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ORIGINAL RESEARCH

Selection for outbreeding in *Varroa* parasitising resistant honey bee (*Apis mellifera*) colonies

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Abstract

Parasitism is expected to select for counter-adaptations in the host: driving a coevolutionary arms race. However, human interference between honey bees (*Apis mellifera*) and *Varroa* mites removes the effect of natural selection and restricts the evolution of host counter-adaptations. With full-sibling mating common among *Varroa*, this can rapidly select for virulent, highly inbred, *Varroa* populations. We investigated how the evolution of host resistance could affect the infesting population of *Varroa* mites.

We screened a *Varroa*-resistant honey bee population near Toulouse, France, for a *Varroa* resistance trait: the inhibition of *Varroa's* reproduction in drone pupae. We then genotyped *Varroa* which had co-infested a cell using microsatellites. Across all resistant honey bee colonies, *Varroa's* reproductive success was significantly higher in co-infested cells but the distribution of *Varroa* between singly and multiply infested cells was not different from random. While there was a trend for increased reproductive success when *Varroa* of differing haplotypes co-infested a cell, this was not significant. This suggests local mate competition, through the presence of another *Varroa* foundress in a pupal cell, may be enough to help *Varroa* overcome host resistance traits; with a critical mass of infesting *Varroa* overwhelming host resistance. However, the fitness trade-offs associated with preferentially co-infesting cells may be too high for *Varroa* to evolve a mechanism to identify already-infested cells. The increased reproductive success of *Varroa* when co-infesting resistant pupal cells may act as a release valve on the selective pressure for the evolution of counter resistance traits: helping to maintain a stable host–parasite relationship.

KEYWORDS

co-evolution, genetics, local mate competition, optimality, reproduction, *Varroa destructor*

1 | INTRODUCTION

The conflicting fitness optima between hosts and parasites means the evolution of a beneficial adaptation in one is expected to select for a counter-adaptation in the other: driving a co-evolutionary arms race (Bell, 1982). The Red Queen Hypothesis predicts that a co-evolutionary arms race will drive oscillations in haplotype frequencies in hosts and parasites influenced by the relative frequency and fitness of matching alleles: selecting for increased genetic diversity, recombination, and evolutionary rates (Bell, 1982; Hamilton, Axelrod, &

Tanese, 1990; Kidner & Moritz, 2013). The typically short reproductive cycles of parasites, compared to their hosts, are expected to increase their rate of evolution. However, the highly inbred lifestyles of some parasites (Beaurepaire, Krieger, & Moritz, 2017) may make it difficult for a population to evolve when their host develops a new defense. This could be the case for the brood-parasitic mite *Varroa destructor*, Anderson & Trueman: a devastating parasite of honey bees (*Apis mellifera*, *L*.). Managed honey bee colonies, untreated with acaricides, typically die within 3 years of *Varroa* infestation. However, management practices can hinder the evolution of *Varroa* resistance in many honey bee populations.

Varroa mothers infest honey bee pupal cells shortly before capping. They then lay one male and up to four female eggs in the pupal cell (Rosenkranz, Aumeier, & Ziegelmann, 2010). The offspring hatch, feed on the developing pupa (Donzé & Guerin, 1994), and mate inside the natal cell. This is the only time in their lives when *Varroa* will mate. Mature, mated, daughter mites leave the cell with their mother and the eclosing bee, carrying all the stored sperms they will ever have for future reproduction. The male and any immature daughter mites desiccate inside the cell (Rosenkranz et al., 2010). The frequent occurrence of full-sibling mating between *Varroa* means populations can rapidly become highly inbred, with low proportions of heterozygotes, particularly in the middle of the brood season (June–July in Europe (Calis, Fries, & Ryrie, 1999)). This is when *Varroa* females are most likely to singly infest a cell and therefore cannot outbreed (Beaurepaire et al., 2017; Fuchs, 1992). This means alleles can rapidly become fixed in a *Varroa* population (Beaurepaire et al., 2017), a potential disadvantage in a co-evolutionary arms race. With the vast majority of *Varroa* infestations in managed honey bee colonies treated with acaricides (Rosenkranz et al., 2010), there is strong positive selection for the evolution of *Varroa* resistance to these chemicals (Beaurepaire et al., 2017; González-Cabrera et al., 2016). However, there has been little research into how *Varroa* can adapt when host resistance traits are allowed to coevolve through natural selection, causing dynamic shifts in *Varroa's* fitness optimum.

