The effect of diet on the development of pheromone signals in *Apis mellifera scutellata* workers

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Significance Statement:

In honey bee colonies, the queen is reproductively dominant and produces pheromonal signals that suppress reproduction in workers. In the absence of a queen, workers compete to gain pheromonal and reproductive dominance over nest mates. Reproductive status and age-related changes modulate pheromone composition in queenless workers and pheromone status (i.e. being worker-like or queen-like) influences diet, but it is not known to what extend diet can modulate the pheromone status of the workers. We present evidence of the role of diet in gaining pheromonal dominance in workers when the queen is absent. We show that differences in diet lead to differences in pheromone composition, modulating pheromone status that ultimately affects reproductive dominance in queenless workers. Understanding the role of diet in establishing pheromonal dominance hierarchies in social insects such as honey bees deepens our understanding of the proximate drivers shaping eusociality.

Abstract

The social organization of honey bee colonies is predominantly controlled by the pheromonal cues produced by the queen, workers and brood. Pheromone composition is dependent on the social environment, context, audience and physiological status of the individual. In the absence of the queen, ontogenic changes and reproductive status modulate pheromone production and composition in workers. In these queenless workers, pheromonal status influence diet with dominant workers consuming less pollen than subordinates as they are fed protein jelly through trophallaxis, which positively correlates with their ovarian activation. However, it is not known to what extent diet affects pheromonal status in queenless workers. To investigate whether diet affects the production of glandular signals, *Apis mellifera scutellata* workers were fed carbohydrate-only or protein-rich diets for twenty-five days. The mandibular and tergal gland secretions were analyzed using gas chromatography and the ovarian activation statuses evaluated. A clear link between diet and pheromone profiles were observed with workers fed a protein-rich diet producing mandibular gland chemical profiles more typical of queens. The effect of diet on tergal gland chemical profiles was less distinct, but a shift from fatty acid to n-alkane components were seen in the older workers irrespective of diet, most likely due to ontogeny. Though mandibular gland profiles were queen-like with high proportions of 9-HDA, the levels of 9-ODA remained in the range of non-reproductive workers and the ovaries were not fully activated. Suggesting that diet could prime queenless workers to become false queens, but do not trigger the transformation into false queens.

Keywords: mandibular gland, tergal gland, pheromones, honey bees, reproductive dominance

1 Introduction

Organization in eusocial insect societies, especially those of the honey bees like *Apis mellifera*, is predominantly governed by the chemical cues produced mainly by exocrine glands and released by the queen, the adult workers, adult drones and the brood. A key component of their social organization is the regulation of the reproductive division of labour. Under typical conditions in a colony, the fertile queen is reproductively dominant and she produces chemical signals that suppress reproductive activity in workers by inhibiting ovarian activation resulting in functional sterility (Slessor et al. 1998; Malka et al. 2006). The other effects of the queen signal include eliciting retinue behaviour, maintenance of worker cohesion, suppression of queen rearing and stimulation of worker activities such as cleaning, building, guarding, foraging, and brood feeding (Winston 1987). The main source of the queen pheromonal signal is the mandibular glands and these secretions are composed of four fatty acids, namely: 9-oxo-2(E)-decenoic acid [9-ODA], (R,E)- or (S,E)-9-hydroxy-2-decenoic acid [9-HDA], 10-hydroxy-2(E)-decenoic acid [10-HDA], 10-hydroxy-decanoic acid [10-HDAA]; and two aromatic compounds methyl p-hydroxybenzoate [HOB] and 4-hydroxy-3-methoxyphenylethanol [HVA] (Crewe and Velthuis 1980; Slessor et al. 1988). These six components are also present in the mandibular gland secretions of workers, however, the relative levels at which these compounds occur in the secretions of the two castes are different with 9-ODA and 9-HDA dominating the pheromone bouquet of queens and 10-HDA and 10-HDAA dominating those of workers (Crewe and Velthuis 1980; Plettner et al. 1996).

Though the secretions from the mandibular glands are considered to be the major queen pheromonal signal, growing evidence suggests that the mandibular gland secretions are working in tandem with the secretions from other sources (Moritz and Crewe 1988; Wossler and Crewe 1999a; Sole et al. 2002; Maisonnasse et al. 2010; Okosun et al. 2017). For example, coniferyl alcohol, methyl oleate,

hexadecane-1-ol and linoleic acid are four compounds that are not produced by the queen's mandibular glands, but they act synergistically with queen mandibular gland components to elicit worker retinue behaviour (Keeling et al. 2003; Maisonnasse et al. 2010). Such evidence suggests that the queen pheromonal signal is multi-sourced with other sources such as the tergal and Dufour's glands contributing to the signal (Moritz and Crewe 1988; Wossler and Crewe 1999a; Sole et al. 2002; Maisonnasse et al. 2010; Okosun et al. 2017).

The tergal gland secretions of both queens and workers consist of hydrocarbons, long-chained fatty acids and long-chained esters (typically C17-20), but similar to the mandibular gland secretions, there are differences in the composition between the two castes (Wossler and Crewe 1999b; Okosun et al. 2015). All *A. mellifera* subspecies do not necessarily exhibit well developed tergal glands in the worker caste. For example, *A. m. mellifera* workers' tergal glands are underdeveloped or even absent, while the workers of the two subspecies found in Southern Africa, *A. m. scutellata* and *A. m. capensis* exhibit well developed tergal glands (Billen et al. 1986; Wossler and Crewe 1999b). Tergal gland secretions function as both primer and releaser pheromones involved in mating behaviour (as attractants for mounting drones), eliciting worker retinue, suppressing ovarian activation in workers and as kin recognition cues (Vierling and Renner 1977; Moritz and Crewe 1988; Wossler and Crewe 1999a, c; Okosun et al. 2019). The specific contributions of the individual components of the tergal gland in executing these functions secretions are still unclear.

