Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Characterizing Honeybee Cuticular Hydrocarbons During Foraging

MS Balbuena[1,2], A González[3*], WM Farina[1,2*]

1 - Laboratorio de Insectos Sociales, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina

2 - Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina

3 - Laboratorio de Ecología Química, Facultad de Química, Universidad de la República, Montevideo, Uruguay

* Senior co-authors

Article History

Edited by
Kleber Del-Claro, UFU, Brazil.
Received 02 March 2018
Initial acceptance 19 May 2018
Final acceptance 16 June 2018
Publication date 25 April 2019

Keywords
Apis mellifera, food source exploitation, cuticular chemistry, chemical communication.

Corresponding author
María Sol Balbuena
Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires
Pabellón II, Ciudad Universitaria
(C1428EHA) - Ciudad Autónoma de Buenos Aires, Argentina.
E-Mail:msbalbuena@bg.fcen.uba.ar

Abstract

Honeybees (*Apis mellifera*) adjust their time and effort during foraging activity. Their metabolic rates together with body temperature rise while gathering profitable resources. These physiological changes may result in a differential cuticular profile, which in turn may bear communicational value. We evaluated if sucrose concentration of collected food affects the cuticular chemistry of honeybees during foraging. We trained bees to artificial feeders with high (2 M) and low (0.5 M) sucrose concentrations, and captured the active foragers for surface extraction of cuticular compounds. We sampled foragers just after feeding, before taking-off towards the hive, and upon landing at the hive entrance, before entering the hive. Through gas chromatography-mass spectrometry analysis of cuticular extracts, we identified and quantified 48 compounds, including cuticular hydrocarbons (CHCs) and volatiles associated with exocrine glands. We found that higher sucrose concentrations resulted in increased amounts of alkanes and alkenes in the surface extracts of foragers captured at the hive entrance, but not at the feeding site. Our results suggest that the differences that have been reported for CHCs in waggle-dancing honey bees can be already found once they return to the hive from profitable food sources.

Introduction

When visiting a floral patch, honeybees have to decide to continue or abandon the feeding site. During this decision-making process, bees evaluate, among many aspects, the profitability of the food source in terms of sucrose concentration and reward rate, its distance from the hive, the difficulty to obtain food and its availability (Seeley, 1995). It has been well studied in *Apis mellifera* that foraging behavior is modulated by the profitability of the exploited nectar source: as food quality increases, so does the frequency of foraging visits and the intensity of nestmate recruitment within the hive; the interval in-between foraging visits is shortened, and the probability of abandoning the source decreases (Núñez, 1970; Núñez, 1982; Seeley et al., 1991; von Frisch, 1967).

The productivity of the food source also correlates positively with the metabolic rate of the forager bee, and higher metabolic rates imply a higher motivational state in the bee (Núñez & Giurfa, 1996). Indeed, the thoracic temperature of forager bees has been also used as a measure of foraging motivation (Schmaranzer & Stabentheiner, 1988; Stabentheiner, 1996; Stabentheiner & Hagmüller, 1991; Stabentheiner et al., 1995), and thermographic measurements have shown that thoracic temperature correlates with higher food quality, both at the feeding site (Schmaranzer & Stabentheiner, 1988; Waddington, 1990) and inside the hive (Farina & Wainselboim, 2001; Stabentheiner & Hagmüller, 1991).

When a forager bee returns to the hive from a profitable nectar source, it dances vigorously to communicate the discovered food source to its nestmates (von Frisch, 1967).

This stereotypic display not only informs about distance and direction to the food source, but also facilitates the conveyance of chemical cues such as odors from the exploited food source (Díaz et al., 2007; Seeley, 1995; von Frisch, 1967), as well as other signals produced during the waggle dance that may attract nestmates (Dyer, 2002; Grüter & Farina, 2009; Thom et al., 2007). The incoming forager also transfers the gathered crop contents through trophallaxis (mouth to mouth contacts) to receiver nestmates. Both dance and trophallaxis affect the body temperature of the bees; waggle dancing increases the body temperature of the recruiting bee (Stabentheiner, 1996; Stabentheiner & Hagmüller, 1991), and trophallaxis raises the body temperature of food receivers, which is also affected by unloading rate and body temperature of the donor bee (Farina & Wainselboim, 2001).

Changes in metabolic rates and/or thoracic temperatures of the active foragers may also promote a passive emission of cuticular hydrocarbons, which in turn may result in a chemical cue to promote foraging. Indeed, four cuticular hydrocarbons (CHCs), *Z*-(9)-tricosene, tricosane, *Z*-(9)-pentacosene and pentacosane, have been reported as putative semiochemicals emitted by waggle-dancing bees (Thom et al., 2007). To mimic a situation that represents intensive dances, three of these compounds were artificially added into a hive, resulting in more foragers exiting the hive and visiting known food sources (Gilley, 2014; Gilley et al., 2012; Thom et al., 2007). These four CHCs were detected by sampling the abdominal surface of dancing bees, and despite their low volatility, they were also found in their surrounding headspace (Thom et al., 2007). Moreover, among several other compounds, the same hydrocarbons were reported not only in the headspace of foraging bees at the feeding site, but also in the cuticular extracts of these entire bees (Schmitt et al., 2007), and have long been known as major cuticular hydrocarbons in forager honeybees (Blomquist et al., 1980b).

If CHCs were chemical signals related to foraging motivation in honeybees, it might be expected that food source profitability modulates this signal, a possibility that has not yet been explored. This hypothesis rests on the fact that sucrose concentration modulates the foraging motivational state, which itself promotes changes in the metabolic rate and the body temperature, potentially promoting the emission of CHCs. Such differential emission of hydrocarbons might be detected not only within the social environment of the hive, but also in the foraging context. In this study, we characterized the cuticular hydrocarbon profiles of forager bees with respect to food source profitability. Specifically, we analyzed the cuticular extracts of forager bees fed with different sucrose concentrations at two different stages during foraging: *i)* at the feeding site, and *ii)* once they land at the hive entrance.

Materials and Methods

Study Site and Animals

Three colonies (H1, H2 and H3) of honeybees *Apis mellifera* L. with a queen, brood and reserves were used. Colonies H1 and H2 were reduced from ordinary commercial hives to four-frame Langstroth hives, containing each about 10000 worker bees. These colonies were used for collecting forager bees at the feeding site. Colony H3, with 3000-3500 honeybees, was housed in a two-frame observation hive, and was used for collecting foragers at the hive entrance, upon returning from the feeder. To prevent interference with other bee colonies, this hive was enclosed within a flight chamber (6 m length x 3 m wide x 2 m height), which consisted of a wooden structure with polyethylene mesh walls. The hive was located at one end of the chamber, allowing for 6 m direct flight from the feeder. Except for the experimental periods, the flight chamber remained open and the bees could forage freely outside. Hence, foragers from all beehives (H1 to H3) had access to a natural environment containing natural flowers. Experiments were performed in spring 2012 (H1), summer 2013 (H2), and summer 2014 (H3) at the Experimental Field of Universidad de Buenos Aires (Argentina). Chemical analyses of cuticular extracts were carried out at the Universidad de la República in Montevideo, Uruguay.

