Chemoecology (2011) 21:75–82
DOI 10.1007/s00049-011-0070-x

CHEMOECOLOGY

RESEARCH PAPER

Age-dependent attractivity of males' sexual pheromones in *Bombus terrestris* (L.) [Hymenoptera, Apidae]

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Received: 10 June 2010 / Accepted: 23 February 2011 / Published online: 23 March 2011

Abstract Males of *Bombus terrestris* (L.) adopt a patrolling behaviour during their nuptial parade using cephalic labial gland (CLG) secretions containing sexual pheromones to attract conspecific virgin queens. The changes in chemical composition of their CLG secretions with age are quite well known. In this study, we investigate the evolution of CLG secretions with age in greater detail and compare behavioural reactions of conspecific virgin queens to the secretions. We show that compounds of CLG secretions follow two profiles. Most of the compounds increase from the first day after emergence until the bees are 15-days-old and then decrease. Others are less abundant in 1 to 15-day-old males and then increase (e.g. tricosane, tricosene, henicosane, tetradecanoic acid, pentacosene, pentacosane, heptacosene, heptacosane, nonacosene and geranylcitronellyl tetradecanoate). Differences in secretion composition lead to preferences of virgin queens for males according to the male's age. Virgin queens prefer the pheromonal gland secretions of bees of the following ages in decreasing order; 1 day = 3 days < 7 days =30 days < 15 days < 10 days. The virgin queens are strongly attracted by secretions containing high amounts of 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, ethyl dodecanoate and hexadecanol. On the contrary, geranylcitronellol is more abundant in 30-day-old males.

Keywords *Bombus terrestris* · Sexual pheromones · Age-dependent variations · Behavioural tests

This article is dedicated to the memory of Dr. Jan Tengö and his significant contribution to the field of chemical ecology of bees.

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Introduction

It is well known that males of *Bombus terrestris* (L.) use cephalic labial gland (CLG) secretions containing sexual pheromones to attract conspecific virgin queens in order to mate (Calam 1969; Kullenberg et al. 1973). Male premating behaviour consists of depositing small amounts of pheromones along a circuit and then patrolling it to find out if a virgin queen has been attracted by their scent marks (Svensson 1979).

The CLG secretions of bumblebees are used as a tool for taxonomical discrimination (Bellés et al. 1987; Coppée et al. 2008; Rasmont et al. 2008). The chemical composition of *B. terrestris*' CLG secretions has been described by many authors since the improvement of the GC/MS technique (Bergman 1997; Bergström 1981; Calam 1969; Coppée et al. 2008). Individual variations were highlighted in different species (Terzo et al. 2005) and are now well

understood since histological (Ågren et al. 1979), physiological (Šobotník et al. 2008) and chemical (Žáček et al. 2009) studies were conducted. These authors showed that in *B. terrestris* ssp. *terrestris*, the CLG secretions evolve with the age of males. Just after males' emergence, the activity of secretory cells is high, but the level of synthesized secretions, as well as the electroantennographic-active (EAG-active) (on virgin queens) compounds concentrations are low (Žáček et al. 2009). At 5 days, the secretory activity stops and the cells degenerate, but the level of secretions only decreases after 7–10 days (Šobotník et al. 2008).

In spite of the numerous studies on the topic, a thorough list of compounds and an examination of the changes of all the compounds with age remains to be published. Moreover, no attention has yet been paid to behavioural reactions of virgin queens to chemical changes of the CLG secretions with age. This might lead to a better understanding of how bumblebees avoid inbreeding which might be hazardous for the population survival since the sex determination in bumblebees is under control of one single locus (Van Wilgenburg et al. 2006; Whitehorn et al. 2009). Copulations between brothers and sisters lead to a progeny made of 50% diploid males that are less adapted than the haploid ones, are smaller (Duchateau et al. 1994; Duchateau and Marien 1995) and have a weaker immune system (Gerloff and Schmid-Hempel 2005).

In this article, we describe the chemical composition of males' CLG secretions of *B. terrestris* ssp. *dalmatinus* between 0 and 40 days old and by means of a simple bioassay we measure preferences of virgin queens for secretions of males of various ages.

Methods and materials

Biological material

Males of *B. t. dalmatinus* were provided by Biobest bvba (Westerlo, Belgium). The colony was raised in a dark room with the following breeding conditions: temperature (*T*) in the range: 25–32°C, relative humidity (RH) in the range: 25–55%. It was fed ad libitum with syrup (1 kg water for 1 kg sugar) and one stock of willow pollen (*Salix* sp.). Every day at a set time, newly emerged males were separated from the mother colony and labelled. They were gathered in small flight cages (13.5 × 12 × 8.5 cm) according to the age they were killed. These males had been maintained in the same breeding conditions as the mother colony for the first 3 days, and then placed in a room with the following conditions: 10,000 lux light for 12 h and min. 20°C temp.

