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Hydroxylation patterns associated with pheromone synthesis and composition in two honey bee subspecies *Apis mellifera scutellata* and *A. m. capensis* laying workers

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ABSTRACT

Colony losses due to social parasitism in the form of reproductive workers of the *Apis mellifera capensis* clones results from the production of queen-like pheromonal signals coupled with ovarian activation in these socially parasitic honey bees. While the behavioral attributes of these social parasites have been described, their genetic attributes require more detailed exploration. Here, we investigate the production of mandibular gland pheromones in queenless workers of two sub-species of African honey bees; *A. m. scutellata* (low reproductive potential) and *A. m. capensis* clones (high reproductive potential). We used standard techniques in gas chromatography to assess the amounts of various pheromone components present, and qPCR to assess the expression of cytochrome P450 genes *cyp6bd1* and *cyp6as8*, thought to be involved in the caste-dependent hydroxylation of acylated stearic acid in queens and workers, respectively. We found that, for both subspecies, the quality and quantity of the individual pheromone components vary with age, and that from the onset, *A. m. capensis* parasites make use of gene pathways typically upregulated in queens in achieving reproductive dominance. Due to the high production of 9-hydroxy-decenoic acid (9-HDA) the precursor to the queen substance 9-oxo-decenoic acid (9-ODA) in newly emerged *capensis* clones, we argue that clones are primed for parasitism upon emergence and develop into fully fledged parasites depending on the colony's social environment.

1. Introduction

Honey bee queens produce a variety of signals that enable them to maintain reproductive control of the colony (Butler and Simpson, 1958; Hoover et al., 2003). This strict control tries to ensure that the role of reproduction is carried out mainly by the queen, with the workers performing the ordinary day-to-day tasks related to nutrition, defence and general colony maintenance (Winston, 1987). To maintain reproductive dominance, the queen employs pheromones from various sources including Dufour's (Katzav-Gozansky et al., 1997; Sole et al., 2002), tergal (Okosun et al., 2015, 2017) and mandibular glands (Hoover et al., 2003), with the mandibular gland pheromone complex providing the queen with perhaps her main weapon in regulating worker reproduction. The main constituents of the mandibular gland pheromone are the aromatic compounds methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) and the fatty acids 9-oxo-2 (E)-decenoic acid (9-ODA), (*R,E*)-9-hydroxy-2-decenoic acid (9-HDA), (*S,E*)-9-hydroxy-2-decenoic acid (9-HDA), 10-hydroxy-2 (E)-decenoic acid and 10-hydroxydecanoic acid (10-HDAA) (Crewe and Velthuis, 1980; Winston and Slessor, 1998).

The biosynthesis of the fatty acid components of the mandibular gland pheromone takes place in a caste-specific fashion, starting with the thioesterification of the precursor molecule octadecanoic (= stearic) acid to octadecanoyl-coenzyme A (= stearyl-CoA). This acylated product is then hydroxylated in a caste-selective manner; hydroxylation at the ultimate (ω) carbon atom mainly occurs in worker mandibular glands while hydroxylation at the penultimate (ω-1) carbon atom mainly takes place in the queen mandibular glands (Plettner et al., 1997). Oxidation of the ω-1 hydroxylation products leads to the fatty acid components 9-ODA and 9-HDA in queens, while that of ω hydroxylation products leads to 10-HDA and 10-HDAA in workers and the respective diacids (Plettner et al., 1998). While the genetic characterisation of this pathway has been carried out in European honey bee subspecies (Malka et al., 2009, 2014; Wu et al., 2017) this has yet to be done in the African honey bees and particularly, the reproductively parasitic *A. m. capensis* clones.

