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ORIGINAL PAPER

Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: *Apidae*)

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Abstract In eusocial Hymenoptera, queen control over workers is probably inseparable from the mechanism of queen recognition. In primitively eusocial bumblebees (*Bombus*), worker reproduction is controlled not only by the presence or absence of a dominant queen but also by other dominant workers. Furthermore, it was shown that the queen dominance is maintained by pheromonal cues. We investigated whether there is a similar odor signal released by egg-laying queens and workers that may have a function as a fertility signal. We collected cuticular surface extracts from nest-searching and breeding *Bombus terrestris* queens and workers that were characterized by their ovarian stages. In chemical analyses, we identified 61 compounds consisting of aldehydes, alkanes, alkenes, and fatty acid esters. Nest-searching queens and all groups of breeding females differed significantly in their odor bouquets. Furthermore, workers before the competition point (time point of colony development where workers start to develop ovaries and lay eggs) differed largely from queens and all other groups of workers. Breeding queens showed a unique bouquet of chemical compounds and certain queen-specific compounds, and the differences toward workers decrease with an increasing development of the workers' ovaries, hinting the presence of a reliable fertility signal. Among the worker groups, the smallest differences were found after the competition point. Egg-laying females contained higher total amounts of chemical compounds and of relative proportions of wax-type esters and aldehydes than nest-searching queens and workers before the competition point. Therefore, these compounds may have a function as a fertility signal present in queens and workers.

Keywords Bumblebees · *Bombus terrestris* · Egg-laying workers · Cuticle surface extracts · Fertility signals · Chemical analyses

Introduction

Colonies of primitively eusocial bumblebees contain two female castes, queens and workers (Michener 1974). At the beginning of the lifecycle of a bumblebee colony, oviposition is limited to the queen. She inhibits ovarian activity and egg laying of most of the workers by pheromonal influence and through physical aggression (Röseler and van Honk 1990). The importance of pheromones versus physical aggression may depend on the number of workers within a colony and may, therefore, vary between different species. A growing worker population inside the nest might have caused the evolution of specific queen pheromones (Velthuis 1976). The queen being unable to control all individuals by agonistic behavior consequently has developed a chemical recognition system to exert her influence. Queen control over workers is strongly connected to the mechanism of queen recognition in all eusocial Hymenoptera (Fletcher and Ross 1985; Keller and Nonacs 1993), and pheromonal queen recognition has already been demonstrated for primitively eusocial halictine bees (Buckle 1982).

In honey bees, queen-specific pheromones are produced in various glands, of which the mandibular and Dufour's

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gland pheromones have been studied in more detail (Slessor et al. 1988; Winston et al. 1990; Katzav-Gozansky et al. 2002, 2004). The queen pheromone is spread within the colony mostly by retinue workers (Seeley 1979). The chemical signals of both glands are thought to act as a fertility signal giving information about the ovarian development of females (Plettner et al. 1993; Katzav-Gozansky et al. 2004; Malka et al. 2006). A fertility signal should have certain traits: It should be honest and thus reliable for its recipients, it should deliver information on a continuous scale such that intermediate stages can be perceived, and it should be selected by potential recipients (Liebig et al. 2000).

In bumblebees, workers exhibit a division of labor into foragers, guards, and nest bees and establish a dominance hierarchy partly connected to the tasks they perform. Worker reproduction is influenced by the presence or absence of a dominant queen or other dominant workers (Röseler and van Honk 1990). A *Bombus* (*Bombus*) *terrestris* queen controls the development of the workers' ovaries using pheromones (van Honk et al. 1980; Röseler et al. 1981; Bloch and Hefetz 1999) and prohibits workers with fully developed ovaries from oviposition (van Honk et al. 1980, 1981). It was suggested that the mandibular glands produce the primer pheromone that is subsequently spread over the queen's body (Röseler et al. 1981). When the queen's dominance ceases toward the end of the colonial cycle, at the so-called competition point (van Doorn and Heringa 1986), workers at the top of the hierarchy start to lay eggs and to dominate other workers (van Doorn 1988; Röseler and van Honk 1990). The timing of the onset of the queen–worker conflict was shown to be influenced by several factors like the number of workers and the sex of the brood (Bloch 1999). However, since these egg-laying workers do not mate, they only produce haploid eggs, the majority of which are eaten by the queen and other workers, sometimes even by workers originating from other colonies (Lopez-Vaamonde et al. 2004). During this stage of the colony development, dominant workers may also eat queen-laid eggs (van Doorn and Heringa 1986). It is so far unknown whether these dominant workers rely on pheromonal control (Katayama 1974; Röseler and Röseler 1977) or rather on specific dominance behavior to suppress the ovarian development of the other workers and to stabilize their dominance.

