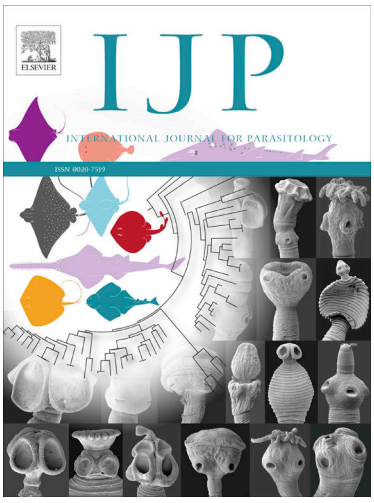

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Host brood traits, independent of adult behaviours, reduce *Varroa destructor* mite reproduction in resistant honeybee populations

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Abstract

The ectoparasitic mite *Varroa destructor* is an invasive species of Western honey bees (*Apis mellifera*) and the largest pathogenic threat to their health world-wide. Its successful invasion and expansion is related to its ability to exploit the worker brood for reproduction, which results in an exponential population growth rate in the new host. With invasion of the mite, wild honeybee populations have been nearly eradicated from Europe and North America, and the survival of managed honeybee populations relies on mite population control treatments. However, there are a few documented honeybee populations surviving extended periods without control treatments due to adapted host traits that directly impact *Varroa* mite fitness. The aim of this study was to investigate if *Varroa* mite reproductive success was affected by traits of adult bee behaviours or by traits of the worker brood, in three mite-resistant honey bee populations from Sweden, France and Norway. The mite's reproductive success was measured and compared in broods that were either exposed to, or excluded from, adult bee access. Mite-resistant bee populations were also compared with a local mite-susceptible population, as a control group. Our results show that mite reproductive success rates and mite fecundity in the three mite-resistant populations were significantly different from the control population, with the French and Swedish populations having significantly lower reproductive rates than the Norwegian population. When comparing mite reproduction in exposed or excluded brood treatments, no differences were observed, regardless of population. This result clearly demonstrates that *Varroa* mite reproductive success can be suppressed by traits of the brood, independent of adult worker bees.

Keywords: *Apis mellifera, Varroa destructor*, Natural selection, Suppressed mite reproduction (SMR), *Varroa*-resistant honey bees

1. Introduction

The *Varroa destructor* mite is an invasive ectoparasite of the Western honey bee (*Apis mellifera*) and undeniably the largest pathogenic threat to honey bee health, severely impacting apiculture and agricultural crop production that relies on honey bees for pollination services. The *Varroa* mite is completely dependent on the honey bee colony for survival with a reproduction cycle tightly synchronized to pupa development inside brood cells (Steiner et al., 1995; Rosenkranz et al., 2010). In the mid-20th century, the *Varroa* mite made a host jump from the Asian honey bee (*Apis cerana*) to the Western honey bee species and has successfully spread throughout the world, with only a few isolated locations remaining mite-free (de Guzman and Rinderer, 1999; Oldroyd, 1999; Rosenkranz et al., 2010).

One of the most significant factors influencing the successful invasion and expansion of the *Varroa* mite with its new host is the ability of the mite to exploit and capitalize on the worker brood for reproduction. In contrast, Asian honey bees exhibit a variety of host traits that limit the ability of mites to reproduce in worker brood cells, acting as a natural control of the mite population growth (Lin et al., 2018; Wang et al., 2020). While some similar host traits exist in Western honey bees, they are far less pronounced and highly variable between subspecies (Corrêa-Marques et al., 2002; Danka et al., 2011; Lin et al., 2016). Unrestricted access to thousands of worker brood cells in colonies of Western honey bees provides the mite with many more opportunities to reproduce, compared with Eastern honey bees. This contributes to an exponential population growth rate of the mite in this new host.. During the mite's reproductive phase, it feeds on developing pupae and vectors detrimental honey bee viruses, in particular *Deformed wing virus* (DWV), causing crippled, flightless adult honey bees with significantly shortened life spans, ultimately resulting in the loss of colony function (de Miranda and Genersch, 2010; Wilfert et al., 2016). To avoid viral infections killing the honey bee colony, mite population control treatments are required in apiculture. The *Varroa*-virus complex has caused a near complete eradication of wild honey bee colonies in Europe and North America (Le Conte et al., 2010). However, there are small sub-populations that have survived extended periods without *Varroa* mite control treatment and have documented resistant and tolerant host phenotypes to both the *Varroa* mite and their viruses (Locke et al., 2012; Locke, 2016a; Oddie et al., 2018).

