Glandular sources of pheromones used to control host workers (*Apis mellifera scutellata)* by socially parasitic workers of *Apis mellifera capensis*

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Abstract

Pheromonal control by the honey bee queen is achieved through the use of secretions from diverse glandular sources, but the use of pheromones from a variety of glandular sources by reproductively dominant workers, has not previously been explored. Using the social parasite, *Apis mellifera capensis* clonal worker, we studied the diversity of glandular sources used for pheromonal control of reproductively subordinate *A. m. scutellata* workers. To determine whether pheromones from different glandular sources are used by reproductively active workers to achieve dominance and evaluate the degree of pheromonal competition between workers of the two sub-species. We housed groups of workers of the two sub-species together in cages and analysed mandibular and tergal gland secretions as well as, ovarian activation status of each worker after 21 days. The results showed that *A. m. capensis* invasive clones used both mandibular and tergal gland secretions to achieve reproductive dominance and suppress ovarian activation in their *A. m. scutellata* host workers. The reproductively dominant workers (false queens) produced more queen-like pheromones and inhibited ovarian activation in subordinate *A. m. scutellata* workers. These results show that tergal gland pheromones working in synergy with pheromones from other glands allow individual workers (false queens) to establish reproductive dominance within these social groups and to act in a manner similar to that of queens. Thus suggesting that, the evolution of reproductively dominant individuals (queens or false queens) and subordinate individuals (workers) in social insects like the honey bee is the result of a complex interplay of pheromonal signals from different exocrine glands.

Keywords: honey bees; tergal gland; social parasites; mandibular gland; queen pheromones

1. Introduction

Successful regulatory mechanisms help to maintain reproductive division of labour in insects' societies. Under normal conditions, there is one fertile queen with a group of workers that are functionally sterile. These workers do not activate their ovaries while there is an active queen, because worker reproduction is suppressed in the presence of queen and brood (reviewed in Robinson, 1992; Reeve and Keller, 2001). Any worker laid eggs are normally removed by other workers (Ratnieks and Visscher, 1989; Pirk et al., 2002; Pirk et al., 2004) even in the absence of relatedness benefits (Pirk et al., 2003). Although, as a deviant state in queenright colonies, some workers do escape pheromonal control by the queen and brood as well as worker policing by their nestmates, to become reproductively active or potentially fertile e.g in honey bees (Oldroyd et al., 1994; Pirk et al., 2003; Châline et al., 2004; Niu et al., 2016), wasps (Foster et al., 2001), bumble bees (Birmingham et al., 2004) and stingless bees (Peters et al., 1999). In the case of honey bees, reproductive workers are able to suppress reproduction in other workers either in their own colonies or colonies containing other sub-species (Hemmling et al., 1979; Malka et al., 2008) and often produce queen glandular pheromones (Sakagami, 1958; Moritz et al., 2004; Malka et al., 2008).

Mandibular gland pheromones of honey bee queens and workers include two aromatic compounds and a series of C10 fatty acids, with the distribution of the compounds between the two castes being different (Crewe and Velthuis, 1980; Plettner et al., 1996). Secretions from the queens are characterised by the abundance of $\omega - 1$ hydroxylated fatty acids (9-ODA and 9HDA) while those of non-laying workers have predominantly ω hydroxylated fatty acids (10-HDA and 10-HDAA) (Plettner et al., 1995). Although queen mandibular pheromone (QMP) is known as the major queen control signal, secretions from other sources such as the Dufour's

and tergal glands work in synergy for the queen to achieve control over the workers (Renner and Vierling, 1977; Saiovici, 1983; Sole et al., 2002; Maisonnasse et al., 2010). Compounds found in the queen and worker tergal glands (fatty acids, alkyl esters, unsaturated and saturated hydrocarbons (see Wossler and Crewe, 1999a; Okosun et al., 2015)) are involved in mating behaviour as attractants for mounting drones, increasing copulatory activity to tergal gland pheromone baited queen dummies, eliciting retinue behaviour from workers, inhibiting worker ovarian development and serve as a kin recognition signal in workers (Renner and Vierling, 1977; Vierling and Renner, 1977; Moritz and Crewe, 1988; Wossler and Crewe, 1999b; c). In both *A. m. capensis* and *A. m. scutellata*, workers have well developed tergal glands unlike other honey bee sub-species such as *A. m. mellifera* (Billen et al., 1986; Wossler et al., 2000), and the pheromones from these tergal glands could be used to enable the workers to function effectively as false queens (Billen et al., 1986; Okosun et al., 2015).

Since the queen substance (9-ODA) is the major pheromonal component used for queen control in the colony, the establishment of dominance by laying over non-laying workers in a queen-less colony is dependent on the dominant workers producing 9-ODA (Crewe and Velthuis, 1980; Moritz et al., 2000; Malka et al., 2008). In queen-less conditions, workers with different reproductive traits are encountered: (1) non-laying workers with worker-like pheromones and inactive ovaries; (2) laying workers with worker-like pheromones and activated ovaries; (3) incipient false queens, workers that have queen-like pheromones and inactive ovaries; (4) false queens, workers with queen-like pheromones and activated ovaries (Sakagami, 1958; Moritz et al., 2004; Malka et al., 2008). In addition, the pheromonal bouquets of honey bee false queens are qualitatively similar to that of a queen and they use these pheromones to exercise physiological and behavioural control akin to that of the queen over other workers (Katzav-Gozansky et al., 2003; Moritz et al., 2004). More importantly, false queens do not participate in lethal fights among themselves which is common in true queens, but otherwise they use

pheromonal contests to control and gain dominance in the colony (Moritz et al., 2000; Moritz et al., 2004). They also behaviourally escape queen control by avoiding her in the colony (Moritz et al., 2002). Once they produce a more queen-like pheromonal bouquet, they are fed via trophallaxis and obtain protein needed to sustain oogenesis (Moritz and Hillesheim, 1985; Schäfer et al., 2006). This false queen phenomenon is most common among but not limited to workers of the Cape honey bee *A. m. capensis*. They develop a QMP bouquet in the presence of queens of other sub-species and suppress production of the QMP in other workers (Hemmling et al., 1979; Moritz et al., 2000; Zheng et al., 2010).