Males were killed by freezing at 0, 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30 and 40 days. These different ages were chosen to see the evolution of secretions during the entire life of males while providing higher temporal resolution during the first days. Twenty males of each age were selected. Both cephalic parts of the labial glands of each specimen were removed after dissection of eyes and placed in a glass vial for extraction in 200 µl of hexane. The vials were left for 24 h at room temperature and then stored at −30°C until chemical analysis (Terzo et al. 2005).

Forty virgin queens of *B. t. dalmatinus* were provided by Biobest bvba (Westerlo, Belgium). These virgin queens were maintained, three by three, in wooden boxes. They were fed with pollen and sugar water ad libitum. The food was replaced every other day. The temperature ranged between 20 and 30°C and the relative humidity 45–55%.

Chemical analyses

Chemical analyses were performed using GC/MS. The mass spectrometer used was an ion trap instrument Finnigan GCQ. The capillary column specifications were as follows: a DB-5ms column (5% phenylmethylploysiloxane stationary phase of 0.25-µm thickness; 30-m column length; 0.25-mm inner diameter). The temperature of the injector was 220°C. The initial temperature of the column was held for 2 min at 70°C, then programmed to 320°C at 10°C/min and held for 3 min at 320°C. Helium was used as carrier gas at a constant velocity of 50 cm/s. Mass spectra were obtained in electron ionisation mode, full scan (m/z 30–600). The extracted samples (1 µl) were injected in the GC in a random order.

Compounds were identified using their mass spectra by comparing to spectra in the National Institute of Standards Technology library (NIST, USA) using Nist MS Search 2.0.

For each chromatogram, the relative area (%) of peaks was integrated using Xcalibur software (v.1.3) according to the following parameters: baseline window = 100; area factor noise = 5; minimum peak width = 5; multiplet resolution = 10; area tail extension = 5 and area scan window = 0.

Quantification of 2,3-dihydrofarnesol

This compound was chosen because of its high relative abundance and its EAG activity (Šobotník et al. 2008). Two males of each age class were used (in case one measurement would fail). Glandular extracts were quantified by gas chromatography coupled with flame ionisation detection (GC–FID) using a Thermo gas chromatograph model Focus. As internal standard, nonyl acetate (400 ng) was added to each sample. Aliquots of 1 µl were injected with a splitless injector held at 240°C. The column (15 m × 0.25 mm i.d.) was maintained at 40°C for

0.5 min before being heated to 180°C at a constant rate of 20°C/min. The final temperature was maintained for 5 min. Quantification of compounds was performed by comparing their GC-peak areas with those of the internal standard using Chrom-Card software (v. 2.3.3.) (Interscience, Louvain-La-Neuve, Belgium).

Behavioural experiments

Secretions of 1-, 3-, 7-, 10-, 15-, and 30-day-old males were tested. Those classes were representative of the main chemical changes in CLG secretions (Table 1). Males aged 0 and 40-days-old were excluded from the behavioural

Table 1 Relative abundance (median, rel. %) of the 36 compounds identified, by age class

