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Cuticular volatiles, attractivity of worker larvae and invasion of brood cells by *Varroa* mites. A comparison of Africanized and European honey bees

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Summary. Africanized honey bees (AHBs) of Brazil and Mexico have proven to be tolerant to *Varroa destructor* mites. In contrast, European honey bees (EHBs: *Apis mellifera carnica*) at the same tropical study site are highly intolerant to these ectoparasites. A lower attractiveness of *Varroa*-tolerant AHB larvae has been hypothesised to be an important trait in reducing the susceptibitlity of AHBs to these mites. Thus, selection for EHB brood that is less attractive to mites is thought to be one possibility for limiting mite population growth and thus increase the tolerance of EHBs to the mite.

In Ribeirão Preto, Brazil, European *A. m. carnica* bees and AHBs were tested with respect to their rate of brood infestation and brood attractiveness to *Varroa* mites. For the comparison of brood infestation rates, we introduced combs with pieces of EHB and AHB brood into honey bee colonies (18 repetitions). The relative infestation rate of EHB brood was significantly higher compared to AHB brood.

The preference behaviour of single *Varroa* mites was tested in a laboratory bioassay where either living host stages were offered or host extracts were presented on dummies. By these tests we could confirm the preference of *Varroa* females for certain developmental host stages and for their corresponding extracts. In contrast to the within-colony results, *Varroa* mites in the laboratory bioassay showed a slight preference for AHB compared to EHB larvae.

The gas chromatographic analysis revealed differences in the chemical spectrum of extracts obtained from different larvae. In accord with the results of the bioassays, we could detect stage-specific odour differences in larval cuticular compounds, including methyl esters and hydrocarbons that have been described as kairomones. None of these substances, however, revealed significant race-specific differences. Therefore, the quantity and composition of certain cuticular compounds seem to be responsible only for the recognition of a suitable host stage by *Varroa* females. The different infestation rates in the colonies, however, seem to be caused neither by race-specific differences in attractiveness of bee larvae nor by an extended attractive period of EHB larvae: both AHB and EHB larvae become attractive approximately 21 h before capping of the brood cell, and thus have the same window of time when they can be parasitised.

Therefore differential *Varroa*-infestation rates are not related to larval attraction but probably are determined by other race-specific and colony-related factors.

Key words. Africanized honey bee – *Apis mellifera carnica* – bee brood infestation – *Varroa* mites – semiochemical – host-finding cues – larval cuticular volatiles – bioassay

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Introduction

The ectoparasitic bee mite *Varroa destructor* (Anderson 2000) represents the most serious problem in beekeeping. Without treatment, colonies of the Western hive bee (*Apis mellifera*, EHB) collapse and die within a few years. The only example of long term tolerance towards varroatosis is the Africanized honey bee (AHB) in Brazil, a hybrid of European and African *Apis mellifera* races, in which infestation rates have remained low over a 20 year period (Guzmán-Novoa *et al.* 1999; Rosenkranz 1999). An explanation for this tolerance is that *Varroa* females have reduced fertility when reproducing in worker brood (Rosenkranz & Engels, 1994; Rosenkranz 1999), but active defence mechanisms by host bees (Boecking & Spivak 1999; Guzmán-Novoa *et al.* 1999; Aumeier 2001) have also been shown to be involved.

A lower attractivity of bee brood to reproductive female mites (Guzmán-Novoa *et al.* 1999) was considered a key feature keeping the level of *Varroa* infesta-

tion in AHB colonies under the hazard threshold (De Guzman *et al*. 1995). The first step in the reproductive cycle of a female mite is to approach a suitable host, a still uncapped brood cell containing a 5th instar larva shortly before capping (Beetsma *et al*. 1999). In this host selection behaviour, mites prefer drone larvae, as shown by *in vitro* testing (Rosenkranz 1993) and as also observed under *in vivo* conditions (Fuchs 1990; Boot *et al*. 1995b). Reduced rates of worker brood invasion were observed in AHB compared with EHB colonies in Mexico (Guzmán-Novoa *et al*. 1996, 1999). Variation in attractiveness of EHB brood to *Varroa* females was also found under temperate climatic conditions (Büchler 1990; De Guzman *et al*. 1995; Guzmán-Novoa *et al*. 1996).

The various factors involved in bee brood infestation by *Varroa* mites have, however, up to now not been examined at the same time and geographical location in AHB and EHB colonies. Therefore, their impact on varroatosis tolerance remains unresolved.

As aliphatic esters and some hydrocarbons produced by last instar bee larvae have been shown to guide host location by the female mite (Le Conte *et al*. 1989; Trouiller *et al*. 1992, 1994; Rickli *et al*. 1994; Rosenkranz 1993; Donzé *et al*. 1998), we analysed the patterns of these cuticular volatiles in bee larvae, bioassayed host selection by the parasite according to Rosenkranz (1993), and measured rates of worker brood infestation in colonies of AHBs and EHBs, both infested with *Varroa* mites of the same origin at the same tropical field site.

Materials and methods

Study site

All experiments were performed during the summer of 1997/98 at the Department of Genetics, University of São Paulo, Ribeirão Preto, a tropical site in Brazil. The chemical analysis of cuticle extracts was performed at Hohenheim and Hamburg, Germany.

Biological material

Strong colonies in Langstroth hives containing 3–5 brood combs were used. Nine AHB (Africanized honey bee) colonies were established from captured swarms with naturally mated queens. Nine EHB colonies (*Apis mellifera carnica*) were headed by queens mated at Hohenheim (Germany). All colonies were infested with *Varroa* mites (Acari; Varroidae) of local (Brazilian) origin.

Cuticular extracts

Worker bee larvae of the 4th larval instar (L4) and 30 mg of body weight as well as larvae of the 5th instar (L5) at 160 mg (for EHBs) or 120 mg (for AHBs) were collected from brood combs with clean forceps and adhering larval food was removed. For each group, 6 pooled samples of 10 staged larvae were extracted for 10 min at room temperature in 6 ml pentane (Merck, uvasol grade) containing nonadecanoic acid methyl ester as an internal standard. The extracts were concentrated to 0.5 or 1 larval equivalents/μl for 5th and 4th instar larvae, respectively, by solvent evaporation.

