Tolerance to virus infections could explain increased winter colony survival observed in

Varroa destructor-resistant honey bees

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Abstract

Honey bee colonies all over Europe and North America have been declining dramatically for over three decades and is continuing to do so which is causing significant threats to economy, agriculture and ecosystems. The main reason behind the declining colonies is an ectoparasitic mite known as *Varroa destructor* and viruses vectored by the mite. In previous studies, it has been suggested that a unique mite-resistant subpopulation of honey bees (*Apis mellifera*) in Gotland, Sweden have developed adaptive tolerance to these viruses as they have managed to survive high mite infestation through natural selection without any mite control treatment. This indicates that there might be a correlation between resistance to *Varroa* destructor and virus tolerance.

This project examined if a correlation between virus resistance and/or virus tolerance can be observed in *Varroa*-resistant honey bees from unique subpopulations in Europe covering Sweden, Norway, France and Netherlands. Results showed that no correlation could be established based on the findings in this project. However, significant differences in winter colony survival numbers between mite-resistant and mite-susceptible honey bees suggest that tolerance mechanisms could be present in these subpopulations. Further studies are required to verify this hypothesis.

Keywords

mite-resistance, mite-infestation, virus dynamics, tolerance mechanisms, *Apis mellifera*
Introduction

Declination of honey bee colonies in Europe and North America poses a significant threat to global agriculture, apiculture and ecosystems. In addition to being producers of honey, bees are one of the most important pollinators of fruits, vegetables and crops which are also used as fodder in meat and dairy production. Over 30% of the world’s food production rely on the pollination from honey bees (Klein A-M, et al. 2006) and the dramatic losses of these pollinators has given major cause of concern for a possible approaching food crisis.

The European honey bee known as *Apis mellifera* is the most common species of honey bee in the world (Lo N. 2010) and the most important pollinating insect species (Bowen-Walker PL, et al. 2006). However, over the last three decades, overwhelming casualties of *A. mellifera* populations have been reported in Europe and North America (Le Conte, et al. 2010). The main contributing factor to these losses is the ectoparasitic brood mite *Varroa destructor* and the pathogens vectored by the mite (Giuseppina T. 2015). Originally, *V. destructor* could only be found in Asia, infesting the Asian honey bees known as *Apis cerana*. After World War II, a large quantity of *A. mellifera* colonies were introduced into Asia for commercial apicultural purposes, which presented *V. destructor* with a new host. Consequently, when the mite-infested *A. mellifera* colonies were brought back to Europe from Asia, the parasites spread rapidly to new locations nearly throughout the entire world (Warrit N. et al. 2011; Martin S. et al. 2002).

Contrary to *A. mellifera* honey bees, an infection of *V. destructor* is not lethal to *A. cerana*. This can be explained by behavioural defences within the *A. cerana* honey bee colonies known as hygienic behaviour and grooming. *Varroa destructor* cannot successfully reproduce in *A. cerana* worker brood cells because the infested pupa and mite are removed and destroyed by hygienic and grooming behaviours of their natural host. This behaviour is a
result from years of evolutionary co-adaptation between host and parasite (Rath, W. 1999) European honey bees have similar behavioural defences although less pronounced and the mites can readily reproduce in worker brood of this new host with an exponential population growth rate (Locke B. 2016).

The consequences of the world-wide infestation by *V. destructor* have resulted in very few mite-free locations which in turn has led to a dramatic declination of honey bee colonies, forcing beekeepers to use mite control treatments which are often chemical and harmful, albeit necessary since the mites cause colony losses within a few years if the bees are left untreated. However, studies show that some populations of honey bees, specifically African subspecies in Africa and Brazil (*Apis mellifera scutellata*) and a few small European subpopulations from Sweden, Norway, Netherlands and France, can survive mite infestation without any mite control treatments (Rosenkranz. 1999; Locke B. 2016). Possible influencing factors to the observed mite infestation resistance could be explained by climate, bee race and genetic variety of *V. destructor* present in honey bee colonies (De Jong, *et al.* 1984; Moretto *et al.* 1991; Camazine. 1986; Rosenkranz. 1986; Moretto *et al.* 1993; Anderson. 2000). The genetics of the race or subspecies of honey bee is the most important factor since honey bees of African origin require no mite control and generally maintain lower infestation rates than other races of *A. mellifera* (Locke B. 2016; Rosenkranz 1999; Moretto *et al.* 1995).