Investigations in *B. hypnorum*, a bumblebee that is thought to be a more primordial species (Röseler and van Honk 1990), showed group specific differences in the bouquets of chemical compounds of females that might have a function as fertility signals (Ayasse et al. 1995). Among the bumblebees, *B. terrestris* belongs to the highly evolved species in which a primer effect of queen pheromones has already been demonstrated (van Honk et al. 1980; Röseler et al. 1981). Since the fertility of workers in this species is not only controlled by the presence of a queen but also by dominant (egg-laying) workers, they may also produce a fertility signal. Whether there is a similar signal released by the queen and of egg-laying workers is still unclear.

This paper explores the following topics:

1. Do queen-specific chemical signals exist in the bumblebee *B. terrestris*?
2. Do egg-laying females (queens and egg-laying workers) release similar signals that can be distinguished from the non-egg-laying individuals and that could have a function as fertility signals?
3. Do the qualitative or quantitative compositions of workers' secretions change at or after the "competition point"?

Materials and methods

Rearing bumblebees

In February and March 2003 and 2005, nest-searching *B. terrestris* L. queens were collected from various locations in the surroundings of Bonn, Germany, and used for founding 19 colonies in the laboratory. Twenty other colonies were founded from virgin queens obtained from colonies bought from Koppert Biological Systems (The Netherlands). Queens were naturally mated in the laboratory and treated to prevent hibernation according to the method described by Röseler (1985). In total, 39 females were transferred into nesting boxes and reared in a dark room at 26–28°C and 70% humidity. Commercial sugar syrup (ApiInvert®) and fresh pollen obtained from Koppert Biological Systems were supplied to all colonies ad libitum. Daily observations of the colonies were made under red light. The initiation of the competition point was identified by the observation of egg-laying workers—specifically by counting ten worker-used egg cells, as these are visually clearly distinguishable from the queen's egg cells.

Sample collection

The following female groups were selected for sample collection: (1) nest-searching queens before colony foundation ($N=10$) and (2) breeding queens were collected before the competition point ($N=39$). Chemical samples of workers were collected before (3) ($N=10$) the competition point. Workers were also collected after the competition point and characterized by their ovarian stages according to Ayasse et al. (1995): (4) Ovarioles small, all follicles beyond the vitellogenic phase ($N=5$), (5) vitellogenesis in a progressive phase (N=8), and (6) ovarioles large, with one or several mature eggs, or females were observed to lay eggs (N=26).

Individual workers and queens were killed by freezing and stored at −40°C until dissection. Cuticle surface extracts were obtained by rinsing an individual abdomen for 30 s in pentane (Merck, Uvasol; worker=0.5 ml; queen=1.0 ml). To gain cuticle surface extracts, we used the abdomen only because we wanted to analyze the head glands separately. Furthermore, former investigations showed that the chemical compounds on the abdominal surface are the same than on the surface of the head and the thorax. The results of the head gland analysis are published elsewhere (Sramkova et al., in preparation). After removing of the abdomen, the samples were stored in the freezer at −40°C until use.

