Trends in Entomology
Vol. 13, 2017

Original Communication

Cuticular hydrocarbon profiles and putative sources of sex pheromones in queens of *Tetragonisca angustula* (Hymenoptera: Apidae: Meliponini)

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ABSTRACT

Sex pheromone production by insects aims to attract the opposite sex for mating. However, not only sexually attractive virgin *Tetragonisca angustula* queens release these compounds, but also mated queens. Herein, the putative sources of *T. angustula* queen pheromones were studied by the gas chromatography coupled to mass spectrometry (GC-MS) analyses of volatile compounds and abdominal gland, cuticular and cephalic extracts of sexually attractive virgin and physogastric queens. Isopropyl and hexyl hexanoates released by *T. angustula* physogastric queens were found both in their Dufour's and tergal glands. Although both esters were present in the tergal glands of sexually attractive virgin queens, only isopropyl hexanoate was detected by the aeration method. Octadecyl octadecenoate was found to be the main constituent of the sexually attractive virgin queen's Dufour's glands, and this same gland contained several esters in physogastric queens. Complex mixtures of normal, branched and unsaturated hydrocarbons were found in the tergal gland and cuticular extracts. The chemical composition of the cephalic physogastric queen extract was similar to the cuticular extract, except for the presence of acids and steroids. In contrast, the virgin queen's head was mostly composed of nerol.

KEYWORDS: *Tetragonisca angustula*, virgin queen, physogastric queen, Dufour's glands, tergal glands

INTRODUCTION

Stingless bees (Hymenoptera: Apidae: Meliponini) are eusocial insects with virgin queens that mate only once during nuptial flights [1, 2]. Such queens do not perform additional copulation after mating with a single male [3]. Some observations, however, show that if their nests are disturbed, the virgin queens or even mated and egg-laying queens (hereafter, physogastric) could be harassed by males attempting copulation inside nests [4, 5]. The mating of stingless bee males, inside nests is believed to be uncommon since they aggregate themselves outside nests, waiting for a nuptial flight of virgin queens for mating with them [6]. These mating swarms and copulation attempts in stingless bees seem to be orchestrated by the queen's pheromones (volatile substances) [7]. Consequently, we need to evaluate how both queens (virgin and physogastric) could be differentiated on the basis of their cuticular hydrocarbon profiles. Further, we need to assess where the main sex pheromones are located to

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comprehend why males are specifically attracted to the abdominal regions of the queens.

The cephalic glands in social bees are the likely candidates to be the main sources of sex pheromones. For example, in *Apis mellifera* (Apini) [8] and *Bombus terrestris* (Bombini) these pheromones are found in their cephalic glands [9]. Similarly, in neotropical stingless bees (Meliponini), the *Scaptotrigona* sp. virgin queens also possess sex pheromones (2-alcohols) that are stored in their cephalic glands [10]. However, in another stingless bee species, *Tetragonisca angustula*, apparently the sex pheromones are located in their abdomen region [11]. These volatile compounds are identified as isopropyl hexanoate (IH), butyl hexanoate (BH) and hexyl hexanoate (HH) [11].

Thus, by assuming that social insects have a broad range of glands (e.g., mandibular, labial, tergal, Dufour's) [12, 13], it is relevant to know where the sex pheromones of *T. angustula* are located, to biologically understand the behavior presented by males toward such queens. Additionally, once *T. angustula* males are attracted to virgin, mated or egg-laying queens [11], we need fine-scale evaluation to verify whether the cuticular hydrocarbon profiles of both queens are similar.

Here, we investigate the cuticular hydrocarbon profiles, as well as the putative sources of sex pheromones of the *T. angustula* queens by gas chromatography coupled to mass spectrometry (GC-MS) analyses of volatile compounds and abdominal gland extracts. The cephalic extracts of sexually attractive virgin and physogastric queens were also included in our analyses to check whether those sex pheromones could be found in the cephalic glands or not, like the other social bee species mentioned above.

MATERIALS AND METHODS

Queens

T. angustula virgin queens were reared *in vitro* and were adapted from another protocol [14]. We transferred 50 larvae (1-2 days old) onto Elisa plate slots with 50 μL of larval food. These specimens were kept in a biochemical oxygen demand (B.O.D.) incubator at 30 ºC in darkness and 100% controlled relative humidity (RH) during the first three days, then at 90% RH with a KCl-saturated solution for 5-6 days, and at 70% RH with NaCl-saturated solution plus glucose until the emergence of the adults.

The virgin queens that emerged were placed in pairs inside Petri dishes containing a brood comb with emergency cells, honey and pollen *ad libitum* from the same species and approximately 20-30 young workers. For chemical analysis, we chose dominant virgin queens (12-14 days old) rather than subordinate ones of same age. For this selection, we used some behavioral displays of dominant queens [15] as indicators of sexual maturity: a) aggressiveness toward other queens, displayed by biting; b) frequent abdomen inflation; c) constant wing vibration, and d) frequent trophallaxy with workers. Physogastric queens were obtained from queenright colonies. The physogastry (enlarged abdomen) of such queens, and torn wings were used to identify them as successfully mated.

Chemicals

All solvents (Synth, Brazil) were dried over anhydrous Na_2SO_4 (Synth, Brazil, 99.0%) and were either distilled or bidistilled. Column chromatographic purifications were performed with silica gel (Acros Organics, USA, 0.035-0.070 mm). Thin-layer chromatographic analyses were achieved using silica gel 60 F254 plates (Merck, Germany), and the spots were visualized with UV light (254 nm) or by spraying with either a $KMnO_4$ solution (Nuclear, Brazil) or a 5% *p*-anisaldehyde (Vetec, Brazil, 98.0%), acetic acid (Chemco, Brazil, 99.7%) and concentrated sulfuric acid (Synth, Brazil, 95.0 - 98.0%) solution and heating to ~ 100 ºC with a heat gun. Sodium thiosulfate (Vetec, Brazil, 98.0%), isopropanol (Tedia, USA, 99.8%), hexanoic acid (Aldrich, Germany, 99.5%), *N*,*N*'-dicyclohexylcarbodiimide (DCC; Merck, Germany, 98.0%), 4-(dimethylamino) pyridine (DMAP; Aldrich, USA, 99.0%), iodine (Rothyl, USA, 99.0%), dimethyl disulfide (Aldrich, Germany, 99.0%) and sodium bicarbonate (Vetec, Brazil, 99.7%) were all purchased. Dodecyl acetate, tetradecyl acetate, tetradecyl butyrate, hexadecyl acetate, hexadecyl butyrate and 2-nonadecanone were synthesized earlier in our laboratory [16].

