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ORIGINAL RESEARCH ARTICLE

Naturally selected honey bee (*Apis mellifera*) colonies resistant to *Varroa destructor* do not groom more intensively

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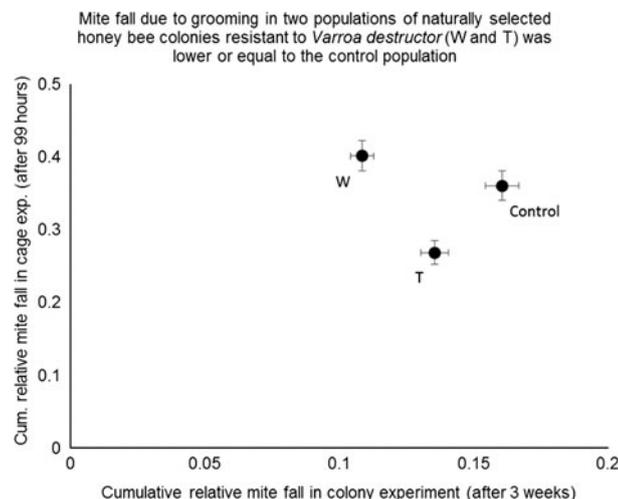
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The ectoparasitic mite *Varroa destructor* is an important cause of high colony losses of the honey bee *Apis mellifera*. In The Netherlands, two resistant *A. mellifera* populations developed naturally after ceasing varroa control. As a result, mite infestation levels of the colonies of these populations are generally between 5–10%. However, the mechanisms behind mite resistance are still unclear. Since grooming behavior is a typical resistance trait that occurs in *A. mellifera*, we compared grooming between colonies of these two resistant populations and control colonies that had been treated against varroa twice a year in previous years. Grooming was investigated by measuring mite fall in broodless colonies in the field and in small cages with a fixed number of mites and bees in the lab. Furthermore, grooming was investigated at the individual level by measuring the effectiveness to remove dust by individual bees from the resistant and control colonies. We found that the grooming behavior of resistant colonies was unexpectedly equally or even less effective than that of control colonies. These results were supported by the effectiveness of individual bees to remove dust. Based on our results, we discuss that the trigger for grooming behavior may be density-dependent: grooming may be only beneficial at high mite infestation levels. Other resistance mechanisms than grooming are more likely to explain the varroa resistance of our two populations.

Colonias de abejas (*Apis mellifera*) seleccionadas naturalmente por su resistencia a *Varroa destructor* no se acicalan más intensamente

El ácaro ectoparasitario *Varroa destructor* es una importante causa de grandes pérdidas de colmenas de la abeja de la miel *Apis mellifera*. En los Países Bajos, dos poblaciones resistentes de *A. mellifera* se desarrollaron naturalmente después de cesar el control de varroa. Como resultado, los niveles de infestación de ácaros en las colonias de estas poblaciones generalmente están entre el 5–10%. Sin embargo, los mecanismos que hay detrás de la resistencia del ácaro todavía no están claros. Dado que el comportamiento de acicalamiento o “grooming” es un rasgo típico de resistencia que sucede en *A. mellifera*, comparamos este comportamiento entre colonias de estas dos poblaciones resistentes y colonias control que habían sido tratadas contra varroa dos veces al año durante los años anteriores. El “grooming” se investigó calculando la caída de ácaros en colonias sin cría en el campo y en pequeñas cajas con un número fijo de ácaros y abejas en el laboratorio. Además, el “grooming” se investigó al nivel individual calculando la efectividad para eliminar el polvo por abejas individuales de las colonias resistentes y del control. Se encontró que el “grooming” de las colonias resistentes era inesperadamente igual o incluso menos eficaz que el de las colonias control. Estos resultados fueron apoyados por la efectividad de las abejas individuales para eliminar el polvo. Basándonos en nuestros resultados, discutimos que el desencadenante del “grooming” puede ser dependiente de la densidad: el comportamiento de acicalamiento sólo puede ser beneficioso con altos niveles de infestación de ácaros. Otros mecanismos de resistencia son más propensos a explicar la resistencia a varroa en nuestras dos poblaciones.



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Introduction

The ectoparasitic mite *Varroa destructor* is considered to be an important cause of the reported high colony losses of the Western honey bee *Apis mellifera* in both Europe and the USA during the last decades (Rosenkranz, Aumeier, & Ziegelmann, 2010). Although the mite does not directly kill the bees, it has large effects by weakening bees through feeding from the haemolymph of the pupae and the adult bees, and by transmitting bee viruses like deformed wing virus and acute bee paralysis virus. Together these effects can shorten the life span of individual bees and subsequently the whole colony may collapse (Boecking & Genersch, 2008; Rosenkranz et al., 2010; van Dooremalen et al., 2012).

The original host of the varroa mite is the Asian honey bee *A. cerana*. Since the mite and *A. cerana* have co-evolved, a balanced host-parasite relationship prevents varroa from becoming a significant pest (Rath, 1999). Hence, *A. cerana* has obtained certain traits that enable them to control mite infestation levels (Peng, Fang, Xu, & Ge, 1987; Rath, 1999). Around 1957, varroa shifted from its original host to *A. mellifera* (Delfinado, 1963). Now it is found worldwide with a few exceptions. *A. mellifera* is vulnerable to the mite because it has had a much shorter co-evolutionary history. As the mite haplotype that switched to *A. mellifera* is very virulent and causes much damage to colonies (Rosenkranz et al., 2010), beekeepers choose to treat colonies against the mites to prevent colony collapse. Although effective acaricide treatment reduces the immediate damage to the colonies, low mite numbers also reduce the selection pressure on bees to adapt to varroa (Fries & Camazine, 2001) and thereby hamper natural selection for mite resistance and *V. destructor* susceptibility.

The original host *A. cerana* has several defensive mechanisms that limit varroa population growth (i.e., resistance): grooming, uncapping and removing of infested worker brood, and entombing of infested drone brood (Peng et al., 1987; Rath, 1999). Furthermore, mites are found to be unable to reproduce in worker brood of *A. cerana*, which is probably due to features of the pupae (de Ruijter, 1987; Rath, 1999). *A. mellifera* shows similar behaviors, like grooming and removal of infested brood, but they are less effective against the mites compared to *A. cerana* (Boecking & Spivak, 1999; Peng et al., 1987). Resistance provides benefits for the host as a result of the reduced parasite load. However, it may also be costly in terms of energy loss. For example, Currie and Tahmasbi (2008) found that bees were able to reduce the mite load by grooming, although there was a cost associated with grooming as bees with

a higher ability to groom had shorter lifespan. Therefore, bees are predicted to only perform defensive behavior towards varroa when it is cost effective.

