A Gland of Many Uses: a Diversity of Compounds in the Labial Glands of the Bumble Bee Bombus impatiens Suggests Multiple Signaling Functions

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Abstract
Communication in social insect colonies depends on signals accurately reflecting the identity and physiological state of the individuals. Such information is coded by the products of multiple exocrine glands, and the resulting blends reflect the species, sex, caste, age, task, reproductive status, and health of an individual, and may also contain caste-specific pheromones regulating the behavior and physiology of other individuals. Here we examined the composition of labial gland secretions in females of the bumble bee Bombus impatiens, of different castes, social condition, age, mating status, and ovarian activation. We show that active queens, gynes, and workers each produce caste-specific compounds that may serve different communicative functions. The composition and amounts of wax esters, mostly octyl esters produced by active queens, differed significantly between castes, mating, and social conditions, suggesting a social signaling role. Farnesyl esters were predominant in gynes and peaked at optimal mating age (6–10 days), suggesting their possible roles as sex pheromone components. Reproductive status of females and age across castes was reflected by the ratio between short- and long-chain hydrocarbons, suggesting that these compounds may serve as fertility signals. Our findings overall suggest that the labial gland composition in B. impatiens reflects different facets of female physiology. While further bioassays are required to determine the functions of these compounds, they are likely to have important roles in communication between individuals.

Keywords Labial glands · Social insects · Reproduction · Sex pheromones · Signals

Introduction

Insect societies rely on chemical signaling for regulating diverse activities ranging from foraging to reproduction, and the outcome of communication depends heavily on how accurately the signals reflect the identity and physiological state of the individuals. Semiochemicals specific to species, caste, age, task, reproductive status, and social status have been identified in numerous eusocial species and mapped to diverse glandular origins (Amsalem 2020; Billen and Šobotník 2015; Blomquist and Bagnères 2010; Keeling et al. 2004; Stokl and Steiger 2017). Some exocrine glands are specific to certain taxa, whereas others are shared across taxa. These ubiquitous glands in insects are useful tools to study the emergence of new signaling functions across species and levels of social organization.

The labial glands are an outstanding example of such glands. Together with the mandibular, hypopharyngeal, and maxillary glands, they constitute the salivary gland complex of insects. The labial glands have thoracic and cephalic compartments, located, respectively, in the thorax and the head (Poiani and Cruz-Landim 2009). These two compartments have the same origin and also likely share the same secretion in hymenopteran species that have a salivary pouch at the intersection of the compartments (Bombus and Meliponinae spp.) (Poiani and Cruz-Landim 2010), making this gland suitable for examining functions associated with social
signaling. Some hymenopteran species (e.g., *Apis mellifera*) lack this pouch and it is still debatable whether the secretion is the same (Katzav-Goazansky et al. 2001) or different (Poiani and Cruz-Landim 2010) between the two compartments, but the cephalic labial glands are well-developed only in eusocial species of Apinae in which the secretion is assumed to be associated with social roles (Poiani and Cruz-Landim 2010).

The thoracic part of the labial gland is ubiquitous across insect orders with the notable exception of Coleoptera and has been studied primarily in the context of larval feeding, digestion, and silk production (Afshar et al. 2013; Musser et al. 2006; Sehnal and Sutherland 2008), with the main components of the larval glandular secretion being various proteins and digestive enzymes (Rivera-Vega et al. 2018).

In social insects, studies have focused on the cephalic labial glands in Hymenoptera and on the thoracic labial glands in Isoptera. In the honey bee, the labial secretion was suggested to be associated with worker tasks, but the functional role was not examined (Katzav-Goazansky et al. 2001). In stingless bees, the cephalic labial glands contain a variety of wax-type esters and terpenes that serve as trail pheromones (Jarau et al. 2006; Stangler et al. 2009), and geraniol, the main compound in the secretions of nurse workers, was found to increase the proportion of larvae differentiating into queens (Jarau et al. 2010). Finally, in termites that lack cephalic labial glands, studies of thoracic labial glands found that those contain a variety of caste-specific defensive compounds, most of them volatile, such as pyrazines and benzoic acid (Sillam-Dussès et al. 2012), but also non-volatile food marking pheromones (Reinhard and Kaib 1995). Although limited to a small number of species, these studies emphasize the varied social roles of the labial gland products in social insects.

Bumble bees are an interesting group for the study of labial gland composition and function. In these species, queens experience both a solitary and a social phase during their life cycle. Newly-emerged queens (gynes) are produced in late summer at the end of the colony annual life cycle. They leave their natal colony and mate before entering a lengthy winter-diapause (Alford 1969). In spring, upon emerging from diapause, they find a nest and live a solitary lifestyle until the first worker emerges. Following that, the queen monopolizes reproduction, but only for a short period. Our previous studies (Orlova and Amsalem 2021; Orlova et al. 2020) show that the ratio between short- and long-chain hydrocarbons on the queen cuticle (below 24 and above 26 carbons, respectively) decreases throughout her life cycle as she progresses towards heading a large colony and producing sexual offspring, and the ability of a cuticular hydrocarbon extract (CHC) to inhibit worker reproduction is dependent on the social context. Workers retain the ability to reproduce and challenge the queen’s reproductive monopoly towards the end of the colony life cycle (Amsalem et al. 2015; Duchateau and Velthuis 1988).

