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SHORT COMMUNICATION

High *Varroa* mite abundance influences chemical profiles of worker bees and mite–host preferences

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ABSTRACT

Honeybee disappearance is one of the major environmental and economic challenges this century has to face. The ecto-parasitic mite *Varroa destructor* represents one of the main causes of the worldwide beehive losses. Although halting mite transmission among beehives is of primary importance to save honeybee colonies from further decline, the natural route used by mites to abandon a collapsing colony has not been extensively investigated so far. Here, we explored whether, with increasing mite abundance within the colony, mites change their behaviour to maximize the chances of leaving a highly infested colony. We show that, at low mite abundance, mites remain within the colony and promote their reproduction by riding nurses that they distinguish from foragers by different chemical cuticular signatures. When mite abundance increases, the chemical profile of nurses and foragers tends to overlap, promoting mite departure from exploited colonies by riding pollen foragers.

KEY WORDS: *Apis mellifera*, *Varroa destructor*, Cuticular hydrocarbons, Parasite transmission, Mite abundance

INTRODUCTION

Parasite success depends not merely on their efficiency in exploiting other organisms but also on their ability to assess the host conditions, in order to abandon the exploited host in search of a more favourable one when it no longer has profitable resources to offer. Transmission to an unexploited host is a crucial step in the life cycle of parasites; high fecundity, as well as behavioural adaptations aimed at increasing the chances of a parasite actually being transmitted, represent some of the traits of a selective process favouring transmission (Combes, 2005). Although the decision to transfer from an exploited host to an unexploited one is critical for parasite success, the factors that allow adaptive transmission timing have seldom been investigated.

Here, we investigated the factors regulating the dispersal of *Varroa destructor*, the ecto-parasitic mite of the honeybee, *Apis mellifera* Linnaeus 1758, which undoubtedly represents an important element in the massive decline of managed bee colonies recorded in recent years (Le Conte et al., 2010). *Varroa destructor* is an obligate parasite of cavity-dwelling honeybees belonging to the genus *Apis*; originally confined to the eastern honeybee, *A. cerana*, it shifted to the new host *A. mellifera* during the last century, after the introduction of *A. mellifera* colonies into the distribution range of *A. cerana* (Martin, 2001). Since then, the parasites have quickly spread following movements of honeybees, reaching nearly a worldwide distribution. Although chemical treatments are used to lower mite load, re-infestation events are common. Mites weaken their hosts by sucking the haemolymph from both adult and immature stages, and transmit RNA viruses that damage bee health (Carreck et al., 2010). Halting mite spread, if it could be achieved, would be an important contribution to saving honeybees from further decline; this goal can be accomplished only by deepening the knowledge of *Varroa* transmission. Although it is documented that beekeeping practices (i.e. moving brood combs among colonies; migrating bee hives for pollination needs) and robbing (stealing of honey from infested/weakened colonies by foragers from nearby colonies) represent important ways of mite transmission, the natural route used by the mites to abandon a collapsing colony has not been extensively investigated.

The mite life cycle can be divided into phoretic and reproductive phases (Rosenkranz et al., 2010). During the reproductive phase, a mature female abandons her phoretic host, an adult bee, to enter a cell containing a host larva, shortly before cell capping occurs, to reproduce. Mother and offspring feed on the immature host haemolymph; mature offspring mate within the cell before abandoning it at the emergence of the adult bee. After leaving the cell, the mite enters the phoretic phase by staying on an adult host for a few days or weeks before entering a new brood cell for the next cycle of reproduction. Previous research (Del Piccolo et al., 2010) has shown that mites, once emerged from the cells, prefer to ride on nurses, distinguishing them from foragers via chemical cues; the nurses will most likely bring the mites in contact with brood susceptible to infestation, a behaviour favouring parasite reproduction. However, this preference is advantageous for mites only when they seek another brood cell in the same colony in which to reproduce. In contrast, in highly infested colonies or those close to collapse, where available host brood is lacking or reduced, we would expect mites to adopt a strategy that allows them to abandon such a compromised situation with scarce or null reproduction possibilities, in search of a more favourable environment. We thus hypothesized that, to maximize the possibility of leaving a highly infested colony, mites should move on to foragers; their dispersal would be even more effective if they moved onto bees from a different colony (i.e. a drifted forager or a robbing bee). The ability to recognize 'non-nestmate hosts' in a highly infested colony might allow the mites to ride on more appropriate carriers that may ensure the parasites abandon the infested colony and transfer directly to a new less-infested one.