Compounds	0 days ($n = 6$)		1 day($n = 10$)		2 days ($n = 8$)		3 days ($n = 10$)		4 days ($n = 9$)		5 days ($n = 10$)		7 days ($n = 10$)	
	M	IQR	M	IQR	M	IQR	M	IQR	M	IQR	M	IQR	M	IQR
Hexadecene	0.31	2.08	0.00	0.05	–	–	0.00	0.00	–	–	–	–	–	–
Ethyl dodecanoate	–	–	0.00	0.00	–	–	0.05	0.14	0.25	0.64	0.46	1.59	0.20	0.22
Tetradecanal	0.00	0.00	0.08	0.45	0.49	0.24	0.57	0.29	0.66	0.63	0.91	0.26	1.04	0.51
2,3-Dihydrofarnesal	0.13	0.97	3.51	2.80	3.15	1.46	2.01	0.66	1.88	0.25	1.90	0.91	1.94	1.04
Dodecyl acetate	0.00	0.00	0.00	0.14	0.05	0.07	0.08	0.06	0.10	0.05	0.11	0.07	0.13	0.04
2,3-Dihydrofarnesol	5.01	21.99	*72.45*	*55.48*	*74.85*	*50.58*	*71.27*	*49.13*	*81.37*	*49.12*	*75.91*	*45.33*	*80.73*	*44.49*
2,3-Dihydrofarnesyl acetate	–	0.00	0.00	0.10	0.00	0.08	0.08	0.08	0.12	0.23	0.08	0.07	0.06	0.04
Tetradecanoic acid	0.19	1.17	0.00	0.43	0.00	0.11	0.07	0.29	0.20	0.31	0.23	0.18	0.20	0.17
Hexadecenal	0.00	0.00	0.29	0.92	0.40	0.15	0.30	0.11	0.28	0.28	0.23	0.13	0.18	0.13
Hexadecanal	–	–	0.19	0.32	0.22	0.12	0.19	0.25	0.13	0.11	0.14	0.12	0.12	0.05
Hexadecan-1-ol	1.15	1.09	1.31	1.96	2.35	1.14	2.35	1.72	2.85	1.46	2.76	0.88	3.12	0.80
Octadecadienal	0.00	0.00	0.00	0.00	–	–	0.00	0.00	0.00	0.00	0.05	0.07	0.06	0.07
Octadecatrienal	0.00	0.05	0.00	0.00	–	–	0.00	0.04	0.06	0.07	0.06	0.08	0.08	0.04
Nonadecadienal	0.04	0.24	0.30	0.27	0.21	0.27	0.24	0.57	0.08	0.12	0.06	0.07	0.07	0.07
Hexadecyl acetate	0.00	0.11	0.33	0.50	0.51	0.45	0.39	0.71	0.12	0.25	0.16	0.14	0.12	0.12
Octadecadienol	–	–	0.66	0.60	1.50	1.82	1.99	2.03	2.53	2.83	1.74	1.55	2.04	3.44
Geranylcitronellal	–	–	0.00	0.00	0.81	1.22	1.35	2.37	1.10	2.40	1.73	1.73	1.79	2.13
Henicosane	*27.61*	*15.04*	*8.04*	11.97	4.27	4.86	4.24	3.16	1.97	2.92	1.79	1.71	1.14	1.65
Geranylcitronellol	–	–	1.21	1.38	1.98	4.49	1.87	6.56	1.80	7.93	2.15	7.95	2.56	8.67
Octadecadienyl acetate	0.95	1.07	0.00	0.00	0.00	0.00	0.00	0.12	0.07	0.35	0.31	1.00	0.50	1.03
Tricos-9-ene	6.34	4.13	0.61	6.40	0.66	2.25	0.82	1.41	0.19	2.36	0.47	1.19	0.15	1.12
Tricosane	16.22	14.62	1.59	15.15	1.57	10.44	1.34	12.51	0.55	11.70	1.19	11.18	0.99	9.32
Tetracosene	0.25	0.57	0.06	0.56	0.00	1.24	0.00	0.58	0.00	0.76	0.00	0.56	0.00	0.00
Pentacos-9-ene	4.53	7.75	0.19	5.50	0.23	2.30	0.28	1.31	0.05	1.56	0.14	1.00	0.03	1.16
Pentacosane	2.48	3.87	0.10	2.60	0.09	1.69	0.00	2.73	0.00	2.16	0.08	2.14	0.07	1.69
Hexacosene	0.00	0.20	0.00	0.35	0.00	0.34	0.00	0.34	0.00	0.32	0.00	0.23	0.00	0.30
Docos-15-enyl acetate	0.57	1.80	0.00	0.82	0.00	0.80	0.00	0.26	0.00	0.34	0.00	0.20	0.00	0.11
Heptacosene	3.73	6.38	0.70	5.08	0.30	3.59	0.47	3.35	0.12	3.43	0.35	2.23	0.14	2.77
Heptacosane	0.57	1.30	0.03	0.77	0.00	0.29	0.00	0.27	0.00	0.23	0.00	0.16	0.00	0.20
2,3-Dihydrofarnesyl dodecanoate	0.50	1.15	0.47	0.67	0.59	3.23	0.30	5.45	0.42	4.85	1.21	4.72	0.51	5.76
Nonacos-9-ene	5.55	7.88	0.39	5.45	0.30	1.19	0.35	0.53	0.06	0.54	0.09	0.32	0.03	0.34
2,3-Dihydrofarnesyl tetradecadienoate + 2,3-dihydrofarnesyl tetradecenoate	0.54	0.29	0.25	0.56	0.36	2.09	0.26	1.90	0.30	1.51	0.44	1.06	0.26	0.82
2,3-Dihydrofarnesyl hexadecanoate + hexadecyl tetradecanoate + tetradecyl hexadecanoate	0.45	0.25	0.06	0.14	0.00	0.17	0.03	0.08	0.00	0.11	0.00	0.06	0.00	0.05
Geranylcitronellyl dodecanoate	0.06	0.71	0.30	0.53	0.25	0.50	0.15	0.30	0.06	0.30	0.07	0.16	0.00	0.11
2,3-Dihydrofarnesyl octadecenoate	0.00	0.05	0.00	0.00	0.00	0.07	0.00	0.05	0.00	0.06	0.00	0.04	0.03	0.07
Geranylcitronellyl tetradecanoate	1.71	0.62	0.59	1.16	0.33	0.53	0.20	0.43	0.19	0.37	0.13	0.18	0.04	0.13