Some of the compounds present in tergal gland secretions are also found in Dufour's gland secretions. Dufour's gland secretions of queens and workers contain long-chained hydrocarbons and long-chained esters (typically C28-32) (Sole et al. 2002), with queens producing relatively larger amounts of the esters and the workers larger amounts of the hydrocarbons. Though the functions of the Dufour's gland secretions are still not fully understood, they have been shown to be involved in

signaling fertility (Dor et al. 2005) and mediating attraction to nest mates (Katzav-Gozansky et al. 2003).

The caste specificity of the pheromones from the different glandular sources is not a rigid trait, but are decidedly plastic within the female phenotype. While queens invariably possess the queen bouquet, workers are able to express queen-like bouquets, often in association with ovarian development (Crewe and Velthuis 1980; Plettner et al. 1993; Katzav-Gozansky et al. 2004; Dor et al. 2005). In workers, pheromone production and composition are predominately dependent on ontogenic changes (i.e. physiological status) and the social environment (e.g. presence or absence of the queen; presence or absence of brood). Under typical colony conditions the queen is reproductively dominant and she produces pheromonal signals that suppress reproductive activity in workers. However, in the absence of the queen the workers compete with each other to gain pheromonal and reproductive dominance over their nest mates. Workers can activate their ovaries and switch from producing high levels of worker substance 10-HDA and 10-HDAA to the production of high quantities of queen-associated compounds 9-ODA and 9-HDA in their mandibular gland secretions (Sakagami 1958; Crewe and Velthuis 1980; Plettner et al. 1993). Reproductive workers can also produce tergal and Dufour's gland bouquets characteristic of queens (Katzav-Gozansky et al. 2004; Dor et al. 2005; Okosun et al. 2015, 2017). Though ovarian activation is usually coupled with changes in pheromonal signaling in workers, pheromonal dominance (expressing a queen-like chemical profile) does not necessary reflect reproductive dominance (Sakagami 1958; Crewe and Velthuis 1980; Plettner et al. 1993; Katzav-Gozansky et al. 2004; Dor et al. 2005). For example, a queenless worker does not necessarily need to have activated ovaries in order to switch her mandibular gland signal from worker- to queen-like and as such become a pheromonal dominant individual (Velthuis 1970; Crewe and Velthuis 1980; Plettner et al. 1993). Conversely, a worker can become reproductively dominant (fully activated ovaries) but express the worker-like mandibular

gland signal of a subordinate individual (Velthuis 1970; Crewe and Velthuis 1980; Plettner et al. 1993; Mumoki et al. 2021). I.e. queen-like mandibular gland secretions are positively correlated with dominance hierarchy, but ovary activation and mandibular gland secretions co-vary (Hepburn 1992). The range of pheromonal and reproductive states intermediate between the worker and queen reproductive phenotypes is easily distinguishable in queenless workers (Sakagami 1958; Okosun et al. 2017; Yusuf et al. 2018), and to some extent even in queenright workers (Zheng et al. 2010). This gave rise to four broad categories of worker reproductive phenotypes (Sakagami 1958; Okosun et al. 2017; Yusuf et al. 2018). These reproductive phenotypes are based on the extent of ovary activation and the status (queen-like or worker-like) of the mandibular, tergal and Dufour's gland chemical signals viz: non-laying workers (with worker-like pheromones and inactive ovaries); laying workers (with worker-like pheromones and activated ovaries); incipient false queens (workers that have queen-like pheromones and inactive ovaries) and false queens (workers with queen-like pheromones and activated ovaries) (Sakagami 1958; Okosun et al. 2017; Yusuf et al. 2018). Queen-like pheromones are defined as 9-ODA and 9-HDA dominating the mandibular gland secretions (Moritz et al. 2000; Moritz et al. 2004) with a ratio of above 0.9 of 9-ODA and 10-HDA (Schäfer et al. 2006), a queen-like blend of tergal gland secretions (described in Okosun et al. (2017) and Wossler and Crewe (1999b)) and high quantities of long-chained esters present in the Dufour's gland secretions (Sole et al. 2002). Even when the full range of queen-like signals is not produced, i.e. when two or even only one of these three glands produce queen-like signals, the pheromone blend is still defined as queen-like (Mumoki et al. 2021). Worker-like pheromonal signals are defined as mandibular gland secretions dominated by 10-HDA and 10-HDAA with a ratio of below 0.5 of 9-ODA and 10-HDA (Schäfer et al. 2006), a worker-like blend of tergal gland secretions (described in Okosun et al. (2017) and (Wossler and Crewe 1999b)) and Dufour's gland secretions dominated by hydrocarbons.

In queenless workers, pheromonal status, that is being worker- or queen-like, influences diet that in turn affects ovarian activation (Schäfer et al. 2006). Ovarian activation is positively correlated with protein consumption (Hoover et al. 2006; Human et al. 2007; Pirk et al. 2010; Altaye et al. 2010). Pheromonal dominant workers, or workers with queen-like pheromones, are fed protein-rich jelly through trophallaxis by the subordinate nest mates, which in turn positively correlates with their ovary activation (Schäfer et al. 2006). Ovary activation in queenless workers is but one of several aspect of honey bee physiology regulated by nutritional stimuli. Different nutrition is the key determinant of female caste or more specifically, diet quantity is the primary driver of the female caste polyphenism (Buttstedt et al. 2016; Slater et al. 2020). Diet is also involved in controlling bee behaviour with nutritionally related pathways regulating the division of labour among the workers in a colony (Ament et al. 2010; Corby-Harris et al. 2022). Despite the central role of nutritional regulation in bee behaviour and phenotypic plasticity in general the regulatory effect of diet on pheromone production and composition has mostly been studied from one direction: the effect of diet on reproductive physiology or fertility and the subsequent effect on pheromone synthesis.