RESULTS AND DISCUSSION

We firstly explored whether, with increasing mite abundance within the colony, mites change their preference for hosts with different tasks, by using binary choice tests. To test our hypothesis, we collected phoretic mites (N=196) from seven hives previously

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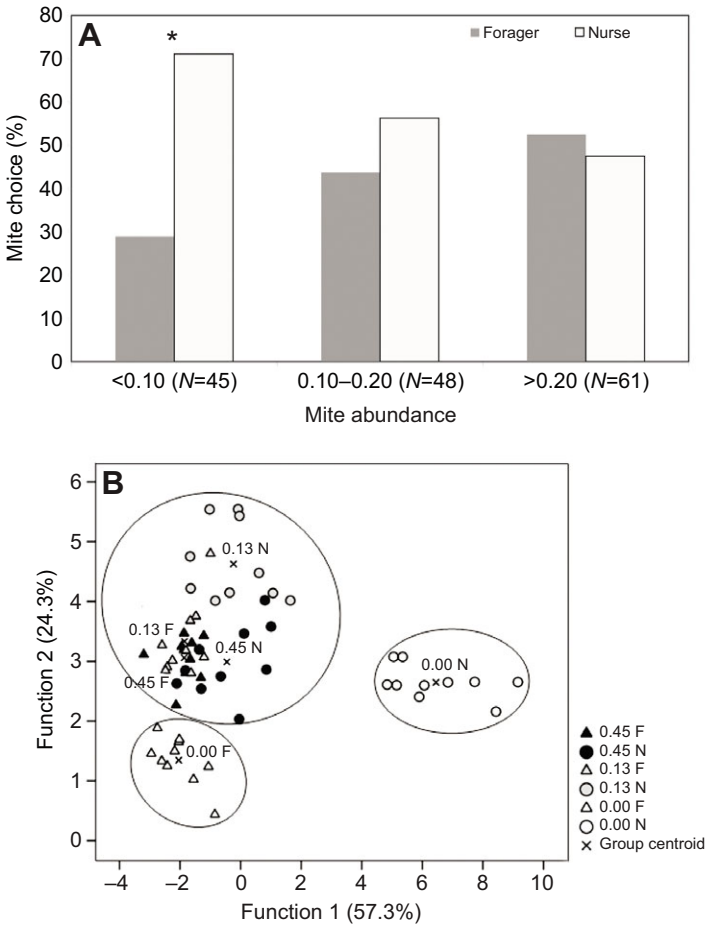

Fig. 1. Mite preference for nurses and foragers and chemical signature of bees with different tasks in beehives with increasing mite abundance. (A) Preference of mites (*N*=154) belonging to colonies with different mite abundance tested through binary choice experiments between a pollen forager and a nurse bee. Mites from a colony with a mite abundance lower than 0.10 significantly preferred to ride on nurse bees than on foragers (*G=8.19, d.f.=1, P=0.01); this preference disappeared when mite abundance was greater. (B) Stepwise discriminant analysis of cuticular chemical signatures of nurse bees (N) and pollen foragers (F) collected from colonies with three different levels of mite abundance (0, 0.13, 0.45). The chemical profiles of nurses and foragers collected from colonies with a mite abundance higher than 0.13 overlap, while those of uninfested colonies are distinct.

sampled for mite abundance and, in a small choice arena, determined the preference of individual mites for bees with different colony tasks – nurse versus forager – from the same colony as the mite itself. Thirty-two mites (16%) chose no bees; 10 mites (5.1%) showed no clear preference for either bee. For the remaining 154 mites, their choice of bee was affected by mite abundance within the colony to which they belonged to (G=6.02, d.f.=2, P<0.05) (Fig. 1A). Choosy mites collected from colonies with a mite abundance of less than 0.10 (*N*=45) significantly preferred to ride on nurses rather than on foragers (G=8.19, d.f.=1, P=0.01). This preference disappeared when we checked the 48 choosy mites collected from colonies with 0.10–0.20 mite abundance (G=0.74, d.f.=1, ns) and the 61 choosy mites collected from colonies with a mite abundance greater than 0.20 (G=0.15, d.f.=1, ns). These results are consistent with those obtained when comparing the total time spent by the mite on the two bees (Z=–2.5, P=0.008; Z=–0.32, ns; Z=–0.55, ns, respectively).

As the mite preference for bees performing different tasks is driven by a specific blend of cuticular hydrocarbons (CHCs) covering the body surface of nurses and foragers (Del Piccolo et al., 2010), we explored whether the chemical signature of nurses and foragers differed among individuals coming from colonies with different mite abundance. CHCs of nurses and foragers collected from three colonies with different mite abundance (no mites, 0.13 and 0.45) were extracted and analysed by gas chromatography coupled with mass spectrometry (GC-MS). Although stepwise discriminant analysis showed that 93.7% of the individuals (Fig. 1B) were correctly assigned to their respective group (function 1: Wilk's λ=0.003, χ^2=308.042, d.f.=40, P<0.001, explaining 57.3% of variance; function 2: λ=0.029, χ^2=184.280, d.f.=28, P<0.001, explaining 24.3% of variance; a cross-validation attribution of specimens revealed that 81.7% of the bees were correctly attributed to their group), a clear-cut difference in CHC profiles between nurses and foragers was detectable only for bees collected from the uninfested colonies (Fig. 1B). This difference disappeared when mite abundance increased: the chemical signature of nurses and foragers collected from colonies with a mite abundance higher than 0.13 overlapped (Fig. 1B). Among pairs of nurses and foragers belonging to the three colonies with different mite abundance, the chemical profile distances between bees performing different tasks were significantly higher in the colony without mites than in the hives with greater mite abundance (0.13 and 0.45) (χ^2=28.599, d.f.=2, P<0.001, *post hoc* test P=0.001).