Table 1 continued

Compounds	10 days ($n = 10$)		15 days ($n = 9$)		20 days ($n = 8$)		25 days ($n = 7$)		30 days ($n = 8$)		40 days ($n = 7$)	
	M	IQR	M	IQR	M	IQR	M	IQR	M	IQR	M	IQR
Hexadecene	–	–	–	–	0.00	0.00	–	–	–	–	0.00	0.00
Ethyl dodecanoate	0.31	0.35	0.47	2.39	0.23	3.54	2.46	7.47	3.25	10.17	0.32	3.56
Tetradecanal	1.39	0.42	1.24	1.02	0.06	0.81	0.00	0.07	0.00	0.12	0.00	0.00
2,3-Dihydrofarnesal	1.61	0.39	2.86	2.58	1.70	4.40	0.90	4.61	0.52	1.92	0.10	0.15
Dodecyl acetate	0.17	0.02	0.27	0.13	0.10	0.27	0.00	0.11	–	–	0.00	0.00
2,3-Dihydrofarnesol	*61.96*	*45.25*	*24.47*	*57.28*	8.58	34.38	0.00	0.66	0.53	0.94	0.65	2.64
2,3-Dihydrofarnesyl acetate	0.08	0.04	0.24	0.24	0.18	0.31	0.00	1.04	0.80	1.73	0.00	0.27
Tetradecanoic acid	0.22	0.12	1.18	1.05	1.34	1.30	2.02	6.93	6.47	13.66	3.07	4.17
Hexadecenal	0.18	0.12	0.30	0.17	0.71	1.17	0.00	0.10	0.00	0.05	0.00	0.17
Hexadecanal	0.15	0.08	0.26	0.50	0.36	0.45	0.00	0.04	0.50	1.00	0.21	0.93
Hexadecan-1-ol	3.27	2.07	4.77	3.07	0.08	1.19	0.00	0.09	0.00	0.20	0.00	0.05
Octadecadienal	0.00	0.06	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Octadecatrienal	0.12	0.15	0.14	0.22	0.00	0.05	0.05	0.48	–	–	0.00	0.00
Nonadecadienal	0.06	0.13	0.17	0.19	0.30	0.32	0.00	0.10	0.17	0.59	0.09	0.14
Hexadecyl acetate	0.09	0.14	0.37	0.31	0.43	0.43	0.01	0.13	0.08	0.95	0.08	0.35
Octadecadienol	1.87	1.85	1.81	0.77	0.00	0.16	0.00	0.00	–	–	–	–
Geranylcitronellal	1.30	0.62	0.97	1.35	0.00	0.22	0.00	0.00	–	–	–	–
Henicosane	1.96	2.04	4.00	2.72	6.51	12.38	4.84	11.11	9.43	10.90	5.62	3.12
Geranylcitronellol	6.41	9.31	4.72	4.76	0.00	1.24	0.00	0.00	0.00	0.00	0.00	0.00
Octadecadienyl acetate	0.00	0.00	–	–	0.80	1.07	0.05	0.40	0.74	1.86	0.73	0.31
Tricos-9-ene	0.50	1.40	0.71	1.83	3.16	4.06	2.07	3.17	2.40	1.37	2.82	1.57
Tricosane	2.82	12.55	2.70	12.60	*17.55*	*20.19*	*10.12*	*18.93*	*18.89*	*12.91*	*36.30*	*31.26*
Tetracosene	0.00	0.39	0.00	0.00	0.00	0.76	0.00	0.15	0.12	0.65	0.00	0.35
Pentacos-9-ene	0.21	1.07	0.13	1.45	1.23	2.13	0.44	2.75	1.83	1.13	2.35	2.35
Pentacosane	0.31	2.31	0.27	2.62	1.95	3.90	0.82	4.62	2.49	2.83	5.42	7.50
Hexacosene	0.04	0.25	0.00	0.28	0.00	0.49	0.08	0.57	0.18	0.46	0.00	0.39
Docos-15-enyl acetate	0.00	0.00	0.00	0.00	0.00	0.39	0.01	0.22	0.12	0.27	0.00	0.09
Heptacosene	0.91	2.39	0.79	2.78	5.48	7.35	3.80	10.48	6.37	6.38	7.13	4.22
Heptacosane	0.00	0.10	0.00	0.00	0.00	1.32	0.31	2.12	0.64	1.57	2.02	2.26
2,3-Dihydrofarnesyl dodecanoate	2.06	4.44	2.22	9.92	3.18	16.12	2.25	5.93	1.92	2.91	2.01	0.88
Nonacos-9-ene	0.07	0.28	0.09	0.41	1.04	2.88	0.26	5.10	1.33	3.12	0.96	1.23
2,3-Dihydrofarnesyl tetradecadienoate + 2,3-dihydrofarnesyl tetradecenoate	0.65	1.42	1.37	4.48	1.16	4.87	0.20	1.44	1.35	2.06	1.49	0.53
2,3-Dihydrofarnesyl hexadecanoate + hexadecyl tetradecanoate + tetradecyl hexadecanoate	0.01	0.03	0.09	0.12	0.43	0.68	0.24	0.65	0.10	0.46	0.00	0.26
Geranylcitronellyl dodecanoate	0.05	0.12	0.26	0.32	0.50	1.24	1.14	1.10	0.88	1.87	1.59	1.40
2,3-Dihydrofarnesyl octadecenoate	0.03	0.04	0.07	0.22	0.10	0.74	0.00	0.26	0.06	0.30	0.00	0.22
Geranylcitronellyl tetradecanoate	0.01	0.09	0.25	0.32	0.61	1.54	2.28	2.68	3.65	5.75	0.00	0.66

