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The relationship between queen execution and cuticular hydrocarbons in stingless bee *Melipona scutellaris* (Hymenoptera: Meliponini)

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Abstract In queenright colonies of stingless bees of the genus *Melipona*, workers recognize, attack, and kill young virgin queens. For *Melipona scutellaris,* we observed that virgin queens were executed when they were between 5 and 9 days old, while newly emerged queens were not attacked. The faster movements of old virgin in relation to newly emerged might be responsible for attacks. It has been also hypothesized that cuticular hydrocarbons are the source of the signal used by workers to recognize virgin queens. We investigated whether newly emerged, 8 days old virgin and physogastric queens of *M. scutellaris* have different cuticular hydrocarbon profiles. Cuticular hydrocarbons of three ages were compared using gas chromatography-mass spectrometry. The cuticular hydrocarbon profiles varied by reproductive status and age. Changes in the cuticular hydrocarbons in virgin queens during aging suggest that these compounds, together with change in movement, may play a role in the recognition of virgin queens by workers prior to regicide.

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Introduction

Meliponini are highly eusocial bees with two female castes: the queens are responsible for reproduction, and the workers carry out the colony's maintenance tasks (Michener 1974). In species of the genus *Melipona,* virgin queens are smaller than the workers and unlike other species of eusocial bees, there is a continuous production of queens across the colony's lifespan. Virgin queens represent 4–7 % of the colony (Imperatriz-Fonseca and Zucchi 1995; Koedam 1999; Sommeijer et al. 2003; Santos-Filho et al. 2006). The main explanation for the high production of queens was as a nest maintenance strategy due to physogastric queen mortality (Engels and Imperatriz-Fonseca 1990; Koedam et al. 1995), or as a future supply for the hive (Koedam et al. 1995). Sommeijer et al. (2003) reported that a considerable proportion of excess queens are not eliminated by the workers but manage to safely leave the nest. They hypothesized that worker fitness was increased by the departure of the excess virgin queens when they reproduce outside their maternal nest.

Triggers are needed for workers to initiate queen execution. Behavior and appearance of virgin queens, including fast body movements, rhythmic wing movements, and an enlarged abdomen, which changes with the queen's age, may encourage workers to attack and subsequently kill them (Silva et al. 1972; Kleinert and Imperatriz-Fonseca 1994; Jarau et al. 2009). Generally, *Melipona* queens mate with a single male when they are 3–8 days old (Kerr et al. 1962; Silva et al. 1972), while excess virgin queens are killed by workers at this age (Imperatriz-Fonseca and Zucchi 1995). In *M. quadrifasciata* colonies with physogastric queens for example, newly emerged queens are not attacked by workers. However, when they reach 3–8 days of age, they begin to move more quickly around the nest, requesting food from workers through antennae contact (Imperatriz-Fonseca and Zucchi 1995). This behavior is likely related to an attempt to be accepted as a new queen (Silva et al. 1972; Van Veen et al. 1999). These virgin queens are attacked and killed or driven from the colony by the workers (Kerr et al. 1962). In *M. marginata* and *M. beecheii*, workers also kill the most active virgin queens, while ignoring queens that move less (Kleinert and Imperatriz-Fonseca 1994; Imperatriz-Fonseca and Zucchi 1995; Van Veen et al. 1999; Jarau et al. 2009).

On the other hand, differences in cuticular hydrocarbons (CHs) produced by virgin queens of different ages might be involved in their recognition by workers. Such age-dependent recognition was reported for the honey bee *Apis mellifera capensis*, where the response of workers toward introduced virgin queens showed a significant increase in hostile reactions as queens aged (Wossler et al. 2006). These authors showed that worker reactions were related to the relative production of (*E*)-9-oxodec-2-enoic acid in the queen's mandibular gland secretions. In fact, queens and workers of the stingless bees have different CH profiles, suggesting an important role for these substances in the recognition of an individual nestmate (Abdalla et al. 2003; Kerr et al. 2004; Ferreira-Caliman et al. 2010; Monnin 2006; Nunes et al. 2009a, b, 2014). However, no study has related virgin queen CH profiles at different ages to queen execution. The stingless bee *Melipona scutellaris* is of particular importance because it appears as "endangered" on the Brazilian agency ICMBio's red list. Therefore, behavioral studies are important for future management of this threatened stingless bee. We collected two colonies of *M. scutellaris* in 2007 and started studies of its biology (Sousa et al. 2013; Santos et al. 2015). We here show that excess virgin queens are killed by workers, a phenomenon triggered by a change in CHC profiles of virgin queens during the first days of their life.

