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Mandibular glands secrete 24-methylenecholesterol into honey bee (*Apis mellifera*) food jelly

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ABSTRACT

Honey bee (*Apis mellifera*) workers feed their larvae with food jelly that is secreted by specialized glands in their heads – the hypopharyngeal and the mandibular glands. Food jelly contains all the nutrients the larvae need to develop into adult honey bees, including essential dietary sterols. The main sterol in food jelly, 24-methylenecholesterol (24MC), is pollen-derived and delivered in food jelly to the larvae in a complex with two proteins, major royal jelly protein 1 (MRJP1) and apisim. Whereas the proteins are synthesized in the hypopharyngeal glands, the sterol-secreting gland has not been identified. We here identified the mandibular glands as sterol-secreting gland for food jelly production by direct detection of the four main honey bee sterols (24MC, campesterol, β-sitosterol and isofucosterol). Furthermore, 24MC seems to be specifically enriched in the mandibular glands, thereby ensuring that food jelly contains the amounts of 24MC necessary for complex formation with MRJP1 and apisimin.

1. Introduction

Honey bee (*Apis mellifera*) queen larvae are fed by young worker bees (nurse bees) with a jelly-like glandular secretion aptly named royal jelly (RJ) which provides the nutrients necessary for development (Huber, 1792; Rembold, 1983; Swammerdam, 1738; von Planta, 1888). As queen cells are vertically oriented on the comb and open downwards (Aristotle, 350 BCE), queen larvae do not only feed on but are also held in place on the cell ceiling by the viscous royal jelly (Buttstedt et al., 2018; Kurth et al., 2019; Pirk, 2018). The basis for royal jelly's viscosity is determined by a complex of two proteins (major royal jelly protein 1 (MRJP1) and apisimin) and a sterol (24-methylenecholesterol (24MC)) (Tian et al., 2018)). The complex polymerizes at the acidic, native pH of royal jelly into long fibrous structures thereby increasing viscosity (Buttstedt et al., 2018; Kurth et al., 2019). Food jelly is not only fed to queen larvae, but also to young worker and drone larvae, albeit in lesser amounts (von Planta, 1888). Thus, food jelly is the only source of sterol for young larvae, and it is assumed that the complex of $MRJP1_4$/$apisimin_4$/$24MC_8$ in the jelly provides queen, worker and drone larvae with this vital sterol for their development.

Food jelly is a product of two specialized head glands; the hypopharyngeal (HPGs) and the mandibular glands (MDGs) (Kratky, 1931; Schiemenz, 1883). It has been repeatedly shown that proteins are contributed by the HPG secretion to food jelly (Dobritzsch et al., 2019; Knecht and Kaatz, 1990; Patel et al., 1960) while fatty acids are added from the MDG secretion (Callow et al., 1959). This secretion contains in worker bees fatty acids with ten carbon atoms (C10), primarily 10-hydroxy-2-decenoic acid (10-HDA) as well as its precursor 10-hydroxy-decanoic acid (10-HDAA) and minor amounts of 9-oxo-2--decenoic acid (9-ODA) and its precursor 9-hydroxy-2-decenoic acid (9-HDA) (Brown et al., 1961; Butenandt and Rembold, 1957; Callow et al., 1959; Wang et al., 2016). [The fatty acid composition in worker MDGs (Crewe and Velthuis 1980) is dependent on *A. mellifera* subspecies and colony state (for reviews see Mumoki et al., 2021; Mumoki and Crewe, 2021).] 10-HDA increases RJ acidity (Mokaya et al., 2020) and facilitates fibril formation of the $MRJP1_4$/$apisimin_4$/$24MC_8$ complex via lowering RJ pH (Buttstedt, 2022). Furthermore, it exhibits antibiotic activity thus preventing the growth of microorganisms in food jelly (Blum et al., 1959). Fatty acids are ingested by nurse bees with their pollen diet mostly as C16 and C18 fatty acids and subsequently shortened in the MDGs to C10 fatty acids (Plettner et al., 1996; for review see Wright et al., 2018). Like fatty acids, sterols are also ingested with the pollen diet (Svoboda et al., 1983a; for review see Wright et al., 2018). Sterols are essential membrane components, that regulate membrane

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permeability and fluidity, and are precursors for the synthesis of hormones (Hartmann, 1998), such as makisterone A in honey bees (Feldlaufer and Svoboda, 1986). Whereas plants predominantly synthesize phytosterols with 28 or 29 carbons (Hartmann, 1998), the most important zoosterol in animals is usually C_{27} cholesterol (for review see Jing and Behmer, 2020). Insects are, with few exceptions, unable to synthesize sterols *de novo* (Ritter and Wientjens, 1967). Thus, they must acquire sterols from their diet. In contrast to many other insects, honey bees are not able to dealkylate C_{28} and C_{29} phytosterols into C_{27} cholesterol for further use, but rather incorporate phytosterols, mainly 24MC, directly into their membranes (for review see Furse et al., 2023; Svoboda et al., 1980, 1983b). Thus, all honey bee membranes contain a mix of phytosterols and 24MC and other phytosterols are integral components of the membranes of HPGs and MDGs. This complicates the identification of the gland that adds 24MC to food jelly as a distinction needs to be made between 'membrane' sterols and 'secreted' sterols. Early studies attempting to identify the gland secreting 24MC, reported a higher relative percentage of 24MC in HPGs (48%) than in MDGs (36%) (Svoboda et al., 1986). However, the inability to distinguish between membrane bound 24MC as opposed to that being secreted, made it impossible to identify the gland that was secreting 24MC. Thus, the origin of 24MC in food jelly remains to be determined and hence it is unclear whether the complex of $MRJP1_4$/$apisimin_4$/$24MC_8$ is preformed in the HPG secretion or whether 24MC encounters MRJP1 and apisimin only after the admixture of both glandular secretions. We have found through direct detection of 24MC in MDGs and HPGs of freshly emerged, brood-rearing and foraging honey bees that the MDG is the gland that secretes 24MC into food jelly.