Cape honey bees have the ability to invade foreign colonies of their own and those of other sub-species. They may engage in lethal fights with the queen of sister sub-species and it is assumed that they may kill the queen in order to gain control of her colony and lay eggs that are not policed (Pirk et al., 2003; Moritz et al., 2003; Härtel et al., 2006 a; b). As the number of eggs laid by the parasites increases, the parasitic worker's larvae are preferentially fed by host workers (Beekman et al., 2000; Neumann and Hepburn, 2002), with an increase in number of parasitic offspring produced (Martin et al., 2002) in infected colonies and the rapid spread of *A. m. capensis* parasitic workers within an apiary (Calis et al., 2002). The most significant impact of these social parasites can be traced to the transport of hundreds of *A. m. capensis* colonies into the endemic range of *A. m. scutellata* in the early 1990s by migratory beekeepers that resulted in workers of *A. m. capensis* invading *A. m. scutellata* colonies (Allsopp and Crewe, 1993). The result of this interaction between the two sub-species was that one highly invasive clonal lineage of *capensis* workers (Baudry et al., 2004; Härtel et al., 2006 a; Oldroyd et al., 2011; Pirk et al., 2012) became established and, is referred to as 'the capensis clone' hereafter. This lineage is still causing losses of several thousands of *A. m. scutellata* managed colonies in the Northern region of South Africa each year. A recent survey of colony losses in South Africa, estimated that 29% of managed colonies of honey bees were lost in 2010 and

even more (46%) were lost in 2011 (Pirk et al., 2014). Moreover, the successful invasion of and establishment of reproductively dominant workers in host colonies is possible because host queens fail to prevent parasitic workers from becoming false queens.

Since the queen uses secretions from a diversity of glandular sources such as mandibular, Dufour's and tergal gland to control her workers (Renner and Vierling, 1977; Maisonnasse et al., 2010). We hypothesise that dominant workers also use secretions from more than one glandular source such as mandibular and tergal glands to exercise dominance over other workers under queenless conditions, especially during social parasitism and usurpation of other colonies. Previously, pheromonal dominance of *A. m. capensis* invasive clones over *A. m. scutellata* workers in mixed colonies was investigated focusing on secretions from the mandibular glands only (see Schäfer et al., 2006). Here we profile the secretions of the mandibular and tergal glands of individual *A. m. capensis* invasive clones and *A. m. scutellata* workers reared together to explore the effects of social parasitism. We also determined ovarian activation status by examination of the ovaries, and mode of feeding by assessing pollen contents in the guts that serves as an indication of trophallactic vs individual feeding by workers. We present the mandibular and tergal gland profiles, and ovarian activation status of individuals showing either queen-like or worker-like pheromone and physiological characteristics.

2. Materials and methods

2.1 Experimental setup

A. m. capensis clone brood was collected from a commercial apiary in Pretoria, South Africa from infested *A. m. scutellata* colonies, while *A. m. scutellata* brood was collected from un-infested colonies from the apiaries at the University of Pretoria experimental farm. The brood

was reared until worker emergence under optimum temperature (34 °C) and relative humidity (60% r. h) (Williams et al., 2013). To investigate reproductive dominance of workers (Ruttner et al., 1976) and establish conditions under which *A. m. capensis* workers can gain reproductive dominance, fifty *A. m. scutellata* workers and ten *A. m. capensis* worker invasive clones in a ratio of 5:1 as reported in Schäfer et al. (2006) were housed in the same wooden hoarding cages (12.5 × 10 × 15 cm) fitted with combs (Kulincevic et al., 1982; Williams et al., 2013). The bees were sampled at intervals of 2, 4, 6, 7, 14 and 21 days (6 sampling intervals) after worker emergence. The experiment was conducted using 18 cages referred to as mixed groups of both *A. m. capensis* invasive clones and *A. m. scutellata* sourced from three experimental hives each for both sub-species. For each of the six sampling intervals, there were replicate cages with mixed groups from different colonies (6 × 3 colonies = 18 cages) and in total, there were 18 sampling points for each sub-species. Bees were fed pollen (5g), 50% sugar-water solution and water as required.

All *A. m. capensis* invasive clones and *A. m. scutellata* workers that survived to the end of particular trial period were frozen and dissected. Although, most *A. m. scutellata* workers did not survive to the end of a particular trial period, two random dissected *A. m. scutellata* workers per cage (18 cages, N=36) and all dissected *A. m. capensis* invasive clones were analysed and included in the final chemical analysis. There was a total of 89 workers, *A. m. capensis* invasive clones (*N=53*) and *A. m. scutellata* (*N=36*) used for the chemical analysis.

2.2 Mandibular and tergal gland extracts

Heads of workers were extracted in 200 µl of dichloromethane (DCM) (Simon et al., 2001), while tergal glands were obtained from the same worker bees by dissecting the intersegmental

membrane dorsally with narrow strips of cuticle on both sides from abdominal tergites (II-V) and kept in 100 µl of DCM (Wossler and Crewe, 1999a; Okosun et al., 2015). These were stored at -20 °C until required for chemical analysis.