We investigated the potential for the evolution of counter resistance traits and selection for outbreeding in *Varroa* infesting resistant honey bee colonies near Toulouse, France (Kefuss, Vanpoucke, Bolt, & Kefuss, 2015). Colonies in this population have survived over 20 years of *Varroa* infestation without treatment using acaricides (Kefuss et al., 2015). Instead, natural selection has resulted in the evolution of a honey bee population with a greatly reduced rate of *Varroa* population growth (Kefuss et al., 2015). This reduced rate of *Varroa* population growth has been linked to the inhibition of *Varroa's* reproduction in pupal cells; particularly the drone brood where *Varroa* normally produces the most offspring (Conlon et al., 2019; Kefuss et al., 2015; Rosenkranz et al., 2010). We focus on the inhibition of *Varroa's* reproduction by infested drone pupae. Having identified colonies which exhibited unusually high rates of nonreproducing *Varroa*, we investigated the reproductive success of *Varroa* between singly and multiply infested cells. We then used genetic analyses to test if reproductive success was linked to genetic polymorphism in founding females. Selection for increased recombination, via outbreeding, could increase evolutionary rates in this *Varroa* population and indicate it is engaged in a co-evolutionary arms race with its host (Beaurepaire, Ellis, Krieger, & Moritz, 2019; Beaurepaire et al., 2017; Bell, 1982; Hamilton et al., 1990).

2 | MATERIALS AND METHODS

2.1 | Honey bee colony screening

Colony screening took place using a *Varroa*-resistant honey bee population near Villemur-sur-Tarn, Haute Garonne, France (Conlon et al., 2019; Kefuss et al., 2015; Rosenkranz et al., 2010). All drone pupal cells in the screened colonies were opened. Cells containing pupae from the white-eyed stage onwards were phenotyped using the number of infesting *Varroa* foundresses and the presence of male and female offspring. By this age, male and female *Varroa* offspring were easily visible to the naked eye. Only cells where *Varroa* had produced male and female offspring were classed as successfully reproducing as this would imply at least one mature, mated, daughter, capable of reproducing herself, will leave the cell with the emerging bee. Cells with no offspring or only sons, which desiccate in the natal cell after the bee has emerged, were classed as nonreproducing. Cells where the mite had died were included when calculating the expected and actual proportions of multiply infested cells, as the mite was presumed to have been alive when entering the cell, but not in the analysis of reproductive success. All mites were collected and stored in ethanol at −20°C until the DNA extraction.

To minimize the effect of the host, we sampled from closely related (one mother queen and three daughter queens) colonies found at four different sites within a 5 km radius. Colonies were considered resistant if >30% of infesting *Varroa* failed to reproduce and susceptible if <15% failed to reproduce. (Martin, Holland, & Murray, 1997). As resistance in the mother colony had been linked to a single locus (Conlon et al., 2019), we also calculated variation from a 50:50 distribution of resistant to nonresistant for all infested drone pupae using a chi-squared test for equal proportions in R (R Core Team, 2017). As we were interested in the potential for coevolution between *Varroa* and resistant honey bee hosts, and a single susceptible colony is too low a number to produce a statistically sound test of resistant versus susceptible colonies, only those colonies classified as resistant were included in subsequent analyses.