One approach to obtain resistant colonies in Europe and North America is through natural selection by ceasing mite control. In Europe successful attempts have been made to make natural resistant populations on the island of Gotland, Sweden (Fries, Imdorf, & Rosenkranz, 2006) by ceasing mite treatment in infested colonies. They found that these populations could reduce *V. destructor* population growth compared to colonies that were treated against mites. Comparable to the population in Gotland, Blacquière, Boot, Calis, and Panziera (n.d.) started in 2007 to select for surviving colonies in which varroa control was ceased. Now, two populations that naturally acquired resistance to varroa mites have been established, in which the mite infestation of the colonies is generally at a constant low level between 5 and 10%. However, the mechanisms behind mite resistance in *A. mellifera* are still unclear. Understanding the mechanisms behind resistance to varroa contributes to the understanding of this relatively new host-parasite relationship and ultimately helps to prevent colony losses. Since grooming behavior is a typical resistance trait of *A. cerana* and also occurs in *A. mellifera*, but with different mite removal success (Aumeier, 2001; Currie & Tahmasbi, 2008; for a review see Pritchard, 2016), we compared the grooming behavior between colonies of these two populations and control colonies.

Grooming can be defined as the cleaning of the bee's body from dust, pollen and ectoparasites (Boecking & Spivak, 1999). The ability to groom depends on environmental factors (Stanimirovic, Stevanovic, Aleksic, & Stojic, 2010). For example, mite removal success increases with temperature: bees with a high grooming ability were most effective at a temperature of 25 °C and a low humidity (Currie & Tahmasbi, 2008). Individual bees can clean themselves (auto-grooming) and also others (allo-grooming). Earlier studies showed that some mites that had been groomed were visibly injured or dead (Aumeier, 2001; Kirrane et al., 2012). So, grooming to remove the mites could be a factor that contributes to suppression of mite populations through removal or (fatally) injuring mites, leading to a stabilization of the host-parasite relationship (Boecking & Spivak, 1999). Besides environmental factors, the bees' genetic make-up affects the ability to groom (Bak & Wilde, 2015). Although different heritabilities have been reported, it is not clear how high the degree is to which grooming is heritable (Pritchard, 2016; Zakar, Jávora, & Kusza, 2014).

The aim of this study was to test the hypothesis that colonies selected for natural resistance against varroa have a higher grooming ability than control colonies. Grooming was investigated by measuring mite fall in: (1) colonies in the field; and (2) small cages in the laboratory. In the small cages we could control the number of bees and mites, which was not possible in the field study. Furthermore, grooming was also measured at the individual level by: (3) measuring the effectiveness of grooming after dusting of individual bees.

Materials and methods

Origin of the resistant honey bee selections

We used resistant colonies from the populations of Tiengemeten (T) and Amsterdamse Waterleidingduinen (W), and compared these colonies with the control group (C). The population of Tiengemeten partly descends maternally from the Gotland (Sweden) population, which is naturally resistant or tolerant (Fries et al., 2006). The population of Amsterdamse Waterleidingduinen is a population of 'hybrid' Dutch colonies, established with 70 colonies in 2008, of which 20 were used as controls and 50 as the starting group to select for resistance. In both selection populations, no varroa control has been done since 2007 (T) and 2008 (W) (Blacqui re et al., n.d.). The 20 control colonies (C), originating from the population of Amsterdamse Waterleidingduinen, were treated with oxalic acid against varroa twice every year, once during summer and once during winter. They were further managed in a similar way to the selection populations.

Every spring, colonies that survived winter without varroa control and that were vital (colony growth, drone production) were selected for the new generation. After the loss of many colonies in the first years (bottleneck), the remaining honey bee colonies showed an average colony loss during winter of $18\% \pm 17$ SD, and a relatively low infestation level of 7–13% (~5% in the treated control colonies). Preliminary additional data showed that this selection, using winter survival, spring colony growth and drone production as critical factors, resulted in two populations with colonies in which adult bees seem to actively suppress the mite population (Blacqui re et al., n.d.). Between winter and summer and *vice versa* mite infestation levels in the selection colonies (W and T) changed with a factor 0.5–2, while in the treated control group (C) infestation levels increased at least tenfold (Blacqui re et al., n.d.). This led us to consider that the T and W colonies were resistant to varroa mites and that the C colonies were susceptible. The possible mechanisms leading to the slow build-up of mite populations in the selection colonies compared to the control, which include increased grooming of varroa mites, reduced reproduction of mites, hygienic behavior and Varroa Sensitive Hygienic behavior (VSH), are now subject of

study. This paper reports about the differences in grooming behavior.

The selection was done by removing the old queen, which resulted in the making of new 'emergency' queens. After maturation of the queens (14 days), the colony was split into 4–5 new colonies that each received a newly emerged queen. Queens were then able to mate in the remote areas of Tiengemeten (a real island) and Amsterdamse Waterleidingduinen, where no other colonies were located within five kilometers. The reason that approximately five kilometers seems to work is because there were only few other colonies around, and because our colonies were scheduled in such a way that they produced a high number of mature drones exactly at the moment the queens were there to mate. A similar case of a naturally surviving and stable honey bee population at a distance of six kilometers from commercial apiaries was published recently (Seeley, Tarpy, Griffin, Carcione, & Delaney, 2015).

Colonies for the present experiments

For the experiments, five colonies from each group (T, W and C), headed by a 2014 queen, were brought to the Grebbedijk apiary, nearby the river Rhine in Wageningen, the Netherlands ($51^{\circ}57'04.0''\text{N}$, $5^{\circ}38'07.5''\text{E}$). These were all treated once in spring with oxalic acid in order to obtain similarly low starting levels of mite infestation. During the study some colonies had to be taken out due to queen failure (more details below).