Chemical analyses

Structural analyses of volatile esters and hydrocarbons were performed on an HP 5890 II gas chromatograph linked to a mass selective detector MSD 5972. Quantitative analysis was carried out on a Hewlett-Packard 5890 II gas chromatograph equipped with a split/splitless injector, electronic pressure control, a FID detector, and an HP 3365 Series II Chemstation. The analyses were performed with a nonpolar fused silica column (DB-5, 30 m × 0.32 mm I.D. × 24 μm), which was operated at the following conditions: 40°C for 5 min, increased to 200°C at a rate of 10°C/min, then increased to 280°C at a rate of 2°C/min, then held at this temperature for 25 min, then increased to 300°C at a rate of 4°C/min. For each analysis, 1 μl of the extract corresponding to 0.5 or 1.0 larvae-equivalents and 10–15 ng IS were injected. Compounds were identified by retention times, co-injections and mass spectra. For statistics, substances are given in percentage ± s.d. of total. Only components making up more than 0.1% of the total volatiles in each of the cuticular extracts were quantified.

Laboratory bioassay

The host preference behaviour of *Varroa* females was bioassayed in the laboratory as follows. Test subjects were placed in 4 depressions in a plexiglass arena maintained at 30°C (Rosenkranz 1993, Fig. 1). For each test, a mite was placed in the arena centre and its movements recorded over 3 min, allowing us to calculate the duration of contacts with the test targets. After four tests, the arena was cleaned with ethanol. Phoretic *Varroa* females were collected from bees randomly brushed from brood combs. Worker bees carrying a mite were anaesthetised with CO_2, the mites were gently wiped off with a needle, stored in a moistened petri dish at 30°C and used for testing within 15 minutes of collection. At least 20 female mites were tested per assay.

Attractiveness of AHB and EHB larvae was compared by offering two live L5 larvae of each bee type or dummies impregnated with corresponding pentane extracts in the depressions of the arena (Fig. 3). Larval extracts, at 3 larval equivalents each, were applied on odour-free dummies which had been prepared by washing L5 larvae for 3 minutes in pentane (Rosenkranz 1993).

To test reactions of *V. destructor* mites to live larvae of different stage and bee type (Figs. 4, 5), 4 larvae of the same body weight and bee type were offered in the bioassay simultaneously. Using larval weight as an index of larval age (Thrasyvoulou & Benton 1982; Michelette 1992; Michelette & Soares 1993), larvae with 25, 50, 65 or 90% of their capping weight were tested. To evaluate whether attractiveness depended upon different cuticular surface chemistry of larval developmental stages or is only a matter of smaller cuticular surface areas, two AHB larvae of 30 mg and 25% capping weight were offered in each depression.

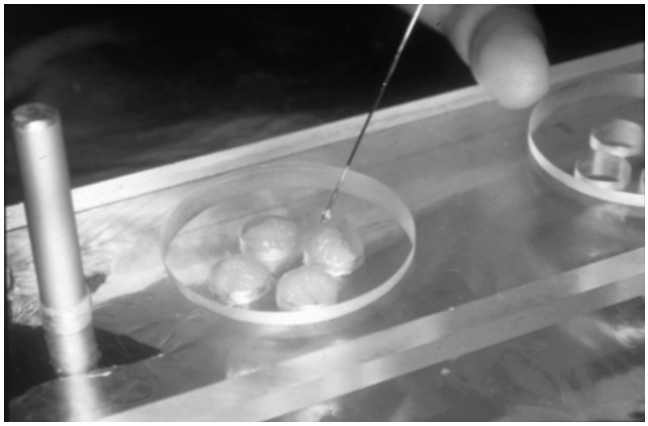

Fig. 1 Laboratory bioassay. Attractiveness of living host larvae or volatile host extracts applied to dummies can be evaluated

Measurement of brood infestation

In vivo infestation rates of worker brood were measured by introducing pieces of comb containing L4 larvae or eggs into colonies of AHBs and EHBs. Two sections of brood comb measuring 10×10 cm from one AHB colony and two identical sized sections from one EHB colony were placed in the centre of an experimental comb. After incubation in the test colony until larvae and eggs reached the 5th larval instar, a total of 800 AHB and 800 EHB L5 larvae were available on the experimental combs for invasion by *Varroa* females. The brood sections originated from 5 EHB and 5 AHB donator colonies, unrelated to the test colonies. The combs from which the brood sections were obtained were of Africanized cell format and had been in the donator colonies for two weeks prior to removal. The *Varroa* population in the test colonies was increased by introducing two brood combs from strongly infested hives. Prior to the testing period, all the open brood in the experimental colonies was destroyed in order to prevent any competition with the larvae in the test sections. Thus, only experimental brood areas were available for invasion by female mites.

The resulting rate of infestation was determined 2 days after capping. Brood cells on the experimental combs were opened by forceps and examined for *Varroa* mites. All invaded *Varroa* mites were used for analysis independently of originating from single or multiply infested cells. All measurements were repeated in 4–5 different AHB and EHB colonies.

Data analysis

Differences in the proportions of semiochemicals on different larval instars and bee types were evaluated by nonparametric Mann-Whitney U-tests because the data did not fulfil the criteria of normality and homoscedasticity. For all classes of cuticular substances analysed (alkanes, alkenes, methyl branched alkanes and esters), a stepwise discriminant function was used to discriminate the four groups of larvae (two age groups and two bee types). Only substances that had an approximate normal distribution in all six replicates when analysed by the normal probability test of David, Pearson and Stephens (Sachs 1999) were used. Only functions with a significant level $P < 0.01$ were computed, yielding 3 discriminant functions (roots). The bioassay data were subjected to χ^2-analysis. Paired t-tests were applied to compare brood infestation rates. For statistics, the programs Microstat (Ecosoft 1985) and Statistica for Windows (Statsoft 1994) were used.

Results

Volatile blends on the larval cuticle

About 100 compounds were identified by gas chromatographic analysis in the larval extracts, mostly hydrocarbons of C_{21}–C_{35} with uneven chain length. Between 40–47% of the total volatiles were saturated, 7–14% unsaturated and 13–21% were methyl-branched alkanes (Table 1). Esters made up 0.5 to 6% of all substances. Aliphatic esters and hydrocarbons represented about 75% of the total volatiles. The total amount of cuticular volatiles increased with larval development from 4th to 5th instar, from 1071 ± 107 ng to 4283 ± 1469 ng in EHBs and from 1948 ± 469 ng to 5034 ± 1400 ng in AHB samples. Between the 4th and 5th larval instar, changes in odour composition also occurred; the amount of methyl-alkanes decreased significantly whereas alkenes and esters increased significantly. In total, 14 hydrocarbons and 5 esters showed significant age-specific differences in both bee types (Table 1). Differences between bee types were less pronounced. They differed in only five substances in the 5th larval instar (Table 1). In EHB larvae, the proportions of methyl alkanes and esters were higher and the proportion of alkanes lower compared to AHB larvae, though differences were not significant.