2. Materials and methods

2.1. Honey bee samples

Honey bees (subspecies: *Apis mellifera scutellata*) were sampled in 2021 from a queen-right brood-rearing colony located on the campus of the University of Pretoria, South Africa (latitude: 25.7530; longitude: 28.2583). To raise worker bees of the same age, a brood frame was removed from the hive and incubated in darkness at 34 °C and 60% relative humidity. A total of 600 newly emerged bees (≤24 h) were paint-marked using Maxx 270 paint markers (Schneider, Germany) on their thoraces and returned to the hive. The marked workers (20–50 per day) were sampled on day 0 (directly after emerging), day six (brood-raising nurse bees with developed HPGs), day 17/18 and day 26/27 (foraging bees with undeveloped HPGs). In addition, pollen foragers were sampled from the same colony. All samples were immediately put-on ice, transported to the laboratory and stored at −80 °C until further processing. The day on which nurse bees were collected was chosen based on Langlands et al. (2022). Enlarged HPG acini of six day old bees were verified during dissections and bees without developed HPGs were omitted from the analysis. To verify onset of foraging, colonies were observed for 30 min per day and marked foraging bees were observed from day 15 onwards. At day 17/18 the developmental stage of the HPGs varied. This day seemed to be in the transition phase when nursing fades out and foraging starts (Seeley, 1982). Thus, we chose on day 17/18 to combine the glands as they were dissected without omitting any developmental stage. On day 26/27 most honey bees had undeveloped HPGs characteristic of foraging bees and bees with developed HPGs at day 26/27 were omitted from the analyses. Dissections of the HPGs and the MDGs were performed according to Carreck et al. (2013) in insect saline and the glands were placed in 150 μl chloroform/methanol (2:1 *v/v*) supplemented with 0.001% (*w/v*) butylated hydroxytoluene (BHT). To specifically extract secretions from the MDGs, 6 day old nurse bees and pollen foragers were used. The bees were placed in insect saline and the head capsule was opened. Both MDGs were penetrated with a fine needle, upon which the secretion leaked from the glands and was simultaneously collected with a 10 μl pipette. In case of the 6 day old nurse bees, the MDG secretion was milky-white, whereas secretion from pollen foragers leaked as clear oil droplets. This difference in the appearance of the secretion has been observed previously by Boch and Shearer (1967) and Haydak (1957). The secretion was added to 150 μl chloroform/methanol/BHT for subsequent lipid extraction. Furthermore, worker jelly was collected from the same hive, placed in HPLC vials, weighed, 150 μl chloroform/methanol/BHT were added, and lipids extracted.

2.2. Detection of 24-methylenecholesterol (24MC)

Lipid extraction was modified after Folch et al. (1956). The glands of three honey bees were placed in 150 μl chloroform/methanol/BHT in glass HPLC vials and lipids extracted for 16 h at room temperature (RT). Subsequently, the mixture was transferred into 1.5 ml safe-lock microcentrifuge tubes (Eppendorf, Hamburg, Germany), 30 μl 0.88% (*w/v*) KCl was added, the mixture vortexed and centrifuged at 4000×*g* for 5 min at RT. The lower chloroform phase was transferred into 250 μl HPLC vial inserts (Agilent, Santa Clara, CA, USA), the chloroform evaporated under a steady stream of nitrogen gas and the lipids re-dissolved in 10 μl hexane containing 0.5 mg/ml 5-α-cholestane as internal standard. To derivatize the sterols as well as the fatty acid pheromones, 10 μl bis-(-trimethylsily) trifluoroacetamide (BSTFA) (Sigma-Aldrich, St. Louis, MO, USA) were added and the samples incubated for at least 5 h at RT before analysis. For each extraction, an extraction blank containing only the chloroform/methanol/BHT mixture was processed with the samples.

Separation of the lipid extracts was performed using an Agilent 6890N gas chromatograph fitted with a flame ionisation detector (FID) in the split-less mode (1 μl inject) and a ZB-5MS column (5% diphenyl/95% dimethyl polysiloxane, 30 m × 0.25 mm × 0.25 μm) (Zebron-phenomenex, Torrance, CA, USA). Helium (with a flow rate of 1 ml per minute) was used as the carrier gas. To develop a gas chromatography method that allowed us to simultaneously detect the four major MDG fatty acids (10-HDA, 10-HDAA, 9-HDA and 9-ODA), the aromatic compound HOB and seven sterols (cholesterol, desmosterol, 24-methylenecholesterol, campesterol, stigmasterol, β-sitosterol and isofucosterol) in a single run, the methods published in Ferioli et al. (2014), Simon et al. (2001), Vanderplanck et al. (2011) and Yusuf et al. (2015) were modified as follows: The oven temperature was programmed from 60 °C for 3 min, then ramped up to 310 °C at 15 °C/min, maintained for 2 min and further increased to 320 °C at 10 °C/min and maintained at this final temperature for 10 min. The method even allowed for separation of campesterol from 24MC which has been reported to co-elute (Vanderplanck et al., 2011) during analysis. Chromatograms were recorded and the peak areas quantified using the software Chemstation® (Agilent). Identification of the MDG components was based on comparisons of retention times of the analytes against those of known external standards (10-HDA, 10-HDAA, 9-HDA, 9-ODA, HOB, HVA, cholesterol, desmosterol, 24-methylenecholesterol, campesterol, stigmasterol and β-sitosterol (Sigma-Aldrich)) (Fig. S1) which were run with each batch.

During analysis, two unknown peaks (UK1, UK2) eluted in all samples shortly after β-sitosterol (Fig. S2). The second unknown peak eluted also in the extraction blanks and was thus not analysed. Earlier publications indicated that besides 24MC, campesterol and β-sitosterol, the fourth major sterol of honey bee membranes is isofucosterol which elutes after β-sitosterol (Svoboda et al., 1980, 1983a, 1986; Vanderplanck et al., 2011; Xu and Gao, 2013). Thus, we expected the first unknown peak to be isofucosterol for which we did not have a standard. The identity of all main sterols in the samples (isofucosterol, 24MC, campesterol and β-sitosterol) was further confirmed with gas chromatography/mass spectrometry (GC-MS) using the same GC oven temperature program described above (Fig. S3).

Quantification was performed by combining external and internal standard quantification methods and comparing the response factor (RF) of each of the compounds in the standard solution mixture relative to the RF of 5-α-cholestane (Table S1). As we did not have a standard for

isofucosterol to calculate the RF, an average of all other sterol RFs was used. Frequently for cholesterol, and occasionally also desmosterol and stigmasterol, very small peaks were detected that were too small to be integrated (see Fig. S2, red line, cholesterol.). These peaks were not detected in the extraction blanks, and quantified as 'traces' during analysis. As the lowest quantifiable amount of a sterol across the gland samples was 17.16 ng, for statistics this trace amount was set to 5 ng (only necessary for some sterols in the MDG secretion of pollen foragers, see section 3.3). Further, in the blank extractions, minor amounts of HOB (1.95 ng/gland pair (GP)), 9-ODA (9.64 ng/GP), 9-HDA (9.62 ng/GP), 10-HDA (21.01 ng/GP) and 10-HDAA (22.82 ng/GP) were detected. For further analysis, these blank values were subtracted from the sample values. Occasionally this resulted in negative values which were replaced with zero.

2.3. Statistical analyses

All statistical analyses were performed with STATISTICA 14.0 (StatSoft, Tulsa, OK, USA). Data were tested for deviations from a normal distribution by Kolmogorov-Smirnov tests. Normally distributed data are reported as means ± standard deviation (SD) and standard error (SE), not-normally distributed data are presented as median ± interquartile range (IQR). Total C10 fatty acid amounts as well as percentage and ratio data were analysed with Kruskal Wallis ANOVA. Data for analysis of individual C10 fatty acid amounts in the MDGs were BoxCox transformed to achieve normal distribution and total sterols, individual sterol amount data as well as sterol and 10-HDA and 10-HDAA data of the MDG secretion were log-transformed to achieve normal distribution. These datasets were subsequently analysed via one-way ANOVA with post hoc Bonferroni test.

