Mating Frequencies of Honey Bee Queens (*Apis mellifera* L.) in a Population of Feral Colonies in the Northeastern United States

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Abstract

Across their introduced range in North America, populations of feral honey bee (*Apis mellifera* L.) colonies have supposedly declined in recent decades as a result of exotic parasites, most notably the ectoparasitic mite *Varroa destructor*. Nonetheless, recent studies have documented several wild populations of colonies that have persisted. The extreme polyandry of honey bee queens—and the increased intracolony genetic diversity it confers—has been attributed, in part, to improved disease resistance and may be a factor in the survival of these populations of feral colonies. We estimated the mating frequencies of queens in feral colonies in the Arnot Forest in New York State to determine if the level of polyandry of these queens is especially high and so might contribute to their survival success. We genotyped the worker offspring from 10 feral colonies in the Arnot Forest of upstate New York, as well as those from 20 managed colonies closest to this forest. We found no significant differences in mean mating frequency between the feral and managed queens, suggesting that queens in the remote, low-density population of colonies in the Arnot Forest are neither mate-limited nor adapted to mate at an especially high frequency. These findings support the hypothesis that the hyperpolyandry of honey bees has been shaped on an evolutionary timescale rather than on an ecological one.

Introduction

The Western honey bee (*Apis mellifera* L.) is incredibly widespread and ecologically successful, as its native range spans all of Africa, Europe, and much of Western Asia [1]. Aside from the now-extinct *Apis nearctica* [2], honey bees are an exotic species in North America, having been introduced by European settlers beginning early in the 17th century. These bees quickly escaped from beekeepers' hives and spread throughout North America, creating two groups of

honey bee colonies: managed colonies living under the supervision of beekeepers in apiaries and feral colonies living on their own in the wild.

In recent decades, many populations of honey bees have experienced unusually high mortality as a consequence of various factors (reviewed in [3]). Research on the possible causes of this mortality has focused on parasites and pathogens, nutritional stress, habitat loss, and toxic and sub-lethal levels of pesticides [4,5,6,7]. One major cause of colony mortality in Europe and North America is the ectoparasitic mite *Varroa destructor*, which was introduced to these continents in the 1970s and mid 1980s, respectively. Conventional wisdom holds that, if a colony of honey bees in the managed population does not receive mite control treatments, the mites (vectoring several associated viruses) will kill the colony within 2 to 4 years [8,9]. The introduction of *Varroa* is widely thought to have decimated the wild colonies in Europe and the feral population in North America [10,11]. However, the increasing reports of feral honey bee colonies surviving in several eastern and southern states in the U.S. [12]—as well as the persistence since at least the 1970s of a population of feral honey bee colonies in the Arnot Forest in New York State [13]—indicate that feral honey bees in North America have survived and may even be rebounding despite the presence of *Varroa* and other disease agents.

How honey bee colonies tolerate pathogens and parasites has been the focus of many studies, and special attention has been paid to several behavioral mechanisms, termed 'social immunity' [14,15], that are inherent to many insect societies. One such mechanism is female multiple mating, or polyandry, which is taxonomically restricted among social insects [16] but occurs at an exceptionally high level in honey bees (genus *Apis*). Estimates of the mating number of *Apis mellifera* queens, based on molecular genotyping of worker offspring, vary between 1 and 59 and average about 14 [17]. Many hypotheses have been proposed to explain why this hyperpolyandry has evolved in honey bees (reviewed by [18]), and the "intracolonial genetic diversity" hypotheses are widely seen as the most plausible [19]. While the benefits of increased intracolonial genetic diversity include an increased behavioral repertoire of the worker force (e. g., [20]), and reduced variation in diploid male production as a consequence of the single-locus sex determination system (e.g., [21]), they also include a lower likelihood of devastating infestations of parasites and pathogens among colony members [22,23,24]. One key to the success of feral honey bee colonies may be, therefore, that their queens have an extremely high mean mating frequency, so their colonies possess unusually high genetic diversity and have exceptional ability to tolerate pathogens and parasites.