Materials and methods

Stingless bees

We keep *M. scutellaris* colonies in the Central Apiary of the Federal University of Viçosa, Viçosa, state of Minas Gerais, Brazil. Bees were obtained from two queenright colonies. Brood combs were transferred to wooden boxes (20 × 20 × 10 cm) maintained at 29–30 °C and observed daily to verify the emergence of queens. The newly emerged queens were marked on the thorax with non-toxic paint of different colors according to their date of emergence. These virgin queens were returned to the nest from which the brood comb was obtained. These nests were observed daily for 1 h throughout the day to ascertain the interactions between workers and the virgin queens, as well as to determine the age of dead virgin queens. Another set of brood combs was used for CH studies of virgin queens with different ages.

Behavioral interactions between workers and virgin queens

Worker/virgin queen behavioral interaction was observed until workers attacked and killed the virgin queens. This generated 300 h of observation for the 30 virgin queens. To verify whether virgin queen deaths were due to attack or natural causes, their carcasses were examined for signs of aggression, such as decapitation.

Analysis of cuticular hydrocarbons

Virgin queens were collected in two queenright colonies as described above. To sample virgin queens at the age when they were killed, we chose 8-day-old virgin queens, given that between 5 and 9 days after emergence, theses queens were executed by workers. Newly emerged virgin queens were placed in a protective metal grid cage with nurse workers, pollen, and honey ad libitum, and then kept in the colonies from which they were obtained for 8 days. On the other hand, two physogastric queens from the two queenright colonies were used for CH analysis. For the other bees, three individuals each were sampled from the same colony.

CHs were extracted individually by immersing the bees in 500 μL of hexane in glass vials for 10 min. The bees were removed with tweezers, and the hexane was evaporated in an N_2 flow. The samples were then diluted in 50 μL of hexane for analysis by gas chromatography-mass spectrometry (GC–MS). This analysis was performed using a gas chromatograph (Hewlett Packard 6890) equipped with a fused silica capillary column (HP-5MS 5 % phenyl 95 % methyl siloxane 30 m × 250 μm × 0.25 μm, Hewlett Packard), coupled directly with a selective mass detector (Hewlett Packard 5973) set to electron impact mode. All analyses were performed under the following conditions: the injected volume was 1 μL at 270 °C in splitless mode; the temperature program used was 60–230 °C at 20 °C/min, 230–300 °C at 2 °C/min, and 300 °C for 10 min; the carrier gas He was set to 1.2 mL/min; ionization energy was 70 eV and a range of 40–600 amu. CHs were identified by their retention index when coinjected with *n*-alkanes in accordance with van den Dool and Kratz (1963), together with fragmentation patterns (Carlson et al. 1998, 1999; Nelson et al. 1981; Pomonis 1989). The positions of double alkene bonds were not assigned. The relative abundance of each peak in the chromatogram was calculated as follow: the relative abundance of single peaks in relation to the peak with the highest area was calculated. The peaks lower than 5 % in this relative abundance were discarded. For the remained peaks, the relative abundance was calculated in relation to the chromatogram total area.

Statistical analysis

A nonparametric, distance-based, permutational analysis of variance (PERMANOVA), using the Bray–Curtis dissimilarity, was carried out to verify if CH profiles differed between physogastric, newly emerged virgin, and 8-day-old virgin queens (Anderson 2005). The pairwise comparison among the three queen groups was assessed by Monte Carlo P-Values, P(MC), given that the low number of groups (three) and low number of replications in each group (two or three) limited the number of possible permutations (Anderson 2005). Together, non-metric multidimensional scaling (NMDS) was carried out to produce an ordination of queen categories based on the Bray–Curtis dissimilarity matrix (Legendre and Legendre 2003).

Results

Behavioral interactions between workers and virgin queens

Of 30 dead virgin queens analyzed, 23 were conclusively determined to have been killed by workers, while the remaining 7 were found dead but showed no signs of aggression. The 23 queens were executed when they were 5–9 days old (median 7 days).