All multivariate discriminant analyses yielded three discriminant functions but only the first two functions were highly significant ($P < 0.001$); all third functions (root 3) were not significant ($P > 0.01$). Functions 1 and 2 together were sufficient to achieved more than 90% of the discrimination among AHB and EHB larvae of different ages. The four classes of substances had different abilities to separate between age groups and bee types (Fig. 2a–d). The patterns of alkanes and esters separated highly significantly only between the 4th and 5th larval instars but not between the AHB and EHB 5th instar larvae. The corresponding Mahalanobis distances (D^2) for bee type were 23.8 (alkanes) and 25.2 (esters), for different larval instars 126.3 and 106.3, respectively (alkanes), and 141.4 and 110.7, respectively (esters). Branched alkanes and alkenes, however, yielded significant discrimination of both bee types and larval instars (Fig. 2b,c).

Bioassayed mite reactions on cuticular extracts

Attractiveness of live L5 larvae as well as larval extracts of both bee types was compared. As dummies we used solvent-extracted L5 larvae. Only 11% of the mites contacted these washed larvae (Fig. 3) briefly spending on them 0.4% of the test time. Topical application of a pentane extract of L4 larvae of both bee types onto the dummies elicited non-specific and short-term reactions. Impregnation with extract of L5 larvae significantly increased the total number of attracted mites (Fig. 3) and contact time (χ^2, $P < 0.001$). Live L5 larvae elicited the most intense reactions, with 91% of mites contacting a test larva and with mites spending a mean of 51.5% of the time in contact with a test larva. Female mites significantly preferred extracts of 5th larval instars and living AHB L5 larvae in comparison to EHB larvae (χ^2, $P < 0.02$). However, there was no difference in the response between L4 extracts of AHB and EHB larvae.

Bioassayed mite reactions on larvae of different stage and type

Simultaneously offering 4 larvae of the same age and bee type revealed differences in their attractiveness to *Varroa* females. In contrast to AHB worker larvae of 30 mg, which is 25% of capping weight, twice as many mites contacted AHB larvae > 60 mg, corresponding to 50% of capping weight (χ^2, $P < 0.05$). Mites expressed more long duration contacts with the heavier AHB larvae (Fig. 4). Moreover, the duration of total contact time tripled on the heavier larval stage (Fig. 5). Attractiveness of the smaller larvae did not increase

Table 1 Pattern of cuticular volatiles identified in pentane extracts of EHB (*A. mell. carnica*) and AHB (Africanized honey bee) larvae of 4th (L4) and 5th (L5) instars, respectively. About $\frac{3}{4}$ of the totals of these substances could be identified as straight-chained and methyl-branched alkanes, alkenes and simple aliphatic esters. Me Cx and DiMe Cx: methyl-substituted hydrocarbons; Cx en and Cx dien: alkenes; ME and EE: methyl and ethylesters; ex substances excluded from the discriminant analysis

	percentages of volatile compounds						
	EHB - L4	sig.	EHB - L5	sig.	AHB - L5	sig.	AHB - L4
C21ex	0.00 ± 0.00	*	1.59 ± 1.15	n.s.	1.79 ± 1.08	*	0.00 ± 0.00
C23	4.48 ± 1.21	**	2.06 ± 0.38	*	2.63 ± 0.65	n.s.	3.63 ± 0.48
C24	0.48 ± 0.12	**	0.19 ± 0.05	n.s.	0.27 ± 0.09	n.s.	0.28 ± 0.04
C25	13.88 ± 2.23	*	9.00 ± 3.38	n.s.	11.62 ± 4.39	n.s.	14.37 ± 2.66
C26	0.66 ± 0.09	n.s.	0.71 ± 0.18	n.s.	0.94 ± 0.33	n.s.	0.68 ± 0.16
C27	12.05 ± 0.81	*	15.93 ± 2.69	n.s.	16.83 ± 5.01	n.s.	14.34 ± 3.22
C28	0.41 ± 0.07	n.s.	0.54 ± 0.11	n.s.	0.58 ± 0.20	n.s.	0.42 ± 0.10
C29	4.37 ± 0.43	n.s.	5.85 ± 1.11	n.s.	6.59 ± 1.42	n.s.	4.67 ± 1.15
C30ex	0.27 ± 0.05	n.s.	0.22 ± 0.04	n.s.	0.26 ± 0.12	n.s.	0.23 ± 0.05
C31	4.14 ± 0.83	*	2.86 ± 0.54	*	4.51 ± 1.25	*	3.18 ± 0.66
C32	0.40 ± 0.03	n.s.	0.37 ± 0.20	n.s.	0.44 ± 0.16	**	0.12 ± 0.07
C33	3.19 ± 1.10	**	0.46 ± 0.13	n.s.	0.98 ± 0.42	*	1.49 ± 0.41
C35	0.69 ± 0.17	**	0.36 ± 0.18	*	0.15 ± 0.07	n.s.	0.18 ± 0.04
alkanes total	45.02 ± 5.55	n.s.	40.14 ± 5.71	n.s.	47.59 ± 11.03	n.s.	43.61 ± 8.47
Me C23	1.11 ± 0.21	**	0.62 ± 0.12	n.s.	0.66 ± 0.30	*	1.01 ± 0.23
Me C25ex	3.59 ± 0.74	n.s.	2.50 ± 0.72	n.s.	2.43 ± 1.09	n.s.	3.08 ± 0.59
DiMe C25	0.21 ± 0.05	*	0.12 ± 0.04	n.s.	0.15 ± 0.06	*	0.20 ± 0.04
Me C26	0.33 ± 0.05	n.s.	0.24 ± 0.08	n.s.	0.24 ± 0.10	n.s.	0.33 ± 0.11
Me C27	4.50 ± 1.34	n.s.	5.26 ± 2.02	n.s.	4.12 ± 1.68	n.s.	4.56 ± 0.96
DiMe C27	0.17 ± 0.04	**	0.08 ± 0.03	n.s.	0.08 ± 0.05	*	0.14 ± 0.03
Me C28	0.69 ± 0.10	n.s.	0.49 ± 0.33	n.s.	0.41 ± 0.09	***	0.83 ± 0.04
Me C29ex	4.60 ± 0.92	*	3.13 ± 0.85	n.s.	2.43 ± 0.96	**	4.21 ± 0.93
DiMe C29	0.48 ± 0.12	n.s.	0.30 ± 0.12	n.s.	0.36 ± 0.12	*	0.50 ± 0.10
Me C30	0.29 ± 0.06	*	0.19 ± 0.05	n.s.	0.15 ± 0.06	**	0.25 ± 0.05
Me C31	3.65 ± 0.79	*	2.24 ± 0.65	n.s.	1.58 ± 0.66	**	2.85 ± 0.71
Me C33	1.92 ± 0.29	*	1.02 ± 0.37	*	0.46 ± 0.21	**	0.79 ± 0.21
methylalkanes total	21.64 ± 4.10	***	16.19 ± 4.50	n.s.	13.06 ± 5.19	*	19.72 ± 3.74
C21eneex	0.04 ± 0.02	*	0.34 ± 0.25	n.s.	0.39 ± 0.35	n.s.	0.04 ± 0.01
C23ene	0.15 ± 0.03	n.s.	0.19 ± 0.06	n.s.	0.25 ± 0.13	n.s.	0.22 ± 0.08
C25ene	0.51 ± 0.10	**	0.98 ± 0.16	n.s.	1.23 ± 0.40	**	0.66 ± 0.27
C27ene	0.75 ± 0.10	***	1.62 ± 0.21	n.s.	1.63 ± 0.51	**	0.87 ± 0.36
C29ene	0.55 ± 0.09	***	1.29 ± 0.24	n.s.	0.99 ± 0.32	**	0.54 ± 0.26
C30ene	0.12 ± 0.04	n.s.	0.07 ± 0.04	n.s.	0.12 ± 0.08	n.s.	0.11 ± 0.04
C31diene	0.05 ± 0.02	**	0.11 ± 0.04	n.s.	0.10 ± 0.05	n.s.	0.06 ± 0.02
C31ene	2.98 ± 0.36	**	3.85 ± 0.52	**	2.31 ± 0.32	*	1.68 ± 0.58
C33dieneex	0.24 ± 0.06	n.s.	0.45 ± 0.18	n.s.	0.47 ± 0.21	n.s.	0.26 ± 0.11
C33ene	3.35 ± 0.65	n.s.	4.34 ± 1.24	n.s.	6.11 ± 1.17	**	2.51 ± 0.63
alkenes total	8.73 ± 0.87	***	13.25 ± 2.41	n.s.	13.60 ± 2.64	***	6.94 ± 2.19
C16 acid ME	0.02 ± 0.03	*	0.31 ± 0.22	n.s.	0.22 ± 0.16	*	0.03 ± 0.02
C16 acid EE	0.06 ± 0.06	*	0.85 ± 0.93	n.s.	0.21 ± 0.09	**	0.04 ± 0.03
C18en acid ME	0.10 ± 0.02	n.s.	0.16 ± 0.12	n.s.	0.21 ± 0.16	n.s.	0.18 ± 0.02
C18 acid ME	0.04 ± 0.02	*	1.13 ± 0.72	n.s.	1.07 ± 0.60	*	0.02 ± 0.01
C18en acid EE	0.07 ± 0.07	*	2.20 ± 1.21	n.s.	0.92 ± 0.36	**	0.12 ± 0.08
C18 acid EEex	0.03 ± 0.02	**	1.28 ± 1.38	n.s.	0.35 ± 0.18	*	0.04 ± 0.02
esters total	0.33 ± 0.12	*	5.93 ± 4.38	n.s.	2.96 ± 1.26	**	0.44 ± 0.11
total of all volatiles	*75.62 ± 11.75*		*75.51 ± 17.00*		*77.23 ± 20.01*		*69.71 ± 14.52*