3. Results

3.1. Gas chromatographic detection of C10 fatty acids (9-ODA, 9-HDA, 10-HDA, 10-HDAA)

We developed a protocol to separate C10 fatty acids and sterols of honey bees in a single gas chromatography (GC) run. This allows for validation of secretion production in the MDGs via the amount of C10 fatty acids. Total C10 fatty acid amounts differed between the days and the glands (Kruskal Wallis ANOVA, $n = 86$, $H = 62.37$, $p < 0.0001$) (Fig. S4A). C10 fatty acid amount ranged between 31.44 ± 53.93 ng (median ± IQR) (HPGs of freshly emerged bees) and 20.09 ± 13.24 µg (MDGs of 18 day old bees). Whereas in freshly emerged bees total C10 fatty acid amounts did not differ between the glands ($p = 0.444$); at day 6, 18 and 26 the MDGs contained a significantly higher amount of C10 fatty acids than the HPGs (219-, 197- and 182-fold, respectively; $p = 0.002$, 0.001 & 0.007) (Fig. S4A). Even though the medians increase in the MDGs from day 0 to day 6 (28-fold), day 0 to day 18 (40-fold) and day 0 to day 26 (29-fold), this is not significant ($p = 0.687$, 0.086 & 0.256, respectively). The amounts of C10 fatty acids that were detected within the HPGs were neglectable (<103 ng) and thus for further C10 fatty acid analysis only the MDGs were considered.

Within the MDGs, individual C10 fatty acid amounts were significantly different when the age groups were pooled (one-way ANOVA, $df = 3$, $n = 168$, $MS = 480.56$, $F = 57.04$, $p < 0.0001$) with 10-HDA detected in higher amounts (8.49 ± 7.76 µg) (mean ± SD) than the other three fatty acids (10-HDAA: 2.16 ± 1.91 µg, $p = 0.022$; 9-HDA: 0.95 ± 0.80 µg, $p < 0.0001$; 9-ODA: 0.02 ± 0.01 µg, $p < 0.0001$) (Fig. S4B). Thus, 10-HDA was the main fatty acid in the MDGs and contributed 73.1% of the total fatty acids (10-HDAA: 18.6%, 9-HDA: 8.2%, 9-ODA: 0.1%).

Furthermore, individual C10 fatty acid amounts were significantly different in the MDGs between the age groups (one-way ANOVA, $df = 15$, $n = 168$, $MS = 152.54$, $F = 43.32$, $p < 0.0001$) (Fig. 1).

Whereas 9-ODA did not show any differences between bees of different ages (all $p = 1.0$), 9-HDA, 10-HDA and 10-HDAA were detected in significantly lower amounts in the freshly emerged bees compared to 6, 18 and 26 day old bees (all $p < 0.0001$, 9-HDA: 0.06 ± 0.05 µg (mean ± SD) & > 1.16 ± 0.81 µg, 10-HDA: 0.35 ± 0.31 µg & > 9.15 ± 3.45 µg, 10-HDAA: 0.11 ± 0.09 µg & > 2.49 ± 0.90 µg) (Fig. 1). None of the fatty acids differed in amounts between day 6, 18 and 26 (all $p = 1.0$). 9-ODA was significantly lower on day 6, 18 and 26 than all other fatty acids (all $p < 0.0001$). Further, 10-HDA was significantly higher on day 6, 18 and 26 than 9-HDA (day 6: 9.15 ± 3.45 µg & 1.45 ± 0.59 µg, $p = 0.039$; day 18: 13.33 ± 6.34 µg & 1.30 ± 0.65 µg, $p = 0.001$, day 26: 12.72 ± 9.63 µg, $p = 0.002$) but not 10-HDAA (all $p = 1.0$) even though the means were 3.7- to 4.2-fold higher for 10-HDA compared to 10-HDAA.

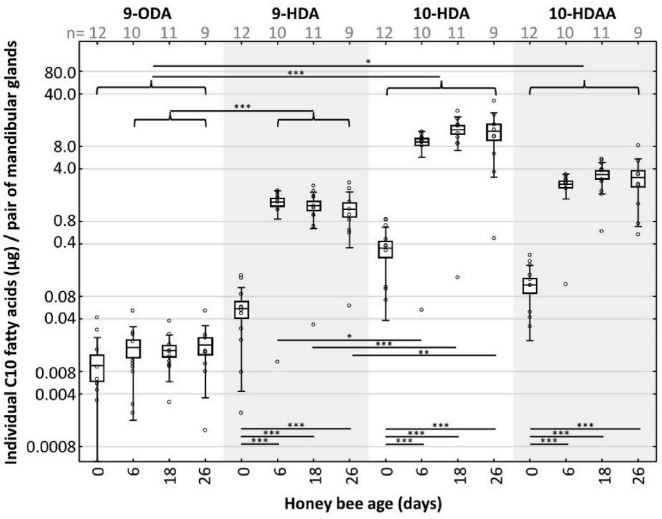

Fig. 1. Individual fatty acids (µg) per pair of mandibular glands (MDGs). Boxes show means ± SE and whiskers show SD. Individual data points are shown as open circles. Statistics have been performed using one-way ANOVA with post hoc Bonferroni tests (Box-Cox-transformed data). Significant differences are indicated by asterisks (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). 9-ODA, 9-oxo-2-decenoic acid; 9-HDA, 9-hydroxy-2-decenoic acid; 10-HDA, 10-hydroxy-2-decenoic acid; 10-HDAA, 10-hydroxy-decanoic acid; n, sample size of analysed pools (each pool contained glands of three bees).

Even though amounts increased for 9-HDA, 10-HDA and 10-HDAA from freshly emerged bees to the older age groups, e.g. 10-HDA was 0.35 ± 0.31 µg at day 0 and 9.15 ± 3.45 µg at day 6, percentages of individual C10 fatty acids per total C10 fatty acids did not, e.g. the 0.35 µg 10-HDA at day 0 correspond to 66.99% of total C10 fatty acids but also the 9.15 µg on day 6 correspond to 69.94% (Fig. S5) ($p = 1.0$) (Kruskal Wallis ANOVA, $n = 164$, $H = 137.81$, $p < 0.0001$). Thus, the composition of C10 fatty acids in the MDGs in freshly emerged bees is in general already the same as in older bees even though they produce lower total amounts. However, the variance is much higher in freshly emerged bees compared to older ones (IQR: 9-ODA, 5.35% & < 0.17%; 9-HDA, 15.14% & < 4.23%; 10-HDA, 18.58% & < 3.35%; 10-HDAA, 4.22% & < 2.28%) (Fig. S5).