Newly emerged queens moved slowly, but were more active than newly emerged workers, which were almost immobile. Initially, virgin queens were found restricted to the region near the brood combs, performing slow movements, which became faster only after contact with workers from the colony. Subsequently, they dwelt in small cavities most likely representing empty brood cells or in spaces between the brood combs. As they aged from day 1 to 3, virgin queens increased their activity and visited other nest regions outside the brood area. From day 5 to 9, virgin queens moved quickly inside the nest and sheltered in corners and among honey pots and brood cells. Only the older workers, characterized by darker body pigmentation, showed aggression toward 5–9 days old virgin queens. These workers contacted the abdomen and head of the virgin queen with their antennae and then used their mandibles to attack her, with some workers holding the legs and wings, while others decapitated her.

Analysis of cuticular hydrocarbons

Analysis of CHs of *M. scutellaris* queens showed a total of 52 peaks and around 103 compounds, including linear, and methyl and dimethyl branched alkanes, alkenes, and alkadienes with 23–41 carbon atoms (Table 1). CH differed significantly between the queen categories [PERMANOVA, Pseudo-$F_{2,5} = 22.410$, Unique Permutation = 275, P(MC) = 0.001]. The pairwise comparison showed significant differences in the CH profiles between newly emerged virgin and 8-day-old virgin queens [$t = 2.707$, Unique Permutation = 10, P(MC) = 0.020, average dissimilarity distance = 66.78], between newly emerged virgin queen and physogastric queens [$t = 5.785$, Unique Permutation = 10, P(MC) = 0.002, average dissimilarity distance = 31.987] and between 8-day-old virgin and physogastric queens [$t = 6.221$, Unique Permutation = 10, P(MC) = 0.002, average dissimilarity distance = 43.075]. These results are supported by a visual assessment of NMDS plot patterns using the Bray–Curtis distance (Fig. 1).

Newly emerged queens showed C_{23} and x-$C_{31:1}$ as main compounds. The 8-day-old virgin queens showed C_{25}, x-$C_{27:1}$, C_{27}, and x-$C_{31:1}$ in high amounts. Physogastric queens exhibited a high diversity of branched alkanes, mainly those with 33 and 35 carbons, which were c.a. 50 % of the CHs. Virgin queens had higher amounts of alkanes and alkenes than physogastric ones, and alkadienes were found only in virgin queens.

Discussion

Our findings show that the aggressive behavior of *M. scutellaris* workers against virgin queens is dependent on virgin queen age. Specifically, workers only attack queens aged at least 5 days or older, the age at which queens become highly active, sheltering in colony corners and honey pots. Similar results have been observed in another bee species. These include *M. compressipes* (Sakagami and Oniki 1963), *M. quadrifasciata* (Sakagami et al. 1965; Silva et al. 1972), *M. marginata* (Kleinert and Imperatriz-Fonseca 1994; Kleinert 2005), *M. beecheii* (van Veen et al. 1999; Wenseleers et al. 2004), and *M. favosa* (Koedam et al. 1995). The ages at which virgin queens are attacked, however, vary according to species.

During the attack against the *M. scutellaris* virgin queens, workers are focused on the head and thorax of the queen, unlike *M. beecheii* workers that attack the

Table 1 Relative abundance (mean ± standard error) of cuticular hydrocarbons in queens of *Melipona scutellaris*