GC-MS analyses

The GC-MS analyses were carried out with an Agilent 6890 gas chromatograph coupled to a

Hewlett-Packard 5973 mass detector; the apparatus was equipped with an Agilent DB5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm i.d.). The mass spectra were recorded within the 40-600 amu range at 3.54 scans.s^{-1}, with the spectrometer operating in the electron ionization (EI) mode at 70 eV. Helium was the carrier gas and flowed at 1 mL.min^{-1}. The injector and interface temperatures were 240 and 280 ºC, respectively. The temperature program used to analyze the volatiles, extracts and synthetic compounds increased the temperature from 50 to 290 °C at 12 °C.min^{-1} and was held for 20 min. The derivatized samples were analyzed using a temperature program that raised the temperature from 50 to 290 °C at 12 °C.min^{-1} and had a final hold time of 50 min. One-microliter samples (1 mg.mL^{-1}) were injected using a 10:1 split ratio for the synthetic samples and the splitless mode for the original samples. The compounds were identified by comparing their mass spectra with the Wiley 275 library database and by comparing the retention indexes and co-elution with synthetic standards and manual interpretation of the mass spectra of the samples.

The retention indexes for the compounds identified in the extracts were obtained by co-injecting the samples with a standard mixture of *n*-alkane (C9-C32), in a GC-MS. The retention indexes were calculated according to the Van den Dool and Kratz equation [17]. The values were compared with those in the literature [18], and the retention index of the compounds were calculated using the equation $RI = 100z + 100[(tr_{(x)} - tr_{(Z)})/(tr_{(Z+1)} - tr_{(Z)})]$, where z = the number of carbon atoms of the n-alkane that elutes before the substance of interest (x); $tr_{(x)}$ = the retention index of the substance of interest; $tr_{(Z)}$ = retention time of the alkane that elutes before the substance of interest and $tr_{(Z+1)}$ is the retention time of the alkane that elutes after the substance of interest. The temperature program linearly increased the temperature at the rate of 12 °C.min^{-1}. Thus, as seen in [18] the retention times of the *n*-alkanes depended linearly on the carbon numbers.

Volatile sampling

Volatile compounds from the virgin (12-14 days old) and physogastric queens were collected using the dynamic headspace method with Porapak Q for 2 h. The queens were removed from the Petri dishes (virgin queens) or colonies (physogastric queens), and analyzed in triplicate, using one queen per analysis. The volatiles were extracted from the Porapak Q, using a mixture of hexane (2 mL) and ethyl acetate (2 mL) as solvent; the extracts were reduced to 30 μL under a weak nitrogen flow. These samples were analyzed by the GC-MS in which heptadecane (1 mg.mL^{-1}) was used as an internal standard.

Synthesis and quantification of volatile compounds

Isopropyl and hexyl hexanoates were synthesized according to the methodology developed by Steglich [19]. Dichloromethane (10 mL), hexanoic acid (10 mmol), DMAP (30-110 mg) and isopropanol or hexanol (20-40 mmol) were added to a round-bottomed flask while stirring. The reaction was cooled to 0 ºC, and DCC (10 mmol) was slowly added for 5 min. The reaction remained at room temperature for 3 hours before being filtered to eliminate the precipitated urea. The organic layer was washed with 0.5 mol.L^{-1} HCl (3 x 10 mL) and saturated $NaHCO_3$ (3 x 10 mL). The combined organic layers were dried over anhydrous magnesium sulfate and filtered before the solvent was evaporated under reduced pressure, yielding a colorless liquid. The product was purified by silica gel column chromatography (30 g, 2 cm column diameter), eluted with hexane and increasing amounts of ethyl acetate, and monitored by thin layer chromatography. These compounds were characterized by GC-MS, ^{1}H- and ^{13}C-NMR analyses (for details, see supplementary information). The ^{1}H- and ^{13}C-NMR analyses were carried out using a Varian INOVA 500 (499.88 MHz) spectrometer equipped with a 5 mm probe using $CDCl_3$ and the solvent with trimethylsilyl (TMS, Cambridge Isotope Laboratories) as a reference standard.

The relative quantification of these two compounds in the natural samples was performed using an internal standard, and a calibration curve was obtained *via* GC-MS for each synthetic ester at different concentrations (0.25; 0.5; 1.0; 3.0 and 5.0 μg.$μL^{-1}$), with heptadecane as an internal standard [20].

Dufour's and tergal gland extracts

The virgin and physogastric queens used in these analyses were immediately removed from their Petri dishes or colonies, respectively, and promptly cooled in a refrigerator (-5 °C). After 10 min, the individual queens were dissected and examined using a Leica stereomicroscope, in a Petri dish containing distilled water cooled by a crushed-ice bath. Dufour's glands were separated from the reproductive tract, macerated in 10 μL of ethyl acetate and analyzed by GC-MS [21, 22]. The tergal glands were dissected by removing all abdominal tergites, macerated in 10 μL of ethyl acetate and analyzed by GC-MS. All procedures were performed in triplicate, using one queen per analysis.