Diet or nutrition is one of the most significant environmental factors shaping pheromone synthesis in insects (reviewed in Henneken et al. 2017). In social insects, there is evidence suggesting that diet can affect cuticular hydrocarbon profiles (the pheromones involved in colony or kin recognition) in certain ant species such as *Wasmannia auropunctata* (Vonshak et al. 2009) *Acromyrmex octospinosus* (Suarez et al. 1999), *Formica aquilonia* (Sorvari et al. 2008) and *Linepithema humile* (Liang and Silverman 2000). In contrast, in *A. mellifera* it has been confirmed that pheromone status can influence a worker's diet in the absence of the queen (Schäfer et al. 2006), but whether diet is a causative factor in the emergence of pheromonal differences in workers (not mediated through its effect on fertility) is not known. To further understand the role of diet in the acquisition of dominant pheromonal status and subsequently reproductive dominance in workers, we investigated the

composition of mandibular and tergal gland pheromones of queenless *A. m. scutellata* workers consuming either a protein-rich or carbohydrate-only diet and further assessed the status of their ovarian development.

2 Materials and Methods

2.1 Honey bees

Frames with capped brood were collected from three queen-right colonies of *Apis mellifera scutellata* Lepeletier headed by a naturally mated queen maintained in standard Langstroth hives at the apiaries of the University of Pretoria (25°44′49″S, 28°15′40″E, Pretoria, South Africa). The collected brood frames were incubated at 34 ± 1 °C and 60% RH in darkness, to simulate conditions within the hive until worker bees emerged. A total of 600 newly emerged workers ≥24 h old were collected from each colony and placed in Perspex® hoarding cages (100 bees per cage) (Köhler et al. 2013) and reared in an incubator using standard honey bee rearing procedures described by Williams et al. (2013) with slight modifications. Bees were fed *ad libitum* using plastic feeding vials (10 ml) with feeding aperture holes (1 x 0.5 cm wide) inserted horizontally into the cages, one with water and one with the experimental diet. Bees were presented with a no-choice diet made to a specific protein to carbohydrate ratio (P:C) by weight. Caseinate (Sodium caseinate, Sigma-Aldrich, St Louis, MO, USA), a milk-derived protein, was used as protein. Caseinate is not part of the natural diet of honey bees but it is commonly used in nutritional studies on animals including honey bees (Archer et al. 2014; Du Rand et al. 2020). The carbohydrate (Sucrose, Sigma-Aldrich, St Louis, MO, USA) concentration was kept constant (50% w/w sucrose) Control groups received a standard diet of 0:1 P:C (sucrose-only) and the experimental group 1:7 P:C. Diet consumption was monitored - diet tubes were weighed before being placed into the cages and were weighed after being replaced. Diets were provided fresh daily. Bees were randomly assigned to cages and cages were randomly assigned to the experimental or control groups (three hoarding cages in total per diet group per colony). After 1, 5, 6,

10, 12, 14, 15, 20 and 25 days, 15 bees per diet group were randomly sampled, immobilized on ice and stored at -20˚C until required for further analyses.

2.2 Extraction of mandibular and tergal gland pheromones

Heads of workers were removed, placed in glass vials and cephalic extracts were made by extracting in 200 µl of dichloromethane for at least 24 h following the methods described in (Yusuf et al. 2015). The tergal glands were collected from the same individuals by dissecting the intersegmental membrane dorsally with narrow strips of cuticle on both sides from abdominal tergites (II–V) and placed in 100 µl dichloromethane following the methods described in (Wossler and Crewe 1999b; Okosun et al. 2015). Extracts were stored at -20 °C until further chemical analysis.

2.3 Chemical analysis

2.3.1 Mandibular gland pheromones

Half of each cephalic extract (~100 µl) was evaporated to dryness under a gentle stream of nitrogen and the other half was stored as a backup for further analysis or confirmation if need be. The residue was re-dissolved in 10 µl of internal standards (containing 0.25 mg/ml octanoic acid and 0.25 mg/ml n-tetradecane in dichloromethane) to which 10 µl of bis-(trimethylsilyl) trifluoroacetamide (BSTFA; Sigma-Aldrich, St Louis, MO, USA) was added and allowed to derivatize for a at least 4 h. One µl of the derivatized extract was injected in splitless mode into an Agilent 6890N gas chromatograph fitted with a HP1-MS (Agilent J&W Santa Clara, CA, USA) capillary column (25 m × 0.20 mm × 0.33 µm) and flame ionization detector (FID). The injector and FID temperatures were set at 230 °C and 320 °C, respectively. Helium was used as carrier gas at a flow rate of 1.0 ml/min and the oven temperature-programmed as follows: 50 °C at 50 °C/min to 100 °C, then increased at 3 °C/min to 220 °C and then held at this temperature for 10 min (Yusuf et al. 2015). All recorded chromatograms were processed with Chemstation® software version B.02.01 (Agilent Technologies, Waldbronn,

Germany). Peaks for the six major components from honey bee mandibular glands (methyl p – hydroxybenzoate (HOB), 9-oxo-2 (E)-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), 9-hydroxy-2 (E)-decenoic acid (9-HDA), 10-hydroxydecanoic acid (10-HDAA) and 10-hydroxy-2 (E)-decenoic acid (10-HDA) were identified based on comparison with retention times of synthetic standards and quantified using their relative mass ratios in relation to those of the internal standards octanoic acid and n-tetradecane. All reagents and standards were of analytical grade with purities of 99% and were purchased from Sigma-Aldrich (St Louis, MO, USA) with the exception of 9-ODA and 9-HDA (PheroTech Inc.) which were custom synthesized.