2.3 Chemical Analyses

2.3.1 Mandibular gland pheromones: Half of each cephalic extract kept in 200 µl of DCM was evaporated to dryness under a gentle stream of nitrogen and the other half was stored as a backup. The residues were re-dissolved in 10 µl of a solution containing the internal standards (~1 mg octanoic acid and 1 mg *n*-tetradecane in 4 ml of DCM). To this, 10 µl of the derivitising agent bis-trimethylsilyl-trifluoroacetamide (Sigma Aldrich, USA) was added and allowed to react for minimum of four hours. One µl of this solution was injected in a splitless mode into an Agilent Technology 6890N gas chromatograph fitted with a 25 m × 0.20 mm × 0.33µm HP1-MS capillary column. The injector and flame ionisation detector 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. The temperature of the oven was set at 50°C and then increased to 100°C at 50°C per min, thereafter increased to 220°C at a rate of 3°C per min and maintained at this temperature for 10 min. The six major mandibular components (HOB, 9-ODA, HVA, 9-HDA, 10HDAA and 10HDA) were quantified using peak areas and their relative mass ratios calculated relative to the internal standard tetradecane (Simon et al., 2001; Yusuf et al., 2015).

2.3.2 Tergal gland pheromones: Prior to injection into an Agilent Technology 6890N gas chromatograph (GC) equipped with HP5-MS capillary column (25m × 0.20mm × 0.33µm). Ten µl of the tergal gland extracts was evaporated to dryness under a gentle stream of nitrogen, to which 10µl of DCM and 10µl of internal standard n-hexadecane (~ 1mg of *n*-hexadecane in 4ml of DCM) solution were added. One µl of this was injected into the GC and the oven temperature was programmed from 50°C to 100°C, ramped at 6°C /min to 300°C, then held

for 10 mins. Temperature for the injection port was set at 230°C while that of the FID was at 310°C. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The quantification of the peaks was achieved relative to the amount of internal standard and tergal gland standard compounds; fatty acids, alkyl esters, unsaturated and saturated hydrocarbons (see Okosun et al., (2015) for details of the tergal gland components). Components were identified based on their retentions times by comparison with those of synthetic compounds.

2.4 Determination of queen-like versus worker-like secretions

We calculated the quantitative ratios for the main queen mandibular gland component 9-ODA and that of the main component in the worker mandibular gland 10-HDA using the following formula: (9-ODA)/ (9-ODA + 10 HDA) (Schäfer et al., 2006). This allowed us to classify the workers into three groups according to their mandibular pheromonal status following Schäfer et al., (2006): worker-like (WL) from 0-0.5; intermediate (IM) from > 0.5-0.9 and queen-like (QL) from > 0.9-1.0.

Based on these three categories of mandibular pheromonal status (QL, IM and WL), tergal gland pheromonal status was calculated. To determine how queen-like the tergal gland secretions were, the seven components described as potential semiochemicals out of the nineteen components from the tergal glands identified earlier by Okosun et al., (2015) were used. The seven compounds used were palmitic acid, oleic acid, n-heneicosene, *n*-tricosene, *n*-pentacosene, *n*-heptacosene and *n*-nonacosene, while the remaining 12 tergal gland compounds were stearic acid, methyl palmitate, methyl stearate, ethyl palmitate, ethyl oleate, ethyl stearate, *n*-heneicosane, *n*-tricosane, *n*-pentacosane, *n*-heptacosane, *n*-nonacosane and *n*-hentricontane**.** The ratios were calculated as amounts of (7 components) / (7 components + 12 components). The confidence interval (CI) and range of the tergal gland ratios were obtained as queen-like (ratio > 0.35), intermediate (ratio > 0.23 ≤ 0.35) and worker-like (ratio ≤ 0.23). To establish

these categories, individuals with known queen-like mandibular profiles were used to classify the queenliness of the tergal gland secretions. Bees with queen-like mandibular glands were grouped and the CI and range of their tergal gland ratios were calculated, this was done for bees with intermediate and also worker-like mandibular glands secretion. Thereafter, tergal gland pheromone bouquet was then set as queen-like (> 0.35), intermediate (> 0.23 ≤ 0.35) and worker-like (≤ 0.23).

These groupings for both mandibular and tergal glands were subsequently referred to in this study as mandibular gland queen-like (MGQL, 0.9-1.0), mandibular gland intermediates (MGIM; 0.5-0.9), mandibular gland worker-like (MGWL; 0-0.5), tergal gland queen-like (TGQL; > 0.35), tergal gland intermediates (TGIM; > 0.23 ≤ 0.35) and tergal gland worker-like (TGWL; ≤ 0.23) secretions.

2.5 *Assessments of ovary activation and pollen consumption*

All frozen honey bee workers at the end of the trial were dissected (*N*=89) and checked for level of ovarian activation. These were classified as inactive ovaries (IO) (stage 1 & 2), intermediate ovaries (INT) (stage 3) and activated ovaries (AO) (stage 4 & 5) (stages described in Hess, 1942; Schäfer et al., 2006; Zheng et al., 2010). We checked for the presence of pollen in the gut in individual bees during dissection, as this enabled us to differentiate between individuals that fed directly on pollen and those that were fed through trophallaxis. This differentiated the workers that utilise the social or individual pathway (Schäfer et al., 2006) to satisfy their protein requirements. The classification into different categories was done following Schäfer et al., (2006): large amount of pollen (LAP) (full distended rectum), small amount of pollen (SAP) (visible in the rectum) and no pollen (NP) in their rectum.