Chemical analysis

For chemical analysis, 1 μl of the extract was injected splitless into a HP 5890 Series II gas chromatograph, equipped with a DB-5 capillary column (30 m×0.25 mm inner diameter, J&W) and a flame ionization detector, using hydrogen as the carrier gas (constant flow, 2.0 ml/min), operating at 50°C for 1 min, after which the split valve was opened and the temperature was increased by 10°C/min up to 310°C. For quantitative analyses, 1 μg of *n*-undecane was added as an internal standard to each sample. Structure elucidation of individual compounds was based on gas chromatography (GC)/mass spectrometry (MS; VG70/250 SE instrument, Vacuum Generators, Manchester, England, linked to a HP 5890; gas chromatographic condition as mentioned above) and comparisons with mass spectra reported in the literature (McLafferty and Stauffer 1989) and GC retention times (coinjection) with those of authentic reference samples. Double bond positions in alkenes were determined by investigation of the corresponding dimethyl disulfide adducts (Buser et al. 1983).

Statistical analysis

Having sorted out peaks below 0.1% of the total bouquet and compounds not separable on the DB5 column, relative and total amounts of chemical compounds were tested for significance of differences between queens and worker groups with an analysis of variances (ANOVA) followed by a multiple comparison test (Tamhane test). The relative proportions of the same compounds were used for a principal components analysis (PCA) followed by discriminant function analyses (DFAs; Backhaus et al. 1987; Norusis 1993a, b) using the SPSS 13.0 statistical system. All DFAs were performed with 15 principal components (PCs) with an Eigenvalue greater than 1. The standardized discriminant function coefficient and the component loadings were used to assess the importance of individual compounds. A compound was considered to have a high component loading when the loading was above 0.5.

Results

In cuticle surface extracts of all females groups, 139 compounds were registered by GC analyses. Eighty-seven of these compounds occurred in quantities of more than 0.1%, 77 of which were registered in breeding queens (Fig. 1), whereas the other ten are produced exclusively by the other groups. Sixty of these 77 substances could be identified by GC/MS analyses and the coinjection technique, while 17 remained unknown. In accordance with literature data (Tengö et al. 1991, Hefetz et al. 1996, Ayasse et al. 1999), alkanes, alkenes, and aldehydes as well as unsaturated and saturated esters of fatty acids were found.

Quantitative differences—absolute amounts In all sample types, the mean total amounts of chemical compounds were found to be highest in breeding queens (1.42±0.1 SE, N=39; Fig. 2) who contained a significantly higher amount than each worker group (ANOVA, F=17.7, $df1$=5, $df2$=92, p<0.001; Tamhane, p<0.001) and than nest-searching queens (ANOVA, F=17.7, $df1$=5, $df2$=92, p<0.001; Tamhane, p<0.001). Furthermore, quantities were higher in workers after the competition point with large ovaries (0.2±0.07 SE, N=26) than in workers before the competition point (0.08±0.03 SE, N=10; ANOVA, F=17.7, $df1$=5, $df2$=92, p<0.001; Tamhane, p<0.001), which were found to contain the smallest amounts of chemical compounds.

Qualitative differences—relative amounts Canonical DFA was performed with 15 PCs with an Eigenvalue above one explaining 86.26% of the variance to test for differences in odor bouquets between female groups. Queens and worker groups were successfully separated with respect to relative amounts of chemical compounds (DFA: discriminant function 1: χ^2=561.79, df=45, p<0.0001; discriminant function 2: χ^2=280.399, df=28, p<0.0001; discriminant function 3: χ^2=96.741, df=13, p<0.0001). All of the nest-searching and breeding queens could be correctly classified according to their odor patterns. On the other hand, some of the dominant workers were classified in the group of subordinate workers based on their odor compound composition, suggesting overlap of dominant and subordinate workers in this aspect (Fig. 3, Table 1). While workers after the competition point with different ovarian sizes showed very similar patterns of chemical compounds, they significantly differed from workers before the competition point and breeding and nest-searching queens (Fig. 3). Dominant