Experimental Procedure

Two experimental settings were employed; one for collecting forager bees at the feeding site (H1 and H2), and another for collecting them at the hive entrance (H3). Non-foraging honeybees from inside the hive (H1 and H2, henceforth "hive bees") were also captured and extracted for comparison purposes. To be sure that they were not foragers, these bees were caught from the center of the hive, in the surroundings of the brood area, where honeybees usually perform tasks as nurses or food processors (Seeley, 1995).

Forager bees were trained to collect unscented 1 M sucrose solution at an artificial feeder located at either 10 (H1 and H2) or 6 m (H3) from the hive. Once a foraging group of approximately 20-30 honeybees was established, unscented 0.5 M and 2 M sucrose solutions were offered in the artificial feeder. The two sucrose solutions were offered alternately, allowing 30 min with no feeding reward in between sucrose solution offerings. After changing the feeder solution, 15 min were allowed before capturing the bees. In this way, bees could make repeated trips from the hive to the feeder. Each honeybee landing at the feeder was allowed to drink *ad libitum* and either captured before taking-off towards the hive (feeding site, H1 and H2) or color-marked on the wings with a dot of permanent marker (Uni, Mitsubishi Pencil Co., Ltd, Tokyo; usually used to mark queen bees) for later identification upon returning to the hive (H3). Returning foragers were captured at the hive entrance, and the time elapsed during homewards flights were recorded. All captured bees were immediately

sacrificed with CO_2 and immersed in dichloromethane to extract cuticular compounds (see below).

Cuticular Chemistry

Cuticular compounds were extracted in dichloromethane (DCM) at room temperature and under gentle hand stirring. In order to detect and quantify less abundant CHCs such as methyl-branched alkanes or alkadienes, the bees were extracted in pooled samples consisting of groups of five bees for H1 and H2 (feeding site and hive bees), and three bees for H3 (hive entrance). In all cases, extractions were done in 1-dram screw-cap vials with either 3 or 2 mL of DCM (for samples containing 5 or 3 bees, respectively). After 1 min, the insects were removed and 100 µL of n-tridecane (0.503 mg/mL in hexane) were added as internal standard (IS). The extracts were then concentrated to 1 mL under a stream of nitrogen for GC-MS analysis.

Chemical analyses were done by gas chromatography coupled to mass spectrometry (GC-MS), using a Shimadzu QP-2010 GC-MS equipped with an AT-5 MS column (Alltech) (30 m × 0.25 mm, 0.25 µm), and operated with a constant carrier flow of 1 mL/min (He). The temperature of the GC oven was programmed from 70 °C (1 min) to 150 °C (1 min) at 10 °C/min, then raised to 300 °C at 5 °C/min, and held for 5 min at 300 °C. The injector temperature was 250 °C and the interphase temperature 310 °C. Injection (1 µL) was in the splitless mode (sampling time 1 min), and mass spectra were acquired from *m/z* 30 to 350, except for the DMDS-derivatized extracts, in which mass spectra were acquired from *m/z* 30 to 550 (70 eV, scan mode). For retention index calculations, a mixture of n-alkanes (100 ppm each, in hexane) was injected in the splitless mode, using the same temperature program.

Cuticular extracts were derivatized with dimethyl disulfide (DMDS) for obtaining additional information for the unsaturated compounds. The extract (100 µL) was mixed in a V-shaped vial with 100 µL of DMDS and 5 µL of an Et_2O solution of I_2 (60 mg/mL). The reaction mixture was kept closed at 50 °C for 2 h, after which 200 µL of hexane and 100 µL of $Na_2S_2O_3$ (5% in water) were added. The organic layer was finally separated and concentrated to 50 µL under a stream of Nitrogen for GC-MS analysis.

Statistical Analysis

For each analyzed sample, peak areas higher than 0.1% of the total ion chromatogram (TIC) were considered for the analysis (excluding the area of the IS). In addition, compounds were excluded if they were not present in at least 3 samples of any given treatment (hive bees, foragers fed with either 0.5 M or 2 M sucrose solution).

In order to analyze the net amounts of all individual CHCs (µg equivalents of IS) in the cuticular extracts of the different treatments, we performed a Principal Component Analysis (PCA) (Quinn & Keough, 2002). In our data analysis, the principal components which explained at least 80% of the variance were then analyzed by MANOVA, and the main components were analyzed by one-way ANOVA/Tukey HSD.

In addition, CHCs were grouped as alkanes and alkenes, and the added amounts for each group were compared among treatments using one-way ANOVA/Tukey HSD. Finally, we also compared the amounts of the two alkanes and the two alkenes that have been reported as foraging promoters (Thom et al., 2007). To reduce the risk of type 1 errors due to the multiple use of the same data, we corrected the significance thresholds using the Bonferroni method ($\alpha' = a / k$), with $\alpha = 0.05$ and $k = 4$. Thus, our significance threshold was $\alpha' = 0.0125$.

To compare flight time between foragers fed with 0.5 M or 2 M sucrose solutions we performed an ANOVA.

Results

We identified a total of 48 compounds by GC-MS (Fig 1 and Table 1), including CHCs (9 through 48, except 13), as well as other compounds previously described in honeybees: 2-nonanol (1) and benzyl acetate (2), known as sting alarm pheromones (Collins and Blum 1982); geraniol (5), geranic acid (6) and farnesol (8), all related to the Nasonov gland; and (*Z*)-11-eicosen-1-ol (13), which has been reported in the sting gland (Pickett et al. 1982; Schmitt et al. 2007). We also found *E*-2-octenyl acetate (3), 2-nonanyl acetate (4) and *E*-2-decenyl acetate (**7**), which have not been reported in *Apis mellifera*, and probably originate from plants previously visited by the bees.

The identification of the compounds was based on mass spectra and retention index comparison with those of databases (Table 1 and Supp 1**)** (Adams, 2007; El-Sayed, 2014; Linstrom & Mallard, 2005). As previously reported (Blomquist et al., 1980a; Carlson et al., 1989; Dani et al., 2004; Francis et al., 1989; Kather et al., 2011; McDaniel et al., 1984), the chromatograms of cuticular extracts were dominated by n-alkanes (C_{23}, C_{25}, C_{27}. Peaks 17, 23 and 28, respectively) and (*Z*)-11-eicosen-1-ol, including lower amounts of alkenes, alkadienes and methyl-branched alkanes (Fig 1). DMDS-derivatives of the alkenes showed double bond positions in C_7 and C_9 for lower molecular weight monoenes, while C_8 and C_{10} unsaturations were found in longer chain alkenes. Methyl-branched alkanes co-eluted as mixtures that were characterized by the higher abundance of diagnostic fragment ions indicating the position of the methyl branches in C_{11}, C_{13} or C_{15} (Table 1).