The aim of this study was to examine the mating frequencies of the queen bees in a remote and persistent population of feral honey bee colonies in rural New York State. We compared the mating frequencies of queens in these feral colonies to those in the nearest managed colonies to see if mating frequency has been shaped by ecological context. One possibility is that queens in a feral population, where colonies are widely spaced, may have lower mating frequencies because of the lower colony density and diminished availability of mates. If so, then this will suggest that their survival depends upon something other than extreme polyandry. An alternative possibility is that queens in a population of feral colonies may have higher mating frequencies. If so, then they might have increased benefits of intracolony genetic diversity which might explain why they have been able to persist despite *Varroa* and other disease agents. The null hypothesis, of course, is that queens in feral colonies and managed colonies have the same mating frequencies, which would suggest that the polyandry of queen honey bees has been shaped by natural selection acting on an evolutionary timescale and has not been tuned on an ecological time scale through intense selection recently for greater resistance to parasites and pathogens.

Materials and Methods

The Arnot Forest (Fig. 1) is a 17-km^2 research preserve owned by Cornell University and located approximately 25 km south of Ithaca, in New York State (42° 17'N, 76° 39 W). It is home to a population of feral honey bee colonies that has been studied since the 1970s. The first census and mapping of this population was performed in 1978 [25]. This work was repeated in 2002, some 15 years after the arrival of *Varroa destructor*, confirming the continued existence of the honey bees living in the forest [13]. In August 2011, we built on this prior work by locating approximately half the colonies in the Arnot Forest and the surrounding state forest lands (an area of some 25 km^2, total) using the same technique ('bee-lining'; see [26] that one of the authors (TDS) used in 1978 and 2002). Consistent with the density of feral colonies found previously in this forest area—about one colony per km^2—we located 10 feral colonies living in trees within or just outside the Arnot Forest (see Fig. 1).

We sampled workers from each feral colony by capturing them from the feeding station when it was moved to within 100 m of the tree that the colony occupied, at which point the feeding station was 1000s of meters from any competing colonies and so was completely
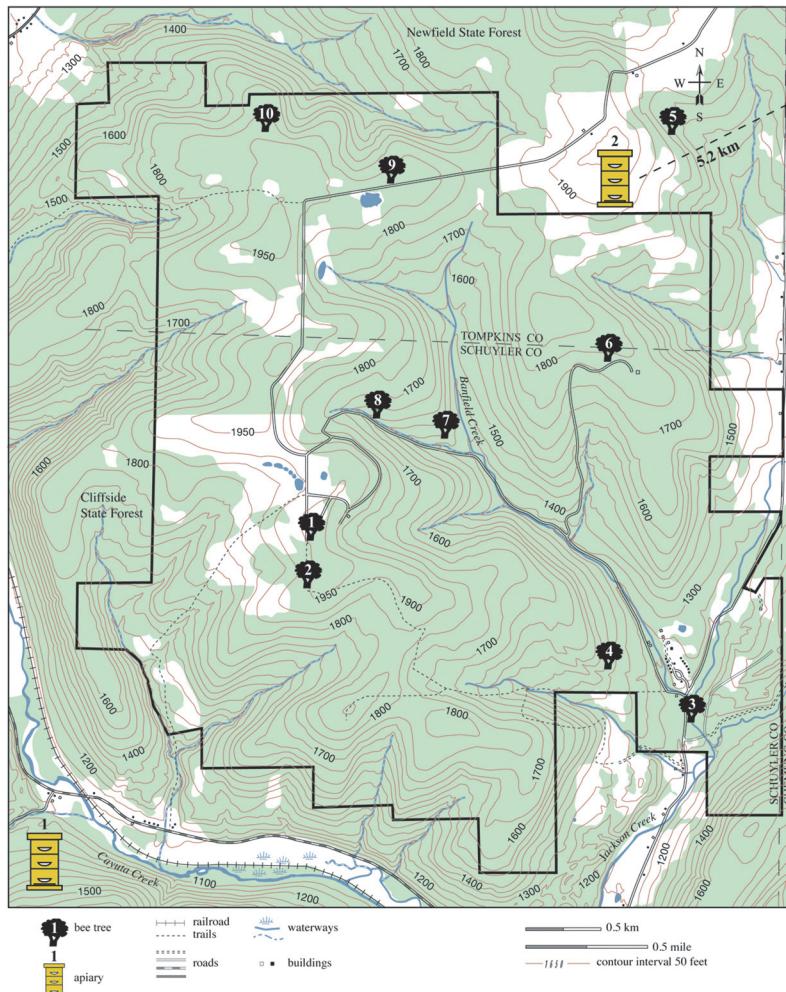

Fig 1. Study population. Map depicting the locations of the honey bee colonies that were investigated in this study. Shown are the locations of the 10 wild colonies of honey bees (bee trees) in the Arnot Forest, and of the two nearest apiaries outside the Arnot Forest.

