Evidence for colony-specific differences in chemical mimicry in the parasitic mite *Varroa destructor*

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Running title: Chemical mimicry in the Varroa mite

Abstract. In social insects, the integrity of a colony is maintained by recognising and removing aliens. Nest-mates use chemical cues on the cuticle of the individual they encounter to determine whether or not it is part of the colony. Parasites have evolved to take advantage of this recognition system by mimicking these chemical cues to gain entry to the colony and therefore avoid being attacked by the host during their stay. Some of these parasites imitate the odour of a particular sub-group of colony members, such as pupae, which makes it more likely that they are accepted into the colony, whereas others mimic the adult colony odour. The ectoparasitic mite *Varroa destructor* uses chemical mimicry to access and remain undetected inside colonies of its honey bee host, *Apis mellifera*. It remains, however, to be tested whether the chemical profile of *V. destructor* mirrors colony-specific cues of the host's chemistry that allows con-specific nest-mate discrimination to occur in honey bees. Here we show that colony-specific differences in the chemical profile of four *A. mellifera* colonies were based on differences in the *n*-alkane:alkene ratio. These colony-specific differences in chemical profile were mirrored by *V. destructor* mites collected from the same four colonies, even though overall chemical mimicry was imperfect.

Key Words Cuticular hydrocarbons; camouflage; *Varroa*; honeybees; alkenes

Introduction

In social insects, a colony provides a safe place to store food and rear brood within a stable micro-climate. For many predators and parasites, however, such colonies are a concentrated source of food, as well as a perfect environment to rear their own offspring. To protect the colony from being exploited social insects have evolved an intricate system for recognising strangers inside the colony and along its borders (Hölldobler and Wilson 1990). Nest-mates constantly compare the odour of other individuals around them to their own to detect strangers. Should the odours differ beyond a certain threshold, the individual is attacked and removed from the colony.

To overcome this system of defence, parasites have evolved a number of chemical strategies to invade and permanently live in social insect colonies (reviewed in: Dettner and Liepert 1994; Lenoir et al. 2001; Bagnères and Lorenzi 2010). Many social parasites mimic host odour by synthesising host-specific compounds (true chemical mimicry) or by acquiring compounds from the host itself (chemical camouflage), e.g. by repeatedly grooming the host. Other parasites reduce their own odour to minute levels, either to mimic the host brood or evade detection altogether (chemical insignificance).

Cuticular hydrocarbons (CHC) have repeatedly been shown to play an important role in the nest-mate recognition behaviour of social insects (reviewed in: Howard and Blomquist 2005). In many insects, CHC are expressed as part of the insect's cuticular lipid layer and differences in the concentration of CHC have been shown to serve as signal of colony-origin

in many species of ants (Wagner et al. 2000; Greene and Gordon 2007; Martin et al. 2013), bees (Arnold et al. 2000; Buchwald and Breed 2005), hornets (Butts et al. 1995; Ruther et al. 1998), termites (Bagnères et al. 1991; Kaib et al. 2004) and wasps (Dani et al. 2004; Dapporto et al. 2006). CHC mimicry has also been shown to be important in the integration of parasites into the host colony (Cini et al. 2011) and there is increasing evidence that a number of parasites mimic the colony-specific fraction of the host odour to be accepted into the colony as a nest-mate (e.g. Guillem et al 2014; Martin and Bayfield 2014). For example, the butterfly *Maculinea rebeli* biosynthesises host-specific compounds before invading the nest of its ant host, *Myrmica schenki*, and, once inside the colony, fine tunes its chemistry to the colony odour by acquiring compounds from the host, possibly through trophollaxis (Akino et al. 1999). The myrmecophilous spider *Cosmophasis bitaeniata* mimics the colony odour and task odour of its ant host's minor workers (*Oecophylla smaragdina*) to avoid aggression (Elgar and Allan 2004, 2006). This social parasite is also able to distinguish between workers of its host colony and those of alien colonies, and chooses the company of the former. This behaviour is extremely important due to *O. smaragdina*'s highly aggressive behaviour, especially that of the major workers.