Mites for the experiments

To obtain the mites needed for both the colony experiment as well as the cage experiment, 10 colonies with high varroa infestation were used: five 'varroa mite showers' (MS) with a high number of phoretic mites, and five accompanying colonies (AC) with a high number of mites in the reproductive phase. These colonies were unrelated to the experimental populations. With an interval of seven days, frames were moved between one MS and one AC in order to force mites to stay on bees (phoretic phase) in the MS and to reproduce in the AC. Both the MS and the AC consisted of two brood stories separated by a queen excluder. The queen was placed in the upper part of the colony. In both the AC and the MS, frames with empty cells (emerged brood cells) from the lower part were moved to the upper part of the colony where new eggs could be laid. In the AC, frames with open brood were then in return moved back to the bottom part. Though, in the MS, open brood cells from the upper part were moved to the AC (bottom part), so no MS mites could enter into these cells, but AC mites could. Then, frames with capped brood from the lower part of the AC were moved into the lower part of the MS. With this method, it was possible to collect a high number of

phoretic mites from the MS, which were replenished with mites from the AC.

To collect mites from the MS, the powdered sugar method was used. This method was found to be highly effective for mite collection ($92.9\% \pm 5.5$ SD) and it has little effect on mite survival (Macedo, Wu, & Ellis, 2002). The method consists of dusting a bee sample with powdered sugar, which causes mites to dislodge from bees. About 400 bees from the MS were taken into a jar with a meshed lid (2×2 mm). Next, one table spoon of powdered sugar was poured through the mesh after which the jar was shaken carefully so no sugar or mites could fall out. After about one minute, the jar was shaken upside down so dislodged mites could fall down into a plastic box. Mites were then collected with a moist paintbrush and put into a container with humid paper which prevented them from desiccation. Mites were used within two hours after collection.

Colony experiment

Grooming behavior at colony level was investigated by comparing mite fall between resistant and control colonies. We started with five colonies per group (Amsterdamse Waterleidingduinen, Tiengemeten and the control group), but some colonies were taken out because of queen failure. The colony experiment was done using 12 colonies: four colonies from Amsterdamse Waterleidingduinen, three of Tiengemeten and five of the control group. As colonies were treated with oxalic acid in spring, mite infestation was low. Therefore, to increase mite numbers in the colonies prior to the experiment and to use similar mite sources for all groups, we transferred mites from the MS to the experimental colonies. Two frames per colony that consisted of a relatively high number of open brood cells were placed into an MS (one frame per colony on 25 July and one on 18 August 2015). After seven days, when the open brood cells were closed, the frames were placed back in the original colony together with the reproducing mites that had entered the now capped brood cells.

To be able to estimate the number of mites present in the colony by measuring the infestation on adult bees, and to exclude mite fall due to VSH, no brood was present during the experiment. In order to stop the queen from laying eggs, the queen was caged three weeks before the start of the experiment and the queen cage was placed in the middle of the hive (11 August 2015). After three weeks, when all already laid eggs had developed into adult bees, the experiment started (1 September 2015). For three weeks, the number of mites was counted that fell on a sticky bottom board placed under each hive. Two weeks after the start of the experiment, the queen was released from the queen cage to minimize colony decline. In order to prevent bees to come into contact with the fallen mites, we covered the sticky bottom board of the hive with a metal wire mesh (0.4×0.4 cm) which allowed the mites to fall through.

Vaseline was put on the bottom board, to prevent mites from escaping from the bottom board and ants from collecting fallen mites. During this period, with intervals of three days, mite fall numbers and injured mite numbers were counted. Mites were checked on injuries by using a microscope (magnification $4\times$). Mites were considered injured when missing one or more legs or when (part of) their idiosoma was missing. Regular dorsal dimples were not taken into account as these are not necessarily caused by grooming but can originate from anomalies during mite ontogeny (Davis, 2009).

In order to compare mite fall between colonies, we calculated mite infestation (number of mites/bee) by estimating the total number of bees and mites per colony. Colony size was estimated by the Liebefeld method (Delaplane, van der Steen, & Guzman, 2013; Imdorf, Buehlmann, Gerig, Kilchenmann, & Wille, 1987). Early in the morning, the area covered (in dm^2) by bees was estimated for all frames of the hive. The size of the colony was then calculated by multiplying the occupied area with the assumed bee density (125 bees/ dm^2). This was repeated every week. After three weeks, at the end of the experiment, all mites in the colony were removed and collected on the sticky bottom board by spraying the bees with oxalic acid (3%), which has been found to be highly effective (97.3–98.8%) with no detrimental effects for bee survival (Rademacher & Harz, 2006). Again, mite fall numbers were counted on the sticky bottom board after 3 and 6 days. The total number of mites present in the colony during the experiment was estimated by adding mite fall numbers as fallen during the experiment to the mite fall numbers as result of the oxalic acid treatment.

Cage experiment

Small cylindrical cages ($\text{Ø} \times \text{H} = 8.4 \times 11.3$ cm) were constructed to investigate grooming behavior at group level of the resistant and control bees. In each cage 12 g bees (between 84 and 110 bees, with on average 98.9 ± 10.3 SD; the exact number of bees was recorded per cage) and 20 varroa mites were put, resulting in a mite infestation of around 20% (on average $21.5\% \pm 2.3$ SD). In total 14 colonies were used, five from the Amsterdamse Waterleidingduinen and control groups each and four from Tiengemeten (1 was taken out because of queen failure). For each colony three cages were made, resulting in 42 cages. Cages consisted of two plastic cups (Figure 1). At the bottom of the inner cup a mesh was made through which only varroa mites could fall (mesh size 0.5×0.5 cm). Fallen mites were then collected on the bottom of the outer cup, which was smeared with Vaseline to prevent mites from escaping. In order to provide sufficient ventilation, air holes were made about one cm above the bottom of the outer cup. Bees and mites were collected on the same day, and used within two hours after collecting. Bees were fed with sugar syrup (50:50 water:sucrose), which was put



Figure 1. Cage consisting of two plastic cups containing bees and varroa mites.

in Eppendorf tubes with small feeding holes and inserted in the lid of the inner cup. In order to put bees into the cage, bees were first anesthetized with CO_2 for a few seconds. When they stopped moving, they were carefully put into the inner plastic cup.

For this experiment, 840 mites were collected from the MS (20 mites per cage \times three cages per colony \times 14 colonies). For each cage, 20 mites were placed onto 20 randomly chosen bees using a small paintbrush. Only mites that automatically walked onto a bee were used. The cages were kept in a dark climate chamber at 25 °C. For four days mite fall numbers were observed twice a day (morning and afternoon). In the pilot study we found that mite damage was low (3 injured mites from 147 fallen mites), therefore mite injury was not measured.

