A gene for resistance to the *Varroa* mite (Acari) in honey bee (*Apis mellifera*) pupae

Running title: A gene for honey bee resistance to *Varroa*

Benjamin H. Conlon[1,2]*, Adriana Aurori[3], Alexandru-Ioan Giurgiu[3], John Kefuss[4], Daniel S. Dezmirean[3], Robin F.A. Moritz[1,3,5], Jarkko Routtu[1]

[1] Molecular Ecology, Institute of Biology/Zoology, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany

[2] Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Copenhagen, Denmark

[3] University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania

[4] Le Rucher D'Oc, 49 Rue Jonas, Toulouse, France

[5] Department of Zoology and Entomology, University of Pretoria, South Africa

Corresponding author*

Benjamin H. Conlon, Current address: Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100, Copenhagen Ø, Denmark. Email: benjamin.conlon@bio.ku.dk, Phone: +45 3532 7009

Keywords: coevolution, social insect, host, parasite, reproduction, *ecdysone*

Abstract

Social insect colonies possess a range of defences which protect them against highly-virulent parasites and colony collapse. The host-parasite interaction between honey bees (*Apis mellifera*) and the mite *Varroa destructor* is unusual, as honey bee colonies are relatively poorly-defended against this parasite. The interaction has existed since the mid-20th Century, when *Varroa* switched host to parasitise *A. mellifera*. The combination of a virulent parasite and relatively naïve host means that, without acaricides, honey bee colonies typically die within 3 years of *Varroa* infestation. A consequence of acaricide use has been a reduced selective pressure for the evolution of *Varroa*-resistance in honey bee colonies. However, in the past 20 years, several natural-selection-based breeding programmes have resulted in the evolution of *Varroa*-resistant populations. In these populations, the inhibition of *Varroa'*s reproduction is a common trait.

Using a high-density genome-wide association analysis in a *Varroa*-resistant honey bee population, we identify an *ecdysone*-induced gene significantly linked to resistance. *Ecdysone* both initiates metamorphosis in insects and reproduction in *Varroa*. Previously, using a less dense genetic map and a Quantitative Trait Loci analysis, we have identified *Ecdysone*-related genes at resistance-loci in an independently evolved resistant population. *Varroa* cannot biosynthesise *ecdysone* but can acquire it from its diet. Using qPCR we are able to link the expression of *ecdysone*-linked resistance genes to *Varroa*'s meals and reproduction. If *Varroa* co-opts pupal compounds to initiate and time its own reproduction, mutations in the host's *ecdysone* pathway may represent a key selection tool for honey bee resistance and breeding.

Introduction

The conflicting fitness optima between host and parasite means that an adaptation in one is expected to lead to a counter-adaptation in the other (Bell, 1982). This, in turn, is expected to drive fluctuations in haplotype frequency; selecting for those which are most effective against the most common virulence or resistance trait (Bell, 1982; Kidner & Moritz, 2013). With the concentration of resources in social insect colonies making them attractive targets for parasites, these fluctuations in the fitness of different defensive traits have typically resulted in social insects evolving a range of anti-parasite defences (Kurze *et al.*, 2016). These social defences, including grooming and hygenic behaviours (Kurze *et al.*, 2016), help to ease the pressure of highly virulent parasites and prevent colonies from collapsing (Hughes *et al.*, 2008). This is not always the case, however, as human interference in managed colonies of the honey bee (*Apis mellifera*) can remove the selective pressure for the evolution of host resistance traits whilst rapidly selecting for highly virulent parasite lineages (Beaurepaire *et al.*, 2017; Fries & Bommarco, 2007). The brood parasitic mite *Varroa destructor*, a devastating parasite of *A. mellifera* and a major cause of honey bee colony collapses (Rosenkranz *et al.*, 2010), is a particularly good example of this.

Varroa switched host from sister bee species *Apis cerana*, to infest colonies of *A. mellifera*, sometime in in the 20th Century (Rosenkranz *et al.*, 2010). The increased virulence of *Varroa* on *A. mellifera*, relative to *A. cerana,* means colonies, untreated with acaricides, typically die within three years of infestation (Rosenkranz *et al.*, 2010). As a brood parasite, a female *Varroa* mite completes her entire reproductive cycle within the pupal cells of *A. mellifera* (Rosenkranz *et al.*, 2010). The mother lays one male and up to five female eggs, which develop sequentially at 30-hour intervals, inside a sealed drone pupal cell (S. J. Martin, 1995);

in the shorter-pupating worker pupal cells, she will only lay up to four female eggs (S. J. Martin, 1994). The *Varroa* mother, and her developing offspring, feed on the fat bodies of the developing pupae (Ramsey *et al.*, 2019). This reduces the bee's adult lifespan and makes it less able to forage and support the colony (Annoscia *et al.*, 2015). The reduction in lifespan of *Varroa*-parasitised pupae translates into negative fitness consequences for the colony as a whole with *Varroa* population size in the autumn acting as a significant predictor of overwinter colony mortality (van Dooremalen *et al.*, 2012). The high rate of colony death due to *Varroa* has led to the widespread use of acaricides in managed honey bee colonies. However, this reduces the selective pressure for the evolution of host defences (Fries & Bommarco, 2007). Although the initial rate of colony loss can be very high, when *A. mellifera* populations are left untreated for *Varroa*, resistance can evolve rapidly (Fries *et al.*, 2006; Kefuss *et al.*, 2015; Locke, 2016b). This combination of a susceptible host, a highly virulent parasite and the rapid evolution of resistance traits makes the interaction between *A. mellifera* and *Varroa* an excellent model for investigating host-parasite co-evolution in social insects.

Despite different geographic and genetic origins, the inhibition of *Varroa*'s reproduction by the infested pupae is a shared trait of many *Varroa*-resistant *A. mellifera* populations across the globe as well as in the original host *A. cerana* (Conlon *et al.*, 2018; Locke, 2016a, 2016b; Nganso *et al.*, 2018; Oddie *et al.*, 2017; Oldroyd, 1999; Rath, 1999; Rosenkranz *et al.*, 2010). Although the inhibition of *Varroa*'s reproduction, seen in these populations, often works in concert with other resistance and tolerance mechanisms (Locke, 2016b; Nganso *et al.*, 2018; Oddie *et al.*, 2017; Rath, 1999), traits such as reduced colony sizes and *Varroa*-Specific Hygiene are complex behavioural traits, which must be expressed at the colony-level (Kurze

et al., 2016; Locke, 2016b). In contrast, the inhibition of *Varroa*'s reproduction by the pupae is an individual-level resistance trait which makes an important contribution to the overwinter survival of untreated colonies, by reducing *Varroa*'s population size in the autumn (Oddie *et al.*, 2017; van Dooremalen *et al.*, 2012). In some resistant populations, it has been shown that the reduced reproduction of *Varroa* is a genetic, heritable, trait of the host pupae (Conlon *et al.*, 2018; Locke, 2016a). Although it is yet to be shown exactly how a honey bee pupa is capable of inducing its parasite not to reproduce (Nazzi & Le Conte, 2016), two genes from the *ecdysone* biosynthesis pathway have been linked to resistance using a Quantitative Trait Loci analysis in a *Varroa*-resistant honey bee population from Gotland, Sweden (Conlon *et al*., 2018). Experimental manipulations show *Varroa* will suspend reproduction when the conditions inside the cell are not optimal (Frey *et al.*, 2013). This suggests the inhibition of *Varroa*'s reproduction may not be a classical immune response but an act of physiological manipulation by the pupa.