#	Compound	RI	Physogastric	Newly emerged	Eight day old virgin	Diagnostic ion (m/z)
1	C_{23}	2300	2.76 ± 0.24	14.51 ± 2.65	2.93 ± 0.26	324
2	11-Me C_{23}	2330	0.32 ± 0.01	0.28 ± 0.12	0.06 ± 0.03	169, 197
	9-Me C_{23}					141, 225
3	C_{24}	2400	0.60 ± 0.05	0.28 ± 0.12	0.13 ± 0.04	338
4	x-$C_{25:1}$	2473	0.56 ± 0.11	4.40 ± 0.72	1.61 ± 0.21	350
5	C_{25}	2500	9.06 ± 0.17	6.05 ± 1.13	8.54 ± 0.41	352
6	13-Me C_{25}	2530	2.76 ± 0.24	0.43 ± 0.19	0.55 ± 0.09	197
	11-Me C_{25}					169, 225
	9-Me C_{25}					141, 253
7	5-Me C_{25}	2548	0.51 ± 0.02	1.19 ± 0.20	0.43 ± 0.18	85, 309
8	x-$C_{27:1}$	2671	0.91 ± 0.13	3.59 ± 0.70	5.98 ± 0.85	378
9	C_{27}	2700	2.92 ± 0.03	3.08 ± 0.62	9.03 ± 1.09	380
10	13-Me C_{27}	2729	1.31 ± 0.14	0.69 ± 0.05	0.79 ± 0.14	197, 225
	11-Me C_{27}					169, 253
	9-Me C_{27}					141, 281
11	5-Me C_{27}	2749	0.07	1.18 ± 0.11	0.47 ± 0.05	85, 337
12	3-Me C_{27}	2773	–	1.01 ± 0.12	0.58 ± 0.02	365
13	Unknown	2781	0.68 ± 0.02	–	–	85, 140
14	5,11-DiMe$_{27}$	2781	–	0.15 ± 0.04	0.10 ± 0.04	85, 183, 253, 351
	5,9-DiMeC_{27}					85, 155, 281, 351
15	x-$C_{29:1}$	2873	0.87 ± 0.10	4.03 ± 0.28	4.36 ± 0.24	406
16	C_{29}	2900	1.87 ± 0.18	1.85 ± 0.29	5.87 ± 0.55	408
17	13-Me C_{29}	2932	2.63 ± 0.28	2.43 ± 0.32	1.74 ± 0.13	197, 253
	11-Me C_{29}					169, 281
	9-Me C_{29}					141, 309
18	7-Me C_{29}	2941	0.40 ± 0.03	2.70 ± 0.35	1.05 ± 0.12	113, 337
19	3-Me C_{29}	2971	–	1.92 ± 0.27	0.84 ± 0.25	393
20	Unknown	2981	0.61 ± 0.01	–	–	–
21	5,11-DiMe$_{29}$	2981	–	0.61 ± 0.06	0.25 ± 0.02	85, 183, 253, 351
	5,9-DiMeC_{29}					85, 155, 281, 351
22	x-$C_{31:1}$	3085	6.91 ± 0.40	25.78 ± 1.50	23.74 ± 1.67	434
23	C_{31}	3100	0.82 ± 0.11	0.60 ± 0.05	2.18 ± 0.19	436
24	15-Me C_{31}	3135	5.52 ± 0.33	1.44 ± 0.15	2.42 ± 0.30	225, 253
	13-Me C_{31}					197, 281
	11-Me C_{31}					169, 309
	9-Me C_{31}					141, 337
25	13,17-DiMeC_{31}	3155	1.24 ± 0.21	0.04 ± 0.03	0.41 ± 0.08	197, 225, 267, 295
	11,15- DiMeC_{31}					169, 239, 253, 323
26	7,17-DiMe C_{31}	3167	0.61 ± 0.01	0.10 ± 0.08	0.27 ± 0.07	113, 155, 337, 379
	7,15-DiMe C_{31}					113, 183, 309, 379
	7,13-DiMe C_{31}					113, 211, 281, 379
	7,11-DiMe C_{31}					113, 239, 253, 379
27	16-Me C32	3228	0.92 ± 0.04	–	0.06 ± 0.05	239
	14-Me C32					211, 281
	12-Me C32					183, 209
28	Unknown	3252	0.71 ± 0.02	–	–	–
29	x-$C_{33:2}$	3255	–	2.00 ± 0.58	1.19 ± 0.07	460
30	x-$C_{33:2}$	3263	–	1.21 ± 0.39	0.67 ± 0.08	460