2.6 Statistical Analyses

Data for chemical profiles of mandibular and tergal glands were not normally distributed (Shapiro Wilks test). Non-parametric Kruskal-Wallis test showed no significance differences for both *A. m. capensis* invasive clones and *A. m. scutellata* workers among the days for mandibular and tergal glands, therefore data were pooled for all the days. To determine the effect of pheromonal status on ovarian activation and pollen consumption, a Kruskal-Wallis test was performed for individual sub-species followed by a post hoc test (multiple comparisons of mean ranks); pheromonal status was the dependent variable while ovarian activation and pollen consumption were independent grouping variables respectively. Paired comparisons were carried out using Mann-Whitney *U* test (M-W U) between *A. m. capensis* and *A. m. scutellata* mandibular, tergal gland pheromonal status, ovarian activation and pollen consumption to determine the differences between the two sub-species. To compare across cages for matched-pairs, average ratios of both mandibular and tergal gland compositions of *A. m. capensis* and *A. m. scutellata* workers in the same hoarding cage (N=18) were obtained respectively. This ratio was then used for each sub-species worker (N=1) in each hoarding cage. Thereafter, Wilcoxon matched-pair tests were performed to evaluate differences between the ratios of both mandibular and tergal gland of workers of the two sub-species in the same hoarding cages. Significance levels were set at *alpha* <0.05 and all analyses were performed using Statistica 12 (StatSoft USA).

3. Results

Data were not significantly different among the days for both glands, for *A. m. capensis* invasive clones mandibular gland [KWA: H (5, N = 53) = 8.91; P > 0.05 and tergal gland KWA: H (5, N = 53) = 4.25; P > 0.05]; for *A. m. scutellata* workers, mandibular gland KWA: H (5, N = 36) = 7.54; P > 0.05 and tergal gland KWA: H (5, N = 36) = 4.75; P > 0.05].

3.1 Pheromonal status and ovarian activation

In the pheromonal bouquets of *A. m. capensis* worker invasive clones, workers with different levels of ovarian activation were not significantly different from each other [KWA: H (2, N = 53) = 1.21; $P > 0.05$] (Fig 1A). Twenty six percent (26%) of *A. m. capensis* worker invasive clones had activated ovaries (AO, stage 4), 68% had intermediate ovaries (INT, stage 3) and 6% had inactive ovaries (IO, stages 1 & 2) (Fig 1A). Overall, for all *A. m. capensis* worker invasive clones dissected ($N = 53$), 98% had MGQL while the remaining 2% had MGIM (Fig 1A). Regardless of whether they had activated ovaries or not, all *A. m. capensis* invasive clonal workers had mandibular queen-like pheromones except for one worker on day 2 with MGIM but with TGQL (Fig 1A).

A. m. scutellata workers showed differences in their pheromonal bouquet at different stages of ovarian activation (MWU, $U = 11$; $Z = -3.019$; $P < 0.001$) (Fig 1B). For *A. m. scutellata* workers ($N = 36$), none had AO ovaries in the presence of *A. m. capensis* worker invasive clones, while there were 14% and 86% with both INT and IO respectively. Only two *A. m. scutellata* workers had MGQL, while one had both MGQL and TGQL (day 14), and the other had MGQL and TGWL (day 21). Overall, there were 5% of the workers with MGQL, 37% MGIM and 58% MGWL secretions (Fig 1B).