Using a commercially-viable *Varroa*-resistant honey bee population from a natural-selection-based breeding programme near Toulouse, France (Kefuss *et al.*, 2015), we investigated the genomic basis for the host-induced non-reproduction of *Varroa* in drone pupae: focusing on a colony for which we identified a ~50% rate of non-reproduction of *Varroa*. Given the haploid genome of honey bee drones, this 50:50 segregation is indicative of single-gene control inherited from a heterozygous mother queen. The unusually high recombination rate in *A. mellifera* (19 cM/MB) (Honeybee Genome Sequencing Consortium, 2006) means that we are able to identify a single gene in the *ecdysone* pathway as linked to the resistance trait. *Varroa* requires *ecdysone* and pupal proteins to initiate vitellogenesis but lacks the genes for *ecdysone* synthesis (Cabrera *et al.*, 2015; Tewarson, 1982). Taking advantage of the differential

fecundity of *Varroa* in worker and drone pupal cells (S. J. Martin, 1994, 1995), we use qPCR of an unrelated *Varroa*-susceptible colony of *A. mellifera* to show that the *ecdysone*-linked genes from the Toulouse and Gotland (Conlon *et al*., 2018) resistant populations are significantly upregulated in young drone pupae, where *Varroa* has the highest fecundity (S. J. Martin, 1994, 1995), at the time that a *Varroa* mother begins to feed and initiate vitellogenesis: providing a functional link between the resistance trait and its associated genes.

Materials and Methods

Toulouse colony screening

Colony screening took place from May-June 2017 near the village of Le Born, Haute Garonne, France (43°53'N, 1°32'E). In the initial screening, covering 15 colonies, 20 *Varroa*-infested drone pupal cells from the white-eyed pupal stage onwards were opened and phenotyped based on the number of *Varroa* with offspring. Cells in which it was not possible to unambiguously phenotype *Varroa* reproduction were excluded from further analyses. *Varroa* were considered to have successfully reproduced if they produced at least one daughter and one son while reproduction was considered unsuccessful if the mite produced no offspring or only sons (Locke, 2016a; S. J. Martin *et al.*, 1997). Based on the initial screening, we selected colonies for further sampling and identified one colony in which ~50% of *Varroa*-infested cells did not reproduce. We performed a Chi-Squared goodness-of-fit test in R (R Core Team, 2017) to identify whether the distribution of successful vs unsuccessful mite reproduction was significantly different to 50:50. Having identified that it was not different to 50:50, we opened every drone pupal cell in this colony. Pupae and mature mites were stored together in 96% ethanol at -80°C for genetic analysis.

DNA extraction, sequencing and genotyping

DNA was extracted from the thorax of *Varroa*-infested brother-drone pupae using a phenol/chloroform extraction (Garnery *et al.*, 1991). The resulting extracts were assessed using a Nanodrop 1000 spectrophotometer (peqlab) and 52 samples underwent 20X, 150bp, paired-end sequencing on Illumina HiSeq with Novogene (Hong Kong).

Variant loci calling and analysis

DNA sequences were mapped to the scaffolds of the *Apis mellifera* 4.5 reference genome (Elsik *et al.*, 2014) using the BWA "MEM" algorithm (Li & Durbin, 2010). Variant loci were identified using Picard (Broad Institute, 2015) and GATK (McKenna *et al.*, 2010; Poplin *et al.*, 2017). Base quality score recalibration, indel realignment, duplicate removal, SNP and INDEL discovery and genotyping was performed for all samples simultaneously using standard hard filtering parameters following GATK best practices (DePristo *et al.*, 2011; van der Auwera *et al.*, 2013). SNPs and INDELs were called into separate files for further analyses.

SNPs and INDELs were filtered to remove loci with fewer than 90% genotyped individuals and where the allelic distribution was greater than 35-65%. F_{ST} was then calculated using windows of 10-20 SNPs and a maximum window size of 50,000 bp in popgenwindows (S. H. Martin *et al.*, 2014). F_{ST} values were analysed in R (R Core Team, 2017) using the qqman package (Turner, 2014). The threshold for suggestive loci ($F_{ST} = 0.306$) was calculated as the 99.99^{th} percentile of F_{ST}.

Candidate SNP and gene analysis

The peak region from the SNP F_{ST} analysis was used to create a list of candidate SNPs, INDELs and genes from the *A. mellifera* Official Gene Set v3.2. (Elsik *et al.*, 2014). SNPeff (Cingolani *et al.*, 2012) was used to identify those SNPs and INDELs which created a change in the amino acid sequence. The functions of genes containing SNPs which caused a functional change in the amino acid sequence were investigated further using the KEGG (Kanehisa *et al.*, 2016), Gene Ontology (Ashburner *et al.*, 2000; Gene Ontology Consortium, 2016) and UniProt (UniProt Consortium, 2018) databases.

Sampling of pupae for qPCR

An *A. mellifera* colony from the apiary of the University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, was monitored hourly for the capping of worker and drone brood. Freshly-capped pupae were marked using a transparent sheet (Human *et al.*, 2013). When enough cells had been capped, the frame was placed in an incubator at 35º C and 70% humidity. The pre-pupae were then collected at five different time points after capping (8 hours, 12 hours, 16 hours, 24 hours and 30 hours), immediately immersed in liquid nitrogen and stored at -80º C. Freshly-capped larvae (0 hours), for which the capping was not yet complete, were also collected from the apiary and instantly frozen in liquid nitrogen. By using this approach we could take advantage of the differential fecundity of *Varroa* in the worker and drone brood of *A. mellifera* (S. J. Martin, 1994, 1995) to test the potential involvement of these genes in the early stages of pupation and *Varroa*'s reproductive cycle.

RNA extraction and qPCR

Total RNA was extracted from the entire body of the worker and drone pupae sampled at each time point (0 hours, 8 hours, 12 hours, 16 hours, 24 hours and 30 hours) using QIAzol lysis reagent (Qiagen) and following the protocol supplied by the manufacturer. The quantity and quality of RNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher). A total of 2µg of RNA per sample was reverse-transcribed using a qPCRBIO cDNA synthesis kit (PCRBiosystems).