Despite recognizing factors that could explain the survivability of honey bees against *V. destructor*, there are fewer explanations for the survivability against varroa-mediated virus transmissions. The mites contribute to weakening colony health and modifying behaviour in bees (Tantillo *G. et al.* 2015). When *V. destructor* attack honey bees, they feed on their circulatory fluid known as hemolymph. This causes a reduction to their body weight, nutrient contents and affects their immunity (DeGrandi-Hoffman *G. et al.* 2015). However, the *Varroa* infestation alone is usually not the direct cause of mortality in honey bees. The mites
also function as vectors and reservoirs for pathogens (Kaftanoglu O, et al. 1992; Tantillo G, et al. 2015). Among pathogens that infect honey bees, viruses are one of the most major threats. The most severe viruses spread by Varroa include deformed wing virus (DWV), acute bee paralysis virus (ABPV), kashmir bee virus (KBV), israeli acute paralysis virus (IAPV), slow bee paralysis virus (SBPV), chronic bee paralysis virus (CBPV) and black queen cell virus (BQCV) (Bakonyi T, et al. 2002; Chen Y, et al. 2004, Bowen-Walker PL, et al. 1999; Di Prisco G, et al. 2011; Tantillo G, et al. 2015), all of which are significant factors in observed honey bee colony disruption.

When viruses enter host cells, they utilize the host’s metabolism and machinery to produce replicates within the cell, which can be harmful and often deadly to the host. Virus transmissions within honey bee colonies can occur vertically and/or horizontally. Vertical transmission occurs when viruses are transferred from mother to offspring, while horizontal transmission includes sexual-, air-borne- and food-borne infections. Horizontal transmission can also occur via an indirect route through an intermediate biological host (Bowen-Walker PL, et al. 1999; Chen Y, et al. 2006) like V. destructor that pass on infections from one host to another. All viruses, even those directly related to V. destructor, can still exist in bee populations without the parasite’s presence due to existing transmission routes, but with an increase in the V. destructor population, infections are spread more efficiently which results in dramatic increases in viral load.

Very little is known about possible resistance or tolerance mechanisms that honey bees may have against Varroa-mediated virus transmissions. However, as shown in previous studies (Locke, B. 2014) mite-resistant European honey bees from Gotland, Sweden that live near mite-susceptible bees have almost identical mite infestation rates as the mite-susceptible bees but they do not die from the virus infections transmitted by them. This is an indication that mite-resistance could be linked with a resistance or tolerance to Varroa-mediated virus
transmissions (Locke B, et al. 2014; U Strauss, et al. 2013), however it has not yet been confirmed if similar virus dynamics exist in other subpopulations of mite-resistant honey bees in Europe.

The method used for diagnosing viral infections in bees consist of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), which quantifies and detects virus genes with fluorescent probes or non-specific fluorescence. When cDNA synthesis and PCR reaction is performed in two separate steps, it is commonly known as a two-step RT-qPCR. During cDNA-synthesis, a single-stranded RNA in each sample is synthesised into double-stranded DNA for increased stability. Viral cDNA is then amplified in a separate PCR-step where a fluorescent signal is emitted from samples containing the target gene. The virus levels are measured by the amount of fluorescence detected after each PCR cycle, where low cycle quantity with high fluorescence indicates a strong positive viral infection. After every performed PCR reaction, the melting curve is analysed to make sure that the products and controls have identical curves, thus containing the same target gene.

The aim of this project was to examine if there is a correlation between mite-resistance and virus tolerance by comparing virus infection dynamics in different mite resistant bee populations against each other and to mite-susceptible bees. To test this, 233 *A. mellifera* honey bee colonies with origins in France, Norway, Netherlands and Sweden that had adapted resistance to *V. destructor* were left to survive only through natural selection. The samples were then collected, processed and screened for late season virus infections including deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), slow bee paralysis virus (SBPV) and chronic bee paralysis virus (CBPV), using Real-Time qPCR techniques.
Materials and methods

Study materials

The material used in this study consisted of adult honey bee colonies of the *Apis mellifera* mite-resistant populations from France, Norway, Netherlands and Sweden, along with a local control group without mite resistance. A total of 233 samples were collected from the agricultural research station in Lövsta, Uppsala during the autumn of 2016 and late spring and summer of 2017. The control group included mite-susceptible bees that have been under regular management and require mite control treatments to survive.

Of the 233 collected samples, 92 were excluded during data processing due to colonies being too weak for sampling, bees dying and samples being extracted before implementation of RNA250-controls.

Ethics

Ethical permits are not applicable on insects and were therefore not a requirement for this study.

