Effects of age and Reproductive Status on Tergal Gland Secretions in Queenless Honey bee Workers, *Apis mellifera scutellata* and *A. m. capensis*

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Abstract

Secretions from tergal glands are part of a queen's pheromonal control of worker reproduction in honey bees. However, in queenless honey bee colonies, workers compete to gain pheromonal, and hence reproductive dominance, over nestmates with ontogenetic changes in their glandular secretions that affect the behavioral or physiological responses of other individuals. Using gas chromatography/mass spectrometry, we investigated for the first time the age-dependent changes in tergal gland secretions of queenless workers of the clonal lineage of *Apis mellifera capensis* and workers of *A. m. scutellata*. The reproductive status of honey bee workers was determined by recording the presence of spermathecae and the level of ovarian activation. The tergal gland chemicals identified in both *A. m. scutellata* workers and *A. m. capensis* clone workers were oleic acid, *n*-tricosene, *n*-pentacosene, and *n*-heptacosene, with three additional compounds, palmitic acid, *n*-heneicosene, and *n*-nonacosene, in *A. m. capensis* clones. We report ethyl esters as new compounds from honey bee worker tergal gland profiles; these compounds increased in amount with age. All *A. m. capensis* clone workers dissected had spermathecae and showed ovarian activation from day 4, while ovarian activation only started on day 7 for *A. m. scutellata* workers that had no spermathecae. Tergal gland secretions were present in higher quantities in bees with activated, rather than inactive ovaries. This suggests that tergal gland secretions from reproductive workers could act as releaser and primer pheromones in synergy with other glandular compounds to achieve pheromonal and reproductive dominance.

Keywords

Tergal glandsQueenless workersAgeOvarian activationEthyl estersReproductive statusHoney beeInvasive beeSocial parasitism

Introduction

Pheromones emitted from exocrine glands are involved in chemical communication in insects. These secretions play an important role in maintenance of colony integrity and

division of labor in social insects (Le Conte and Hefetz 2008). Primer pheromones have long-term effects by altering development, physiology, and neural systems of the receivers, while releaser pheromones have short-term effects that influence a receiver's behavior, including mate attraction, orientation, and alarm in social insects such as ants (Richard and Hunt 2013), termites (Prestwich 1983), bumblebees (Ayasse and Jarau 2014), and honey bees (Pankiw 2004; Winston and Slessor 1998;). A queen uses secretions from her exocrine glands, inclduing mandibular, tergal, or Dufour's, to control the activities and reproductive hierarchy in the colony (Katzav-Gozansky et al. 1997; Keeling et al. 2003; Sole et al. 2002; Winston and Slessor 1998). Not being in direct contact with a queen does not preclude pheromone transfer, as messenger worker bees help to disseminate the queen's pheromones within the colony (Juska et al. 1981; Seeley 1979).

The tergal gland pheromone is an important part of honey bee queen control in a colony. Some of the compounds in the tergal gland secretions of the honey bee include hexadecanoic acid methyl ester (methyl palmitate), (Z)-9-hexadecenoic acid (palmitoleic acid), hexadecanoic acid (palmitic acid), (Z)-9-octadecenoic acid (oleic acid), (*E*)-11-octadecenoic acid (vaccenic acid), octadecanoic acid methyl ester (methyl stearate), octadecanoic acid (stearic acid), decanoic acid (lauric acid), decyl decanoate, *n*-alkanes and alkenes (Al-Qarni et al. 2005; Espelie et al. 1990; Smith et al. 1993; Wossler and Crewe 1999a). A honey bee queen's tergal gland secretions act as contact pheromones that elicit retinue behavior in workers (Wossler and Crewe 1999b). This is achieved in synergy with the mandibular glands' secretions that attract workers for retinue behavior with the help of messenger bees, with the tergal gland secretions helping to maintain the court (Seeley 1979; Velthuis 1985; Vierling and Renner 1977). In some instances, in the absence of mandibular glands, both tergal and Koschewnikow gland secretions are attractive to workers (Butler and Simpson 1965; Vierling and Renner 1977). A queen's tergal gland secretions also are involved in mating behavior, functioning as a source of attractant in mounting drones, initiating fighting among virgin queens, and supressing ovarian activation in workers (Butler 1971; Pflugfelder and Koeniger 2003; Renner and Vierling 1977; Vierling and Renner 1977; Wossler and Crewe 1999c).

Ontogenetic changes in honey bee, *Apis mellifera* L, queen tergal gland secretions also occur, as well as differences in secretions due to mating status (Al-Qarni et al. 2005; Espelie et al. 1990; Smith et al. 1993; Wossler and Crewe 1999a). Ontogenetic changes in a queen's glandular secretions lead to modulation in both primer and releaser effects on the receiver that can enable the queen to assert her dominance over workers within a colony (Crewe and Velthius 1980; Velthuis 1985). The queen asserts her dominance in the colony through effective regulatory mechanisms in place to maintain the reproductive division of labor (Mohammedi et al. 1998). Nonetheless, some honey bee workers evade reproductive dominance and reproduce, such as in Cape honey bees (Onions 1912; Velthuis 1970; Velthuis et al. 1990).

The Cape honey bee *A. m. capensis* and the Savannah honey bee, *A. m. scutellata* are subspecies found in South Africa. *Apis mellifera scutellata* is found in the northern region, while *A. m. capensis* is found in the Western Cape coastal region. Cape honey bee workers have unique traits, such as thelytokous worker reproduction (Onions 1912), possession of spermathecae (Ruttner 1977), and high ovariole numbers (Phiancharoen et al. 2010).

Additionally, Cape honey bee workers have short latency periods before becoming reproductive, and produce queen-like pheromones rapidly after queen loss (Crewe and Velthius 1980; Velthuis et al. 1990). The successful reproduction of *A. m. capensis* laying workers in the presence of a queen is possible if they escape the reproductive suppression of the resident queen and brood, and causes reproductive competition that leads to reproductive anarchy (reviewed in Neumann and Hepburn 2002; Neumann and Moritz 2002). All these traits enable *A. m. capensis* workers to become social parasites in colonies of their own sub-species (Härtel et al. 2006) or other sister sub-species like *A. m. scutellata* (Neumann and Hepburn 2002; Zheng et al. 2010), despite the presence of queen and worker policing (Neumann et al. 2003; Pirk et al. 2002, 2003).

Since the introduction of an *A. m. capensis* parasitic lineage (clones) (Baudry et al. 2004) into the native range of *A. m. scutellata* in the northern region of South Africa (Allsopp and Crewe 1993; Johannsmeier 1997) by beekeepers, *A. m. capensis* clonal workers have been able to invade queenright *A. m. scutellata* colonies successfully. The infestations of *A. m. capensis* laying worker clones cause annual losses of numerous commercial Savannah honey bee colonies (Du Toit 2001; Johannsmeier 2001). This has forced some commercial beekeepers out of business, resulting in an increase in the price of honey and other bee products as well as the cost of pollination services (Du Toit 2001; Johannsmeier 2001). Furthermore, as a social parasite, *A. m. capensis* clones increase in numbers in *A. m. scutellata* colonies upon infestation and, as they do not forage or contribute to the work of the colony, result in eventual death of the infested colony as the number of host workers declines (Neumann and Moritz 2002). In queenless situations, *A. m. capensis* clones take advantage of the absence of a queen in a colony. This results in strong competition for production of queen-like pheromone bouquets among workers, with some workers becoming reproductive and laying eggs (Crewe and Velthius 1980; Moritz et al. 2000; Velthuis et al. 1990). The success of social parasitism by *A. m. capensis* laying workers is enhanced by their ability to mimic the queen's mandibular and Dufour's gland secretions (Moritz et al. 2000; Sole et al. 2002).

