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Pheromonal predisposition to social parasitism in the honeybee *Apis mellifera capensis*

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In honeybees, worker reproduction is mainly regulated by pheromones produced by the brood and the queen. The source of one of the queen pheromones influencing worker reproduction has been located in the mandibular glands. In nonlaying workers, this gland's profile is dominated by fatty acids that are incorporated into the food given to the brood and to nest mates. After queen loss and onset of reproductive activity, workers are able to synthesize different fatty acids, which are normally only produced by queens and that contribute to their reproductive success. *Apis mellifera capensis* workers have the ability to rapidly produce queen-like mandibular profiles that could represent an important factor in their ability to behave as facultative intraspecific social parasites. Indeed, *A. m. capensis* workers can take over reproduction from the host queens in colonies of other subspecies. Here, we show that in the presence of their own queen, the mandibular gland profile of *A. m. capensis* workers is dominated by the precursor of the major compound of the queen pheromone. This is a unique trait among honeybee workers and suggests that *A. m. capensis* workers are primed for reproduction and that this phenomenon represents a pheromonal predisposition to social parasitism. We identified geographical variation in the ratio of queen- to worker-specific compounds in the mandibular gland profile of *A. m. capensis* workers, which corresponds with the introgression with the neighboring subspecies *A. m. scutellata*. *Key words: Apis mellifera capensis*, mandibular gland, queen pheromone, social parasitism, worker reproduction. *[Behav Ecol]*

In honeybee colonies, pheromonal and behavioral mechanisms normally ensure that workers do not threaten the reproductive monopoly of the queen (Fletcher and Ross 1985; Winston 1987). Under these conditions, few workers have ovaries that are developed (Visscher 1989; Visscher 1996) and only about 0.1% of all adult males derive from worker-laid eggs (Visscher 1989). In contrast, in *Apis mellifera capensis*, which is native to the southern tip of Africa (Hepburn and Radloff 1998), workers show a unique set of traits related to reproduction and exhibit queen-like characteristics (Neumann and Hepburn 2002). A variable proportion of *A. m. capensis* workers possess a spermatheca (Hepburn and Crewe 1991; Phiancharoen et al. 2009), whereas in other subspecies, only queens retain this sperm storage organ that allows them to conserve male gametes during their whole reproductive life. After queen loss, honeybee workers can produce male offspring (Velthuis et al. 1965; Page and Erickson 1988). *A. m. capensis* workers do so more rapidly and therefore have a higher reproductive potential than those of other subspecies (reviewed by Neumann and Hepburn 2002 and Neumann and Moritz 2002). In addition, *A. m. capensis* workers are able to reproduce in the presence of a laying queen to a greater extent than workers of other subspecies (e.g., Pettey 1922; Moritz et al. 1999; Pirk et al. 2002; Jordan et al. 2008; Beekman et al. 2009).

Mandibular pheromone secretions are associated with reproductive activity in the honeybee. In queens, these secretions are dominated by 9-keto-(*E*)-2-decenoic acid (9ODA) and (*E*)-9-hydroxy-2-decenoic acid, whereas worker glands produce 10-hydroxy-2-decenoic acid (10HDA) and 10-hydroxydecanoic acid (10HDAA) that are incorporated into the food given to the brood and to nest mates (Plettner et al. 1996). However, when workers acquire reproductive status, they are also capable of producing the compounds normally found in queens (Plettner et al. 1993; Crewe and Velthuis 1980). This (Schäfer et al. 2006; Dietemann et al. 2007) and the fact that workers of *A. m. capensis* are able to produce diploid female offspring (Onions 1912) via thelytokous parthenogenesis (Verma and Ruttner 1983; Moritz and Haberl 1994) contribute to their ability to behave as facultative social parasites. *A. m. capensis* workers can exploit colonies of other honeybee subspecies by usurping the host queen, leading to her death and colony dwindling (Johannsmeier 1983; Woyke 1995; Martin, Beekman, et al. 2002; Martin, Wossler, et al. 2002; Neumann et al. 2003; Moritz et al. 2008).