2.2 | Mite density and distribution

Using the screening data, we calculated the expected proportion of multiply infested drone cells, if mites were distributed randomly, using a Poisson distribution:

$$p_i = \frac{L^i}{i!} \times e^{-L}$$

| Colony | Uninfested cells | Infested cells | | | Number of mites |
		Single	Multiple	Dead	
Mother	385	78	20	9	119
Daughter 1	41	13	6	1	30
Daughter 2	117	15	8	3	35
Daughter 3	22	14	18	1	61

TABLE 1 Screening data for honey bee colonies with the number of uninfested cells, singly infested cells, multiply infested cells, the number of cells in which the *Varroa* had died and the total number of *Varroa* collected (Resistant colonies: Mother, Daughter 1, and Daughter 2; Susceptible colony: Daughter 3)

where p is the probability that a mite will enter a cell already infested by i mites and L is the mean infestation calculated from the number of mites (V) and the number of available drone cells (C) (Fuchs, 1992).

$$L = \frac{V}{C}$$

The difference between the expected and observed proportions was tested using a chi-squared test in R (R Core Team, 2017). As the calculation for the expected proportion of multiply infested drone cells is a function of the number of available drone cells in a colony, we calculated the observed and expected proportions separately for each colony.

2.3 | Reproductive success

We tested for a link between the number of infesting *Varroa* and reproductive success in the drone pupae of resistant honey bee colonies using a binomial GLMM in the *lme4* package (Bates, Mächler, Bolker, & Walker, 2015) for R (R Core Team, 2017). We used reproductive success as a binary response variable, the number of foundresses as a fixed variable, and colony as a random effect.

2.4 | Genetic polymorphism

For each resistant colony, DNA for mites from multiply infested drone cells ($n = 34$) was isolated by washing the mites twice with double-distilled H_2O and dried before being crushed in a PCR plate containing 100 μl of 5% Chelex solution. 5 μl of Proteinase K was then added to each sample. Total mite DNA was isolated from the individuals using standard Chelex thermocycling conditions (Walsh, Metzger, & Higuchi, 1991) for genotyping at LMU Biozentrum (Munich, Germany). Seven microsatellite markers (Vdes01, Vdes04, VD112, VD152, VD307, VJ292, and VJ294) (Cornman et al., 2010; Evans, 2000; Solignac et al., 2003) were tested.

The minimum number of haplotypes across all sampled mites was inferred for multiply infested drone cells. We counted the number of drone cells where only one haplotype was identified (monomorphic; *i.e.*, genetically identical foundresses) and those with at least two haplotypes identified (polymorphic). For missing data or heterozygous alleles, we assumed the minimum possible number of genotypes were present; we therefore risked underestimating, rather than overestimating, genetic diversity within a cell.

We tested whether the number of polymorphic cells was significantly different than expected if the mites were distributed randomly using a chi-squared test. The expected number of polymorphic cells was calculated by randomly permuting the observed genotypes between cells in each colony and averaging the number of polymorphic cells obtained over 10,000 permutations. We then tested whether *Varroa* in polymorphic cells were more likely to reproduce than *Varroa* in monomorphic cells using a GLMM in the *lme4* package (Bates et al., 2015) for R (R Core Team, 2017). We used reproductive success as a binary response variable, genetic polymorphism between the founding *Varroa* as a fixed variable, and colony as a random effect.

3 | RESULTS

3.1 | Colony screening

We screened four honey bee colonies, three (Mother; Daughter 1; and Daughter 2) resistant and one (Daughter 3) susceptible (Table 1). The mother colony was produced in 2015 while the 3 daughters came from 2016. None of the resistant colonies varied significantly from a 50:50 distribution of resistant:susceptible pupae (Mother: resistant= 32, susceptible = 38, $\chi^2 = 0.357$, $df = 1$, $p = .550$; Daughter 1: resistant = 8, susceptible = 5, $\chi^2 = 0.308$, $df = 1$, $p = .579$; Daughter 2: resistant = 4, susceptible = 9, $\chi^2 = 1.231$, $df = 1$, $p = .267$) while the susceptible colony exhibited significant variation (resistant = 1, susceptible = 12, $\chi^2 = 7.692$, $df = 1$, $p = .006$). In total, we identified 140 infested drone cells in resistant colonies; of which 34 (24%) were multiply infested. Of these 13 *Varroa* foundresses had died after infesting the cell leaving 127 cells for further analyses.