n number of males, – the compound is absent in every specimens of the age class. The main compound of each age class is marked in italics and the EAG-active compounds are in bold. Compounds are listed in the retention order on a DB5-ms column. *M* median, *IQR* interquartile Range (Quartiles 3–1)

tests; freshly emerged males do not scent-mark inside the colony and males do not survive until 40 days in natural conditions. The behavioural tests were performed in an olfactometer made of a glass tray (70 × 70 × 8 cm) covered with a polycarbonate plate with a central hole allowing the introduction of a Petri dish (9.2-cm diameter) containing a virgin queen. The arena was divided into 4 quarters (35 × 35 cm). A circular wire mesh (8-cm height)

was set in the arena to avoid direct contact between the female and the scent marks. Males' secretions were put down in the corners of the arena. The different age secretions were tested two by two. The four corners contained respectively and randomly: (1) a blank filter paper, (2) a filter paper with 2.5 µl of hexane, (3) a filter paper with 2.5 µl of secretions from a bee aged of 'X' days, (4) a filter paper with 2.5 µl of secretions from a bee aged of 'Y' days (Fig. 2). The different age pairs to be tested (33 times each) were randomly chosen. Forty-three control tests were performed following the same protocol. A digital camera (Philips SPC 900NC PC camera) connected to a PC was set and centred above the experimental arena, allowing the recording of the virgin female moves. The experimentation room was kept at 20–30°C temp., and 45–55% RH. Red light was used since bumblebees are unable to perceive this wavelength.

The following protocol was used to perform each test: The corner positions of blank, solvent and males' extracts in the olfactometer were chosen randomly. Light was then switched to red. A virgin female was placed in a Petri dish (9.2-cm diameter) and set free 1–2 min later after she has calmed down. As soon as the virgin female was free, her position was recorded every 5 s, during 7 min (= 84 successive positions of the queen being tested). Each possible pair of ages was tested 33 times, each time with a different queen. After each test, the entire olfactometer was cleaned using acetone.

Virgin queens' positions were quantified by the total number (the sum) of approaches to each odour source (i.e. the presence of the female in each quarter of the arena). These positions were statistically compared with a χ^2 test, the null hypothesis being an identical number of approaches in the four zones (33 queens × 84 positions: $n = 2772$). A Fishers exact test was used to compare queens' approaches in the four zones two-by-two.

Results

Chemical composition of males' CLG secretions

The chemical study is based on 112 interpretable chromatograms distributed amongst the 13 age classes (Tab. 1). Thirty-six compounds have been identified amongst the secretions: alkanes, alkenes, aliphatic alcohols, aldehydes, esters, acyclic sesqui- and diterpenic alcohols and aldehydes. The 2,3-dihydrotransfarnesol (DHF) is the main component (in relative abundance) in males aged of 1 to 15 days old. In younger males (0 days old), it is henicosane that dominates while in older males (20–40 days old), tricosane is the most abundant component. There are two profiles of the relative concentration of the compounds with age. On one hand, most compounds follow the same pattern as DHF (i.e. high abundance in 1 to 15-day-old males, then decreasing). On the other hand, tetradecanoic acid, tricosene, pentacosene, pentacosane, heptacosene, heptacosane, nonacosene and geranylcitronellol tetradecanoate have the same profile as tricosane (i.e. low abundance in 1 to 15-day-old males, then increasing).