The mite *Varroa destructor* is an ectoparasite of the European honey bee *Apis mellifera*. The mite uses chemical mimicry to blend in with the host's CHC chemistry whilst sitting on the bee (Nation et al. 1992). This way, the body chemistry of the *V. destructor* and the bee are so similar that the mite evades being detected by the host despite the close contact. In addition to that, mites often hide in between the bee's 3rd and 4th ventro-lateral tergites of the abdomen (Boecking and Spivak 1999), where they are difficult to reach by the host. Female mites move around and between host colonies by hitching a ride on adult bees. By switching hosts *V. destructor* gains access to a particular area of the colony, for example,

by moving from a foraging bee onto nurse bee it gains access to the brood area (Kraus et al. 1986). Evidence presented in Nation et al. (1992) suggests that the mite's CHC profile changes as it moves onto a new host, since bees of different ages differ in their CHC profile (Nation et al. 1992; Arnold et al. 2000; Aumeier et al. 2002; Kather et al. 2011). Nevertheless, it remains to be tested whether the cuticular chemistry of *V. destructor* also matches colony-specific differences in host odour. Therefore, we investigate whether *V. destructor* mites collected from different *A. mellifera* colonies have adjusted their chemical mimicry to match the small colony-specific differences in CHC of their host colony.

Methods and Materials

Sample Collection and Chemical Analysis

Samples were collected from two apiaries (Sheffield and York; 100km apart) and from two *Varroa*-infested hives per apiary. Within each apiary, hives were 1m apart. For each hive, at least 10 bees were scooped off a brood frame into a vial and frozen for analysis. A *Varroa* board was placed underneath the hive and frames containing adult honey bees were covered in icing sugar. After 15-20 min., mites (60 per hive) were collected straight from the *Varroa* board using a fine, moist brush that was cleaned and dried after each mite. Mites were gently wiped with water once or twice to remove excess sugar and placed in Eppendorf tubes. All samples were frozen at -20ºC until extraction.

For the extraction, mites were pooled into groups of six mites per sample whereas bees due to their size were extracted individually. Samples were immersed in high-performance liquid chromatography-grade hexane (bees: 0.5 ml; mites: 300µl) containing a C_{20} standard (1mg/100ml HPLC grade hexane). Samples were left at room temperature for 15 min. before transferring 30 µl of extract to a glass insert, which was then left to evaporate to dryness before being stored at-20ºC until analysis. Immediately before analysis, samples were re-suspended in 30µl hexane and analysed on an HP6890-GC (equipped with an HP-5MS

column; length: 30m; ID: 0.25mm; film thickness: 0.25µm) connected to an HP5973-MSD (quadrupole mass spectrometer with 70-eV electron impact ionization). Samples were injected in the splitless mode. The oven was programmed from 70ºC to 200ºC at 40ºC/min and then from 200ºC to 320ºC at 25ºC/min and, finally, held for 5 min at 350ºC. The carrier gas helium was used at a constant flow rate of 1.0ml min-1. Compounds were identified using standard MS databases, diagnostic ions and Kovats indices.

Statistical Analysis

A number of chromatograms (17.5%) had to be discarded due to poor quality, leaving an average of nine *A. mellifera* chromatograms and eight *V. destructor* chromatograms per colony for statistical analysis. The peak area of each compound was determined by manual integration of each total ion chromatogram (TIC) and compound concentration (mg/ml HPCL grade hexane) was calculated using the standard C_{20} peak. The profiles of *A. mellifera* consisted of several homologue series of odd-chained *n*-alkanes (C_{23} - C_{31}); alkenes (C_{23} - C_{33}); dienes (C_{31} - C_{33}) and 9-, 11-, 13-mono-methylalkanes (C_{25} - C_{31}), which is in agreement with previous reports (Dani et al. 2004, Blomquist et al. 1980). The pooled *V. destructor* samples contained the same compounds listed above. Compounds which on average contributed less than 1% to the overall chemical profile (i.e. *n*-alkanes + alkenes + methylalkanes) were excluded from the analysis.