Within populations of *A. mellifera* there is large natural variation in the mite's reproductive success, which is rarely 100% (Gregorc et al., 2016; Mondet et al., 2020). Mite reproductive success is defined as the ability of a mother mite to produce a viable mated female offspring before the bee emerges from its brood cell as an adult. Suppressed mite reproduction (SMR), is a term first coined by Harbo and Harris (1999), referring to a hereditary phenotype of a honey bee colony that causes *Varroa* mites to have a reduced reproductive success rate. This phenotype will undoubtedly have a significant influence on mite population growth and thus the development of virus infections and the life-span of the colony. It is also a trait of economic importance as a selection criterion for honey bee mite-resistant breeding programs. In naturally adapted mite-resistant honey bee populations, the mite's reproductive success rate has been recorded to be as low as 50% (Locke et al., 2012; Locke, 2016a; Oddie et al., 2018). However, the underlying host mechanisms responsible for expression of the SMR phenotype in any honey bee population, those in breeding programs or those that are naturally mite-resistant, remain elusive. It has been proposed that SMR is related to adult honey bee hygienic behaviors (Harbo and Harris, 2005; Harris, 2007). An example is Varroa Sensitive Hygiene (VSH) behavior, where adult bees selectively remove brood parasitized with reproducing mites while ignoring brood with non-reproductive mites. This behavior results in the appearance of a higher rate of non-reproducing mites (Ibrahim and Spivak, 2006; Danka et al., 2011; Harris et al., 2012). Another honey bee behviour that could relate to the SMR phenotype is uncapping

and recapping of the wax cap placed over the brood cell by adult workers. This behavior could potentially disrupt the timing of mite reproduction, or even physically displace or damage the mites in the brood cell (Oddie et al., 2018, 2021). Another explanation for the SMR phenotype is related to traits of the worker brood such as altered volatile expression patterns that could inhibit mite reproduction (Locke et al., 2012; Frey et al., 2013). The mite uses volatile compounds from the cuticle of the larvae and pupae, that vary during specific developmental stages through pupation, as the signal to either initiate or inhibit the onset of egg laying (Frey et al., 2013; Nazzi and Le Conte, 2016).

The aim of this study was to gain a better understanding of the honey bee host mechanisms responsible for the SMR phenotype. This was approached by separating the adult bee behaviors from brood traits and measuring the rate of *Varroa* mite reproductive success. We examined three naturally adapted mite-resistant honey bee populations from Sweden, Norway and France that express SMR (Locke and Fries 2011; Locke et al., 2012; Oddie et al., 2017) and compared them with a local mite-susceptible population as a control group. The origin and phenotypes of the three naturally surviving honey bee populations examined in this study have been abundantly described (Locke, 2016a; Oddie et al., 2017). Briefly, these populations have evolved independently without mite control since 1994 (Avignon, France; (Le Conte et al., 2007)), 1999 (Gotland, Sweden; (Fries et al., 2003)) and 2001 (Oslo, Norway; (Oddie et al., 2017)). Adult bees were restricted from sections of brood on the same hive frame as brood that was exposed to adult bees. The hypothesis was that if mite reproductive success was reduced in the worker brood that was excluded from adult bees, then brood traits would be a significant contributor to the SMR expression in these populations, independent of the adult worker behaviors. Specific reasons for failed mite reproduction were also examined to compare and identify differences between the mite-resistant populations.

2. Materials and methods

2.1. Genetic background and colony establishment

During the summer of 2016, queens from each of these three populations were produced, mated in their original geographic locations and transported to Sweden according to European Union (EU) legislation guidelines. Queens from a local Swedish mite-susceptible honey bee population were similarly produced and used as controls. All queens were established in Swedish standard hives (Lågnormal, LP Biodling, Sweden) at a single apiary located at the Swedish University of Agricultural Sciences, Uppsala, at the Lövsta research station (GPS Coordinates: 59° 50’ 2.544”N, 17° 48’ 47.447”E). In the autumn of 2016, all colonies were treated against *Varroa* mites using tai-fluvalinate (ApistanRegisted, Vita Europe, UK) to equalize the mite infestation pressure.