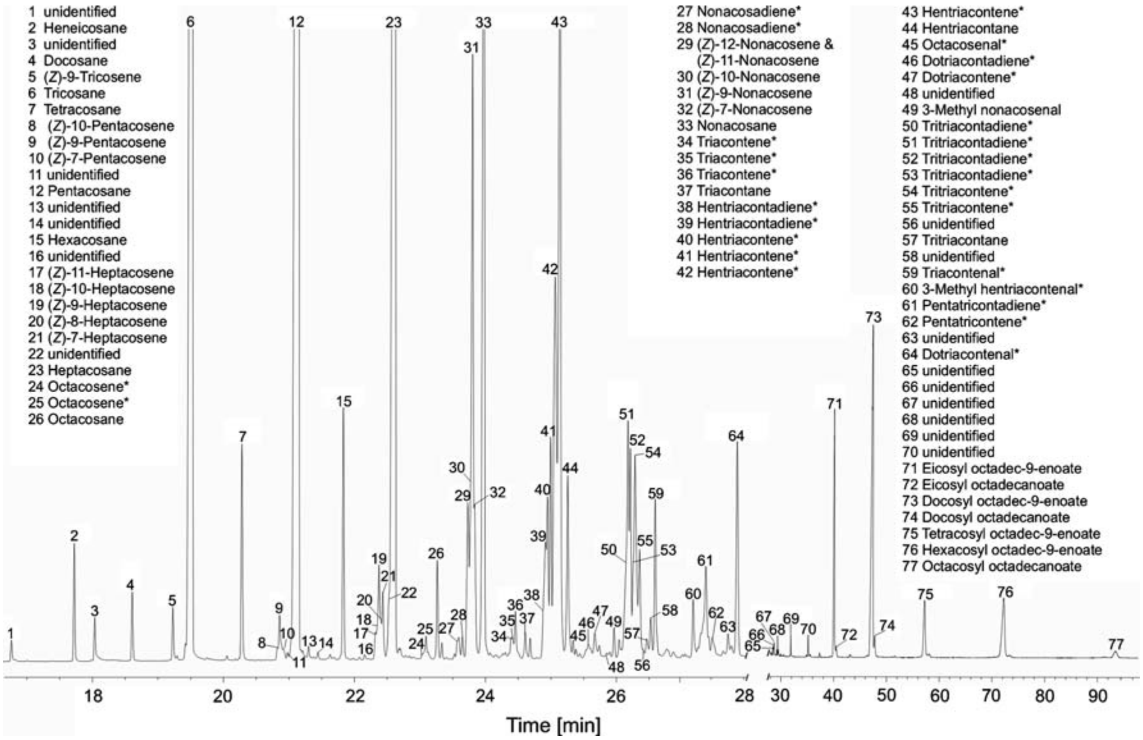

Fig. 1 Gas chromatogram of the cuticle surface extract of a breeding queen (*asterisk*: unknown double bond position)

workers showed an odor bouquet differing less from breeding queens than the bouquet of all the other female groups including nest-searching queens.

The standardized discriminant function coefficients and the components loadings in a PCA revealed that wax-type esters, aldehydes, and hydrocarbons are most important for discrimination of egg-laying and non-egg-laying females. The mean relative proportions of these compounds are shown in Fig. 4. Workers before the competition point showed the highest relative proportions of all alkenes and of most of the alkanes besides tetracosane and pentacosane (Fig. 4a).

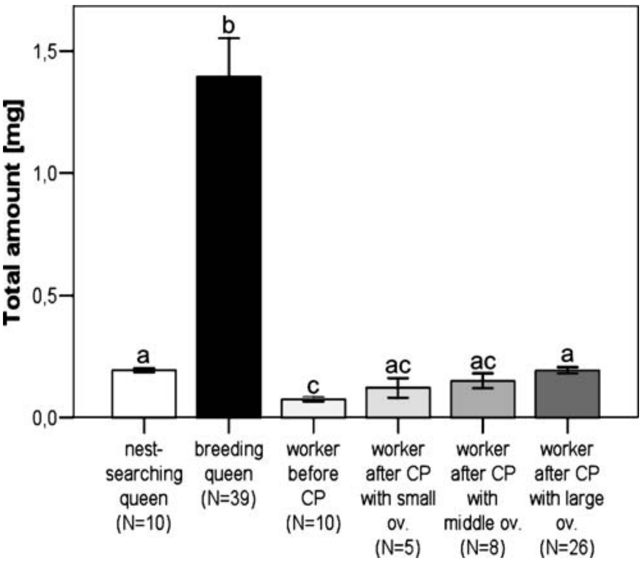

Fig. 2 Comparison of the total amounts of chemical compounds in cuticle surface extracts of nest searching and breeding queens and workers before and after the competition point (*CP*). Different *letters* denote statistical differences (ANOVA, Tamhane test, $p<0.001$)