A. m. capensis is separated from the neighboring subspecies *A. m. scutellata* by an introgression zone in which there is a gradual mixture of traits of both taxa (Figure 1; Hepburn et al. 1998). The introduction of *A. m. capensis* from the Western Cape province of South Africa into the range of *A. m. scutellata* by beekeepers in 1990 resulted in large-scale colony usurpations by *A. m. capensis* parasitic workers (Allsopp and Crewe 1993). This event has raised considerable attention because it has led to significant damage to South African apiculture with losses due to the *A. m. capensis* parasite counted in tens of thousands of colonies per year in the area occupied

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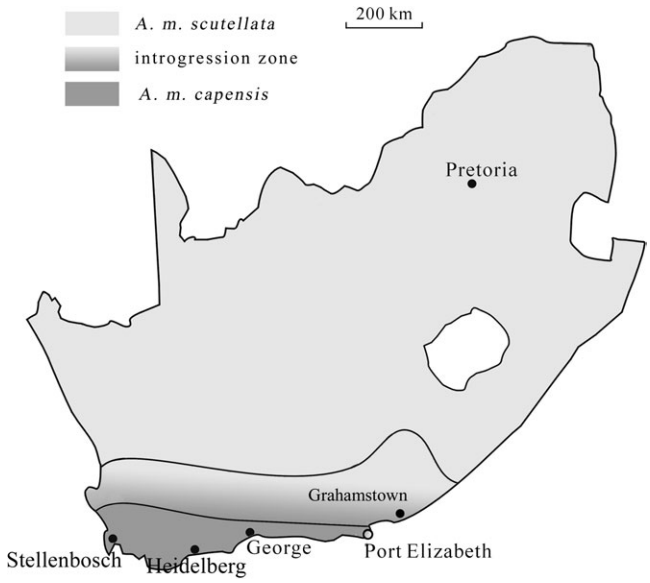

Figure 1
Map of South Africa showing the sampling locations (full circles) and the limit of the *Apis mellifera capensis* and *A. m. scutellata* distribution areas as well as the introgression zone between these subspecies. Reece (2002) sampled at Port Elizabeth (empty circle).

by *A. m. scutellata* (Allsopp and Crewe 1993; Dietemann, Lubbe, et al. 2006). The ability of *A. m. capensis* workers to behave as facultative social parasites has spurred much research to understand how these workers escape reproductive regulation in a host colony (e.g., Simon et al. 2001; Reece 2002; Moritz et al. 2003; Neumann et al. 2003; Dietemann, Pflugfelder, et al. 2006). The importance of the profiles of the mandibular glands for social organization has been demonstrated many times (reviewed by Winston and Slessor 1998), but in particular, it is thought to play an important role in achieving reproductive dominance (Hepburn and Allsopp 1994; Moritz et al. 2000; Moritz et al. 2002; Moritz et al. 2004).

In order to further understand the role of the secretion of the mandibular gland in the acquisition of reproductive status and in the proximate mechanisms of social parasitism, we investigated the composition of mandibular gland profiles of *A. m. capensis* workers from queenright colonies. Mandibular profiles of *A. m. capensis* workers have until now mainly been characterized from individuals in queenless social units and described only partially in queenright situation (Moritz et al. 2002; Reece 2002). We investigated whether the products of the mandibular gland of the workers of this peculiar subspecies corresponds to those known for other honeybees (Crewe 1982; Plettner et al. 1996; Plettner et al. 1997). Because variations in anatomical and physiological traits of *A. m. capensis* workers have been reported (Hepburn and Crewe 1991; Jordan et al. 2008; Phiancharoen et al. 2009), we surveyed the geographical variation of the mandibular gland composition in several locations within the distribution area of this subspecies. Recent studies suggest regulation of the expression of queen-like traits in *A. m. capensis* workers as being determined by independent genes (e.g., ovarian development and spermatheca size; Jordan et al. 2008; Phiancharoen et al. 2009). We thus considered whether the presence of a spermatheca is linked to differences in mandibular gland profiles to determine whether these 2 traits were developmentally or functionally linked. We discuss the consequences of the uniqueness of *A. m. capensis* workers mandibular gland profiles for the evolution of social parasitism and for our understanding of social organization in the honeybee colony.