Cuticular and cephalic extracts

Virgin and physogastric *T. angustula* queens were killed in a freezer (T = -18 °C) and washed twice with 1 mL of distilled hexane for 1 min to remove the cuticular wax. The volumes of the cuticular hexane extracts were reduced to 30 μL under a slow nitrogen flow and analyzed using GC-MS. The cephalic extracts were macerated using 30 μL of ethyl acetate and analyzed *via* GC-MS. Additionally, the total extract of the physogastric queen abdomen was also analyzed and prepared by maceration with ethyl acetate (30 μL) and later analyzed by GC-MS. The procedure was performed in triplicate, using one queen per analysis [23, 24].

DMDS/I_2 derivatization to determine alkene double-bond positions

The samples were dissolved in 2 mL of bidistilled hexane, treated with 200 mL of dimethyl disulfide (DMDS) and 100 mL of iodine solution (32 mg of I_2 in 2 mL distilled ether) and stirred at 50 °C for 24 h under reflux. The reactions were stopped with 2 mL of aqueous sodium thiosulfate ($Na_2S_2O_3$, 1 g in 10 mL of distilled water). The organic phase was extracted, dried over anhydrous magnesium sulfate, filtered through treated cotton and evaporated under flowing nitrogen. The derivatized samples were dissolved in bidistilled hexane and analyzed by GC-MS [25].

RESULTS AND DISCUSSION

The analysis of the volatile compounds adsorbed by the aeration method showed that under stress and outside their nests, the *T. angustula* physogastric queens released two esters, the IH (0.80 $\mu g.h^{-1}$ for a 393 mg bee, R^2 (coefficient of determination or R-square) = 0.9824) and HH (2.58 $\mu g.h^{-1}$, R^2 = 0.9921). On the other hand, the sexually attractive virgin queens released only IH (0.03 $\mu g.h^{-1}$ for a 100 mg bee). The IH, therefore, was present at approximately six-fold higher levels in the physogastric queens than in the virgin queens. This finding may be ascribed to the fact that stingless bee physogastric queens often have tergal and Dufour's glands that are more developed than those of virgin queens [26-29].

We demonstrated in fine scale, that IH and HH were present in both the Dufour's and tergal glands of *T. angustula* physogastric queens, but in virgin queens they were present only in the tergal glands. (Tables 1 and S1). Currently, it is not possible to argue that the hemolymph carried these volatile compounds (IH and HH) and stored them in the Dufour's and tergal glands or, alternatively, they were synthesized independently. Some studies with other bee species have found that determining the origin of these compounds is very complex [27-30]. We were unable to determine which gland was responsible for the release of IH and HH to attract males. However, knowing that virgin queens contain these two esters only in their tergal glands (as shown by both dynamic headspace and glandular dissection – table 1), we can infer that the tergal glands of both queens are potential candidates as the sources of IH and HH release. An additional proxy corroborates this evidence. Both queens (virgin and physogastric) presented erratic behaviors when they were outside nests (i.e., under stress), during volatile sampling, such as prolonged abdomen inflation and wing vibration. It was clear to us that under such situations the queens did expose their tergal glands to the environment while releasing volatiles, supporting the above hypothesis.

The Dufour's gland extracts of *T. angustula* physogastric queens were mostly composed of esters (93.00% of the Dufour's gland compounds), predominantly tetradecyl acetate (21.63%) and tetradecyl butyrate (21.83%) (Tables 1 and S1). In contrast, the Dufour's glands of *T. angustula* virgin queens were dominated by octadecyl octadecenoate (67.34%). The presence of *n*-pentacosane in the

Table 1. Chemical compositions of cephalic, cuticular, abdominal and glandular extracts of virgin and physogastric *Tetragonisca angustula* queens.

No.	Compounds	RI (calc.)	RI (lit.)	Physogastric queen					Virgin queen (12-14 days old)			
				Head (%)[a]	Abdomen (%)[b]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]	Head (%)[a]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]
***n*-Alkanes**												
1	Heneicosane	2100	2100	-	0.27 ± 0.11	-	0.33 ± 0.09	-	-	-	0.66 ± 0.15	0.20 ± 0.28
2	Tricosane	2300	2300	5.59 ± 0.67	12.34 ± 4.91	0.96 ± 1.35	10.27 ± 1.51	11.87 ± 3.01	-	-	3.89 ± 1.61	8.08 ± 1.60
3	Pentacosane	2500	2500	2.39 ± 0.41	5.61 ± 1.49	1.92 ± 3.33	5.15 ± 1.63	6.26 ± 8.84	-	19.22 ± 15.31	44.82 ± 4.87	20.03 ± 3.83
4	Heptacosane	2700	2700	1.94 ± 0.24	4.75 ± 1.23	2.38 ± 4.12	5.64 ± 2.31	16.72 ± 3.99	-	-	7.31 ± 3.73	17.32 ± 0.54
5	Nonacosane	2900	2900	-	0.65 ± 0.13	-	1.06 ± 0.73	2.73 ± 0.82	-	-	0.23 ± 0.23	2.58 ± 0.56
Methylalkanes												
6	9-, and 11-methyltricosane[c]	2338	-	2.46 ± 0.69	3.60 ± 0.48	-	2.39 ± 1.53	2.52 ± 0.98	-	-	0.42 ± 0.24	0.65 ± 0.10
7	9-, 11-, and 13-methylpentacosane[c]	2536	-	2.56 ± 0.72	4.87 ± 1.47	-	2.37 ± 1.15	1.76 ± 0.61	-	-	1.15 ± 0.71	1.04 ± 0.05
8	9-, 11-, and 13-methylheptacosane[c]	2735	-	1.79 ± 0.44	3.93 ± 1.72	-	1.90 ± 0.39	4.46 ± 3.43	-	-	0.58 ± 0.18	0.91 ± 0.06
9	9-, 11-, 13-, and 15-methylnonacosane[c]	2935	-	1.72 ± 0.28	3.45 ± 1.26	-	2.72 ± 0.81	4.38 ± 2.45	-	-	0.09 ± 0.15	0.50 ± 0.32
10	11-, 13-, and 15-methylhentriacontane[c]	3133	-	2.55 ± 0.99	3.70 ± 2.44	-	4.27 ± 1.15	4.25 ± 0.47	-	-	0.47 ± 0.43	0.70 ± 0.95
Alkenes												
11	Heneicosene	-	-	-	-	-	-	-	-	-	0.10 ± 0.16	-
12	Tricosene	-	-	-	-	-	0.39 ± 0.34	-	-	-	0.11 ± 0.18	-

Table 1 continued..