We focused our quantitative analysis only on the CHCs. The net amount of each CHC was calculated from the TIC peak areas as mg-equivalents of internal standard per bee, as shown in Table 2 (see Supp 2 for more details) for the different treatments, namely, hive bees, foragers fed with 0.5 M or 2 M sucrose solution concentrations.

An initial PCA analysis of CHC net amounts was done for foragers captured at the feeding site (from hives H1 and H2), resulting in two principal components (PC) that represented 48.81 and 16.39% of the overall variance.

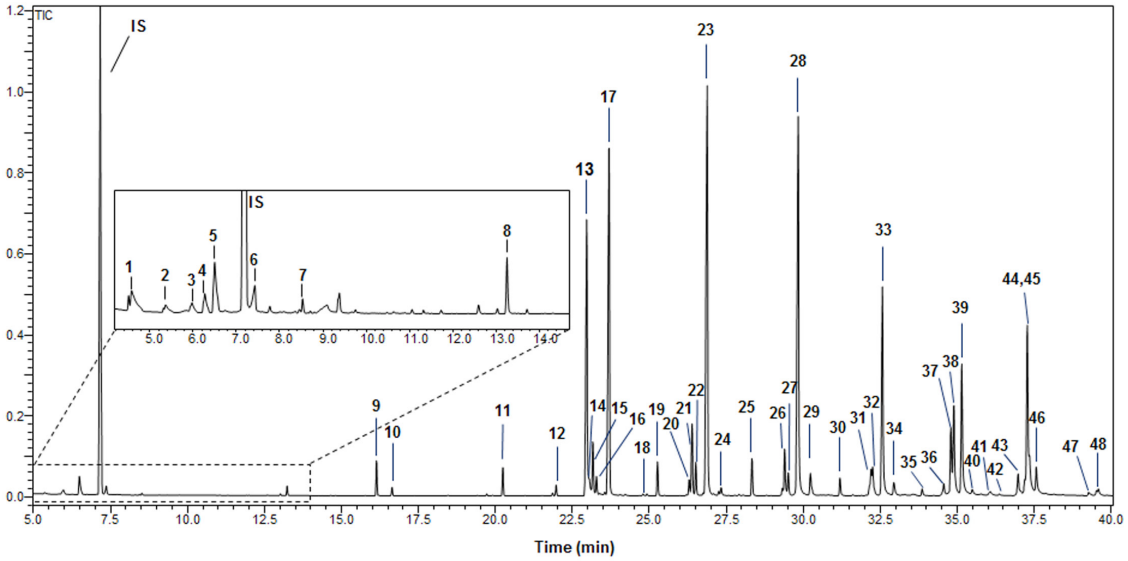

Fig 1. Typical total ion chromatogram of cuticular extracts of *Apis mellifera* foragers. The expanded portion (peaks **1** – **8**) includes more volatile glandular compounds. Peaks **9** through **48** correspond to cuticular hydrocarbons, except for the abundant (*Z*)-11-eicosen-1-ol (**13**). See text for GC and MS conditions, Tables 1 and S1 for characterization details, and Table 2 for quantitative data (IS: internal standard).

The major contributing component (PC1) clearly segregated the two honeybee groups: hive bees and forager bees, with no obvious separation of forager bees fed with 0.5 or 2 M sucrose solutions (Fig 2). Although there was a significant interaction between the factors hive and treatment (MANOVA: $F_{10,120}$= 4.375, $p < 0.01$), LSD *post-hoc* comparisons for individual PCs showed no interaction between these two factors in PC1. The graphic groupings observed in Fig 2 for PC1 were corroborated by a significant difference in the composition of CHCs for hive and forager bees (ANOVA PC1: F = 121.22, df = 2, $p < 0.01$), and not for foragers fed with 0.5 or 2 M (Tukey: hive bees vs. foragers: df = 67, $p < 0.01$; 0.5 M vs. 2 M: df = 67, p = 0.906, Fig 2).

Table 1. Retention indices and mass spectrum diagnostic ions of CHCs and glandular compounds in the cuticular extracts of honeybees (see Supp 1 for full ion tables)

Peak[1]	Compound(s)	Retention Index[2]		Diagnostic ions (%)	S.I.[3]
		Exp.	Lit.		
1	2-Nonanol	1098	1098	129 (M^+-CH_3, 4), 126 (M^+-H_2O, 44), 45 (100), 31 ($CH3O^+$, 2)	94
2	Benzyl acetate	1166	1157	150 (M^+, 30), 108 (100), 91 (60), 79 (33), 77 (19)	95
3	*E*-2-Octenyl acetate	1208	1208	128 (M^+-CH_2CO, 8), 110 (11), 43 (100)	94
4	2-Nonanyl acetate	1232	n.a.[4]	126 (M^+-CH_3COOH, 11), 87 (M^+-C_7H_{15}, 41), 43 (100)	n.a.
5	Geraniol	1254	1249	154 (M^+, 1), 139 (M^+-CH_3, 3), 123 (M^+-CH_2OH, 11), 69 (100), 31 ($CH3O^+$, 1)	94
6	Geranic acid	1345	1342	168 (M^+, 2), 150 (1), 123 (M^+-CO_2H, 15), 100 (M^+-C_5H_8,20), 69 (100)	92
7	*E*-2-Decenyl acetate	1404	1408	156 (M^+-CH_2CO, 5), 138 (M^+-CH_3COOH, 6), 110 (15), 43 (100)	96
8	(*E*,*Z*)-(2,6)-Farnesol	1724	1722	136 (M^+-$C_5H_{10}O$, 10), 69 (100)	92
9	9-Nonadecene[5]	1874	1875	266 (M^+, 13), 97 (86), 83 (98), 69 (84), 55 (100) DMDS: 360 (M^+), 187, 173	96
10	Nonadecane	1898	1900	268 (M^+, 3), 85 (53), 71 (70), 57 (100)	95
11	Heneicosane	2100	2100	296 (M^+, 2), 85 (56), 71 (71), 57 (100)	97
12	Docosane	2199	2200	310 (M^+, 2), 85 (58), 71 (74), 57 (100)	96
13	11-Eicosen-1-ol	2265	2260	278 (M^+-H_2O, 8), 250 (2), 222 (2), 96 (84), 82 (100), 55 (89), 31 (CH_3O^+,5) DMDS: 390 (M^+), 217, 173	91
14	X,Y-Tricosadiene[6]	2268	n.a.	320 (M^+, 15), 96 (94), 82 (79), 81 (95), 67 (100)	n.a.
15	9-Tricosene	2273	2272	322 (M^+, 12), 97 (100), 83 (93), 69 (71) DMDS: 416 (M^+), 243, 173	95
16	7-Tricosene	2280	2280	322 (M^+, 11), 97 (100), 83 (95), 69 (78) DMDS: 271, 145	96
17	Tricosane	2300	2300	324 (M^+, 5), 85 (66), 71 (84), 57 (100)	92