The relative amounts of aldehydes and wax-type esters were twice the amounts in egg-laying females (queens and workers after the competition point) than in workers before the competition point and in nest-searching queens (Fig. 4b). The relative proportions of aldehydes were always highest in reproductive queens, while relative amounts of the most important esters were found to be highest in workers after the competition point except for hexacosyloctadec-9-enonate. The proportion of all considered aldehydes and wax-type esters discovered in nest-searching queens always remained below that of workers before the competition point.

Discussion

Bumblebees are model organisms to study chemical interactions between queens and workers in social insects. Depending on the species, queen pheromones as well as chemical signals produced by workers play important roles in the establishment of dominance hierarchies (Röseler and van Honk 1990). In *B. terrestris*, dominance is established of the first workers at their emergence by an aggressive behavior of the queen (van Doorn 1989) and later, when the

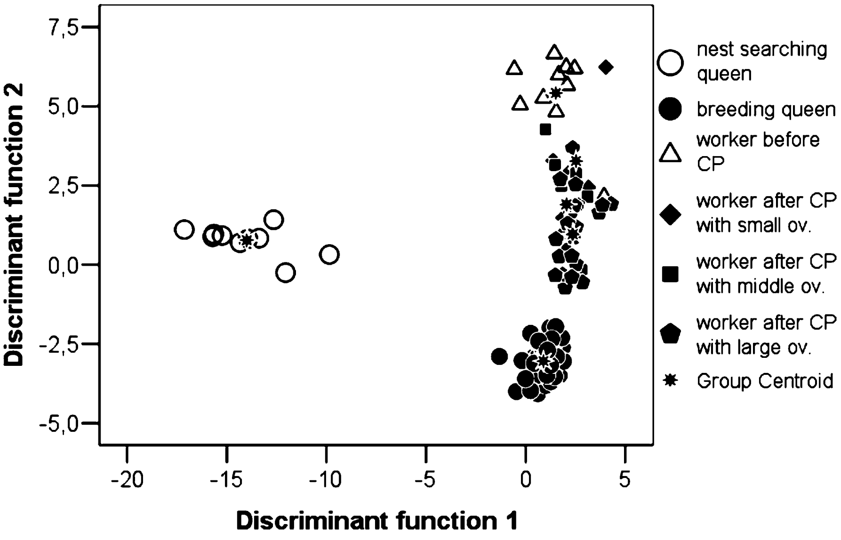

Fig. 3 Comparison of the odor bouquets of cuticle surface extracts of *B. terrestris* females in different stages in the colony cycle before and after the competition point having various ovary (*ov.*) sizes using a DFA

colony size exceeds the number of workers controllable by direct interactions, maintained by the influence of queen pheromones (van Honk et al. 1980).

In primitively eusocial bees forming more populous nests, queen and worker recognition is assumed to be enabled by changes in the composition of chemical compounds (Hölldobler and Michener 1980).The DFA performed in the present study with the volatile patterns of various female groups revealed a complete change of the odor profiles from nest-searching queens to workers and queens living in a nest, which supports the aforementioned hypothesis. The origin and purpose of the substances is so far completely unknown.

Nest-searching queens produce very small amounts of chemical compounds and only a few alkanes and alkenes. This composition can be viewed as a very basic pattern. At this stage, it is not rewarding for the queen to spend energy for producing signals without potential recipients.

This situation changes completely during colonial buildup: The emerging workers must be directed to invest into the queen-laid eggs. The queen may achieve worker control by generating a different bouquet of substances. She especially increases production of aldehydes and wax type esters, though absolute amounts of alkanes and alkenes are very high.