2.3.2 Tergal gland pheromones

Ten µl of internal standard (0.25 mg/ml n-hexadecane in dichloromethane) were added to 10 µl of the tergal gland extract. One µl of the extract was injected in splitless mode into an Agilent 6890N gas chromatograph equipped with a HP5-MS (Agilent J&W Santa Clara, CA, USA) capillary column (25 m × 0.20 mm × 0.33 µm) and FID. The injector and FID temperatures were set at 230 °C and 310 °C, respectively. Helium was used as carrier gas at a flow rate of 1.0 ml/min and the oven temperature-programmed as follows: 50 °C at 50 °C/min to 100 °C, then increased at 6 °C/min to 300 °C and then held at 300 °C for 10 min (Okosun et al. 2015). Peaks for five of the major components from honey bee tergal glands (methyl palmitate, methyl stearate, stearic acid, tricosane, pentacosane) were identified based on comparison with retention times of synthetic standards and quantified using their relative mass ratios in relation to those of the internal standard n-hexadecane. All reagents and standards were of analytical grade and were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.4 Assessment of ovary activation

The level of ovarian activation was assessed from the same bees used for the pheromone analyses. The bees were dissected under a microscope and ovaries classified as: inactive - thread like ovarioles

(stage 1 and 2), intermediate - ovaries showing development of oocytes (stage 3), and activated - clearly developed oocytes (stage 4 and 5) as described in (Hess 1942; Schäfer et al. 2006; Pirk et al. 2010; Zheng et al. 2010; Okosun et al. 2015).

2.5 Statistical analyses

All data were evaluated for normality using the Shapiro–Wilks and Lilliefors tests and homoscedasticity using Levene's test prior to analysis. As the data was not normally distributed, Kruskal-Wallis ANOVA (KWA) and median test with multiple comparisons were applied to determine differences in the individual mandibular and tergal gland components among workers of different ages with diet as the independent (grouping) variable and the mandibular and tergal gland components as dependent variables. The percentage of workers with undeveloped, intermediately developed and fully developed ovaries were compared with a chi-square test. The alpha level was set to 0.05 for all analyses. All analyses were performed using IBM SPSS® Statistics v27.

3 Results

3.1 Mandibular gland pheromone profile

The queenless workers fed different diets for a period of 25 days showed marked differences in their mandibular gland pheromone bouquets (see Table 1 and Fig. S1).

HOB and HVA (the aromatic compounds): At day 6 (i.e. 6 day-old) workers that received the protein-rich diet produced significantly higher proportions of HOB [KWA: H = (1, N = 38) = 4.754, $P < 0.05$] than those fed the carbohydrate-only diet (see Table 1 and Fig. S1). While the differences in the proportions of HVA produced by the workers receiving different diets were significant at day 15, with the workers fed the protein-rich diet producing significantly higher proportions of HVA [KWA: H (1, N = 38) = 3.859, $P < 0.05$, see Table 1 and Fig. S1].

Table 1 The proportions of the mandibular gland components from caged, queenless workers of *A. m scutellata* fed different diets for 25 days The mandibular gland components are: HOB, p-hydroxybenzoate; 9-ODA, 9-oxo-2(E)-decenoic acid; 9-HDA, 9-hydroxy-2(E)-decenoic acid; 10-HDAA, 10-hydroxy-decanoic acid; and 10-HDA, 10-hydroxy-2(E)-decenoic acid; HVA, 4-hydroxy-3-methoxyphenylethanol. Data is presented as the mean proprotion ± SE. Significant differences in the proportion of a compound between dietary groups on a specific day are indicated with an asterisk (*) (Kruskal-Wallis ANOVA, $P<0.05$)

		HOB (%)		9-ODA (%)		HVA (%)		9-HDA (%)		10-HDAA (%)		10-HDA (%)	
Day	**N**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**
1	38	0.75 ± 0.75	1.44 ± 0.97	n.d.	n.d.	2.66 ± 1.47	2.36 ± 1.15	4.57 ± 3.51	1.19 ± 1.16	2.80 ± 1.62	3.50 ± 2.68	89.21 ± 4.56	91.50 ± 3.58
5	34	0.17 ± 0.17	0.16 ± 0.14	0.93 ± 0.59	0.16 ± 0.14	n.d.	n.d.	34.39 ± 6.36	37.49 ± 5.72	5.82 ± 1.14	5.78 ± 1.67	58.70 ± 7.17	56.05 ± 6.95
6	38	3.02 ± 1.73*	9.21 ± 2.55*	1.65 ± 1.34	0.04 ± 0.04	5.47 ± 1.35	3.67 ± 1.65	43.36 ± 7.62*	17.23 ± 2.77*	2.47 ± 0.97	7.41 ± 2.07	44.03 ± 7.24	62.44 ± 5.92
10	37	n.d.	0.24 ± 0.23	n.d.	n.d.	12.40 ± 4.51	7.99 ± 2.02	6.45 ± 2.80	10.35 ± 4.97	n.d.	1.91 ± 1.05	81.18 ± 4.63	79.50 ± 5.02
12	35	n.d.	1.15 ± 0.98	n.d.	n.d.	n.d.	n.d.	3.36 ± 2.10*	22.88 ± 5.60*	11.83 ± 5.37	n.d	84.81 ± 5.74	75.97 ± 6.02
14	36	0.67 ± 0.36	0.52 ± 0.51	0.59 ± 0.31	0.19 ± 0.13	0.90 ± 0.62	n.d.	36.87 ± 8.57	37.82 ± 9.24	4.40 ± 2.40	0.06 ± 0.06	56.57 ± 8.61	61.41 ± 9.30
15	38	4.65 ± 2.65	0.61 ± 0.41	0.17 ± 0.17	0.72 ± 0.44	0.89 ± 0.87*	3.57 ± 1.33*	24.59 ± 7.54*	50.34 ± 9.64*	7.39 ± 2.98	2.89 ± 0.93	62.31 ± 7.47*	41.87 ± 8.64*
20	31	n.d.	n.d.	n.d.	n.d.	5.94 ± 2.30	7.91 ± 2.76	8.36 ± 4.68	16.92 ± 6.47	3.32 ± 1.72	6.64 ± 2.43	82.39 ± 5.30	68.52 ± 7.27
25	21	n.d.	n.d.	0.61 ± 0.41	4.25 ± 3.29	n.d.	1.01 ± 0.78	39.26 ± 9.04	11.23 ± 6.15	3.93 ± 1.76	5.22 ± 3.08	56.20 ± 9.48	78.30 ± 8.20