2.2. Experimental design

The study was performed during August of 2017 with additional data collected in August 2019. The experiemental mite-resistant colonies had their genetic origin in Norway (n = 3), Sweden (n = 5) and France (n = 4), meaning the queens of these colonies were produced, mated and transported from their country of origin. A control group of colonies was included in the study with their origin being a Swedish mite-susceptible population (n = 5). The queens from each colony were confined to a single frame of drawn-out wax using a queen-excluder frame-cage in order to obtain frames with brood of uniform age. After 48 – 72 h, when the frames were full of eggs, the queen excluder was removed. Then, frames were checked daily to monitor the brood development and observe when the brood started to be capped. At ~8-9 days after queen egg laying, when the majority of the larval brood cells had just been sealed for

pupation, a section covering an estimated 500 sealed brood cells was designated for the exclusion treatment and isolated from contact with adult workers. Initially a metal cage was pressed into the wax around the designated brood to exclude adult bee access (Fig. 1A). While this metal cage generally served its purpose in excluding adult bees, it was inconsistant and adult bees managed to dig through the wax to get inside the caged area in a few colonies, which were then excluded from the analysis. Therefore, the brood exclusion method was adapted to use a nylon covering stapled to the wooden frame (Fig. 1B). This method was more consistent and effective at excluding adult bees from the brood. Approximately 500 worker brood cells on the same frame were used as the adult honey bee exposure treatment group.

2.3. Frame dissection and mite reproduction evaluation

When the brood cells were ~9 days post capping, at which time mite reproductive success is possible to assess, the frames were removed from the colonies for dissection. In order to evaluate the mite reproductive success in individual brood cells, cell caps were removed using a scalpel, and the pupa and mite families were carefully removed from the cell using forceps and a fine paint brush according to standard methods (Dietemann et al., 2013; Table 1). Individual cell content was analyzed using a stereoscopic microscope (Leica MZ75, 6.5X magnification, Leica Microsystems, Germany). The pupal developmental stage, the number of mite offspring and their developmental stage, were recorded and compared with each other to evaluate mite reproductive success (Supplementary Table S1). A mite was considered to have successfully reproduced if it had produced a male offspring and a viable female offspring that would mature and mate with each other before the bee emerges from the brood cell as an adult (Dietemann et al., 2013). If a mite failed to reproduce, the reason for failure (absence of a male, delayed egg laying, dead progeny or infertility of the mother mite) was recorded (Supplementary Table S1), together with mite fecundity (total number of offspring produced; Dietemann et al., 2013). Brood cells were opened until a minimum of 30 infested cells were uncovered, or until all available cells were opened.

2.4. Statistical analyses

Statistical analyses were performed using R version 4.0.1 R Development Core Team, 2010. A language and environment for statistical computing: reference index. R Foundation for Statistical Computing, Vienna) and R Studio Version 1.3.959 (R Studio Team, 2020. RStudio: Integrated Development for R). Data was shown to be normally distributed using a Shapiro normality test. A linear mixed-effect model was performed with rate of mite reproductive success as the response variable, population origin and excluder treatment as the independent variables and colony and year as random effect variables. This was done to compare treatments across populations, to compare treatments within each population, and to compare fecundity using the packages “multcomp”, “lme4”, “nlme”, “car”, “lmertest”, “lsmeans”, and “dplyr”. Least-square means of the model were used to compare treatments between individual populations using the package “emmeans”. Interactions were included in the model and sequentially removed when significance was not detected. *P* value threshold of 0.05 was used to determine significance. All graphs were made using the package “ggplot2”.

2.5. Data accessibility

The datasets generated and/or analysed during the current study are available at the Swedish National Data Service, https://doi.org/10.5878/znc2-9b12.

3. Results

Mite reproductive success rates did not significantly differ between treatment groups of either caged brood or brood exposed to adult bees and their possible removal behaviors, irrespective of the population's genetic background ($\chi^2 = 2.45$, degrees of freedom (df) = 1, $P > 0.11$). The only variable that did influence *Varroa* mite reproductive success was the population's genetic background, irrespective of treatment ($\chi^2 = 44.51$, df =3, $P < 0.005$).