MATERIALS AND METHODS

Sampling

Honeybee workers were sampled from queenright colonies along a south west to north-east transect within the endemic range of *A. m. capensis* (Figure 1; Hepburn et al. 1998) ($N = 71$ workers from 1 colony from Stellenbosch; $N = 109$, 300, and 210 workers from 3 colonies from Heidelberg; and $N = 49$ workers from 1 colony from George) and the introgression zone ($N = 196$, 245, and 158 workers from 3 colonies from Grahamstown) and reaching the *A. m. scutellata* territory ($N = 28$ workers from 4 colonies from the apiary of the University of Pretoria). The *A. m. scutellata* samples serves as reference pattern for mandibular gland products of workers with low reproductive potential (Dietemann et al. 2007). All the workers were randomly sampled from the surface of randomly chosen frames after taking off the hive lid.

The presence or absence of a spermatheca in workers was determined by dissection using a binocular microscope. The abdomen of each bee was opened by pulling the sternites back, thereby exposing the ovaries, and the area close to the junction of the ovarioles was investigated for the occurrence of the spermatheca. In addition, depending on the observed frequency of spermathecae in workers of each colony, from 3 to 10 workers with spermathecae and a similar number of workers without spermathecae were selected from each sample (with/without spermatheca—Stellenbosch: 10/10; Heidelberg colony 1: 6/6, colony 2: 3/9, and colony 3: 8/8; George: 5/5; and Grahamstown colony 1: 12/12, colony 2: 3/8, and colony 3: 4/7) and their level of ovary activation was evaluated. Their stage of activation was visually scored using a 5-point scale, modified from Hess (1942): stage 1 is inactive; stage 2 is swollen ovarioles without vitellus; stage 3 is swollen ovarioles with visible vitellus; in stage 4, the ovarioles contain distinct but immature oocytes; and stage 5 is fully activated ovaries with distinct mature oocytes.

Gas chromatography

For chemical analysis of mandibular gland composition, the workers that were used to measure ovarian development were decapitated, and each head was extracted in 200 µl dichloromethane for at least 24 h before gas chromatographic analysis. Half of the extract was used and evaporated to dryness under a stream of nitrogen (the other half was retained as a backup). The residue was redissolved in 10 µl internal standard solution (containing 1 mg of octanoic acid and 1 mg of tetradecane in 4 ml dichloromethane), and 10 µl bis(trimethylsilyl)trifluoroacetamide was added to derivatize the fatty acids and allow their binding to the matrix of the gas chromatography column. One microliter of this solution was injected into a gas chromatograph (Hewlett Packard 6890) fitted with a split–splitless inlet and a 25 m × 0.32 mm methyl silicone–coated fused silica capillary column. The carrier gas was helium with a flow rate of 1 ml/min; the oven temperature was programmed as follows: 60 °C for 1 min, then heated at 50 °C/min to 110 °C, then 3 °C/min to 220 °C, and then held at 220 °C for 10 min following standard analytical procedures (Dietemann, Pflugfelder, et al. 2006). Chromatograms were recorded and peak areas quantified using HP ChemStation software. The following 5 major mandibular gland components were identified based on the retention times of synthetic compounds: 9ODA, 9-hydroxy-2(*E*)-decenoic acid (9HDA), methyl p-hydroxybenzoate (HOB), 10-hydroxy-2(*E*)-decenoic acid (10HDA), and 10-hydroxy-2-decanoic acid (10HDAA).

These compounds were chosen among those that constitute the queen pheromone (Engels et al. 1997) because they have been shown to trigger behavioral and physiological responses in workers comparable with total queen extracts (Hoover et al. 2003). In addition, the compounds 9HDA, 9ODA, 10HDA, and 10HDAA characterize the 2 alternative biosynthetic pathways for queen and worker mandibular gland products (Plettner et al. 1996) and are commonly used to quantify the pheromonal status of individuals (Moritz et al. 2004). HVA (4-hydroxy-3-methoxyphenylethanol), another component of the queen pheromone, was not detected in any of the worker samples and was thus not included in the further analysis.

Because both absolute quantities and proportions between components in a multicomponent pheromone can encode information, we compared both these variables in the groups tested.