9-ODA and 9-HDA (the queen compounds): There were no significant differences in the proportions of the queen substance 9-ODA produced by workers fed different diets during the 25 days [KWA: Day 1 H (1, N = 38) = 0.000, P > 0.05; Day 5 H (1, N = 34) = 0.431, P > 0.05; Day 6 H (1, N = 38) = 0.317, P > 0.05; Day 10 H (1, N = 37) = 0.000, P > 0.05; Day 12 H (1, N = 35) = 0.000, P > 0.05; Day 14 H (1, N = 36) = 0.536, P > 0.05;Day 15 H (1, N = 38) = 1.028, P > 0.05; Day 20 H (1, N = 31) = 0.000, P > 0.05; Day 25 H (1, N = 21) = 0.019, P > 0.05; see Table S1]. In contrast, there were marked differences in the proportions of its precursor, 9-HDA. At day 6, the workers on the carbohydrate-only diet produced significantly higher proportions of 9-HDA [KWA: H (1, N = 38) = 4.197, P < 0.05], their mandibular gland pheromone bouquets consisted of 43.36 ± 7.62 % 9-HDA compared to the 17 ± 2.77 % present in the bouquets of the workers fed the protein-rich diet (Table 1 and Fig. S1). At days 12 and 15 the inverse was observed, the bouquets of the workers fed the protein-rich diet exhibited significantly higher proportions of 9-HDA than the workers fed the carbohydrate-only diet [KWA: H (1, N = 35) = 6.666, P < 0.05 and KWA: H (1, N = 38) = 4.984, P < 0.05, respectively]. Interestingly, at day 15 the bouquets of protein fed workers were composed of 50.34 ± 7.54 % 9-HDA, while 9-HDA constituted only 24.59 ± 7.54% of the bouquets of the workers fed the carbohydrate-only diet (Fig. S1), the opposite of what was observed at day 6.

10-HDAA and 10-HDA (the worker compounds): At day 12, the proportions 10-HDAA (the precursor of 10-HDA) produced by workers on the protein-rich diet were significantly lower than the workers fed the carbohydrate-only diet [KWA: H (1, N = 38) = 3.961, P < 0.05] (Table 1 and Fig. S1). Three days later at day 15, the proportion of 10-HDA were significantly lower [KWA: H (1, N = 35) = 6.678, P < 0.05] in the workers feeding on the protein-rich diet.

3.2 Tergal gland pheromone profile

The tergal gland pheromone bouquets of the workers fed on protein-rich or carbohydrate-only diets for a period of 25 days also exhibited marked differences (see Table 1 and Fig. S2).

The fatty acid and esters (MEP, MES and STA): Workers that received the protein-rich diet produced significantly lower proportions of methyl palmitate at day 1 [KWA: H (1, N = 40) = 6.714, P < 0.05] and day 5 [KWA: H (1, N = 40) = 4.221, P < 0.05]. At day 10, the proportions of methyl stearate and stearic acid were significantly lower in workers fed the protein-rich diet [KWA: H (1, N = 18) = 5.755, P < 0.05 and KWA: H (1, N = 18) = 5.755, P < 0.05, respectively].

Long-chained hydrocarbons or n-alkanes (C23 and C25): In contrast to the fatty acid and esters, the workers fed the protein-rich diet produced significantly higher proportion of tricosane at day 1 [KWA: H (1, N = 40) = 7.317, P < 0.05] and day 10 [KWA: H (1, N = 18) = 5.337, P < 0.05] than the workers fed the carbohydrate-only diet. Likewise, workers fed the protein-rich diet also produced significantly higher levels of pentacosane at day 1 [KWA: H (1, N = 40) = 4.338, P < 0.05] and at day 10 [KWA: H (1, N = 18) = 5.337, P < 0.05].

A noticeable shift in the chemical profile of the tergal gland secretions were observed during the 25 day experimental period that were not linked to diet, but seemed to be related to age. Up to and including day 10, the fatty acid and esters (stearic acid, methyl palmitate and methyl stearate) accounted for the largest portion of the pheromonal bouquets of workers in both the dietary groups. However, after day 10 the proportions of stearic acid, methyl palmitate and methyl stearate produced decreased drastically, leaving the n-alkanes (tricosane and pentacosane) to dominate the pheromonal bouquets in both the dietary groups alike (see Table 2 and Fig. S2).

3.3 Ovarian development

Results from the ovarian assessments revealed that the ovaries of the workers fed the protein-rich and carbohydrate-only diets were inactive (stages 1 and 2) until they reached 14 days old. In both dietary groups, 10 % of workers had intermediate developed ovaries (stages 3) at day 14 (see Fig. 1). Ten percent, 15 % and 20 % of workers fed the carbohydrate-only diet had ovaries classified as

Table 2 The proportions of five major tergal gland components from caged, queenless workers of *A. m scutellata* fed different diets for 25 days The tergal gland components are: MEP, methyl palmitate; MES, methyl stearate; STA, stearic acid; C23, tricosane; and C25, pentacosane. Data is presented as the mean proprotion ± SE. Significant differences in the proportion of a compound between dietary groups on a specific day are indicated with an asterisk (*) (Kruskal-Wallis ANOVA, $P<0.05$)

		MEP (%)		MES (%)		STA (%)		C23 (%)		C25 (%)	
Day	**N**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**	**Sucrose-only diet**	**Protein diet**
1	40	12.98 ± 2.74*	5.14 ± 1.53*	52.37 ± 6.36	37.53 ± 6.23	12.16 ± 2.67	14.74 ± 3.14	13.68 ± 2.49*	27.48 ± 3.91*	8.81 ± 1.76*	15.10 ± 2.16*
5	38	8.94 ± 2.19*	2.96 ± 0.78*	28.40 ± 4.47	31.85 ± 4.46	19.63 ± 3.06	16.05 ± 2.72	27.40 ± 2.71	27.63 ± 2.59	15.62 ± 1.57	21.52 ± 2.27
10	18	2.00 ± 0.11	1.00 ± 0.15	29.55 ± 1.45*	22.10 ± 2.17*	58.26 ± 2.86*	43.57 ± 4.27*	6.96 ± 2.52*	20.20 ± 3.70*	5.02 ± 1.84*	13.98 ± 3.08*
15	20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	52.13 ± 1.10	43.81 ± 6.04	47.88 ± 1.15	56.19 ± 6.14
20	20	4.40 ± 4.40	n.d.	n.d.	n.d.	n.d.	n.d.	56.31 ± 4.27	59.60 ± 0.79	39.29 ± 3.42	40.40 ± 0.80
25	20	4.08 ± 2.16	2.27 ± 2.27	2.90 ± 1.50	n.d.	5.73 ± 2.97	n.d.	54.07 ± 4.85	60.48 ± 1.54	33.22 ± 3.19	37.25 ± 1.60