Samples were standardised by transforming CHC concentrations into relative proportions based on the total CHC concentration. To provide a metric of colony separation based on CHC profiles, three Fisher canonical discriminant analyses (DA) were conducted. For this, the proportion of each compound (relative to the total compound abundance of that

chemical class per individual) was transformed before the multi-variate analysis according to the formula;

$$Z = \ln[Ap/g(Ap)]$$

to avoid complications arising from analysing compositional data (Aitchison 1986). Ap is the proportion of the compound and g(Ap) is the geometric mean of all compounds to be included in the multi-variate analysis. The first two DAs were used to examine colony separation within the *V. destructor* mites and *A. mellifera* workers, while the final DA combined CHC profiles of the two species to test how closely parasites cluster to their host colony. For the final DA, each of the eight host-parasite groups (four parasite groups and four host groups) were treated as a separate group to investigate the relative separation of parasites and hosts according to their colony origin. As a cross-validation technique, a jack-knife (leave-one-out) sampling scheme was employed, in which each case was classified by the functions derived from all cases other than the case itself. A priori probabilities of assignment were calculated based on group sizes. All DAs were run in the statistical software R (v 2.81).For each of the three DAs described above, we also ran an ordination analysis (detrended correspondence analysis, DCA) followed by a goodness of fit test on the transformed data to test whether the group separation observed was indeed significantly associated with colony origin. All ordination analyses were conducted in R (v. 2.81) as part of the statistical package 'vegan' (Oksanen 2013).

To investigate whether parasite and host profiles were similar in the relative proportions of their CHC, CHC proportions were divided into the three main chemical classes that make up the host CHC profile: *n*-alkanes, methylalkanes and alkenes (alkenes + alkadienes). An ANOVA with post-hoc Tukey's test was run separately for each chemical class on the arcsine-transformed proportions. All significance tests were conducted using the statistical software R (v 2.81).

193

194

195 **Results**

196 There was significant colony separation amongst *A. mellifera* workers based on their CHC
197 profiles (Goodness of fit; R2=0.63, $p < 0.001$) (Fig. 1a). In the DA, 88% of bees were
198 correctly assigned to their colony. There was some overlap in the CHC profile of workers in
199 colonies 1 and 2, which led to the miss-assignment of three bees (two individuals in colony 1
200 and one individual in colony 2. All bees in colonies 3 and 4 grouped with their respective
201 colony.

202 A similar pattern of separation was found amongst *V. destructor* mites. Mites were
203 clearly separated according to their host colony (Goodness of fit; R2=0.63, $p < 0.001$) (Fig.
204 1b). Based on the DA, 67% of mites were 'correctly' assigned their colony and, as was the
205 case with *A. mellifera* workers, there was overlap between colonies 1 and 2. In this case, five
206 individuals of colony 1 were mis-assigned to colony 2 and four individuals of colony 2 were
207 mis-assigned to colony 1. All mites of colony 4 clustered together and only one individual of
208 colony 3 grouped with colony 1.

209 When combining host and parasite profiles, *A. mellifera* bees and *V. destructor* mites
210 still grouped according to colony (Goodness of fit; R2=0.72, $p < 0.001$) but this time mites
211 also grouped closely to bees of the same colony (Fig. 1c). Overall, colonies 1 and 2 clustered
212 separately from colonies 3 and 4, as had been observed previously when host and parasite
213 profiles were run separately. Based on the DA, mites of colony 1 not only grouped with mites
214 of colony 2 (as described above), but 33% also grouped with bees of colony 1. Furthermore,
215 9% of mites from colony 2 grouped with bees from colony 2, whereas 14% of mites from
216 colony 3 clustered with bees from colony 3. There was no mis-assignment of mites from

colony 4 with bees from colony 4, but the mites still clustered more closely to bees of colony 4 on the DA than to bees of colonies 1 and 2. There were no cases where mites had been mis-assigned to bees of a different colony.

This divide in chemical profiles between the colonies was also found when looking at the relative proportions of alkene, methylalkane and *n*-alkane in *A. mellifera* and *V. destructor* individuals. The *A. mellifera* colonies varied in the relative proportion of alkene (ANOVA: $F = 15.8$, d.f. = 7, 59, $p < 0.001$) and *n*-alkane (ANOVA: $F = 23.64$, d.f. = 7, 59, $p < 0.001$) (Fig. 2a). Colonies 3 and 4 were more '*n*-alkane rich', whereas colonies 1 and 2 had higher levels of alkenes. As observed in the DCA (Fig. 1a), colonies 1 and 2 were very similar in their CHC profile. In comparison, *V. destructor* mites had similar relative proportions of alkene to *n*-alkane compared to host individuals of the same colony (Tukey's test: $p > 0.1$), with the exception of mites from colony 4, whose alkene proportions were significantly higher compared to their host (post-hoc Tukey's test: $p < 0.0001$) (Fig. 2a, b). Overall, methylalkane proportions were higher in the parasite compared to the host (post-hoc Tukey's test: $p < 0.001$).