3.2. Sterols within the mandibular and hypopharyngeal glands

Since our analysis used whole glands complete with their membranes, we detected sterols in both glands at all four time points. Total amounts of sterols varied between the groups (one-way ANOVA, $df = 7$, $n = 86$ $MS = 0.81$, $F = 56.70$, $p < 0.0001$) (Fig. 2). The highest amount of sterols was detected in the HPGs of 6 day old nurse bees (1.91 ± 0.51 µg; mean ± SD) and the lowest in the MDGs of 26 day old forager bees (0.25 ± 0.05 µg). Both glands contained at day 6 during the nurse bee period a significantly higher amount of sterols than at any other day (MDGs = 0.79 ± 0.23 µg; 1.9-fold higher than day 0, 2.2-fold higher than day 18, 3.2-fold higher than day 26; all $p < 0.0001$) (HPGs; 3.3-fold

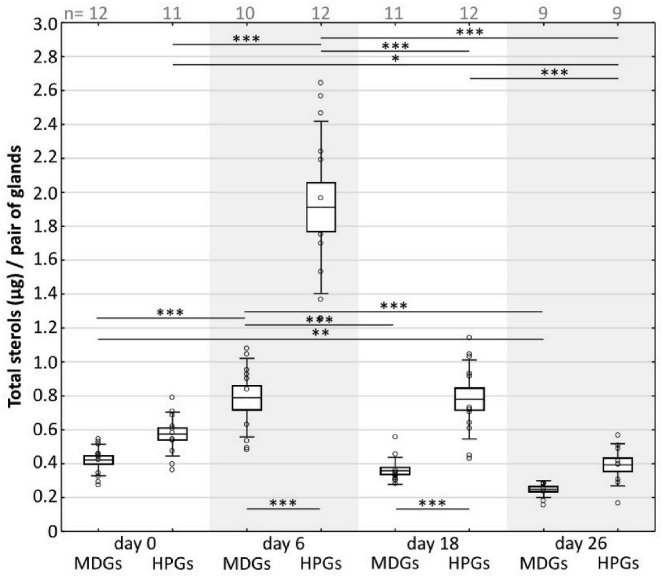

Fig. 2. Total sterols (µg) per pair of glands. Boxes show mean ± standard error and whiskers show standard deviation. Individual data points are shown as open circles. Statistics have been performed using one-way ANOVA with post hoc Bonferroni tests (log-transformed data). Significant differences are indicated by asterisks (***p < 0.001, **p < 0.01, *p < 0.05). HPGs, hypopharyngeal glands; MDGs, mandibular glands; n, sample size of analysed pools (each pool contained glands of three bees).

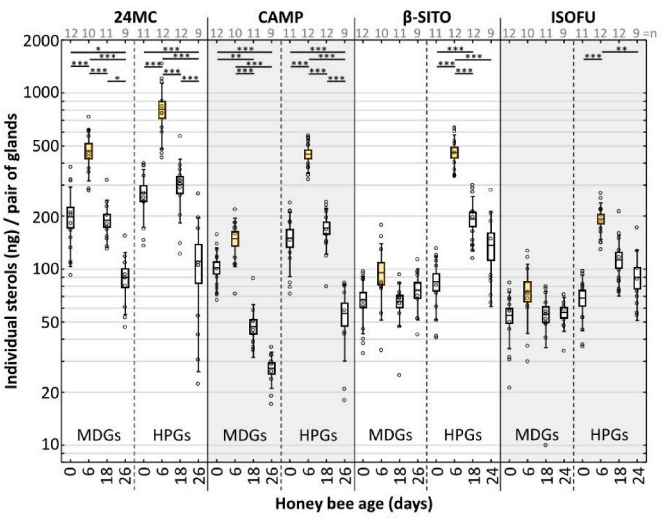

Fig. 3. Individual sterols (ng) per pair of glands. Boxes show mean ± standard error and whiskers show standard deviation. Individual data points are shown as open circles. Statistics have been performed using one-way ANOVA with post hoc Bonferroni tests (log-transformed data). Within-group significant differences are indicated by asterisks (***p < 0.001, **p < 0.01, *p < 0.05). HPGs, hypopharyngeal glands; MDGs, mandibular glands; 24MC, 24-methylenecholesterol; CAMP, campesterol; β-SITO, β-sitosterol; ISOFU, isofucosterol; n, sample size of analysed pools (each pool contained glands of three bees). Boxes representing sterols within the nurse bee period at day 6 were highlighted in yellow.

higher than day 0, 2.5-fold higher than day 18, 4.9-fold higher than day 26; all $p < 0.0001$). Whereas in the freshly emerged bees and in the 26 day old bees total sterol amount did not significantly differ between the glands ($p = 0.248$ and $p = 0.051$, respectively), at day 6 and 18 the HPGs contained a higher amount of sterols than the MDGs (2.4- and 2.2-fold, $p < 0.0001$) (Fig. 2).

Of the seven individual sterols analysed, cholesterol, desmosterol, and stigmasterol were only detected in trace amounts in a subset of the samples (71, 4 and 19 out of 86 pools, respectively) and thus were not considered further. 24-methylenecholesterol (24MC), campesterol, β-sitosterol and isofucosterol were detected in all samples. When bees of different ages were pooled there were different quantities present (one-way ANOVA, $df = 3$, $n = 344$, $MS = 3.55$, $F = 31.21$, $p < 0.0001$) with 24MC detected in higher amounts (317.14 ± 270.02 ng) than the other three sterols (campesterol: 152.13 ± 139.11 ng, β-sitosterol: 152.83 ± 143.46 ng, isofucosterol: 90.16 ± 54.95 ng; all $p < 0.0001$) and isofucosterol being significantly lower than β-sitosterol ($p = 0.011$) but not campesterol ($p = 0.058$).

Furthermore, individual sterol amounts were significantly different between the glands and between bees of different ages (one-way ANOVA, $df = 31$, $MS = 1.28$, $F = 42.12$, $p < 0.0001$) (Fig. 3). In the MDGs, only 24MC and campesterol were different between the days, whereas the amounts of β-sitosterol (65.57 ± 18.14 to 95.45 ± 44.12 ng) and isofucosterol (54.56 ± 19.24 to 74.89 ± 31.75 ng) did not vary (all $p = 1.0$).

Campesterol was detected in equally high amounts in freshly emerged and 6 days old bees (101.76 ± 29.78 ng and 148.88 ± 45.67 ng, $p = 1.00$) and decreased towards a similar level at day 18 (47.23 ± 15.65 ng, $p = 0.003$ & < 0.0001) and day 26 (27.37 ± 6.38 ng; both $p < 0.0001$). 24MC increased 2.4-fold from freshly emerged bees (198.06 ± 94.52 ng) to 6 day old bees (469.43 ± 152.79 ng, $p = 0.0001$) and decreased again on day 18 (189.33 ± 56.49 ng, $p = 0.0003$) to the same level as in freshly emerged bees ($p = 1.00$) (Fig. 3). A further significant decrease occurred on day 26 (89.56 ± 34.45 ng, $p = 0.011$). Thus, in the MDGs 24MC was the only sterol that was detected in greater amounts on day 6 compared to freshly emerged bees and therefore the increase in total sterol amounts reported earlier (Fig. 2) is primarily caused by higher amounts of 24MC but not the other sterols.