Queen tergal gland secretions and their roles in the organization of the colony have been extensively investigated (Al-Qarni et al. 2005; Espelie et al. 1990; Renner and Vierling 1977; Smith et al. 1993; Vierling and Renner 1977; Wossler and Crewe 1999a), but little is known about the tergal gland secretions of workers and their role in the colony. Although Wossler and Crewe (1999a) reported that queenright workers of *A. m. scutellata* and *A. m. capensis* in their native range can be separated, based on tergal gland profile, the tergal gland secretions of queenless workers, and especially those of the *A. m. capensis* parasitic worker clonal lineage, have not been investigated. Moreover, ontogenetic changes and the effects of reproductive status on glandular secretions have been reported for the mandibular gland (Simon et al. 2001, 2005) and Dufour's gland (Sole et al. 2002) of *A. m. capensis* workers, but not for queenless worker tergal gland secretions or whether these are affected by reproductive status of queenless workers. This study investigated what changes occur in tergal secretions of *A. m. capensis* clone workers and *A. m. scutellata* workers with age, and whether reproductive status of honey bee workers has an effect on tergal gland secretions.

Methods and Materials

Worker Rearing

Worker brood frames from three colonies were collected from a commercial apiary in Pretoria in November, 2012 for *A. m. capensis* clones, and from three colonies of *A. m. scutellata* from the University of Pretoria apiary between August 2012 and March 2013. All frames were placed in an incubator at 34 °C and 60 % r.h until workers emerged. Fifty newly emerged bees from the different colonies then were placed in standard 12.5 × 10 × 15 cm wooden hoarding cages fitted with comb (Pirk et al. 2010) and kept for 2, 4, 6, 7, 14, or 21 days. There were three cages (from the three different colonies) for each day with 21 cages each for *A. m. capensis* clones and *A. m. scutellata*. Bees were fed on pollen, 50 % sugar water solution, and water *ad libitum*.

To compare tergal gland and cuticular profiles of queenless caged honey bee workers with queenright workers, queenright *A. m. scutellata* workers were collected from the University of Pretoria apiary (August 2014). Ten honey bee workers of unknown age were collected per colony from three different colonies.

Dissections

At the end of each trial period (2, 4, 6, 7, 14, and 21 days), the remaining bees were frozen until required for dissection. Ten bees were randomly selected and dissected for each treatment (day and cage), with a total of 400 bees dissected. The tergal glands of bees at different ages were obtained by dissecting the intersegmental membrane longitudinally with narrow strips of cuticle on both sides from abdominal tergites II-V (Wossler et al. 2000) [mean length (SD) x mean width (SD) 5.34 ± 0.21 × 0.51 ± 0.04 mm]. To allow for identification of the tergal gland components, the most anterior portion of abdominal tergite II (4.06 ± 0.11 × 0.67 ± 0.03 mm) was used to obtain a cuticular extract. These abdominal tergite strips were placed in 100 µl of dichloromethane (DCM) in 2 ml screw top sample vials and stored at −20 °C until chemical analysis.

Assessment of Ovary Activation and Presence of Spermatheca

The level of activation of ovaries in each bee dissected was assessed and ranked following Hess (1942): workers with thread-like ovarioles (stages I-II), workers with intermediate ovaries showing early development of oocytes (stage III), and workers with clearly defined oocytes (stages IV-V) (Hess 1942; Schäfer et al. 2006; Velthuis 1970). For statistical analysis, the ovaries were classified as either inactive (stages I-II) or activated (stages III-V). The presence or absence of spermathecae in the workers also was recorded (Phiancharoen et al. 2010; Ruttner 1977).

Gas Chromatography (GC)

From the tergal gland and cuticular extracts, an aliquot of 10 µl of each was placed in a 150 µl GC vial insert and evaporated to dryness under a gentle stream of nitrogen; the residue was redissolved in 10 µl of internal standard solution (~1 mg of *n*-hexadecane in 4 ml of DCM) and 10 µl of DCM. One microliter of this was injected into an Agilent

Technology 6890 N gas chromatograph fitted with an HP5 capillary column (25 m × 0.20 mm × 0.33 μm; Agilent) and a flame ionization detector (FID). The oven temperature was programmed from 50 to 100 °C at the maximum heating rate and then ramped to 300 °C at 6 °C.min^{-1}, and held for 10 min. The injection port was 230 °C and the FID 310 °C. Helium was the carrier gas at a flow of 1.0 ml.min^{-1}. Peaks were quantified relative to the internal standard. The amount (μg) of each component produced by the tergal gland was obtained by subtracting its amount in the cuticular extract from its amount in the tergal gland extract. Both tergal gland and cuticular compounds were identified based on retentions times in comparison with those of synthetic compounds.

GC-Mass Spectrometry (GC/MS)

To confirm the identities of the compounds, extracts were analyzed on a Shimadzu QP2010 ultra GC/MS in electron impact ionization mode. One microliter of each sample was injected (at 230 °C) with a split ratio of 10:1 onto an InertCap 5MS/NP capillary column (30 m × 0.25 mm × 0.25 μm; GL Sciences, Tokyo, Japan). Helium was the carrier gas at 1.0 ml.min^{-1} and the oven was programmed from 50 to 100 °C at the maximum heating rate and then at 6 °C.min^{-1} to 300 °C, and held for 10 min. The ion source was operated at 200 °C. Mass spectral data between *m*/*z* 50–700 were recorded at 70 eV with a scan speed of 2500. Compounds were identified based on a comparison of mass spectra with those in mass spectral libraries [NIST08 and Wiley (10th edition)] and confirmed with pure synthetic fatty acids, alkyl esters, and hydrocarbon standards purchased from Sigma Aldrich (St. Louis, MO, USA).

Statistical Analyses

Since data for chemical profiles of tergal glands and cuticular extracts were not normally distributed (Shapiro Wilks tests), non-parametric tests were employed (Pirk et al. 2013). The Mann–Whitney *U* test (MWU) was used to compare honey bee tergal gland and cuticular extracts, as well as honey bee workers with activated and inactive ovaries, for both *A. m. capensis* clones and *A. m. scutellata* workers. The Kruskal-Wallis test (KWA) was used to compare quantitative changes with age in tergal gland and cuticular extracts for each subspecies. To separate the tergal gland profiles of *A. m*. scutellata workers and *A. m. capensis* clones, the means of the relative proportions of putative semiochemicals were subjected to principal components analysis. All statistical analyses were performed using Statistica 12 (StatSoft USA).

Results

Chemical Profiles of Tergal Gland and Cuticular Extracts of A. m. capensis Clone Workers and A. m. scutellata Workers

The compounds identified in the tergal gland and cuticle extracts of both *A. m. capensis clones* and *A. m. scutellata* workers were fatty acids, alkyl esters, unsaturated (*n*-alkenes), and saturated hydrocarbons (*n*-alkanes).

Unless otherwise specified, MWU values are for differences between tergal gland and cuticular profiles on day 21, and KWA values for differences during adult development. In *A. m. capensis* clones, for all days, greater quantities of palmitic acid (MWU, $U = 125$; $P < 0.001$) and oleic acid (MWU, $U = 56$; $P < 0.001$) were found in tergal gland, compared to cuticular extract (Supplementary Fig. 1). Similarly, for all days, greater amounts of unsaturated hydrocarbons were found in tergal gland, than in cuticular extracts, for which they were only present in trace amounts: *n*-heneicosene (MWU, $U = 72$; $P < 0.001$), *n*-tricosene (MWU, $U = 80$; $P < 0.001$), *n*-pentacosene (MWU, $U = 37$; $P < 0.001$), *n*-heptacosene (MWU, $U = 29$; $P < 0.001$), and *n*-nonacosene (MWU, $U = 70$; $P < 0.001$) (Supplementary Fig. 2). The opposite was true for saturated hydrocarbons, with low amounts in the tergal glands extract compared to the cuticular extract. The amounts of *n*-heptacosane, *n*-nonacosane, and *n*-hentriacontane were greater in cuticular than in tergal gland extracts (MWU, $U = 85$; $P < 0.001$) (Supplementary Fig. 3).