when offering two instead of one 30 mg larva in each depression of the test arena. We could also observe a clear increase in attractiveness of EHB larvae above a certain age. However, EHBs of 50% capping weight ($=75$ mg) were clearly less attractive than larvae at 65% capping weight (100 mg; χ^2, $P < 0.001$, Fig. 4). The ratio of long-duration contacts was seven times higher on 100 mg vs 75 mg larvae (Fig. 4), and the total contact time on larvae increased from 8% to 42% respectively (Fig. 5). Thus, EHB larvae took more time after hatching from the egg to become attractive.

Rates of brood invasion by Varroa *females*

The number of brood cells available to female mites for infestation differed according to the age of brood introduced into the test colony: a mean of $46.5 \pm 24\%$ of brood introduced as L4 larvae were accepted whereas

only 18.2 ± 10.6% of the introduced eggs were reared to L5 larvae (*t*-test: $P < 0.001$).

However, neither bee type nor the total amount of available brood cells nor the ratio of EHB to AHB brood influenced brood infestation rates. In EHB and AHB hives, the infestation of EHB L5 larvae, formerly

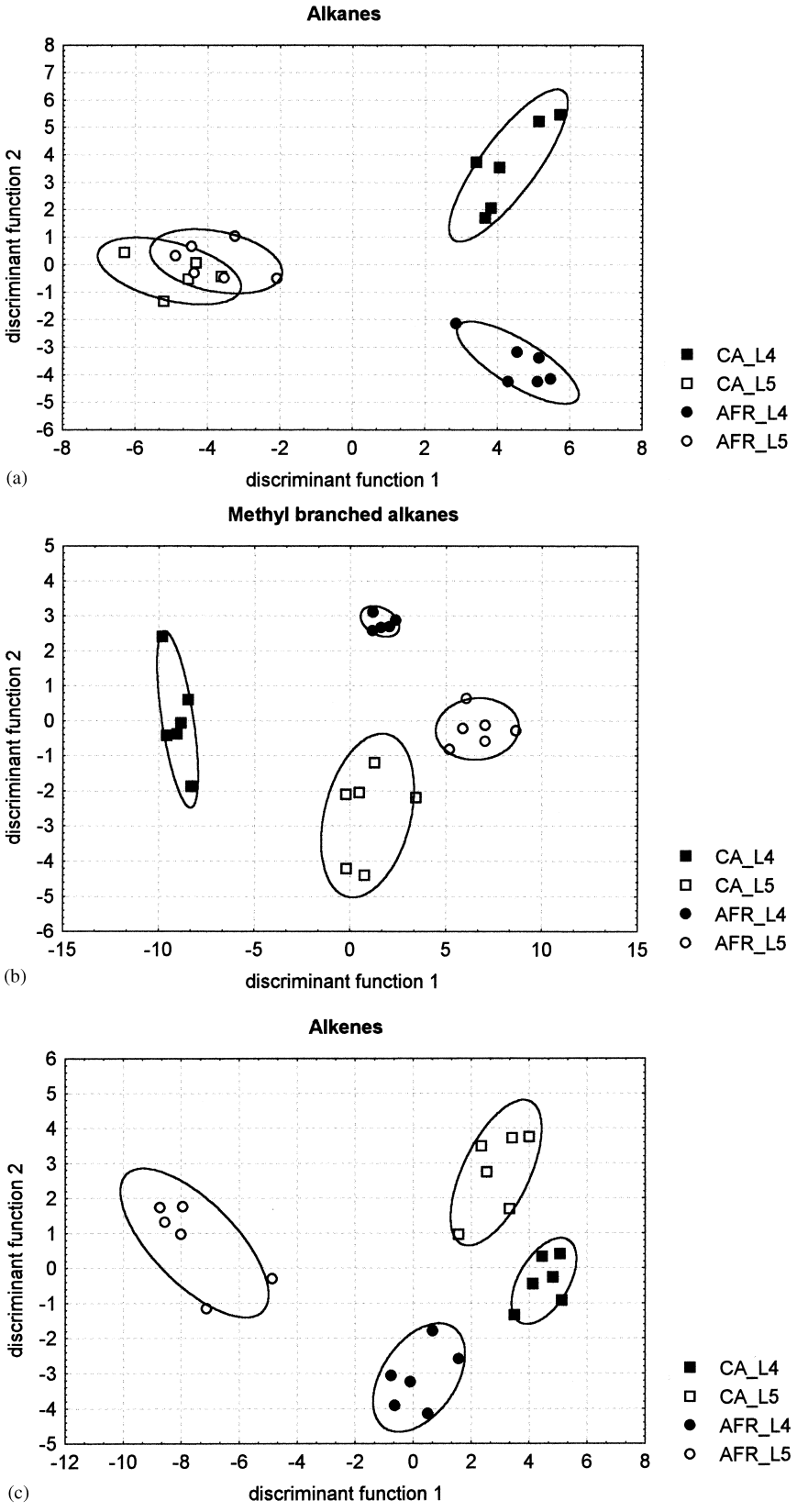

Fig. 2 Scatterplots of discriminant function analysis with 95% confidence ellipses using alkanes (a), methyl branched alkanes (b), unsaturated alkenes (c) and aliphatic esters (d) as variables. CA = *A. mell. carnica* larvae, AFR = Africanized honey bee larvae, L4 = 4th larval instar, L5 = 5th larval instar before sealing