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dominated by foragers from the nearby colony (as confirmed by separate genetic analyses, i.e. Seeley et al., in press). More than 100 foragers visiting the feeder were collected, stored, and preserved in 95% ethanol. We also sampled ~100 workers from each of 20 managed colonies (i.e., 10 colonies per apiary in the two nearest apiaries located outside of the Arnot Forest; these apiaries contained 22 and 24 colonies, respectively). Apiary 1 was 1.0 km from the southwest boundary of the Forest and Apiary 2 was 5.2 km from the northwest boundary (see Fig. 1). These were the only managed colonies found within 6 km (the typical flight range of honey bees) of the Arnot Forest and were used to provide information on the mating frequency of commercially produced queens. Apiary 1 was started in April 2011 with mated queens newly purchased from Wooten's Golden Queens, Palo Cedro, California. Apiary 2 was established in the early 2000s and its colonies received, from time to time, new queens purchased from Wooten's Golden Queens.

After collecting the adult worker bees from each feral and managed colony, we individually extracted their DNA following the Chelex method (see [27]). We then analyzed each bee at 12 microsatellite loci (A24, A28, A79, A88, A107, Ap43, Ap66, Ap81, AC006, B124, HB-THE-03, and HB-THE-04). One multiplex PCR was used to amplify 10 µl reactions of extracted DNA containing 1.0–4.0 mM of fluorescent dye-labeled primer (Applied Biosystems), 1x reaction buffer, 1.5 units *Taq* polymerase, 3mM dNTP mixture, 0.001 mg bovine serum albumin, 1 µl DNA, and 25 mM MgCl$_2$. We ran the PCR products from all individuals on a Perkin-Elmer ABI Prism 3730XL automated capillary DNA sequencer and determined paternal inheritance with GeneMapper 4.0 software. This practice has become standard across most studies investigating mating number (more precisely, the effective paternity frequency of the workers) by queens and the resultant genetic structure of honey bee colonies (e.g., [28,29,30,31,32]).

We tabulated a paternal marker set for each worker to estimate the genetic structure within each colony using COLONY 1.2 [33]. The total number of different marker sets within a colony signified the observed paternity frequency of the queen (N_o), or the total number of drone fathers that are represented in the offspring. We also determined the proportion of each subfamily within a colony so that we could calculate the effective paternity frequency (m_e) using the sample statistic proposed by Nielsen et al. [34],

$$m_e = \frac{(n-1)^2}{\sum_{i=1}^{N_o} p_i^2 (n+1)(n-2) + 3 - n}$$

where n is the number of worker samples and p_i is the proportion of subfamily i within the sample population. This unbiased measure is used for calculating skews in paternity and intracolony genetic relatedness.

We then compared the mating frequencies across the three groups of colonies (Apiary 1, Apiary 2, and feral colonies) using analysis of variance. We used Tukey post-hoc comparisons to identify differences among the means of the three groups of colonies with respect to observed and effective mating numbers. All statistical tests are two-tailed with α = 0.05, and all means are reported as ± SD unless otherwise specified.

We entered the drone alleles from each population into the microsatellite toolkit 3.1.1 in Excel [35] using the haploid data option, from which we determined the allelic frequencies, sample size, and the mean number of alleles by population. We calculated drone diversity by population using Nei's unbiased gene diversity.

Results

We analyzed 1,089 worker bees, hence 36.2 ± 8.35 individuals per colony (range 13–47). We excluded microsatellite loci that produced consistent PCR products in fewer than 25% of the individuals from a given colony. Interestingly, there was an unusually high frequency of null alleles in all three groups for several of the microsatellite loci, and these loci were also excluded to avoid potential scoring errors and inflated estimates of effective paternity. Nonetheless, most colonies were analyzed with 6–10 loci, each with high allelic variability, so that the average non-detection error [36] was extremely low (2.49×10^{-11}).