Sample preparation

A sample size of 233 adult honey bees stored at -20°C were mechanically ground to a compound followed by addition of 4.6 ml Milli-Q water to form a homogenate liquid. From this, 100 µl was mixed with 350 µl of RLT buffer containing 10 µl/ml β-mercaptoethanol and 10 ng/ml RNA Control 250 (Invitrogen, Thermo Fisher Scientific) in a collection tube. The samples were centrifuged for 3 minutes at 13 x g and the supernatant was transferred to a new collection tube and briefly vortexed.
RNA extraction

The lysates were placed in the Qiacube automated extraction robot (QIAGEN) for complete RNA extraction using the RNEasy protocol for plant tissues (QIAGEN) with elution volume of 50 µl.

Nucleic acid quality assessment

The quality and yield of the extracted RNA was assessed using NanoDrop ND-1000 (NanoDrop Technologies Inc.) with absorbance read at 230 nm (peak absorbance for nucleic acids), 260 nm (peak absorbance for proteins) and 280 nm (peak absorbance for phenolic compounds). Absorbance ratio values A280/A260 and A260/A230 were used to assess the purity of RNA, with values under 1.0 considered contaminated as per the manufacturer’s instructions\(^1\). Nuclease free water was added to adjust the concentration of all samples to 100 ng/µl. The samples were stored at -80°C.

cDNA synthesis

First strand cDNA-synthesis was performed by mixing template RNA with random hexamer primers, 5X Reaction Buffer, RiboLock RNase Inhibitor (20 U/µl), 10 mM dNTP mix and M-MuLV Reverse Transcriptase (20 U/µl) using the Thermo Scientific First Strand cDNA Synthesis’ Kit #K1612 protocol. After briefly centrifuging the samples, they were incubated for 5 minutes at 20°C followed by 60 minutes at 37°C. After the final incubation, the cDNA-mixture was diluted 10-fold with nuclease free water to make a diluted stock mixture for future qPCR and additional analyses.

Real-time qPCR

Real-time qPCR was used to screen 6 RNA-viruses including DWV, ABPV, SBPV, SBV, BQCV, and CBPV plus an additional RNA Control 250 (Invitrogen, Thermo Fisher Scientific) in all samples. A PCR mixture was prepared by mixing cDNA template with 1X SsoFast EvaGreen supermix, forward primer and reverse primers (table 1). Each PCR reaction included a positive calibration curve containing a 10-fold dilution series covering 7 orders of magnitude for each targeted virus and a negative control of H2O. The PCR products were amplified using the BIO-RAD CFX Connect™ Real-Time System with cycling conditions at 30 seconds at 95°C for enzyme activation, 1-5 seconds at 95°C for denaturation (30-40 cycles), 5 seconds at 58°C for annealing/extension (30-40 cycles) followed by a melt curve for 2-5 sec/step at 65°C-95°C.

Table 1. Primer sequences for 6 RNA-viruses including RNA Control 250.
**Statistical analysis**

Student’s t-test was used to test the hypothesis if there was a difference in deformed wing virus titres during May, deformed wing virus titres during August, number of mites during May and number of mites during August between mite-resistant and mite-susceptible populations.

**Results**

The aim of this project was to examine whether there is a correlation between mite resistance and virus tolerance by comparing samples from different mite-resistant subpopulations from Sweden, Norway, Netherlands and France to each other and to a mite-susceptible control group. In practice, this was accomplished by screening 233 samples of honey bees that were left to survive only through natural selection to 6 RNA-viruses. Out of these 233 samples, 30 were excluded from the study during data processing due to colonies swarming, being too weak for sampling or dying. Additionally, 62 samples were excluded as they had been extracted prior to the study’s implementation of a RNA250-control in the protocol.

Remaining 141 samples were screened for viruses including deformed wing virus (DWV), black queen cell virus (BQCV), saebrood virus (SBV), acute bee paralysis virus (ABPV), slow bee paralysis virus (SBPV) and chronic bee paralysis virus (CBPV). Titres of these viruses were analysed over 4 months starting from September 2016, followed by May 2017, June 2017 and ending with August 2017. Alongside this, number of mites per 100 bees were analysed over the same period. All honey bees had been chemically treated against mites before the start of the project. Due to some negative samples being detected as false positives for DWV and SBV, the cycle quantity threshold was adjusted for these viruses, which
entailed that samples with low titres of DWV and SBV were not detected as positive. Only one sample in the control group contained ABPV titres.