These results showed that, at low mite abundance, mites stay within the colony where they are born and promote their reproduction by riding nurses. This preference ensures that mites are quickly transferred to another host larva within the same colony where they can reproduce. When mite abundance increases within the colony, the lack of differences in chemical cues between nurses and foragers probably does not allow mites to discriminate between bees with different tasks and causes mites to ride on both of them. As the honeybee possesses a flexible conditional age determination system that is socially regulated (Huang and Robinson, 1996), it is possible that, in conditions of high mite abundance, when the bee population decreases and few new bees emerge, foragers revert from foraging to brood care, homogenizing the chemical signatures of workers with different tasks. This chemical profile homogenization of bees with different tasks, induced indirectly by abundant mite

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presence, does not provide mites with the cues for discrimination and promotes mite emigration from infested colonies.

Another possibility is that honeybees have evolved to modify their chemical profiles when they are in colonies with moderate/high mite abundance, inducing mites to move on to foragers and lowering in this way the colony mite load.

We next explored whether, with increasing mite abundance within the colony, mites are able to discriminate between homocolonial foragers (foragers from the same colony as the mite) and heterocolonial foragers (alien foragers coming from a different colony). We tested this hypothesis by presenting phoretic mites (N=309) collected from eight hives (five out of the seven above-mentioned colonies and three additional ones) with a binary choice between homocolonial and heterocolonial foragers. As mites are able to recognize different hydrocarbon blends on the bee cuticle (Del Piccolo et al., 2010), they may discriminate differences in the chemical signature among foragers belonging to different colonies. We found that 224 choosy mites [65 mites (21%) did not make any choice], regardless of the mite abundance of the colony, did not show nestmate host recognition ability (G=4.84, d.f.=2, ns) or, at least, they did not express it in our experimental setup (Fig. 2). In fact, they did not show any preference between homocolonial and heterocolonial foragers at any mite abundance of the colony (<0.10, G=3.1, d.f.=1, ns; 0.10–0.20, G=1.35, d.f.=1, ns; >0.20, G=0.62, d.f.=1, ns, respectively). These results are consistent with the total time spent by the mite on each of the two forager types (Z=–1.42, ns; Z=–0.89, ns; Z=–0.97, ns, respectively).

Although the results of the second behavioural assay did not support our hypothesis that mites could maximize their dispersal by moving onto bees from a different colony (i.e. a drifted forager), we must keep in mind that, possibly, within our sample of homocolonial foragers collected from colonies with high mite abundance, there could be heterocolonial foragers entering the colonies for robbing. This might have biased our result by apparently lowering the percentage of mites choosing foreign foragers. In any case, by riding both nestmate foragers and foreign foragers, mites increase their probability of leaving the exploited colonies. An infested colony, progressively weakened by parasites and mite-transmitted diseases, finally becomes an easy target for robbing foragers from foreign colonies; these robber foreign bees might represent excellent vectors to directly transfer mites from the old exploited beehive to a new one.

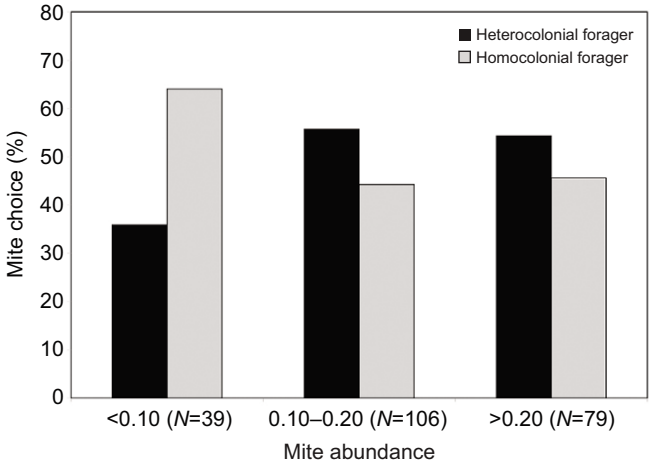

Fig. 2. Mite preference for homocolonial and heterocolonial foragers in beehives with increasing mite abundance. Preference of mites (N=224) belonging to colonies with different infestation rates tested through binary choice experiments between two foragers, one from the same colony and one from a foreign colony (G=4.84, d.f.=2, ns).

