

Cephalic and Dufour gland secretions of *Scaptotrigona mexicana* queens: Chemical composition and biological activity*

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Abstract – Cephalic and Dufour gland secretions of virgin and physogastric queens of *Scaptotrigona mexicana* were analyzed by GC-MS. Worker responses to extracts and selected compounds identified in these secretions were evaluated in experimental hives. Cephalic secretions of virgin queens and 1-month-old physogastric queens are constituted of 2-ketones, 2-alcohols and esters, while the cephalic secretions of 1-year-old physogastric queens are constituted of 2-alcohols and esters. Dufour gland secretions from virgin queens are mainly constituted of 2-alcohols, ketones, hydrocarbons and esters, while Dufour gland secretions from physogastric queens are mainly constituted of esters, where hexyl hexanoate is the major compound. Workers were significantly attracted to natural extracts and synthetic blends of selected compounds of the 1-year-old physogastric queen cephalic secretion as well as to a blend of enantiomeric 2-alcohols. Dufour gland secretion was not attractive to workers.

Scaptotrigona mexicana / queens / cephalic secretions / Dufour gland / stingless bees / Apidae

1. INTRODUCTION

The stingless bees species of the Neotropical areas show an astonishingly great diversity and many aspects of their biology, ecology and behavior are so far unknown. Stingless bees, like all other eusocial bees, use different ways of communication inside and outside the nest, but so far most studies have focused on visual, auditory and chemical communication (Michener, 1974; Nieh, 1999). One of the most advanced eusocial bees is the western honeybee, *Apis mellifera*. In this species, queen substances are involved in the inhibition of queen rearing and swarming, the prevention of worker ovary development and queen

retinue behavior (Winston, 1993). In contrast, little is known about the chemical composition and role of queen substances in stingless bees (Engels et al., 1987, 1988, 1990, 1993, 1997; Abdalla et al., 2004; Gracioli-Vitti et al., 2004). For instance, there is no evidence so far that queen pheromones guide or induce queen retinue (Engels et al., 1990) or inhibit the ovary development of workers. It is clear that physogastric queens use chemical communication to organize work inside the colony, caste differentiation and nest homeostasis (Nogueira-Neto, 1997). Also virgin queens use a sexual pheromone to attract drones (Engels et al., 1990). Behavioral observations in *Scaptotrigona mexicana*, a species widely distributed in Mexico showed that workers of queenless colonies are attracted to physogastric queens, whereas the workers in queenright colonies are not attracted

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to physogastric queens (Grajales et al., 2005). This previous study, however, did not identify the chemical composition or glandular origin of the pheromones involved in queen-worker interaction in *S. mexicana*, and many questions concerning this topic remain to be investigated. In this study we chemically identified the cephalic and Dufour gland secretions in virgin and physogastric queens of *S. mexicana* and evaluated the behavioral responses of worker to these substances.

2. MATERIALS AND METHODS

2.1. Bees

One-year-old ($n = 12$) and 1-month-old physogastric queens ($n = 10$), as well as virgin queens of different ages ($n = 15$) of *S. mexicana* were collected from different hives of the meliponary of El Colegio de la Frontera Sur (ECOSUR) located in Cacahoatan ($14^{\circ}59'N$, $92^{\circ}10'W$, altitude 480 m), Chiapas from January to February, 2005. Immediately after collection, queens were transferred to 2 mL glass vials and placed at $-20^{\circ}C$ for 10 min to immobilize them and avoid the emptying of their glands at the moment of dissection.

2.2. Sample preparation

The heads of the queens were carefully removed and the Dufour glands were dissected in distilled water with two pairs of fine forceps under a stereomicroscope. Individually and separately, the heads and the Dufour glands were crushed in 2 mL of hexane, and then concentrated with nitrogen to 100 μ L. The secretion samples were stored at $-20^{\circ}C$ for further analysis and bioassays.