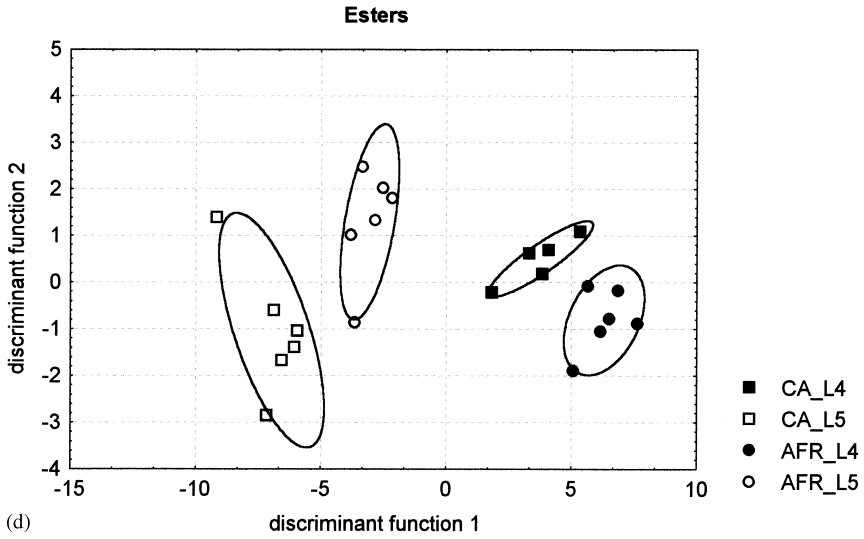

Fig. 2 (*Continued*)

introduced as L4 larvae, was 2.2 ± 1.0 times (t-test: $P = 0.02$) and 2.5 ± 1.0 times (t-test: $P = 0.01$) higher than simultaneously offered AHB larvae (Table 2). When introduced as eggs, a 3.2 ± 1.5 and 2.6 ± 1.3 times higher proportion of brood cells containing EHB L5 larvae was infested with *Varroa* females in EHB (t-test; $P = 0.03$) and AHB (t-test; $P = 0.03$) colonies, respectively (Table 3).

Discussion

Stage-specific patterns in the cuticular volatiles of late larval instars in the honey bee

Our comparison of the cuticular volatiles in honey bee larvae of the 4th and 5th instar clearly revealed stage specific differences in the pattern of hydrocarbons and

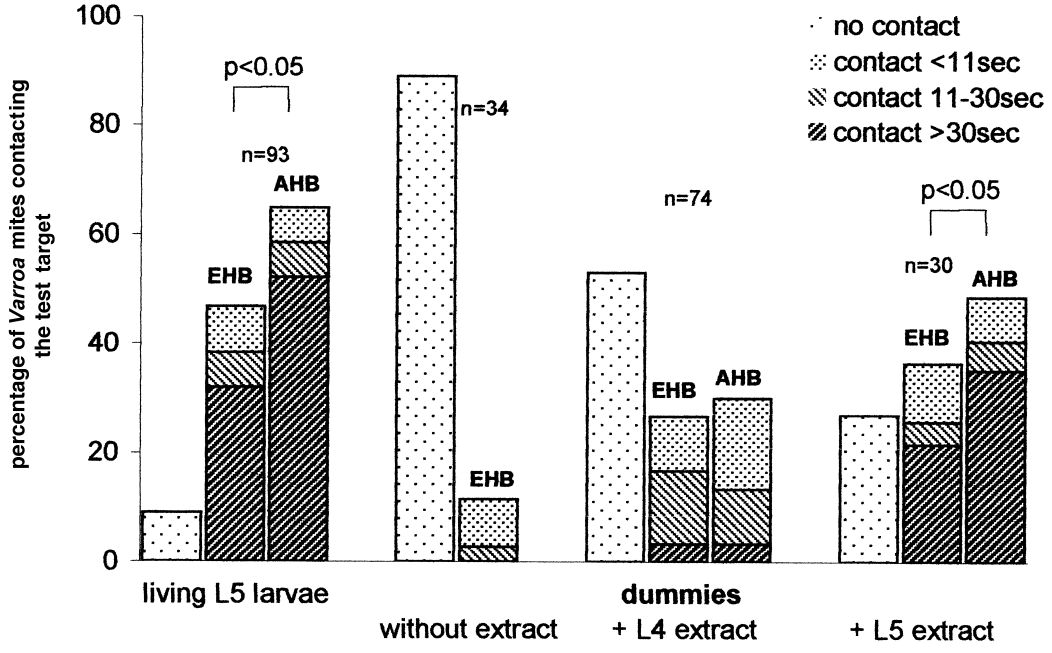

Fig. 3 Attractiveness of AHB and EHB larvae and larval extracts to *Varroa* mites. Two larvae or cuticular extracts of each bee type were offered simultaneously in a bioassay. The percentage of mites with no, short, middle and long-term contacts on larvae were recorded. AHB L5-larvae and the corresponding extracts were significantly more attractive compared to EHB larvae (χ^2; $P < 0.05$). N = number of tested mites. L4 = 4th larval instar, L5 = 5th larval instar before sealing