Discussion

The CHC profiles of *V. destructor* mites varied according to host colony. This phenomenon has also been shown in *Braula* flies, another honey bee parasite (Martin and Bayfield 2014) and in a number of other social insect parasites (Akino et al. 1999; Sledge et al. 2001; Elgar and Allan 2004, 2006; Guillem et al 2014). On the whole, *A. mellifera* workers and *V. destructor* mites from different colonies were clearly distinguishable based solely on their cuticular chemistry, both when compared on their own and in combination.

Each *A. mellifera* colony varied in the relative proportion of alkene and *n*-alkane, with colonies 1 and 2 having significantly higher levels of alkenes compared to colonies 3 and 4.

Colonies 1 and 2 were indeed extremely similar in CHC profile, as were colonies 3 and 4. This chemical similarity could be due to close relatedness because the former two colonies were from the Sheffield apiary, whereas the latter two belonged to the York apiary. This similarity in CHC was also reflected in the CHC profiles of their respective mites, with mites from colonies 1 and 2 clustering closely together in the DCA/DA as did mites from colonies 3 and 4.

When hosts and parasites were analysed together, mites still formed the same groups as described above but this time mites from colonies 1 and 2 clustered closely to bees from colonies 1 and 2, whereas mites from colonies 3 and 4 clustered closely to bees from colonies 3 and 4. Even though there was some overlap between mites and bees of the same colony, mites and bees of the same colony did not form a distinct cluster. This could be because the relative proportions of alkene and *n*-alkane observed in the mites mirrored but were not a perfect replicate of those observed in the CHC profile of bees belonging to the same colony. Especially, mites from colony 4 had significantly lower levels of alkene compare to their host colony, which explained why, according to the DA, there were no mis-assignments of individuals between these two groups. This result suggests that *V. destructor* mites mirror the colony-specific shifts in *n*-alkane:alkene ratios of their host colony; although not perfectly. Alkenes in particular have been linked to nest mate recognition in *A. mellifera* (Breed 1998; Dani et al. 2005) and behavioural evidence suggests that the bees are particularly susceptible to this particular hydrocarbon class (Châline et al. 2005). Compared to their bee host, mites had relatively high amounts of methyalkane. This could be explained by the fact that *V. destructor* spends part of its reproductive cycle on the developing brood that have high methyalkane levels (Nation et al., 1992). Throughout its life, *V. destructor* switches between sitting on adult bees and sitting on the brood. The CHC profile of adult bees is very low in methylalkane relative to the brood. Therefore, it is likely that the majority of study mites that

have just left a brood cell and moved onto an adult bee to hitch a ride to the next suitable brood cell, since the experiment was conducted during the summer when the mites are actively reproducing.

This constant switching from mimicking adult bees to mimicking brood, and *vice versa*, could explain the imperfect alkene:*n*-alkane ratios described above. As *A. mellifera* brood only has minute quantities of alkene in their CHC profile (Blomquist et al. 1980; Kather et al. 2011), the mites need to change from an alkene-rich and methylalkane-poor 'adult bee' profile to an alkene-poor and methylalkane-rich 'brood' profile and *vice versa*. So, during this switching period the mite's chemical mimicry may be imperfect as time is required to alter its profile between the two extremes.

The difference in methylalkane levels between host and parasite are greater than the difference in the alkene:*n*-alkane ratio. As alkenes play a key role in nestmate recognition of honey bees (Breed 1998; Dani et al. 2005) there will be greater pressure on mites to closely mimic the alkene:*n*-alkane ratio of the host, rather than levels of methylalkanes, since these may allow bees to distinguish brood or newly emerged adults from adult bees. Consequently, adult bees will be more sensitive to differences in alkene:*n*-alkane ratios and hence flag up potential invaders than to differences in methylalkane levels. Furthermore, if differences in methylalkane levels do function as a 'brood' signal then there is no advantage to the mite to remove all traces of methylalkanes, or it could that methylalkanes have a high (40°C and higher) melting temperature (Gibbs, 2002), meaning that it takes longer for the methylalkanes to 'wear off', than the alkanes and alkenes.