3.2 | Reproductive success and mite density

Varroa were significantly more likely to reproduce successfully in multiply infested drone cells in the resistant honey bee colonies (Figure 1a; Multiple 80% success, Single 54% success; GLMM, Multiple versus Single infestation: $\chi^2 = 7.396$, $df = 1$, $p = .007$). However, we identified no significant differences in the expected versus observed rates of singly infested cells in resistant colonies (Figure 1b; Mother: $\chi^2 = 0.045$, $df = 1$, $p = .833$; Daughter 1: $\chi^2 = 0.206$, $df = 1$, $p = .650$; Daughter 2: $\chi^2 = 1.475$, $df = 1$, $p = .225$). The number of sons in cells classified as successfully reproducing always matched
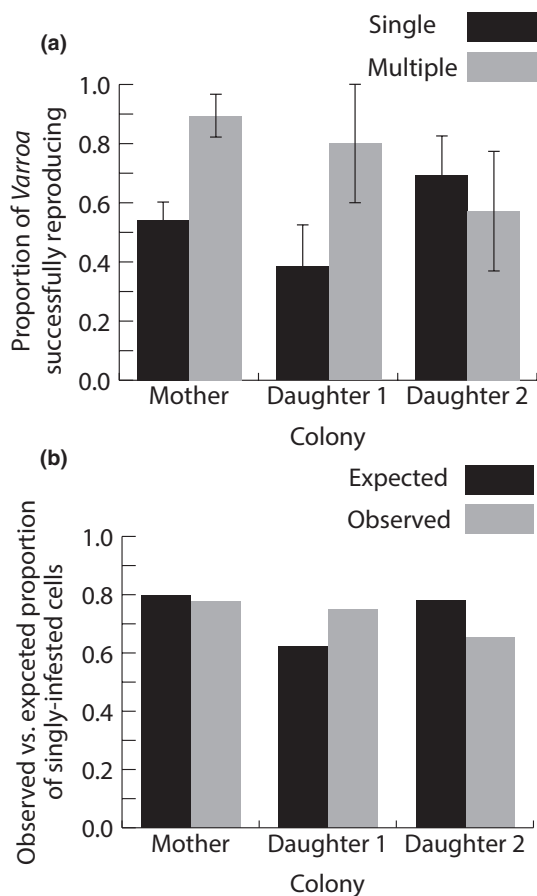
FIGURE 1 (a) *Varroa* is significantly more likely to successfully reproduce in multiply infested cells in resistant colonies (Resistant: Mother, Daughter 1, and Daughter 2). Mother: *n* = single 78:20 multiple; Daughter 1: *n* = single 13:6 multiple; Daughter 2: *n* = single 15:8 multiple. GLMM, Multiple versus Single infestation: $\chi^2 = 7.396$, *df* = 1, *p* = .007. Error bars show ± Standard Error. (b) The proportion of observed versus expected singly infested cells does not differ significantly for any colony (Resistant colonies: Mother, Daughter 1, and Daughter 2)

the number of foundresses. As *Varroa* mothers lay only one male egg per reproductive episode (Rosenkranz et al., 2010), this indicates all mothers laid at least one egg in multiply infested cells where *Varroa* successfully reproduced (Colony screening data, available on Dryad).

3.3 | Genetic polymorphism

Across the 3 resistant colonies, DNA extraction and microsatellite amplification was successful for 31 multiply infested pupal cells. Three microsatellites were identified as polymorphic (VJ294, VD307, and Vdes02; two alleles per locus) and retained for further analyses.