Table 1. Retention indices and mass spectrum diagnostic ions of CHCs and glandular compounds in the cuticular extracts of honeybees (see Supp 1 for full ion tables). (Continuation)

Peak[1]	Compound(s)	Retention Index[2]		Diagnostic ions (%)	S.I.[3]
		Exp.	Lit.		
18	X-Tetracosene	2374	2372	336 (M^+, 13), 97 (100), 83 (97), 69 (74)	n.a.
19	Tetracosane	2400	2400	338 (M^+, 2), 85 (59), 71 (75), 57 (100)	95
20	X,Y-Pentacosadiene	2470	n.a.	348 (M^+, 23), 96 (100), 82 (97), 81 (93), 67 (82)	n.a.
21	9-Pentacosene	2474	2474	350 (M^+, 8), 97 (100), 83 (83), 69 (67) DMDS: 444 (M^+), 271, 173	94
22	7-Pentacosene	2481	2481	350 (M^+, 7), 97 (100), 83 (81), 69 (69) DMDS: 299, 145	94
23	Pentacosane	2500	2500	352 (M^+, 3), 85 (59), 71 (78), 57 (100)	94
24	13+11-Me-pentacosane	2533	2533	351 (M^+-CH_3, 1), 225 (13-methyl, 4), 224 (13-methyl, 5), 197 (11-methyl, 3), 196 (11-methyl, 4), 169 (13-methyl, 7), 168 (13-methyl, 12), 85 (66), 71 (82), 57 (100)	n.a.
25	Hexacosane	2600	2600	366 (M^+, 3), 85 (63), 71 (83), 57 (100)	93
26	9-Heptacosene	2675	2675	378 (M^+, 6), 97 (100), 83 (80), 69 (63) DMDS: 299, 173	n.a.
27	7-Heptacosene	2683	2683	378 (M^+, 5), 97 (100), 83 (76), 69 (67) DMDS: 327, 145	n.a.
28	Heptacosane	2700	2700	380 (M^+, 3), 85 (61), 71 (81), 57 (100)	91
29	13+11-Me-heptacosane	2731	2733	379 (M^+-CH_3, 2), 253 (11-methyl, 3), 252 (11-methyl, 3), 225 (13-methyl, 5), 224 (13-methyl, 7), 197 (13-methyl, 5), 196 (13-methyl, 8), 183 (2), 169 (11-methyl, 6), 168 (11-methyl, 8), 85 (65), 71 (80), 57 (100)	n.a.
30	Octacosane	2799	2800	394 (M^+, 2), 85 (64), 71 (80), 57 (100)	93
31	9-Nonacosene	2878	2876	406 (M^+, 7), 97 (100), 83 (78), 69 (60) DMDS: 327, 173	n.a.
32	8-Nonacosene	2882	n.a.	406 (M^+, 8), 97 (100), 83 (77), 69 (60) DMDS: 341, 159	n.a.
33	Nonacosane	2900	2900	408 (M^+, 2), 85 (62), 71 (81), 57 (100)	95
34	15+13+11-Me-nonacosane	2931	n.a.	407 (M^+-CH_3, 1), 281 (11-methyl, 2), 280 (11-methyl, 2), 253 (13-methyl, 3), 252 (13-methyl, 4), 225 (15-methyl, 7), 224 (15-methyl, 10), 211 (1), 197 (13-methyl, 4), 196 (13-methyl, 7), 183 (3), 169 (11-methyl, 7), 168 (11-methyl, 7), 85 (64), 71 (80), 57 (100)	n.a.
35	Triacontane	3000	3000	422 (M^+, 1), 85 (65), 71 (81), 57 (100)	92
36	X,Y-Hentriacontadiene	3063	3077	432 (M^+, 8), 96 (100), 82 (85), 69 (71)	n.a.
37	10-Hentriacontene	3077	n.a.	434 (M^+, 8), 97 (100), 83 (76), 69 (60) DMDS: 341, 187	n.a.
38	8-Hentriacontene	3084	3086	434 (M^+, 8), 97 (100), 83 (78), 69 (62) DMDS: 369, 159	n.a.
39	Hentriacontane	3100	3100	436 (M^+, 2), 85 (61), 71 (79), 57 (100)	92
40	15+13+11-Me-hentriacontane	3130	n.a.	435 (M^+-CH_3, 1), 309 (11-methyl, 1), 308 (11-methyl, 1), 281 (13-methyl, 4), 280 (13-methyl, 4), 253 (15-methyl, 4), 252 (15-methyl, 6), 225 (15-methyl, 4), 224 (15-methyl, 6), 197 (13-methyl, 5), 196 (13-methyl, 7), 169 (11-methyl, 6), 168 (11-methyl, 4), 85 (67), 71 (82), 57 (100)	n.a.
41	X-Dotriacontene	3180	n.a.	448 (M^+, 4), 97 (100), 83 (81), 69 (62)	n.a.
42	Dotriacontane	3199	3200	450 (M^+, 1), 85 (66), 71 (83), 57 (100)	94
43	X,Y-Tritriacontadiene	3258	n.a.	460 (M^+, 10), 96 (100), 82 (84), 69 (73)	n.a.
44	X-Tritriacontene	3278	n.a.	462 (M^+, 9), 97 (100), 83 (79), 69 (61)	n.a.
45	Y-Tritriacontene	3285	n.a.	462 (M^+, 7), 97 (100), 83 (75), 69 (59)	n.a.
46	Tritriacontane	3300	3300	464 (M^+, 1), 85 (66), 71 (83), 57 (100)	93
47	X,Y-Pentatriacontadiene	3384	n.a.	488 (M^+, 9), 96 (100), 82 (84), 69 (69)	n.a.
48	X-Pentatriacontene	3395	n.a.	490 (M^+, 5), 97 (100), 83 (77), 69 (55)	n.a.

[1] Peak numbers as in Figure 1
[2] Experimental linear retention indices calculated according to (Adams 2007). Literature retention indices from (Adams 2007; El-Sayed 2014; Herzner et al., 2013; Linstrom and Mallard 2005).
[3] Similarity index according to (Adams 2007; Linstrom and Mallard 2005).
[4] Not available.
[5] The geometry of double bonds in *A. mellifera* CHCs was assumed to be *Z* as previously reported (McDaniel et al. 1984).
[6] Letters (X,Y) indicate that the double bond position could not be determined unambiguously.