We found no significant differences in mean mating frequency among the three groups (Fig. 2). The mean effective mating frequencies (m_e) of the Apiary 1 queens (20.8 ± 5.9) and the Apiary 2 queens (16.0 ± 6.4) were not statistically distinguishable from that of the Arnot Forest queens (17.4 ± 9.8; $F_{2,27} = 1.04$, P = 0.36). Moreover, all three estimates lie well within the range of mating frequencies reported previously for *Apis mellifera* queens [17,31].

We obtained genotypic data for 574 drone fathers total among the three groups (Table 1). The unbiased gene diversity was highest in drones from Apiary 1 (0.76 ± 0.03) followed by the drones from the Arnot Forest (0.73 ± 0.04) and the drones from Apiary 2 (0.61 ± 0.07). The mean numbers of alleles per locus for drones from Apiary 1, the Arnot Forest, and Apiary 2 were 9.90 ± 4.77, 9.70 ± 3.47, and 8.10 ± 4.36, respectively. Closer inspection of the allele frequencies of drone fathers from the different populations (Table 2) reveals that the number of unique alleles (i.e., those not found in either other population) follows a similar pattern. Apiary 1 had 1–7 unique drone alleles in eight of the 10 loci analyzed (HB-THE-03, A107, A113, AC006, A28, AP66, AP81, and B124), Apiary 2 had 1–5 unique drone alleles in 5 of the 10 loci analyzed, and the Arnot Forest was intermediate with 1–9 unique drone alleles in 7 of the 10 loci analyzed (Table 2).

Discussion

Feral colonies of honey bees, especially ones living more or less isolated from managed colonies, provide insights into the basic ecology of honey bees that cannot be gained from their managed counterparts. This study of the feral honey bee colonies living in the remote Arnot Forest was undertaken to gain insights into their mating biology and to explore how they have been able to survive while the population of managed honey bee colonies in North America has experienced heavy mortality each year and has depended on the aid of beekeepers to survive.