At the end of the experiment bees were washed with soapy water to dislodge and count the remaining mites. This was done because bees might already have been infested with mites beforehand, and therefore the number of mites in the cage could have been more than 20. When less than 20 mites were found back (during the experiment and after washing) it was assumed that 20 mites had been put in initially (it happened seven times that one mite was missing, likely due to miss counting of the remaining mites). Washing of bees was done by putting all bees from a cage into a jar with soapy water for 20 min, after which they were shaken for 30 s. Next, the content of the jar was poured over a sieve (0.4 \times 0.4 cm) through which only mites could fall. Below this sieve a second one was placed to catch and count the removed mites. Bees were then washed with water of high pressure until no mites could be removed (Dietemann et al., 2013).

Individual grooming experiment

Dust removal efficiency was quantified and compared between individual bees from the resistant and control colonies (5 colonies of the Amsterdamse Waterleidingduinen and the control each, and 4 colonies of Tiengemetten). Starch (CAS 9005-84-9) was used to dust bees because it can be easily quantified by iodine. About 80 ± 15 SD bees from a colony were collected into a plastic box ($L \times B \times H = 8 \text{ cm} \times 5 \text{ cm} \times 5 \text{ cm}$). The bottom of this box contained a mesh (0.2 \times 0.2 cm) through which starch could pass but no bees. Before the start of the experiment the box was put upside down (the mesh up) and four grams of starch was poured through the mesh (Figure 2, panel 1). To minimize differences in initial starch cover between bees of the same box and between different boxes, a standard method for starch distribution was performed. In order to distribute starch equally over the bees, a second box of the same size but without mesh was placed upside down onto the box and they were both turned around five times. Starch that fell out the meshed box was collected into the second box and weighed to determine the initial amount of starch on the bees. Eventually, the box was placed with the mesh below (Figure 2, panel 3). The second box was immediately removed after the starch distribution, and a third box was placed under the meshed box to collect removed starch fallen through the mesh during the experiment. This was the start of the experiment: for three minutes bees were able to groom and clean themselves from dust. Removed starch fell through the mesh which prevented bees from dusting themselves again. After three minutes, the experiment was stopped by anesthetizing the bees with CO_2 for one minute after which they were put in the freezer at -20 °C until the quantification of the amount of starch left on the bees. In advance a small hole was made ($\varnothing = 1 \text{ cm}$) in the lid of the meshed box through which CO_2 could flow. During the experiment the hole was covered with tape (Figure 2, panel 2). CO_2 ensured that bees stopped grooming within two seconds and did not resume activity. We repeated the experiment four times: 24 July, 23 August, 26 August and 28 August 2015.

The quantification of the remaining starch was based on the indication of starch by iodine. Through dissolving the remaining starch on the bee in water and adding iodine, the staining value of the blue starch-iodine complex could be measured. From each experimental box, 5–15 bees were randomly used and their starch was removed (on average 12 bees \pm 3.3 SD) (Figure 2, panel 4). We determined the remaining starch for each of these bees individually. This was done by soaking each bee in an Eppendorf tube with 1.3 ml water, followed by carefully moving the bee up and down in the water and then let it stand for 15 minutes. Because starch aggregates in water, the starch solution was heated at 100 °C for 20 min using a heating block to make it water soluble (solubility: 50 g



Figure 2. Method of starch experiment to determine individual grooming behavior: (1) meshed box upside down; (2) meshed box with tape covering CO₂ hole in the lid; (3) meshed box put into third box during the experiment; (4) bees covered with starch.

starch/l at 90 °C; estimated starch/bee: around 30 g/l). After heating, each tube was vortexed for 10 s and cooled down at -20 °C for 10 min. Next, starch solution was diluted by adding 0.7 mL water to each tube. From each tube a 50 µl sample was transferred to a flat bottom 96 well micro plate, together with 150 µl water and 10 µl iodine solution (10 mg/ml Povidone-iodine (Betadine)). The absorbance was measured at 580 nm using a microplate reader (TECAN instruments). The Tecan program involved 120 sec shaking the plate, 12 measurements per well and 25 flashes per measurement. Absorbance of the starch-iodine complex from bees from resistant and control colonies was compared and each colony was tested four times.

Data analysis

Colony experiment

First, a generalized linear mixed model (GLMM) with binomial distribution and logit link function was used to compare mite fall of the colonies of the three treatment groups (fixed factor) (in R using the function `glmer` from the “lme4” package), followed by a post hoc test (Hothorn, Bretz, & Westfall, 2008) to determine pairwise differences between means of each group. In the GLMM, colony and measurement day were taken as random factors. Next, Cox regression was used to compare the timing of mite fall between the resistant and control groups. From each colony, the time (days) to event (mite fallen) was compared. Mites that were not found fallen during the experiment were treated as censored data. Furthermore, a linear mixed model

(LMM) was used to compare the percentage of injured mites (number of injured mites as percentage of the total mite fall numbers) per day per colony from each treatment. The injured mite percentage was not normally distributed, and a Gamma distribution was used with log link, followed by a post hoc test (Sidak). Colony and measurement day were used as random factors in the LMM. We choose the best model by selecting the covariance matrix and the method (restricted maximum likelihood method (REML) or maximum likelihood (ML)) with the lowest Aikake's information criteria (AIC).

Next, the correlations between mite infestation (number of mites per bee) and the mite fall numbers, injured mite numbers and injured mite percentages were investigated using Spearman's rank test because data was not normally distributed. Furthermore, the ratio between bee and mite mortality was analyzed. From the weekly bee colony size measurements, the daily bee mortality was calculated as the percentage of the total number of bees that died each week, divided by seven (no new bees emerged as we prevented the queen to lay eggs). This was compared to the daily mite mortality calculated as the percentage of the total number of mites present in the colony that fell during three days, divided by three. Differences in daily mortality (dependent variable) between bees and mites (fixed factor) were tested using a LMM. We tested these differences for three levels of mite infestation. Colony was used as random factor. Data were not normally distributed, and a Gamma distribution was used with a log link, followed by a post hoc test (Sidak).

