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

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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

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76 **Introduction**

77 In social insects, a colony provides a safe place to store food and rear brood within a stable
78 micro-climate. For many predators and parasites, however, such colonies are a
79 concentrated source of food, as well as a perfect environment to rear their own offspring.
80 To protect the colony from being exploited social insects have evolved an intricate system
81 for recognising strangers inside the colony and along its borders (Hölldobler and Wilson
82 1990). Nest-mates constantly compare the odour of other individuals around them to their
83 own to detect strangers. Should the odours differ beyond a certain threshold, the individual
84 is attacked and removed from the colony.

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

92 Cuticular hydrocarbons (CHC) have repeatedly been shown to play an important role
93 in the nest-mate recognition behaviour of social insects (reviewed in: Howard and Blomquist
94 2005). In many insects, CHC are expressed as part of the insect's cuticular lipid layer and
95 differences in the concentration of CHC have been shown to serve as signal of colony-origin

96 in many species of ants (Wagner et al. 2000; Greene and Gordon 2007; Martin et al. 2013),
97 bees (Arnold et al. 2000; Buchwald and Breed 2005), hornets (Butts et al. 1995; Ruther et al.
98 1998), termites (Bagnères et al. 1991; Kaib et al. 2004) and wasps (Dani et al. 2004;
99 Dapporto et al. 2006). CHC mimicry has also been shown to be important in the integration
100 of parasites into the host colony (Cini et al. 2011) and there is increasing evidence that a
101 number of parasites mimic the colony-specific fraction of the host odour to be accepted into
102 the colony as a nest-mate (e.g. Guillem et al 2014; Martin and Bayfield 2014). For example,
103 the butterfly *Maculinea rebeli* biosynthesises host-specific compounds before invading the
104 nest of its ant host, *Myrmica schenki*, and, once inside the colony, fine tunes its chemistry to
105 the colony odour by acquiring compounds from the host, possibly through trophallaxis
106 (Akino et al. 1999). The myrmecophilous spider *Cosmophasis bitaeniata* mimics the colony
107 odour and task odour of its ant host's minor workers (*Oecophylla smaragdina*) to avoid
108 aggression (Elgar and Allan 2004, 2006). This social parasite is also able to distinguish
109 between workers of its host colony and those of alien colonies, and chooses the company of
110 the former. This behaviour is extremely important due to *O. smaragdina*'s highly aggressive
111 behaviour, especially that of the major workers.

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

120 by moving from a foraging bee onto nurse bee it gains access to the brood area (Kraus et al.
121 1986). Evidence presented in Nation et al. (1992) suggests that the mite's CHC profile
122 changes as it moves onto a new host, since bees of different ages differ in their CHC profile
123 (Nation et al. 1992; Arnold et al. 2000; Aumeier et al. 2002; Kather et al. 2011).
124 Nevertheless, it remains to be tested whether the cuticular chemistry of *V. destructor* also
125 matches colony-specific differences in host odour. Therefore, we investigate whether *V.*
126 *destructor* mites collected from different *A. mellifera* colonies have adjusted their chemical
127 mimicry to match the small colony-specific differences in CHC of their host colony.

128 **Methods and Materials**

129 Sample Collection and Chemical Analysis

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

138 For the extraction, mites were pooled into groups of six mites per sample whereas
139 bees due to their size were extracted individually. Samples were immersed in high-
140 performance liquid chromatography-grade hexane (bees: 0.5 ml; mites: 300µl) containing a
141 C₂₀ standard (1mg/100ml HPLC grade hexane). Samples were left at room temperature for 15
142 min. before transferring 30 µl of extract to a glass insert, which was then left to evaporate to
143 dryness before being stored at -20°C until analysis. Immediately before analysis, samples were
144 re-suspended in 30µl hexane and analysed on an HP6890-GC (equipped with an HP-5MS