2.3. Chemical analysis

Gas chromatography-mass spectrometry of cephalic and Dufour gland secretions was carried out with a Varian CP-3800 (Palo Alto, CA) chromatograph linked to a Varian Saturn 2200 mass spectrometer (Palo Alto, CA). The samples were analyzed using a non polar capillary column (DB-5MS, 30 m \times 0.25 mm i.d. 0.25 μ m, J&W Scientific). The oven was programmed from $50^{\circ}C$ to $280^{\circ}C$ at $15^{\circ}C/min$. The carrier gas was helium at 1 mL/min. Chiral separations were carried out with a cyclodextrin-based capillary column (Cyclosil-B,

30 m, 0.25 mm i.d., 0.25 μ m, J&W Scientific) using helium as carrier gas (275.8 KPa) at $65^{\circ}C$. Identification of the natural compounds was done by comparison of retention times and mass spectral data with those of synthetic compounds. Tentative identification of unknown compounds was carried out using the NIST 2002 spectral library or mass spectral data reported in literature. The relative percentage of the components was calculated from the sum of the recorded peaks. Quantification of the glandular secretion was performed by using tridecane (1 μ g/sample) as an internal standard. Synthetic compounds were obtained from Fluka and Sigma-Aldrich (Toluca, Mexico). The purity of these compounds was $>95\%$ by GC. Hexyl hexanoate and hexyl butanoate were prepared according to Attygalle and Morgan (1986).

2.4. Behavioral tests

The bioassays were carried out in the meliponary of ECOSUR in a special room designed to exclude all possible external noises and odors. The tests were performed in 15 hives (30 \times 20 cm) based on the model of Engels et al. (1987), and were subdivided into a brood nest and a storage part, connected only by two narrow tunnels to avoid rapid odor exchange. The hives were prepared in November 2004 to obtain strong colonies in March 2005 (hives with an at least four months old physogastric queen). The nest boxes were covered with a plexiglass plate for the experiments. Three days before the tests, the queens were removed from the hives, because in preliminary observations workers of this condition showed a higher response to queens (Grajales et al., 2005). The reserves were removed from the storage area to clear the arena. Behavioral tests were performed from March to August, 2005. Two different experiments were carried out at different times; (a) in the first one, the extracts of one queen equivalent of cephalic and Dufour gland secretions of physogastric and virgin queens were evaluated; and (b) in the second one, the effect of natural secretions was compared with synthetic blends of compounds identified from cephalic secretions. The synthetic blends used in the experiments were prepared according to the natural proportions of each component from the extract using hexane as a solvent; (a) a blend representing one head equivalent of selected components identified in 1-year-old physogastric queens (5 μ L of 29% 2-heptanol, 9% decane, 2% limonene, 55% 2-nonanol, 2% hexyl hexanoate

Table I. Mean (\pm SE) composition (%) of the cephalic volatiles of *S. mexicana* queens; virgin (VQ), 1-month old physogastric (PQ1m) and 1-year old physogastric (PQ1y).

Peak	Compound	VQ (n = 11)	PQ1m (n = 6)	PQ1y (n = 10)
1	2-Heptanone	0.8 \pm 0.19	(t)	(-)
2	2-Heptanol	15 \pm 3.18	17 \pm 4.48	29 \pm 4.87
3	Decane	2 \pm 1.04	(-)	9 \pm 2.03
4	Limonene	(-)	(-)	(t)
5	Nonen-2-ol	(t)	(-)	(-)
6	2-Nonanone	0.5 \pm 0.13	2 \pm 1.15	(-)
7	Isomer of peak 5	(t)	(-)	(-)
8	2-Nonanol	62 \pm 4.35	64 \pm 2.23	55 \pm 4.88
9	Hexyl butanoate	(t)	1 \pm 0.31	(t)
10	Isomer of peak 11	0.5 \pm 0.11	1 \pm 0.22	2 \pm 0.49
11	Undecen-2-ol	17 \pm 2.38	6 \pm 1.59	(-)
12	2-Undecanol	2 \pm 0.41	8 \pm 5.36	5 \pm 3.03
13	Hexyl hexanoate	(-)	(t)	0.5 \pm 0.11