We grouped the CHCs into alkanes and alkenes, and compared their net amounts in extracts of forager bees captured at the feeding site, finding no differences with respect to the two rewarding treatments (alkanes: ANOVA; $F_{1,54}$= 69.719, p = 0.857; alkenes: ANOVA; $F_{1,54}$= 11.715, p = 0.948; Fig 3a). Similarly, the amounts of the individual CHCs reported by Thom et al. (2007) as semiochemicals involved in the waggle dance did not show differences in the cuticular extracts from forager bees collected at the feeding site after feeding on 2 M or 0.5 M sucrose solutions (ANOVA; 9-tricosene: $F_{1,36}$ = 1.238, p = 0.273; tricosane: $F_{1,36}$ = 0.096, p = 0.7579; 9-pentacosene: $F_{1,36}$ = 0.003, p = 0.9536; pentacosane: $F_{1,36}$ = 0.058, p = 0.8109; Fig 3b).

When foragers fed with 0.5 M or 2 M sucrose solutions were allowed to fly back to the hive, the former took 13.2 ± 2.5 sec, while bees fed with high sucrose concentration flew faster, arriving after 7.2 ± 0.5 sec (ANOVA: $F_{1,68}$ = 5.24, p = 0.025).

Table 2. CHCs for hive bees and foragers collected under different rewarding programs. Numbers and color scale inside cells represent the amounts of each CHC expressed as µg-equivalents of internal standard (µg eq IS/bee). For more details see Online Resource 1. ND: not detected.

Hydrocarbon amounts (mg eq IS/bee)

	Hive entrance		Feeder reward		Hive bees
9-Nonadecene	0.95	0.80	0.26	0.22	0.24
Nonadecane	0.07	0.25	0.17	0.15	ND
Heneicosane	1.52	1.52	0.73	0.73	0.20
Docosane	1.10	0.93	0.32	0.32	0.11
Tricosadiene	1.08	1.00	0.45	0.48	0.47
9-Tricosene	7.01	6.14	3.37	3.58	0.51
7-Tricosene	2.16	1.63	0.97	1.03	0.15
Tricosane	24.79	21.27	13.77	13.98	3.13
Tetracosene	0.49	0.32	0.16	0.13	ND
Tetracosane	2.55	2.05	1.19	1.15	0.23
Pentacosadiene	ND	ND	ND	1.38	ND
9-Pentacosene	9.98	8.83	5.60	5.62	1.11
7-Pentacosene	2.88	2.24	1.67	1.68	0.20
Pentacosane	25.52	22.02	16.72	16.53	6.22
Me-pentacosane	0.61	0.60	0.39	0.38	0.32
Hexacosane	1.75	1.47	1.52	1.45	0.51
9-Heptacosene	3.72	3.49	3.36	3.41	0.58
7-Heptacosene	1.06	0.89	1.29	1.29	0.17
Heptacosane	14.64	13.50	15.28	14.86	8.90
Me-heptacosane	0.90	0.88	0.75	0.73	0.76
Octacosane	0.60	0.51	0.80	0.73	0.54
9-Nonacosene	1.21	1.20	2.29	2.33	0.63
7-Nonacosene	0.83	0.93	0.83	0.98	0.92
Nonacosane	6.39	6.16	8.78	8.43	9.11
Me-nonacosane	0.48	0.48	0.51	0.48	0.53
Triacontane	0.22	0.24	0.33	0.30	0.36
Hentriacontadiene	0.42	0.41	0.48	0.44	0.79
9-Hentriacontene	2.59	2.54	2.32	2.22	3.95
7-Hentriacontene	3.31	3.13	3.65	3.48	5.22
Hentriacontane	3.70	3.47	6.51	6.20	7.91
Me-hentriacontane	0.24	0.26	0.18	0.19	0.22
Dotriacontene	ND	ND	0.21	0.17	0.40
Dotriacontane	ND	ND	0.14	ND	0.13
Tritriacontadiene	0.85	0.75	0.85	0.78	2.10
Tritriacontene	4.86	4.61	5.82	5.49	10.06
Tritriacontene	0.72	0.51	0.82	0.84	ND
Tritriacontane	0.41	0.37	0.81	0.75	1.41
Pentatriacontadiene	ND	ND	ND	ND	0.26
Pentatriacontene	ND	ND	ND	ND	0.21
	2 M	0.5 M	2 M	0.5 M	
	Sucrose concentration				

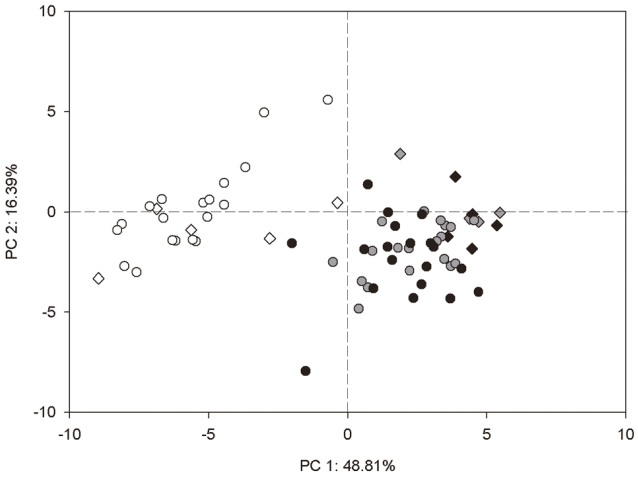

Fig 2. Relationship between cuticular hydrocarbons and sucrose concentration of hive bees and foragers. Scores plot showing the relationship of cuticular hydrocarbons extracted from honeybees from different hives [diamonds: Hive 1, spring 2012 (N = 5 samples per treatment); circles: Hive 2, summer 2013 (N = 19 samples per treatment)], task groups (hive bees: white; forager bees: gray and black), and feeding treatments (gray: 0.5 M, black: 2 M). The two main principal components (PC) account for 48.81 and 16.39% of the overall data variance, respectively.

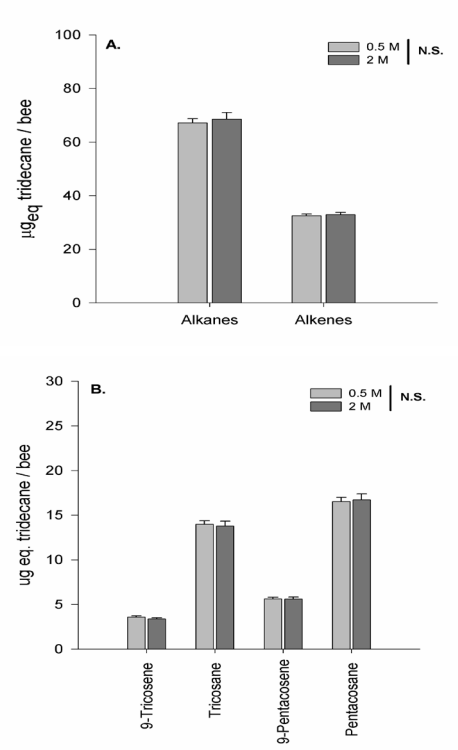

Fig 3. Hydrocarbon amounts extracted from foragers captured at the feeding site. (A) Net hydrocarbon amounts (µg equivalents of tridecane per bee) grouped as alkanes or alkenes, extracted from forager bees of different rewarding programs (light gray bars: 0.5 M; dark gray bars: 2 M). (B) Net amounts of specific hydrocarbons (9-tricosene, tricosane, 9-pentacosene, and pentacosane) could be involved in the recruitment in forager bees. Error bars represent standard error of the mean. Asterisks indicate significant differences ($p < 0.05$). N.S. indicates no significant differences ($p > 0.05$).