Primers for the three genes: *Mblk-1* (NM_001011629.1), *Cyp18a1* (XM_393885.4) and *Phantom* (not isoform specific) were designed using Primer Blast (Ye *et al.*, 2012). The resulting primers: *Mblk-1* F-5151 (5'-TGGACGCGTGGATTTGATT-3'), R-5255 (5'-GGAGAAAGGGAAAGCGGAG-3'), *Cyp18a1* F-578 (5'- TCAGCAACGTAATCTGCTCC-3)', R- 686 (5'-CTGCCGAACAATTTGAACCC-3', *Phm* F-5'-ATGATCGTGCCCATGCAAT-3'), R-5'- TTTGGAACGGAAGAAACGACT-3' were then used for qPCRs with the LightCycler 480 Real-Time PCR System (Roche) and the 2x qPCRBIO SyGreen Mix Lo-Rox kit (PCRBiosystems). The qPCR conditions were as follows: a denaturation step at 95 °C for 2 min, followed by 40 PCR cycles of 95 °C for 10 s, 56 °C for 10 s and 72 °C for 20 s.

qPCR analysis

The relative gene expression levels were estimated after normalisation using the reference gene RPS5 (Evans, 2006) for 5 biological replicates and 2 technical replicates per time point. We tested each gene for differences in sex, time and the interaction of sex and time using General Linear Models and Tukey's post-hoc test in R (R Core Team, 2017).

Results

One colony exhibited a Mendelian 1:1 segregation for the resistance trait

In screening the haploid drone offspring of queens in the resistant population from Toulouse, we identified 1 colony, from which we collected 69 singly-infested drone pupae, where 45% of mites did not reproduce. This was not significantly different from a Mendelian 1:1 segregation expected under single locus control ($X^2 = 0.710$, df = 1, p = 0.399) and contrasts with a mean of 31.5% non-reproducing *Varroa* (±6.5 SE) for the remaining population. The drone pupae collected from this colony were then used for DNA extraction and subsequent genomic analysis.

Sequencing of drone pupae

After mapping and filtering to remove individuals with low coverage, markers with <90% genotype coverage, a sequencing depth outside of 15-50 reads per individual and an allelic distribution greater than 35-65%, our dataset contained 45 individuals (19 Resistant, 26 Susceptible) and 112,976 SNPs for analysis using the Fixation Index (F_{ST}).

Identification of a single-locus linked to resistance

The presence of a single locus for resistance, predicted by the Mendelian 1:1 segregation in our colony screening, was supported by our identification of one peak from 7.42-7.45 Mbp on Chromosome 15 in the F_{ST} analysis (Figure 1A; SNPs = 20; Mean F_{ST} = 0.338).

The F_{ST} peak identified from the Toulouse population is 2.27 Mbp from, and outside the interval of, a QTL peak previously identified in the Gotland population of *Varroa*-resistant *A. mellifera* (Figure 1B) (Conlon *et al.*, 2018). As the best-segregating markers in the Toulouse

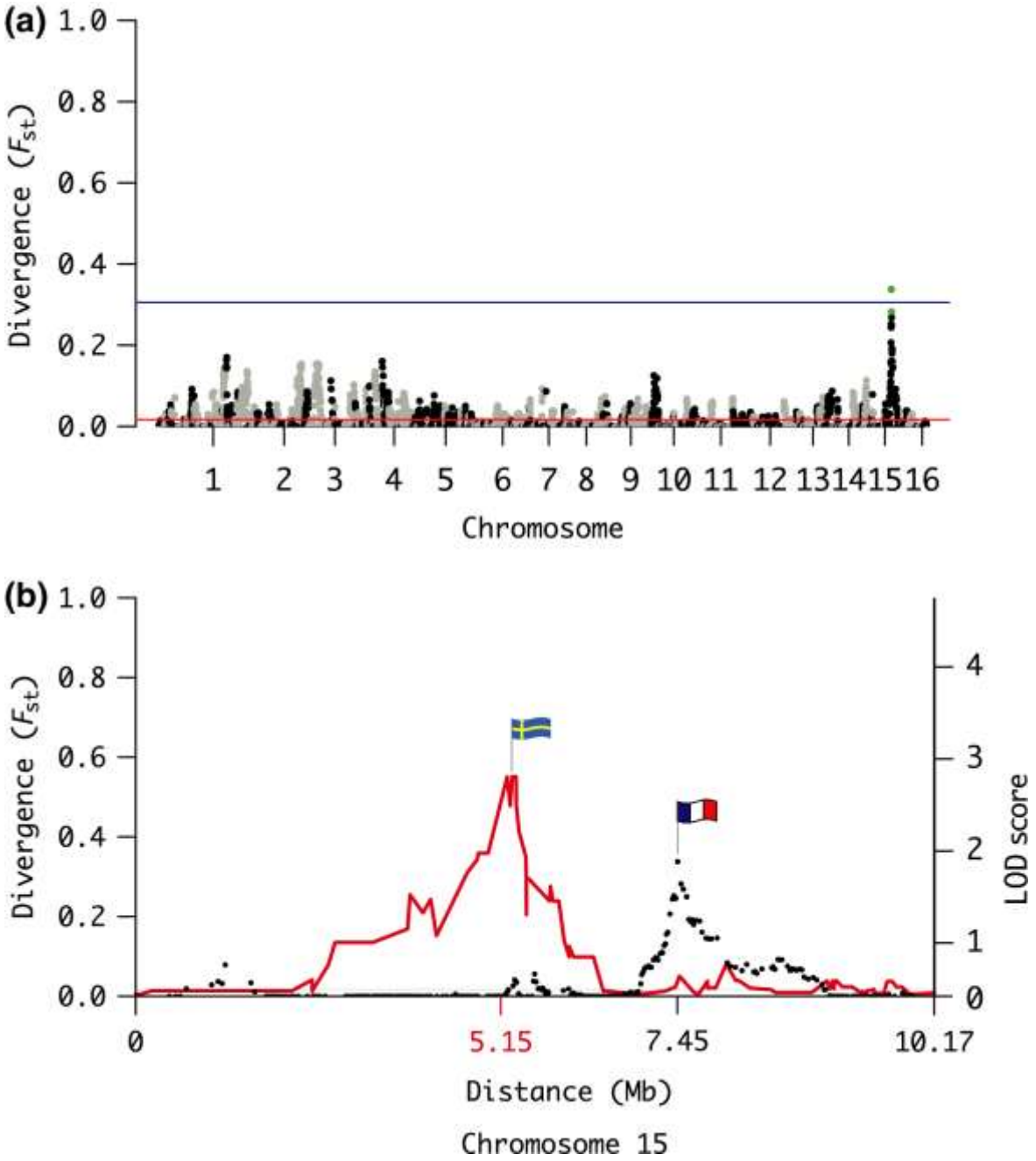

Figure 1. Comparision of estimated genetic differentiation (FST), using 20 SNP windows, between susceptible and resistant pupae reveals one suggestive locus on Chromosome 15.