(t) = traces, (-) = chemical compound not found.

and 5% 2-undecanol) and (b) a blend of chiral (R)-(-)-2-alcohols (5 μ L of 29% (R)-(-)-2-heptanol, 55% (R)-(-)-2-nonanol and 5% 2-undecanol), and (c) chiral (S)-(+)-2-alcohols (5 μ L of 29% (S)-(+)-2-heptanol, 55% (S)-(+)-2-nonanol and 5% 2-undecanol). In all experiments hexane was used as a control. The extracts or the blends were applied onto cotton dummies made of sterile plain wrapped cotton rolls (4 cm \times 1 cm, Richmond, USA). After evaporation of the solvent for 30 s the cotton dummies were placed in small wire cages (5 cm \times 1 cm) inside the hives storage areas. The activity of workers was video-taped during five minutes with a handycam (Sony DCR-DVD101/DVD201). Workers attraction was measured by counting the number of bees in direct contact with the cage in each minute, during the five minutes taped.

2.5. Statistical analysis

Two way-ANOVA of repeated measures and Tukey's test ($\alpha = 0.05$) were used to compare the behavioral responses of workers to different extracts and synthetic blends. We used a transformation of the data set (\sqrt{x}) because our data did not meet with assumption of normality and homoscedasticity. A correspondence analysis was performed for the compounds identified in queen cephalic secretions in order to analyze the association between the groups of queens and the compounds. All statistical tests were performed with Statistica version 7.1 (Stat soft, Inc 2005) and SAS (The SAS system for windows, release 8.02) software.

3. RESULTS

3.1. Chemical analysis

3.1.1. Cephalic secretions of queens

The chemical composition of cephalic secretion of *S. mexicana* queens of different physiological condition is listed in Table I. All secretions contain the same 2-alcohols composition with 2-nonanol as the major component. In addition, there are several differences: both ages of physogastric queens are characterized by hexyl hexanoate, whereas virgin queen secretion is distinguished by nonen-2-ol, which was tentatively identified according to the mass spectrum shown by Kozlov et al. (1996), and isomer of nonen-2-ol (peak 7) whose mass spectrum resembled an unsaturated alcohol (Tab. I). Comparison between the cephalic gland secretion composition of 1-month-old and 1-year-old physogastric queens shows that both types of queens differ in the number of volatile compounds identified, with 10 and 8 volatile compounds, respectively (Tab. I). It was noteworthy that limonene was present only in 1-year-old physogastric queen secretions while some other compounds such as 2-heptanone, 2-nonanone and undecen-2-ol (the latter was identified according to the mass spectrum provided by Baeckström et al. (1989)) were found in virgin and 1-month-old

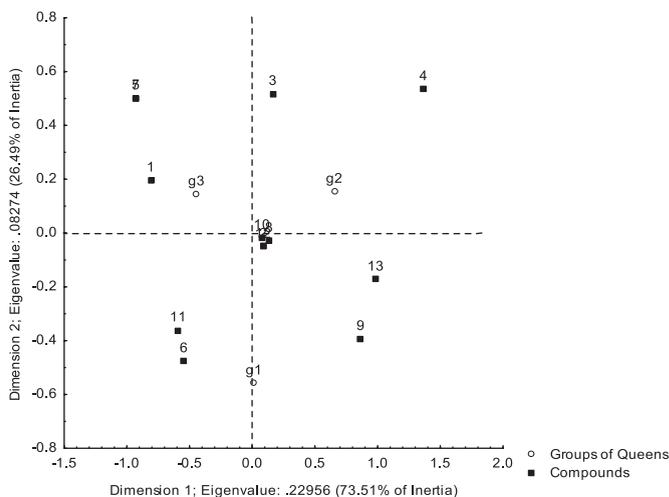


Figure 1. Correspondence analysis of compounds identified in 1-year-old queen cephalic secretions. (g1) 1-month-old physogastric queens, (g2) 1-year-old physogastric queens and (g3) virgin queens. The numbers correspond to the compounds identified in queen cephalic secretions (see Tab. I).