The CHC profiles of bees captured at the hive entrance also showed some differences. PCA analysis to compare CHCs did not show a clear segregation between bees fed with 0.5 or 2 M sucrose solutions (Supp 3). However, when grouping CHCs into alkanes and alkenes, CHC extracts from bees that returned from a feeder with higher sucrose reward showed higher net amounts of both, alkanes (ANOVA; $F_{1,28} = 6.595$, $p = 0.016$) and alkenes (ANOVA: $F_{1,28} = 4.496$, $p = 0.043$; Fig 4a). This difference was also found for the individual alkanes (tricosane and pentacosane) reported as foraging promoters (Thom et al., 2007), but not for the individual alkenes, which only showed a non-significant trend (ANOVA: tricosane: $F_{1,28} = 7{,}127$, $p = 0.012$; pentacosane: $F_{1,28} = 7.621$, $p = 0.010$; 9-tricosene: $F_{1,28} = 2.477$, $p = 0.127$; 9-pentacosene: $F_{1,28} = 1.73$, $p = 0.199$; Fig 4b).

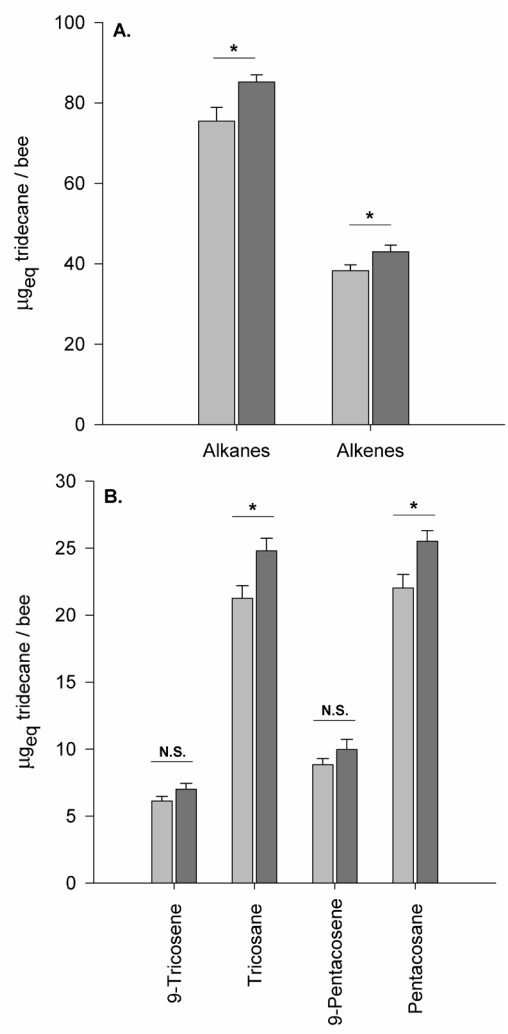

Fig 4. Hydrocarbon amounts extracted from bees captured at the hive entrance. (A) Net hydrocarbon amounts (µg equivalents of tridecane per bee) grouped as alkanes or alkenes, extracted from forager bees of different rewarding programs (light gray bars: 0.5 M; dark gray bars: 2 M) captured upon arrival to the hive entrance (N = 15 for both sucrose concentrations). (B) Net amounts of specific hydrocarbons (9-tricosene, tricosane, 9-pentacosene, and pentacosane) could be involved in the recruitment in forager bees. Error bars represent standard error of the mean. Asterisks above bars indicate significant differences ($p < 0.05$). N.S. indicates no significant differences ($p > 0.05$).

Discussion

In this study, we assessed whether the profitability of the exploited food source promotes changes in the cuticular hydrocarbon profile of honeybees during foraging. To do so, we analyzed the CHCs of foragers fed with sucrose solutions of low and high concentrations. For comparison purposes, we also analyzed non-forager bees collected within the hive, and found that CHC profiles of hive bees and foragers differ, independently of the hive from which the bees originated. In general, we found higher amounts of CHCs in forager bees, but relatively more abundant high molecular weight CHCs (> C31) in hive bees, as has been previously reported (Del Piccolo et al., 2010). In line with these results, task-related cuticular differences have been reported in *Apis mellifera* (Dani et al., 2004; Kather et al., 2011), and at least in part, these differences are consistent with higher exposure of foraging bees to more variable environmental conditions such as temperature and humidity (Heinrich, 1993), which differ from the highly controlled conditions experienced within the hive.

When comparing the effect of sucrose concentration on the CHCs of forager bees, we found that bees captured at the feeder, just after feeding and before taking-off back to the hive, showed similar CHC profiles regardless of the concentration of the sucrose solution they had ingested. However, when the bees were captured just after arriving to the hive entrance, CHC extracts from bees that returned from a highly profitable food source (2 M sucrose) contained more alkanes and alkenes than those that arrived from a poorer source (0.5 M sucrose). This difference was also observed for the two specific alkanes (n-tricosane and n-pentacosane) that have been reported as foraging promoters in dancing bees (Thom et al., 2007). The two alkenes similarly reported, (*Z*)-9-tricosene and (*Z*)-9-pentacosene, were found to be unaffected by food profitability, showing only a non-significant trend in line with that of the alkanes.

It has been shown that honeybees increase their metabolic rates and thoracic temperature when collecting sugar solutions of higher concentrations and reward rates (Schmaranzer & Stabentheiner, 1988). Metabolic rate and body temperature correlate with the motivational state of forager bees, which in turn depends on external stimuli such as food profitability (Balderrama et al., 1992; Moffatt, 2001; Schmaranzer & Stabentheiner, 1988). Moreover, there is evidence that the flight velocity of foragers returning to the hive is higher when more profitable food (*i.e.* higher sugar concentration) is offered at the feeding site (von Frisch & Lindauer, 1955). In the same line, we found that bees fed with 0.5 M sucrose solution took about twice the time to return to the hive compared to bees fed with 2 M. Due to the intense activity of the flight muscles, a bee flying back from a food source probably reaches higher temperatures if flying faster (von Frisch & Lindauer, 1955). If body temperature is at least partially responsible for the difference in the CHC

profiles of bees returning from high and low profitable food sources, as we hypothesize here, this would explain that bees captured at the feeding site did not show the differences in CHC profiles that were observed later, after flying back to the hive. In line with this argument, a comparison of net amounts of CHCs extracted from bees captured before and after flying shows higher amounts for the later, regardless of the sucrose solution treatment. Worth of note, our methodology involved capturing the bees and immediately extracting them, without freezing for later extraction to avoid neutralizing the possible effect of body temperature in the cuticular chemistry.