Examining the labial gland composition during the transitions in reproductive roles throughout the life cycles of queens and workers may shed light on the function of the gland products, and adaptive changes that the glandular secretion has acquired.

In bumble bees, the content of cephalic labial glands in females has only been studied in *Bombus terrestris*. There, the cephalic labial glands exhibit quantitative differences in the amounts of fatty acid dodecyl esters between queens and workers. These esters are produced in larger quantities by sterile compared to fertile females in both castes (Amsalem et al. 2014).

Cephalic labial glands of bumble bee males, however, have been studied extensively. Males produce various terpenes and fatty alcohols that serve for territory marking (Appelgren et al. 1991; Svensson and Bergström 1977; Valterova et al. 2019), and the secretion is highly variable across species and is often used as a chemotaxonomy tool to distinguish between cryptic species (Bertsch et al. 2005).

Here we examined the cephalic labial gland secretion across different castes, social conditions, ages, and life stages in the bumble bee *Bombus impatiens*. Previous studies show that despite similarities in the life cycles of *B. terrestris* and *B. impatiens*, the Dufour’s gland and the cuticular lipid compositions are different and may have different roles (Amsalem et al. 2014, 2009; Derstine et al. 2021; Orlova et al. 2020). We examined the composition of the cephalic labial gland contents in gynes and active queens, and in workers under queenright and queenless conditions, across different ages. We discuss possible functions of these secretions in *B. impatiens* females.

**Methods and Materials**

**Bumble Bee Rearing**

Source colonies for experimental bees were obtained from Koppert Biological Systems (Howell, Michigan, USA) or Biobest Canada Ltd. (Leamington, Ontario, Canada). They were approximately 3–4 wk old with less than 30 workers each, a queen, and all stages of brood. Colonies were maintained in closed 30×30×22.5 cm nest-boxes in a growth chamber at 28–30 °C, 60% relative humidity, and constant darkness, and were supplied ad libitum with a 60% sugar solution and honeybee-collected pollen (Koppert Biological Systems, Howell, Michigan, USA). Queens and workers used in the study were the same as in Derstine et al. (2021). Briefly, all workers were collected upon emergence (<24 h old) from 10 colonies before the colonies produced gynes and males. Newly emerged workers were individually...
marked at the time of collection and randomly assigned to one of three treatments: queenright (QR, n = 70), queenless (QL, n = 70), and queenless broodless (QLBL, n = 70). QR workers were returned to their natal QR colony until they reached the desired age, while QL and QLBL workers were housed in plastic cages (11 cm diameter × 7 cm height) in groups of 3–6 workers without a queen until they reached the desired age of sampling. Queenless groups of workers typically lay eggs within 6–8 d (Amsalem et al. 2015), and because the presence of brood affects worker reproduction (Starkey et al. 2019), we included a group without brood. In the QL groups, eggs laid by workers were left intact, while in the QLBL groups, eggs laid by workers were removed daily. We collected 5 workers of each age (days 1–14) in each treatment (70 workers/treatment). All workers were stored at −80°C until dissection. Twenty active queens that were all mated and laying eggs (hereafter, “active queens”) were obtained from twenty full-sized colonies with >100 workers. These queens were several months old and were actively producing female workers prior to sampling. Newly emerged, unmated queens (hereafter “gynes”; n = 20) were collected from 3 colonies. Gynes were separated from their natal colonies upon emergence to prevent mating, housed in small cages in groups of 3–5 gynes, and sampled at 4 time points: 3, 6, 10, and 14 d after emergence (5/time point). All sampled individuals were examined for reproductive status and labial gland composition.

**Ovarian Activation**

Ovaries were dissected under a stereomicroscope in distilled water, and the largest three terminal oocytes across both ovaries (at least one from each ovary) were measured with an eyepiece micrometer. The mean of these three oocyte measurements was recorded as mean terminal oocyte size and used in all analyses except when PERMANOVA was conducted (see below). This analysis required the use of a categorical variable for ovaries. Therefore, ovary stages were classified into four categories using the mean terminal oocyte size as follows: 1 – undeveloped ovaries (oocytes < 1 mm), 2 – partial development (1–2 mm), 3 – advanced development (2–3 mm) and 4 – full development (>3 mm).

**Preparation and Analysis of Labial Gland Extracts**

After freeze killing, both cephalic labial glands were dissected out of the head capsule by opening the sclerotized cuticle of the head capsule with a forceps and separating the two clusters of gland acini (i.e., small saclike cavities that form the glands) from the surrounding tissue using a fine forceps. The clusters of acini were then placed in a vial with 50 µl hexane with 100 ng pentadecane as an internal standard. The vials were stored at -20°C. Prior to GC analysis, samples were evaporated to a volume of 10 µl, of which 1 µl was analyzed with an Agilent 7890A GC equipped with a HP-5 ms column (0.25 mm id × 30 m × 0.25 µm film thickness, Agilent, Santa Clara CA, USA) and interfaced to an Agilent 5975C mass selective detector operated in electron impact ionization mode (70 eV). The temperature program was 60°C to 120°C at 15°C/min, then 4°C/min to 300°C (5 min hold). The injector port and FID were held at 250°C and 320°C, respectively. Compounds were tentatively identified based on diagnostic ions in the resulting spectra and retention indices relative to straight-chain alkanes. For unsaturated compounds, the locations of double bonds were not determined in this study, and the double bond positions and geometries for compounds listed in Table 1 are tentative. Where possible, supporting evidence for tentative identifications was obtained by matching retention times and mass spectra with those of authentic standards of known structure. Mass spectra of selected compounds are provided as supplementary material (Figure S1).