No.	Compounds	RI (calc.)	RI (lit.)	Physogastric queen					Virgin queen (12-14 days old)			
				Head (%)[a]	Abdomen (%)[b]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]	Head (%)[a]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]
13	9-, 10-, 11- and 12-Pentacosene[c]	-	-	0.92 ± 0.15	1.72 ± 0.44	-	2.56 ± 1.73	6.19 ± 7.54	-	-	2.46 ± 1.07	3.12 ± 2.17
14	9-, 10-, 11-, 12- and 13-Heptacosene[c]	-	-	0.42 ± 0.11	0.66 ± 0.24	-	0.82 ± 0.41	0.63 ± 0.90	-	-	2.94 ± 1.05	2.11 ± 1.59
15	10-, 11-, 12-, 13- and 14-Nonacosene[c]	-	-	2.24 ± 0.39	3.87 ± 0.95	-	6.06 ± 3.17	3.62 ± 0.32	-	-	3.11 ± 2.04	6.51 ± 1.58
16	Hentriacontadiene	-	-	5.22 ± 1.38	6.49 ± 4.61	-	12.77 ± 3.48	6.76 ± 0.13	-	-	1.37 ± 1.51	10.13 ± 3.83
17	10-, 11-, 12-, 13-, 14- and 15-Hentriacontene[c]	-	-	7.01 ± 0.58	11.37 ± 5.17	-	19.84 ± 9.88	11.84 ± 1.78	-	-	4.43 ± 2.59	13.21 ± 4.29
18	Tritriacontadiene	-	-	5.86 ± 0.18	5.96 ± 3.67	-	11.52 ± 3.11	6.95 ± 2.13	-	-	-	2.76 ± 3.91
19	11-, 12-, 13-, 14-, 15-, and 16-Tritriacontene[c]	-	-	-	2.19 ± 1.11	-	3.41 ± 1.84	1.98 ± 1.77	-	-	-	1.04 ± 1.47
Dimethylalkanes												
20	9,13-Dimethylpentacosane	2564	-	-	0.98 ± 0.69	-	0.48 ± 0.56	0.22 ± 0.32	-	-	-	0.54 ± 0.15
Acids												
21	Hexadecanoic acid	1969	1984	0.23 ± 0.11	0.31 ± 0.54	-	-	-	-	-	-	-
22	Octadec-9-enoic + octadecadienoic acids[c]	2137	2161	31.06 ± 8.92	6.84 ± 10.52	-	-	-	-	-	-	-
23	Octadecanoic acid	2149	2124	4.64 ± 1.24	1.98 ± 2.68	-	-	-	-	-	-	-
Alcohols												
24	1-Nonanol	1169	1171	-	0.36 ± 0.39	-	1.33 ± 1.28	-	-	-	0.03 ± 0.05	-

Table 1 continued..

No.	Compounds	RI (calc.)	RI (lit.)	Physogastric queen					Virgin queen (12-14 days old)			
				Head (%)[a]	Abdomen (%)[b]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]	Head (%)[a]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]
25	Nerol	1266	1228	-	-	-	-	-	92.45 ± 5.68	-	-	-
Ketones												
26	2-Nonadecanone[d]	2104	2106	1.09 ± 0.70	3.25 ± 0.47	0.24 ± 0.25	3.22 ± 1.81	1.07 ± 0.89	-	-	14.95 ± 9.26	4.57 ± 2.12
Esters												
27	Ethyl hexanoate	-	-	-	-	0.10 ± 0.13	-	-	-	-	-	-
28	Isopropyl hexanoate[d]	1035	1040	-	0.42 ± 0.69	0.25 ± 0.20	0.33 ± 0.36	-	-	-	0.03 ± 0.06	-
29	Butyl hexanoate	1188	1188	-	0.12 ± 0.22	0.64 ± 0.13	0.03 ± 0.05	-	-	-	-	-
30	Hexyl hexanoate[d]	1383	1352	-	1.48 ± 1.78	0.90 ± 0.85	1.16 ± 1.26	-	-	-	0.10 ± 0.14	-
31	Decyl acetate	-	1408	-	-	0.08 ± 0.11	-	-	-	-	-	-
32	Ethyl dodecanoate	-	1595	-	-	0.05 ± 0.07	-	-	-	-	-	-
33	Dodecyl acetate[d]	1603	1609	-	-	14.13 ± 6.86	-	-	-	-	0.13 ± 0.19	-
34	Isopropyl dodecanoate	1623	-	-	-	1.76 ± 1.73	-	-	-	-	-	-
35	Butyl dodecanoate + dodecyl butyrate[c]	1783	-	-	-	6.12 ± 2.16	-	-	-	-	-	-
36	Tetradecyl acetate[d]	1805	1811	-	-	21.63 ± 5.13	-	-	-	-	-	-
37	Isopropyl tetradecanoate	-	1814	-	-	0.11 ± 0.16	-	-	-	-	-	-

Table 1 continued..