Cage experiment

First, mite fall was analyzed using a GLMM (in R using glmer) with binomial distribution and logit link, with treatment (fixed factor) and colony and date of testing (random factors), followed by a post hoc test (Hothorn et al., 2008). In order to compare the differences in timing that mites fell off during four days between the cages from the resistant and control groups, Cox regression was performed next. The time (hours) to event (mite fallen) was compared between the cages of the treatment groups. Mites that were not found fallen at the end of the experiment were treated as censored data. Treatment was used as factor and date of testing (July or August) as covariate.

Individual grooming experiment

A LMM was used to compare the absorbance, as a measure of grooming efficiency, between resistant and control groups. From each treatment group, bees from different colonies were repeatedly tested over time. Therefore, treatment group and measurement date were used as fixed factors and colony, the bees' weight and box (to account for the group of bees that were dusted at once) as random factors. We included bee weight in the analysis because we found a negative correlation between the bee's weight and starch absorption (Spearman's rank correlation $r = -1.04$, $N = 651$, $p = 0.008$). As the random factor box was not significant, we assumed that possible variation in the initial

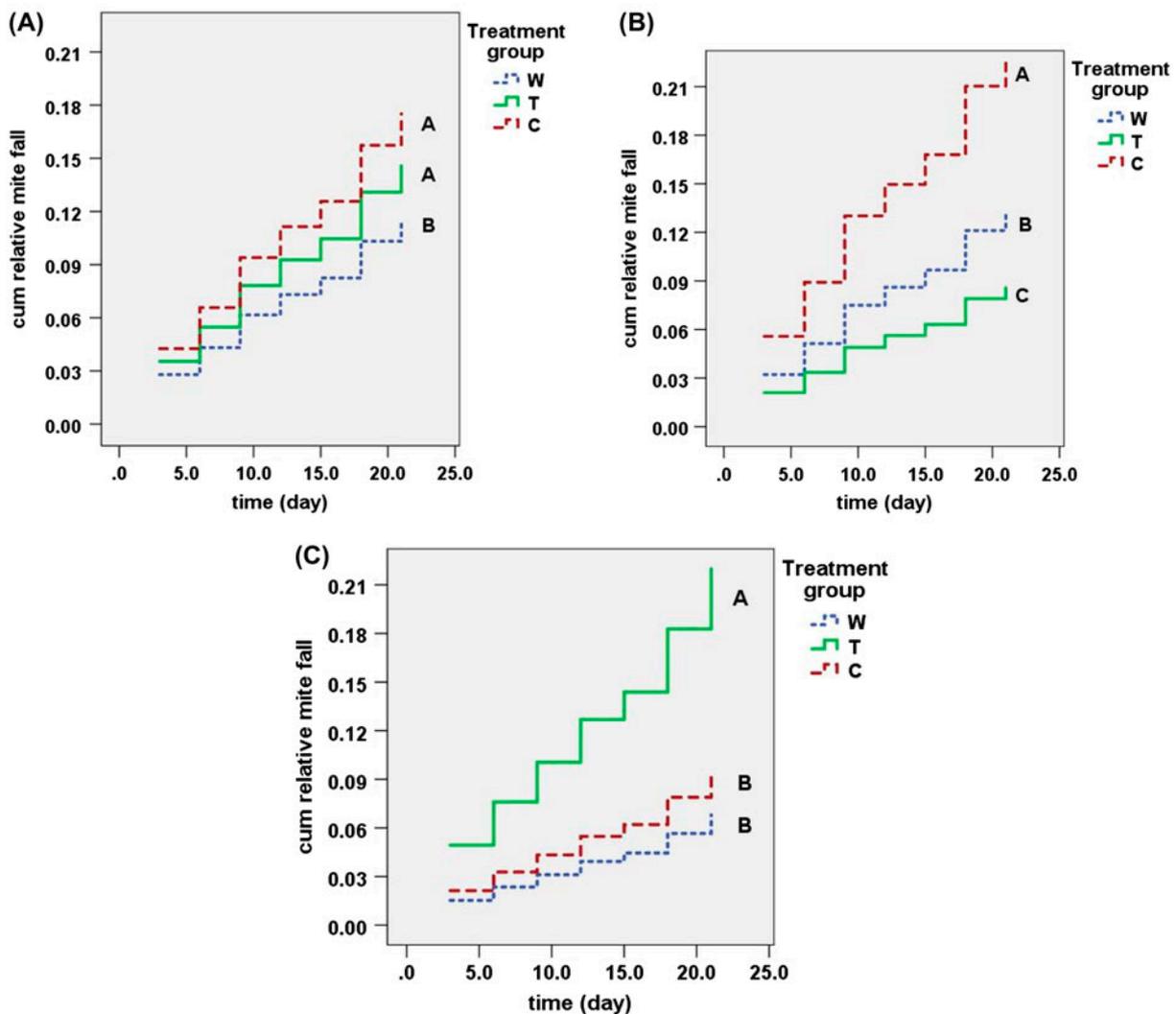


Figure 3. (A) Cumulative relative mite fall (fraction mite fall in relation to initial mite numbers) as function of time in days since the start of the experiment for all colonies from the three groups (initial mite infestation 2–10%). Panel (B) gives cumulative relative mite fall of colonies with high mite infestation level (8–10%) (for W: $N = 2$, C: $N = 2$, T: $N = 1$), whereas panel (C) gives Cumulative relative mite fall of colonies with low mite infestation level (<8%) (for W: $N = 2$, C: $N = 3$, T: $N = 2$). Note: W refers to the colonies from the Amsterdamse Waterleidingduinen, T to the colonies from Tiengemetten, and C to the control colonies. A higher cumulative mite fall after 21 days (a steeper slope) results from a higher daily mite fall during those 21 days. Letters indicate significant differences between treatment groups.

amount of starch added to the boxes (4 g) did not matter. The LMM was followed by a post hoc test (Sidak).

All statistical analyses were performed in IBM SPSS statistics 22, except when written differently.