Compounds in labial gland extracts were quantified on a Trace 1310 GC (Thermo Fisher, Waltham, MA, USA) equipped with a flame-ionization detector (FID) and a TG-5MS column (0.25 mm id × 30 m × 0.25 µm film thickness, Thermo Fisher). The temperature program and conditions were the same as above.

**Synthesis of Ester Standards**

Approximately 40 wax esters and terpenoid esters were synthesized by one of three methods, as represented by the following examples. A full list of the esters, and the methods used to synthesize and purify each one is provided in Table S1. Depending on their properties, synthesized compounds were purified by one or more of vacuum flash chromatography, vacuum distillation, or low-temperature recrystallization (see Table S1).

Method A (example, (E,E)-farnesyl linoleate): (E,E)-Farnesol (0.222 g, 1 mmol), linoleic acid (0.281 g, 1 mmol), 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (0.384 g, 2 mmol), and a few crystals of dimethylaminopyridine catalyst were dissolved in 20 ml CH₂Cl₂ and stirred overnight at room temp. The following morning, the solvent was removed by rotary evaporation, and the residue was portioned between hexane and water. The hexane layer was washed sequentially with 1 M aqueous HCl and brine, dried over anhydrous Na₂SO₄, and concentrated. The residue was purified by vacuum flash chromatography on silica gel, eluting with 7.5% EtOAc in hexane.

Method B (example decyl myristate): Myristoyl chloride (1.24 g, 5 mmol) was added by syringe pump over 30 min to a solution of decanol (0.95 g, 6 mmol), pyridine (0.4 g, 5 mmol), and a few crystals of dimethylaminopyridine catalyst in 25 ml CH₂Cl₂ at room temp, and the mixture was
Table 1  Relative percentages of compounds in labial gland secretion presented as mean ± SE. For all compounds in bold, identification has been confirmed with authentic standards. UD stands for undetectable. RI stands for retention index. The double bond positions and geometries in compounds marked with asterisks are tentative, although their retention times and mass spectra matched those of standards with the double bond positions and configurations shown.