Among the groups of breeding females, the queens produce a distinct odor signal and can be separated from all worker groups by the sheer amount as well as by a unique qualitative composition containing four so far unidentified queen-specific substances. The difference we found in total amounts is probably not a result that can be explained by the larger size of the queen as was shown in *B. hypnorum*, where the size of a worker also determined her dominance status (Ayasse et al. 1995) and workers clustered into defined “odor groups” according to their dominance rank and glandular composition (Hefetz et al. 1993). As in our findings, the total amounts of substances decreased from breeding queens via dominant and subordinate workers and foragers to newly emerged workers (Ayasse et al. 1995). Also in accordance with our results, dominant and subordinate workers differed from each other with respect to relative amounts of single compounds. We found that breeding females especially increase production of aldehydes, wax-type esters, and pentacosene. Bioassays with mixtures of synthetic compounds have to prove their potential role as a fertility signal.

Table 1 Classification table with cross validation results for group memberships of *B. terrestris* females

Original/predicted	Nest-searching queen	Breeding queen	Worker before competition point	Worker with small ovaries	Worker with middle ovaries	Worker with large ovaries
Nest-searching queen	10 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Breeding queen	0 (0.0%)	38 (97.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.6%)
Worker before competition point	0 (0.0%)	0 (0.0%)	9 (90.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Worker with small ovaries	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (20.0%)	4 (80.0%)	0 (0.0%)
Worker with middle ovaries	0 (0.0%)	0 (0.0%)	1 (12.5%)	3 (37.5%)	2 (25.0%)	2 (25.0%)
Worker with large ovaries	0 (0.0%)	0 (0.0%)	1 (3.8%)	2 (7.7%)	3 (11.5%)	20 (76.9%)