In the HPGs, all sterols showed significantly increased amounts on day 6 compared to freshly emerged bees (all $p < 0.0001$, 24MC: 3.0-fold from 270.51 ± 97.61 ng to 809.54 ± 326.38 ng, campesterol: 3.0-fold from 150.43 ± 59.65 ng to 449.58 ± 97.47 ng, β-sitosterol: 5.4-fold from 84.84 ± 33.39 ng to 459.14 ± 119.74 ng, isofucosterol: 2.8-fold from 68.59 ± 23.95 ng to 192.70 ± 45.04 ng) (Fig. 3, yellow boxes). Thus, in the HPGs the increase in total sterol amounts is caused by larger amounts of all sterols and not only 24MC. From day 6, amounts of all sterols significantly decreased again until day 26 ($p < 0.0001$ except isofucosterol ($p = 0.0040$)) (Fig. 3).

None of the individual sterols differed between the glands in the freshly emerged bees and in the 26 day old bees ($p = 0.683$–1.0). At day 6 and 18 the HPGs contained a higher amount of campesterol, β-sitosterol and isofucosterol than the MDGs ($p < 0.0001$ except isofucosterol ($p = 0.0040$)) but not of 24MC ($p = 1.0$). This indicates that the difference reported in total sterol amount between the glands on day 6 and 18 is caused by primarily campesterol, β-sitosterol and isofucosterol but not by 24MC which is equally high in both glands. Indeed on day 6 and 18 in the MDGs, 24MC was detected in significantly higher amounts than the three other sterols (all $p < 0.0001$) whereas in the HPGs on the same days 24MC did not differ from campesterol ($p = 0.581$ & 0.631) and β-sitosterol ($p = 0.752$ & 1.0).

When age and gland were pooled, individual sterols did not only vary in their total amount but also as percentage of total sterols (Kruskal Wallis ANOVA, $N = 344$, $H = 165.96$, $p < 0.0001$). Across all groups 24MC made up 41.20 ± 16.90% (median ± IQR) of the total sterols, a significantly higher percentage than all other sterols (all $p < 0.0001$). Campesterol 20.19 ± 11.90% and β-sitosterol 20.00 ± 10.08% were detected in similar percentages ($p = 1.0$), both significantly higher than isofucosterol 13.84 ± 6.63% ($p = 0.004$ & $p < 0.0001$). Furthermore, percentages of individual sterols differed between the groups (Kruskal Wallis ANOVA, $N = 344$, $H = 269.40$, $p < 0.0001$) (Fig. S6). At day 0, 24MC made up 38.32 ± 18.56% in the MDGs and 38.75 ± 31.86% in the HPGs whereas at day 6, 24MC made up 59.16 ± 13.61% in the MDGs

and 38.43 ± 11.94% in the HPGs. This prompted us to have a closer look at the percentage ratios of the individual sterols which differed between the time points (Kruskal Wallis ANOVA (KWA), N = 160, H = 100.11, p < 0.0001) (Fig. 4).

At day 0 and day 26 individual sterol ratios did not differ between the groups (KWA, all p = 1.0) and were also not significantly different from one (One-sample Wilcoxon signed rank (WSR) test (multiple comparisons adjusted p-value = 0.0031), W = 12-44, p > 0.054). At day 6 and 18, the percentage ratio of 24MC was significantly different from campesterol and β-sitosterol (KWA, day 6: p = 0.0008 & p < 0.0001, day 18: p < 0.0001 & p = 0.0001). Further, these three sterols deviated at both days from a ratio of one (WSR, day 6: 24MC 1.50 ± 0.09, campesterol 0.79 ± 0.12, β-sitosterol 0.50 ± 0.12, all p = 0.002, day 18: 24MC 1.49 ± 0.37, campesterol 0.59 ± 0.25, β-sitosterol 0.69 ± 0.17, all p = 0.001). Thus, whereas at day 0 and 26 none of the individual sterols was predominated in one of the glands, on day 6 and 18 24MC was enriched in the MDGs and campesterol as well as β-sitosterol in the HPGs (Fig. 4).

3.3. Mandibular gland secretion and worker jelly

In the previous sections (3.1 & 3.2), lipid extractions of complete MDGs and HPGs were analysed. Thus, the sterols present are a combination of those located within the cell and organelle membranes ('membrane' sterols) and those possibly present directly within the lumen of the glands as part of the glandular secretion ('secreted' sterols). Whereas it is not possible to isolate pure HPG secretion due to the structure of the HPG, in the MDG the glandular cells surround the glandular reservoir which stores the secretion. Upon penetration of the gland with a fine needle, the secretion of the MDG can be obtained. However, this is accompanied with a loss of glandular secretion and thus usually complete MDGs are preferred to the secretion for analysis. As the C10 fatty acids, e.g. 10-HDA, are only present in the MDG secretion but not in the membranes, analysing the amount of 10-HDA makes it possible to determine the amount of secretion that is lost during secretion extraction from the MDG. Analysing complete MDGs, 9.15 ± 3.45 μg (mean ± SD) of 10-HDA were detected within paired glands of a 6 day old nurse bee. Analysing only MDG secretion, 10-HDA amount reduced to 5.09 ± 1.73 μg per bee (Student's t-test, df = 12, n = 14 p = 0.047). Thus, approximately half (44.38%) of the secretion is lost during extraction of the secretion from the glands.