| Compound name                  | Class               | RI  | Mean percentage (%) ± SE | Specificity |
|--------------------------------|---------------------|-----|--------------------------|-------------|
|                                |                     |     | Active queens (n = 19)    | Gynes (n = 20) | QL workers (n = 67) | QLBL workers (n = 70) | QR workers (n = 69) |
| (E)-β-farnesene myristic acid  | terpenoid           | 1511| 0.79 ± 0.07              | UD          | UD          | UD          | Gynes          |
| 2-heptadecanone                | free fatty acid     | 1728| 0.13 ± 0.04              | 1.02 ± 0.12 | UD          | UD          | Queens         |
| unknown                        | unknown             | 1898| 0.34 ± 0.05              | 0.08 ± 0.01 | 0.41 ± 0.03 | 0.40 ± 0.02 | 0.55 ± 0.03 |
| unknown 1                      | unknown             | 1909| 0.09 ± 0.02              | UD          | UD          | UD          | Workers        |
| unknown 2                      | terpenoid           | 1914| 0.07 ± 0.02              | 0.07 ± 0.02 | UD          | UD          | Queens         |
| methyl palmitate               | methyl/ethyl ester  | 1921| 1.22 ± 0.16              | 1.61 ± 0.37 | 0.88 ± 0.13 | 2.10 ± 0.28 |               |
| palmitic acid                  | free fatty acid     | 1970| 0.39 ± 0.11              | 0.53 ± 0.07 | 0.10 ± 0.01 | 0.04 ± 0.01 | 0.13 ± 0.01 |
| heneicosane                    | hydrocarbon         | 2100| 0.94 ± 0.09              | 1.12 ± 0.12 | 1.38 ± 0.16 | 1.13 ± 0.12 | 1.25 ± 0.11 |
| methyl oleate*                 | methyl/ethyl ester  | 2108| 0.90 ± 0.26              | 0.08 ± 0.02 | 0.08 ± 0.01 | 0.07 ± 0.02 | 0.22 ± 0.03 |
| oleic acid* + stearic acid     | free fatty acid     | 2199| 9.76 ± 1.29              | 2.20 ± 0.28 | 2.05 ± 0.22 | 3.20 ± 0.33 | 2.81 ± 0.34 |
| (Z)-9-tricosene*               | hydrocarbon         | 2273| 15.3 ± 1.5               | 4.18 ± 0.60 | 24.1 ± 0.7  | 21.8 ± 0.7  | 22.1 ± 0.70 |
| tricosane                      | hydrocarbon         | 2300| 8.28 ± 0.50              | 2.68 ± 0.30 | 10.5 ± 0.3  | 10.7 ± 0.4  | 8.31 ± 0.20 |
| tetracosane*                   | hydrocarbon         | 2373| 1.17 ± 0.11              | 0.27 ± 0.03 | 1.51 ± 0.03 | 1.40 ± 0.04 | 1.33 ± 0.03 |
| tetracosane                    | hydrocarbon         | 2400| 0.22 ± 0.02              | 0.05 ± 0.00 | 0.42 ± 0.01 | 0.43 ± 0.01 | 0.34 ± 0.01 |
| (Z)-9-penta-cosene*            | hydrocarbon         | 2477| 18.1 ± 1.3               | 9.73 ± 0.86 | 29.9 ± 0.4  | 28.9 ± 0.5  | 25.7 ± 0.50 |
| pentacosane                    | hydrocarbon         | 2500| 3.52 ± 0.29              | 1.15 ± 0.06 | 7.50 ± 0.20 | 7.36 ± 0.20 | 6.06 ± 0.18 |
| octyl palmitoleate* + decyl myristoleate* | wax ester | 2536| 0.24 ± 0.03              | UD          | UD          | UD          |               |
| octyl palmitoleate + decyl myristate | wax ester | 2569| 1.37 ± 0.20              | UD          | UD          | UD          |               |
| hexacosene*                    | hydrocarbon         | 2571| 0.24 ± 0.01              | 0.35 ± 0.01 | 0.38 ± 0.02 | 0.33 ± 0.02 |               |
| hexacosane                     | hydrocarbon         | 2600| 0.07 ± 0.01              | 0.26 ± 0.03 | 0.11 ± 0.00 | 0.1 ± 0.00  | 0.08 ± 0.00 |
| heptacosane                    | hydrocarbon         | 2676| 2.47 ± 0.43              | 4.55 ± 0.27 | 5.29 ± 0.28 | 5.04 ± 0.31 | 4.62 ± 0.33 |
| heptacosane                    | hydrocarbon         | 2700| 0.70 ± 0.10              | 0.97 ± 0.08 | 1.72 ± 0.09 | 1.56 ± 0.09 | 1.44 ± 0.09 |
| octyl oleate*                  | wax ester           | 2755| 9.38 ± 1.19              | 0.07 ± 0.02 | 0.13 ± 0.04 | 0.32 ± 0.10 | 0.16 ± 0.03 |
| unknown 3                      | unknown             | 2763| 0.01 ± 0.00              | UD          | UD          | UD          | Gynes          |
| dodecyl myristate              | wax ester           | 2764| 0.01 ± 0.00              | 0.02 ± 0.00 | 0.02 ± 0.00 | UD          | Workers        |
| decyl palmitate + octyl stearate| wax ester           | 2770| 0.32 ± 0.07              | UD          | UD          | UD          | Active Q       |
| Octacosene*                    | hydrocarbon         | 2771| 0.11 ± 0.01              | 0.31 ± 0.04 | 0.26 ± 0.03 | 0.26 ± 0.03 |               |
| unknown 4                      | unknown             | 2780| 0.17 ± 0.03              | UD          | UD          | UD          | Gynes          |
| (E,E)-farnesyl dodecanoate*    | terpenoid           | 2799| 0.14 ± 0.04              | 0.70 ± 0.10 | UD          | UD          | Queens         |
| octacosane                     | hydrocarbon         | 2800| UD                       | 0.06 ± 0.01 | 0.08 ± 0.01 | 0.04 ± 0.00 |               |
| squalene                       | terpenoid           | 2815| 0.09 ± 0.01              | UD          | 0.11 ± 0.02 | 0.16 ± 0.03 | 0.14 ± 0.02 |
| nonacosene*                    | hydrocarbon         | 2878| 1.56 ± 0.25              | 2.81 ± 0.22 | 2.71 ± 0.13 | 2.68 ± 0.18 | 2.38 ± 0.17 |
| nonacosane                     | hydrocarbon         | 2900| 0.31 ± 0.04              | 0.24 ± 0.02 | 0.83 ± 0.03 | 0.77 ± 0.04 | 0.74 ± 0.04 |
| geranyl linoleate*             | terpenoid           | 2944| 0.45 ± 0.04              | 0.03 ± 0.00 | 0.03 ± 0.00 | 0.08 ± 0.00 |               |
| Compound name                      | Class                  | RI     | Mean percentage (%) ± SE | Specificity       |
|-----------------------------------|------------------------|--------|--------------------------|-------------------|
| (E,E)-farnesyl myristate*         | terpenoid              | 2945   | 0.20 ± 0.06               | Active Q          |
| decyl oleate*                     | wax ester              | 2955   | 3.92 ± 0.52               | UD                |
| dodecyl palmitate                 | wax ester              | 2966   | 0.18 ± 0.15               | UD                |
| (E)-dihydro-farnesyl myristate*   | terpenoid              | 2966   | 0.05 ± 0.01               | UD                |
| triacontene*                      | hydrocarbon            | 2977   | 0.08 ± 0.02               | UD                |
| unknown 5                         | unknown                | 2987   | 0.12 ± 0.09               | 0.10 ± 0.01       |
| unknown 6                         | unknown                | 2994   | 0.06 ± 0.02               | 0.10 ± 0.01       |
| triacontane                       | hydrocarbon            | 3000   | UD                        | 0.04 ± 0.01       |
| unknown 7                         | unknown                | 3003   | 0.05 ± 0.01               | 0.04 ± 0.01       |
| hentriacontene*                   | hydrocarbon            | 3089   | 1.06 ± 0.17               | 1.65 ± 0.1        |
| unknown 8                         | unknown                | 3118   | 0.10 ± 0.03               | 0.29 ± 0.01       |
| unknown 9                         | unknown                | 3126   | UD                        | 0.01 ± 0.00       |
| unknown 10                        | unknown                | 3139   | 2.77 ± 0.33               | 0.32 ± 0.01       |
| (E)-dihydro-farnesyl palmitolate* | terpenoid              | 3145   | 0.05 ± 0.01               | 0.31 ± 0.01       |
| dodecyl oleate*                   | wax ester              | 3145   | 0.52 ± 0.08               | 0.43 ± 0.07       |
| (E)-dihydro-farnesyl palmitate*   | terpenoid              | 3168   | 0.15 ± 0.02               | UD                |
| dodecyl linoleate*                | wax ester              | 3174   | UD                        | 0.03 ± 0.00       |
| (E,E)-farnesyl palmitoleate*      | terpenoid              | 3183   | 0.25 ± 0.04               | UD                |
| dotriacontane                     | hydrocarbon            | 3200   | UD                        | 0.08 ± 0.01       |
| (E,E)-farnesyl palmitate*         | terpenoid              | 3208   | 0.18 ± 0.05               | UD                |
| unknown 11                        | unknown                | 3237   | 0.15 ± 0.09               | 0.12 ± 0.02       |
| unknown 12                        | unknown                | 3248   | 0.17 ± 0.11               | 0.09 ± 0.01       |
| unknown 13                        | unknown                | 3260   | UD                        | 0.20 ± 0.02       |
| Tritriacontene*                   | hydrocarbon            | 3276   | 0.11 ± 0.02               | 0.16 ± 0.01       |
| Myristoleyl oleate* + oleyl myristoleate* | wax ester         | 3334   | 0.58 ± 0.04               | 0.31 ± 0.01       |
| (E)-dihydro-farnesyl linoleate*   | terpenoid              | 3351   | 1.41 ± 0.20               | UD                |
| (E,E)-farnesyl oleate*            | terpenoid              | 3402   | 1.95 ± 0.33               | UD                |
| unknown 14                        | unknown                | 3409   | UD                        | 0.25 ± 0.04       |
| (E,E)-farnesyl linoleate*         | terpenoid              | 3461   | 0.38 ± 0.05               | UD                |
| unknown 15                        | unknown                | 3461   | UD                        | 0.18 ± 0.05       |
| palmitoyl oleate*                 | wax ester              | 3605   | 5.92 ± 1.72               | 1.82 ± 0.19       |
| unknown 16                        | unknown                | 3655   | 2.78 ± 0.75               | 0.19 ± 0.02       |
| unknown 17                        | unknown                | 3668   | 0.62 ± 0.20               | UD                |