Results

Colony experiment

As a result of introducing mites from the MS into the colonies, the initial mite infestation of the colonies varied between 2 and 10% (number of mites/bee) (See Online Supplementary Information Table S1). The probability that a mite fell did not differ between the groups (GLMM, $F_{2,27739} = 0.205$, $p = 0.815$). Mean mite fall percentage (from the estimated total mite numbers in the colony) during three days for the Amsterdamse Waterleidingduinen colonies was $1.4\% \pm 1.2$ SE, for the Tiengemetten colonies $2.5\% \pm 1.2$ SE and for the control colonies $2.6\% \pm 1.9$ SE. Also the percentage of injured mites did not differ between the treatments (LMM, $F_{2,59} = 0.797$, $p = 0.456$) (mean injured mite percentage per day for Amsterdamse Waterleidingduinen was $10.4\% \pm 10.9$ SE, for Tiengemetten $10.1\% \pm 6.6$ SE and for the control $9.7\% \pm 8.5$ SE). There was a significant difference in cumulative mite fall over time between the treatments (Cox regression, $\chi^2 = 18.7$, $df = 2$, $p < 0.001$). The daily mite fall was highest for both the control and Tiengemetten group, and lowest for the Amsterdamse Waterleidingduinen group (Figure 3(a)). When comparing the cumulative mite fall over time for only colonies with the highest mite infestation levels (8–10%), the daily mite fall was highest for the control colonies, and higher for the colonies of Amsterdamse Waterleidingduinen than for Tiengemetten (Cox regression, $\chi^2 = 39.174$, $df = 2$, $p < 0.001$) (Figure 3(b)). When comparing the cumulative mite fall over time for only colonies with the lowest mite infestation level (<8%), the Tiengemetten group had a higher daily mite fall over time than the control and Amsterdamse Waterleidingduinen group (Cox regression, $\chi^2 = 41.196$, $df = 2$, $p < 0.001$) (Figure 3(c)).

Mite fall numbers and injured mite numbers were positively correlated with mite infestation (Spearman's rank correlation, $r = 0.415$, $p < 0.001$; $r = 0.490$, $p < 0.001$ resp.). Though, injured mite percentage was neither correlated with mite infestation (Spearman's rank, $r = 0.183$, $p = 0.095$) nor with the number of bees present in the hive (Spearman's rank, $r = -0.135$, $p = 0.220$). Also mite fall percentage was not correlated with mite infestation (Spearman's rank, $r = 0.084$, $p = 0.447$). When comparing these factors within each treatment group, a negative correlation was found between mite fall percentage and mite infestation for Tiengemetten (mite infestation range 5–8%) (Table 1). A positive correlation was found between mite fall numbers and mite infestation, though no correlation

Table 1. Correlations (Spearman's rank) between mite infestation level (number mites as percentage of number of bees) and mite fall numbers, mite fall percentage (as percentage of initial mite numbers), injured mite numbers and injured mite percentage (as percentage of mite fall numbers) for each group.

	No. fallen	Perc. fallen	No. injured	Perc. injured
W	$r = 0.615$ $p < 0.001$	$r = 0.358$ $p = 0.06$	$r = 0.746$ $p < 0.001$	$r = 0.519$ $p = 0.005$
C	$r = 0.409$ $p = 0.015$	$r = 0.086$ $p = 0.625$	$r = 0.182$ $p = 0.294$	$r = -0.103$ $p = 0.555$
T	$r = -0.20$ $p = 0.933$	$r = -0.601$ $p = 0.004$	$r = -0.20$ $p = 0.933$	$r = -0.171$ $p = 0.458$

Notes: The correlation coefficient r is given with the corresponding p -value. Significant correlations are in bold, borderline significant in italics. W refers to the colonies from the Amsterdamse Waterleidingduinen, T to the colonies from Tiengemetten, and C to the control colonies.

between mite fall percentage and mite infestation was found for Amsterdamse Waterleidingduinen group (mite infestation range: 4–15%). Furthermore, the Amsterdamse Waterleidingduinen group showed a positive correlation between both injured mite numbers as well as injured mite percentage with mite infestation. The control group did not show any correlation between the mite fall percentage or injured mite percentage with mite infestation, but did show a positive correlation between mite fall numbers and mite infestation (mite infestation range 2–15%) (Table 1).

The mite infestation level increased during three weeks of the experiment, because bee mortality was higher than mite mortality (GLMM, $F_{1,162} = 79.066$, $p < 0.001$). Mean daily bee mortality was 1.78 ± 0.96 SE, while mean mite mortality was 0.75 ± 0.5 SE. When also taking mite infestation level into account, a significant interaction effect between infestation level (low (<4%), middle (4–8%) high (8–15%)) and bee and mite mortality was found (GLMM, $F_{5,158} = 19.203$, $p < 0.001$) (Figure 4). Daily bee mortality was always higher than mite mortality at every mite infestation level. Furthermore, bee mortality was highest at low mite infestation (<4%), although it was not significantly higher compared to the bee mortality at the highest mite infestation level (Figure 4). Mite mortality did not differ between mite infestation levels.

Cage experiment

The mite fall percentage did not differ between treatment groups (GLMM, $F_{2,868} = 0.507$, $p = 0.603$). The mean daily mite fall for the Amsterdamse Waterleidingduinen colonies was $8.9\% \pm 7.7$ SE, for the Tiengemetten colonies $6.9\% \pm 7.9$ SE and the control colonies $7.8\% \pm 7.1$ SE. The cumulative mite fall over time differed significantly between the treatment groups (Cox regression, $\chi^2 = 16.48$, $df = 3$, $p = 0.001$); the hourly mite fall over time for the Tiengemetten group was lower than for the Amsterdamse Waterleidingduinen and control

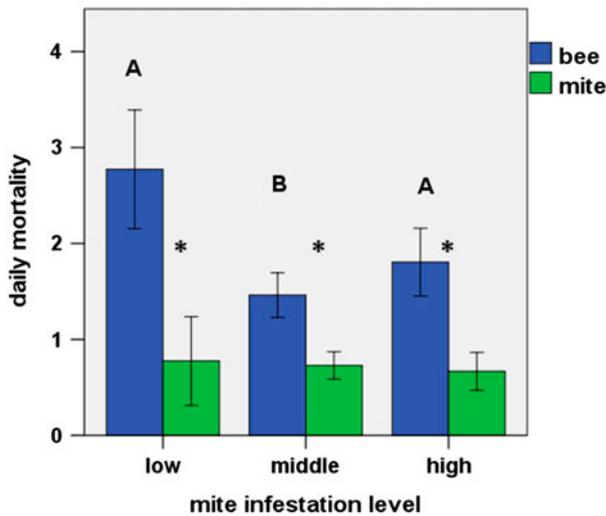


Figure 4. Mean daily bee and mite mortality as percentages of the population present at start of every week (bees) and three days (mites) at different mite infestation levels: low (<4%), middle (4–8%) and high (8–15%). Error bars indicate the 95% confidence interval. Daily mite mortality was calculated as $100 \times (\text{mite fall numbers per three days}/\text{total mites present at the start of the three days})$, divided by three. Daily bee mortality was calculated as $100 \times (\text{number of bees in week } i - \text{number of bees in week } i + 1)/\text{number of bees in week } i$, divided by seven.