81.6% of cross validated grouped cases were classified correctly

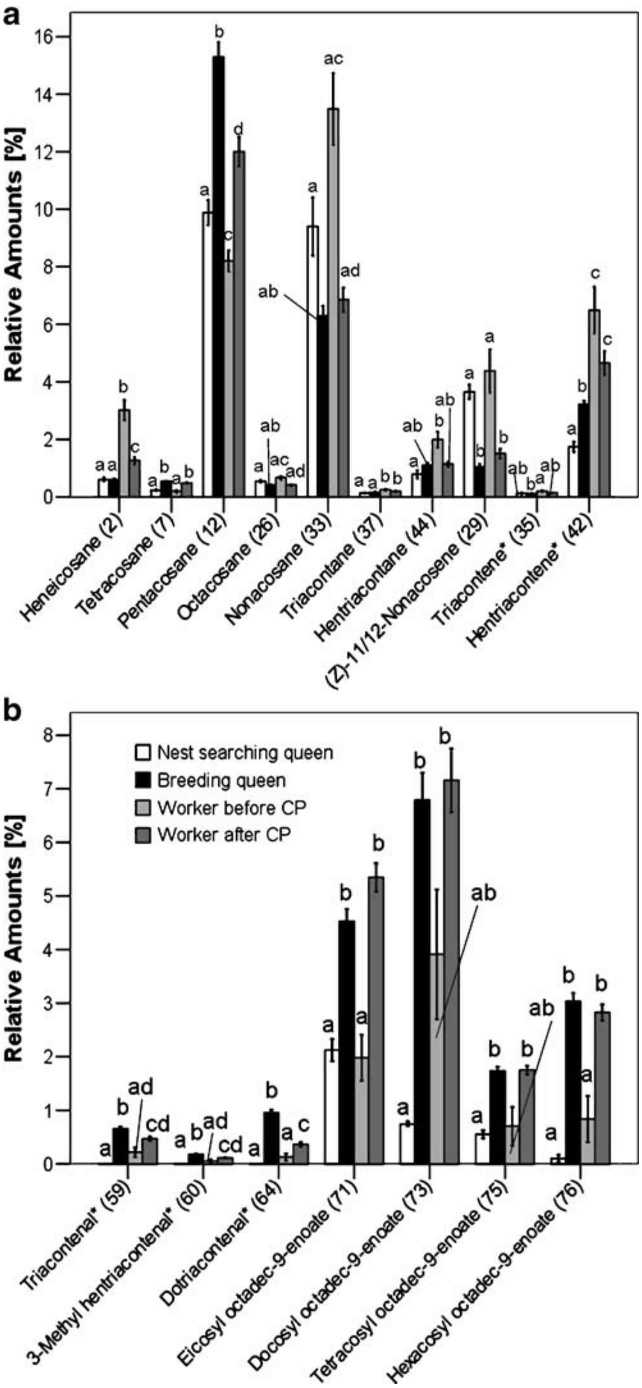

Fig. 4 Relative amounts of selected alkanes and alkenes (**a**) and aldehydes and wax-type esters (**b**) in cuticle surface extracts of nest-searching and breeding queens and workers before and after the competition point (*CP*). Selection was based on the degree to which substances contribute to group separation in DFA. Different *letters* denote statistical differences of compounds between female groups (ANOVA, $p<0.01$; Tamhane test, $p<0.05$; *asterisk*: unknown double bond position)

The *B. terrestris* queen pheromones are suggested to originate in the mandibular glands and are subsequently spread over the body surface (Röseler et al. 1981). However, chemical analyses of the mandibular glands and other glands like the hypopharyngeal glands, labial glands, tarsal glands, and Dufour's glands performed during our own investigations (Sramkova et al., unpublished) and by other groups (Hefetz et al. 1996; Cahlíková et al. 2004) indicate that none of the most important aldehydes and the wax-type esters typical for breeding queens can be found in any of these glands. Further investigations are required, and we have indications that the wax type esters may originate from intersegmental glandular cells.

In Dufour's gland and head extracts, cuticular washings, and headspace samples of *B. hypnorum*, distinctive patterns of chemical compounds were found in queens, dominant and subordinate workers, and foragers, which were also characterized by the size of their ovarioles (Ayasse et al. 1995). Our results confirm the findings for *B. terrestris*: Qualitative and quantitative chemical analyses revealed that workers after the competition point that possess ovarioles with developed eggs produce a mixture of volatile compounds most similar to that of queens, while workers without developed ovaries showed a different odor bouquet. Also in accordance with the findings for *B. hypnorum*, the smallest—but still a significant—difference existed between workers after the competition point having developed ovarioles with and without eggs, while the volatile patterns of workers before the competition point largely differed from all other groups. In total, our results give evidence for a honest fertility signal in *B. terrestris* females and support the idea that fertility signals in workers of social bees are produced not only in the highly eusocial honey bees (Katzav-Kozansky et al. 2006) but also in primitively eusocial bumblebees.

Differences between secretions of workers and queens were also found on the egg surface. In *B. terrestris*, eggs show caste- and colony-specific chemical signals consisting mostly of mixtures of alkanes, alkenes, and alkadienes (Ayasse et al. 1999). Patterns of chemical compounds on eggs consisting of alkanes and alkenes, not even transferable to individuals by sustained contact, were also discovered on eggs of ant queens. These signals render cooperation cheaper than the pursuit of individual interests in social insect colonies as they facilitate policing (D'Ettorre et al. 2006).

In the ponerinae ant *Pachycondyla inversa*, 3,11-dimethylheptacosane was the predominant signal of queens (D'Ettorre et al. 2004), and *Harpegnathos saltator* reproductives used mainly 13,23-dimethylheptatriacontane (Liebig et al. 2000). In the highly social honeybee *Apis mellifera*, worker reproduction was recently found to be suppressed by a nonvolatile ester produced in the Dufour's gland (Katzav-Gozansky et al. 2002, 2004).

In honeybees and ants, the production of these caste- and species-specific substances is supposed to be an "honest signal" indicating to workers the benefits of investing into rearing of the current offspring (D'Ettorre et al. 2006). As soon as the signal ceases, the production of own offspring becomes the best strategy for workers starting to lay eggs as they have a closer genetic relationship to their own offspring than to the queen's sons, and it would not even be guaranteed that the reared brood originates from the queen and not from potentially even less related fellow workers. However, only some dominant workers eventually manage oviposition. In future investigations, the function of wax-type esters and aldehydes as fertility signals of egg-laying females has to be studied in behavioral experiments.

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