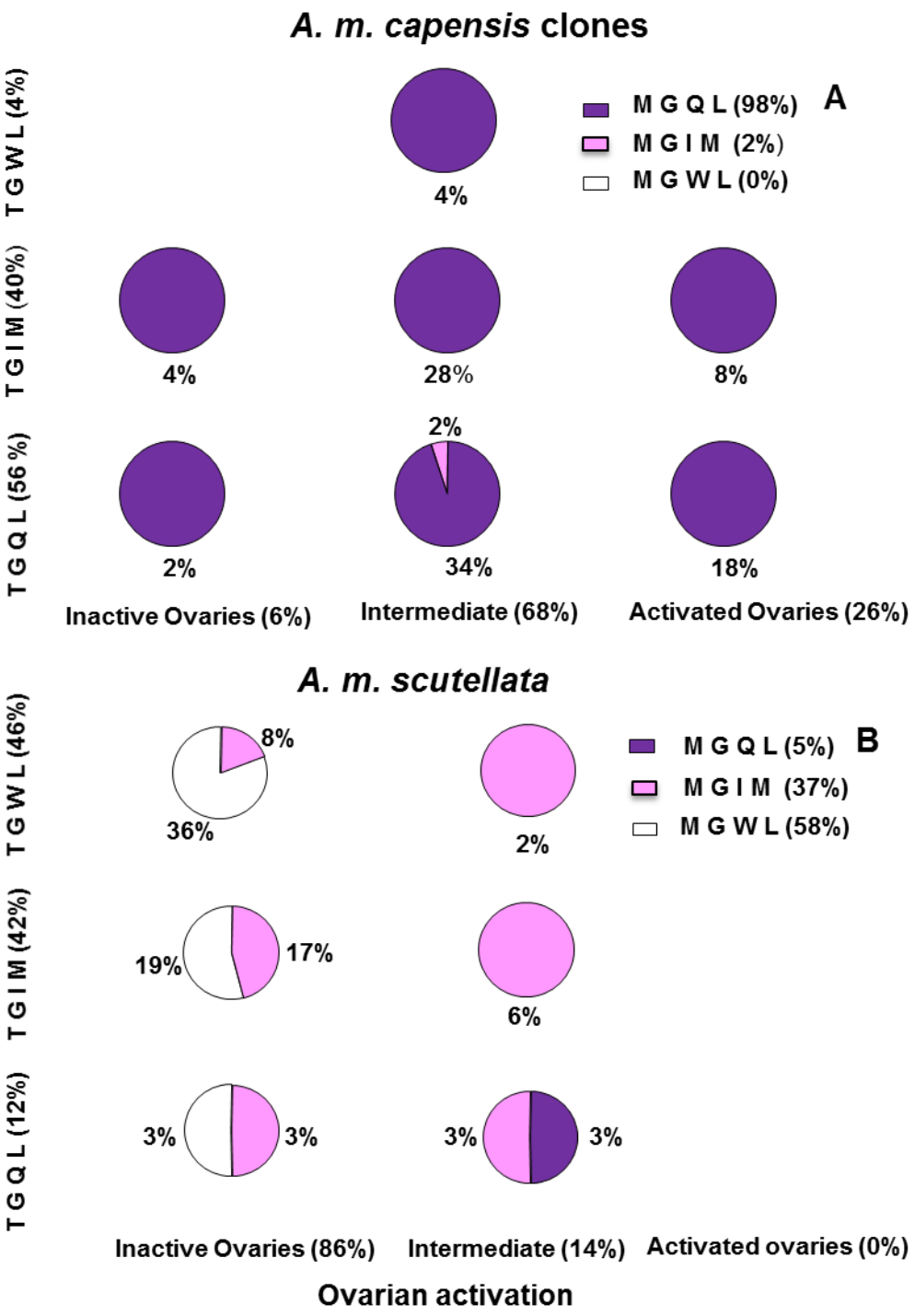

Fig. 1 Mandibular and tergal gland pheromonal status of *A. m. capensis* invasive clones (A) and *A. m. scutellata* (B) with their level of ovarian activation (TGWL = tergal gland worker-like secretions, ≤ 0.23; TGIM = tergal gland intermediate secretions, > 0.23 ≤ 0.35; TGQL = tergal gland queen-like secretions, > 0.35; MGWL = mandibular gland worker-like secretions, 0-0.5; MGIM = mandibular gland intermediate secretions, 0.5-0.9 and MGQL mandibular gland queen-like secretions, 0.9-1).

3.2 *Pheromonal status and Pollen consumption*

There were significant differences in the tergal gland secretions based on the different amounts of pollen consumed by workers [KWA: H (2, $N = 53$) = 19.57; $P < 0.001$] (Fig 2). *A. m. capensis* invasive worker clones with no pollen in their rectum had significantly more queen-like tergal gland secretions compared to workers with small amounts of pollen (Multiple comparisons of mean rank $Z = 3.465$; $P < 0.001$) or large amount of pollen (Multiple comparisons of mean rank $Z = 3.399$; $P < 0.05$), while there was no difference in the tergal gland secretions of workers with a small amount of pollen and a large amount of pollen (Multiple comparisons of mean rank $Z = 0.76$; $P > 0.05$) (Fig 2A). Of the *A. m. capensis* invasive clones 64% had no pollen (NP) in their rectum, 25% small amount of pollen (SAP) and 11% had large amounts of pollen (LAP). Irrespective of the pollen consumption level, 98% had MGQL and 2% had MGIM (Fig 2A).

Among the *A. m. scutellata* workers that consumed different amounts of pollen, they also showed differences in their pheromonal bouquet [KWA: H (2, $N = 36$) = 11.587; $P < 0.05$] (Fig 2B). Workers with large amounts of pollen were significantly different from workers with small amounts of pollen (Multiple comparisons of mean rank $Z = 3.079$; $P < 0.05$). For the *A. m. scutellata* workers there were (6%) with NP, (35%) with SAP, and (59%) with LAP (Fig 2B).