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

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152 Statistical Analysis

153 A number of chromatograms (17.5%) had to be discarded due to poor quality, leaving an
154 average of nine *A. mellifera* chromatograms and eight *V. destructor* chromatograms per
155 colony for statistical analysis. The peak area of each compound was determined by manual
156 integration of each total ion chromatogram (TIC) and compound concentration (mg/ml HPCL
157 grade hexane) was calculated using the standard C₂₀ peak. The profiles of *A. mellifera*
158 consisted of several homologue series of odd-chained *n*-alkanes (C₂₃ - C₃₁); alkenes (C₂₃ -
159 C₃₃); dienes (C₃₁ - C₃₃) and 9-, 11-, 13-mono-methylalkanes (C₂₅ - C₃₁), which is in
160 agreement with previous reports (Dani et al. 2004, Blomquist et al. 1980). The pooled *V.*
161 *destructor* samples contained the same compounds listed above. Compounds which on
162 average contributed less than 1% to the overall chemical profile (i.e. *n*-alkanes + alkenes +
163 methylalkanes) were excluded from the analysis.

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

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

$$170 \quad Z = \ln[A_p/g(A_p)]$$

171 to avoid complications arising from analysing compositional data (Aitchison 1986).

172 A_p is the proportion of the compound and $g(A_p)$ is the geometric mean of all compounds to
173 be included in the multi-variate analysis. The first two DAs were used to examine colony
174 separation within the *V. destructor* mites and *A. mellifera* workers, while the final DA
175 combined CHC profiles of the two species to test how closely parasites cluster to their host
176 colony. For the final DA, each of the eight host-parasite groups (four parasite groups and four
177 host groups) were treated as a separate group to investigate the relative separation of parasites
178 and hosts according to their colony origin. As a cross-validation technique, a jack-knife
179 (leave-one-out) sampling scheme was employed, in which each case was classified by the
180 functions derived from all cases other than the case itself. A priori probabilities of assignment
181 were calculated based on group sizes. All DAs were run in the statistical software R (v
182 2.81). For each of the three DAs described above, we also ran an ordination analysis
183 (detrended correspondence analysis, DCA) followed by a goodness of fit test on the
184 transformed data to test whether the group separation observed was indeed significantly
185 associated with colony origin. All ordination analyses were conducted in R (v. 2.81) as part
186 of the statistical package ‘vegan’ (Oksanen 2013).

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

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194

195 **Results**

196 There was significant colony separation amongst *A. mellifera* workers based on their CHC
197 profiles (Goodness of fit; $R^2=0.63$, $p < 0.001$) (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; $R^2=0.63$, $p < 0.001$) (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; $R^2=0.72$, $p < 0.001$) 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

217 colony 4 with bees from colony 4, but the mites still clustered more closely to bees of colony
218 4 on the DA than to bees of colonies 1 and 2. There were no cases where mites had been mis-
219 assigned to bees of a different colony.

220 This divide in chemical profiles between the colonies was also found when looking at
221 the relative proportions of alkene, methylalkane and *n*-alkane in *A. mellifera* and *V.*
222 *destructor* individuals. The *A. mellifera* colonies varied in the relative proportion of alkene
223 (ANOVA: $F = 15.8$, d.f. = 7, 59, $p < 0.001$) and *n*-alkane (ANOVA: $F = 23.64$, d.f. = 7, 59, p
224 < 0.001) (Fig. 2a). Colonies 3 and 4 were more '*n*-alkane rich', whereas colonies 1 and 2 had
225 higher levels of alkenes. As observed in the DCA (Fig. 1a), colonies 1 and 2 were very
226 similar in their CHC profile. In comparison, *V. destructor* mites had similar relative
227 proportions of alkene to *n*-alkane compared to host individuals of the same colony (Tukey's
228 test: $p > 0.1$), with the exception of mites from colony 4, whose alkene proportions were
229 significantly higher compared to their host (post-hoc Tukey's test: $p < 0.0001$) (Fig. 2a, b).
230 Overall, methylalkane proportions were higher in the parasite compared to the host (post-hoc
231 Tukey's test: $p < 0.001$).

232

233 **Discussion**

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

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

242 Colonies 1 and 2 were indeed extremely similar in CHC profile, as were colonies 3 and 4.
243 This chemical similarity could be due to close relatedness because the former two colonies
244 were from the Sheffield apiary, whereas the latter two belonged to the York apiary. This
245 similarity in CHC was also reflected in the CHC profiles of their respective mites, with mites
246 from colonies 1 and 2 clustering closely together in the DCA/DA as did mites from colonies
247 3 and 4.