(A) Genome-wide comparision of FST, using 20 SNP windows, between susceptible and resistant pupae. The suggestive line was calculated as the 99.99th percentile of FST. The suggestive window, at the 99.99th percentile, has a mean FST of 0.338. SNP windows used in the functional analysis are highlighted in green. (B) FST, calculated using 20 SNP windows, on Chromosome 15 (blue dots) compared to the LOD score for the same resistance trait on Chromosome 15 in the Gotland resistant population (red line). The mean FST for the Toulouse population was 0.338. The mean FST in the Gotland QTL region was 0.04.

209x296mm (300 x 300 DPI)

population were found at the end of the SNP window, we included the adjacent window in subsequent analyses. This created a window of 27 SNPs from 7.42-7.46 Mbp on Chromosome 15.

Although the reasons are so-far poorly understood, 10-20% of *Varroa* are expected not to reproduce regardless of host genotype. As this could be due to environmental influences or damage or senescence of the mite (Bienefeld *et al.*, 1998; S. J. Martin *et al.*, 1997), we would therefore expect an unavoidable 10-20% error rate in the identification of resistant pupae. This is reflected in our data (Supplementary Figure 1) and means that an F_{ST} of 0.338 is likely to be an underestimation.

Three functional SNPs within the gene Mblk-1 are linked to resistance

The analytical power of using the haploid honey bee drones (Honeybee Genome Sequencing Consortium, 2006) allowed us to narrow our resistant locus down to 9 functional SNPs, in 4 genes, across a 43 Kb window (7.423-7.465 Mbp) which result in a non-synonymous change in the amino acid sequence (Supplementary Table 1). This list of 9 SNPs could then be reduced further by considering recombination events within the 43 Kb window. We found a 2.2 centiMorgan (cM) gap, which caused 3 SNPs (Table 1) to segregate better between the two phenotypes than the other 6 SNPs. These 3 SNPs are all located within the *ecdysone*-regulated gene *Mblk-1*.

Expression of Mblk-1 and Cyp18a1 varies significantly between worker and drone pupae

To test the link between the *ecdysone*-linked genes, at resistance loci from Toulouse and Gotland (Figure 2), the initiation of *Varroa*'s reproductive cycles and the *Varroa* mother's

Table 1. The three best‐segregating SNPs in the FST window with their genomic position, bases, gene name and amino acid change

Chromosome	Position (Mbp)	Resistant Allele	Susceptible Allele	Gene	Amino Acid Change
15	7.454459	A	G	*Mblk-1*	Asn -> Thr
15	7.454648	T	C	*Mblk-1*	Gln -> Arg
15	7.464915	A	G	*Mblk-1*	Leu -> Pro

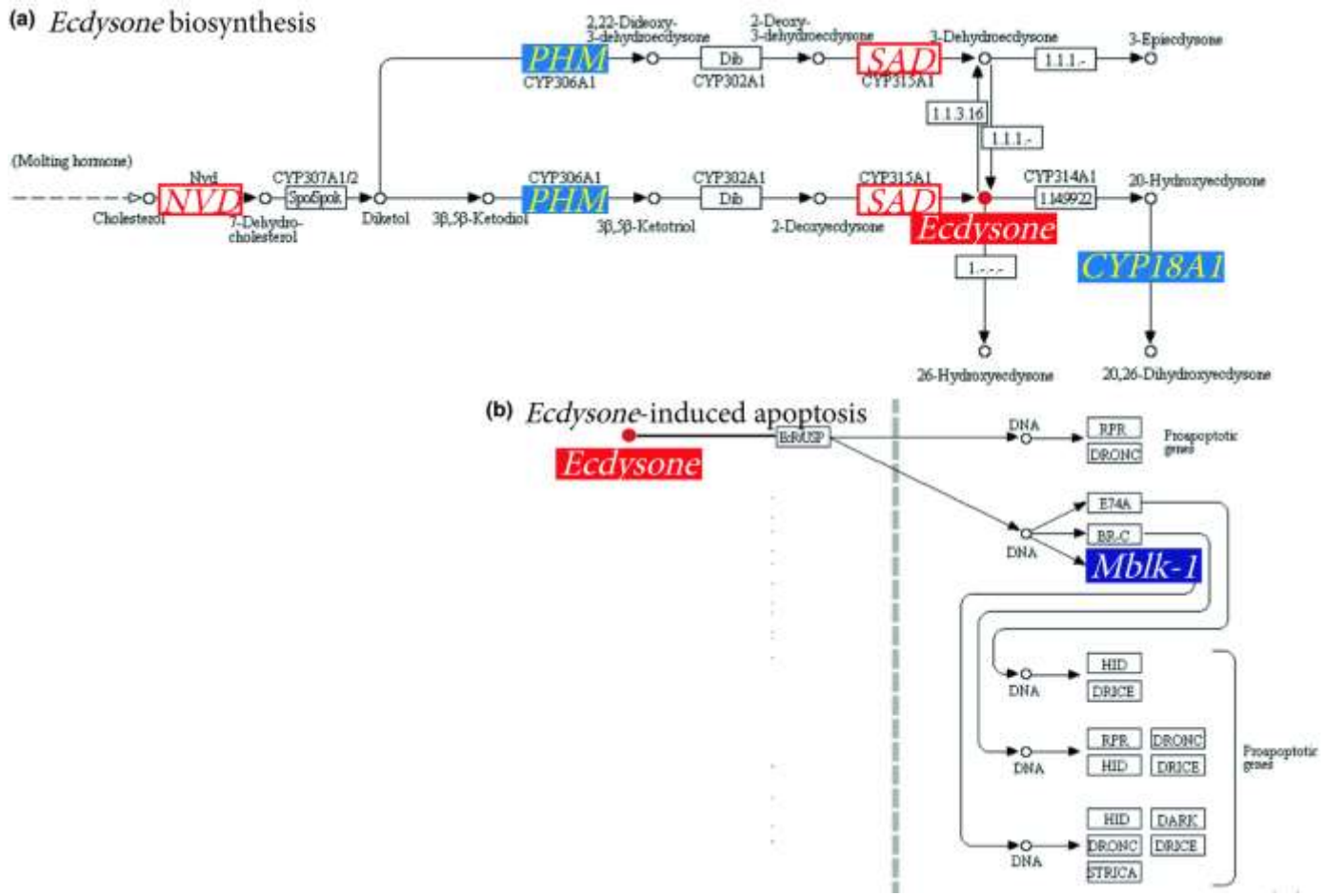

Figure 2. The role of ecdysone (white on red) and genes linked to the inhibition of Varroa reproduction in the Toulouse (white on blue) and Gotland (yellow on blue) resistant populations.