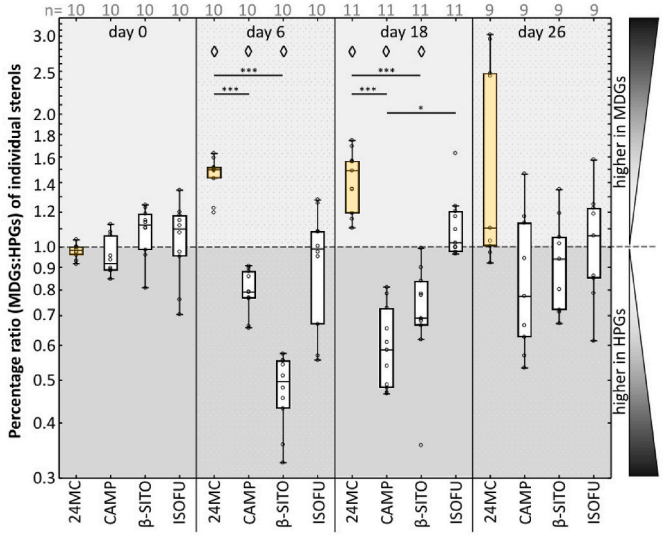

Fig. 4. Percentage ratio (MDGs:HPGs) of individual sterols within the same pool. Boxes show median ± IQR and whiskers show the non-outlier range. Individual data points are shown as open circles. Statistics have been performed using Kruskal Wallis ANOVA (KWA) and one-sample Wilcoxon signed rank (WSR) test. KWA within-group significant differences are indicated by asterisks (***p < 0.001, **p < 0.01, *p < 0.05) and deviations from 1 as determined via WSR (p < 0.0031, adjusted for multiple comparisons) are indicated by diamonds. HPGs, hypopharyngeal glands; MDGs, mandibular glands; 24MC, 24-methylenecholesterol; CAMP, campesterol; β-SITO, β-sitosterol; ISOFU, isofucosterol. Boxes representing 24MC are highlighted in yellow. n, sample size of analysed pools (each pool contained glands of three bees).

We could detect all four sterols in all MDG secretion samples of 6 day old nurse bees. In the pollen foragers the sterols have only been detected in quantifiable amounts in some of the pools (24MC: 2/4 pools, campesterol: 2/4 pools, β-sitosterol: 3/4 pools, isofucosterol: 1/4 pools) and in the others only in traces (<5 ng). Thus, total sterol amounts were significantly higher (5.0-fold) in the secretion of MDGs of nurse bees (191.25 ± 62.48 ng (mean ± SD) compared to in foragers (38.67 ± 18.35 ng) (Student's t-test, df = 6, n = 8, p = 0.003).

Amounts of individual sterols varied between the MDG secretion of 6 day old nurse bees and pollen foragers (one-way ANOVA, df = 7, n = 32, MS = 0.69, F = 16.83, p < 0.0001) (Fig. 5A). 24MC, campesterol and isofucosterol were detected in larger amounts in the MDG secretion of 6 day old nurse bees compared to the secretion of pollen foragers (24MC: 9.8-fold, 95.81 ± 28.59 ng & 9.78 ± 5.93 ng, p < 0.0001; campesterol: 4.4-fold, 34.93 ± 13.86 ng & 8.01 ± 4.22 ng, p = 0.004; isofucosterol: 4.1-fold, 22.74 ± 7.61 ng & 5.59 ± 1.19 ng, p = 0.010). β-sitosterol did not show any significant difference (37.78 ± 13.30 ng & 16.53 ± 8.29 ng, p = 0.264) (Fig. 5A).

While 24MC decreased 9.8-fold from nurses to foragers, the individual C10 fatty acids did not differ between nurses and foragers (all p = 1.0) (Fig. 5B) (9-ODA: 0.01 ± 0.01 μg (day 6) & 0.02 ± 0.02 μg (pollen forager), 9-HDA: 0.85 ± 0.21 μg (day 6) & 0.53 ± 0.66 μg (pollen forager), 10-HDA: 5.09 ± 1.73 μg (day 6) & 4.55 ± 5.37 μg (pollen forager), 10-HDAA: 1.53 ± 0.48 μg (day 6) & 1.26 ± 1.19 μg (pollen forager)) (one-way ANOVA, df = 7, n = 32, MS = 4.54, F = 12.07, p < 0.0001) showing that the MDGs of pollen foragers were producing a secretion but this secretion contained significantly reduced amount of sterols.

In addition, we determined the amounts of fatty acids and sterols in worker jelly (WJ) of the same colony (n = 3). The most abundant fatty acid was 10-HDA (5.28 ± 1.46 mg/g WJ) being significantly higher than 10-HDAA (1.65 ± 0.33 mg/g, p = 0.0021), 9-HDA (0.59 ± 0.04 mg/g, p = 0.0004) and 9-ODA (0.08 ± 0.04 mg/g, p = 0.0002) (one-way ANOVA, df = 3, MS = 16.48, F = 26.22, p = 0.0001). Even though the differences in abundance between 10-HDAA, 9-HDA and 9-ODA were not significant (likely due to the low sample size), they still followed the order detected for the fatty acids in the MDGs with 10-HDAA > 9-HDA > 9-ODA. The same was found for the sterols with 24MC (0.18 ± 0.14 mg/g) being more abundant than the other sterols (campesterol: 0.05 ± 0.03 mg/g, β-sitosterol: 0.03 ± 0.01 mg/g, isofucosterol: 0.02 ± 0.01 mg/g) but this was not significantly different (one-way ANOVA, df = 3, MS = 0.02, F = 3.42, p = 0.0731). The ratio between the main fatty acid 10-HDA and the main sterol 24MC was 53.53 ± 41.81 (median ± IQR) in worker jelly, 63.29 ± 61.49 in the MDG secretion of 6 day old nurse bees and 283.90 ± 522.26 in the MDG secretion of the pollen foragers. Thus, the ratio in worker jelly is more similar to the ratio in the secretion of nurse bees than to the secretion of pollen foragers. Again, no significant differences could be detected due to low sample size and very high variations especially in the pollen foragers (Kruskal Wallis ANOVA, N = 11, H = 1.96, p = 0.375).

4. Discussion

4.1. C10 fatty acids

The first C10 fatty acid to be identified in honey bees was 10-HDA discovered in MDGs of workers (Butenandt and Rembold, 1957; Callow et al., 1959) and 9-ODA was subsequently discovered in MDGs of queens (Butler and Simpson, 1958; Butler et al., 1959; Callow and