physogastric queens. In order to analyze and identify an association between the groups of queens and the compounds identified, we performed a correspondence analysis, where the first axis explained 73.5% of variability within the results. We found an association between the groups of queens and compounds identified in cephalic secretions that was significantly correlated ($X^2 = 58.088$, $P = 0.0001$). Figure 1 shows that the groups of queens are arranged by age and physiological status; first virgin queens (g3), then 1-month-old physogastric queens (g2) and finally 1-year-old physogastric queens. The enantiomer configuration for 2-heptanol and 2-nonanol was determined as (S)-(+ for both compounds.

3.1.2. Dufour gland secretion

Virgin and physogastric queens differed in their chemical composition of the Dufour gland secretion. The GC-MS analysis showed that the virgin queen Dufour gland secretion contained 11 volatile compounds consisting of 2-alcohols, ketones, hydrocarbons and esters (Tab. II). The secretions of 1-year-old and 1-month-old physogastric queens contain only three esters, hexyl butanoate, hexyl hexanoate and hexyl octanoate; the former with both es-

ters found in traces and the latter with a large amount of hexyl hexanoate.

3.2. Worker behavioral response to different extracts

3.2.1. Cephalic and Dufour gland secretions

The number of workers attracted to the cage was significantly different depending on the extracts tested ($F = 1.71$; D.F. = 20,120; $P = 0.04$) (Fig. 2). Worker response also varied significantly over time ($F = 2.54$; D.F. = 5,30; $P = 0.04$), and diminished in the last two minutes of bioassays. The workers showed the highest responses to extracts of physogastric queens of both ages, and the lowest to hexane, Dufour gland secretions, and virgin queen cephalic extract (Fig. 2).

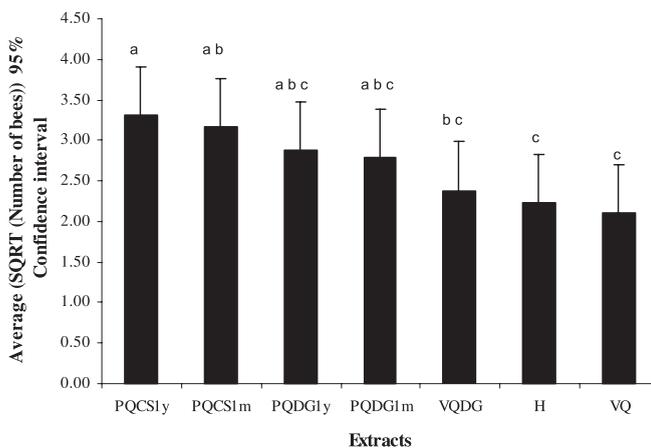
3.2.2. Cephalic secretions vs. synthetic blends

There were significant differences in worker responses to the synthetic blends, cephalic extracts, and hexane ($F = 2.03$; D.F. = 16,75; $P = 0.02$). Workers were more attracted to queen cephalic secretions (QCS),

Table II. Mean (\pm SE) composition (%) of the Dufour gland volatiles of *S. mexicana* queens; virgin queens, 1-month old physogastric queens (PQ1m) and 1-year old physogastric queens (PQ1y).

Peak	Compound	VQ (n = 8)	PQ1m (n = 9)	PQ1y (n = 12)
1	2-Heptanone	2 \pm 6.95	(-)	(-)
2	2-Heptanol	5 \pm 2.63	(-)	(-)
3	Decane	8 \pm 5.79	(-)	(-)
4	2-Nonanone	16 \pm 12.02	(-)	(-)
5	2-Nonanol	17 \pm 11.64	(-)	(-)
6	Hexyl butanoate	(-)	(t)	(t)
7	Undecadien-2-ol	7 \pm 2.74	(-)	(-)
8	Undecen-2-ol	3 \pm 1.30	(-)	(-)
9	2-Undecanol	0.2 \pm 0.12	(-)	(-)
10	Hexyl hexanoate	38 \pm 10.58	(t)	78 \pm 7.26
11	Hexyl octanoate	4 \pm 1.77	(-)	22 \pm 2.30
12	Unknown ester	1 \pm 0.85	(-)	(-)

(t) = traces, (-) = chemical compound not found.