Whatever the reason for the imperfect mimicry it appears to not exceed the discriminant threshold of the honey bee (Hölldobler and Carlin 1987), since *V. destructor* is generally ignored by the host indicating that the parasite's chemical mimicry of a colony is within the accepted threshold of its *A. mellifera* host. How this mimicry is achieved, for

example by synthesising host cues (Howard et al. 1990), actively grooming or licking the host or trophollaxis (e.g., Lenoir et al., 1997) or by passively adsorbing the host CHCs profile (Vander Meer and Wojcik, 1982), remains unknown. The close and constant contact between host and parasite in this system would make transfer of CHCs between the two very likely. However, there is no evidence that *V. destructor* licks or grooms *A. mellifera* and the mite's legs are too short to potentially spread host compounds across its body, so biosynthesis cannot be ruled out. Whatever, the mechanism the ability of *V. destructor* to mimic its host's colony odor helps explains why despite years trying to eradicate it or selecting for hygienic bees to detect it, the mite remains the beekeepers 'number one enemy'.

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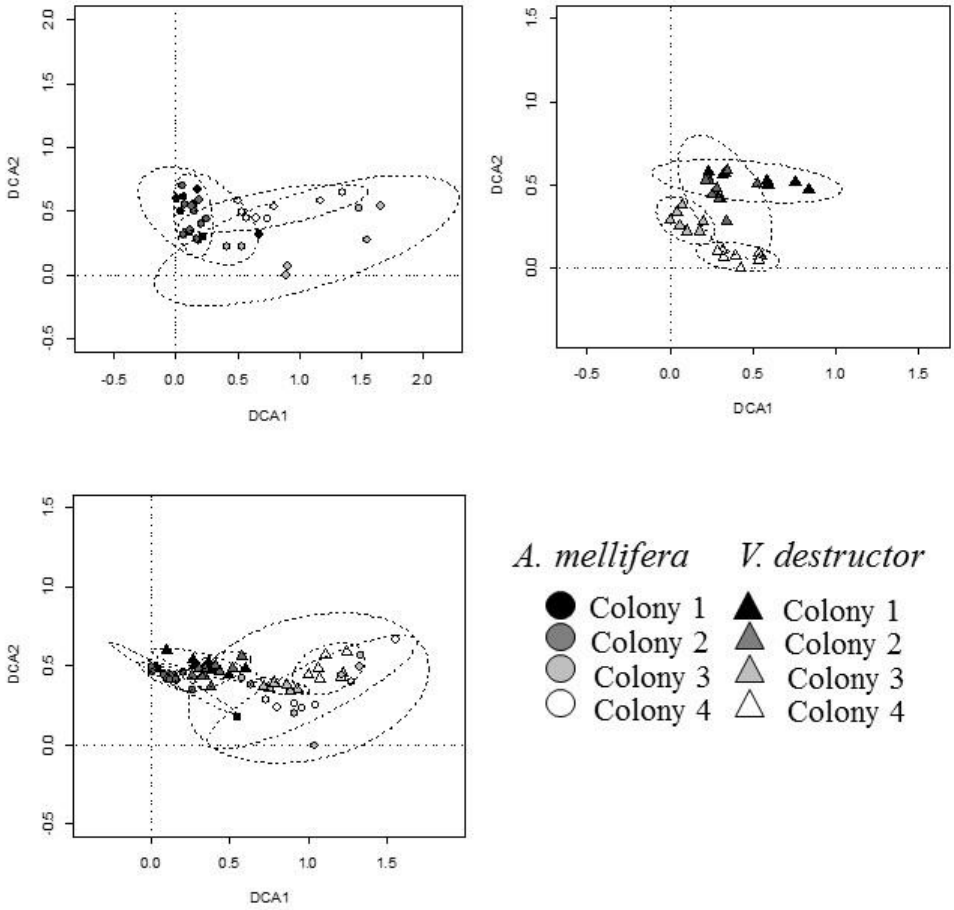
Fig.1 Colony separation based on the CHC profiles of a) *A. mellifera* workers, b) *V. destructor* mites and c) the two species combined. The ellipses correspond to the 95% confidence limit for each group.