The average mite reproductive success rates were significantly lower in the French (estimate = 0.326, df = 14, t.ratio = 3.89, $P = 0.008$) and Swedish (estimate = 0.125, df = 14, t.ratio = 0.0784, $P < 0.005$) mite-resistant populations compared with the mite-susceptible control group (Fig. 2). The mite reproductive success in the Norwegian population was slightly lower than in the mite-susceptible controls, but was not significantly different (estimate = 0.125, df = 14, t.ratio = 1.35, $P = 0.55$; Fig. 2), while the average mite reproductive success rates were not different between the French and Swedish colonies (estimate= 0.121, df = 14, t.ratio = 1.57, $P = 0.42$; Fig. 2). Mite fecundity was also not affected by treatment ($\chi^2 = 0.806$, df = 1, $P = 0.37$), but was significantly affected by the colony background ($\chi^2 = 31.11$, df = 3, $P < 0.001$). The mite fecundity in the French and Swedish populations were similar to each other (estimate = 0.045, df = 14, t.ratio = 0.194, $P = 0.997$), but both were significantly different from the controls (Control-Sweden: estimate = 1.05, df = 14, t.ratio = 4.52, $P = 0.002$; Control-France: estimate = 1.01, df = 14, t.ratio = 4.00, $P = 0.006$), while the mites in the Norwegian colonies had similar fecundity rates to those in the control group (estimate = 0.38, df = 14, t. ratio – 1.41, $P = 0.52$).

Failed mite reproductive success, either due to the absence of a male mite, delayed egg laying, dead progeny or mite infertility was excluded from statistical analysis due to the small and uneven sample size (Table 2). Delayed egg laying was the most common reason for failed mite reproduction across all populations, while the absence of male mites occured more often in the French and Swedish colonies than in the Norwegian and control colonies (Fig. 3).

4. Discussion

The mite reproductive success rates and mite reproductive fecundity in this study were similarily low whether the parasitized brood was exposed to, or blocked off from, adult worker bees. This clearly demonstrates that *Varroa destructor* mite reproductive success can be suppressed by traits of the honey bee host brood, independent of adult worker behavioral traits.

With host-parasite relationships being particularly complex and intertwined, we do not exlude the potential for an additive effect of adult bee behavior on the expression of the SMR phenotype in any of these populations. However we believe these results eloquently reveal significant information regarding adaptations of host resistance and the SMR phenotype, in particular highlighting the role of host brood in *Varroa*-resistant honey bee populations.

The SMR phenotype has been widely considered to be an effect of the adult bee VSH behaviour (Harbo and Harris, 1999). The results of this study suggest that either VSH is not expressed to a significant degree in these colonies or that removal behaviors such as VSH do not specifically target the reproducing mites. A recent study examined the link between VSH and SMR, and found that the presence of mite offspring was not a crucial trigger for the VSH behaviour (Sprau et al., 2021).

The evolution of novel behaviors such as VSH is a complex and difficult process, even in the face of a strong natural selection such as high parasite load (Sokolowski, 2001). However,

many honey bee mite-resistant breeding programs focus on behaviors such as VSH, but have had difficulty in producing sustainable mite resistance. Selecting for these behavioral traits is laborious and their genetic basis is not entirely understood, with one study only able to explain 10% of variance in the trait (VSH) measured with two quantitative trait loci (Tsuruda et al., 2012). Other studies looking at the genetic basis for VSH found different genes associated with the trait, implying that this a multi-loci complex, most likely involving many genes of small effect (Spötter et al., 2016; Scannapieco et al., 2017).

Frey et al. (2013) showed that the reproductive cycle of the mite is highly sensitive to changes in the cuticular pheremonal compound profiles of the brood. Honey bees use a variety of pheromonal compounds, functioning as complex releaser and primer signals, to regulate social organization in the colony (Nazzi and Le Conte, 2016). Some of these compounds are exploited by the mites, who use them to locate targets for feeding and reproduction. Fatty acid esters (FAE) such as methyl palmitate, ethyl palmitate, and methyl linolenate, are pheromones that signal adult nurse bees to cap the cells of developing bee larvae and have been shown to also attract mites to the brood cells (Nazzi and Le Conte, 2016). Small changes in brood volatile quantities or timing could therefore reduce the fitness of the parasites by interrupting their reproduction cycle. This could potentially be a simpler adaptive strategy for honey bee resistance as opposed to adult bee behaviors.