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South Africa has two honey bee subspecies; *Apis mellifera scutellata* (the savannah honey bee) and *A. m. capensis* (the Cape honey bee). The Cape honey bee is native (and its distribution limited) to the western Cape region of South Africa, confined largely to the fynbos biome in the southern-western corner of the country (Hepburn and Crewe, 1991) while the savannah honey bee extends northwards across various parts of South Africa, Botswana, Namibia and into East Africa (Hepburn and Radloff, 1998). The main difference between these two subspecies lies in the mode of reproduction of their workers. As with most other social insects, reproduction in *A. m. scutellata* workers is through arrhenotokous parthenogenesis where unfertilised eggs give rise to haploid male offspring. In contrast, workers of the *capensis* subspecies that possess the thelytoky gene have the unique ability to reproduce through thelytokous parthenogenesis, producing diploid female offspring from unfertilised eggs (Verma and Ruttner, 1983). Further still, a specific invasive lineage of *capensis* laying workers has evolved through a short-sighted evolutionary process (Moritz et al., 2008) into a facultative reproductive social parasite of both *A. m. capensis* and *A. m. scutellata* (Härtel et al., 2006; Onions, 1912; Pirk et al., 2014). Aumer et al. (2019) recently reported that a single non-synonymous single nucleotide polymorphism (SNP) occurring in the heterozygous dominant thelytoky locus (*Th*) is the genetic switch that enables *A. m. capensis* social workers to turn into social parasites. The social parasite seeks out susceptible host colonies (Neumann et al., 2001), gains entry and if successful, sets itself up as a false queen by producing queen-like glandular secretions (Crewe and Velthuis, 1980; Okosun et al., 2017; Sole et al., 2002; Zheng et al., 2010), activating its ovaries and laying eggs (Neumann and Hepburn, 2002).

The mandibular gland pheromone biosynthetic pathway (Fig. 1) allows for the queen-mediated regulation of queen-typical mandibular gland products in workers. One key regulation point is at the hydroxylation step where queenright workers hydroxylate stearyl- CoA at the ω position while queens and some queenless workers hydroxylate the same precursor at the ω-1 position (Malka et al., 2009, 2014; Wu et al., 2017). Should some workers manage to bypass regulation by the queen at the hydroxylation level, recent studies have highlighted a second point of regulation at the oxidation step where the honey bee queen prevents oxidation of ω-1 products in workers by inhibiting the oxidation of 9-HDA to the queen substance 9-ODA, a process catalysed by the enzyme alcohol dehydrogenase, ADH (Malka et al., 2014; Mumoki et al., 2018; Wu et al., 2017).

Hydroxylation of stearic-acyl CoA is catalysed by Cytochrome P450 (CYP) enzymes (Malka et al., 2009, 2014; Plettner et al., 1998; Wu et al., 2017) also known as mixed function oxidases (MFO) or Cytochrome P450 monooxygenases. P450s are a superfamily of heme-thiolate enzymes that perform a wide range of functions in insects, from the metabolism of foreign chemicals to the synthesis and degradation of hormones and ecdysteroids as reviewed by Feyereisen (1999). These biochemical processes confer on the insect useful traits such as resistance to insecticides (Mao et al., 2011; Scott, 1999), an ability to degrade and synthesise chemical signals such as pheromones and cuticular hydrocarbons (Calla et al., 2018; MacLean et al., 2018; Wojtasek and Leal, 1999); traits crucial to the insects' survival, growth, development and reproduction. With about 46 putatively functional P450s, the honey bee genome has a greatly reduced number of *CYP*s, as compared to *Drosophila* (85 genes) and *Anopheles gambiae* (106 genes) (Claudianos et al., 2006; Weinstock et al., 2006). In *Apis mellifera*, the P450 enzymes have mainly been associated with detoxification of mycotoxin (Niu et al., 2011), flavonoids, acaricides and insecticides (Berenbaum and Johnson, 2015; du Rand et al., 2017; Mao et al., 2009; Mao et al., 2011) and synthesis of pheromones in the honey bee mandibular glands (Malka et al., 2009, 2014; Wu et al., 2017).