No.	Compounds	RI (calc.)	RI (lit.)	Physogastric queen					Virgin queen (12-14 days old)			
				Head (%)[a]	Abdomen (%)[b]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]	Head (%)[a]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]
38	Dodecyl Hexanoate	1978	-	-	-	14.91 ± 4.65	-	-	-	-	-	-
39	Tetradecyl butyrate[d]	1985	-	-	-	21.83 ± 3.98	-	-	-	-	-	-
40	Hexadecyl acetate[d]	2004	2009	-	-	0.95 ± 0.75	-	-	-	-	-	-
41	Tetradecyl hexanoate	2177	-	-	-	5.72 ± 2.69	-	-	-	-	-	-
42	Hexadecyl butyrate[d]	2183	-	-	-	1.03 ± 0.97	-	-	-	-	-	-
43	Hexadecyl hexanoate	2373	-	-	-	0.14 ± 0.15	-	-	-	-	-	-
44	Dodecyl dodecanoate	2565	-	-	-	1.69 ± 1.11	-	-	-	-	-	-
45	Tetradecyl dodecanoate	-	-	-	-	0.85 ± 0.69	-	-	-	-	-	-
46	Tetradecyl tetradecanoate	2764	-	-	-	0.11 ± 0.15	-	-	-	-	-	-
47	Octadecyl octadecenoate	2803	-	-	-	-	-	-	-	67.34 ± 24.73	2.76 ± 2.40	-
Steroids												
48	Ergostadien-3β-ol	3282	-	2.55 ± 0.11	-	-	-	-	-	-	-	-
49	Campesterol	3290	3305	4.37 ± 3.78	-	-	-	-	-	-	-	-
50	β-Sitosterol	3419	3408	5.64 ± 2.24	3.69 ± 2.95	-	-	-	-	-	-	-

Table 1 continued..

No.	Compounds	RI (calc.)	RI (lit.)	Physogastric queen					Virgin queen (12-14 days old)			
				Head (%)[a]	Abdomen (%)[b]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]	Head (%)[a]	Dufour's gland (%)[b]	Tergal gland (%)[b]	Cuticular lipid (%)[a]
51	Fucosterol	3447	-	7.10 ± 1.60	4.87 ± 3.67	-	-	-	-	-	-	-
Unknown compounds		-	-	0.09	0.27	1.50	-	5.79	7.45	13.44	7.86	4.00
Total		-	-	100	100	100	100	100	100	100	100	100

RI (calc.) = calculated retention index, RI (lit.) = retention index from the literature (http://www.pherobase.com/). [a,b]Average (relative abundance in %) ± DP of *T. angustula* queens from (a) two and (b) three colonies. [c]Compounds co-eluted in a single peak. [d]Synthetic standards were used to confirm the structures.

Dufour's gland was attributed to contamination from the cuticle [31]. This gland is poorly developed in the *T. angustula* virgin queens, similar to other stingless bee species [28]. However, the presence of octadecyl octadecenoate as the major component in *T. angustula* Dufour's gland is surprising and contrasts with the chemical analyses of virgin queens from other species, such as *Melipona bicolor* and *Scaptotrigona mexicana*, which contained more components in their Dufour's gland [22, 32]. Nevertheless, the causes of this variation remain unknown. The tergal gland extracts of the *T. angustula* queens were similar to their cuticular lipid extracts (Table 1), except for the presence of alcohol and esters; the hydrocarbons of the tergal glands can be derived from the cuticle. The total extract of the physogastric queen abdomen showed a composition similar to that of its correspondent tergal gland and cuticular extracts, except for the additional presence of acids, steroids, and the more volatile esters (IH, BH, and HH) also found in the Dufour's gland.

The cuticular lipids from *T. angustula* queens were complex mixtures of normal, branched, and unsaturated hydrocarbons containing 21 to 33 carbon atoms that corresponded to 91.43 and 93.14% of the total samples of virgin and physogastric queens, respectively (Table 1). *n*-Alkanes were more abundant in *T. angustula* virgin queens (48.21%) than in physogastric queens (37.58%). Interestingly, the relative amounts of monomethylalkanes were higher in physogastric queens (17.37%) than in virgin queens (3.80%). The monomethylalkanes of *T. angustula* queens consisted of isomeric mixtures (Tables 1 and S1). These mixtures were also detected in *Formica argentea* ants [33], *Cardiocondyla obscurior* ant queens [31], *Schwarziana quadripunctata* stingless bees [34], *Osmia lignaria* and *Megachile rotundata* solitary bees [35] and others. Branched alkanes have been associated with fecundity in ants, e.g., *Diacamma ceylonense* [36] and *Camponotus floridanus* [37]. In *T. angustula*, monounsaturated alkenes also occurred as isomeric mixtures (Tables 1 and S1). The DMDS derivatives of the *n*-alkenes revealed unsaturation in several positions from C-9 to C-16 (Table S2). Hentriacontene (13.21% and 11.89% in virgin and physogastric queens, respectively) eluted as a broad chromatographic peak; a closer analysis of the DMDS derivatives revealed a mixture of positional isomers with double bonds located at carbons 10-, 11-, 12-, 13-, 14- or 15- (Table S2). Isomeric mixtures of alkenes were also observed in other species, such as *Drosophila pseudoobscura* [38], *Osmia lignaria* and *Megachile rotundata* [35]. According to Blomquist *et al*. [38], double bonds may be found at almost any position in insect-derived alkenes. Additionally, examining the relative abundances of the constituents in the cuticular lipidic extracts of virgin and physogastric queens, it was found that 4.57% and 1.07% of the extracts, respectively, was 2-nonadecanone.

The primary function of the cuticular lipids is to protect the insects from desiccation; however, they could also be involved in the insect-recognition systems [39]. The cuticular hydrocarbon profiles in stingless bees can be used to distinguish species, castes, and sexes [24, 34, 40]. Understanding the strong attraction of stingless bee males to physogastric queens and previously observed mating attempts [4-5, 11, 41] is difficult, considering the wide variety of bee-associated components and signals that are present. The chemical cues released by queens appear to be very strong, and males who are stimulated by such pheromones cannot discriminate the reproductive status of these queens when the queens release volatile compounds, such as IH and HH. Therefore, additional research is needed to understand this issue better.