There were increases in amounts of methyl palmitate [KWA: H (6, $N = 202$) = 25.77; $P < 0.001$], palmitic acid [KWA: H (6, $N = 202$) = 31.54; $P < 0.001$], ethyl palmitate [KWA: H (6, $N = 202$) = 36.64; $P < 0.001$], methyl stearate [KWA: H (6, $N = 202$) = 19.23; $P < 0.001$], oleic acid [KWA: H (6, $N = 202$) = 46.49; $P < 0.001$], and ethyl oleate [KWA: H (6, $N = 202$) = 30.41; $P < 0.001$] (Supplementary Fig. 1) in tergal gland extracts with age. A similar result was found for unsaturated hydrocarbons [KWA: H (6, $N = 202$) = 28.93; $P < 0.001$] (Supplementary Fig. 2), and for saturated hydrocarbons, except for *n*-tricosane [KWA: H (6, $N = 202$) = 5.32; $P > 0.503$] and *n*- pentacosane [KWA: H (6, $N = 202$) = 9.55; $P > 0.145$] (Supplementary Fig. 3).

In the cuticular profiles, there were increases in methyl stearate [KWA: H (6, $N = 202$) = 14.34; $P = 0.026$], oleic acid [KWA: H (6, $N = 202$) = 49.42; $P < 0.001$], and stearic acid [KWA: H (6, $N = 202$) = 17.18; $P = 0.008$] with age (Supplementary Fig. 1). However, there was no increase in amount of unsaturated hydrocarbons with age, except for *n*-pentacosene [KWA: H (6, $N = 202$) = 31.13; $P < 0.001$] (Supplementary Fig. 2). The amounts of *n*-heptacosane [KWA: H (6, $N = 202$) = 57; $P < 0.001$], *n*-nonacosane [KWA: H (6, $N = 202$) = 64.88; $P < 0.001$], and *n*-hentriacontane [KWA: H (6, $N = 202$) = 72.07; $P < 0.001$] all increased with age (Supplementary Fig. 3).

In *A. m. scutellata* workers, there were no differences in amounts of fatty acids and alkyl esters between the tergal gland and cuticular extract for most days, except for oleic acid on days 2, 4, 6, 7, and 14 (MWU, $U = 191$; $P < 0.001$) (Supplementary Fig. 4). There were greater amounts of unsaturated hydrocarbons in the tergal gland than in the cuticular extract: *n*-heneicosene (MWU, $U = 47$; $P < 0.001$), *n*-tricosene (MWU, $U = 92$; $P < 0.001$), *n*-pentacosene (MWU, $U = 111$; $P < 0.001$), *n*-heptacosene (MWU, $U = 229$; $P < 0.001$), and *n*-nonacosene on day 7 (MWU, $U = 173$; $P < 0.001$) (Supplementary Fig. 5). There were lower amounts of saturated hydrocarbons in tergal gland extracts than in cuticular extracts (MWU, $U = 94$; $P = 0.001$) (Supplementary Fig. 6), similar to the trend in *A. m. capensis* clones.

In the tergal gland extracts, there were increases with age in the amounts of methyl palmitate [KWA: H (6, $N = 198$) = 30.26; $P < 0.001$], palmitic acid [KWA: H (6, $N = 198$) = 36.72; $P < 0.001$], methyl stearate [KWA: H (6, $N = 198$) = 15.53; $P = 0.017$], oleic acid [KWA: H (6, $N = 198$) = 15.29; $P = 0.018$], and ethyl oleate [KWA: H (6, $N = 198$) = 22.71; $P < 0.001$] (Supplementary Fig. 4). For *n*-alkenes, *n*-heneicosene increased and peaked on

day 21 [KWA: H (6, *N* = 198) = 17.18; *P* = 0.008], while greater amounts of *n*-heptacosene were found on days, 4, 6, and 7 [KWA: H (6, *N* = 198) = 28.38; *P* = 0.008] (Supplementary Fig. 5). Of the *n*-alkanes, only *n*-heptacosane [KWA: H (6, *N* = 198) = 13.12; *P* = 0.041], *n*-nonacosane [KWA: H (6, *N* = 198) = 44.29; *P* < 0.001], and *n*-hentricontane [KWA: H (6, *N* = 198) = 15.02; *P* = 0.020] increased with age in the tergal gland extract (Supplementary Fig. 6).

In the cuticular profiles, there were increases with age in methyl palmitate [KWA: H (6, *N* = 198) = 25.52; *P* < 0.001], palmitic acid [KWA: H (6, *N* = 198) = 28.51; *P* < 0.001], oleic acid [KWA: H (6, *N* = 198) = 33.83; *P* < 0.001], and stearic acid [KWA: H (6, *N* = 198) = 24.05; *P* < 0.001] (Supplementary Fig. 4). All the unsaturated hydrocarbons, except *n*-heneicosene [KWA: H (6, *N* = 198) = 18.86; *P* = 0.06], increased with age (Supplementary Fig. 5). There were increases with age in the amount of *n*-heptacosane [KWA: H (6, *N* = 198) = 70.81; *P* < 0.001], *n*-nonacosane [KWA: H (6, *N* = 198) = 83.25; *P* < 0.001], and *n*-hentricontane [KWA: H (6, *N* = 198) = 70.52; *P* < 0.001]. However, the highest amounts of *n*-heneicosane, *n*-tricosane, and *n*-pentacosane were recorded on day 7 [KWA: H (6, *N* = 198) = 21.46; *P* < 0.001] (Supplementary Fig. 6)

Potential semiochemicals from tergal glands of both *A. m. scutellata* and *A. m. capensis* clone workers that were different for at least 5 times during development were oleic acid (Supplementary Figs. 1, 4), *n*-tricosene, *n*-pentacosene, and *n*-heptacosene (Supplementary Figs. 2, 5). In *A.m. capensis* clones, an additional three compounds, palmitic acid (Supplementary Fig. 1), *n*-heneicosene and *n*-nonacosene were greater in tergal glands for all 7 times of development recorded (Supplementary Fig. 2).

The percentage contribution of fatty acid and alkyl esters to the total tergal gland profiles was high for all days, except day 4 for both *A.m. capensis* clones (Table 1) and *A. m. scutellata* workers (Table 1). The percentage contribution of *n*-alkenes to tergal gland profiles was highest on day 6 for both *A.m. capensis* clones and *A. m. scutellata* workers (Table 1). The percentage contribution of *n*-alkanes to total cuticular profiles was greatest on day 21 for both *A.m. capensis* clones and *A. m. scutellata* workers.

To confirm that the three new compounds found in this study were not artefacts, we sampled honey bee workers from queenright *A. m. scutellata* colonies, and we confirmed the presence of the ethyl esters, albeit in low amounts (Table 2).