Quantification of the DHF

The mean absolute quantity of DHF varies from 0.2 to 330 µg (Fig. 1). Its variation follows the observed relative abundance (Table 1). It increases from 1 to reach its maximum at 7 days of age and decreases from 10 to 20 days of age. Moreover, it is nearly totally absent in males of 0 and 25–40 days old. The trend line, moving with average is also shown in Fig. 1 (automatically built in Excel 2003).

Behavioural experiments

Thirty-three control tests were performed previous to olfactory tests in order to check that no external stimuli could disturb the virgin queens. The χ^2 result of the control test has a non-significant P value (H0 accepted).

Results of the behavioural tests are shown in Fig. 2, the sum of recorded approaches (Table 2) of virgin queens in each quarter is shown here as percentages. The virgin queen always shows a highly significant preference for one of the four olfactory stimuli presented (Fig. 2a–f). Results of the χ^2 tests are presented in Table 2, the χ^2 results being statistically highly significant in each case (H0 rejected), contrary to the control tests. We then tested (Fishers exact test) two-by-two the significance of females' occurrences

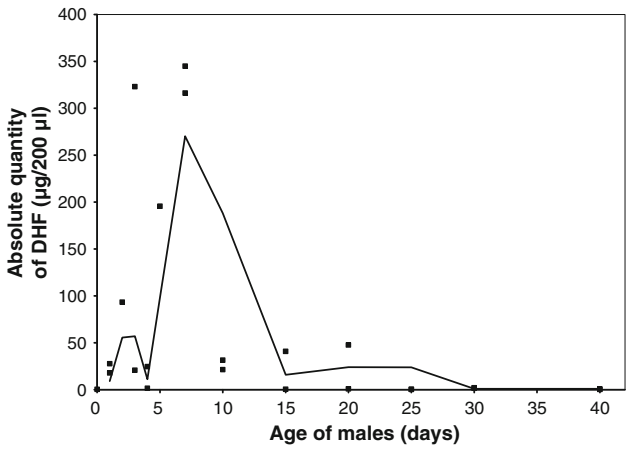

Fig. 1 Quantification of the DHF in the different age classes, using nonyl acetate as internal standard. Two measurements by age class, except for 1- and 5-day-old males in which one measurement failed. The *black curve* represents the trend line moving with average