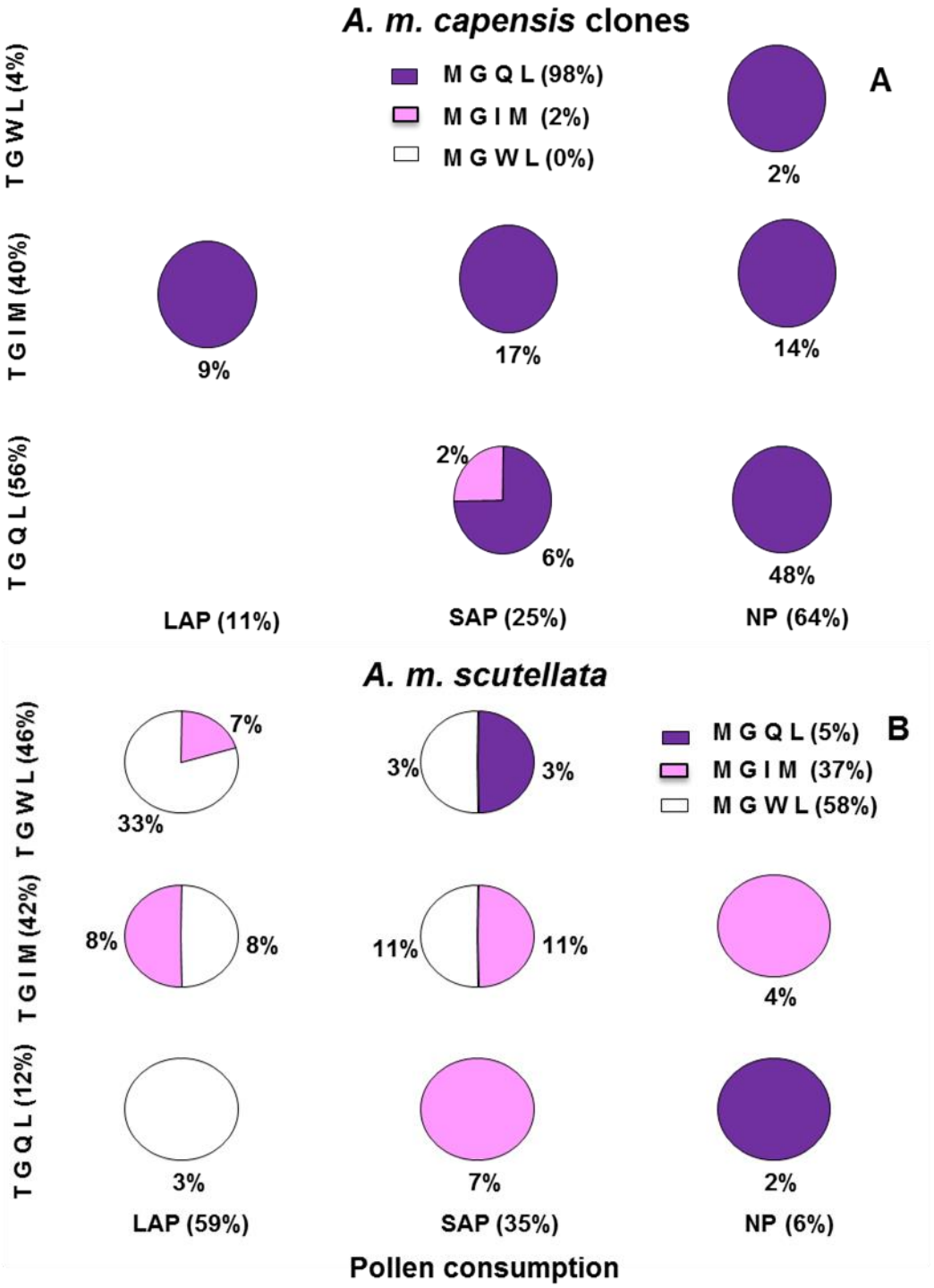

Fig. 2 Mandibular and tergal gland pheromonal status of *A. m. capensis* invasive clones (A) and *A. m. scutellata* (B) with their pollen consumption (LAP = large amount of pollen; SAP = small amount of pollen and NP = no pollen). (TGWL = tergal gland worker-like secretions, ≤ 0.23; TGIM = tergal gland intermediate secretions,> 0.23 ≤ 0.35; TGQL = tergal gland queen-like secretions, > 0.35; MGWL = mandibular gland worker-like secretions, 0-0.5; MGIM = mandibular gland intermediate secretions, 0.5-0.9 and MGQL mandibular gland queen-like secretions, 0.9-1.0).

When compared between sub-species (Fig 3), *A. m. capensis* clonal workers (*N = 53*) had significantly more queen-like mandibular ratios (MWU, *U* = 61*; Z* = 7.461; *P* < 0.001) and tergal gland ratios (MWU, *U* = 356*; Z* = 4.991; *P* < 0.001) than *A. m. scutellata* workers (*N = 36*). Also there were significantly more *A. m. capensis* workers with activated ovaries (MWU, *U* = 131*; Z* = 5.563; *P* < 0.001) and no pollen consumed (MWU, *U* = 288*; Z* = 7.461; *P* < 0.001) compared to *A. m. scutellata* workers.

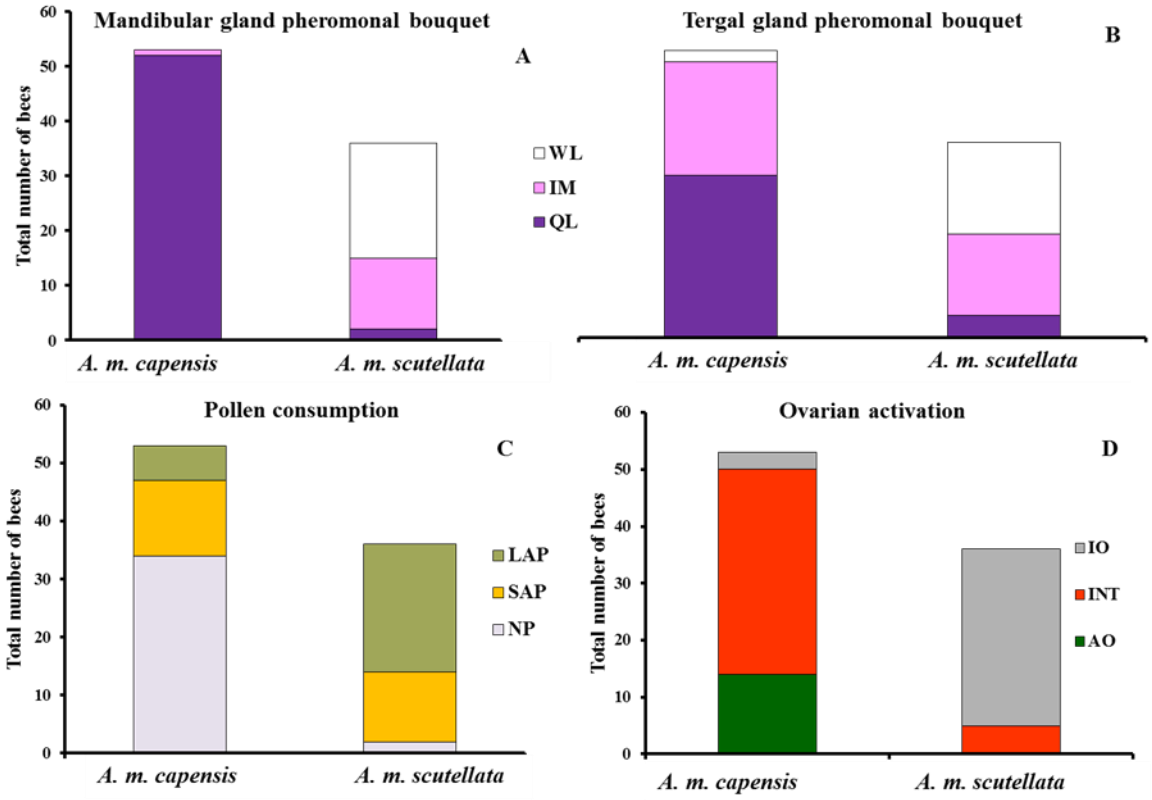

Fig. 3 *A. m. capensis* and *A. m. scutellata* workers and their mandibular gland pheromonal classification (A), tergal gland pheromonal classification (B), pollen consumption (C) and ovarian activation (D) statuses. WL = worker-like secretions; IM = intermediate secretions; QL = queen-like secretions; LAP = large amount of pollen; SAP = small amount of pollen; NP = no pollen; UD = undeveloped ovaries; INT = intermediate ovaries and FD = fully developed ovaries.