Table 1. Relative proportions of compounds in *Apis mellifera capensis* and *A. m. scutellata* tergal glands and cuticular extracts on different days

Age (days), Relative proportions (± SE)														
	0 (*N* = 30)		2 (*N* = 30)		4 (*N* = 30)		6 (*N* = 29)		7 (*N* = 29)		14 (*N* = 26)		21 (*N* = 24)	
	Tergal gland	Cuticular profile	Tergal gland	Cuticular profile	Tergal gland	Cuticular profile	Tergal gland	Cuticular profile	Tergal gland	Cuticular profile	Tergal gland	Cuticular profile	Tergal gland	Cuticular profile
A. *m. capensis*														
Fatty acids and esters	50.74 ± 1.51	53.60 ± 1.46	45.42 ± 1.52	48.59 ± 1.00	37.34 ± 1.25	42.37 ± 1.22	40.97 ± 0.96	42.82 ± 1.15	49.73 ± 1.06	37.80 ± 1.06	45.84 ± 1.27	39.94 ± 1.04	45.49 ± 1.11	32.15 ± 0.88
n-alkenes	17.5 ± 0.35	7.05 ± 0.14	26.01 ± 1.17	9.96 ± 0.71	22.19 ± 0.46	7.63 ± 0.28	32.03 ± 0.38	8.44 ± 0.27	15.3 ± 0.30	3.82 ± 0.39	25.44 ± 1.37	1.34 ± 0.02	23.39 ± 0.83	4.92 ± 0.27
n-alkanes	31.76 ± 0.97	39.34 ± 0.77	28.58 ± 1.47	41.45 ± 0.87	40.46 ± 0.80	49.99 ± 0.75	27.00 ± 0.61	48.73 ± 0.80	35.08 ± 0.97	58.38 ± 1.33	27.81 ± 1.03	61.73 ± 2.40	31.12 ± 0.66	62.94 ± 2.62
A. *m. scutellata*														
Fatty acids and esters	48.16 ± 1.22	50.49 ± 1.20	48.49 ± 1.19	49.93 ± 1.16	41.11 ± 1.11	46.91 ± 1.60	42.20 ± 0.96	44.36 ± 1.20	43.91 ± 1.27	44.04 ± 1.50	54.14 ± 1.05	50.08 ± 1.32	45.04 ± 0.79	31.05 ± 0.54
n-alkenes	18.62 ± 0.57	10.54 ± 1.26	17.32 ± 0.38	9.46 ± 0.50	14.37 ± 0.31	5.38 ± 0.45	18.8 ± 0.56	4.79 ± 0.73	18.03 ± 0.53	2.69 ± 0.12	14.73 ± 0.39	8.02 ± 0.30	18.35 ± 0.24	12.63 ± 1.59
n-alkanes	33.21 ± 1.08	5.38.97 ± 1.07	37.18 ± 1.34	40.61 ± 1.01	44.43 ± 1.01	47.70 ± 1.26	39.00 ± 1.14	50.85 ± 1.45	38.05 ± 1.33	52.33 ± 1.05	31.13 ± 0.84	41.90 ± 0.99	36.61 ± 0.81	56.33 ± 2.90

Table 2. The amounts of compounds (µg ± SE) in tergal gland and cuticular extracts of *Apis mellifera scutellata* queen right workers

Compounds	Tergal gland (*N* = 30)	Cuticular profile (*N* = 30)
Fatty acids and esters		
Methyl palmitate	0.07 ± 0.02	0.09 ± 0.01
Palmitic acid	0.05 ± 0.01	0.02 ± 0.004
Ethyl palmitate	0.10 ± 0.03	0.04 ± 0.01
Methyl stearate	0.15 ± 0.04	0.09 ± 0.02
Oleic acid	0.06 ± 0.01	0.02 ± 0.01
Stearic acid	0.19 ± 0.05	0.10 ± 0.03
Ethyl oleate	0.07 ± 0.03	0.01 ± 0.04
Ethyl stearate	0.13 ± 0.04	0.12 ± 0.02
n-alkenes		
Heneicosene	0.08 ± 0.02	0.03 ± 0.01
Tricosene	0.16 ± 0.05	0.01 ± 0.002
Pentacosene	0.15 ± 0.04	0.03 ± 0.01
Heptacosene	0.09 ± 0.03	0.01 ± 0.003
Nonacosene	0.07 ± 0.02	0.01 ± 0.01
n-alkanes		
Heneicosane	0.03 ± 0.01	0.03 ± 0.003
Tricosane	0.20 ± 0.04	0.24 ± 0.03
Pentacosane	0.15 ± 0.05	0.16 ± 0.01
Heptacosane	0.11 ± 0.10	0.14 ± 0.02
Nonacosane	0.09 ± 0.04	0.06 ± 0.01
Hentricontane	0.08 ± 0.03	0.05 ± 0.01

Assessment of Ovary Activation and Presence of Spermathecae

Of the 400 bees dissected (*A. m. capensis clones N = 202*, *A. m. scutellata* workers *N = 198*), all *A. m. capensis* clones had spermathecae present, but none of the *A. m. scutellata* workers did. We used the presence of spermathecae in *A. m. capensis* workers to confirm that they were clones. In *A. m. capensis* clone workers, activation of the ovaries started as early as day 4, while in *A. m. scutellata* activation started only on day 7 (Supplementary Fig. 7).

Tergal Gland Compounds and Ovarial Status of *A. m. capensis* Clones and *A. m. scutellata*

In *A. m. capensis* clones and *A. m. scutellata*, there were differences (MWU, $U = 12$; $P < 0.001$) between honey bee workers with activated and inactive ovaries for all the tergal gland fatty acids and esters (Supplementary Fig. 7A, B), unsaturated hydrocarbons (Supplementary Fig. 7C, D), and saturated hydrocarbons (Supplementary Fig. 7E, F).

The principal component analysis of *A. m. capensis clones* and *A. m. scutellata* workers with activated or inactive ovaries is shown in Fig. 1. *Apis mellifera capensis clones* and *A. m.*

scutellata workers could be separated based on age, ovarian activation and subspecies. Oleic acid and *n*-heptacosene had factor loadings greater than 0.5 for the first component, and described 25.23 % of the variation, while the second factor described 23.64 % of the variation with high loadings for *n*-tricosene.