Table 1 continued

#	Compound	RI	Physogastric	Newly emerged	Eight day old virgin	Diagnostic ion (m/z)
31	x-$C_{33:1}$	3285	1.79 ± 0.05	4.01 ± 0.17	4.94 ± 0.35	462
32	17-Me C_{33}	3335	11.66 ± 0.05	0.13 ± 0.11	2.26 ± 0.35	253
	15-Me C_{33}					225, 281
	13-Me C_{33}					197, 309
	11-Me C_{33}					169, 337
33	15,19-DiMe C_{33}	3355	8.54 ± 0.02	–	–	225, 295
	13,17-DiMe C_{33}					197, 253, 267, 323
	11,15-DiMe C_{33}					169, 239, 281, 351
34	Unknown	3408	0.32	–	–	–
35	17-Me C_{34}	3424	1.88 ± 0.03	–	–	253, 267
	16-Me C_{34}					239, 281
	15-Me C_{34}					225, 295
	14-Me C_{34}					211, 309
	13-Me C_{34}					197, 323
	12-Me C_{34}					183, 337
36	Unknown	3445	1.24 ± 0.05	–	–	–
37	x-$C_{35:2}$	3461	–	5.41 ± 2.12	2.34 ± 0.98	488
38	x-$C_{35:2}$	3467	–	1.78 ± 0.41	0.93 ± 0.33	488
39	x-$C_{35:1}$	3478	–	1.38 ± 0.16	0.72 ± 0.07	490
40	x-$C_{35:1}$	3482	0.27 ± 0.01	1.12 ± 0.24	–	490
41	17-Me C_{35}	3535	11.12 ± 0.72	–	0.61 ± 0.11	253, 281
	15-Me C_{35}					225, 309
	13-Me C_{35}					196, 337
	11-Me C_{35}					169, 365
42	15,19-DiMe C_{35}	3355	8.85 ± 0.48	–	0.11 ± 0.09	225, 253, 295, 323
	13,17-DiMe C_{35}					197, 267, 281, 351
	11,15-DiMe C_{35}					169, 239, 309, 379
43	17-Me C_{36}	3626	1.00 ± 0.07	–	–	253, 295
	16-Me C_{36}					239, 309
	15-Me C_{36}					225, 323
	14-Me C_{36}					211, 337
	13-Me C_{36}					197, 351
	12-Me C_{36}					183, 365
44	Unknown	3650	0.68 ± 0.04	–	–	–
45	x-$C_{37:2}$	3655	–	1.18 ± 0.05	1.30 ± 0.52	516
46	x-$C_{37:2}$	3661	–	1.14 ± 0.16	0.92 ± 0.07	516
47	x-$C_{37:2}$	3668	–	0.76 ± 0.33	1.48 ± 0.79	516
48	x-$C_{37:1}$	3690	–	0.67 ± 0.23	0.38 ± 0.12	518
49	17-Me C_{37}	3735	2.65 ± 0.25	0.49 ± 0.22	1.18 ± 0.12	253, 309
	15-Me C_{37}					225, 337
	13-Me C_{37}					197, 365
	11-Me C_{37}					169, 393
50	15,19-DiMe C_{37}	3755	3.69 ± 0.35	–	3.38 ± 0.39	225, 281, 295, 351
	13,17-DiMe C_{37}					197, 267, 309, 379
	11,15-DiMe C_{37}					169, 239, 337, 407

Table 1 continued

#	Compound	RI	Physogastric	Newly emerged	Eight day old virgin	Diagnostic ion (m/z)
51	17-Me C_{38}	3826	0.05 ± 0.04	0.19 ± 0.16	1.08 ± 0.20	253, 309
	16-Me C_{38}					239, 323
	15-Me C_{38}					225, 337
	14-Me C_{38}					211, 351
	13-Me C_{38}					197, 365
	12-Me C_{38}					183, 393
52	15,19-DiMe C_{39}	3955	0.77 ± 0.04	0.20 ± 0.17	2.04 ± 0.21	225, 295, 309, 379
	13,17-DiMe C_{39}					197, 267, 337, 407
	11,15-DiMe C_{39}					169, 239, 365, 435

C_n Number of carbon atoms in alkanes, x-$C_{n:y}$ number of carbon atoms in alkenes or alkadienes; "x" denotes the double bond position, and "y" the number of unsaturation, for compounds with high molecular weight was not possible to assign the double-bond position, *Me* methyl, *DiMe* dimethyl, *RI* retention index

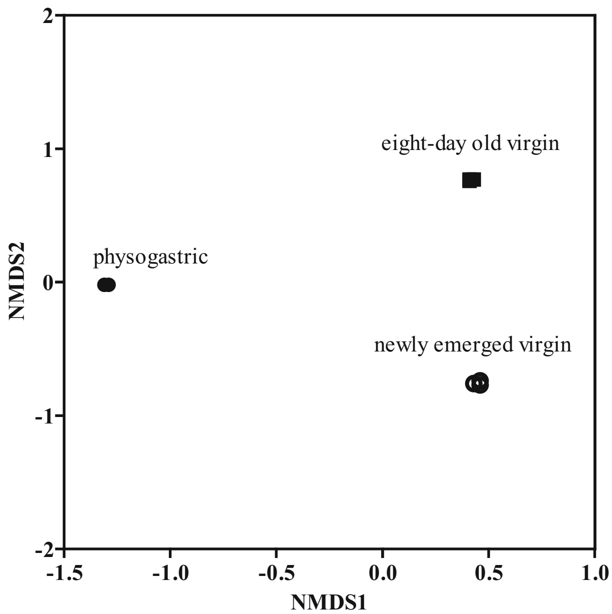

Fig. 1 Discrimination of queens of *Melipona scutellaris* based on cuticular hydrocarbon composition, using non-metric multi-dimensional scaling (NMDS) ordination based on Bray–Curtis distance