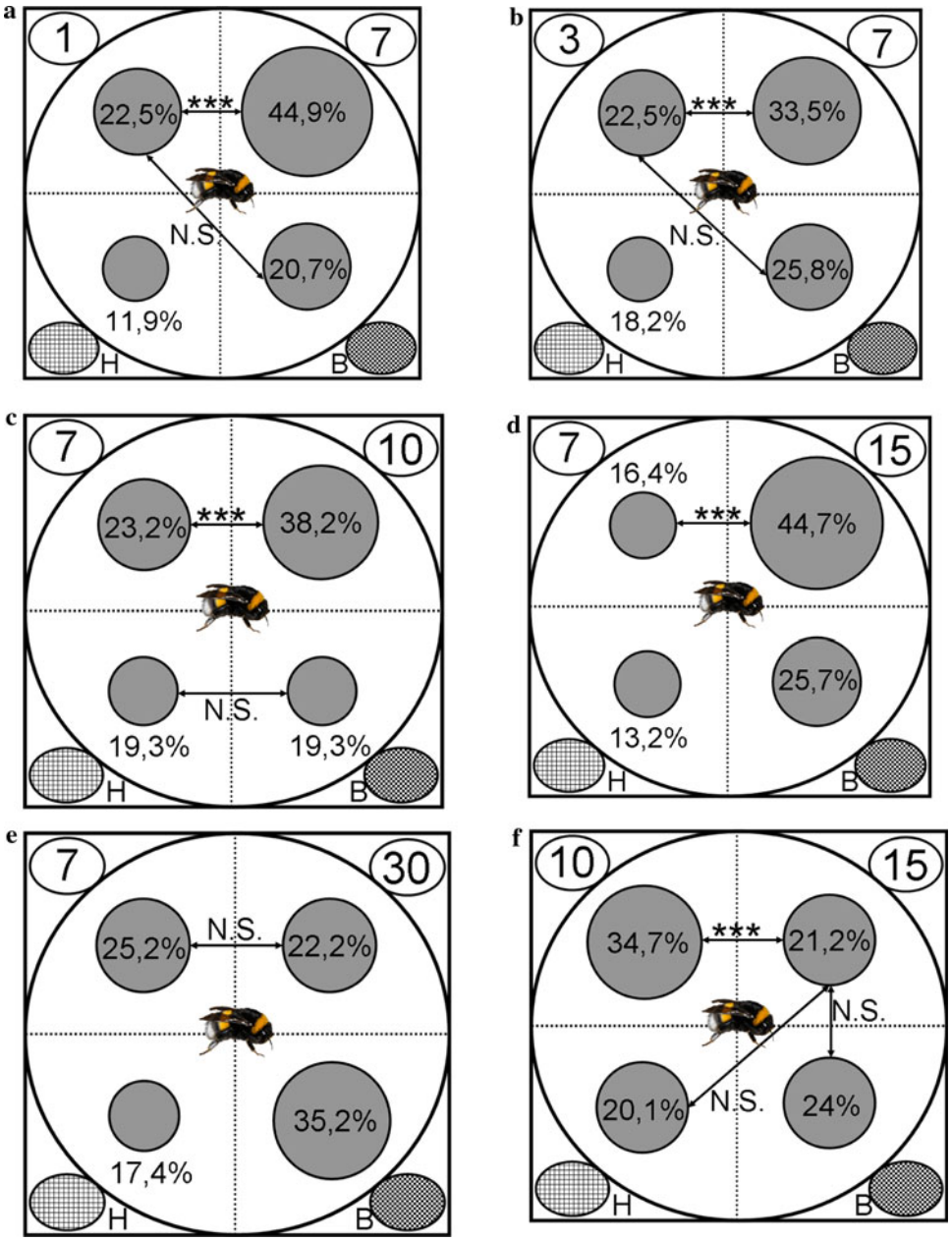

Fig. 2 Approach (in %) of virgin queens in each quarter of the olfactometer, containing blank (the *grey spot*; *B*); pure hexane (the *hatched spot*, *H*) and two different ages secretions as follows: **a** 1 versus 7 days old, **b** 3 versus 7 days old, **c** 7 versus 10 days old, **d** 7 versus 15 days old, **e** 7 versus 30 days old, **f** 10 versus 15 days old. N.S. means the Fishers exact test result is non-significant (P value > 0.05). The result of the Fishers exact test applied to the zones containing the sexual pheromones extract is given on the arrow linking the two zones (*NS*: P value > 0.05, $^{*}P$ value < 0.05, $^{**}P$ value < 0.01, $^{***}P$ value < 0.001)

near the odour sources: hexane versus blank, hexane versus extracts, blank versus extracts, extract 'X' versus extract 'Y' (Table 3). The males' secretions are preferred to pure solvent and blank, with two exceptions: there is no significant difference between the blank versus 1- and 3-day-old males' secretions (Fig. 2a, b), neither between hexane and blank versus 15-day-old males' secretions (Fig. 2f).

The χ^2 results for the extract 'X' versus extract 'Y' tests show that 7-day-old males secretions were more attractive than 1- and 3-day-old ($P < 0.001$) ones (Fig. 2a, b) but less attractive than 10- and 15-day-old ones ($P < 0.001$) (Fig. 2c, d). No significant difference was detected between 7- and 30-day-old males' secretions (Fig. 2e). Ten-day-old males are more attractive than 15-day-old males ($P < 0.001$) (Fig. 2f). To summarise the attractivity of the secretions of males of different ages, we can order them from the least to the most potent: 1 day = 3 days < 7 days = 30 days < 15 days < 10 days.

Discussion

With a simple bioassay, we showed that chemical changes in ageing males elicit different responses of virgin queens. Progressive changes in chemical composition of males' CLG secretions are related to preferences of virgin queens for a certain bouquet. The chemical composition described here, as well as, the virgin queens preferences are matching

Table 2 Results of the behavioural tests conducted on virgin queens to detect their preference for sexual pheromones of males from different ages

Ages tested (days)		Sum of females' presence in zone ($n = 2772$)				$\chi 2$ applied on four zones	
Extract "X"	Extract "Y"	Blank	Hexane	Extract "X"	Extract "Y"	*P* value	Significance
1	7	574	330	623	1,245	6.25E−65	***
3	7	716	505	623	928	1.35E−14	***
7	10	534	535	643	1,060	9.24E−26	***
7	15	712	366	454	1,240	5.47E−66	***
7	30	975	482	698	617	2.2E−19	***
10	15	665	558	963	586	1.08E−14	***

The age (in days) tested are shown in the two first columns. The sum of the queens presence in each quarter are given (queens' position recorded every 5 s during 7 min; 33 queens × 84 positions = 2772 data). A χ^2 was applied to the result and the *P* value and significance are shown in the two last columns (***$P < 0.001$)