MATERIALS AND METHODS

Mite abundance evaluation

Mite abundance for each experimental colony was evaluated by randomly sampling 300 bees within the colony on the same day of the mite/bee collection for bioassays. The sampled bees were killed by freezing and then inspected for the presence of phoretic mites. This sampling procedure will yield colony mite abundance with adequate precision (Lee et al., 2010). Variation in the mite abundance of the 10 sampled colonies used for the bioassays was remarkable, ranging from less than 0.05 up to about 0.53. We thus divided the tested mites into three different categories according to the mite abundance of the colony from which they were collected: (1) mites (N=126) belonging to three colonies with a mite abundance lower than 0.10; (2) mites (N=214) belonging to four colonies with a mite abundance higher than 0.10 but lower than 0.20; (3) mites (N=165) belonging to three colonies with a mite abundance higher than 0.20. At least 20 mites per colony were tested for each treatment in the two bioassays. We chose the threshold of 0.10 mite abundance as it has been reported (Lee et al., 2010) to be the maximum limit after which a colony needs to be treated.

Bee and mite collection

Between June and July, for the first bioassay, we collected a total of 196 nurse bees (bees were collected while they had their head/thorax in cells containing larvae), 196 foragers (bees collected while returning from foraging flights) and 196 phoretic mites by individually brushing them off the adult bees. For the second bioassay, we collected a total of 309 foragers and 309 phoretic mites from the bodies of adult bees; finally, 309 foragers were collected from five additional colonies located far from the experimental ones. Mites and bees were transferred to the laboratory and stored in glass containers, and provided with bee pupae and small sweets, respectively, until they were used for bioassays (within 24 h).

Experimental procedure

Each mite was singly tested by placing it in the centre of a small arena (a Petri dish of 6 cm diameter) where two live bees were confined in two adjacent small areas (about 2.5 cm^2 each) defined by a metallic net. The bees were provided with small sweets and allowed to move freely in each small area and interact with each other through the metallic net. Each mite was also free to walk inside the arena and to ride on either bee. Trials lasted for 90 min. All mites and bees were tested once.

Data collection and analysis

For each mite, we recorded the total time spent on either bee, as well as the position on the body of either bee or anywhere else inside the arena, every 15 min for a total of six subsequent checks. Mite preference was evaluated through two alternative ways. Firstly, we considered the number of times the mite was recorded on the body of the two offered bees: a mite was considered choosy when it was found ≥2 times more frequently on one bee than on the other. A G-test with William correction was used to compare the mite preference for the two offered bees within each treatment group. Secondly, mite preference was established on the basis of the amount of time spent on the two bees; data were analysed with the Wilcoxon test. All the mites that did not make a choice during the trial and/or did not show a clear preference were excluded from the analysis.

Chemical analyses

CHCs of 10 nurses and 10 foragers collected from three colonies with different mite abundance (0, 0.13, 0.45) were extracted and analysed by GC-MS. An extract of each bee was obtained by washing its four wings in 100 µl of pentane in an ultrasonic bath for 10 min. Preliminary analyses showed that extraction from the wings gives the same chemical profile obtained with extraction of the entire body; this procedure avoided any contamination from internal body fluid or pollen. Solvent was then evaporated under a nitrogen stream and extracts were re-suspended in 10 µl of heptane. Analyses of 1 µl of cuticular compound extract for each specimen were performed using a

Hewlett Packard (Palo Alto, CA, USA) 5890A gas chromatograph coupled to an HP 5971 mass selective detector (using 70 eV electronic ionization source) following the standard procedure reported elsewhere (Cappa et al., 2013). Identification of the compounds and data processing were carried out before conducting statistical analyses, as described previously (Cappa et al., 2013). Discriminant analysis was used to determine whether the predefined groups of bees (foragers and nurses) could be discriminated on the basis of their profiles. The significance of Wilk's λ and the percentage of correct assignments were used to estimate the validity of the discriminant function. A cross-validation test (leave-one-out) was also performed. Moreover, we calculated the chemical Euclidean distances (Turillazzi et al., 2000), i.e. the chemical distance between forager–nurse pairs of bees, by standardizing peak percentages with *Z*-scores. The differences among groups were analysed with the Kruskal–Wallis test.

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Competing interests
The authors declare no competing financial interests.

Author contributions
R.C. designed the study, performed the field work, and followed and analysed the behavioural data. C.B., F.C. and S.M. performed the field work, the bioassays in the lab, the chemical analysis and the statistical analysis of chemical data. G.P. provided support with GC-MS. D.P. provided support with beehives in the field. All authors prepared and edited the manuscript prior to submission.

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