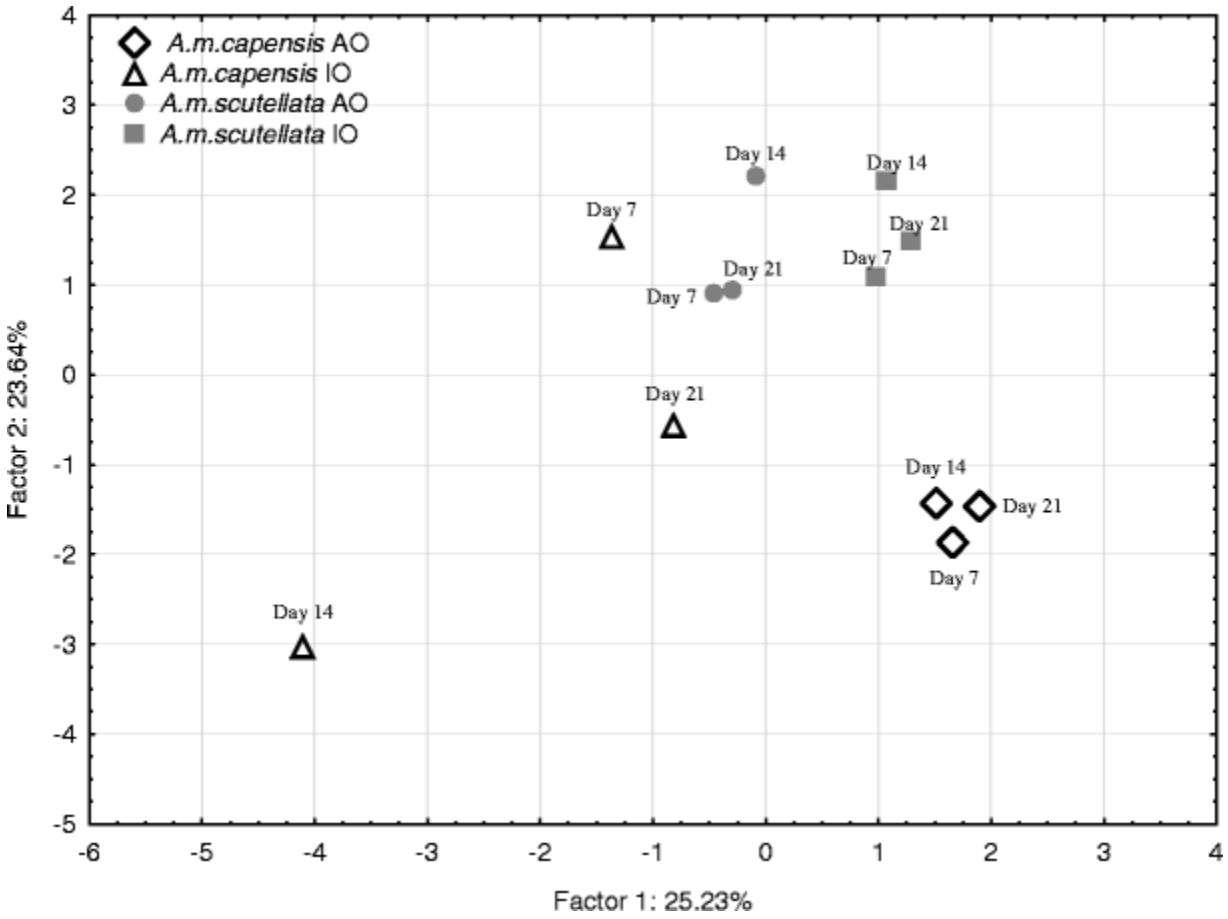

Fig. 1. Separation by principal component analysis, of potential tergal gland semiochemicals of *Apis mellifera capensis* clones and *A. m. scutellata* workers (days 7, 14, and 21) with activated ovaries (AO) and inactive ovaries (IO)

Discussion

Tergal gland profiles of *A. m. capensis* clones were distinctive from those of *A. m. scutellata* workers. The compounds identified were mostly fatty acids, alkenes, and alkyl esters. Both *A. m. scutellata* and *A. m. capensis* clones contained oleic acid, *n*-tricosene, *n*-pentacosene, and *n*-heptacosene, while an additional three compounds, palmitic acid, *n*-heneicosene, and *n*-nonacosene, were found in *A. m. capensis* clones. These compounds increased with age for queenless caged workers of both sub-species.

All compounds found in this study, except for the three ethyl esters, ethyl palmitate, ethyl oleate, and ethyl stearate, have been reported previously in tergal gland profiles of virgin, mated queens and queenright workers of both *A. m. capensis* and *A. m. scutellata* (Wossler and Crewe 1999a). The ethyl esters have been reported as part of honey bee brood pheromone (Le Conte et al. 1990). Since workers were kept in cages without brood, we can exclude contamination as the source of these compounds. Traces of ethyl oleate have been

found on honey bee worker cuticle (Leoncini et al. 2004), while ethyl palmitate and ethyl oleate have been found on the cuticle of stingless bees *Frieseomelitta vari* (Nunes et al. 2008). Ethyl palmitate has been implicated in inhibition of ovary activation in honey bee workers (Mohammedi et al. 1998), while ethyl oleate delays transition of nurse honey bees into foragers (Castillo et al. 2012). The latter causes nurse bees to spend more time in the hive to tend to the brood and the queen (Leoncini et al. 2004). Since reproductively dominant bees need more subordinate individuals to tend to their needs (Hillesheim et al. 1989), one would expect that the signal for dominance should be more abundant in bees with activated ovaries. Thus, the greater amounts of these ethyl esters in the tergal gland of reproductive workers is consistent with their role as primer pheromones that suppress ovarian activation of their nestmates, thus achieving reproductive dominance over other workers in queenless situations.

Fatty acids and esters are most abundant in the tergal gland extracts of *A. mellifera scutellata* and *A. m. capensis* queenright workers (Wossler and Crewe 1999a). Similarly, here, fatty acid and alkyl esters were most abundant in the tergal gland extracts, on all days sampled, of caged queenless workers for both *A. m. capensis* clones and *A. m. scutellata*. In honey bees, mated queens have larger amounts of fatty acids and esters in their tergal gland compared to virgin queens (Espelie et al. 1990; Wossler and Crewe 1999a). Additionally, Al-Qarni et al. (2005) have reported that palmitoleic, palmitic, and oleic acids were present in greater quantities in naturally mated queens than in artificially inseminated ones. We found a similar trend with reproductive status of queenless workers: palmitic and oleic acids were present in greater quantities in ovary-activated workers than in non-reproductive workers. As honey bees with activated ovaries have more tergal gland compounds than non-reproductively active bees, this suggests that these fatty acids work with secretions from other glands to achieve pheromonal and reproductive dominance among queenless workers.

Alkenes also are abundant in the tergal gland extracts of *A. m. scutellata* and *A. m. capensis* queenright (Wossler and Crewe 1999a) and queenless workers (our study). Reproductive status and age are known to affect tergal gland secretions of honey bee queens (Smith et al. 1993). Similarly, we found increases in the amount of tergal gland alkenes with regard to reproductive status and age for both *A. m. capensis* worker clones and *A. m. scutellata* workers. This suggests that tergal gland alkenes play a role in establishing pheromonal dominance of reproductively active honey bee workers in the colony.

Clone workers of *A. m. capensis* have the ability to achieve reproductive dominance as they increase in numbers and take over host colonies (Neumann and Moritz 2002), but do not participate in standard hive duties (Hillesheim et al. 1989), and they have high ovariole numbers and spermathecae (Phiancharoen et al. 2010). Thus, the presence of spermathecae in all *A. m. capensis* workers in this study confirms that they are *A. m. capensis* workers. Additionally, initiation of ovarian activation as early as day 4 in *A. m. capensis* clones, the presence of spermatheca in all clones, and the higher percentage of bees with activated ovaries compared to *A. m. scutellata* workers, could aid *A. m. capensis* clones in achieving complete reproductive dominance when the host queen is lost during host colony infestation.

The role of the identified compounds, in particular the three new esters, in regulating social interactions among workers will be explored using behavioral bioassays.