Ecdysone biosynthesis genes (other than Phm – Phantom) missing in the Varroa genome (Nvd and Sad) are outlined in Red. Hormone biosynthesis (A) and ecdysone-induced apoptosis (B) pathways are modified from KEGG (Kanehisa et al., 2016).

209x296mm (300 x 300 DPI)

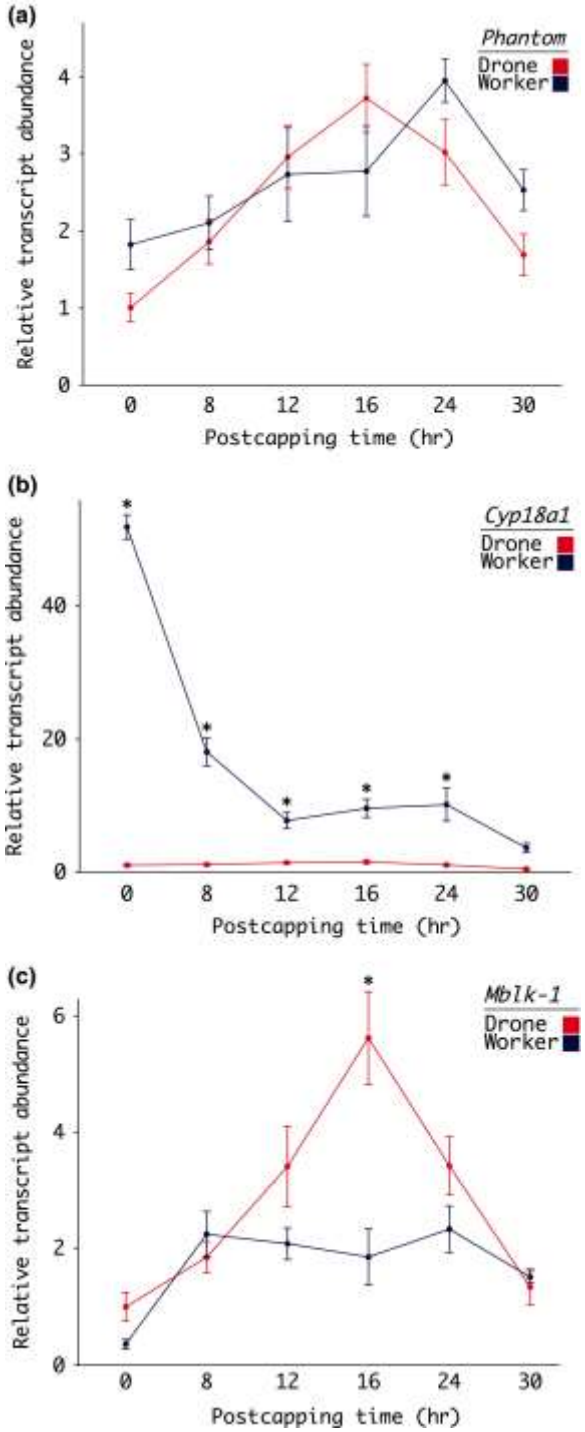

Figure 3. Relative gene expression of Phantom (Phm) (A), Cyp18a1 (B) and Mblk-1 (C) varies over time and between drones and workers.

An asterisk (*) indicates expression differed significantly at $p = 0.05$ between drones and workers. The results of statistically significant tests between sexes and within time points are as follows: Cyp18a1(3b): 0, 8, 16 and 24 hours post-capping $p < 0.001$; 12 hours post-capping $p = 0.013$; Mblk-1(3c): 16 hours post-capping $p < 0.001$. Detailed results of all statistical analyses and pairwise comparisons are in Supplementary Table 2.

209x296mm (300 x 300 DPI)

early meals, we performed qPCR of *Mblk-1, Cyp18a1 and Phantom* in non-resistant drone and worker pupae from the apiary at the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, Romania, at six different time points post-capping (0 hours, 8 hours, 12 hours, 16 hours, 24 hours and 30 hours). Although we saw a peak in the expression of *Phantom*, with significant individual effects for both sex and time, the interaction between the two was not significant (Supplementary Table 2): suggesting there are no significant differences in the production of *ecdysone* between drones and workers within a specific time point (Figure 3A). However, for *Cyp18a1* there was a significant interaction between sex and time (Supplementary Table 2). Tukey's test indicated significant differences in expression between workers and drones at p=0.05 for the first 24 hours post-capping (Figure 3B). With no apparent differences in the initiation of the *ecdysone* biosynthesis pathway by *Phantom*, this suggests that the titre of *ecdysone* may be higher in drones in the early stages of pupation. This is reflected in the expression of the *ecdysone*-regulated gene *Mblk-1* (Figure 3C). Here we see a relatively constant expression of the gene in workers during early pupation but a significant interaction between sex and time is driven primarily by an increase in the expression of *Mblk-1* in drones relative to workers around 16 hours post-capping (Figure 3C; Supplementary Table 2).

Discussion

We sought to investigate the genomic basis for the inhibition of *Varroa* reproduction in a population of *Varroa*-resistant honey bees near Toulouse, France. We identified one colony exhibiting a 1:1 Mendelian segregation of the resistance trait in haploid drones. Our prediction of single-gene control for the trait in this colony was supported by the genomic analysis identifying a single gene, *Mblk-1*, as linked to resistance.

The presence of the *ecdysone*-regulated gene *Mblk-1* (Figure 2) as the best-segregating gene at the significant locus suggests a potential pathway for resistance in the Toulouse population of *Varroa*-resistant *A. mellifera*. The regulation of metamorphosis by *ecdysone* and *Mblk-1* is conserved across both holo- and hemi-metabolous insects (Takayanagi-Kiya *et al.*, 2017; Ureña *et al.*, 2014). As *Varroa* incorporates honey bee pupal proteins into its developing oocytes (Tewarson, 1982) and has been experimentally shown to suspend reproduction when pupal cues, related to the initiation of morphogenesis, are not optimal (Frey *et al.*, 2013), a small change in the structure or expression of *Mblk-1* could induce *Varroa* to suspend reproduction. We have previously identified two *ecdysone*-related genes (*Cyp18a1* and *Phantom*), linked to resistance, using reduced-representation whole-genome sequencing for a QTL analysis of reduced *Varroa* reproductive success in a resistant honey bee population from the island of Gotland, Sweden (Conlon *et al.*, 2018). *Phantom* is a gene involved in *ecdysone* biosynthesis (Cabrera *et al.*, 2015) while *Cyp18a1* is involved in lowering the titre of *ecdysone* during the transition from pre-pupa to pupa (Rewitz *et al.*, 2010).