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stirred overnight. The solvent was then removed by rotary evaporation and the residue was partitioned between water and hexane. The hexane layer was washed successively with 1 M aqueous HCl and brine, dried over anhydrous Na₂SO₄, and concentrated. The residue was purified by vacuum flash chromatography on silica gel, eluting with 7.5% EtOAc in hexane. The purified compound was then recrystallized from 15 ml acetone at -20 °C overnight, filtering the resulting mixture in a cold room, producing the purified compound as low-melting white plates.

### Statistical Analysis

Statistical analyses were performed using SPSS v.21 and R Studio. Permutational multivariate analysis of variance (PERMANOVA, adonis, and adonis2 functions in R) were used to compare chemical profiles in their entirety between groups. Similarity percentage analysis (simper function in R) was used to identify the components contributing to distinction between groups. Prior to analysis, relative amounts of compounds were Z-transformed with a mean of 4 and standard deviation of 1 to avoid negative values. Colony identity was always used as the first term in PERMANOVA with the term of interest being added second to avoid overestimation of its contribution to variance. Pseudo-F and p-values for terms of interest are reported in the results after accounting for variance between colonies.

Generalized Linear Mixed Model analysis was performed to assess the effect of continuous and categorical factors on the relative amounts of major classes of compounds in the gland. Robust estimation was used to handle violations of model assumptions (Ghosh and Basu 2016). In all analyses, we used treatment group (QL workers, QLBL workers, QR workers, gynes, and active queens) as the main effect followed by post-hoc contrast estimation using the Least Significant Difference (LSD) method. Colony identity was used as a random effect in all analyses involving workers. Satterthwaite correction was employed to account for small and unequal sample sizes (Loh 1987; Yau and Kuk 2002). Generalized Linear Mixed Model analysis was performed on standardized values (Z-scores) of oocyte size and relative amounts of compounds to obtain standardized beta coefficients. Statistical significance was accepted at \( \alpha = 0.05 \).