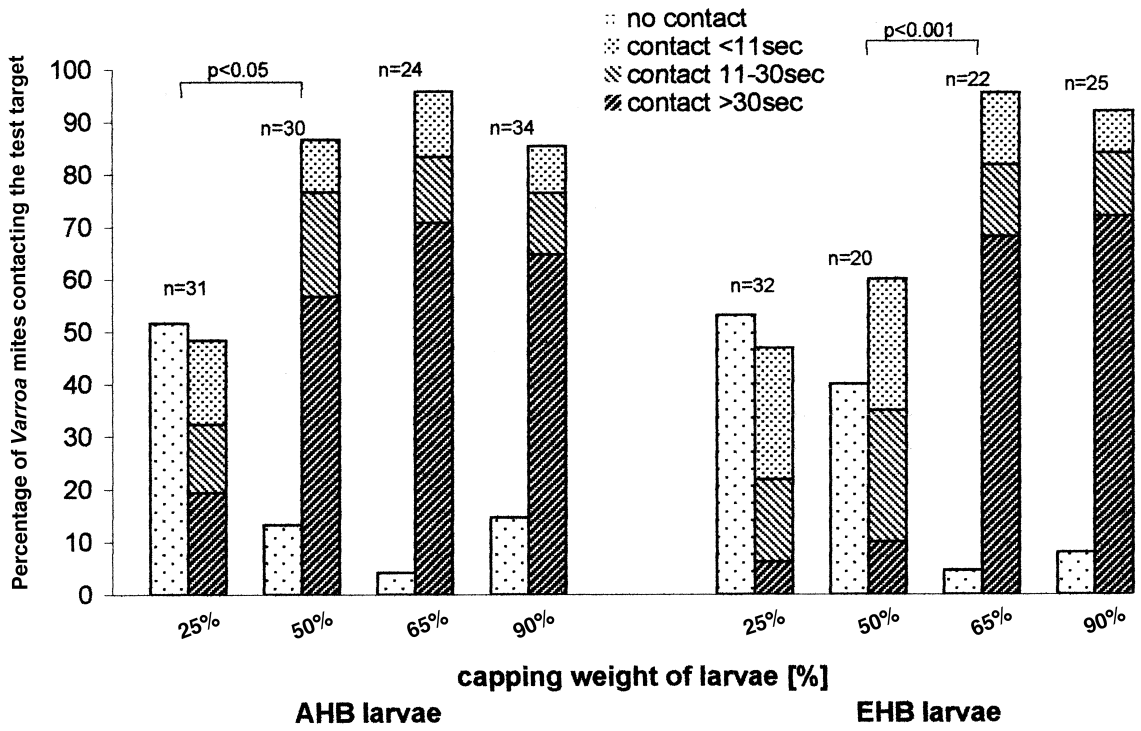

Fig. 4 Attractiveness of honey bee worker larvae of different ages to female *Varroa* mites in a laboratory bioassay. Four larvae of each age were offered simultaneously. Larval weight in relation to weight at capping was used to form larval age groups. The percentage of mites with no, short, middle and long-term contacts during the bioassays revealed differences between the age groups. N = number of tested mites

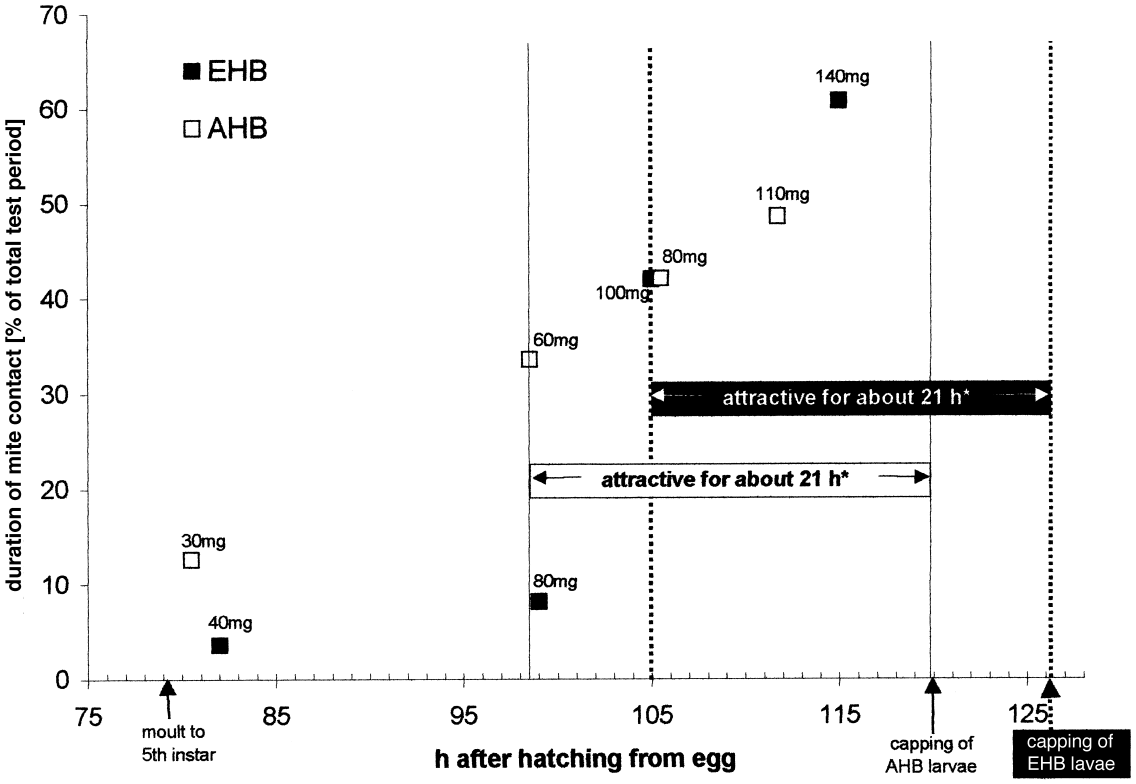

Fig. 5 Attractiveness of honey bee worker larvae of different ages to *Varroa* mites in a laboratory bioassay. Larvae were divided into age groups according to body weight and time after hatching from the egg (*data from Michelette 1992). The duration of contact of *Varroa* females to larvae of the same age that were simultaneously offered was recorded in relation to the total test period. Both bee types are attractive over a 21 h period but AHB larvae are attractive at an earlier stage (and body weight) relative to EHB larvae

