R). Immunocompetence was quantified as the relative abundance (RA) of the transcript expression of three antimicrobial peptides: **a, d** abaecin, **b, e** defensin, and **c, f** hymenoptaecin. *Blue* and *red* denote data from feral and managed colonies, respectively. *Solid lines* indicate significant linear relationships. (Color figure online)

correlate with immunocompetence. Our results support that management increases genetic diversity in honey bees probably as a result of admixture among progenitor populations as honey bee queens are shipped among regions (Harpur et al. 2012). Domesticated species are generally thought to be less genetically diverse than are their wild relatives (Wang et al. 2014). However, honey bees are unique in that feral bees are derived from domesticated lines in their nonnative range. In addition, honey bees, even when managed, undergo a mix of local breeding, regional dispersal, and natural reproduction among hives from different sources. Yet, what is interesting is that despite this diversity of managed bees, they are less rather than more immunocompetent than the feral populations, suggesting that while diversity matters to immune function, so may the ability of natural selection to increase the frequency of resistant varieties.

Our third major finding is that genetic diversity has a significantly positive correlation with the expression of AMPs in feral colonies. Associations between higher genetic diversity and fine ss traitsós uch as colony productivity (Mattila and Seeley 2007), pathogen resistance (Tarpy 2003), survival (Tarpy et al. 2013), and reduced likelihood of diploid male production (Tarpy and Page 2001)óa re well-established in the literature for *A. mellifera* and have been the subject of many studies of managed hives. However, our study is among the fis t showing a direct positive association between genetic diversity and immunocompetence in honey bee populations, particularly in feral unmanaged populations that subject to natural selection. Simone-Finstrom et al. (2016) recently found that colonies with higher genetic diversity exhibit reduced intra-colonial variance of AMP production at the larval stage in managed honey bees. Our findings demonstrate a positive correlation between colony genetic diversity and average AMP expression in adult individuals in feral colonies, and should be followed up with studies that link functional genetic diversity and immune gene expression to colony-level survival or fine ss advantages.

Assuming that neutral genetic diversity is linked to loci under selection (Chapman et al. 2009), we show that high genetic diversity at the colony level appears to be associated with increased immunocompetence of honey bee colonies exposed to natural selective pressures. We hypothesize that this association is the result of immune variants that better respond to certain pathogens being maintained in managed colonies at very low frequencies, and only increasing in frequency as they become selectively advantageous in unmanaged conditions. It is also possible that artificial selection for beekeeper-favored traits (e.g., reduced swarming behavior) may exhibit trade-offs with immune response. Studies have investigated trade-offs between selection acting at the individual and social level on the honey bee immune system (Harpur et al. 2014), but little is understood about how selection for desirable apicultural traits has impacted beesí ability to cope with pathogens and parasites. To this extent,

many domesticated plant species also show reduced defenses to herbivores, and our results indicate that this might also be true more generally (Turcotte et al. 2014). In addition, our findings highlight the limitations of using managed colonies to understand honey bee responses to diseases, as these colonies lack adaptive mechanisms that honey bees have naturally used to mitigate pathogens and environmental stressors (Mikheyev et al. 2015).

Evolutionary trade-offs within the immune systems of honey bees may drive differential responses in different aspects of immune function. Previous studies have shown no effect of genetic diversity on cellular immune function of individuals (Lowe et al. 2011) and colonies (Lee et al. 2013; Wilson-Rich et al. 2012). However, in the systemic immune system, antimicrobial peptides appear to be differentially affected by genetic diversity. This and other studies have also shown a positive correlation between genetic diversity and expression levels of defensins and hymenoptaecin (Simone-Finstrom et al. 2016; Evison et al. 2016). At the molecular level, one possible mechanism linking higher genetic diversity with stronger immune response is alternative splicing. Higher allelic richness could increase the exonic variants of AMPs and, consequently, their levels of expression after an immune challenge (as discussed in Evans et al. 2006).

Overall, this study demonstrates that feral and managed colonies can show small genetic differentiation in sympatry under high levels of admixture, which most likely is the product of unidirectional gene ow from the managed to the feral population. Higher genetic variation in feral bees was associated with higher immunocompetence. These results point to a possible important role of adaptive genetic variation resulting from strong selective pressures from pathogens and parasites in wild populations. Future studies should use genomic approaches to reveal possible candidate genes responsible for the genetic basis of the different immune phenotypes found in feral and managed colonies. If such studies confirm the presence of adaptive genetic variation in feral North American populations, then these bees could be incorporated into breeding programs that focus on improving overall honey bee health.

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