**Figure 2.** Mean number of bees attracted to cephalic secretion extracts from virgin queen (VQ), virgin queen Dufour gland (VQDG), cephalic secretion from one year old physogastric queen (PQCS1y), Dufour gland from 1-year old physogastric queen (PQDG1y), cephalic secretion from 1-month old physogastric queen (PQCS1m), Dufour gland secretion from one month old physogastric queen (PQDG1m) and hexane (H). Bars marked by different letters are significantly different ($P < 0.05$; Tukey test).

synthetic blends (SB), (R)-(-)-2-alcohols, (S)-(+)-2-alcohols than to the hexane control during the three minutes of the behavioral test, but the workers did not show any difference in their responses during the last two minutes of the bioassay (Fig. 3).

4. DISCUSSION

The chemical composition of cephalic secretions of *S. mexicana* varies with the age

and the physiological status of the queens. Nonen-2-ol and the isomer of nonen-2-ol were detected only in virgin queens, while 2-heptanone and nonen-2-ol were found in virgin queens and 1-month-old physogastric queens. The 2-ketones and 2-alcohols composition of virgin queen cephalic secretion resembles that of workers of *S. mexicana* although in workers 2-ketones are the main volatile components rather than 2-alcohols (Luby et al., 1973; Barrera-Gordillo, 2005). Hexyl hexanoate was identified in 1-year and

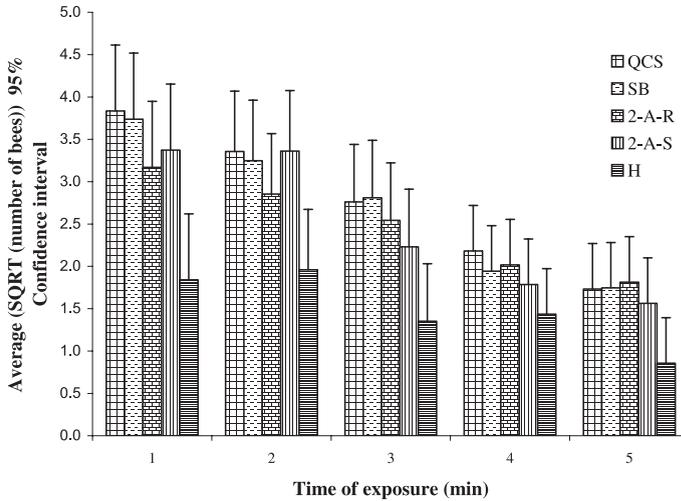


Figure 3. Mean number of bees attracted to extracts and synthetic blends; queen cephalic secretion (QCS), synthetic blend of physogastric queen cephalic secretion (SB), (R)-(-)-2-alcohols (2-A-R), (S)-(+)-2-alcohols (2-A-S) and hexane (H).

1-month-old physogastric queens as one of the compounds which could confer the physiological status of queens. Similarly, Engels et al. (1993) showed that the 2-alcohols composition of cephalic secretions of *S. postica* queens varied with age. In addition, the correspondence analysis helped to identify which of the volatile compounds may indicate the status of queens. Some compounds identified in virgin queens did not appear in 1-year-old physogastric queens, but 1-year-old and 1-month-old physogastric queens are similar in their pheromone compounds because of the esters they share. The chiral configuration of the 2-alcohols of *S. mexicana* was congruent with the cephalic secretions of *S. postica* queens (Engels et al., 1990). It is interesting to note that the pattern of volatile compounds identified in queen cephalic secretions qualitatively resembles that of *S. postica* (Engels et al., 1990). Therefore, this could be a pattern in a genus-specific of stingless bees.