Test colonies	Number of available cells		Number of invaded mites		Infestation rate [%]		EHB/AHB
	EHB	AHB	EHB	AHB	EHB	AHB	
AHB1	189	62	37	3	19.6	4.8	4.0
AHB2	168	184	35	11	20.8	6.0	3.5
AHB3	274	149	18	6	6.6	4.0	1.6
AHB4	90	470	21	61	23.3	13.0	1.8
AHB5	128	297	14	20	10.9	6.7	1.6
EHB1	217	217	115	52	53.0	24.0	2.2
EHB2	201	99	48	6	23.9	6.1	3.9
EHB3	108	189	5	7	4.6	3.7	1.3
EHB4	164	55	53	9	32.3	16.4	2.0
EHB5	139	322	37	58	26.6	18.0	1.5
	total				mean (± s.d.)		
AHB	849	1162	125	101	16.3 (± 6.4)	6.9 (± 3.2)	2.5 (± 1.0)
EHB	829	882	258	132	28.1 (± 15.6)	13.6 (± 7.6)	2.2 (± 1.0)

Table 2 Proportion of capped brood cells infested with *Varroa destructor*. AHB and EHB brood had been introduced as L4-larvae into AHB and EHB test colonies, respectively. Infestation rates in EHB brood was more than twice as high as in AHB brood independent of the type of host colony (*t*-test, $P = 0.0111$ for AHB and $P = 0.0185$ for EHB colonies)

Test colonies	Number of available cells		Number of invaded mites		Infestation rate [%]		EHB/AHB
	EHB	AHB	EHB	AHB	EHB	AHB	
AHB6	141	108	10	4	7.1	3.7	1.9
AHB7	101	74	13	2	12.9	2.7	4.8
AHB8	172	78	16	3	9.3	3.8	2.4
AHB9	115	61	8	3	7.0	4.9	1.4
EHB6	40	24	27	3	67.5	12.5	5.4
EHB7	37	52	11	11	29.7	21.2	1.4
EHB8	45	24	36	8	80.0	33.3	2.4
EHB9	33	58	8	4	24.2	6.9	3.5
	total				mean (± s.d.)		
AHB	529	321	47	12	9.1 (± 2.4)	3.8 (± 0.8)	2.6 (± 1.3)
EHB	155	158	82	26	50.4 (± 23.9)	18.5 (± 10.0)	3.2 (± 1.5)

Table 3 Proportion of capped brood cells infested with *Varroa destructor*. AHB and EHB brood had been introduced as eggs into AHB and EHB test colonies, respectively. Infestation rates in EHB brood were more than twice as high as in AHB brood independent of the type of host colony (*t*-test, $P = 0.0298$ for AHB and $P = 0.0326$ for EHB colonies)

C_{16} and C_{18} monoesters. Fifth instar larvae had significantly higher amounts of esters and unsaturated alkenes but lower amounts of methyl branched alkanes compared to 4th instar larvae. In total, 18 substances showed significant stage specific differences in both bee types. Three of these 18 substances, namely methyl palmitate, ethyl palmitate and heneicosane, have been described as kairomones attractive to *Varroa* females (Le Conte *et al.* 1989; Trouiller *et al.* 1992; Rickli *et al.* 1994), and methyl palmitate and methyl oleate are components of a pheromone which trigger the capping of the brood cells by nurse bees (Le Conte *et al.* 1990). Our data confirm the significant increase in the quantities of methyl and ethyl esters in the 5th larval instar. However, the amounts and relative proportions of the components differ from those published by Trouiller *et al.* (1991). This might be due to the shortened extraction period and the use of a different solvent (pentane instead of hexane) in our experiments.

We report for the first time a clear ontogenetic pattern of cuticular hydrocarbons in honey bee larvae. Using the relative amounts of either alkanes, branched alkanes or unsaturated alkenes as variables, one can significantly discriminate among the two larval instars. In addition to the increase in the concentration of some aliphatic esters, the relative proportions of alkanes, alkenes and methyl alkanes showed an age specific signal which could be used either by nurse bees or by *Varroa* females. It is known that hydrocarbons of middle chain length (C_{21}–C_{29}) are perceived by worker honey bees (Fröhlich *et al.* 2000) and that the same substances elicit arrestment behaviour in *Varroa* females (Rickli *et al.* 1994). High relative amounts of alkenes and low relative amounts of methyl alkanes could, therefore, be used as a means of recognition by host nurse bee or parasite of a 5th instar larva.

Differences in cuticular volatile concentrations between AHB and EHB larvae

In contrast to the stage-specific patterns of volatiles, merely small differences were found between AHB and EHB larvae of the 5th instar. None of the substances

with a demonstrated pheromonal or kairomonal activity revealed significant differences among bee types. This result is supported by the multivariate discrimination analysis. The clusters of AHB and EHB 5th instar larvae could only be significantly discriminated when using alkenes and methyl branched alkanes as variables. The groups of substances with proven kairomonal activity were less able (esters and alkanes: Le Conte *et al*. 1989, Rickli *et al*. 1994) or even unable (alkanes) to discriminate AHB from EHB 5th instar larvae.

Therefore, our chemical analyses indicated a higher attractiveness to *Varroa* females of 5th instar compared to the 4th instar larvae but did not reveal those differences that lead *Varroa* females to have a reduced attractiveness toward AHB compared to EHB 5th instar larvae.