intermediate developed ovaries (stages 3) at days 10, 20 and 15, respectively. In comparison, 15%, 20 % and 33 % of workers fed the protein-rich diet had ovaries classified as intermediate developed ovaries (stages 3) at days 15, 20 and 25, respectively. At day 25, the percentage of workers with intermediate developed ovaries (stages 3) were significantly higher ($\chi^2 = 2.133$, df = 1, $P < 0.05$) in the group that were fed the protein-rich diet (33 %) than the group that received the carbohydrate-only diet (20 %). No worker developed fully activated ovaries (stage 4 and 5) during the experiments (Fig. 1).

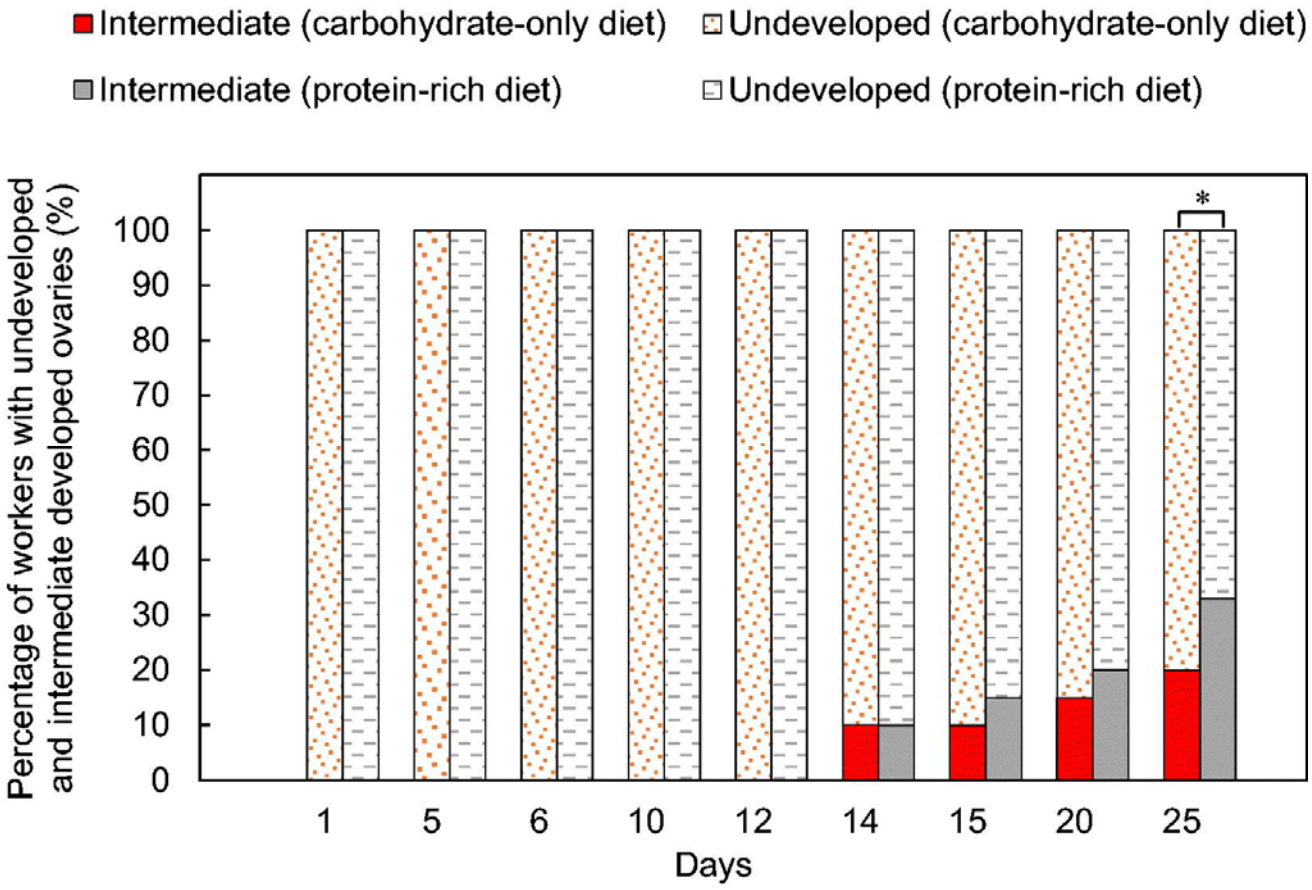

Figure 1. Percentage of bees with different stages of ovarian activation in queenless workers of *A. m. scutellata* fed different diets for 25 days At day 25, the percentage of workers with intermediate developed ovaries (stages 3) were significantly higher in the group that were fed the protein-rich diet. Significant differences are indicated with an asterisk (*). ($\chi^2 = 2.133$, df = 1, $p < 0.05$).

4 Discussion

Pheromones are chemicals that elicit behavioural and physiological responses in conspecific. Usually, both the chemical composition of a pheromone and the responses of other individuals to it are thought to be highly stereotyped, so that any deviation in composition can affect the responses. Here we present evidence of the role of diet in gaining pheromonal dominance in honey bee workers in the absence of the queen. We show that differences in diet can lead to differences in the composition of mandibular and tergal gland chemicals in queenless workers and that it is not mediated through diet's effect on fertility. Specifically, the results show the influence of diet on pheromone profiles and the link between diet and the proportions of specific pheromonal components in queenless workers (see Tables 1 and 2, Fig. S1 and S2).