Fig.2. Relative proportions (percentages) of the main chemical families that make up the CHC profile of a) *A. mellifera* workers from different colonies and how these compare to b) their respective *V. destructor* parasites. Significance levels were calculated within chemical families and bars with different letters are significantly different from one another within their chemical family.


DCA2
DCA1
-0.5
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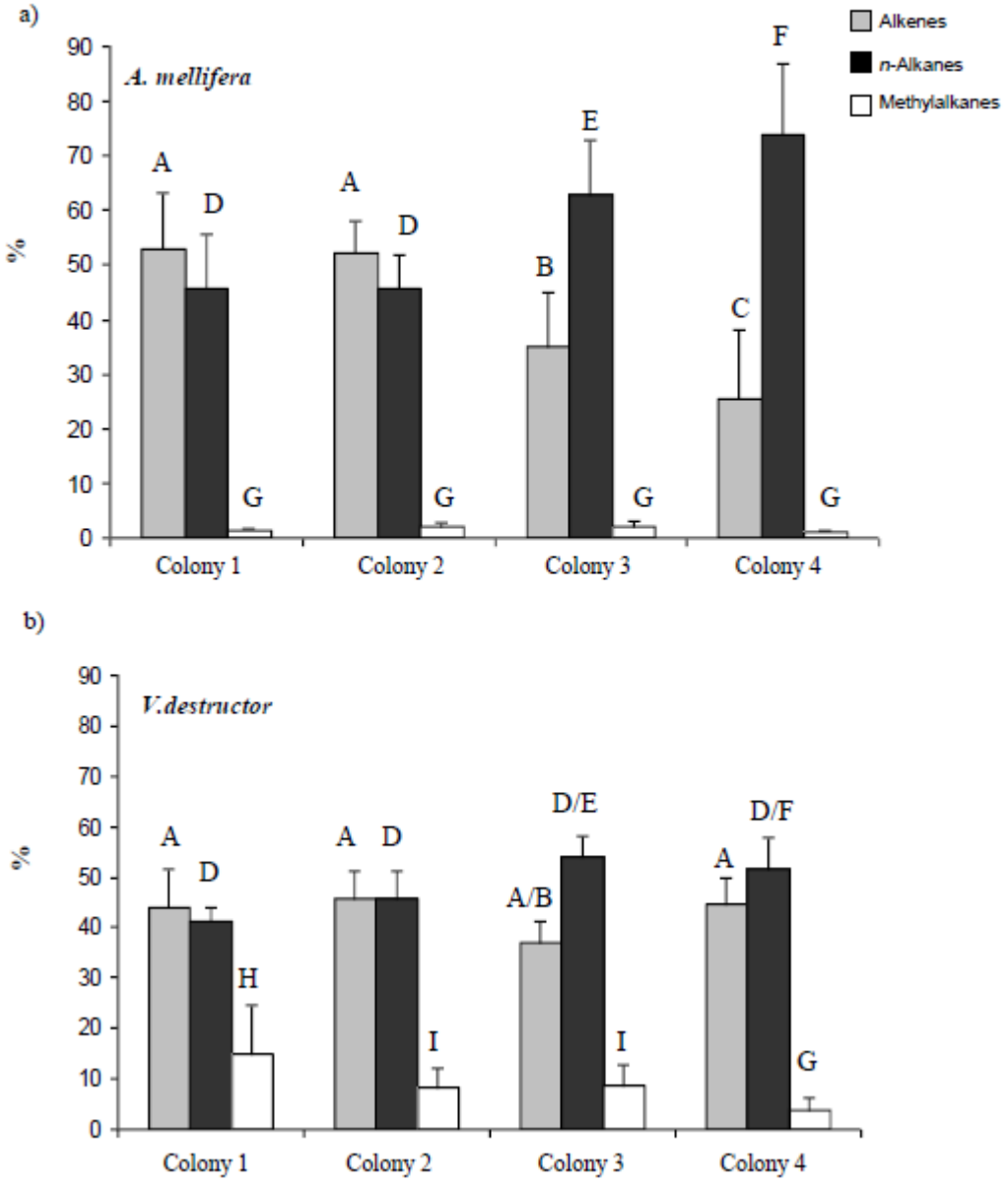
A. mellifera
V. destructor
Colony 1
Colony 2
Colony 3
Colony 4
Colony 1
Colony 2
Colony 3
Colony 4

Fig. 1



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437 Fig. 2

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