These temperature changes would not be expected to upregulate CHC biosynthesis, given that they occur within a very short time window. Returning bees had to fly only 6 m in our experimental setup, and they did so right after feeding, so only a few seconds elapsed between feeding at the feeder and capture at the hive entrance. However, an increase in body temperature may affect the physicochemical properties of the honeybee cuticular envelope, for instance its density and viscosity, possibly making the CHCs more available for surface extraction, but also possibly promoting their passive release (Schmitt et al., 2007; Thom et al., 2007). In fact, most of the CHCs identified in our study have been previously described not only as components of cuticular extracts but also as volatiles sampled in the headspace of forager honeybees at the feeding site, thus indicating that CHCs are slightly volatile (Schmitt et al., 2007).

A similar temperature effect on CHC surface chemistry may also occur within the hive. It has been shown that during recruitment by waggle dancing, or when exchanging food with receiver nestmates, returning foragers adjust their thoracic temperatures in relation to the food source profitability (Farina & Wainselboim, 2001; Stabentheiner, 1996; Stabentheiner & Hagmüller, 1991). Moreover, four common honeybee CHCs, two alkanes and two alkenes, have been reported on the body surface of waggle dancing honeybees (Thom et al., 2007), and they are known to be perceived, learned and discriminated by workers (Chaline et al., 2005; Getz & Smith, 1987). Furthermore, when a subset of these compounds [(*Z*)-9-tricosene, tricosane and pentacosane] were injected into the hive, the number of exiting bees increased, and the authors postulated that a passive emission of these compounds could be promoted by the high body temperature reached by the intense movement of the dancer bee (Thom et al., 2007). Indeed, Thom et al. (2007) not only found chemical differences between dancer and non-dancer foragers returning from the same food source, but also between intense and less intense dancers. Thus, our results are complementary with this notion, since we show that honeybees with different foraging motivational state present different CHC profiles, but they do so even before entering the hive, and obviously before any possible temperature effect caused by the waggle dance.

Our results show that, when bees were captured at the hive entrance, some CHCs (i.e. tricosane and pentacosane) are present in higher amounts in the surface extracts of foragers fed with high sugar concentration, compared to those from bees fed with low sugar concentration. This small but significant chemical difference in CHC profiles could alert nestmates about the presence of good food sources, and may be enough to activate or reactivate unemployed foragers to forage. In the same line, Thom and Dornhaus (2007) suggest that volatile compounds of active foragers could promote an increase in foraging behavior. Since CHCs are only faintly volatile, body contacts may also be significant, and we have shown that simple body contacts with active foragers are enough to motivate experienced nestmate foragers to return to known feeding sites (Balbuena et al., 2012). Such social interactions are unrelated to dance-following or trophallaxis, and likely involve the perception of cuticular compounds. Our results are in line with previous studies that suggest that cuticular chemistry may possess a signaling role during the recruitment process in honeybees, certainly secondary, but still potentially relevant.

Acknowledgments

We thank Héctor Verna for technical assistance during the experimental period and Carolina Mengoni Goñalons for helping us with the statistical analysis. We also thank anonymous referees for their comments. This study was partly supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires, and Universidad de la República.

References

Adams, R.P. (2007). Identification of essential oil components by Gas Chromatography/Mass Spectrometry. Carol Stream, IL: Allured Publishing. 804 p.

Balbuena, M.S., Molinas, J. & Farina, W.M. (2012). Honeybee recruitment to scented food sources: correlations between in-hive social interactions and foraging decisions. Behavioral Ecology and Sociobiology, 66: 445-452. doi 10.1007/s00265-011-1290-3

Balderrama, N.M., de Almeida, L.O.B. & Núñez, J.A. (1992). Metabolic rate during foraging in the honeybee. Journal of Comparative Physiology B, 162:440-447.

Blomquist, G.J., Chu, A.J. & Remaley, S. (1980a). Biosynthesis of wax in the honeybee, *Apis mellifera* L. Insect Biochemistry, 10: 313–321.

Blomquist, G.J., Chu, A.J. & Remaley, S. (1980b). Biosynthesis of wax in the honeybee, *Apis mellifera* L. Insect Biochemistry, 10: 313-321.

Carlson, D.A., Roan, C.S., Yost, R.A. & Hector, J. (1989). Dimethyl disulfide derivatives of long-chain alkenes,

alkadienes, and alkatrienes for gas chromatography - mass spectrometry. Analytical Chemistry, 61:1564–1571.

Chaline, N., Sandoz, J.C., Martin. S.J., Ratnieks, F.L. & Jones, G.R. (2005). Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). Chemical Senses, 30(4):327-35. doi: 10.1093/chemse/bji027

Collins, A.M. & Blum, M.S. (1982). Bioassay of compounds derived from the honeybee sting. Journal of Chemical Ecology Bioassay, 8:463-470.

Dani, F.R., Corsi, S., Pradella, D., Jones, G.R. & Turillazzi, S. (2004). GC-MS analysis of the epicuticle lipids of *Apis mellifera* reared in central Italy. Insect Social Life, 5:103–109.

Del Piccolo, F., Nazzi, F., Della Vedova, G. & Milani, N. (2010). Selection of Apis mellifera workers by the parasitic mite *Varroa destructor* using host cuticular hydrocarbons. Parasitology, 137(06): 967-973. doi: 10.1017/S0031182009991867

Díaz, P., Grüter, C. & Farina, W. (2007). Floral scents affect the distribution of hive bees around dancers. Behavioral Ecology and Sociobiology, 61(10):1589-1597. doi: 10.1007/s00265-007-0391-5

Dyer, F.C. (2002). The biology of the dance language. Annual Review of Entomology, 47:917–49.

El-Sayed, A.M. (2014). The Pherobase: Database of Insect Pheromones and Semiochemicals.

Farina, W.M. & Wainselboim, A.J. (2001). Changes in the thoracic temperature of honeybees while receiving nectar from foragers collecting at different reward rates. Journal of Experimental Biology, 204:1653–1658.

Francis, B.R., Blanton, W.E., Littlefield, J.L. & Nunamaker, R.A. (1989). Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera, Apidae). Annual Entomology Society of America, 82:486–494.