Acknowledgments

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Supplementary material

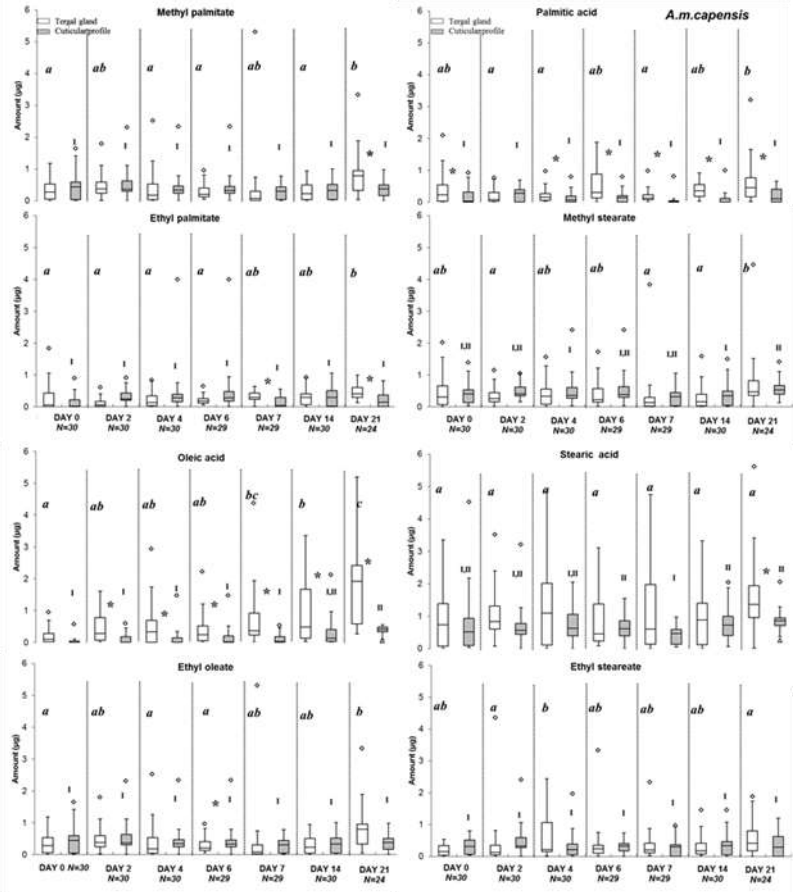

Figure 1. The amounts of fatty acids and esters in tergal gland and cuticular profiles of *Apis m. capensis* clones (days 0, 2, 4, 6, 7, 14, and 21, *N*=202).The median line, quartile, whisker, minimum △ and ◇ maximum outliers are shown. * denotes significant difference at $P < 0.05$ (Mann -Whitney *U* test) of the pairwise comparison between tergal gland and cuticular profile for each day. Different letters within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for tergal gland profile. Different roman numerals within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for cuticular profile

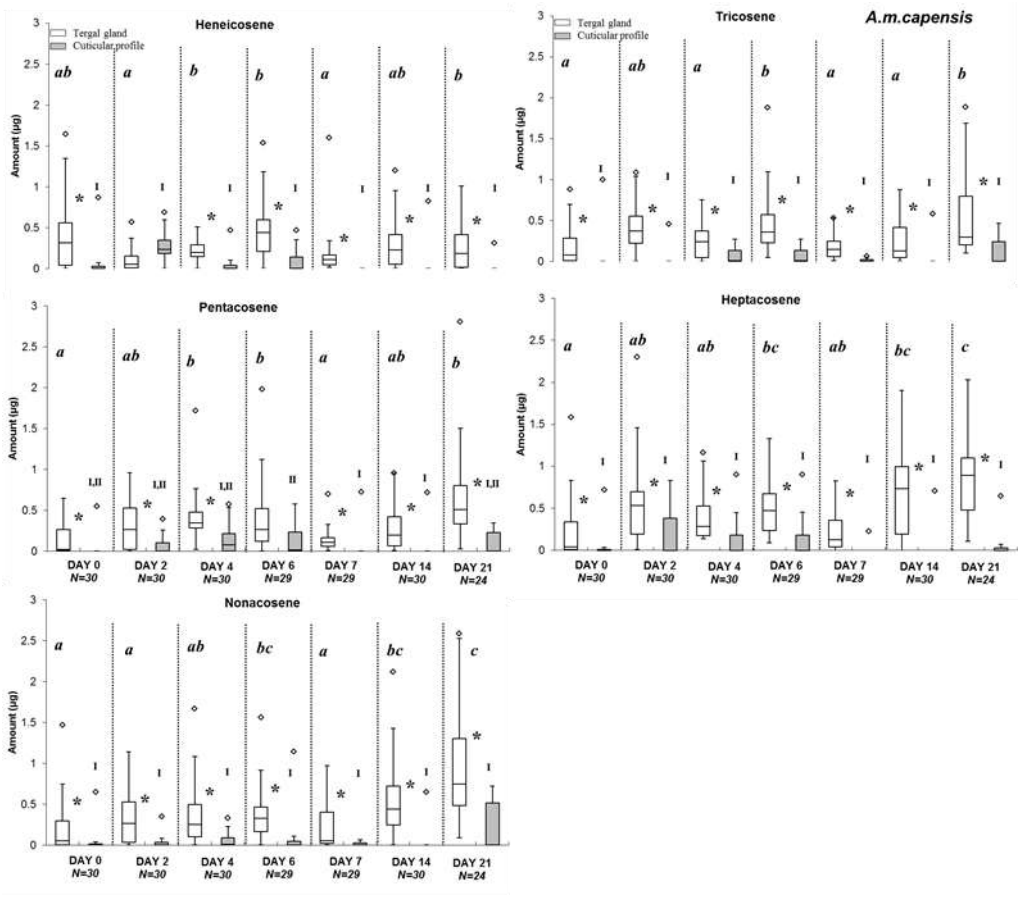

Figure 2. The amounts of *n*-alkenes in tergal gland and cuticular profiles of *Apis m. capensis* clones (days 0, 2, 4, 6, 7, 14, and 21, *N*=202).The median line, quartile, whisker, minimum △ and maximum ◇ outliers are shown. * denotes significant difference at $P < 0.05$ (Mann -Whitney *U* test) of the pairwise comparison between tergal gland and cuticular profile for each day. Different letters within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for tergal gland profile. Different roman numerals within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for cuticular profile