248 When hosts and parasites were analysed together, mites still formed the same groups
249 as described above but this time mites from colonies 1 and 2 clustered closely to bees from
250 colonies 1 and 2, whereas mites from colonies 3 and 4 clustered closely to bees from colonies
251 3 and 4. Even though there was some overlap between mites and bees of the same colony,
252 mites and bees of the same colony did not form a distinct cluster. This could be because the
253 relative proportions of alkene and *n*-alkane observed in the mites mirrored but were not a
254 perfect replicate of those observed in the CHC profile of bees belonging to the same colony.
255 Especially, mites from colony 4 had significantly lower levels of alkene compare to their host
256 colony, which explained why, according to the DA, there were no mis-assignments of
257 individuals between these two groups. This result suggests that *V. destructor* mites mirror the
258 colony-specific shifts in *n*-alkane:alkene ratios of their host colony; although not perfectly.
259 Alkenes in particular have been linked to nest mate recognition in *A. mellifera* (Breed 1998;
260 Dani et al. 2005) and behavioural evidence suggests that the bees are particularly susceptible
261 to this particular hydrocarbon class (Châline et al. 2005). Compared to their bee host, mites
262 had relatively high amounts of methylalkane. This could be explained by the fact that *V.*
263 *destructor* spends part of its reproductive cycle on the developing brood that have high
264 methylalkane levels (Nation et al., 1992). Throughout its life, *V. destructor* switches between
265 sitting on adult bees and sitting on the brood. The CHC profile of adult bees is very low in
266 methylalkane relative to the brood. Therefore, it is likely that the majority of study mites that