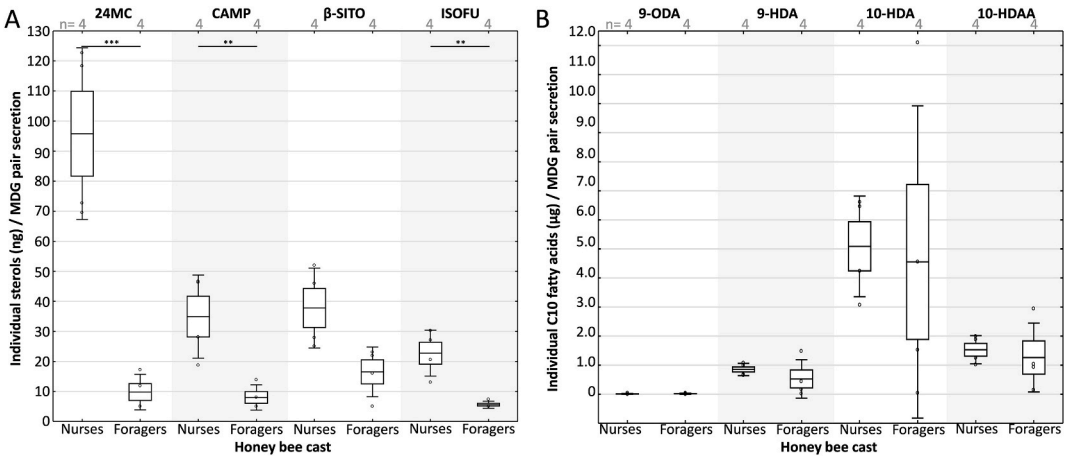

Fig. 5. Individual sterols (ng) and C10 fatty acids (µg) per MDG pair secretion. Boxes show mean ± standard error and whiskers show standard deviation. Individual data points are shown as open circles. Statistics have been performed using one-way ANOVA with post hoc Bonferroni tests (log-transformed data). Within-group significant differences are indicated by asterisks (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). MDGs, mandibular glands; 24MC, 24-methylenecholesterol; CAMP, campesterol; β-SITO, β-sitosterol; ISOFU, isofucosterol; 9-ODA, 9-oxo-2-decenoic acid; 9-HDA, 9-hydroxy-2-decenoic acid; 10-HDA, 10-hydroxy-2-decenoic acid; 10-HDAA, 10-hydroxy-decanoic acid; n, sample size of analysed pools (each pool contained glands of three bees).

Johnston, 1960). As 9-ODA is part of the MDG queen pheromone (Callow and Johnston, 1960; Callow et al., 1964; Gary, 1962), C10 fatty acids are mostly studied regarding their presence in queen compared to worker MDGs (Crewe and Velthuis, 1980; for review see Mumoki and Crewe, 2021).

Freshly emerged honey bee workers only secrete small amounts of 10-HDA, with quantities increasing rapidly to the nurse bee period and high amounts are still detected in foragers (Boch and Shearer, 1967; Haydak, 1957). This is confirmed in the present study with only 0.51 ± 0.68 µg C10 fatty acids being produced in MDGs of freshly emerged *A. mellifera scutellata* workers while older workers (≥6 days) produced more than 14.09 ± 2.77 µg C10 fatty acids. In MDGs of queen-right workers C10 fatty acids (10-HDA, 10-HDAA, 9-HDA & 9-ODA) amount to anything between 8.8 and 65.8 µg (*Apis mellifera capensis*, 8.8 µg (Zheng et al., 2010); *A. m. scutellata*, 10.4 µg (Zheng et al., 2010); *A. m. adansonii*, 12.6 µg (Yusuf et al., 2015); *A. m. intermissa*, 13.7 µg (Crewe and Moritz, 1989); *A. m.* unknown subspecies, USA, 14.6 µg (Plettner et al., 1993); *A. m.* unknown subspecies, China, 65.8 µg (Yang et al., 2017). In China beekeepers have selected *A. mellifera* colonies for high RJ production since the 1960s (Cao et al., 2016) which might be the reason why these workers produce also higher amounts of 10-HDA.) Indeed, subspecies level variations in worker C10 fatty acids have been described before (for review see Mumoki and Crewe, 2021) and thus also location and breeding-line might influence age-dependent C10 fatty acid production. 9-HDA, 10-HDA and 10-HDAA amounts found in this study did not differ between 6, 18 and 26-day old bees. Thus, forager bees do produce high amounts of 10-HDA in all studies considering worker age (Boch and Shearer, 1967; Hu et al., 2021; Yang et al., 2017). As the only known functions of 10-HDA are as a component of food jelly (Blum et al., 1959; Kinoshita and Shuel, 1975; Spannhoff et al., 2011; Yousefi et al., 2012; Buttstedt, 2022) it is surprising that foragers have such high amounts of 10-HDA in their MDGs. It is assumed that MDG as well as HPG secretions are added to other bee products while processing them (Isidirov et al., 2011). The presence of MRJPs originating from HPGs in honey or by bees processed sugar solution has been repeatedly shown (Erban et al., 2019 (and references therein); Lewkowski et al., 2019; Šimúth et al., 2004). Contrary to that, only a single study reports on 10-HDA in honey samples in varying amounts (not detected – 16.9 µg/g) (Isidirov et al., 2011). Furthermore, MRJPs were detected in pollen stores (Scarselli et al., 2005; Bílikova and Šimúth, 2010) whereas 10-HDA was not detected in bee-collected pollen (Standifer et al., 1980; Verhoef and Hoekstra, 1986). Human and Nicolson (2006) reported that total lipid content of fresh pollen is significantly higher than in bee-collected or stored pollen. This shows that there is currently no data supporting the notion that the MDG secretion is added to pollen. It seems to be occasionally added to nectar but not mandatorily (Isidirov et al., 2011). Thus, it remains mysterious why foragers bear such high amounts of 10-HDA in their MDGs and further studies are needed to unravel the role of 10-HDA in forager MDGs.

4.2. Sterols

Across samples, 24MC was detected as main sterol of honey bees (317.1 ng, 41.2% of total sterols). This is in accordance with several studies detecting sterols in honey bee larvae, prepupae, workers, MDGs, HPGs and queens (Barbier and Schindler, 1959; Svoboda et al., 1980, 1983, 1986a, 1986b, Vanderplanck et al., 2011, Xu and Gao, 2013). In royal jelly, 24MC makes up between 49.0 and 83.5% of total sterols with sitosterol (5.3–24.6%), isofucosterol (9.0–28.3%) and campesterol (6.3–7.7%) also being present (Ferioli et al., 2014; Kodai et al., 2007; Svoboda et al., 1986). We show for the first time that 24MC, campesterol, β-sitosterol and isofucosterol are present in worker jelly.

As food jelly production is split up between HPGs and MDGs, both glands can be considered as the glands that secrete sterols into food jelly. And indeed, total sterol amount is higher in 6 days-old nurse bees compared to freshly hatched and older bees (>18 days-old) in both glands. In addition, on day 6 total sterol amount is higher in HPGs compared to MDGs (2.4-fold). This finding could lead to the conclusion that both glands may be secreting 24MC. However, there is a peculiar difference in relation to the structure of the two glands as the workers age that is important. The HPGs have their largest size and highest activity in nurse bees (about day 6–14) (for review see Ahmad et al., 2021; Crailsheim and Stolberg, 1989; Deseyn and Billen, 2005; Dobritzsch et al., 2019; Knecht and Kaatz, 1990; Langlands et al., 2022; Soudek, 1927), while the cells of the MDGs become smaller as the workers age (Kratky, 1931) even though 10-HDA production increases (Boch and Shearer, 1967; Hu et al., 2021; Yang et al., 2017). In addition, nurse bee HPGs are filled with secretory vesicles that are membrane enclosed (Kratky, 1931; for review see Ahmad et al., 2021). As sterols are essentially present in membranes, their presence in HPGs of the highest sterol amount in 6-day old nurse bees is expected simply due to the proliferation of membrane enclosed secretory vesicles. In contrast, in MDGs the high amount of sterol reached in 6-days old nurse bees is not due to membrane enclosed secretory vesicles since the mode of secretion is into the lumen of the glandular reservoir.