### Results

#### Ovarian Activation

As expected, active queens had fully activated ovaries (mean oocyte length, 3.4 ± 0.04 mm) while gynes had inactivated ovaries (0.82 ± 0.04 mm). In gynes, oocyte size significantly changed with age (GLMM, \( F_{3,16} = 68.3, p < 0.001 \)), being smallest on day 3 (0.54 ± 0.02 mm) and peaking on day 10 after emergence as adults (1.03 ± 0.02 mm).
Oocyte size in workers was significantly affected by social condition and age, with a significant interaction between the two (GLMM, $F_{13,118} = 18.86$, $p < 0.001$ for age, $F_{2,69} = 38.08$, $p < 0.001$ for social condition, $F_{26,125} = 2.13$, $p = 0.003$ for interaction). QLBL workers had larger oocytes than both QL and QR workers (mean oocyte length, $1.97 \pm 0.15$ mm for QLBL, $1.35 \pm 0.13$ mm for QL and $1.11 \pm 0.12$ mm for QR groups, $n = 70$ per each group) (post-hoc LSD pairwise contrast, $p < 0.01$) and QL workers, on average, had larger oocytes than QR workers (post-hoc LSD pairwise contrast, $p < 0.01$). Oocyte size in all worker groups started increasing on day 2 and reached a plateau on day 8 (post-hoc LSD pairwise contrast, $p < 0.01$ for all comparisons between days 1–8, $p > 0.05$ for later time points).

**Identification of Gland Constituents**

The chemical analyses of the cephalic labial glands showed a total of 79 compounds in queens and workers, of which 53 were conclusively or tentatively identified, and 26 remain unknown at present.

![Fig. 1 Beta-dispersion of the relative proportions of the cephalic labial gland components considering caste (A), worker treatment (B) and worker age (C) as grouping variables. The plots display the first two principal coordinates](image-url)
(Table 1, Figure S2). All compounds were used in subsequent discriminant analyses (Fig. 1) but only known compounds were used in further analyses (Figs. 2, 3 and 4). The main ions of the unknown compounds are provided as supplementary material (Table S2). The secretion was composed mainly of hydrocarbons ranging from 21 to 33 carbons, fatty acids, wax esters, and terpenoid esters. Of these, 41 were ubiquitous in all groups while 38 compounds (20 of them identified) were present only in specific groups. Nine compounds were specific to active queens (mostly wax esters), ten to gynes (mostly terpenoids), nine to the queen caste as a whole (mostly terpenoids), and another ten to workers (mostly hydrocarbons and wax esters) (Table 1). The mean relative percentages and the absolute amounts of individual compounds in each of the examined groups are provided in Table 1 and the mean relative percentages of the main classes of compounds are provided in Table S3.

**Discriminant Analyses**

The cephalic labial gland profiles of all bees were analyzed by PERMANOVA using standardized relative quantities of substances. Active queens, gynes, and workers differed significantly from one another (Pseudo-$F_4 = 30.39$, $R^2 = 0.16$, $p = 0.001$) (Fig. 1A). Compounds contributing to the difference between gynes and other females included

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**Fig. 2** Relative proportions of terpenoid compounds in bees of different castes and treatment groups (A), and in gynes of different ages (B). Letters above columns denote significant differences at $\alpha = 0.05$.
various terpenoid compounds (cumulative percentage of variance > 40% for all comparisons), while compounds differentiating between active queens and other females included octyl esters (cumulative percentage of variance > 20% for all comparisons).
Because large differences between castes may have obscured differences between treatments and ages in workers, we analyzed the data of workers separately. Worker groups also differed significantly (Pseudo-F2 = 11.63, R2 = 0.08, p = 0.001) with QR workers being distinguished from other workers by a series of unknown compounds characterized by a base peak at m/z = 95 (unknowns 14 and 16 in Table 1, cumulative percentage of variance > 10%) (Fig. 1B).

Workers differed significantly by age (Pseudo-F13 = 5.88, R2 = 0.22, p = 0.001), with one-day-old workers being separated from other ages by the hydrocarbon compounds (cumulative percentage of variance > 30%) (Fig. 1C). Workers with different stages of ovary activation also differed significantly (Pseudo-F3 = 10.92, R2 = 0.11, p = 0.001) with workers having undeveloped ovaries (stage 1) being distinguished from all other workers by the most abundant hydrocarbon compounds (cumulative percentage of variance > 30%).

Based on the discriminant analysis information, further analyses of the labial gland secretions were done using three major classes of compounds: 1) terpenoid compounds, comprising farnesene, farnesyl esters, dihydrofarnesyl esters, and geranyl esters, 2) wax esters, with the alcohol moiety chain lengths ranging from 8 to 18 carbons and the acid moiety chain lengths ranging from 14 to 22 carbons, 3) hydrocarbons with chain lengths ranging from 21 to 33 carbons. The relative proportion of each compound class in the total secretion was calculated and used in further analyses. The ratio of short- (≤ 24 carbons) to long-chain hydrocarbons (≥ 26 carbons) was also calculated.