Table 3 Two-by-two Fishers exact tests applied to queens presence

Ages tested ($n = 33$)		Fishers exact test results											
		Blank vs. hexane		Blank vs. extract "X"		Blank vs. extract "Y"		Hexane vs. extract "X"		Hexane vs. extract "Y"		Extract "X" vs. extract "Y"	
Extract 'X'	Extract 'Y'	*P* value	Sign.	*P* value	Sign.	*P* value	Sign.	*P* value	Sign.	*P* value	Sign.	*P* value	Sign.
1	7	6.98E−09	***	3.26E−01	NS	1.27E−29	***	1.29E−11	***	5.77E−65	***	5.71E−25	***
3	7	1.66E−05	***	6.91E−02	NS	2.11E−04	***	1.29E−02	*	1.58E−15	***	4.09E−07	***
7	10	1.00E+00	NS	2.59E−02	*	3.54E−21	***	2.59E−02	*	5.14E−21	***	6.85E−13	***
7	15	4.37E−14	***	7.63E−08	***	1.48E−17	***	2.95E−02	*	8.40E−58	***	8.10E−44	***
7	30	1.62E−20	***	1.42E−06	***	1.71E−10	***	8.01E−06	***	4.19E−03	**	1.10E−01	NS
10	15	2.88E−02	*	1.57E−07	***	1.10E−01	NS	1.46E−13	***	5.58E−01	NS	7.82E−12	***

P values and their significance are given for each test. *NS* non statistically significant—$P > 0.05$

* Statistically significant—$P < 0.05$

** Highly statistically significant—$P < 0.01$

*** Very highly statistically significant—$P < 0.001$

The ages (in days) tested are shown in the two first columns

the previous descriptions of histological, physiological and electrophysiological changes with age in *B. terrestris* (Šobotník et al. 2008; Žáček et al. 2009). The virgin queens are strongly attracted to males with a higher amount of EAG-active compounds (Žáček et al. 2009), i.e. ethyl dodecanoate, 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, hexadecanol and geranylcitronellol (Table 1), i.e. 7-day-old and older males. Those males secrete a lower relative concentration of tricosane. Attractiveness of the bouquet seems to be due to a high level of EAG-active compounds (listed in Žáček et al. 2009) even if they are not at their highest level of relative abundance. In other words, attractivity seems to be due to the total ratio of EAG-active compounds. The preference of virgin females for males of at least 7 days old may be due to a selection of males that are well fit and able to survive out of the nest.

The absolute configuration of 2,3-dihydrofarnesol in *B. terrestris* was described independently in two articles: First by Ställberg-Stenhagen (1970) and much later by Luxová et al. (1970). In both articles, the absolute configuration was found to be pure (*S*) regardless the fact that the first report was based on wild males from Sweden while the second study was done with males from laboratory colony established in the Czech Republic. However, the males were of different (undefined) age. Therefore, we believe that differences in the attractiveness of the secretion are due to changes in the quantity of 2,3-dihydrofarnesol not due to a change of absolute configuration.

Ten-day-old males are most attractive to queens and their secretions show a high fraction of EAG-active compounds. This includes a high DHF relative abundance (but not the highest), as well as 2,3-dihydrofarnesal, hexadecanol and geranylcitronellol. Ethyl dodecanoate increases until the age of 30 days, which does not correspond to the decreasing trend of virgin queens' response. One could

speculate that this compound may have a different function in the secretions (e.g. repellent instead of attractant). Males older than 30 days are rare in the wild and a female should avoid losing her energy mating with them for their possibly poor reproductive abilities (if they already mated, their sperm reserves may be low). It is also interesting to note that one EAG-active compound described in the subspecies *B. t. terrestris* (Žáček et al. 2009), i.e. octadecatrienol was not detected in *B. t. dalmatinus.*

The profile of CLG secretions in *B. t.* ssp. *dalmatinus*, with a peak of secretions at 7 days old, could be linked to having a maximal attraction potential to virgin queens. This might be an advantage in species that are monoandrous such as *B. terrestris* (Baer and Schmid-Hempel 2001; Duvoisin et al. 1999). Having only few chances to mate, they will be favoured by being as attractive as possible. In multiple mating species on the other hand, males could be expected to be attractive during their entire life without age-dependent changes in their CLG secretions.

It is clear that the strategy of 'age-dependent secretions composition' could play a role in inbreeding avoidance. As virgin queens are not attracted to males younger than 5 days old, they will not mate with their brothers in the parental nest (Ågren et al. 1979). Furthermore, as bumblebees sex determination is pledged to sl-CSD (single locus-Complementary Sex Determination) (Van Wilgenburg et al. 2006; Whitehorn et al. 2009), inbreeding is more than hazardous for the population survival by leading to diploid and triploid progeny.

Acknowledgments The authors acknowledge the 'Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture', which awarded a grant to A.C., and the 'Fonds de la Recherche Fondamentale et Collective (projet 2.4.564.06.F)'. They are also grateful to Biobest for having provided the biological material. I.V. thanks the Czech Science Foundation (project no. 203/09/1446) for financial support. A.C. is grateful to Mike Wykes for his kind proof reading.

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