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

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

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

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

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

301

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

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

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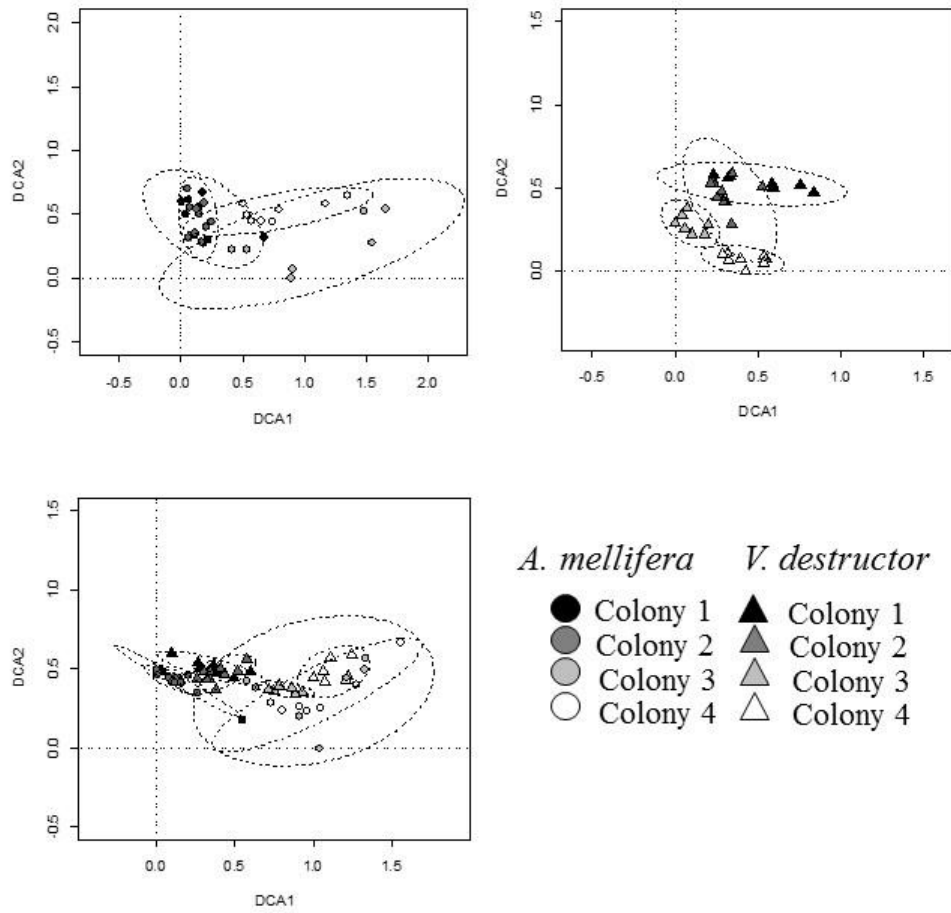
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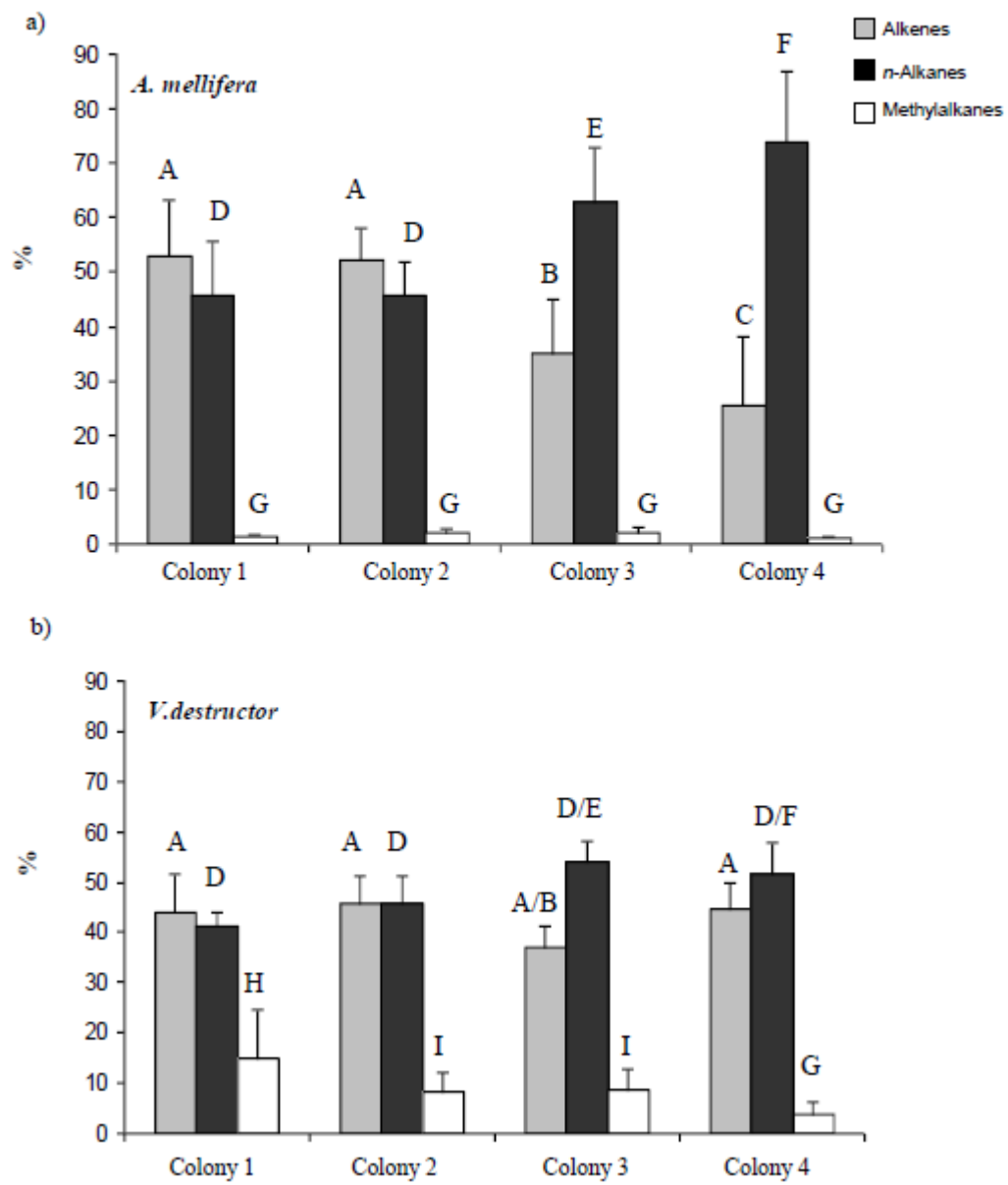
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434 Fig. 1



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437 Fig. 2

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