**Terpenoid Components**

GLM analysis revealed that the proportions of terpenoid components differed significantly between all groups and were highest in gynes, where they comprised up to 68% of the total secretion, and lowest in QL workers (0.1% of the total secretion) (GLMM, F4,227 = 75.66, p < 0.0001 for group, p < 0.0001 for all post hoc comparisons) (Fig. 2A). Among gynes of different ages, terpenoid compound proportions peaked on days 6 and 10 and declined on day 14, without covariance with oocyte size or interaction between oocyte size and age (GLMM, F3,12 = 9.19, p = 0.002 for age, F1,12 = 1.49, p = 0.24 for oocyte size, F3,12 = 0.4, p = 0.75 for interaction) (Fig. 2B).

**Wax Ester Components**

The proportion of wax esters was highest in active queens (on average 23%) and lowest in QL workers (on average 2.9%) (GLMM, F4,240 = 52.20, p < 0.0001 for group, p < 0.05 for all post hoc comparisons). However, the composition of wax esters differed between groups, with octyl esters being almost exclusively present in active queens and dodecyl ester proportions being highest in active queens, QR, and QLBL workers and undetectable in gynes, which almost exclusively produced long-chain esters (> 32 carbons in total) (Fig. 3). Wax ester proportion in gynes was not significantly explained by either age or by oocyte size (GLMM, F3,12 = 1.82, p = 0.19 for age, F1,12 = 1.1, p = 0.31 for oocyte size, F3,12 = 0.47, p = 0.70 for interaction).

Following our findings on caste differences in the abundance of different wax esters, we performed a PERMANOVA based solely on ester compounds. The groups differed from one another significantly (Pseudo-F2 = 21.59, R2 = 0.10, p = 0.001). Active queens differed from all other females by the proportions of octyl esters (cumulative percentage of variance > 30%), gynes differed from all other females by the proportions of very long chain esters (> 32 carbons) (cumulative percentage of variance > 30%), and workers of different social conditions differed in the proportions of dodecyl esters and palmityl octadecenoate (cumulative percentage of variance > 70%).

**Differences in Compound Classes Between Workers of Different Ages and Treatments**

Based on the results of the discriminant analysis, we tested whether treatment group, age, and oocyte size predicted the relative proportion of wax esters and the series of unknown compounds with m/z 95 mass spectral base peak. Wax ester proportion was significantly predicted by age and treatment group, being highest in QR workers and at later ages (day 11 and later) but not by oocyte size, with significant interaction between age and treatment (GLMM, F13,138 = 1.96, p = 0.028 for age, F2,114 = 23.12, p < 0.0001 for treatment, F26,120 = 2.23, p = 0.002 for interaction between age and treatment, p > 0.05 for covariance with oocyte size and interaction between oocyte size and other terms). The proportion of unidentified compounds with m/z 95 base peak compounds was significantly predicted by age, treatment group (highest in QR workers), and oocyte size, with significant interaction between age and treatment and age and oocyte size (GLMM, F13,135 = 2.58, p = 0.003 for age, F2,112 = 24.17, p < 0.0001 for treatment, F26,111 = 2.33, p = 0.001 for interaction between age and treatment, F1,147 = 5.08, p = 0.026 for covariance with oocyte size, F2,147 = 0.94, p = 0.39 for interaction between treatment and oocyte size and F3,147 = 1.81, p = 0.046 for interaction between age and oocyte size).
Hydrocarbon Composition and Ovarian Development

In line with a previous study (Orlova et al. 2020), the short- to long-chain hydrocarbon ratio was highest in active queens (5.21 ± 0.16) and lowest in gynes (0.82 ± 0.09) (GLMM, F_{4,240} = 96.65, p < 0.0001 for group, post-hoc LSD: p < 0.0001 for active queen vs. other groups, p < 0.0001 for gyn vs. other groups, p > 0.05 for comparisons between worker treatments). In workers, short- to long-chain hydrocarbon ratio was on average 3.17 ± 0.08 and was significantly predicted by age and treatment group and oocyte size, peaking on day 8 and being initially higher in QL and QL workers, and then in QR workers at later ages with significant interaction between age and treatment (GLMM, F_{3,142} = 2.37, p = 0.007 for age, F_{2,88} = 4.65, p = 0.012 for treatment, F_{26,134} = 1.92, p = 0.009 for interaction between age and treatment, F_{1,141} = 8.14, p = 0.005 for covariance with oocyte size, p > 0.05 for interaction of oocyte size with other terms). In gynes, short- to long-chain hydrocarbon ratio peaked on day 14 and displayed no covariance with oocyte size, but there was significant interaction between age and treatment (GLMM, p = 0.001 for interaction). When the relationship between age and treatment (GLMM, p = 0.012 for age, F_{1,12} = 51.04, p < 0.0001 for age, F_{1,12} = 3.2, p = 0.098 for oocyte size, F_{1,12} = 12.16, p = 0.001 for interaction). When the relationship between short to long CHC ratio was analyzed separately using regression curve estimation, polynomial regression with cubic fit proved the best fitting curve (R = 0.57, R^2 = 0.325, F_{3,202} = 32.39, p < 0.0001) (Fig. 4).