There have also been studies indiciating that brood developmental traits influence the SMR phenotype. Two ecdysone-related genes (*Cyp18a1* and *Phantom*) have been linked to mite resistance in the Swedish naturally adapted honey bee population using whole-genome sequencing for a quantitative trait locus analysis of reduced mite reproductive success (Conlon et al., 2018). These genes regulate important enzymes for pre-pupal development and metamorphosis by controlling steroid levels (Rewitz et al., 2010). Unusual concentrations of steroid compounds during the pre-pupal phase could make the age of the pupae appear suboptimal and the mother mite would suspend oogenesis (Frey et al., 2013; Conlon et al., 2018). Additionally, the *Ecdysone*-regulating gene *Mblk-1* has been linked with mite resistance in another honey bee population from Toulous, France (Conlon et al., 2019) and is responsible for both initiating metamorphosis in insects and initiating the reproduction in *Varroa* mites, once they acquire it from their host during feeding (Ureña et al., 2014; Cabrera et al., 2015; Mondet et al., 2018; Takayanagi-Kiya et al., 2017; Mondet et al., 2018).

Delayed egg laying was the most common reason for failed mite reproduction across all populations in this study, similar to a pan-European study assessing mite reproduction (Mondet et al., 2020). However, the absence of male mite offspring was significantly higher in the Swedish and French populations, which also have on average higher overall mite reproductive failure, compared with the Norwegian and control populations. The first egg laid by the mother mite develops into the male offspring (Donzé and Guerin, 1994). Adaptations by the honey bee brood that disrupt the oviposition or development of the male mite would need to occur early during the mite reproductive phase. Future research could investigate if differences in the brood pheromones that mites use to syncronize reproductive timing specifically influence ovipositioning and timing in relation to the first male egg (Frey et al., 2013). Previous research on the French and Swedish populations found that the most likely cause for failed reproductive success was delayed egg laying for the Swedish population and infertility for the French population (Locke et al., 2012). In this study there were no apparent differences between these population in the reasons for reproductive failure. This could be due to the different environmental conditions between this and earlier experiments, the minimal number of examined brood cells or colonies, or changes in the population phenotypes since last investigated. Recent studies have found that the *Varroa* mite has more genetic diversity than previously thought and therefore is potentially capable of adapting through a host-parasite evolutionary arms race. (Moro et al., 2020). Further research looking into how honeybees

interrupt *Varroa* mite reproduction would be beneficial in understanding the fluidity of this system, and what type of selection both the mites and honey bees are undergoing.

The differences between the French and Swedish mite-resistant honey bee populations and the mite-susceptible control population in this study mirror previous work and suggest the heritability and fixed genetic nature of the SMR phenotype in these naturally adapted mite-resistant populations (Locke et al., 2012; Locke, 2016b). The Norwegian honey bee population mite reproductive success rates were not significantly different from the mite-susceptible control population, in contrast with the French and Swedish populations which were significantly different from the control.

This contrasts previous work on the Norwegian population showing more dramatic differences in SMR between them and susceptible populations, when examined in Norway (Oddie et al., 2017). This could suggest that either Norwegian honey bees express mite-resistant phenotypes better in their local environment which they have adapted to, that they are specifically adapted for Norwegian mites that genetically differ from the mites they were exposed to in this study (Moro et al., 2020), or there has been a loss of the genetic heritability of the SMR phenotype in this population. Local adaptation has been shown to be important for colony survival when exposed to *Varroa* mite infections (Büchler et al., 2014; Meixner et al., 2015). Additionally, gene versus environment interaction studies have shown that mite-resistant populations do not necessarily maintain their resistant traits when moved to a new environment (Büchler et al., 2014; Meixner et al., 2015; Kovačić et al., 2020). This could mean that the Norwegian population has some factor that increases their SMR in Norway that is not present in Sweden. Further, while previous studies found that the mites showed little to no adaptation since their transition from *A. cerana* to *A. mellifera* (Kraus and Hunt, 1995; Solignac et al., 2005), a recent study has shown that it is possible for mite populations to change their reproductive strategies in resistant populations (Moro et al., 2021). They investigated an isolated artificially selected Dutch honey bee population that once displayed VSH (Panziera et al., 2017), but now shows no signs of VSH 4 years later. Genetic variation in mite genotypes exist in mite-resistant honey bee populations (Beaurepaire et al., 2019; Moro et al., 2020) which could potentially influence their reproductive success. However, this variation does not explain the differences in the SMR phenotype between the colonies examined in this study, since all the test colonies were managed in the same apiary, originally established from the same local bees and mites, where drifting of mites between colonies is expected (Frey and Rosenkranz, 2014; Nolan and Delaplane, 2017).