Figure 1. Four European countries with mite-resistant honey bee subpopulations including A) Sweden, B) Norway, C) Netherlands and D) France, were analysed and compared for virus titres and number of mites to each other and to E) the local control group consisting of mite-susceptible honey bees. On the left y-axis, number of virus replicates were analysed for RNA-viruses including DWV (dark blue), BQCV (green), SBV (orange), ABPV (red), SBPV (purple) and CBPV (light blue) in all populations over 4 months. The number of samples during each month is presented above the set of columns on the x-axis. On the right y-axis, number of *Varroa* per 100 bees were analysed.
**Deformed wing virus**

Lower virus titres of DWV were observed in the Swedish subpopulation from September followed by missing titres in May and June and a sudden peak in August (Fig 1, A). A similar exponential growth can be observed in the mite-susceptible bees over the same period with lower titres, also lacking titres in May and June (Fig. 1, E). A slight inverse exponential growth can be observed in the Norwegian subpopulation with initial titres peaking in September followed by a slight decrease in May and August, however missing titres in June (Fig. 1, B). In the Dutch and French subpopulation (Fig. 1, C; D), virus titres ranged around $10^6$ and $10^8$.

**Black queen cell virus**

BQCV was detected in all populations except for the Dutch subpopulation in May (Fig. 1, C). Lower titres $\leq 10^4$ were observed during September in the Swedish, Norwegian and mite-susceptible population and during May and August in the Norwegian subpopulation. The highest titres were observed in June for the Norwegian subpopulation.

**Sacbrood virus**

SBV was detected in all populations (Fig. 1, A; B; C; D; E). Lower titres could be observed in May for the Norwegian and the Dutch subpopulation (Fig. 1, B; C). All populations experienced a peak of SBV in June with titres generally ranging highly around $10^9$ to $10^{10}$.

**Slow bee paralysis virus, chronic bee paralysis virus and acute bee paralysis virus**

No SBPV or CBPV was detected in any population. Lower ABPV titres were detected during June in the mite-susceptible population (Fig. 1, E), however no titres were found in any other month or in any other population.
Mite infestation

All countries with mite-resistant honey bees had similar mite infestation numbers around 2-6 *Varroa* per 100 bees in August (Fig. 1, A; B; C; D), a significant difference compared to the mite-susceptible population with 26 *Varroa* per 100 bees in August (Fig. 1, E).

Comparison of deformed wing virus between mite-resistant and mite-susceptible honey bees

A direct comparison between mite-resistant honey bees and mite-susceptible honey bees for mite infestation numbers and virus titres in targeting DWV was of specific interest since DWV is one of the more widely distributed honey bee viruses. All samples from each European mite-resistant subpopulation was pooled into one group and analysed for DWV titres and number of mites, which was then compared to those of mite-susceptible bees. A significant difference in virus titres can be observed in May between the two groups (p=0.0339) and in mite numbers in August (p=0.0240). Due to some negative samples being detected as positive for DWV, the cycle quantity threshold was adjusted for these viruses which entailed that some samples with lower titres of DWV were not detected as positive.
Figure 2. Deformed wing virus titres analysed for all mite-resistant subpopulations pooled together (MR, in blue) directly compared to mite-susceptible colonies (MS, in red) are shown here. Virus replicates are displayed on the left y-axis and number of Varroa mites per 100 bees is displayed on the right y-axis, both covering a period of 4 months including September, May, June and August. Number of samples per month is displayed above each column.

**Winter colony survival**

Number of surviving colonies after winter were counted in April 2018 and compared to the number of colonies in August 2018 for European mite-resistant subpopulations and the local control group including mite-susceptible honey bees. The most significant decrease in winter colony survival for mite-resistant honey bees was observed in the Swedish subpopulation at 50 %, however hardly any differences could be observed in other subpopulations. In total, percentage of colony survival for all mite-resistant subpopulations was 83 %. In the mite-susceptible control group, percentage of colony survival was 33 %.
Figure 3. Winter colony survival was assessed for mite-resistant subpopulations including France (blue), Sweden (purple), Netherlands (red) and Norway (yellow) which were compared to each other and the mite-susceptible control group (green). Percentages of colony survival was calculated for each subpopulation and the control group.

Discussion

Honey bee colonies in Europe and USA have been in dramatic decline for over three decades with *Varroa destructor* and RNA-viruses vectored by the mite being the most significant causes. Recent studies have observed higher winter colony survival in a *Varroa*-resistant Swedish subpopulation from Gotland, however no correlation between mite-resistance and virus-resistances was established (Locke, B. 2014). The aim of this project was to determine if a correlation between mite-resistance and virus-resistances could be observed in other European mite-resistant subpopulations compared to mite-susceptible honey bees.