Statistics

Data of chemical composition of mandibular glands were not normally distributed and log transformation did not normalize their distribution. Moreover, variances were not homogeneous according to Levene's test. We therefore used nonparametric statistics to compare the composition of glandular products between locations from which honeybees were sampled.

A Kruskall–Wallis one-way analysis of variance was performed on absolute as well as relative amounts of each glandular compound. Relative proportions were arcsine transformed to prevent dependence of the variance on the mean. The analysis showed that differences in glandular profiles of workers with a spermatheca existed between sampling locations. The same was true for workers without spermathecae. Data could therefore not be pooled across locations for comparison of glandular profiles of workers with and without a spermatheca. Absolute as well as relative amounts of 9ODA, 9HDA, 10HDAA, and 10HDA between *A. m. capensis* workers with and without spermathecae within each of the different localities (George, Heidelberg, Stellenbosch, and Grahamstown) were therefore compared with Mann–Whitney test. A Bonferroni correction was applied when data were used repeatedly in paired comparisons.

Frequencies of spermatheca occurrence in workers were averaged for the different colonies sampled from Heidelberg and Grahamstown. The frequencies for the 4 locations (George, Grahamstown, Heidelberg, and Stellenbosch) were compared with a chi-square test.

Statistical tests were performed using the program Systat 12.0.

RESULTS

Ovarian development

Dissection revealed that the ovaries of all *A. m. capensis* workers ($n = 117$) were undeveloped (score of 1).

Presence of spermathecae and mandibular gland profile

The frequency of spermathecae ranged from 1% to 23% between colonies (Figure 2) and was significantly different across the 4 regions where *A. m. capensis* was collected (χ^2 test, df = 3, $\chi^2 = 20.0$, $P < 0.01$). There was no significant difference in the relative composition of glandular profiles between workers with or without spermathecae for any of the sampling locations following Bonferroni correction for multiple tests (Mann–Whitney *U*-test, $P > 0.02$ in all comparisons). This was also true when absolute amounts were compared, with the exception of the samples collected from Grahamstown. Those workers with spermathecae produced a significantly higher amount of each of the 4 compounds (Mann–Whitney *U*-test, $N_{\text{with spermatheca}} = 18$, $N_{\text{without spermatheca}} = 27$; $U_{9\text{-ODA}} = 386$; $U_{9\text{-HDA}} = 384$, $U_{10\text{-HDAA}} = 427$; and $U_{10\text{-HDA}} = 427$; $P < 0.002$ for all compounds).

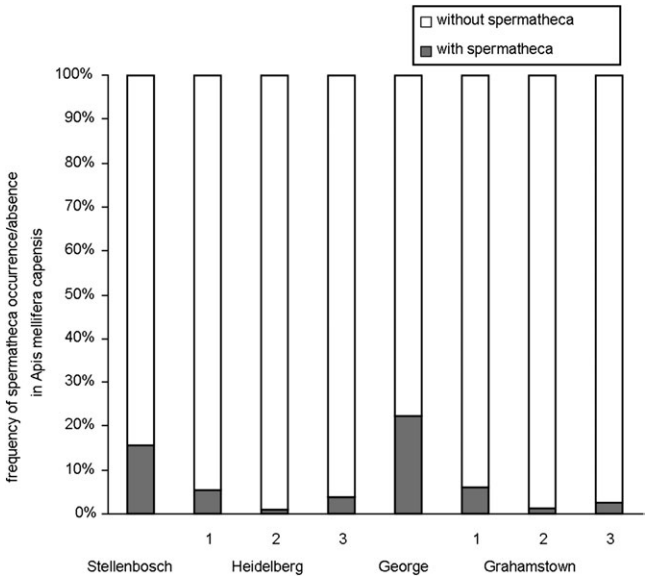

Figure 2
Frequency of spermatheca occurrence or absence in the *Apis mellifera capensis* colonies sampled from the core distribution area of the subspecies (Stellenbosch, Heidelberg, and George) and from the introgression zone (Grahamstown) with *A. m. scutellata*.