While there was a trend for increased reproductive success in polymorphic cells, it was not significant (Figure 2a; 77.8% success in polymorphic cells, 58.3% in monomorphic cells; GLMM-Monomorphic vs. polymorphic: $\chi^2 = 1.667$, *df* = 1, *p* = .197). The number of polymorphic cells in each colony was slightly higher than expected (Figure 2b), except for Daughter 1, although the difference
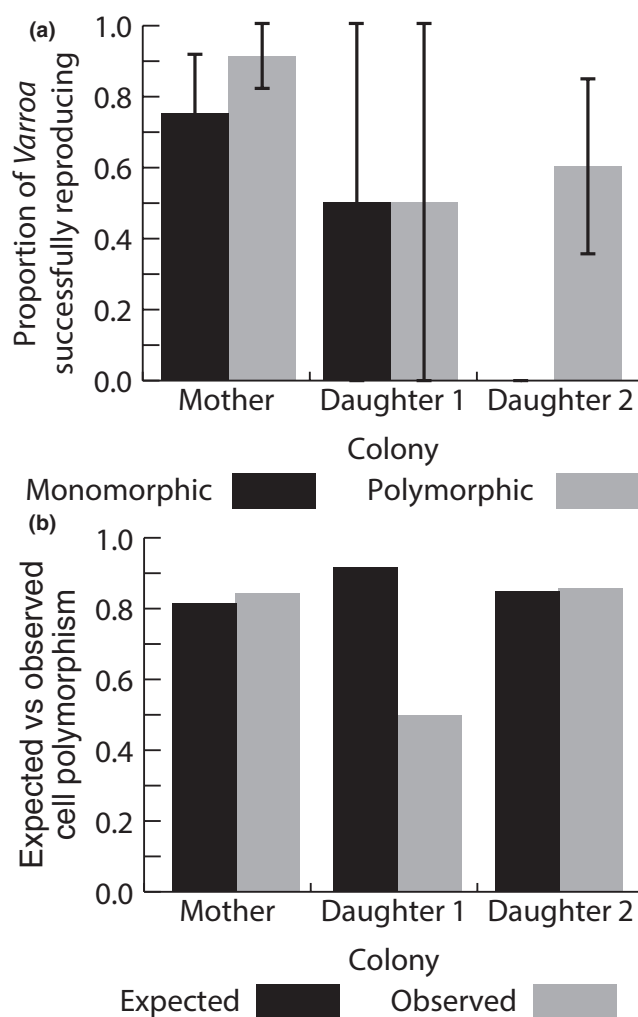
FIGURE 2 (a) A nonsignificant trend for increased *Varroa* reproductive success in polymorphic cells in resistant honey bee colonies (Resistant colonies: Mother, Daughter 1, and Daughter 2). Mother: *n* = monomorphic 3:16 polymorphic; Daughter 1: *n* = monomorphic 2:2 polymorphic; Daughter 2: *n* = monomorphic 1:6 polymorphic. Error bars show ± Standard Error. (b) The proportion of observed versus expected polymorphic cells did not differ significantly for any colony (Resistant colonies: Mother, Daughter 1, and Daughter 2)

was not significant ($\chi^2 = 0.49$, *df* = 3, *p* = .92). While the nonsignificant result could be due to sample size, it was not possible to collect more samples as every drone cell in the colonies was opened during screening.

4 | DISCUSSION

We investigated the potential for the evolution of counter-adaptations and selection for outbreeding in *Varroa* parasitising *Varroa*-resistant honey bee colonies: focusing on the inhibition of *Varroa's* reproduction by host drone pupa. During our initial colony screening, we identified 3 resistant colonies (1 mother, 2 daughter) and 1 nonresistant colony (Daughter 3). That only the

nonresistant colony exhibited significant variation from a 50:50 ratio of Resistant:Susceptible pupae, with no intermediate levels of resistance, supported the previous identification of a single resistance-linked locus in this population (Conlon et al., 2019) and suggests Mendelian inheritance of the resistance trait.

Varroa were significantly more likely to successfully reproduce when multiply infesting drone pupal cells in a resistant honey bee colony, with a nonsignificant trend for this to increase further when foundresses were polymorphic. This could suggest that *Varroa* can overcome the host resistance trait by multiply infecting a resistant drone cell. However, *Varroa's* distribution between multiply and singly infested drone cells was not significantly different from random. This suggests the mites either cannot identify or do not preferentially enter already-infested cells and cannot discriminate between co-foundresses based on relatedness.

The increased reproductive success for *Varroa* when foundresses co-infest a cell could suggest selection for an increased evolutionary rate, through outbreeding and recombination, in *Varroa* (Bell, 1982; Hamilton et al., 1990). This could be particularly important in our colonies where the evolution of host defenses means successful reproduction is less likely in singly infested cells but still possible in multiply infested cells. By contrast, the artificial selection pressure of human-applied acaricide treatments, where *Varroa's* reduced reproductive success is not a trait of the infested pupa, means resistant haplotypes can rapidly approach fixation in a largely inbred population (Beaurepaire et al., 2017; González-Cabrera et al., 2016). As the distribution of *Varroa* in our colonies was not significantly different from random, it appears *Varroa* cannot detect or does not preferentially enter already-infested cells. However, particularly when mite density is low, a prospective foundress faces several trade-offs in deciding whether or not to infest a cell.