African honey bees have been shown to have a very different mandibular pheromone repertoire as compared to their European and Asian counterparts. Queenright *A. m. capensis* and *A. m. scutellata* workers expressed higher amounts of the queen substance 9-ODA as compared to the populations found in Europe, North America and the Middle East (Crewe and Velthuis, 1980; Plettner et al., 1993; Velthuis et al., 1990). Even within the African races, subspecies-specific differences have been well documented (Crewe, 1982; Crewe and Velthuis, 1980; Hepburn and Crewe, 1991; Simon et al., 2001; Yusuf et al., 2015; Zheng et al., 2010) although much less is known about the biosynthesis of these mandibular gland secretions. Here, we examined the development of reproductive dominance in the two sub-species of South African honey bees, with differing reproductive potentials. We assessed the pheromone profiles and ovarian activation in queenless *A. m. scutellata* and *A. m. capensis* laying workers, and measured the levels of gene expression of selected cytochrome P450 genes, the enzymes responsible for the catalysing the caste-specific hydroxylation of stearic-acyl CoA. We hypothesised that regardless of the subspecies, pheromone expression by African honey bee workers would be dependent on the social environment of the host colonies where young queenless workers would produce worker-like acids and their older counterparts produce more queen-like secretions by utilising the same cytochrome P450 genes usually upregulated in queen mandibular glands. Further, we predicted that adult *capensis* parasites from queenless host colonies would upregulate the queen-typical mandibular gland pheromone genes in producing queen-like fatty acids. In contrast, their counterparts obtained from queenright host colonies would produce worker-typical acids by upregulating genes typically highly expressed in worker mandibular glands.

2. Materials and methods

2.1. Honey bee samples

A. m. scutellata colonies with naturally mated queens were maintained on the University of Pretoria experimental farm, using standard beekeeping practices while the *A. m. scutellata* colonies infested with *A. m. capensis* clones were donated by local beekeepers from the Gauteng and Limpopo Provinces in South Africa. The infested colonies were kept in isolation under a restriction tent and all colonies were managed using standard beekeeping procedures (Williams et al., 2013) and terminated at the end of the experiment.

2.1.1. Sampling adult clones from clone-infested A. m. scutellata colonies

Adult (age unknown) *A. m. capensis* clones were collected from the queen-right and queen-less colonies of *A. m. scutellata* as described in (Mumoki et al., 2018). Briefly, clone-infested *A. m. scutellata* colonies (having approximately 15, 000–20, 000 honey bees) were visually inspected for the presence of the host (*A. m. scutellata*) queen or indirectly by searching for presence of recent queen-laid eggs. Identified as black bees among the typical yellow-black *scutellata*, adult *capensis* clones were collected from the hive frames by aspirating into collection jars.

In total, eight clone-infested colonies were queen-right at the time of sample collection and from these 24 adult clones were collected. 16 clone-infested *scutellata* colonies were queenless at the time of sampling. Six of these queenless colonies were utilised for the collection of adult bees while the rest we used in collection of clone-laid brood for the laboratory rearing experiments. The number of adults collected per colony was not uniform for the clone-infested colonies due to variation in the actual number of *capensis* clone adults available in the infested colonies. In total, 48 *capensis* clone adults were collected for this work, 24 from queen-right colonies (termed queen-right, QR clones) and 24 from queen-less colonies (termed queen-less, QL clones).

2.1.2. Sampling of laboratory-reared queen-less capensis clones and scutellata workers

Brood combs with late-stage *A. m. capensis* clone pupae were collected from queenless *A. m. scutellata* clone-infested colonies while the brood combs for the *scutellata* samples were sourced from *A. m. scutellata* colonies with naturally mated queens. The combs were taken to