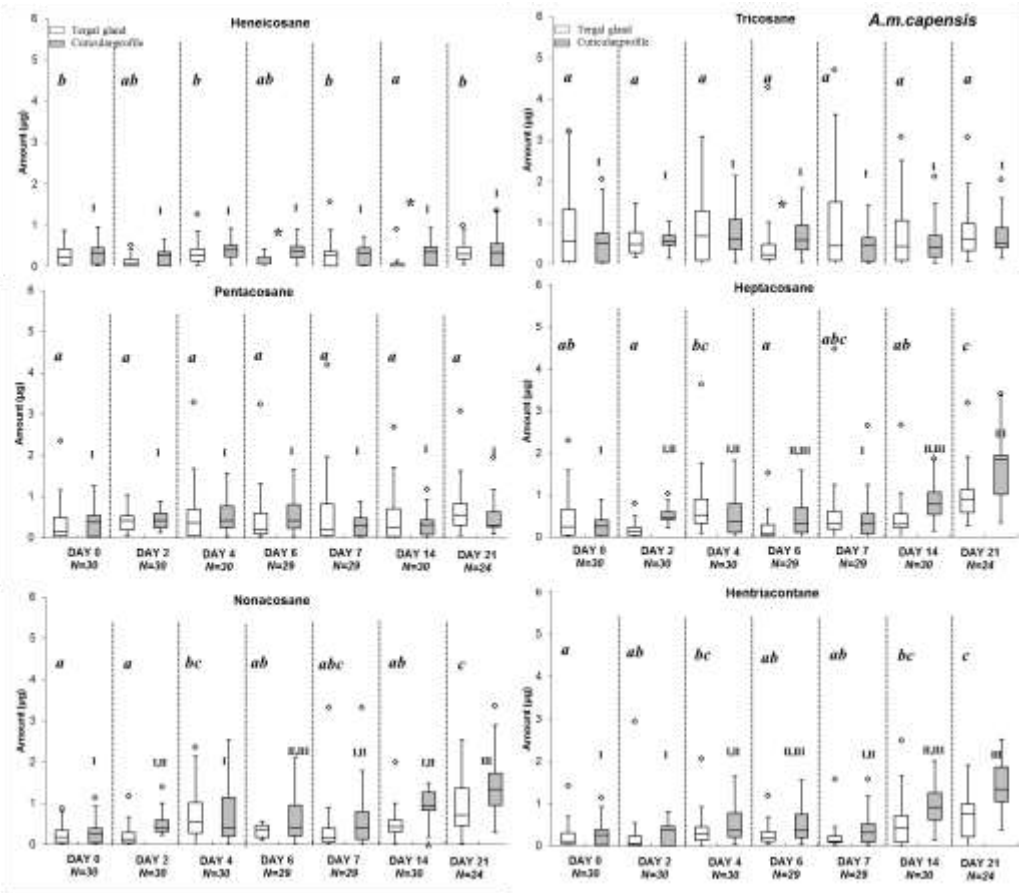

Figure 3. The amounts of *n*-alkanes in tergal gland and cuticular profiles of *Apis m. capensis* clones (days 0, 2, 4, 6, 7, 14, and 21, *N*=202).The median line, quartile, whisker, minimum △ and maximum ◇ outliers are shown. * denotes significant difference at $P < 0.05$ (Mann -Whitney *U* test) of the pairwise comparison between tergal gland and cuticular profile for each day. Different letters within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for tergal gland profile. Different roman numerals within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for cuticular profile

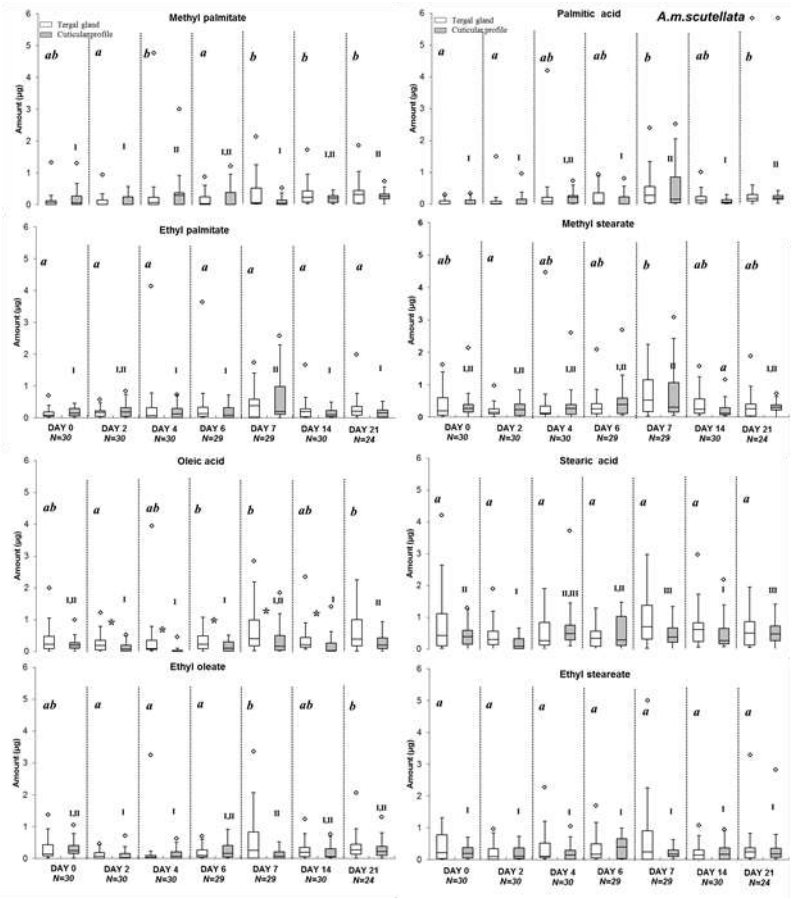

Figure 4. The amounts of fatty acids and esters in tergal gland and cuticular profiles of *Apis m. scutellata* workers (days 0, 2, 4, 6, 7, 14, and 21, *N*=198).The median line, quartile, whisker, minimum and maximum outliers are shown. * denotes significant difference at $P < 0.05$ (Mann -Whitney *U* test) of the pairwise comparison between tergal gland and cuticular profile for each day. Different letters within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for tergal gland profile. Different roman numerals within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for cuticular profile

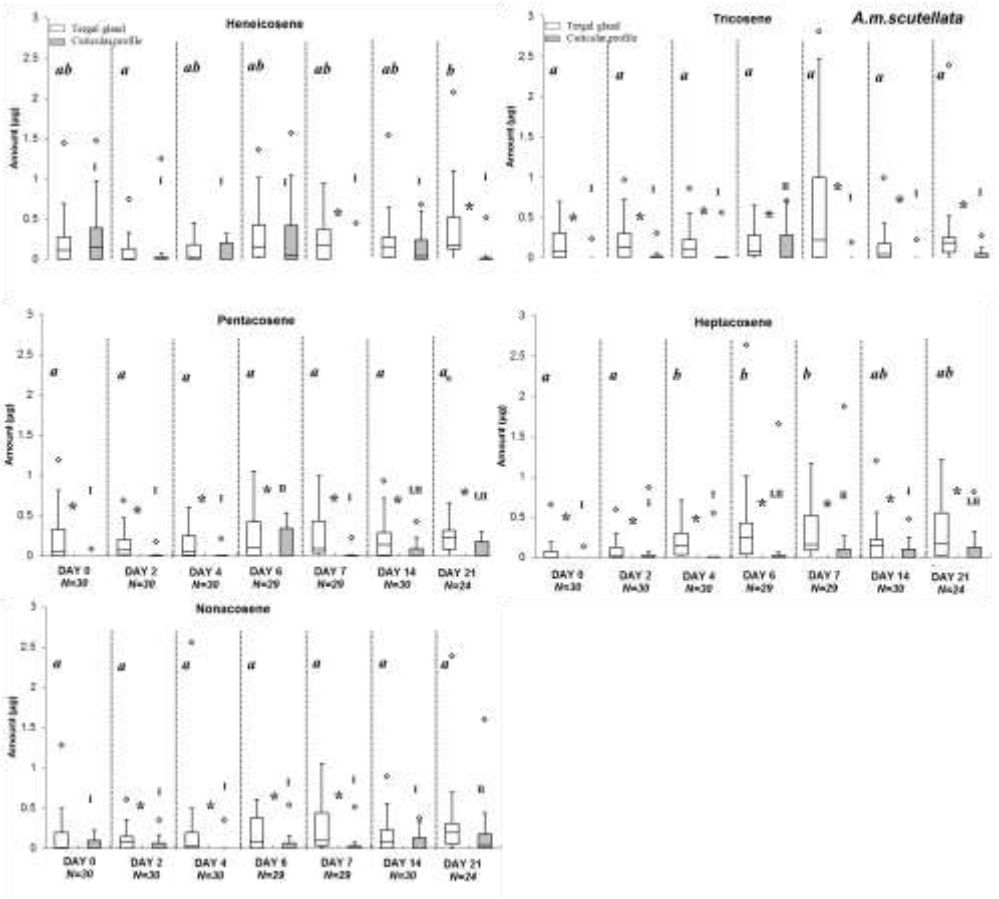

Figure 5. The amounts of *n*-alkenes in tergal gland and cuticular profiles of *Apis m. scutellata* workers (days 0, 2, 4, 6, 7, 14, and 21, *N*=198).The median line, quartile, whisker, minimum and maximum outliers are shown. * denotes significant difference at $P < 0.05$ (Mann -Whitney *U* test) of the pairwise comparison between tergal gland and cuticular profile for each day. Different letters within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for tergal gland profile. Different roman numerals within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for cuticular profile