As well as being involved in the initiation of pupation in honey bees, *ecdysone* and its derivatives also act as a trigger for vitellogenesis in the Acari (Cabrera *et al.*, 2015; Mondet *et al.*, 2018; Roe *et al.*, 2008). Like many specialised parasites, *Varroa* exhibits reduced metabolic pathways even when compared to other parasitic Acari and Arthropods (Cabrera *et al.*, 2015; Grbić *et al.*, 2011). One of these functionally reduced pathways is the *ecdysone* biosynthesis pathway (Cabrera *et al.*, 2015; Roe *et al.*, 2008). This suggests that *ecdysone*-linked genes could represent a common pathway for the inhibition of *Varroa* reproduction across independently-evolved *Varroa*-resistant honey bee populations. Fascinatingly,

although *Varroa* shows increased expression of some genes involved in the production and reception of *ecdysone* when initiating reproduction (Cabrera *et al.*, 2015; Mondet *et al.*, 2018), the pathway is incomplete with only three of the seven genes from the *ecdysone* biosynthetic pathway present in the *V. destructor* genome (Figure 2) (Cabrera *et al.*, 2015; Cornman *et al.*, 2010). Despite this, *ecdysone* is one of the most prevalent ecdysteroids detected in extracts of *Varroa* feeding on drone pupae (Feldlaufer & Hartfelder, 1997).

Varroa mothers can ingest functional forms of *ecdysone* and use them to initiate vitellogenesis (Cabrera *et al.*, 2017). With similar ecdysteroid compounds found in *Varroa* extracts to those identified from the drone pupae they fed on (Feldlaufer & Hartfelder, 1997), and the incorporation of honey bee proteins into *Varroa*'s oocytes (Tewarson, 1982), this suggests the reduced number of genes (Cabrera *et al.*, 2015) may be an adaptation of the mite to its parasitic lifestyle and missing compounds are acquired through *Varroa*'s diet (Cabrera *et al.*, 2015). *Varroa* feeds on the pupa's fat bodies (Ramsey *et al.*, 2019) where expression of the *Drosophila* homolog of the *Mblk-1* gene increases during the pre-pupal phase (Baehrecke & Thummel, 1995). With the role of *Mblk-1* in metamorphosis being higly conserved in insects (Takayanagi-Kiya *et al.*, 2017; Ureña *et al.*, 2014), this raises the possibility that *Mblk-1* and the pulse of pre-pupal ecdysteroids are not a cue but a necessary physiological component for the successful initation and timing of reproduction in *Varroa*. Our findings support this as the expression of *Mblk-1*, *Phantom* (Figure 3A, Figure 3C) and, by proxy, *ecdysone* titres in the drone pupae peaks around the same time as the *Varroa* mother's early meals and the initation of vitellogenesis. Although the gene expression study focused on a non-resistant colony of *A. mellifera*, *Varroa* still exhibits differential fecundity between worker (S. J. Martin, 1994) and drone (S. J. Martin, 1995) pupal cells. This means that

variation in the expression of these genes, already linked to the successful reproduction of *Varroa*, between sexes can provide further evidence for their involvement in the resistance trait in drones.

We also found that the decrease in gene expression occurs between 16 and 30 hours; around the same time post-capping that an artificially-infested *Varroa* mother will no longer successfully initiate reproduction in worker pupae (Frey *et al.*, 2013). Although *Varroa* artificially infested into drone cells can still successfully reproduce at this point (Frey *et al.*, 2013), the expression of *Mblk-1* is so much higher in drones (Figure 3C) that it is possible the titres of *Mblk-1* and *ecdysone* remain high even though the number of transcripts has decreased: particularly as we see little change in the expression of *Cpy18a1*, which negatively-regulates *ecdysone* titres, in drones over this time. With only mature, mated, *Varroa* daughters surviving to leave the cell with the emerging bee (Rosenkranz *et al.*, 2010), the co-option of host hormones and proteins to initiate reproduction could aid *Varroa*'s fitness; by preventing the mite from wasting its finite number of stored sperms fertilising eggs which will not survive to leave the pupal cell (Rosenkranz *et al.*, 2010; Tewarson, 1982). However, a change in the regulation of genes involved in the production of, or induced by, *ecdysone* could reduce the amount available for ingestion by *Varroa* rendering it incapable of initiating oogenesis. In this sense, the host-induced inhibition of *Varroa* reproduction may represent a case of the host wresting back control of its extended phenotype: preventing its exploitation by the parasite and increasing the pupa's own fitness.

The independent evolution of the same resistance trait from different genetic, but similar physiological, backgrounds in both the Toulouse and Gotland (Conlon *et al.*, 2018)

populations of *Varroa*-resistant *A. mellifera* suggests this trait is highly evolvable under the right selective pressures. Although we do not have data for workers from the Toulouse population, there is evidence from the Gotland population that the resistance trait is expressed, and exhibits dominance, in worker pupae too (Locke, 2016a). Although there are likely to be fitness costs associated with a resistance trait so closely linked to successful pupation (Rewitz *et al.*, 2010), the virulence of *Varroa* on colonies of *A. mellifera* suggests the parasite pressure is high enough to outweigh the costs.

While the parasite pressure from *Varroa* may be enough to help *A. melifera* overcome any costs associated with resistance, this only tells half of the story as a reduced rate of reproduction would be expected to select for counter adaptations in *Varroa* (Bell, 1982). While it is beyond the scope of the current study to say whether this is happening, the high proportion of non-reproducing *Varroa*, combined with the survival of the Toulouse population for over 20 years, suggests that the colonies of *A. mellifera* must either be able to respond to, or reduce, the likelihood of *Varroa* evolving counter-adaptations. While it has been suggested that epistatic interactions in the Gotland population may help to prevent the evolution of counter-adaptations in *Varroa* (Conlon *et al.*, 2018), this may not be the case with the Toulouse population where we have a 50:50 segregation of phenotypes in the drone pupae of one colony, and a 31.5:68.5 segregation of resistant:susceptible in the population, and the identification of a single resistance-linked locus.

The 50:50 segregation of resistant:susceptible phenotypes means there is still the possibility for *Varroa* to successfully reproduce in 50% of the avaliable drone pupae. The social nature of *A. mellifera* means this individual-level resistance could aid colony-level survival even

when it is not expressed by all pupae (Kurze *et al.*, 2016). If *Varroa* is restricted in its reproduction to only half the available brood, this could reduce the population growth-rate; preventing it from reaching the critical mass which would cause colony death, but not to the extent that it favours the evolution of counter adaptations to the resistance trait. This could help a colony overcome any fitness costs associated with the trait as it would still produce some susceptible drones, which were not parasitised by *Varroa* as pupae and reproduce successfully, whilst also reducing the selective pressure for the evolution of counter adaptations in *Varroa*.