The chemical composition of the cephalic physogastric queen extract was similar to the cuticular extract, with the exception of the additional presence of acids and steroids. In contrast, the cephalic virgin queen extract was mostly composed of nerol (Tables 1 and S1), which was also a major component in the heads of *Trigona fulviventris* workers and was described as an alarm pheromone [42].

CONCLUSION

The attraction of males toward *T. angustula* physogastric queens outside nests suggests the possibility of multiple roles for the queen's pheromones, depending on the context. We found IH and HH in both the Dufour's and tergal glands of physogastric queens, and only in the tergal glands

of virgin queens. However, we were unable to determine whether the hemolymph carries the volatile compounds and stores them in the Dufour's and tergal glands or whether the compounds are independently synthesized. The behavior of the virgin and physogastric queens observed during the release of the volatiles, which included abdomen inflation and wing vibration while exposing their tergal glands to the environment, suggests that these glands are potential candidates as the sources of the IH and HH release which attracts males and is likely to play another undetermined role. Additional studies are required to elucidate the functions of the compounds found in the tergal and Dufour's glands, and cuticular, abdominal and cephalic extracts of virgin and physogastric *T. angustula* queens to determine their biological activities in all social contexts.

ACKNOWLEDGEMENTS

The authors acknowledge the Conselho Nacional do Desenvolvimento Científico e Tecnológico (CNPq) and CAPES-PROAP-2010 *via* the Graduate Program in Zoology of the Departamento de Zoologia, Universidade de São Paulo, for the financial support. This publication was also supported by FAPESP-GSK 2014/50249-8.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

SUPPLEMENTARY INFORMATION

Isopropyl hexanoate

Overall yield: 24.16%. R_f 0.68 (silica, hexane:ethyl acetate 8:2). GC-MS (IE, 70 eV) *m/z* 143 ($M^{+\bullet}$, 6), 117 (43), 99 (75), 87 (14), 71 (22), 60 (44), 55 (12), 43 (100), and 41 (29). IV (neat) ν_{max} 2958, 2933, 2873, 2861, 1733, 1457, 1373, 1245, 1174, 1108, 968, and 823 cm^{-1}. ^1H NMR (499.88 MHz, $CDCl_3$, TMS): δ 0.90 (t, 3H, *J* 7.0 Hz, H-6), 1.23 (d, H-1' and H-2'), 1.31 (m, H-5 and H-4), 1.62 (quint, H-3), 2.25 (t, *J* 7.5 Hz, H-2), 5.00 (sept, H-1'). ^{13}C NMR (125.69 MHz, $CDCl_3$, TMS): δ 13.9 (C-6), 21.9 (C-2' and C-3'), 22.3 (C-5), 24.7 (C-4), 31.3 (C-3), 34.7 (C-2), 67.3 (C-1'), and 173.5 (C-1).

Hexyl hexanoate

Overall yield: 73.34%. R_f 0.73 (silica, hexane:ethyl acetate 8:2). GC-MS (IE, 70 eV) *m/z* 129 ($M^{+\bullet}$, 4), 117 (100), 99 (73), 84 (63), 71 (25), 69 (24), 61 (16), 56 (49), 43 (78), and 41 (37). IV (neat) ν_{max} 2956, 2931, 2860, 1737, 1467, 1245, 1172, 1099, 1008, 997, and 902 cm^{-1}. ^1H NMR (499.88 MHz, $CDCl_3$, TMS): δ 0.89 and 0.90 (2t, 3H, *J* 7.0 Hz, H-6 and H-6'), 1.32 (m, H-3', H-4, H-4', H-5 and H-5'), 1.62 (m, H-3 and H-2'), 2.29 (t, *J* 7.5 Hz, H-2), 4.06 (t, *J* 7.0 Hz, H-1'). ^{13}C NMR (125.69 MHz, $CDCl_3$, TMS): δ 13.9 and 14.0 (C-6 and C-6'), 22.3 (C-5), 22.5 (C-5'), 24.7 (C-4), 25.6 (C-4'), 28.6 (C-2'), 31.4 (C-3), 31.5 (C-3'), 34.4 (C-2), 64.4 (C-1'), and 174.0 (C-1).

Table S1. Diagnostic ions for the compounds found in *T. angustula* queens.

Compounds	**Diagnostic ions (*m/z*)[a]**
***n*-Alkanes**	
Heneicosane	296($M^{+\cdot}$, 5)
Tricosane	324($M^{+\cdot}$, 8)
Pentacosane	352($M^{+\cdot}$, 4)
Heptacosane	380($M^{+\cdot}$, 4)
Nonacosane	408($M^{+\cdot}$, 2)
Methylalkanes	
9-methyltricosane +11-methyltricosane	225(11)/224(14), 141(14)/140(32) 197(5)/196(9), 169(5)/168(9)

Table S1 continued..