Workers that received the protein-rich diet produced more queen-like mandibular profiles than the workers that received the carbohydrate-only diet. Development of the more queen-like mandibular gland profiles at day 12 and onwards, coincided with an increased percentage of workers with activated ovaries (Fig. 1), confirming that the workers are acquiring more queen-like attributes. There were no significant differences in the levels of 9-ODA (the major queen pheromone compound) produced by workers consuming different diets, but 9-HDA (the precursor of 9-ODA) became more prominent in the glandular profile of workers on the protein-rich diet from day 12 (i.e. from when workers were 12 days old). At day 15, the relative proportions of 9-HDA increased to 50.34 ± 9.64 % in workers consuming the protein-rich diet, double the proportion observed in the same aged workers that fed on the carbohydrate-only diet and higher than those reported in queen-right *A. m. scutellata* workers of mixed ages (Zheng et al. 2010) and queenless workers that consumed sucrose solution and pollen in the presence of synthetic queen mandibular gland pheromones (Yusuf et al. 2018). In queens, 9-HDA is the second most abundant fatty acid in mandibular gland secretions (Slessor et al. 1988) and are functionally active in maintaining swarm clusters (Winston et al. 1982), inhibiting

queen rearing (Butler and Callow 1968) and triggering retinue response (Slessor et al. 1988), either acting alone or synergistically with 9-ODA. As such, the presence of high levels of 9-HDA in workers is expected to disturb the social organization within a colony (Zheng et al. 2010) and is a strong indication that the workers' pheromonal bouquets are becoming more queen-like than worker-like. We also observed that the production of higher levels of 9-HDA in the protein fed workers coincided with these workers producing significantly lower proportions of the worker compound 10-HDA and its precursor, 10-HDAA (at days 12 and 15). Relative proportions of 10-HDA and 10-HDAA decreased to below 42% and 2% (respectively) in the workers consuming the protein-rich diet, well below those previously reported in queen-right *A. m. scutellata* workers of mixed ages (Zheng et al. 2010) and queenless *A. m. scutellata* workers consuming sucrose solution and pollen in the presence of synthetic queen mandibular gland pheromones (Yusuf et al. 2018). The 10-HDAA proportions remained lower than the previously reported proportions up to day 25, while the proportions of 10-HDA increased again after day 15 to within the ranges previously reported for *A.m. scutellata* workers (Zheng et al. 2010; Yusuf et al. 2018). The mandibular gland profiles of the protein fed workers with the high proportions of 9-HDA, together with the lower proportions of the worker compounds 10-HDA and 10-HDAA, markedly indicate a more queen-like mandibular gland pheromone profile, but it has not yet acquired all the characteristic to be classified as queen-like mandibular gland pheromones, such as high proportions of 9-ODA, as defined by Crewe and Velthuis (Crewe and Velthuis 1980), Sakagami (Sakagami 1958) and Yusuf et al. (Yusuf et al. 2018). This, together with the observation that the ovaries are not fully activated, seem to suggest that diet could prime queenless workers to become false queens but do not trigger the transformation into false queens in terms of their pheromonal status.

It is unlikely that the observed effect of diet on pheromone status is mediated by through its effect on fertility (Hoover et al. 2006; Human et al. 2007; Pirk et al. 2010; Altaye et al. 2010). The results

show that there are no significant differences in ovary activation between dietary groups for workers of the same age (Fig. 1). Yet, significant differences in those same workers' chemical profiles were observed (Tables 1 and 2, Fig. S1 and S2). Taken together, this strongly suggests that diet directly affects the chemical profiles and that the observed effect in this specific social context is not mediated through the effect of diet on reproductive status. If diet were driving ovary activation, we would have expected to see significant differences in ovary activation between the dietary groups by Day 15 (Hess 1942) coupled with the observed significant differences in the chemical profiles.

Regarding the production of the other mandibular gland pheromone components, HOB and HVA which are both queen-specific, the relative proportions of both these aromatic fatty acids varied with different diets, however, the proportions reflected the levels found in worker-like pheromonal bouquets of *A. m. scutellata* (Zheng et al. 2010; Yusuf et al. 2018).

The observed fluctuations in the pheromone profiles of the workers during the 25-day experimental period are not unexpected. Honey bee worker pheromone profiles within the social structures of colonies and micro-colonies are highly dynamic (Allan et al. 1987; Crewe et al. 1989; Simon et al. 2001; Okuson et al. 2015). These fluctuations in the pheromone profiles can be due to the natural progression or transitioning of the workers to the next age-associated task. Workers perform a series of tasks in the colony linked to their age with workers in the early stages of their life performing tasks inside the nest such as cleaning, brood care, feeding of the queen, wax production, building comb, progressively taking over tasks at the periphery of the brood nest to the outside of the nest such as guarding and foraging (Rösch 1925, 1930; Lindauer 1952). The workers performing the different tasks can normally not be distinguished by external morphology, but these age-associated tasks are accompanied by various changes in nutritional requirements, anatomy and physiology, including changes in exocrine glands because of changing functions (Huang et al. 1994; Huang and

Robinson 1996; Naiem et al. 1999). For example, when workers are 6 days old, they transition to nurse bees. The production of the mandibular gland fatty acids like 9-HDA and 10-HDA are expected to increase as these fatty acids are not only important components of the mandibular gland pheromone bouquet, but also function as nutritional fatty acids present in the protein-rich food jelly produced by nurse bees that are fed to the larvae, queen and other nest mates (Barker et al. 1959; Crailsheim et al. 1992; Isidorov et al. 2009; Buttstedt et al. 2023). The protein components of the protein-rich food jelly are synthesized in the hypopharyngeal glands (Knecht and Kaatz 1990; Patel et al. 1960). During this time when workers assume the role of nurse bees and fatty acid production in the mandibular glands are upregulated due to the task at hand (Buttstedt et al. 2023), the workers competing to gain pheromonal dominance when there is no queen signal to regulate the increased fatty acid production becomes evident in the pheromone profiles (Sakagami 1958; Crewe and Velthuis 1980; Plettner et al. 1993; Moritz et al. 2000, 2004).