The chemical composition of Dufour glands of *S. mexicana* was different for virgin and physogastric queens. The secretions of virgin queens were mainly constituted of 2-alcohols, 2-ketones, hydrocarbons and esters, while the Dufour gland of physogastric queens contained esters as major compounds.

It is evident that some of the compounds found in virgin Dufour gland secretions were also present in virgin queen cephalic secretions, but these were not present in the Dufour glands of physogastric queens. Thus, these compounds could be related to the physiological status of queens in the colony. Hexyl hexanoate was the major compound found in 1-year-old physogastric queens and this was also identified as a major compound of cephalic secretions of *S. postica* physogastric queens (Engels et al., 1990). Esters seem to be the common components of the Dufour gland secretions of Apidae (Abdalla et al., 2004; Dor et al., 2005), and they may function as a pheromone to attract workers for provisioning processes.

So far, few studies have investigated the chemical composition and biological activity of Dufour glands in queens of stingless bees. For instance, Abdalla et al. (2004) reported the chemical composition of the Dufour gland secretion in queens of *Melipona bicolor* but the behavioral activity of its components was not evaluated. Studies in honey bees (*Apis mellifera*) showed that esters are the main components identified in queen Dufour gland secretions, and they may function as an egg discriminator pheromone or as a reliable fertility signal among honeybee

workers (Katzav-Gozensky et al., 2001). Our behavioural tests showed that workers of *S. mexicana* were attracted to 1-year-old physogastric queen cephalic secretions but not to virgin queen cephalic secretions. Furthermore, workers were attracted to the synthetic blend equivalent to one head and to a blend of (R)-(-)-2-alcohols and (S)-(+)-2-alcohols, during the five minutes of the bioassay. These results are similar to those found in honey bee queens (Kaminski et al., 1990; Pankiw et al., 1994; Plettner et al., 1997), where workers are more attracted to cephalic and mandibular secretions. These authors suggested that queen mandibular gland secretion is related to the control of several aspects of colony functioning in honey bees. To our knowledge there is no information on the function of the mandibular gland of stingless bee physogastric queens. Engels et al. (1990) reported that the mandibular gland secretion of *S. postica* virgin queens attracted males, but no information was provided on the role of the mandibular gland secretion of physogastric queen secretions inside the nest. In relation of the worker response of *S. mexicana* to (R)-(-) and (S)-(+) enantiomers of the 2-alcohols these results provide evidence that the ability of stingless bees to detect some compounds is limited. However the attraction may have different functions, workers of *S. postica* showed different behavioral responses to the (R)-(-) and (S)-(+) isomers, such as attraction and alarm (Engels, 1987). In some studies performed in *A. mellifera carnica* (Laska and Galizia, 2001) bees were able to discriminate between the optical isomers of several terpenes, but failed to distinguish between the (+) and (-) forms of α -terpineol, camphor, rose oxide, fenchone, and 2-butanol; therefore these and our results in stingless bees support the assumption that enantioselective molecular odor receptors may exist for some but not all volatile enantiomers.

In conclusion, our results suggest that the cephalic gland secretions of *S. mexicana* may function as a queen-signal pheromone, but further studies are needed to elucidate the complete chemical identification of the component secretion and its role on the behavior of the workers inside the nest.

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Les sécrétions des glandes céphaliques et de Dufour chez les reines de *Scaptotrigona mexicana* : composition chimique et activité biologique.