Because relative proportions of compounds did not vary between workers with and without spermathecae, data from these groups were pooled and the differences in glandular profiles between localities were tested again with Mann–Whitney *U* tests (see MATERIALS AND METHODS) based on this larger sampling size. Gland composition of *A. m. capensis* workers from George, Heidelberg, and Stellenbosch showed few significant differences in the composition of individual components (Figure 3). *A. m. capensis* workers from Grahamstown were different when compared with these 3 localities (Figure 3). Similarities in proportions of HOB between most localities are due to the low number of workers producing this compound in detectable quantities.

Mandibular gland profile of *A. m. capensis*

The analysis of the mandibular gland products of *A. m. capensis* workers collected from queenright colonies in Heidelberg, George, and Stellenbosch showed that in most workers (63 of 70), 9HDA, the precursor of the major queen pheromone compound (9ODA), dominated the glandular profile (Figure 3). The percentage composition of 9HDA in workers from these 3 locations varied from 2.5% to 91.5% of the total fatty acids, with an average (±standard deviation [SD]) of 68.6 ± 20.1%. Taking into account the percentage of both precursor and final product (9ODA + 9HDA) of the 5 compounds, the value varied from 25.5% to 100%, with an average (±SD) of 82.9 ± 15.8%.

Most workers from Heidelberg (33 of 40, i.e., 82.5%) had 9HDA-dominated profiles with 64.8 ± 24.0% (mean ± SD) of the total amount of the 5 fatty acids detected in these workers. In 4 workers (10%), the profile was dominated by 9ODA. The percentage of 9ODA averaged 86.2 ± 11.5% (mean ± SD) of the total amount of the 5 fatty acids detected in these workers.

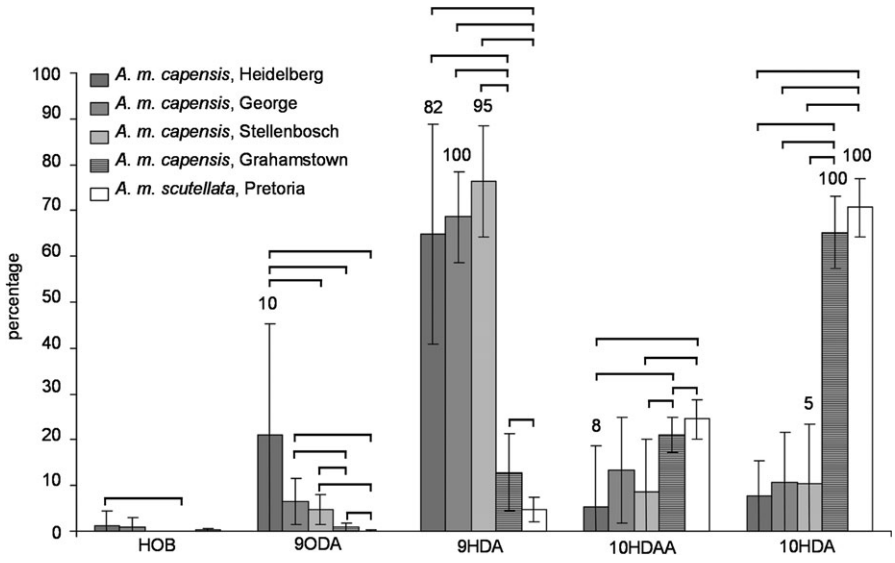

Figure 3
Comparison on the percentage composition of the 5 compounds among 2 categories of *Apis mellifera capensis* workers and *A. m. scutellata* workers. Workers from the center of endemism of *A. m. capensis* (George, Heidelberg, and Stellenbosch) have similar profiles, whereas that of workers from Grahamstown in the introgression zone between *A. m. capensis* and *A. m. scutellata* resemble *A. m. scutellata*, with 10HDA dominating the glandular profile. The numbers given above the columns indicate the proportion of workers, which have their mandibular gland profile dominated by the corresponding compound. Zero values are not shown for clarity. The bars linked by horizontal lines are significantly different following Bonferroni correction (Mann–Whitney *U* test, with $P < 0.0025$).