Numerous studies have investigated the mating frequencies of *Apis mellifera* queens living in managed colonies crowded in apiaries (e.g., [29,32]). However, little is known about the mating frequencies of queens of European descent living in feral colonies dispersed across the landscape. Tilley and Oldroyd [37] analyzed three colonies in Australia that were abandoned and thus left unmanaged for several years, although their goal was to compare the paternity distributions of workers and queens rather than the mating frequency of the putatively feral queens. Nielsen [38] used RAPD markers to directly identify worker paternity in six feral colonies of European descent in California. Taken together, these two studies suggest that the queens in feral honey bee colonies of European descent have relatively low mating frequencies (6.6 ± 3.0) compared to queens in managed colonies of European descent (10.1 ± 3.6). In the southern United States, there is also a thriving population of colonies of African descent, and their queens are known to have among the highest mating frequencies in the species (20.0 ± 8.5; [31]). The fact that our estimate of queen mating frequency for the feral Arnot Forest colonies (17.4 ± 9.8) is not different from our estimates of queen mating frequency for the two nearest groups of managed colonies (20.8 ± 5.9, and 16.0 ± 6.4), suggests that the previous estimates
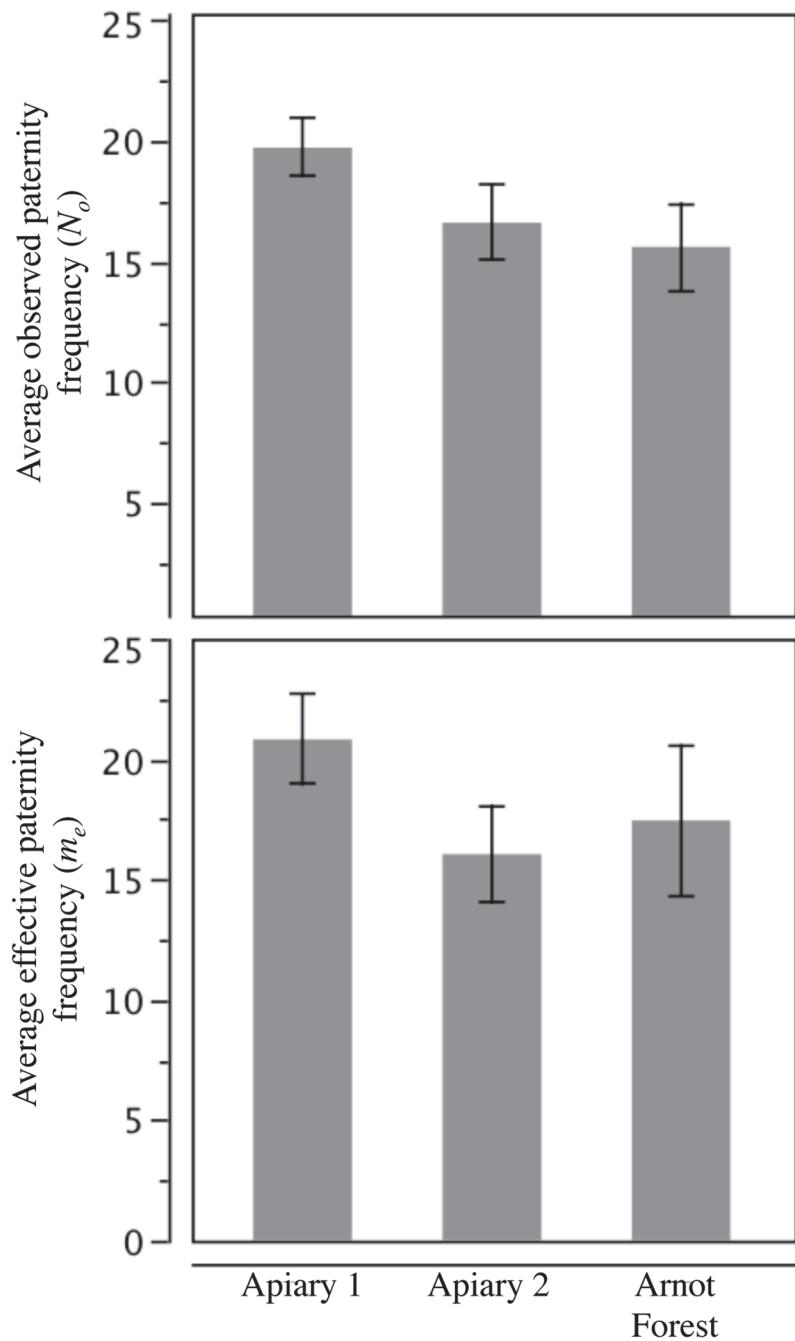
Fig 2. Mating frequencies of queens from feral and managed colonies. Average ± SEM of the observed (top) and effective (bottom) mating frequencies of the 10 honey bee queens in each of the three groups of colonies. No significant differences (at α = 0.05) were found among the three groups.

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for feral colonies of European descent are underestimates because of low allelic diversity [38], small marker number [37], or small colony sample size. Whatever the reason, our results show that queen honey bees in dispersed, feral colonies do not necessarily mate with significantly fewer males than queens in crowded, managed colonies.

It might seem surprising that the low density of honey bee colonies in the Arnot Forest (approximately one colony per square kilometer) does not result in a decreased level of polyandry

Table 1. Population statistics for drone fathers.

Population	Sample Size	Unbiased Gene Diversity and SD	Mean No. of Alleles and SD
Arnot Forest	156	0.73 ± 0.04	9.90 ± 4.77
Apiary 1	197	0.76 ± 0.03	9.70 ± 3.47
Apiary 2	166	0.61 ± 0.07	8.10 ± 4.36

Population genetics statistics for drone fathers from the Arnot Forest, Apiary 1, and Apiary 2, based on alleles from 10 variable microsatellite loci. Drone alleles inferred from worker and queen genotypes using the program COLONY 1.2 [33].

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of the queens in these colonies. This result is, however, consistent with the experiences of queen producers and is understandable given the reproductive biology of honey bees. For example, Neumann et al. [29] examined the mating frequencies of queens at various commercial mating sites in Europe, some of them isolated on islands or in mountain valleys. They found significant differences among some sites, but they found no association between number of nearby drone-source colonies (a proxy for drone abundance) and queen mating frequency. These results, combined with the results reported here, suggest that drone availability is generally not a significant constraint on the mating frequency of honey bee queens. A typical colony raises hundreds if not thousands of drones for each queen that it rears [39], thus the numerical sex ratio is extremely male-biased (even though there is a 1:1 investment into the two sexes because of the resources required to raise a queen and issue a swarm; [40]). Thus, even in regions with low densities of colonies, honey bee queens evidently encounter plentiful drones. Because queens and drones mate in discrete sites ("drone congregation areas"; [41]), the low density of colonies in a feral population does not necessarily create low drone densities where matings occur.