Scaptotrigona mexicana / abeille sans aiguillon / reine / sécrétion céphalique / gland de Dufour

Zusammenfassung – Sekrete der Kopf- und Dufourdrüsen von *Scaptotrigona mexicana* Königinnen: Chemische Zusammensetzung und biologische Aktivität. Frühere Untersuchungen an *Scaptotrigona mexicana* zeigten, dass Arbeiterinnen aus weisellosen, aber nicht die aus weiselrichtigen Völkern, von physogastrischen Königinnen angezogen wurden. In der vorliegenden Studie identifizierten wir mittels GC-MS die chemische Zusammensetzung der Kopf- und Dufourdrüsensekrete von unverpaarten Jungköniginnen und von physogastrischen *S. mexicana* Königinnen und bewerteten in Versuchsvölkern die Verhaltensantworten von Arbeiterinnen auf diese Substanzen. In zwei unterschiedlichen Experimenten untersuchten wir: (a) die Wirkung der Extrakte eines Königinnenäquivalents der Kopf- und Dufourdrüsensekrete von physogastrischen und von Jungköniginnen und (b) den Effekt der natürlichen Sekrete physogastrischer Königinnen und den von synthetischen Mischungen der Sekretkomponenten. Die Extrakte oder Mischungen wurden auf Watteattrappen appliziert und die Aktivität der Arbeiterinnen wurde über einen Zeitraum von fünf Minuten auf Video aufgezeichnet.

Die chemische Zusammensetzung der Kopfdrüsensekrete von *S. mexicana* variiert in Abhängigkeit vom Alter und physiologischen Status der Königinnen (Tab. I). Nonen-2-ol und das Isomer von Nonen-2-ol wurden nur bei unverpaarten Jungköniginnen gefunden, während 2-Heptanon und Nonen-2-ol sowohl in Jungköniginnen als auch in einem Monat alten physogastrischen Königinnen vorkam. Hexylhexanoat wurde in einem Monat und in ein Jahr alten physogastrischen Königinnen gefunden und stellen Komponenten dar, die den physiologischen Status der Königinnen widerspiegeln. Einige Komponenten, die in Jungköniginnen auftraten, waren in ein Jahr alten physogastrischen Königinnen nicht zu finden, wohingegen

einen Monat und ein Jahr alte physogastrische Königinnen sich chemisch ähnlich waren, vor allem in Hinblick auf die Esterkomponenten. Die Enantiomerkonfiguration für 2-Heptanol und 2-Nonanol wurde in beiden Fällen als (S)-(+)- bestimmt. Hinsichtlich der Dufourdrüsensekrete waren *S. mexicana* Jungköniginnen klar unterschiedlich von physogastrischen Königinnen (Tab. II). Die Sekrete der unverpaarten Jungköniginnen enthielten vor allem 2-Alkohole, 2-Ketone, Kohlenwasserstoffe und Ester, während die Dufourdrüsen von physogastrischen Königinnen hauptsächlich Ester aufwiesen. Einige der Komponenten der Dufourdrüsensekrete von Jungköniginnen waren auch in ihren Kopfdrüsensekreten zu finden, aber nicht in den Dufourdrüsen von physogastrischen Königinnen, was darauf hinweist, dass diese Komponenten in Zusammenhang mit dem physiologischen Status einer Königin in der Kolonie stehen könnten. Hexylhexanoat war die Hauptkomponente in ein Jahr alten physogastrischen Königinnen.

Verhaltenstests zeigten dass *S. mexicana* Arbeiterinnen die Kopfdrüsensekrete ein Jahr alter physogastrischer Königinnen attraktiv fanden, aber nicht die von unverpaarten Jungköniginnen und ebenso wenig Dufourdrüsensekrete (Abb. 2). Des weiteren fanden Arbeiterinnen die synthetischen Mischungen eines Kopfüquivalents und eine Mischung aus (R)-(-)-2-Alkoholen and (S)-(+)-2-Alkoholen attraktiv (Abb. 3).

Unsere Ergebnisse weisen darauf hin, dass die Kopfdrüsensekrete von *S. mexicana* ein Königinnenpheromon mit Signalfunktion darstellen können.

***Scaptotrigona mexicana* / Königinnen / Kopfsekrete / Dufourdrüse / Stachellose Bienen / Apidae**

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