Compounds	**Diagnostic ions (*m/z*)[a]**
9-methylpentacosane +11-methylpentacosane +13-methylpentacosane	253(7)/252(7), 141(11)/140(21) 225(7)/224(10), 169(8)/168(16) 197(5)/196(8)
9-methylheptacosane +11-methylheptacosane +13-methylheptacosane	281(12)/280(11), 141(14)/140(33) 253(6)/252(8), 169(6)/168(10) 225(2)/224(3), 197(3)/196(2)
9-methylnonacosane +11-methylnonacosane +13-methylnonacosane +15-methylnonacosane	309(4)/308(4), 141(9)/140(15) 281(8)/280(6), 169(8)/168(14) 253(5)/252(6), 197(6)/196(9) 225(6)/224(9)
11-methylhentriacontane +13-methylhentriacontane +15-methylhentriacontane	309(1)/308(1), 169(5)/168(5) 281(6)/280(6), 197(7)/196(13) 253(12)/252(18), 225(12)/224(23)
***n*-Alkenes**	
Heneicosene	294($M^{+\cdot}$, 9)
Tricosene	322($M^{+\cdot}$, 11)
9-, 10-, 11- and 12-Pentacosene	350($M^{+\cdot}$, 21)
9-, 10-, 11-, 12- and 13-Heptacosene	378($M^{+\cdot}$, 15)
10-, 11-, 12-, 13- and 14-Nonacosene	406($M^{+\cdot}$, 15)
Hentriacontadiene	432($M^{+\cdot}$, 25)
10-, 11-, 12-, 13-, 14- and 15-Hentriacontene	434($M^{+\cdot}$, 12)
Tritriacontadiene	460($M^{+\cdot}$, 26)
11-, 12-, 13-, 14-, 15-, and 16-Tritriacontene	462($M^{+\cdot}$, 12)
Dimethylalkanes	
9,13-dimethylpentacosane	267(12)/268(3), 211(14)/210(5), 197(7)/196(11), 141(11)/140(19)
Acids	
Hexadecanoic acid	256($M^{+\cdot}$, 65), 73(100)
Octadec-9-enoic acid + octadecadienoic acid	282($M^{+\cdot}$,7), 264(34), 69(100) 280($M^{+\cdot}$, 17), 264(17), 55(100)
Octadecanoic acid	284($M^{+\cdot}$, 64), 73(100)
Alcohols	
1-Nonanol	98(58), 70(100)
Nerol	154($M^{+\cdot}$, 1), 136(5), 121(12), 69(100)
Ketone	
2-Nonadecanone	282($M^{+\cdot}$,14), 58(100)

Table S1 continued..

Compounds	**Diagnostic ions (*m/z*)[a]**
Esters	
Ethyl hexanoate	117(8), 88(100)
Isopropyl hexanoate	143(6), 117(43), 43(100)
Butyl hexanoate	129(3), 117(100), 99(99)
Hexyl hexanoate	129(4), 117(100)
Decyl acetate	140(6), 43(100)
Ethyl dodecanoate	228($M^{+\cdot}$, 5), 101(49), 88(100)
Dodecyl acetate	228($M^{+\cdot}$, 0.1), 43(100)
Isopropyl dodecanoate	242($M^{+\cdot}$, 2), 200(96), 43(100)
Butyl dodecanoate + dodecyl butyrate	256($M^{+\cdot}$, 4), 213(3), 201(86), 183(52), 168(2), 56(100) 213(4), 183(1), 168(13), 89(100)
Tetradecyl acetate	256($M^{+\cdot}$, 0.1), 43(100)
Isopropyl tetradecanoate	229(48), 43(100)
Dodecyl Hexanoate	284($M^{+\cdot}$, 2), 117(100)
Tetradecyl butyrate	284($M^{+\cdot}$, 0.1), 89(100)
Hexadecyl acetate	224(7), 196(8), 43(100)
Tetradecyl hexanoate	312($M^{+\cdot}$, 0.1), 117(100)
Hexadecyl butyrate	269(6), 224(8), 89(100)
Hexadecyl hexanoate	224(10), 117(100)
Dodecyl dodecanoate	368($M^{+\cdot}$, 7), 201(100)
Tetradecyl dodecanoate	396($M^{+\cdot}$, 12), 201(100)
Tetradecyl tetradecanoate	424 ($M^{+\cdot}$, 26), 257(68), 229(100)
Octadecyl octadecenoate	534 ($M^{+\cdot}$, 5), 283(11), 264(61), 57(100)
Steroids	
Ergostadien-3β-ol	398($M^{+\cdot}$, 8), 314(100)
Campesterol	400($M^{+\cdot}$, 100)
β-Sitosterol	414($M^{+\cdot}$, 100)
Fucosterol	412($M^{+\cdot}$, 7), 314(100)

[a]*m/z* (abundance of the peak).

Table S2. Mass spectral identifications of double bond positions in alkenes from the *T. angustula* queens.

Compounds	**Double bond position**	**Diagnostic ions (*m/z*) of the DMDS derivatives**
Pentacosene *m/z* 444 ($M^{+\cdot}$, 13)	9 10 11 12	271(7), 173(10) 257(53), 187(51) 243(45), 201(49) 229(12), 215(11)

Table S2 continued..

Compounds	Double bond position	Diagnostic ions (*m/z*) of the DMDS derivatives
Heptacosene *m/z* 472 ($M^{+\cdot}$, 8)	9	299(12), 173(12)
	10	285(39), 187(43)
	11	271(27), 201(27)
	12	257(8), 215(11)
	13	243(14), 229(10)
Nonacosene *m/z* 500 ($M^{+\cdot}$, 16)	10	313(100), 187(95)
	11	299(17), 201(21)
	12	285(12), 215(15)
	13	271(15), 229(16)
	14	257(24), 243(24)
Hentriacontene *m/z* 528 ($M^{+\cdot}$, 27)	10	341(14), 187(14)
	11	327(18), 201(16)
	12	313(56), 215(60)
	13	299(48), 229(43)
	14	285(100), 243(99)
	15	271(92), 257(88)
Tritriacontene *m/z* 556 ($M^{+\cdot}$, 21)	11	355(2), 201(9)
	12	341(11), 215(15)
	13	327(17), 229(29)
	14	313(74), 243(80)
	15	299(71), 257(80)
	16	285(100), 271(95)
Octadecenoic acid *m/z* 376 ($M^{+\cdot}$, 27)	9	203(75), 173(100)