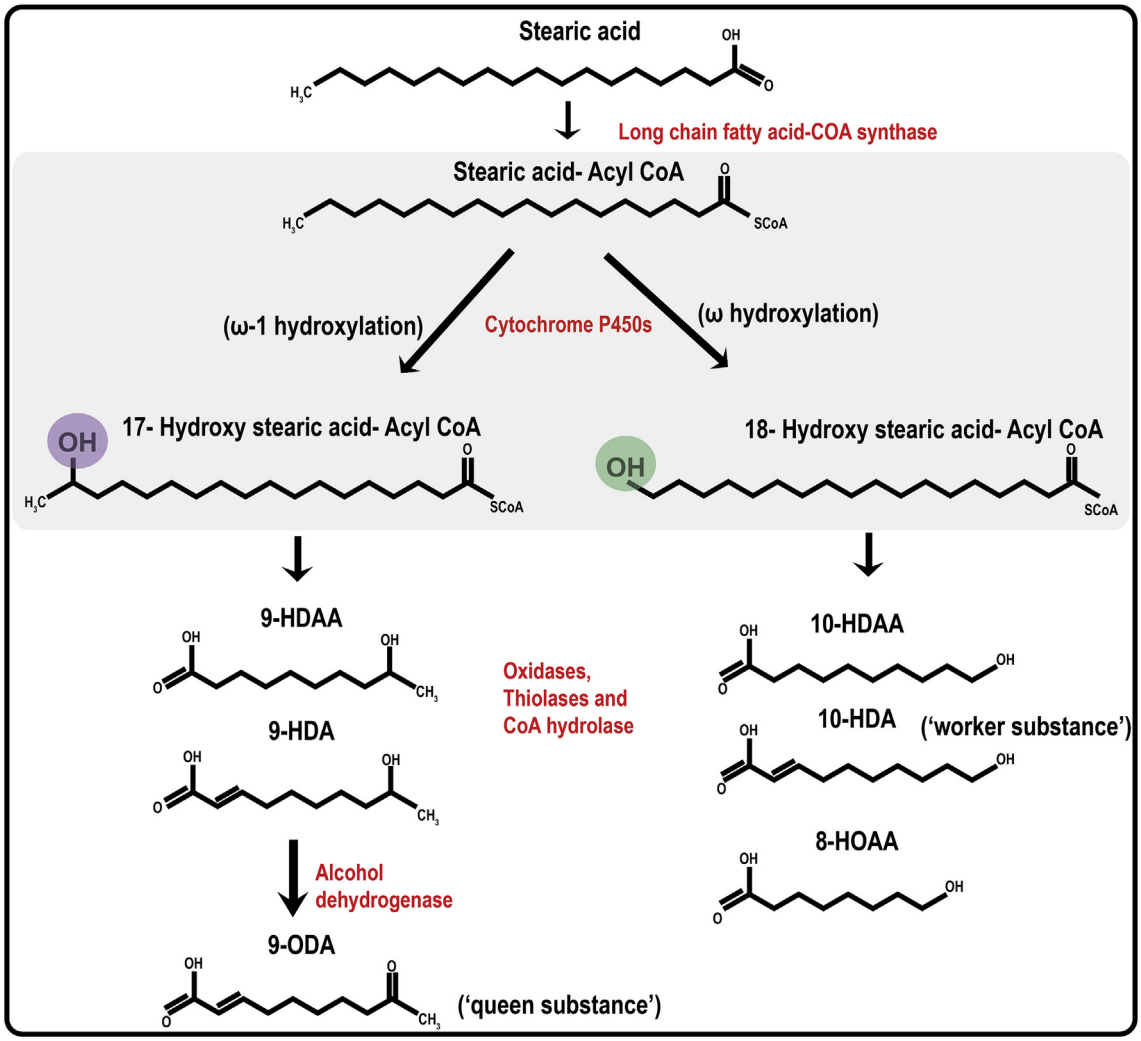

Fig. 1. A schematic representation of the biosynthetic pathway of the main fatty acid components of mandibular gland pheromone in honey bees. The biosynthetic pathway bifurcates at the hydroxylation step leading to ω-1 hydroxylation (typical of queens) and ω hydroxylation (typical of workers).

the laboratory, where they were reared in incubators at 34 °C and 60% relative humidity (Williams et al., 2013).