The inhibition of *Varroa*'s reproduction appears to play an important role in colony survival for the Toulouse and other *Varroa*-resistant populations (Conlon *et al.*, 2018; Locke, 2016a, 2016b; Oddie *et al.*, 2017). While inhibition in the Toulouse and Gotland resistant populations may be linked to the manipulation of *Varroa* using the *ecdysone* hormone, they appear to achieve the same result using different methods. This raises the possibility that *ecdysone* represents a common link for the inhibition of *Varroa* reproduction in independently-evolved resistant populations and that the co-option of the pre-pupal ecdysteroids is an important physiological trigger for the initiation of *Varroa* reproduction. Although there are likely to be fitness costs associated with the resistance trait, that it apparently survives in the population suggests the fitness cost of resistance is outweighed by the fitness cost of *Varroa* parasitism. Our results also highlight the ability of social insect societies to rapidly evolve resistance to a novel parasite and how the evolution of a resistance trait expressed in an individual could also benefit the non-resistant individuals in a colony by restricting the growth of the parasite population. This colony-level defence could then reduce the selective pressure for the evolution of increased virulence in the parasite population: reducing the risk of colony death.

Acknowledgements:

We would like to thank M. Kardell for her help with the initial colony screening, P. Leibe and D. Kleber for their help in the lab and C. Jenkins and E. Stolle for helpful discussions on the data. This research was funded by the Deutsche Forschungsgemeinschaft (RO 5121/1-1 to J.R.) and the Unitatea Executiva pentru Finantarea Invatamantului Superior, a Cercetarii, Dezvoltarii si Inovarii (Proiecte de Cercetare Exploratorie GRAL to R.F.A.M. and D.S.D.).

Data and materials availability:

Sequence data have been deposited in the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) under the BioProject accession number: PRJNA473430. Correspondence and requests for materials should be addressed to B.H.C. (benjamin.conlon@bio.ku.dk).

References

Annoscia, D., Del Piccolo, F., Covre, F., Nazzi, F. (2015). Mite infestation during development alters the in-hive behaviour of adult honeybees. *Apidologie, 46*(3), 306-314.

Ashburner, M., Ball, C. A., Blake, J. A., et al. (2000). Gene Ontology: tool for the unification of biology. *Nature genetics, 25*(1), 25.

Baehrecke, E. H., Thummel, C. S. (1995). The *Drosophila* E93 gene from the 93F early puff displays stage-and tissue-specific regulation by 20-hydroxyecdysone. *Developmental biology, 171*(1), 85-97.

Beaurepaire, A. L., Krieger, K. J., Moritz, R. F. (2017). Seasonal cycle of inbreeding and recombination of the parasitic mite *Varroa destructor* in honeybee colonies and its implications for the selection of acaricide resistance. *Infection, Genetics and Evolution, 50*, 49-54.

Bell, G. (1982). *The Masterpiece Of Nature: The Evolution and Genetics of Sexuality*. Berkeley, USA: University of California Press.

Bienefeld, K., Haberl, M., Radtke, J. (1998). Does the genotype of honeybee brood influence the attractiveness for *Varroa jacobsoni* and/or the reproduction of this parasite? *Hereditas, 129*(2), 125-129.

Broad Institute. (2015). Picard Tools. Retrieved from http://broadinstitute.github.io/picard/

Cabrera, A. R., Shirk, P. D., Evans, J. D., et al. (2015). Three Halloween genes from the *Varroa* mite, *Varroa destructor* (Anderson & Trueman) and their expression during reproduction. *Insect molecular biology, 24*(3), 277-292.
Cabrera, A. R., Shirk, P. D., Teal, P. E. (2017). A feeding protocol for delivery of agents to assess development in *Varroa* mites. *PLOS ONE, 12*(4), e0176097.
Cingolani, P., Platts, A., Wang, L. L., et al. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly, 6*(2), 80-92.
Conlon, B. H., Frey, E., Rosenkranz, P., et al. (2018). The role of epistatic interactions underpinning resistance to parasitic *Varroa* mites in haploid honey bee (*Apis mellifera*) drones. *Journal of evolutionary biology, 31*(6), 801-809.
Cornman, R. S., Schatz, M. C., Johnston, J. S., et al. (2010). Genomic survey of the ectoparasitic mite *Varroa destructor*, a major pest of the honey bee *Apis mellifera*. *Bmc Genomics, 11*(1), 602.
DePristo, M. A., Banks, E., Poplin, R., et al. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature genetics, 43*(5), 491.
Elsik, C. G., Worley, K. C., Bennett, A. K., et al. (2014). Finding the missing honey bee genes: lessons learned from a genome upgrade. *Bmc Genomics, 15*(1), 86. Evans, J. (2006).
Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. *Journal of Inverterbrate Pathology, 93*(135-139).
Feldlaufer, M., Hartfelder, K. (1997). Relationship of the neutral sterols and ecdysteroids of the parasitic mite, *Varroa jacobsoni* to those of the honey bee, *Apis mellifera*. *Journal of Insect Physiology, 43*(6), 541-545.
Frey, E., Odemer, R., Blum, T., Rosenkranz, P. (2013). Activation and interruption of the reproduction of *Varroa destructor* is triggered by host signals (*Apis mellifera*). *Journal of invertebrate pathology, 113*(1), 56-62.
Fries, I., Bommarco, R. (2007). Possible host-parasite adaptations in honey bees infested by *Varroa destructor* mites. *Apidologie, 38*(6), 525-533.
Fries, I., Imdorf, A., Rosenkranz, P. (2006). Survival of mite infested (*Varroa destructor*) honey bee (*Apis mellifera*) colonies in a Nordic climate. *Apidologie, 37*(5), 564-570.
Garnery, L., Vautrin, D., Cornuet, J., Solignac, M. (1991). Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie, 22*(1), 87-92.
Gene Ontology Consortium. (2016). Expansion of the Gene Ontology knowledgebase and resources. *Nucleic acids research, 45*(D1), D331-D338.
Grbić, M., Van Leeuwen, T., Clark, R. M., et al. (2011). The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. *Nature, 479*(7374), 487.
Honeybee Genome Sequencing Consortium. (2006). Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature, 443*(7114), 931.
Hughes, D. P., Pierce, N. E., Boomsma, J. J. (2008). Social insect symbionts: evolution in homeostatic fortresses. *Trends in Ecology & Evolution, 23*(12), 672-677.
Human, H., Brodschneider, R., Dietemann, V., et al. (2013). Miscellaneous standard methods for Apis mellifera research. *Journal of Apicultural Research, 52*(4), 1-53.
Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic acids research, 44*(D1), D457-D462.
Kefuss, J., Vanpoucke, J., Bolt, M., Kefuss, C. (2015). Selection for resistance to *Varroa destructor* under commercial beekeeping conditions. *Journal of Apicultural Research, 54*(5), 563-576.