Discussion

Our analysis of the cephalic labial gland secretions revealed a diversity of compounds representing a number of different chemical classes. This structural diversity, and the substantial differences in composition between bees of differing caste, age, and social condition allude to diverse roles played by the different compounds. Some of these differences, such as the abundance of terpenoids in gynes and the octyl esters in queens, parallel those found in other secretions of *B. impatiens* and *B. terrestris* (Amsalem et al. 2014, 2009; Derstine et al. 2021). Overall, we showed strong associations of terpenoid compounds with caste and mating status, of esters with social condition, and of the hydrocarbon profile with reproductive status.

Terpenoid compounds were predominant in gynes. These compounds comprised 40–60% of the total secretion, and their amounts peaked in gynes aged 6 to 10 days, coinciding with the age range optimal for mating (Treanore et al. 2021). This finding suggests that terpenoid compounds may play a role in mating in bumble bee queens. Terpenoid compounds were also found to play a role in territory marking and mating in bumble bee males (Bergman and Bergström 1997), although males produce predominantly low molecular weight terpenes like farnesol, whereas, in queens (this study), terpenoids are mainly represented by farnesyl esters of unsaturated fatty acids. The low volatility of these esters suggests that if they do have a signaling role, they are likely short-range signals that are perceived upon contact. Interestingly, terpenoid compounds, albeit of a different structure, were found to be the distinguishing feature of the Dufour’s gland secretion of *B. impatiens* gynes (Derstine et al. 2021), where they may also serve as sex pheromones. The similarity in compounds across species, sexes, and castes may point to evolutionary constrains on chemical diversity and perhaps an adoption of the same chemicals for different functions. For example, previous studies found that terpenoid compounds were produced by the same metabolic pathway as juvenile hormones in non-social insects (Engel et al. 2016). We know very little about the levels of juvenile hormone in bumble bee queens before and after mating and exploring the relationship between juvenile hormone level and terpenoid production (and the changes caused in these parameters by mating) could be a productive avenue of research.

The amount and identity of wax ester components were a differentiating factor across castes. Specifically, active queens, gynes, and workers differed in the composition of non-terpenoid esters, and the differences we observed in the labial glands mirror trends previously determined for Dufour’s gland secretions of *Bombus impatiens*. Workers were characterized by dodecyl esters, whereas gynes produced no dodecyl esters at all, but produced predominantly longer esters with 14–18 carbons in the alcohol moiety and 18–20 carbons in the acid moiety. This suggests that common biosynthetic pathways are activated in different glands, or alternatively, that esters are produced outside of the glands, possibly in the fat body, and are transported separately to different glands. Mechanisms regulating ester biosynthesis are not yet well characterized in bumble bees. The predominance of dodecyl esters in workers and octyl esters in queens of *B. impatiens* mirrors the contents from analyses of cephalic labial gland secretions of *B. terrestris* (Amsalem et al. 2014). Overall, aliphatic esters were by far most abundant in the labial glands of active queens and QR workers, and least abundant in gynes. The common characteristic of active queens and QR workers is, perhaps, due to the fact that they were sampled from a fully functional large colony, unlike gynes and QL and QLBL workers, which were reared in small groups. The abundance of esters in these
bees might suggest a social communication function, but, alternatively, esters might be used for their physical properties, for example, in building and repair of wax cells. Labial gland esters have been implicated in nest building in solitary bees but their function in social species is as yet unknown.

Hydrocarbons made up a large part of the cephalic labial gland secretions in all castes. The ratios of short- to long-chain hydrocarbons in the labial glands displayed the same trend as hydrocarbons on the cuticle, where active queens have the highest short- to long-chain hydrocarbon ratio, and gynes have the lowest (Orlova et al. 2020). Additionally, in both gynes and workers, the change in the ratio occurs in tandem with ovarian development, and the terminal oocyte size is significantly correlated with the short- to long-chain hydrocarbon ratio. This suggests that in bumble bees, hydrocarbon synthesis is associated with oogenesis and might serve as a fertility signal, as was previously shown in solitary insects (Blomquist and Bagnères 2010).

Finally, we observed an intriguing set of unidentified relatively heavy (likely molecular weights 430–530 amu) compounds characterized by a base peak at m/z 95. The proportions of these compounds were not large (0.5–4% of total secretion) but they discriminate significantly between castes and between different treatment groups in workers, in a similar manner to esters. As with the ester components, the proportion of these compounds increased with age, and their amounts significantly correlated with ester amounts. Further attempts are in progress to try and identify these compounds and understand the cause of their co-occurrence with esters.

Overall, our analysis of labial gland secretion compositions revealed differences between castes, social conditions, and physiological states in both queens and workers, and allowed us to formulate several hypotheses about the possible functions of the cephalic labial gland compounds. The terpenoid esters which are abundant in gynes may act as a sex pheromone, while the wax esters may have a social signaling function. The ratio of short- to long-chain hydrocarbons may be associated with or regulated by oogenesis and may signal fertility. Testing these hypotheses will require further research involving behavioral assays, and elucidation of the physiological and molecular mechanisms underlying the biosynthesis of different classes of compounds.

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