Comparison between hoarding cages showed that the mandibular gland ratios (Fig 4A) of *A. m. capensis* clonal workers was significantly higher than those of *A. m. scutellata* workers (Wilcoxon matched pairs test, $N = 18$, $T = 0.0$, $P < 0.001$). The tergal gland ratios (Fig 4B) of *A. m. capensis* clonal workers was significantly higher than *A. m. scutellata* workers' with which they were paired (Wilcoxon matched pairs test, $N = 18$, $T = 0.0$, $P < 0.001$).

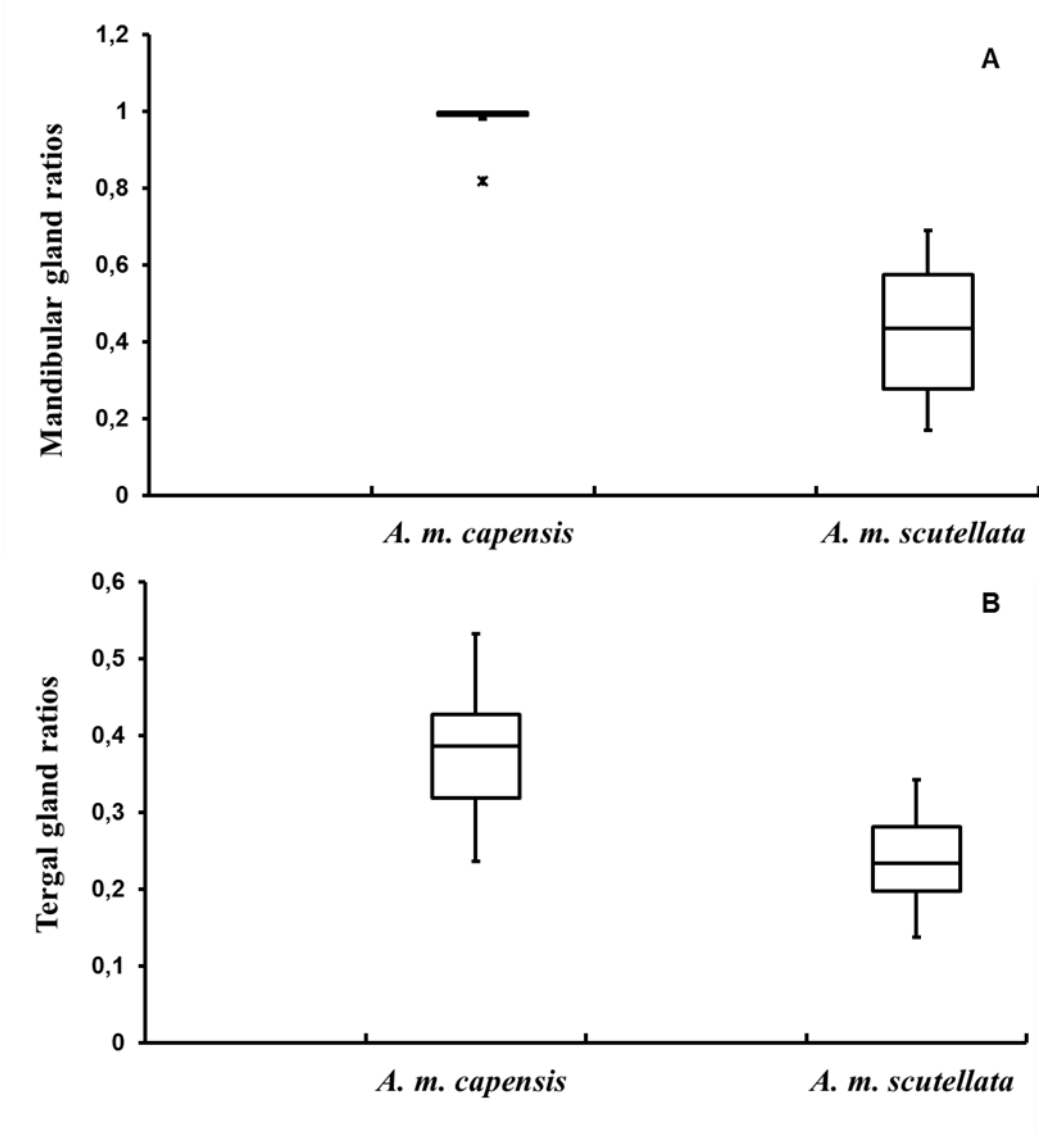

Fig. 4 Mandibular gland (A) and tergal gland (B) ratios of *A. m. capensis* invasive clonal workers and *A. m. scutellata* workers reared in the same hoarding cage