This study clearly distinguishes that adult bee behaviors are not involved in the expression of the SMR phenotype in these naturally adapted mite-resistant honey bee populations. Although we hypothesise that the reduced reproduction of mites is influenced by brood factors in these populations, there could still be factors that we have not examined, such as hive environment, that could be influencing mite reproduction. Brood transfer experiments could be used to identify such environmental effects and further studies testing the hypothesis that brood traits alone regulate the SMR phenotype are ongoing.

The distinction made in this study is an important first known step towards understanding the mechanisms behind SMR and more generally mite resistance, and opens the door for future research to discover more precisely what specific brood features are important for the SMR phenotype. A deeper understanding of the ecological interactions between *Varroa* mites and their hosts are also important for efforts in developing mite-resistant breeding programs. This could potentially simplify selection criteria evaluation methods, selection strategies, and help develop more efficient and sustainable efforts towards long-term genetic stock improvements for mite resistance in honey bees.

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Legend to Figures

Fig. 1. Photographs of the two types of experimental frames used to exclude approximately 500 sealed worker brood cells from adult bees (*Apis mellifera)*. (A) Wire mesh cage; (B) nylon mesh cage. The frame size used is called Swedish Lågnormal, with dimensions 222 mm height x 366 mm width.

Fig. 2. The average rates of *Varroa destructor* mite reproductive success (means +/- SE) examined in four honey bee (*Apis mellifera)* populations (*n* indicates number of colonies) with error bars indicating standar error. Bars represent the three mite-resistant populations examined from: Sweden (*n* = 6), France (*n* = 5), and Norway (*n* = 3), and the mite-suspectable control group (*n* = 4). Within each population, treatment groups were differentiated between caged brood excluded from adult bees (light color) and brood exposed to adult bees (dark color).

Fig. 3. Average rate of reasons for the failed *Varroa destructor* reproductive success in the three naturally adapted honey bee (*Apis* mellifera) populations and control group, exposed and exluded groups pooled. The recorded reasons are: i) absence of a male; ii) delayed egg laying as mite offspring were too young to successfully reproduce; and iii) infertility of the foundress.

Highlights

- *Varroa* reproductive success was reduced in three mite-resistant honey bee populations
- Host brood traits reduce mite reproduction, independent of adult bees
- The added presence of adult bees did not increase the rate of reduced mite reproduction
- Fundamental understanding of the host brood–parasite relationship is required for future work

Table 1. Number of examined honey bee (*Apis mellifera)* worker brood cells, how many were opened, examined, naturally infested by mites (Varroa destructor), and how many had mites that reproduced successfully.

GENETIC BACKGROUND	MEASUREMENT	EXPOSED BROOD	CAGED BROOD
NORWAY	opened cells	772	937
	infested cells	89	73
	reproductive mites	70	58
FRANCE	opened cells	1965	1135
	infested cells	81	76
	reproductive mites	46	39
SWEDEN	opened cells	1204	796
	infested cells	161	133
	reproductive mites	76	59
CONTROL	opened cells	536	797
	infested cells	120	94
	reproductive mites	115	83

Table 2. The total number of mites (Varroa destructor) with failed reproduction presented for each population together with the number of failed reproductions due to the specific reasons observed and recorded.

Background	Total failed reproduction	Infertile mother	Delayed egg laying	Absence of male	Dead progeny
Sweden	160	43	59	56	2
France	72	19	33	20	0
Norway	34	10	21	3	0
Control	16	6	8	2	0