It would be interesting to estimate the density of the honey bee colonies in the Arnot Forest using the techniques proposed by Arundel et al. [42]. To do so, one could mate 10+ queens in the Arnot Forest and quantify the number of drone fathers from distinct colonies among the worker offspring. In the current study, however, we estimated the mating frequencies of the queens in the Arnot Forest population itself, rather than of queens of known pedigrees outside of the Arnot Forest population. Moreover, the frequency of null alleles and the lack of allelic diversity at particular loci rendered us unable to conduct a sufficient pedigree analysis within and among the 10 feral colonies. We were also unable to use the molecular data to infer the effective population size of the feral bees in our study [43] because our sample size was just 10 colonies, too low for the analytical tools currently available.

Previous research on feral populations of honey bees has focused on population genetics, trying to determine what level of genetic difference exists between the feral and managed colonies in a region. Delaney et al. [44] have shown that the genetics of the feral colonies and the managed colonies nearby can be distinct (see also Seeley et al., in press). In the present study, for example, the queens from Apiary 1 were from a single commercial source, and it is apparent from the number of unique drone alleles that these colonies were not immediately introducing a significant number of novel alleles into the surrounding feral population. This separation in genetic identity is even more apparent when comparing pairwise F_{st} analysis between individuals from the Arnot Forest and the two managed apiaries. As such, we can conclude that the queens of the feral colonies in the Arnot Forest rely considerably, although not exclusively, on the drones produced by other feral colonies in this population.

Our findings support the null hypothesis that honey bee queens in feral colonies mate at the same frequency as those in managed colonies. Therefore, our evidence does not support the

Table 2. Alleic frequencies of drone fathers.

Locus HB-TDE-03				Locus 107				Locus A113			
	Arnot Forest	Apiary 1	Apiary 2		Arnot Forest	Apiary 1	Apiary 2		Arnot Forest	Apiary 1	Apiary 2
104	1.03			144		3.13		146		15.43	
144		4.00		152	9.09			148		1.14	
146	1.03			154	3.64		0.91	154		0.57	
156	6.19			156	3.64		1.36	166			0.53
158	3.09			158	1.82	5.21	4.09	176			0.53
160	2.06			160	10.91		3.64	208	4.00		1.59
162	10.31	13.00		162	36.36	8.33	15.91	214	28.00	14.86	32.28
164	4.12			164	5.45	2.08	10.00	216	4.00	4.00	3.17
166	1.03	1.00		166	1.82	3.13	4.09	220	39.33	40.57	50.26
168	7.22	5.00		168	1.82	20.83	16.36	222	4.00	8.00	1.06
172	4.12			170	3.64	7.29	4.09	226	12.67	8.00	4.23
178		5.00		172	5.45	1.04	11.82	228	6.00	3.43	5.29
180	1.03			174			0.91	230	2.00	4.00	
182	7.22	6.00	3.57	176	1.82		2.27	234			1.06
184	1.03	1.00	12.50	178	12.73	6.25	8.64				
186		1.00		180			2.73				
188	4.12		2.68	182	1.82						
190	1.03	1.00		183		1.04					
192	4.12	24.00	11.61	184		11.46	11.82				
194	31.96	32.00	51.79	196			0.45				
196	4.12	7.00	5.36	198			0.45				
198	2.06		10.71	214		3.13					
200	2.06		1.79	216		6.25					
220	1.03			220		10.42					
				222		5.21					
				228		5.21					

Locus AC006				Locus A024				Locus A28			
	Arnot Forest	Apiary 1	Apiary 2		Arnot Forest	Apiary 1	Apiary 2		Arnot Forest	Apiary 1	Apiary 2
100		0.58		92	10.64	12.90	13.55	92		6.09	
102		1.73		94	3.19	9.68	25.81	94		2.61	
104		12.72		96	2.13			100		0.87	
146	46.43	49.13	58.80	98	1.06			102		12.17	
148	17.26	2.89	6.48	100			4.52	104		25.22	
150	3.57		3.70	102	17.02	4.84	15.48	106		1.74	
154	11.90	20.81	14.81	104	19.15	37.10	35.48	126	0.63		
156	17.86	9.25	13.43	106	6.38		3.87	128	6.96	11.30	17.03
158	2.38	2.89	0.93	128	7.45			132	0.63		
160			0.46	134	32.98	35.48		134	74.05	40.00	82.97
164			0.46	146			1.29	136	3.16		
168	0.60							140	1.90		
214			0.46					144	6.33		