abdominal region (Jarau et al. 2009). Our data show that the abdominal region of *M. scutellaris* queens seems to be important for recognition, as workers engage in aggressive behavior only after contacting the abdomen of the virgin queen with their antennae. Class III unicellular glands occur dorsally on the abdomen of stingless bees and are more developed in queens than in workers, suggesting that gland secretions may have a pheromone function (Abdalla and Cruz-Landim 2002; Guerino and Cruz-Landim 2003). Moreover, the Dufour gland located at the abdominal edge in queen bees seems to be responsible for the production of cuticular hydrocarbons, which are important for recognition of queens by workers in the stingless bee *M. bicolor* (Abdalla et al. 2004).

Our PERMANOVA analysis shows that the cuticular hydrocarbons' profile differs between *M. scutellaris* virgin and physogastric queens in a clade isolated from newly-emerged and 8-day-old queens. Differences in cuticular hydrocarbon profiles between *M. scutellaris* physogastric and virgin queens may be a physiological change that occurs after mating, given that mating in insects has been associated with changes in the profile of these compounds (Cuvillier-Hot et al. 2001; Polerstock et al. 2002; Abdalla et al. 2003; Howard and Blomquist 2005; Hora et al. 2008; Nunes et al. 2009a; Oppelt and Heinze 2009; Everaerts et al. 2010; Izzo et al. 2010; Will et al. 2012).

In the social insects, the cuticle is not yet mature after the last change. The body cuticle is completely mature only in older individuals and the more abundant CHs are alkanes, followed by alkenes, alkadienes, and methyl alkenes (Elias-Neto et al. 2013; Falcon et al. 2014; Kather and Martin 2015). Our findings for the CH profiles in *M. scutellaris* virgin queens show differences from those reported for this stingless bee by Kerr et al. (2004), which were rich in tricosane contrasting with hentricontene as described here, which may be due to different CH extraction procedures, given that those authors only used the bee's wings.

Our findings show a different CH profile in 8-day-old *M. scutellaris* virgin queens, which may be a trigger for workers to kill the queens. The literature information about CH profile for virgin queens of different ages is scarce. Newly emerged queens and 2-day-old *M. marginata* virgin queens have similar profiles, but these are different from the 8 day old virgin queen profiles, which are more likely to be attacked by workers (Caliman 2008). *Melipona scutellaris* virgin queens have larger amounts of long-chain alkenes than physogastric queens, compounds that may play a pheromone role. *A. mellifera* workers sprayed with alkenes are more frequently attacked by their nest mates than those treated with alkanes (Dani et al. 2005). The alkenes in the body cuticle of *M. scutellaris* may be the trigger for workers to attack the virgin queens, because the double-bond position and the methyl groups are recognized by bees (Dani et al. 2005; Chaline et al. 2005; Kather and Martin 2015).

In the bumble bee *Bombus terrestris*, van Oystaeyen et al. (2014) claim that long chain hydrocarbons, likely pentacosane, function as queen pheromone, inhibiting ovary activation and/or oosorption in workers. However, Amsalem et al. (2015) stated that there is no experimental evidence that proves long chain alkanes affect worker reproduction. The CH profile in *M. scutellaris* physogastric queen with high amount of long chain and branched alkenes suggest its function as a reliable signal of queen presence in the colony.

CH profile differences between virgin and mated queens, and between sterile workers and reproductive queens have been reported in social insects (Bonavita-Cougourdan et al. 1991; Peeters et al. 1999; Cuvillier-Hot et al. 2001; Abdalla et al. 2003; Dietemann et al. 2013; Biseau et al. 2004; Oppelt and Heinze 2009; Nunes et al. 2010). *M. bicolor* and *M. marginata* physogastric queens have different CH profiles from virgin queens (Abdalla et al. 2003; Caliman 2008) and *Friesella schrottkyi* workers with active ovaries differ from workers with atrophied ovaries (Nunes et al. 2010). The influence of ovary activation in the CH profile has also been reported in ants (Peeters et al. 1999; Biseau et al. 2004).

In conclusion, our results show that recognition of virgin queens by *M. scutellaris* workers occurs at a median age of 7 days, and this timeframe matches changes in the behavior and cuticular hydrocarbon profiles of these bees. We suggest that CHs may be the trigger for virgin queen recognition by workers in the nest, resulting in attacks by workers on virgin queens, which may be subsequently killed.

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