In the HPGs all four of the detected sterols significantly increased

from freshly emerged to 6 day-old bees, whereas in the MDGs only the amount of 24MC increased significantly. Furthermore, despite a significantly higher amount of total sterols in the HPGs compared to MDGs at day 6, at the individual sterol level only campesterol, β-sitosterol and isofucosterol are significantly higher in the HPGs. The amount of 24MC on day 6 in the MDGs is not significantly different from that in the HPGs. All of this suggests that from emergence to the nurse bee stage, the increase in the MDGs may be due to secreted sterols, mainly 24MC. This becomes especially clear when considering the percentage ratio. In freshly emerged bees and in 26 day-old foragers, all four sterols do have a MDG:HPG percentage ratio not different from one, meaning they contribute the same percentage of total sterols. In 6 day-old nurse bees and in 18-day old workers that are likely in the transition phase between nursing and foraging (Seeley, 1982) 24MC MDG:HPG percentage ration increases to 1.5. Thus, in the MDGs 24MC makes up a higher percentage of total sterols than in the HPG. 24MC may be specifically enriched in the MDGs of food jelly producing bees whereas other sterols, such as campesterol and β-sitosterol, are primarily shuttled to the HPGs and maybe even other organs. Further studies are needed to substantiate this hypothesis.

The amount of 24MC in pollen collected from plants accounts on average for 23% of total sterol content but depends largely on the plant species (Zu et al., 2021). Given that in royal jelly, 24MC makes up 49–84% of total sterols (Ferioli et al., 2014; Kodai et al., 2007; Svoboda et al., 1986) there is a discrepancy between average 24MC percentage in pollen and in royal jelly. It has been shown that 24MC is always the predominant sterol in prepupae (37.6–65.6%) even when raised from nurse bees fed with pollen in which 24MC was as low as 1.5% (Svoboda et al., 1983a, 1986). This further points to the fact that honey bee workers either selectively transfer sterols to the developing brood (Svoboda et al., 1983a) and/or transfer sterols from their own accumulated sterol reserves as colonies have been shown to be able to raise brood on a sterol free diet for up to 8 weeks (Herbert et al., 1980) in which the main sterol in the prepupa is still 24MC (Svoboda et al., 1980). That nurse bees use body reserves to raise larvae has been shown in the absence of pollen for proteins already (Haydak, 1935). Although not shown before for honey bees, tissue-specific sterol characteristics are known from other phytophageous insects such as *Drosophila melanogaster* (Carvalho et al., 2012) and *Bombyx mori* (Takeshima et al., 2019). If honey bees specifically enrich 24MC in their MDG secretion, then there must be a process or receptor that is specific for 24MC. In general, sterols are ingested by insects with their diet and subsequently transported to different organs in the aqueous hemolymph bound to transport particles, e.g. lipophorin (Lp) (for review see (Jing and Behmer, 2020)). Lp shuttles lipids from the gut to peripheral organs where the lipids are either delivered at the cell surface or Lp binds to Lp receptors (LpR) upon which it is taken up via endocytosis (for review see Rodenburg and Van der Horst, 2005). In *A. mellifera*, one LpR was identified with two transcripts being detected only in worker heads (Guidugli-Lazzarini et al., 2008). Strikingly in the HPGs only one of the transcripts was detected in a very low amount. Other organs located in the head were not examined in this study and thus it remains to be determined whether the additional transcript is associated with the MDGs (Guidugli-Lazzarini et al., 2008).

Another possibility for providing 24MC to larvae, when 24MC amount is low in the available pollen source, would be by interconversion of phytosterols, e.g. campesterol into 24MC, as has been shown for *Manduca sexta* (Lepidoptera) (Svoboda et al., 1972). Whether honey bees are able to do this has not yet been studied. However, if the foragers have a choice, they collect pollen with higher proportions of 24MC (Vanderplanck et al., 2020). Furthermore, caged bees with 24MC in their diet consumed more food compared to bees fed a lipid-free diet (Chakrabarti et al., 2020). This points to the fact that honey bees may recognize which pollen/diet contains higher amounts of 24MC. Ruedenauer et al. (2021) reported that forager bees are not able to discriminate between pollen differing in sterol concentration with their antennae. However, the sterol mixture used did not contain 24MC (Ruedenauer et al., 2021) and thus it might be that indeed 24MC is needed by honey bees to assess pollen quality in terms of sterols.

That HPG development is induced in worker bees by pollen feeding has been known for almost a century (Soudek, 1927). This has been almost exclusively ascribed to the protein in pollen which enables the HPGs to produce proteinaceous secretion (for review see Ahmad et al., 2021; Soudek, 1927; Standifer et al., 1960). Lipids and MDG development have only recently taken into account. Nurse-age honeybees in cages confined to a high lipid diet had larger HPGs than those receiving less lipids (Stabler et al., 2021). Chakrabarti et al. (2020) fed artificial diets containing 0–1% 24MC to caged bees and found a higher head protein content that followed increased 24MC concentration which was ascribed to better developed brood food-producing glands. Pollen fed caged bees increased MDG protein compared to groups that did not receive pollen (Peters et al., 2010) as well as 10-HDA and 10-HDAA contents in honey bee heads (Zhang et al., 2022). All of this indicates that protein and lipid intake influence the development of both, HPGs and MDGs. Indeed, it has been shown that adult caged workers regulate lipid intake around optimal values relative to protein (Stabler et al., 2021).

The presence of a 9.8-fold higher amount of 24MC in the isolated MDG secretion of 6 day-old nurse bees compared to foragers provides clear evidence that the MDGs secrete 24MC into food jelly. At the same time the total amount of C10 fatty acids in the MDG secretion did not differ with age. While C10 fatty acid production is not age dependent in workers more than 6 days old, sterol secretion is primarily confined to young nurse bees.

The fact that 24MC is a component of royal jelly has been known for a long time (Brown et al., 1961), but that 24MC occurs in a protein complex ($MRJP1_4$/apisimin$_4$/$24MC_8$) is a rather recent finding (Tian et al., 2018; Xu and Gao, 2013). If 24MC is the only sterol that can bind to the complex and if full complex formation is only possible in the presence of 24MC, this would explain why nurse bees even when deprived of sufficient pollen enrich 24MC in the jelly. The $MRJP1_4$/apisimin$_4$/$24MC_8$ complex is needed for fibril formation that increases the viscosity of royal jelly (Buttstedt et al., 2018). This makes 24MC an essential sterol for successful honey bee queen rearing.

Data availability statement

The data that support the findings of this study (ng amounts of substances as evaluated by gas chromatography) are available in the methods and appendix of this article.

Author contributions

All authors conceived and designed the study and contributed to reviewing the final manuscript. AB collected the data, AB and AAY analysed the data, AB wrote the first version of the manuscript.

Animal welfare

All experiments were performed in accordance with relevant guidelines and regulations.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.104011.

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