Getz, W.M. & Smith, K.B. (1987). Olfactory sensitivity and discrimination of mixtures in the honeybee *Apis mellifera*. Journal of Comparative Physiology A, 160:239-245.

Gilley, D.C. (2014). Hydrocarbons emitted by waggle-dancing honey bees increase forager recruitment by stimulating dancing. PLoS ONE, 9(8):e105671. doi: 10.1371/journal.pone.0105671

Gilley, D.C., Kuzora, J.M. & Thom, C. (2012). Hydrocarbons emitted by waggle-dancing honey bees stimulate colony foraging activity by causing experienced foragers to exploit known food sources. Apidologie, 43(1):85-94.

Gruter, C. & Farina, W.M. (2009). The honeybee waggle dance: can we follow the steps? Trends in Ecology and Evolution, 24(5):242-7. doi:10.1016/j.tree.2008.12.007

Heinrich, B. (1993). The hot-blooded insects: Strategies and mechanisms of thermoregulation. Cambridge, MA: Harvard University Press. 600 p.

Herzner, G., Kaltenpoth, M., Poettinger, T., Weiss, K., Koedam, D., Kroiss, J. & Strohm, E. (2013). Morphology, chemistry and function of the postpharyngeal gland in the South American digger wasps *Trachypus boharti* and *Trachypus elongatus*. PLoS ONE, 8(12):e82780. doi: 10.1371/journal.pone.0082780

Kather, R., Drijfhout, F.P. & Martin, S.J. (2011). Task group differences in cuticular lipids in the honey bee *Apis mellifera*. Journal of Chemical Ecology, 37(2):205-12. doi: 10.1007/s10886-011-9909-4

Linstrom, P. & Mallard, W.G. (2005). NIST Standard Reference Database Number 69. Gaithersburg, MD: National Institute of Standards and Technology.

McDaniel, C.A., Howard, R.W., Blomquist, G.J. & Collins, A.M. (1984). Hydrocarbons of the cuticle, sting apparatus, and sting shaft of *Apis mellifera* L.: Identification and preliminary evaluation as chemotaxonomic characters. Sociobiology, 8:287-298.

Moffatt, L. (2001). Metabolic rate and thermal stability during honeybee foraging at different reward rates. Journal of Experimental Biology, 204(4):759-766.

Núñez, J.A. (1970). The relationship between sugar flow and foraging and recruiting behavior of honey bees (*Apis mellifera* L.). Animal Behaviour, 18:527-538.

Núñez, J.A. (1982). Honeybee foraging strategies at a food source in relation to its distance from the hive and the rate of sugar flow. Journal of Apicultural Research, 21:139-150.

Núñez, J.A. & Giurfa, M. (1996). Motivation and regulation of honey bee foraging. Bee World, 77(4):182-196.

Pickett, J.A., Williams, I.H. & Martin, A.P. (1982). (*Z*)-11-Eicosen-1-ol, an important new pheromonal component from the sting of the honey bee, *Apis mellifera* L. (Hymenoptera, Apidae). Journal of Chemical Ecology, 8(1):163-175.

Quinn, G.P. & Keough, M.J. (2002). Experimental design and data analysis for biologists. Cambridge: Cambridge University Press.

Schmaranzer, S. & Stabentheiner, A. (1988). Variability of the thermal behavior of honeybees on a feeding place. Journal of Comparative Physiology B, 158:135-141.

Schmitt, T., Herzner, G., Weckerle, B., Schreier, P. & Strohm, E. (2007). Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals. Apidologie, 38(2):164-170. doi: 10.1051/apido:2006067

Seeley, T.D. (1995). The wisdom of the hive. Cambridge (Massachusetts): Harvard University Press. 295 p.

Seeley, T.D., Camazine, S. & Sneyd, J. (1991). Collective decision-making in honey bees: How colonies choose among nectar sources. Behavioral Ecology and Sociobiology, 28: 277-290.

Stabentheiner, A. (1996). Effect of foraging distance on the thermal behaviour of honeybees during dancing, walking and trophallaxis. Ethology, 102: 360-370.

Stabentheiner, A. & Hagmüller, K. (1991). Sweet food means "hot dancing" in honeybees. Naturwissenschaften, 78: 471-473.

Stabentheiner, A., Kovac, H. & Hagmüller, K. (1995). Thermal behavior of round and wagtail dancing honeybees. Journal of Comparative Physiology B, 165: 433-444.

Thom, C. & Dornhaus, A. (2007). Preliminary Report on the Use of Volatile Compounds by Foraging Honey Bees in the Hive (Hymenoptera: Apidae: *Apis*). Entomologia Generalis, 29: 299-304. doi: 10.1127/entom.gen/29/2007/299

Thom, C., Gilley, D.C., Hooper, J. & Esch, H.E. (2007). The scent of the waggle dance. PLoS Biol, 5: e228. doi: 10.1371/journal.pbio.0050228

von Frisch, K. (1967). The dance language and orientation of bees. Cambridge (Massachusetts): The Belknap Press of Harvard University Press. 566 p.

von Frisch, K & Lindauer, M. (1955). Über die Fluggeschwindigkeit der Bienen und über ihre Richtungsweisung bei Seitenwind. Naturwissenschaften, 42: 377–385.

Waddington, K.D. (1990). Foraging profits and thoracic temperature of honey bees (*Apis mellifera*). Journal of Comparative Physiology B, 160: 325-329.

Conflict of Interest: The authors declare that they have no conflict of interest.

Author contributions: MSB, AG and WMF conceived and designed the experiments. MSB performed the experiments. AG. performed chemical analysis. MSB, AG and WMF performed data analysis. MSB, AG and WMF drafted the manuscript. All authors revised and commented on the manuscript.

Supplementary files

Supplementary file 1. Complete fragment ion profiles (> 2%) of the main compounds identified in surface extracts of honeybees.

http://periodicos.uefs.br/index.php/sociobiology/rt/suppFileMetadata/2977/0/1914
http://dx.doi.org/10.13102/sociobiology.v66i1.2977.s1914

Supplementary file 2. Quantitative analysis of CHCs for hive bees and foragers collected under different rewarding programs.

http://periodicos.uefs.br/index.php/sociobiology/rt/suppFileMetadata/2977/0/1915
http://dx.doi.org/10.13102/sociobiology.v66i1.2977.s1915

Supplementary file 3. Relationship (scores plot) between CHCs and fed sucrose concentration in honeybees upon arrival to the hive entrance (gray: 0.5 M, black: 2 M). The two main principal components (PC) account for 36.33 and 23.04% of the overall data variance, respectively. N = 15 for both treatments (MANOVA: $p = 0.191$, $F = 1.628$).

http://periodicos.uefs.br/index.php/sociobiology/rt/suppFileMetadata/2977/0/1916
http://dx.doi.org/10.13102/sociobiology.v66i1.2977.s1916