In the present study, at the onset of transitioning to nurse bees at day 6, protein fed workers produce significantly less 9-HDA compared to carbohydrate-only fed workers. However, at day 15 (in the older nurse bees), the protein fed workers produce significantly higher amounts of 9-HDA. This is noteworthy since the increased production of 9-HDA, a queen compound, is coupled with a significant decrease in the production of 10-HDA, a worker compound. If the higher production of 9-HDA was due to the upregulation of protein jelly production induced by the protein in the diet, a coinciding increase in 10-HDA production would have been observed as well given that 10-HDA is one of the major fatty acids found in the protein-rich jelly produced by nurse bees (it is present at much higher levels in the jelly than 9-HDA) (Barker et al.1959; Isidorov et al. 2009; Buttstedt et al. 2023). However, the results do not reflect this, but rather increased production of 9-HDA (a queen compound) and decreased production of the worker compound 10-HDA (see day 15, Table 1). An indication that the pheromone profile is becoming more queen-like in character than worker-like in

character, though it is not a queen signal yet as it has not yet acquired all the characteristics to be classified as a queen mandibular gland signal, and as such the signal is not sufficient to suppress to age-related transition to the next age-associated worker task that causes a change in behaviour and the accompanying change in physiology. [Queen-like pheromones are defined as 9-ODA and 9-HDA dominating the mandibular gland secretions (Moritz et al. 2000; Moritz et al. 2004) with a ratio of above 0.9 of 9-ODA and 10-HDA (Schäfer et al. 2006)]. After day 15, the workers transition into the next age-associated task followed by the subsequent fluctuation in the pheromone profiles (see Table 1). Transitioning from a nurse bee to the next task is accompanied by physiological changes in the mandibular glands (and the hypopharyngeal glands) reflecting the shift in emphasis from producing food jelly components, to increased synthesis of the ketone 2-heptanone, an alarm pheromone to enhance colony defense or scent marker for foraging efficiency produced by the mandibular glands (Vallet et al. 1991; Hua et al. 2016; Buttstedt et al. 2023). Though older workers (> 15 days) do not cease to produce 9-HDA, 10-HDAA and 10-HDA after transitioning from being nurse bees under both queen-right and queenless condition, which is also reflected in the results (Table 1) (Allan et al. 1987; Crewe et al. 1989; Simon et al. 2001; Zheng et al. 2010; Yusuf et al. 2015; Mumoki et al. 2021; Buttstedt et al. 2023). It is also worth noting that the ability to digest consumed protein drastically decreases after transitioning. Nurse bees have high levels of midgut proteolytic activity to digest consumed protein and produce the protein-rich jelly (Crailsheim and Stolberg, 1989). Proteolytic activity in the midgut declines to minimal amounts in older workers, though sufficient proteolytic activity remains for the digestion of the proteins in the jelly received through trophallaxis (Crailsheim and Stolberg 1989; Crailsheim et al. 1992; Brodschneider and Crailsheim 2010).

Regarding the influence of diet on pheromone production in the tergal glands: the tergal gland pheromone bouquets of the workers fed different diets also presented marked differences (Table 2 and Fig. S2). Though the proportions of the individual components varied with diet, there were no

clear shift in the profiles to be coming less worker-like and more queen-like as observed in the mandibular gland pheromone profiles. Apart from methyl stearate at days one and five and stearic acid at day 10 that exhibited proportions that are considered to be more queen-like, the other components remained within the ranges of the tergal gland pheromone blends that are considered worker-like (Wossler and Crewe 1999b; Okosun et al. 2015). An unexpected shift in the chemical profiles of the tergal gland secretions were observed at day 15 that were seemingly not related to diet or the workers acquiring dominant pheromonal status, but seemed to be an age-related change in the workers' profiles. Up to and including day 10, the fatty acid and fatty acid esters (stearic acid, methyl palmitate and methyl stearate) accounted for the largest portion of the pheromonal bouquets of the workers (from both the dietary groups), reflecting the trend previously reported for queen-right and aw well as queenless *A. m. scutellata* workers (Wossler and Crewe 1999b; Okosun et al. 2015). However, at day 15 the proportions of the fatty acid and fatty acid esters (stearic acid, methyl palmitate and methyl stearate) produced decreased drastically, leaving the n-alkanes (tricosane and pentacosane) to dominate the pheromonal bouquets of the workers from both the dietary groups alike (see Table 2 and Fig. S2). Curiously, this shift from fatty acid and fatty acid esters to the n-alkanes coincided with an increase percentage of workers exhibiting activated ovaries (see Fig. 1). This shift was unexpected as it was previously found that the fatty acid and fatty acid ester pheromone components are present in higher proportions in ovary-activated queenless workers (Okosun et al. 2015). The reasons for the shift from fatty acid to n-alkane pheromone components in the tergal glands could likely be attributed to ontogeny. It could be that younger (0 to 10 days old) workers predominately rely and use the more volatile cues (fatty acid esters) that could transmit information further in the hive without contact, while older (15 to 25 days old) workers undertaking more mobile tasks rely more on the less volatile contact cues (the hydrocarbons) for communication.

In conclusion, we present evidence of the role of diet in gaining pheromonal dominance in workers in the absence of the queen. We show that diet is a causative factor in the emergence of pheromonal differences, consequently modulating pheromone status that ultimately affect reproductive dominance in queenless workers. Understanding the role of diet in establishing pheromonal dominance hierarchies in social insects, like the honey bee, deepens our understanding of the proximate drivers shaping eusociality.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

AAY and CWWP conceived the study. OOO and EEDR performed the experiments. EEDR performed data analysis and wrote the first draft of the manuscript. AAY, CWWP, OOO and EEDR contributed to revisions. AAY and CWWP secured funding. All authors gave final approval for publication.

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8 Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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