Specific host selection behaviour of Varroa *females in vitro*

In our laboratory bioassay (Rosenkranz 1993), attractiveness of living host larvae as well as host extracts can be evaluated under constant environmental conditions and independently from colony-specific influences like nurse bees, wax or other larvae. In contrast to airstream olfactometer tests (Le Conte *et al*. 1989), our bioassay reflects the natural situation of invasion: the distance between the odour source within the depressions of the arena and the test mites is small (Goetz & Koeniger 1993; Boot *et al*. 1995a). Furthermore, only direct contacts with live larvae or prepared dummies are recorded as positive choices. This arrestment behaviour indicates a real acceptance of the target by the mite instead of non-specific reactions to chemotactic stimuli. The results of our bioassays with living larvae confirmed that *Varroa* females only arrest on 5th instar larvae of the age of about 21 h pre-cell capping. Larvae of 30–40 mg, that is, those just after the moult from 4th to 5th instar, merely elicited some non-specific and short-term contacts. Bioassays with larval extracts clearly showed that this arrestment behaviour is influenced by host cuticular volatiles: *Varroa* females significantly distinguished among extracts of 4th and 5th larval instars. However, impregnated dummies did not regain full attractiveness of living larvae, possibly due to the lack of additional, non-chemical stimuli (Le Conte & Arnold 1988; Colin *et al*. 1992; Boot *et al*. 1995a).

Fifth instar AHB larvae and the extract derived from them were significantly more attractive to host searching mites than the equivalent EHB stimuli. Obviously, the esters (Le Conte *et al*. 1989, Trouiller *et al*. 1992) and hydrocarbons (Rickli *et al*. 1994) so far described as attractive for *Varroa* females are not exclusively responsible for this preferential behaviour as we could not detect any differences between bee types in these components. Possibly the somewhat higher proportion of unbranched saturated alkanes in the AHB larvae were of relevance because this fraction proved to elicite strong responses of female mites in bioassays (Rickli *et al*. 1994). But even Rickli *et al*. (1994) assumed synergistic effects of different components of the non-polar fraction of cuticular volatiles. *Varroa* females may use a gestalt pattern of host odors to discriminate host larval stage suitable for reproduction. Further tests with distinct mixtures of compounds must clarify whether the differences in volatile patterns, as identified by discriminant analysis, are responsible for the preference of *Varroa* mites for AHB larvae in the bioassays.

Infestation of AHB and EHB brood: results of experiments within colonies

In our within-colony experiments, we offered a real choice situation between EHB and AHB brood to female mites. We used *Varroa*-infested host colonies of both bee types to test whether mites in AHB or EHB colonies preferred brood of the host colony. In some experiments, the test combs were provided with eggs to guarantee a long-term adaptation of the adult bees to the introduced brood and, therefore, prevent unspecific reactions of the nurse bees at the time of invasion of *Varroa* mites.

Contrarily to the bioassay, brood cells containing EHB larvae were invaded at higher rates than neighbouring cells with AHB larvae. In all 18 experiments with mixed comb sections, the infestation rates of EHB brood were 1.3 to 5.4 fold higher those of AHB brood. Similar results were obtained by Guzmán-Novoa *et al*. (1996, 1999) in Mexico. The preference for EHB brood was independent from the total number of available 5th instar larvae and from the mean rate of *Varroa*-infestation of the test colony. Furthermore, the results were not affected by the bee type of the test colony. This indicates that effects caused by previous host contact do not influence the preference behaviour of *Varroa* females.

Factors assumed to influence the invasion of brood cells by female mites

Our contrasting results of the bioassays and the within-colony tests clearly indicate that, under natural conditions, the invasion behaviour of *Varroa* females is not exclusively triggered by host volatiles. In contrast to our bioassay, the following factors cannot be standardised within a bee hive: the duration of the attractive period could reveal differences according to bee type, *i.e*. that EHB larvae become attractive earlier before the capping of the brood cell compared to AHB larvae. An extended attractive period has been reported for drone larvae, in which the duration of the 5th larval stage is significantly longer compared to worker larvae (Ifantidis 1988; Boot *et al*. 1992, 1995b; Fuchs, 1992). The developmental periods of AHB and EHB worker larvae also show race-specific differences (Rosenkranz & Engels 1994; Michelette 1992). EHB larvae are capped at an average weight of 150 mg about 120 h after hatching from the egg. In contrast, AHB larvae reach a capping weight of only 120 mg and are sealed

about 6 h earlier than EHB larvae (Rembold *et al*. 1980; Rachinsky 1990; Michelette & Soares 1993). In contrast to these differences in larval development, our bioassay confirmed that AHB and EHB larvae are attractive for similar periods of time: both bee types became attractive to *Varroa* females approximately 21 hours before the capping of the brood cell. In the smaller AHB larvae, this corresponds to an average body weight of 60 mg (or 50% of the body weight at cell capping), whereas the EHB larvae became attractive at a body weight of 100 mg (or 65% of the body weight at cell capping). Others (Ifantidis 1988; Boot *et al*. 1992) have reported a similar period of attraction of brood to *Varroa* mites. Therefore the increased invasion of brood cells with EHB larvae within the honey bee colony is not related to the duration of their attractive period.

It is more likely that differences in the size of larvae may influence directly and indirectly the invasion rates of *Varroa* mites. EHB larvae are significantly larger in size compared to AHB ones (this study; Michelette & Soares 1993). Because both larval types were reared in the same combs within uniformly sized brood cells, we assume a shorter distance of the larvae from the cell rim in EHB larvae due to their higher body volume. A shorter distance of the larval body from the cell rim is known significantly to increase *Varroa* infestation rates (Goetz & Koeniger 1993; Boot *et al*. 1995a; Beetsma *et al*. 1999). Additionally, the feeding frequency of larvae by nurse bees will differ according to the size of the larvae. The smaller AHB larvae may receive a lower number of feeding visits which leads to a lower probability for female mites to be transported to suitable brood cells (Boot *et al*. 1992; Lindauer 1952).

Semiochemicals, kairomonal host detection and varroatosis tolerance: Africanized lessons for bee breeding programs?

Varroa population dynamics depend on many factors, of which the invasion rate of mites into brood cells, mite reproductive capacity and mite mortality are regarded as crucial (Calis *et al*. 1999). A lower invasion rate could prolong the phoretic phase of mites (Fries & Rosenkranz 1996) and, therefore, reduce the reproductive output of a given population. However, we do not know whether the observed lower infestation rates of AHB larvae under within-colony choice conditions may have a real impact on mite population growth under undisturbed condition. Unfortunately, it is hardly possible to evaluate exclusively the factor "invasion rate".

The proportion of "mites on bees" to "mites within brood cells" seems to be the only factor that separates honey bee strains with lower invasion rates under practical beekeeping aspects.

Our comparisons of laboratory bioassays and within colony data clearly demonstrate the difficulties of finding tolerance factors which could be used in breeding programs aimed at reducing the impact of *Varroa* mites on *Apis mellifera* colonies. The question as to whether chemical cues that are used in host selection behaviour by *Varroa* females also contribute to the established varroatosis tolerance of the AHB in Brazil remains as yet unanswered.

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