Notes: Letters mark significant differences between bee mortality. The differences between mite mortality are not significant between mite infestation levels. Asterisks indicate significant differences between bee and mite mortality for every mite infestation level.

groups (Figure 5). Furthermore, the hourly mite fall was higher in July than in August ($p = 0.004$), but mostly for

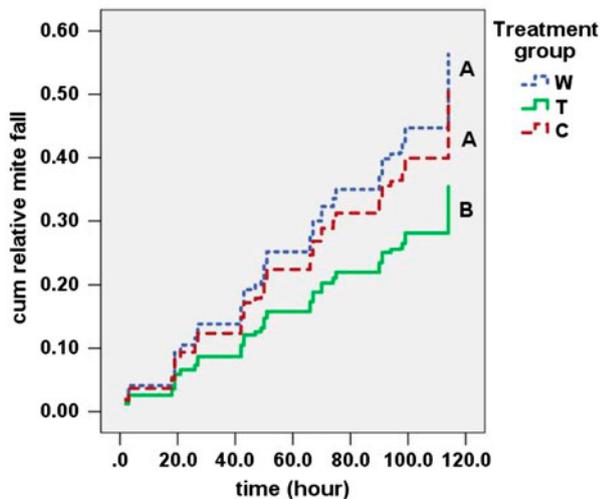


Figure 5. Mite fall in cages as a function of time in hours for colonies of each treatment group during July and August.

Notes: W refers to the colonies from the Amsterdamse Waterleidingduinen, T to the colonies from Tiengemeteten, and C to the control colonies. Letters indicate significant differences between treatment groups.

the control group (higher in July than in August ($p < 0.001$)). No differences were found between July and August in the Amsterdamse Waterleidingduinen and Tiengemeteten group ($p = 0.652$ and $p = 0.368$ resp.).

Individual grooming experiment

The average amount of starch per bee was $0.04 \text{ g/bee} \pm 0.005 \text{ SE}$. The absorbance of the blue staining, as a negative indicator of grooming efficiency, differed between the treatment groups and moment of testing (LMM: treatment $F_{2,15,684} = 5.936$, $p = 0.012$; moment $F_{3,42,915} = 21.111$, $p < 0.001$). There was no interaction between testing moment and treatment ($F_{3,45,286} = 1.379$, $p = 0.244$). The Amsterdamse Waterleidingduinen group had a significantly higher absorbance than the control group (Figure 6). All testing moments differed significantly ($p < 0.05$) except the first and last moment of testing ($p = 0.737$).

Discussion

In this study the grooming behavior was compared between colonies that were naturally selected for varroa resistance and control colonies that are treated against varroa twice a year. This was tested by: (1) comparing mite fall in broodless colonies in the field; (2) in small cages with a fixed number bees and mites; and (3) by comparing the dust removal efficiency of individual bees. Method 1 and 3 are new methods, added to the four so far used methods listed in Bąk and Wilde (2015), and are both quantitative in nature. Because no brood was present in the cage and the colony experiment, all mites fell due to mite removal activity of bees (grooming) and to natural mite mortality.

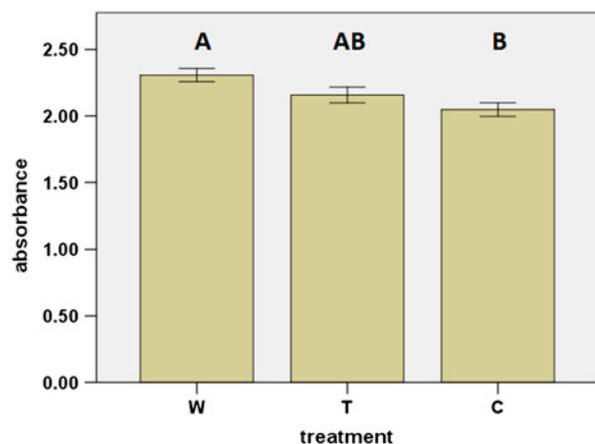


Figure 6. Mean absorbance of the blue staining of each treatment group. The higher the absorbance the higher the amount of starch left on the bee.

Notes: W refers to the colonies from the Amsterdamse Waterleidingduinen, T to the colonies from Tiengemeteten, and C to the control colonies. Letters indicate significant differences between treatment groups.

We found that bees from the two naturally resistant populations, Amsterdamse Waterleidingduinen and Tiengemetten, showed grooming behavior towards the parasitic varroa mite, though they were equally or even less effective in mite removal than bees from the control colonies. Especially the comparison between the control colonies and the colonies from the Amsterdamse Waterleidingduinen is relevant as they have the same origin. Mite fall did not differ between resistant and control colonies in the experiment, neither for the colonies in the field nor in the experiment with the small cages. Furthermore, in both experiments the cumulative mite fall over time in the control colonies was higher or equal to both groups of resistant colonies. These results were supported by the effectiveness of individual bees to remove dust. Bees from the control colonies were more or equally effective in dust removal as bees from resistant colonies. The absence of increasing grooming behavior in the selected Tiengemetten colonies is not surprising, as grooming has not been found to be important in the origin colonies of Gotland (Locke, 2016). Our results suggest that other resistance traits than grooming are responsible for the observed resistance of colonies of both resistant populations, such as control of mite reproduction success (e.g., attractiveness of brood, infertility, decreased fecundity of mites and availability of brood) and uncapping and removal of parasitized brood (VSH) (Rosenkranz et al., 2010; Zakar et al., 2014).