The initial hypothesis for this project was that mite-resistant honey bees would have lower virus titres compared to mite-susceptible honey bees to establish a correlation between mite-
resistance and virus resistance to explain the differences in winter colony mortality observed in the study on the Swedish honey bee subpopulation (Locke, B. 2014). Findings in previous studies have shown a positive correlation between number of mites and levels of viral RNAs (Shen, M. 2005). However, contradicting results in this project demonstrate that virus titres were not lower in the European subpopulations compared to the control group (Fig. 1). Virus titres in June and August were mostly equal for SBV and BQCV titres between populations and higher DWV titres can be observed in all subpopulations when compared to the control group. This was further demonstrated when comparing the pooled mite-resistant subpopulations to the mite-susceptible subpopulation for DWV since virus titres were higher in the mite-resistant population during all months (Fig. 2). Similar observations have been made in previous studies on virus dynamics between mite-resistant and mite-susceptible honey bees (Locke, B. 2014) where slightly higher DWV titres were observed in July in mite-resistant Swedish honey bees compared to mite-susceptible honey bees, and equal DWV titres were observed for other months.

These findings indicate that there are no signs of virus resistance in mite-resistant subpopulations. However, despite high virus titres, observed winter colony survival in mite-resistant subpopulations was significantly higher than in mite-susceptible honey bees (Fig. 3). In one study, a significant correlation between DWV viral load and overwintering colony losses was observed (Highfield, A. et al. 2009), however in this project, such correlation was only established in the mite-susceptible population. There is evidence that *V. destructor* increases the virulence of DWV infections which results in decreased winter colony survival (L. Wilfert, et al. 2016). This could possibly explain the differences in observed winter colony survival in mite-susceptible honey bees compared to mite-resistant subpopulations. Additionally, tolerance mechanisms that may make the mite-resistant honey bees more capable of dealing with an infection of DWV may be present in these populations. This
hypothesis is also suggested in the study on the mite-resistant Swedish subpopulation from Gotland (Locke, B. 2014). Similar speculations could be made about SBV and BCQV titres since they were almost equally high in mite-resistant and mite-susceptible colonies.

Most high virus titres for all populations were observed in autumn, which is expected due to the mite’s exponential population growth over the season, causing increased virus transmissions that lead to virus infections which peak during autumn and winter (SJ, Martin, et al. 2002; Locke, B. 2014).

Variations in virus titres between the mite-resistant subpopulations were observed, specifically in DWV titres. In colonies that are heavily infested with V. destructor, nearly 100% of adult workers are estimated to be infected with high titres of DWV since it is the most common Varroa transmitted virus (de Miranda, et al. 2011; de Miranda, et al. 2010), however, as was observed in May and June for the Swedish subpopulation and control group, and June for the Norwegian subpopulation (Fig. 1, A; B; E), some honey bees were completely negative for DWV. All subpopulations were sampled during four different months however the colonies were always the same ones being sampled, so it is curious that titres would exist initially in September, then disappear over the spring and summer to suddenly reappear in autumn. Results regarding missing titres of DWV could be explained by the adjustment of the cycle threshold for DWV and SBV, meaning samples with low virus titres may have been detected as negative. Similar missing titres of BQCV was observed in the Dutch subpopulation during May which could be due to laboratory errors.

No samples were positive for SBPV, CBPV and ABPV except for one positive sample for ABPV in the control group during June (Fig. 1, E). This was expected since these viruses are not as common and wide-spread in honey bees compared to DWV, BQCV and SBV.
The method used for diagnosing virus infections in bees consist of two-step RT-qPCR. Both advantages and disadvantages are found within this method. In this case, it is the preferred method since it provides possibility to stock cDNA and quantify several targets. Furthermore, it is highly sensitive, making it possible to detect very low titres of virus infections which also increases the chance of detecting contaminants within samples. However, when working with wide-spread honey bee viruses like DWV and SBV, contaminants can be found in many locations in the lab. Additionally, two-step RT-qPCR includes more pipetting steps which increases the chance of errors and contamination. Therefore, sterile laboratory work is essential to provide accurate results.

During the project, contamination was found in negative samples for DWV and SBV which made it necessary to change the detection threshold for those samples, consequently making the interpretation of data for DWV and SBV uncertain.

In conclusion, no correlation between high mite-resistance and virus-resistance could be established for European mite-resistant subpopulations based on the findings in this project. However, significant differences in winter colony survival numbers between mite-resistant and mite-susceptible honey bee colonies indicate that possible virus tolerance mechanisms not yet understood or other means of survival are present in these subpopulations. Additional studies are required to further investigate the cause of higher colony winter survival observed in mite-resistant honey bees and how this correlates to *Varroa destructor*-resistance.

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References