(Continued)

Table 2. (Continued)

	Arnot Forest	Apiary 1	Apiary 2		Arnot Forest	Apiary 1	Apiary 2		Arnot forest	Apiary 1	Apiary 2
220			0.46					**150**	1.90		
								152	4.43		

Locus A88	Arnot Forest	Apiary 1	Apiary 2	*Locus AP66*	Arnot Forest	Apiary 1	Apiary 2	*Locus AP81*	Arnot forest	Apiary 1	Apiary 2
128	3.15	6.99		**91**	29.03	4.65	87.21	**93**		2.76	
132	2.36			**93**	41.94	41.86	4.65	**124**	5.56	2.76	
134	19.69	42.66	6.50	**96**	12.90	1.55	6.98	**126**	9.26	34.48	16.20
136	43.31	21.68	41.46	**99**	16.13	1.55	1.16	**134**	64.20	52.41	73.74
140	2.36	10.49	10.57	**101**		0.78		**135**		0.69	
142	3.15			**134**		4.65		**136**	12.35	1.38	7.82
144	13.39	14.69	29.27	**136**		17.83		**138**	7.41	4.83	2.23
146	1.57		2.44	**140**		2.33		**150**	1.23	0.69	
150	1.57	0.70	0.81	**142**		3.88					
152	9.45	2.80	8.94	**144**		17.05					
				146		0.78					
				150		3.10					

Locus B124	Arnot Forest	Apiary 1	Apiary 2
134		2.80	
214	5.13		
216	29.49	39.16	42.00
218	10.26	17.48	12.00
220	5.13	9.79	10.00
222	8.97	3.50	9.33
224	20.51	14.69	8.67
226	5.13	8.39	4.67
230	2.56	2.10	10.00
232	6.41		1.33
234	1.28	2.10	0.67
236	5.13		1.33

Allele frequencies for drone fathers from each population (Arnot Forest, Apiary 1, and Apiary 2) for 10 variable microsatellite loci. Unique alleles for each population are underlined.

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hypothesis that the population of feral colonies living in the Arnot Forest is able to persist despite the known infestation with *Varroa destructor* [13] and its associated viruses because of heightened levels of polyandry by their queens. Prior studies that have demonstrated strong effects of high intracolonial genetic diversity on resistance to devastating infections have generally tested the effects of extreme differences in queen mating frequency (i.e., single-mating vs. multiple-mating) rather than the differences that are typical of the normal variation in mating frequency (e.g., [45,46]). Neumann and Moritz [47] looked at the correlation between *Varroa* infestation level and queen mating frequency across a typical range of queen mating frequencies and found no significant association. The fact that even a rather low level of polyandry ($\geq$7 mates) strongly enhances a colony's phenotype suggests that there is some threshold benefit of polyandry above which there are diminishing returns to additional genetic diversity, at least with respect to resisting parasites and pathogens (c.f., [48]).

Exceptionally high levels of queen polyandry are universal among species of the honey bee genus *Apis* [17]. The results of our comparison of feral and managed honey bees in *Apis mellifera* support the view that it is unlikely that the mean mating frequency of queens is a labile trait than can rapidly evolve to adapt to ecological challenges. Franck et al. [49] compared two subspecies in different habitats in Africa and found differences in mating frequency between them, but these populations probably diverged long ago and therefore have experienced protracted selection for higher or lower mating frequencies. The results reported here, which found no difference in mating frequency of queens in the feral colonies in the Arnot Forest vs. queens in the nearest managed colonies outside this forest, supports the notion that mating frequency of queen honey bees is shaped by natural selection acting on an evolutionary, not an ecological, timescale.

Acknowledgments

Author Contributions

Conceived and designed the experiments: TDS DRT DAD. Performed the experiments: TDS DAD. Analyzed the data: DRT DAD. Contributed reagents/materials/analysis tools: DRT DAD. Wrote the paper: TDS DRT DAD.

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