In 3 individuals (7.5%), the typical worker glandular compound 10HDAA dominated the profile. However, a high proportion of 9HDA was present in their profiles (31.8 ± 7.1% on average). The fatty acid 10HDA was undetectable in 5 workers, including 2 of the workers with 9ODA-dominated patterns and 10HDAA was not detected in 24 workers. In 4 of them, both 10HDA and 10HDAA were undetectable. Thus except for the 4 workers having 9ODA-dominated profile, all workers had a high proportion of 9HDA. All but one of the workers from George and Stellenbosch had 9HDA-dominated profiles. Nine workers from Stellenbosch and 1 from George lacked 10HDAA. One worker from George lacked 10HDA.

The analysis of gland products of *A. m. capensis* workers from Grahamstown ($n = 45$) showed that 10HDA dominated the composition of the gland (Figure 3). The percentage of 9ODA + 9HDA of the 5 compounds varied from 2.8% to 40.1%, with an average (±SD) of 13.7 ± 9.0%.

Mandibular gland profile of *A. m. scutellata*

All *A. m. scutellata* workers had the typical worker-like profile dominated by 10HDA, followed by 10HDAA, with small quantities of 9HDA (4.7 ± 2.7% of the total amount of the 5 fatty acids, mean ± SD; Figure 3) and a few individuals (6 of 28, 21.4%) had traces of 9ODA (0.6 ± 0.5% mean ± SD, Figure 3). The percentage of 9ODA + 9HDA of the 5 compounds varied from 0% to 10.4%, with an average (±SD) of 4.8 ± 2.7%.

The comparison of the mandibular gland bouquets between *A. m. capensis* workers from Stellenbosch, Heidelberg, and George and *A. m. scutellata* workers showed that the former had more 9ODA and 9HDA and less 10HDA and 10HDAA. *A. m. capensis* workers from Grahamstown showed an intermediate status: They had higher proportions of 9ODA and 9HDA and lower proportions of 10HDA and 10HDAA than *A. m. scutellata* but lower proportions of 9ODA and 9HDA and higher proportions of 10HDA and 10HDAA than *A. m. capensis* collected from the other 3 locations (Figure 3). Despite significant differences between their glandular profiles *A. m. capensis* from Grahamstown are more similar to *A. m. scutellata* than to other *A. m. capensis* (Figure 3).

DISCUSSION

The results showed that nonlaying workers of *A. m. capensis* from queenright colonies produce in their mandibular glands, irrespective of the presence of a spermatheca, more compounds typical of queens than expected for nonreproductive individuals based on our knowledge of mandibular gland profiles in other subspecies. We also found geographical variation in this trait with workers of *A. m. capensis* from Grahamstown producing slightly more queen-like mandibular profiles than *A. m. scutellata* but distinctly less queen-like profiles than *A. m. capensis* workers George, Heidelberg, and Stellenbosch (Figure 3). Moreover, frequency of spermatheca occurrence in workers varied between locations sampled, according to the findings of Phiancharoen et al. (2009).

Variations in mandibular gland composition between Grahamstown and the other populations sampled in our study are therefore likely to be linked to subspecific differences. Grahamstown is situated close to the introgression zone between the subspecies *A. m. capensis* and *A. m. scutellata*, on the Eastern border of the natural distribution area of *A. m. capensis* defined on morphological characteristics (Hepburn et al. 1998). Because there are no clear cut geographical boundaries between the neighboring subspecies, but rather a continuum (Hepburn and Radloff 2002) of variations of different traits (morphology, reproduction, and genetics), it is also likely that mandibular gland profiles vary in composition along this continuum (Hepburn et al. 1998). At a more local scale, we could also observe small but significant variations in glandular profiles between Heidelberg, George, and Stellenbosch populations. The biological significance of these differences remains to be elucidated because it is not clear whether they are due to variation between localities, as in the case of spermatheca size and frequency (Phiancharoen et al. 2009) or variation between colonies.

Alongside the production of queen-like mandibular profiles, the presence of a spermatheca in certain *A. m. capensis* individuals suggests a queen-like character. We expected these queen-like traits to be linked developmentally or functionally in individuals with a high reproductive potential, possessing a spermatheca and producing more queen-like mandibular gland profiles. This does not correspond to our observations, as there was no correlation between presence of spermathecae and the ratio of queen- to worker-typical compounds in individual workers. Similarly, the workers from the *A. m. capensis* core area had equal absolute amounts of each compound irrespective of the presence of spermathecae. In contrast, in the introgression zone (Grahamstown), workers possessing a spermatheca produced significantly higher amounts of the 5 compounds analyzed. This is unexpected because presence of spermatheca (a queen-like trait) is in this case associated with a worker-like higher capacity to produce food destined to the brood. These results thus support the idea that the traits associated with reproduction in honeybee workers are under independent genetic control (Jordan et al. 2008).