Kidner, J., Moritz, R. (2013). The red queen process does not select for high recombination rates in haplodiploid hosts. *Evolutionary Biology, 40*(3), 377-384.
Kurze, C., Routtu, J., Moritz, R. F. (2016). Parasite resistance and tolerance in honeybees at the individual and social level. *Zoology, 119*(4), 290-297.
Li, H., Durbin, R. (2010). Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics, 26*(5), 589-595.
Locke, B. (2016a). Inheritance of reduced *Varroa* mite reproductive success in reciprocal crosses of mite-resistant and mite-susceptible honey bees (*Apis mellifera*). *Apidologie, 47*(4), 583-588.
Locke, B. (2016b). Natural *Varroa* mite-surviving *Apis mellifera* honeybee populations. *Apidologie, 47*(3), 467-482.
Martin, S. H., Davey, J. W., Jiggins, C. D. (2014). Evaluating the use of ABBA–BABA statistics to locate introgressed loci. *Molecular biology and evolution, 32*(1), 244-257.
Martin, S. J. (1994). Ontogenesis of the mite *Varroa jacobsoni* Oud. in worker brood of the honeybee *Apis mellifera* L. under natural conditions. *Experimental & applied acarology, 18*(2), 87-100.
Martin, S. J. (1995). Ontogenesis of the mite *Varroa jacobsoni* Oud. in drone brood of the honeybee *Apis mellifera* L. under natural conditions. *Experimental & applied acarology, 19*(4), 199-210.
Martin, S. J., Holland, K., Murray, M. (1997). Non-reproduction in the honeybee mite *Varroa jacobsoni*. *Experimental & applied acarology, 21*(8), 539-549.
McKenna, A., Hanna, M., Banks, E., et al. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome research, 20*(9), 1297-1303.
Mondet, F., Rau, A., Klopp, C., et al. (2018). *Transcriptome profiling of the honeybee parasite* Varroa destructor provides new biological insights into the mite adult life cycle. *Bmc Genomics, 19*(1), 328.
Nazzi, F., Le Conte, Y. (2016). Ecology of *Varroa destructor*, the major ectoparasite of the western honey bee, *Apis mellifera*. *Annual Review of Entomology, 61*, 417-432.
Nganso, B. T., Fombong, A. T., Yusuf, A. A., et al. (2018). Low fertility, fecundity and numbers of mated female offspring explain the lower reproductive success of the parasitic mite *Varroa destructor* in African honeybees. *Parasitology, 17*, 1-7.
Oddie, M. A., Dahle, B., Neumann, P. (2017). Norwegian honey bees surviving *Varroa destructor* mite infestations by means of natural selection. *PeerJ, 5*, e3956.
Oldroyd, B. P. (1999). Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. *Trends in Ecology & Evolution, 14*(8), 312-315.
Poplin, R., Ruano-Rubio, V., DePristo, M. A., et al. (2017). Scaling accurate genetic variant discovery to tens of thousands of samples. *bioRxiv*, 201178.
R Core Team. (2017). R: A language and environment for statistical computing. Retrieved from https://www.R-project.org/
Ramsey, S. D., Ochoa, R., Bauchan, G., et al. (2019). *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proceedings of the National Academy of Sciences*, 201818371.
Rath, W. (1999). Co-adaptation of *Apis cerana* Fabr. and *Varroa jacobsoni* Oud. *Apidologie, 30*, 97-110.
Rewitz, K. F., Yamanaka, N., O'Connor, M. B. (2010). Steroid hormone inactivation is required during the juvenile-adult transition in *Drosophila*. *Developmental cell, 19*(6), 895-902.

Roe, R. M., Donohue, K. V., Khalil, S., Sonenshine, D. E. (2008). Hormonal regulation of metamorphosis and reproduction in ticks. *Front Biosci, 13*, 7250-7268.
Rosenkranz, P., Aumeier, P., Ziegelmann, B. (2010). Biology and control of *Varroa destructor*. *Journal of invertebrate pathology, 103*, S96-S119.
Takayanagi-Kiya, S., Kiya, T., Kunieda, T., Kubo, T. (2017). Mblk-1 Transcription Factor Family: Its Roles in Various Animals and Regulation by NOL4 Splice Variants in Mammals. *International journal of molecular sciences, 18*(2), 246.
Tewarson, N. C. (1982). Immunocytochemical localization of host (*Apis mellifera*) proteins in growing oocytes of a hemophagous mite (*Varroa jacobsoni*) by the unlabeled antibody—enzyme (PAP) method. *International Journal of Invertebrate Reproduction, 5*(6), 345-348.
Turner, S. D. (2014). qqman: an R package for visualizing GWAS results using QQ and manhattan plots. *bioRxiv*, 005165.
UniProt Consortium. (2018). UniProt: the universal protein knowledgebase. *Nucleic acids research, 46*(5), 2699.
Ureña, E., Manjón, C., Franch-Marro, X., Martín, D. (2014). Transcription factor E93 specifies adult metamorphosis in hemimetabolous and holometabolous insects. *Proceedings of the National Academy of Sciences*, 201401478.
van der Auwera, G. A., Carneiro, M. O., Hartl, C., et al. (2013). From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. *Current protocols in bioinformatics, 43*(1), 11.10. 11-11.10. 33.
van Dooremalen, C., Gerritsen, L., Cornelissen, B., et al. (2012). Winter survival of individual honey bees and honey bee colonies depends on level of *Varroa destructor* infestation. *PLOS ONE, 7*(4), e36285.
Ye, J., Coulouris, G., Zaretskaya, I., et al. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*(1), 134. doi:10.1186/1471-2105-13-134

Authors contributions

B.H.C. designed the study, performed the field work, DNA extractions and data analysis, contributed to the interpretation of the results and wrote the first draft of the manuscript. A.A performed the fieldwork, RNA extractions and the qPCR, analysed the data and contributed to the interpreting of the results and manuscript. A-I.G. performed the fieldwork, RNA extractions and the qPCR, provided input on the interpretation of the results and contributed to the manuscript. J.K. bred the resistant honey bee population, performed fieldwork, provided input to the interpretation of the results and contributed to the manuscript. D.S.D. contributed to the experimental work and sequencing, provided input to the interpretation of the results and contributed to the manuscript. R.F.A.M. designed the study, contributed to the

interpretation of the results and to writing the final manuscript. J.R. designed the study, provided input to the interpretation of the results and to writing the final manuscript.