This study showed for the first time that removal of dust might be related to the behavior for removing varroa mites. Dusting bees with powdered sugar is a common method to dislodge mites from bees (Dietemann et al., 2013) in order to measure varroa infestation levels or to use the dislodged mites in (laboratory) experiments. Dusting stimulates bees to groom (Land & Seeley, 2004), which might (partly) cause enhanced mite removal after dusting (Stevanovic, Stanimirovic, Lakic, Djelic, & Radovic, 2012). Therefore, the dust removal efficacy might be useful as an indicator of grooming behavior towards varroa mites, which has never been used before. Given the small differences between the groups, we recommend that the study should be repeated on colonies with known varying degrees of grooming behavior to verify that dust removal can indeed be used as a proxy for bees' ability to remove mites by grooming. In contrast to dusting, our results show that mite injury was not a good proxy for grooming behavior. The idea of mite injury as a proxy for grooming behavior is indeed controversial (Bienefeld, Zautke, Pronin, & Mazed, 1999; Boecking & Spivak, 1999; Fries, Huazhen, Wei, & Jin, 1996; Rinderer, De Guzman, & Frake, 2013). However, mite injury is thought to occur due to allo-grooming (Pritchard, 2016) and the group size in our cage experiment and individual grooming experiment may be too small to trigger allo-grooming and could thus explain the absence of mite injuries.

The mite fall in the cages was in line with mite fall experiments with comparable cages at 25 and 35 °C

(Currie & Tahmasbi, 2008; Kirrane et al., 2012). However, mite fall in our colonies was about three times lower than in the cage experiment. Differences in mite recognition might explain these differences in mite removal. Bees can recognize mites by their cuticular hydrocarbons (CHCs), which passively change after being transferred to another colony (Kather, Drijfhout, & Martin, 2015). Incomplete or mismatching CHCs profiles might explain the higher mite fall in our cages compared to our colonies. In the cage experiment, mites were collected from the mite shower colonies and therefore had a different origin than the bees from the three treatment groups. In the colony experiment, additional mites were introduced by 'showering' ready-to-be-capped-brood with phoretic mites from the mite showers. However, this trapping already occurred 14 days before the start of the experiment. By the time the colony experiment started, the mimicry of the mite CHC profiles was probably complete and thus mite camouflage was in place. In addition, probably half of the mites present had been raised in the capped brood inside the colonies (first generation daughters of the captured mites from the 'mite shower' colonies). A second explanation for the lower mite fall in our colonies than in the cage experiment might be differences in the number of tasks of bees in the colonies and bees in the cages. The number of tasks in the cages might be limited (e.g., no nursing larvae or foraging), while in the colonies bees may have to prioritize many tasks. With more tasks to prioritize, chances are higher that several of these tasks may have been more urgent than grooming of mites from bees, resulting in less mites removed from the colony than from the cages.

Mite fall numbers were positively correlated with mite infestation level. This is in line with findings of different studies to use the daily mite fall to calculate the mite infestation level (Branco, Kidd, & Pickard, 2006; Calatayud & Verdu, 1993; Martin, 1998). Hence, differences in mite fall numbers reflect then differences in mite infestation rather than differences in grooming behavior. This is a problem in the colony experiment, because colonies differed in mite infestation level. Therefore, when studying grooming behavior, the mite fall percentage may be a better indicator and could be used instead. In the cage experiment, this was not a problem as we used a fixed mite infestation level (20%). In contrast to the positive correlation between mite infestation level and mite fall numbers, the mite infestation level was not related to the mite fall percentage (mite fall number as percentage of the total mite population), indicating that bees did not become more efficient in mite removal at higher or lower mite infestations (and grooming was not mite density dependent). This suggests that the mite fall percentage as a measure for grooming activity can be compared between groups, independently of their mite infestation level. However, analyzing only the data of the Tiengemetten group suggested that, in contrast to the above, the

mite fall (removal) was density dependent. Namely, a negative correlation was found between mite fall percentage and mite infestation level. Therefore more research is needed to investigate this hypothesis.

Daily bee mortality did not differ between the treatment groups for a given mite infestation level. Interestingly, more bees died than mites, and thus mite infestation levels grew. The relatively high bee mortality compared to mite mortality may be partially explained by the ability of phoretic mites to avoid removal from the colony by preferring younger bees over older bees like foragers that will most likely die outside the nest (Kraus, 1993; Pernal, Baird, Birmingham, Higo, & Slessor, 2005). The relatively high bee mortality compared to mite mortality may also be explained by bees not efficiently removing mites, except perhaps at high levels of mite infestation. Our speculation is that grooming is only beneficial at high levels of mite infestation, but the pattern was not very consistent over treatments (only in control colonies). This lack of investment in grooming is in line though with the studies of Vandame, Colin, Morand, and Otero-Colina (2000), Vandame, Morand, Colin, and Belzunces (2002) who investigated another resistance behavior of the bees than we did in this study, namely the ability of bees to remove infested brood. They suggested that this behavior might only be beneficial above a certain threshold. Below this threshold the best strategy would be to accept the damage of the parasite (tolerance), only beyond this threshold it would be beneficial to be perform active defensive behavior (resistance). The results of our colony experiment were based on a limited number of colonies and a relative small mite infestation range. Perhaps mite infestation in our colony experiment was too low for grooming to be cost effective. Our higher infestation levels in the cage experiments were additionally difficult to include due to the use of foreign mites. It would be interesting to investigate the costs and benefits of grooming at a large range of mite infestation levels to see whether grooming becomes more cost effective with increasing mite infestation levels. Note, it should be kept in mind that the infestation of the selected colonies in recent years in general was between 5 and 10% under broodless conditions (Blacqui re et al., n.d.).

In conclusion, the results of this study show that the observed resistance of Tiengemeten and Amsterdamse Waterleidingduinen colonies towards *V. destructor* cannot be explained by grooming. Contrasting results about the importance of grooming have been reported, some indicate grooming as an important resistance trait (Aumeier, 2001; B chler, Drescher, & Tornier, 1992; Fries et al., 1996; Guzman-Novoa, Emsen, Unger, Espinosa-Monta no, & Petukhova, 2012; Rinderer et al., 2001, 2013) but others do not (Harbo & Harris, 1999; Locke & Fries, 2011; Vandame et al., 2002). These conflicting results indicate that depending on the interaction between factors like the genetic make-up of the bees and the mites, different strategies evolve that confer resistance towards

V. destructor (Guzman-Novoa et al., 2012). Hence, other resistance strategies than grooming, such as removal of infested brood and the inhibition of mite reproduction, are more likely to explain resistance of bees from Tiengemeten and Amsterdamse Waterleidingduinen.

Author contributions

AK, FvL, CvD and TB designed the research, AK and TB carried out the experiments, AK and FvL analyzed the data, and all authors wrote the paper.

Supplementary material

Supplementary material is available for this article at: <http://dx.doi.org/10.1080/00218839.2017.1329797>.

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