Varroa foundresses were more likely to reproduce successfully when multiply infesting a cell. However, the increased likelihood of mortality and reduced fertility after a long phoretic phase, outside of the honey bee pupal cell (Nazzi & Le Conte, 2016; Rosenkranz et al., 2010), could explain why mite-distribution was not significantly different from random in our colonies. When mite density in a colony is low, the search time for an already-infested cell increases (Fuchs, 1992). Eventually, it could be expected that the fitness cost of entering an uninfested cell, and undergoing an inbred or failed reproductive cycle, will become less than that of continuing the search and risking reduced fertility or mortality (Charnov, 1976; Nazzi & Le Conte, 2016; Parker & Maynard Smith, 1990; Rosenkranz et al., 2010). This could explain why we detected significant differences in *Varroa's* reproductive success between singly and multiply infested cells but not in the distribution of *Varroa* among cells; any mechanism to detect and preferentially enter already-infested cells may not be used often enough to warrant its maintenance by selection. Therefore, the only criterion *Varroa* uses when deciding to enter a cell is whether it is of the right age, although this leaves open the question of why *Varroa* is still more likely to successfully reproduce when infesting a cell with other foundresses, that could be the result of local mate competition (Hamilton, 1967).

It has previously been shown that the number of *Varroa* daughters per-mite decreases as the number of infesting mites per-cell increases; a consequence of fewer eggs per-foundress rather than complete nonreproduction by some mites (Fuchs & Langenbach, 1989). With *Varroa's* first egg being male, this shifts the sex ratio from 22:78 toward 50:50 (Fuchs & Langenbach, 1989; Hamilton, 1967). It has been hypothesized that *Varroa* require the presence of specific host compounds, above a minimum threshold, in their diet in order to successfully initiate a reproductive cycle (Conlon et al., 2019). However, the presence of another female mite may reduce this threshold. *Varroa* could then invest the limited resources at its disposal in at least one unfertilized egg, a haploid male, in an attempt to benefit from the reproductive investment of another female. This reduced investment in offspring could be an adaptation to overcome the fitness costs associated with increased competition for food, and valuable host compounds, when co-infesting a cell. Reduced individual investment in a reproductive cycle, when co-infesting pupal cells, could therefore help *Varroa* overcome host resistance when mite density is high.

Acaricide treatment prevents *Varroa* resistance from evolving in many managed honey bee colonies, thereby preventing the evolution of a stable host–parasite relationship (Bell, 1982; Hamilton et al., 1990). Our results show that, when *Varroa* resistance is allowed to develop by natural selection (Fries & Bommarco, 2007; Kefuss et al., 2015), it is possible for a host–parasite relationship to evolve. The increased reproductive success we identify when *Varroa* co-infests the drone pupae of resistant honey bee colonies means that, in contrast to acaricide-treated colonies (Beaurepaire et al., 2017; González-Cabrera et al., 2016), there may be selection for outbred offspring. This, combined with a small proportion of *Varroa* reproducing in each generation, could reduce the selective pressure for the evolution of more virulent counter resistance traits and result in a more stable host–parasite relationship.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Benjamin H. Conlon: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (lead); writing – review & editing (equal). **Chedly Kastally:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – review & editing (equal). **Marina Kardell:** Investigation (supporting); writing – review & editing (supporting). **John Kefuss:** Investigation (supporting); methodology (supporting); writing – review & editing (supporting).

Robin F. A. Moritz: Conceptualization (equal); methodology (equal); resources (equal); supervision (supporting); writing – review & editing (supporting). **Jarkko Routtu:** Conceptualization (equal); funding acquisition (equal); methodology (equal); project administration (equal); supervision (lead); writing – review & editing (equal).

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