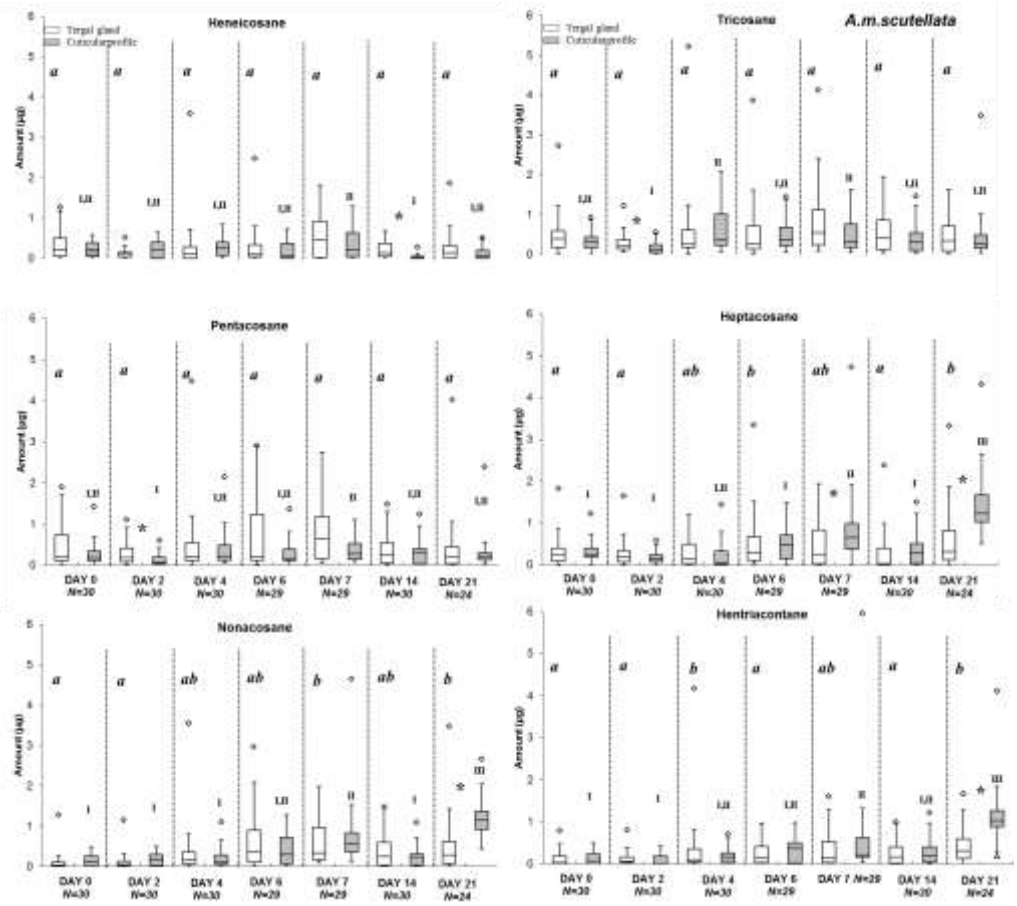

Figure 6. The amounts of *n*-alkanes in tergal gland and cuticular profiles of *Apis m. scutellata* workers (days 0, 2, 4, 6, 7, 14, and 21, *N*=198).The median line, quartile, whisker, minimum and maximum outliers are shown. * denotes significant difference at $P < 0.05$ (Mann -Whitney *U* test) of the pairwise comparison between tergal gland and cuticular profile for each day. Different letters within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for tergal gland profile. Different roman numerals within the row represent significant differences at $P < 0.05$ (Kruskal Wallis-test) on different days for cuticular profile

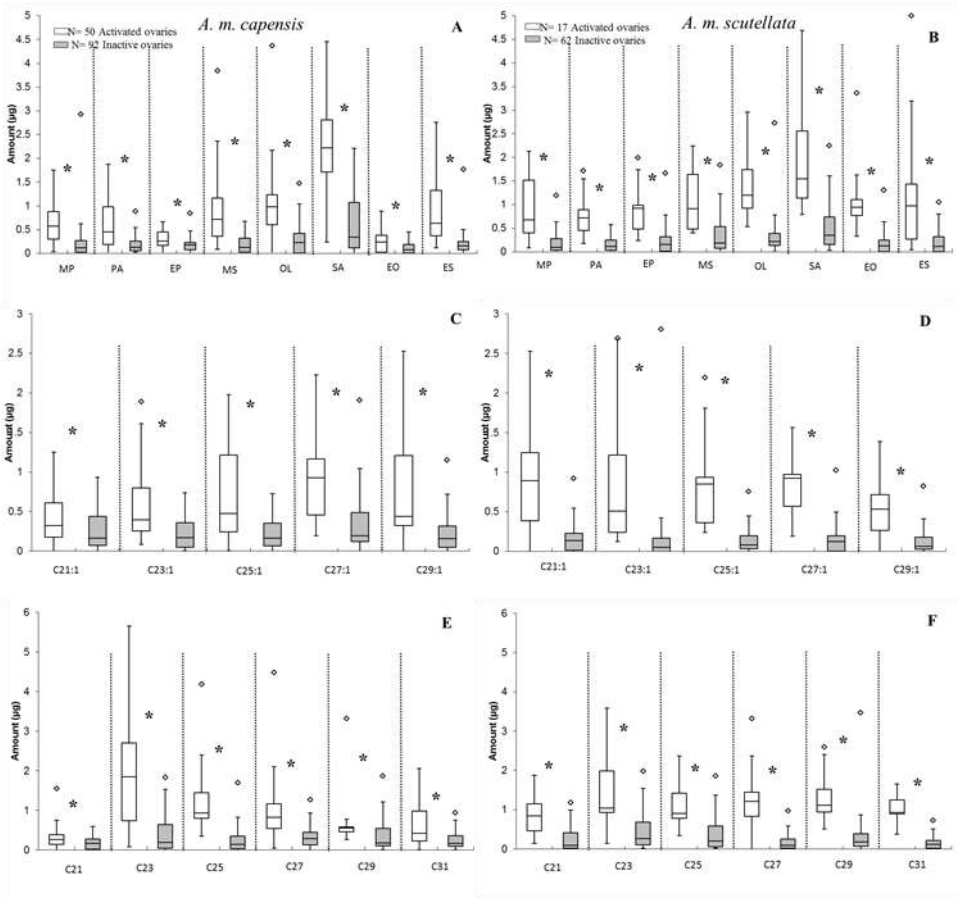

Figure 7. The amounts of compounds in tergal gland profiles of *Apis m. capensis* clones (days 4, 6, 7, 14, and 21, *N*=142) and *A. m. scutellata* workers (days 7, 14, and 21, *N*=79) with activated and inactive ovaries. Fatty acids and esters (Figs. A, B); unsaturated hydrocarbons (C, D) and saturated hydrocarbons (E, F) *denotes significant difference at $P < 0.05$ (Mann -Whitney *U* test) of the pairwise comparison between workers with activated and inactive ovaries. The median line, quartile, whisker and the outliers ◇ are shown. MP= methyl palmitate; PA= palmitic acid; EP= Ethyl palmitate; MS= Methyl stearate; OL= Oleic acid; SA= Stearic acid; EO=Ethyl oleate; ES= Ethyl stearate; $C_{21:1}$= Heneicosene; $C_{23:1}$= Tricosene; $C_{25:1}$= Pentacosene; $C_{27:1}$= Heptacosene; $C_{29:1}$= Nonacosene; C_{21}= Heneicosane; C_{23}= Tricosane; C_{25}= Pentacosane; C_{27}= Heptacosane; C_{29}= Nonacosane; C_{31}= Hentricontane