According to Reece (2002), workers of queenright *A. m. capensis* colonies have a high amount of 10HDA with a ratio of 9ODA to 10HDA of 0.20 ± 0.10. This study analyzed gland composition of workers from Port Elizabeth, which is close to the introgression zone between *A. m. capensis* and *A. m. scutellata* (Figure 1). This ratio is therefore in line with our results for workers originating from the same area (Grahamstown) with *A. m. scutellata*-like profile dominated by 10HDA and ratio of 9ODA to 10HDA of 0.02 ± 0.02 (mean ± SD, $n = 45$).

The higher amount and proportions of 9ODA and 9HDA found in the mandibular gland profile of *A. m. capensis* workers from the core distribution area of the subspecies indicate a unique biosynthetic capability among honeybee workers. *A. m. capensis* workers accumulate more of the 10-carbon (ω-1)-functional acids (9HDA) by preferentially following the "queen-specific" synthetic pathway, in contrast to workers of other subspecies that produce the ω-hydroxy acids (10HDA) (Plettner et al. 1996; Plettner et al. 1998). However, oxidization of 9HDA into 9ODA as in reproductive workers or queens is rare. The regulation of queen-like pheromone production in *A. m. capensis* workers is therefore not taking place at the early functionalization step of the synthesis as in other subspecies, but later, at the hydroxyl group oxidation (Plettner et al. 1996; Plettner et al. 1998) so that a proportion of the precursor stearic acid is already directed into the biosynthetic pathway typical of queens.

An aspect to consider when workers produce unusually high quantities of 9HDA is how what we interpret as a queen-like signal (high proportions of 9ODA and 9HDA) is perceived by nest mates and functionally interpreted in the colony. As the second most abundant product of the mandibular glands (Slessor et al. 1988; Plettner et al. 1997), 9HDA has been shown to be active either alone or synergistically with 9ODA in maintaining swarm clusters (Butler and Fairey 1964; Butler et al. 1964; Winston et al. 1982), inhibiting queen rearing (Butler and Callow 1968), attracting drones (Brockmann et al. 2006), and eliciting retinue response (Slessor et al. 1988). It can therefore be expected that the presence of workers producing this queen-like compound disturbs the social organization within the colony. Because colonies of *A. m. capensis* apparently function as efficiently as colonies of other subspecies, we conclude that the particular mandibular gland profile of *A. m. capensis* workers represents the baseline profile of non-laying workers in this subspecies. The behavior associated with the perception of this profile might thus be context dependent (e.g., it may change in the absence of a queen). Individuals producing compounds usually typical for queens could be accepted in the colony as hopeful reproductives that would initiate oogenesis once the social context becomes favorable, but would not trigger particular behaviors such as caring or policing in the presence of the queen.

The accumulation of 9HDA, the precursor of 9ODA, in *A. m. capensis* workers from the center of endemism could be interpreted as physiological priming for pheromone production and reproduction. The fact that only 1 step remains before synthesis of the final compound 9ODA could allow a swift switch over to a queen-like signal (Hepburn and Allsopp 1994) dominated by 9ODA and the onset of reproduction. This normally occurs once they are released from the queen's or brood's presence (Schäfer et al. 2006; Dietemann et al. 2007) or once they enter colonies of other subspecies in which the queens are not able to regulate them (Neumann and Hepburn 2002; Neumann and Moritz 2002; Dietemann, Pflugfelder, et al. 2006).

Our results confirm that the reproductive control system in *A. m. capensis* is unique when compared with other honeybee subspecies. This finding challenges our knowledge of the chemical ecology of honeybees derived from studying European races but enriches our understanding of the mechanisms of pheromonal regulation of reproduction in social insects and of the evolution of social parasitism.

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