4. Discussion

Reproductively dominant workers use diverse pheromone signals from both mandibular and tergal glands to dominate subordinate workers in queenless situations. These workers achieved false queen status and consumed little or no pollen unlike subordinate workers that had large amounts of pollen in their guts. Regardless of whether they had activated ovaries or not, all *A. m. capensis* invasive clonal workers emerging from their cells already showed queen-like attributes as observed in workers from day 2, while in *A. m. scutellata* even workers with activated ovaries did not show such queen-like attributes except for one worker on day 14. This adds a level of detail to our knowledge that dominance interactions are mediated by pheromones from mandibular gland secretions (Hemmling et al., 1979; Crewe and Velthuis, 1980; Schäfer et al., 2006) and Dufour's gland (Katzav-Gozansky et al., 2003; Malka et al., 2008). Indeed, all categories of queenless workers described earlier, false queens, incipient false queens, laying workers and normal workers (Hemmling et al., 1979; Crewe and Velthuis, 1980; Schäfer et al., 2006) were found in our study. False queens are workers with both mandibular gland queen-like secretions (MGQL) and tergal gland queen-like secretions (TGQL) with activated ovaries. Incipient false queens are those workers with both MGQL and TGQL but inactive ovaries. Laying workers on other hand, are workers that had mandibular gland worker-like secretions (MGWL) and tergal gland worker-like secretions (TGWL) but activated ovaries; while normal workers are those with MGWL, TGWL and inactive ovaries (Fig. 1).

The presence of laying workers in social insect colonies may be overlooked by their fellow workers if they do not produce chemical cues related to their reproductive potential such as in the myrmicine ant, *Aphaenogaster cockerelli* (Smith et al., 2011) or in honey bees, *A. m.*

mellifera (Malka et al., 2008). Such chemical crypsis may explain the presence of a category of laying workers that do not have either MGQL or TGQL but have activated ovaries. In addition, there were no *A. m. capensis* clonal workers with MGWL (Fig. 1A; 3A), this could be due to the coupling of ovary activation and queen-like glandular secretions in the *capensis* clone (Lattorff et al., 2007; Jarosch et al., 2011). The 58% of *A. m. scutellata* workers with undeveloped ovaries in the presence of reproductively dominant *A. m. capensis* clonal workers (Fig. 1B), shows that *A. m. capensis* workers readily pheromonally and reproductively dominate sister sub-species *A. m. scutellata* workers. While the 3% of *A. m. scutellata* workers false queens (Fig. 1B) indicate that *A. m. scutellata* workers can still compete pheromonally in an arms race by upregulating their pheromone production in events of social parasitism. The false queens in this study also inhibited ovarian activation in subordinate workers as only 14% were able to activate their ovaries (Fig. 1B). This proportion of workers with activated ovaries when reared in groups with Cape honey bee clonal workers were low compared to 27% of *A. m. scutellata* workers that had activated ovaries when reared within their own group (Okosun et al., 2015). Notably, the number of *A. m. capensis* invasive clone false queens and incipient false queens (54%) in this study (Fig. 1A) shows that our experimental set-up indeed simulated conditions under which *A. m. capensis* workers in their native range can gain reproductive dominance, in a manner similar to what happens under field conditions during usurpation of foreign colonies (Martin et al., 2002; Neumann and Hepburn, 2002; Härtel et al., 2006 b).

As false queens produce mandibular and Dufour's glandular secretions that are queen-like, they can prevent other workers from switching the biosynthesis of their glandular secretions and in the process regulate reproduction (Moritz et al., 2000; Sole et al., 2002; Katzav-Gozansky et al., 2003; Zheng et al., 2010). This could have been the case here, as only one *A. m. scutellata* worker was able to achieve pheromonal dominance in the presence of *A. m. capensis* invasive clones. This study provides evidence for reproductive dominance being established through the

use of pheromones from a number of glandular secretions acting synergistically or additively to regulate various processes in the colony either by the queen or by reproductively dominant workers (Renner and Vierling, 1977; Saiovici, 1983; Maisonnasse et al., 2010). The synergist effect in this study could result from mandibular gland components being used to attract the retinue workers, while the tergal glands secretions act to maintain the retinue around the false queens as is found for queens (Renner and Vierling, 1977; Wossler and Crewe, 1999b).

The queen-like pheromones of the *A. m. capensis* invasive clone workers, resulted in them being fed predominantly through trophallaxis by their nest mates rather than having to consume pollen directly as the majority of the *A. m. scutellata* workers had to do (Altaye et al., 2010; Archer et al., 2014). Regarding pollen consumption, reproductively dominant *A. m. capensis* invasive clones had more workers that were false queens that consumed little or no pollen (Fig. 3C). The *A. m. scutellata* host workers that were subordinate could have supplied the dominant workers with pre-processed food through trophallaxis as these *A. m. scutellata* workers had large amounts of pollen in their hindguts (Fig. 2B). This is consistent with reports that dominant workers assume dominance status via the social pathway (Schäfer et al., 2006) as they are fed through trophallaxis by their subordinates in their own colonies or in colonies of other sub-species (Korst and Velthuis, 1982; Neumann and Hepburn, 2002; Schäfer et al., 2006). Also, the dominant workers are able to activate their ovaries more rapidly by being fed royal jelly by subordinate workers rather than consuming pollen directly themselves with its associated digestive processes (Moritz and Crailsheim, 1987). Moreover, the presence of reproductively active workers with no pollen in their recta indicates that oogenesis is not dependent on pollen consumption alone but rather on royal jelly obtained through trophallaxis from subordinates (Lin and Winston, 1998; Pirk et al., 2010).

In summary, analysis of both tergal and mandibular gland secretions from the same bees in this study, allowed us to classify them based on their tergal gland profiles and shows that tergal

gland and mandibular gland profiles could be used to classify dominance status in queenless honey bee workers. This provides additional insights into how pheromones from various glandular secretions contribute to the evolution of reproductive dominance and reproductive division of labour within social insect societies.

Conflict of interests

The authors declare no conflict of interests

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