REFERENCES

1. Peters, J. M., Queller, D. C., Imperatriz-Fonseca, V. L., Roubik, D. W. and Strassmann, J. E. 1999, Proc. R. Soc. B Biol. Sci., 266, 379.
2. Jaffé, R., Pioker-Hara, F. C., Santos, C. F., Santiago, L. R., Alves, D. A., Kleinert, A. M. P., Francoy, T. M., Arias, M. C. and Imperatriz-Fonseca, V. L. 2014, Naturwissenschaften, 101, 261.
3. Kerr, W. E., Zucchi, R., Nakadaira, J. T. and Butolo, J. E. 1962, J. New York Entomol. Soc., 70, 265.
4. Cortopassi-Laurino, M. 1979, Rev. Bras. Entomol., 23, 177.
5. Sakagami, S. F. and Laroca, S. 1963, J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool., 15, 319.
6. Santos, C. F., Menezes, C., Vollet-Neto, A. and Imperatriz-Fonseca, V. L. 2014, Sociobiology, 61, 115.
7. Engels, W. and Imperatriz-Fonseca, V. L. 1990, In Social Insects. An evolutionary approach to castes and reproduction, W. Engels (Ed.), Springer Berlin Heidelberg: Berlin, pp. 167-230.
8. Gary, N. E. 1962, Science, 136, 773.
9. Krieger, G. M., Duchateau, M.-J., van Doorn, A., Ibarra, F., Francke, W. and Ayasse, M. 2006, J. Chem. Ecol., 32, 453.
10. Verdugo-Dardon, M., Cruz-Lopez, L., Malo, E. A., Rojas, J. C. and Guzman-Diaz, M. 2011, Apidologie, 42, 543.
11. Fierro, M. M., Cruz-López, L., Sánchez, D., Villanueva-Gutiérrez, R. and Vandame, R. 2011, J. Chem. Ecol., 37, 1255.
12. Ayasse, M., Paxton, R. J. and Tengö, J. 2001, Annu. Rev. Entomol., 46, 31.
13. Provost, E., Blight, O., Tirard, A. and Renucci, M. 2008, In Insect Physiology: New Research, R. P. Maes (Ed.), Nova Science Publishers, Inc., pp. 19-72.

14. Santos, C. F., Santos, P. D. S. and Blochtein, B. 2015, Apidologie, 47, 539.
15. Imperatriz-Fonseca, V. L. and Zucchi, R. 1995, Apidologie, 26, 231.
16. Pianaro, A. 2012, Chemical Ecology of *Melipona quadrifasciata* Lepeletier, *Scaptotrigona* aff. *depilis* Moure and *Solenopsis saevissiva* Smith, State University of Campinas.
17. van den Dool, H. and Kratz, P. D. 1963, J. Chromatogr. A, 11, 463.
18. Adams, R. O. 2001, Identification of essential oil components by gas chromatography/ quadrupole mass spectrometry. Allured, Illinois.
19. Neises, B. and Steglich, W. 1978, Angew. Chemie Int., 17, 522.
20. Raffa, K. F. and Steffeck, R. J. 1988, J. Chem. Ecol., 14, 1385.
21. Abdalla, F. C., Jones, G. R., Morgan, E. D. and Cruz-Landim, C. 2004, J. Braz. Chem. Soc., 15, 621.
22. Patricio, E. F. L. R. A., López, L. C., Maile, R. and Morgan, E. D. 2003, Apidologie, 34, 359.
23. Francke, W., Lübke, G., Schroeder, W., Reckziegel, A., Imperatriz–Fonseca, V. L., Kleinert, A. M. P., Engels, E., Hartfelder, K., Radtke, R. and Engels, W. 2000, J. Braz. Chem. Soc., 11, 562.
24. Pianaro, A., Menezes, C., Kerr, W. E., Singer, R. B., Patricio, E. F. L. R. A. and Marsaioli, A. J. 2009, J. Chem. Ecol., 35, 1117.
25. Buser, H.-R., Arn, H., Guerin, P. and Rauscher, S. 1983, Anal. Chem., 55, 818.
26. Abdalla, F. C., Cruz-Landim, C. and Gracioli, L. F. 2005, Neotrop. Entomol., 34, 41.
27. Cruz-Landim, C., Abdalla, F. C. and Gracioli, L. F. 2006, Apidologie, 37, 164.
28. Cruz-Landim, C., Abdalla, F. C. and Gracioli-Vitti, L. F. 2005, Insect Sci., 12, 467.
29. Abdalla, F. C. 2006, Rev. Bras. Entomol., 50, 153.
30. Abdalla, F. C., Velthuis, H., Duchateau, M. J. and Cruz-Landim, C. 1999, Netherlands J. Zool., 49, 139.
31. Will, S., Delabie, J. H. C., Heinze, J., Ruther, J. and Oettler, J. 2012, J. Insect Physiol., 58, 1245.
32. Grajales-Conesa, J., Rojas, J. C. and Guzman-Diaz, M. 2007, Apidologie, 38, 38.
33. Krasnec, M. O. and Breed, M. D. 2013, J. Chem. Ecol., 39, 59.
34. Nunes, T. M., Turatti, I. C. C., Mateus, S., Nascimento, F. S., Lopes, N. P. and Zucchi, R. 2009, Genet. Mol. Res., 8, 589.
35. Buckner, J. S., Pitts-Singer, T. L., Guédot, C., Hagen, M. M., Fatland, C. L. and Kemp, W. P. 2009, Comp. Biochem. Physiol., 153, 200.
36. Cuvillier-Hot, V., Cobb, M., Malosse, C. and Peeters, C. 2001, J. Insect Physiol., 47, 485.
37. Endler, A., Liebig, J., Schmitt, T., Parker, J. E., Jones, G. R., Schreier, P. and Hölldobler, B. 2004, Proc. Natl. Acad. Sci. USA, 101, 2945.
38. Blomquist, G. J., Toolson, E. C. and Nelson, D. R. 1985, Insect Biochem., 15, 25.
39. Singer, T. L. 1998, Am. Zool., 38, 394.
40. Nunes, T. M., Nascimento, F. S., Turatti, I. C., Lopes, N. P. and Zucchi, R. 2008, Anim. Behav., 75, 1165.
41. Campos, L. A. O. and Melo, G. A. R. 1990, Brazilian J. Genet., 13, 491.
42. Johnson, L. K. and Wiemer, D. F. 1982, J. Chem. Ecol., 8, 1167.