Sample collection for *capensis* clones: 10 newly-emerged *capensis* clones were transferred to standard (11 × 10 × 14 cm) Plexiglas hoarding cages fitted with a small piece of comb as described by (Köhler et al., 2013), where they were housed with 40 newly-emerged *scutellata* honey bees to form combination/mixed cages. Reproductively dominant honey bees obtain the protein needed for oogenesis by being fed by worker honey bees, through trophylaxis (Crailsheim, 1991). We therefore housed the *capensis* clones with *scutellata* workers in proportions that ensured that there were sufficient non-reproductive (*scutellata*) workers to feed the reproductively dominant *capensis* clones (Okosun et al., 2017; Schäfer et al., 2006).

For the *A. m. scutellata* samples: 50 callow bees were transferred to the aforementioned standard Plexiglas hoarding cages fitted with a piece of comb as was done for the *capensis* clone samples.

The cages from the two groups were then placed in separate incubators at 34 °C and 60% relative humidity and maintained on 50% sucrose solution (w/v), pollen and water, *ad libitum* (Pirk et al., 2010). Five honey bees were then randomly sampled from these hoarding cages after 0 days (< 24 h; *capensis* clones), 3 days (*scutellata*), and 7 days (*capensis* clones and *scutellata*). These ages were chosen based on the known physiological changes associated with pheromone biosynthesis for the two sub-species. While the *A. m. capensis* clones commence pheromone biosynthesis upon emergence (< 24 h old) their *A. m. scutellata* counterparts take much longer than this (Okosun et al., 2015). Based on this difference, we therefore decided to examine the young *A. m. scutellata* at day 3, and comparatively follow this up at day 7 where the worker bees would be committed to production of queen-like or worker like glandular secretions.

Clone-laid brood was collected from 10 queenless clone-infested *A. m. scutellata* colonies while four *scutellata* colonies with naturally mated queens were utilised in the sampling of the *A. m. scutellata* worker brood. For each age there were four replicates (thus n for each day = 20 individuals).

2.2. Dissection of mandibular glands

At the aforementioned ages, the honey bees were immobilised by freezing at −20 °C. The heads were removed and put on dry ice while the thorax and abdomen were pinned on wax plates containing Insect Ringer pH 7.4 (6.4 mL of 5 M NaCl, 3.75 mL of 0.1 M $CaCl_2$, 1.25 mL of 1 M KCl). Heads were dissected along the dorso-ventral plane, with each half containing one mandibular gland as described by (Zheng et al., 2012). The mandibular gland of one half-head carefully dissected out and placed in an Eppendorf Tube® (Hamburg, Germany) containing 200 µL of TRIzol® Reagent (Invitrogen, Carlsbad 92008, USA) and stored at −80 °C for RNA isolation. The second half of the honey bee head was placed in a glass vial containing 200 µL of dichloromethane (DCM) HPLC grade (Sigma-Aldrich, Chemie Gmbh, Munich, Germany)

and pheromones extracted for at least 24 h.

2.3. Assessment of ovary activation and presence of spermatheca

Abdominal dissection was carried out using standard techniques to expose the ovaries and spermathecal (Carreck et al., 2013). Ovaries were classified into one of five developmental stages as described by (Hess, 1942); stage I & II having threadlike ovarioles, III being intermediate with early oocyte development, IV & V with clearly developed oocytes (Schäfer et al., 2006; Velthuis, 1970). Presence or absence of a spermatheca, a queen-associated trait, was also recorded (Phiancharoen et al., 2010).

2.4. Pheromone analyses and gene expression

2.4.1. Gas chromatographic analysis of mandibular gland pheromones

Head extracts obtained from 2.2. above were analysed by Gas Chromatography as described by (Dietemann et al., 2006; Simon et al., 2001; Yusuf et al., 2015) where 100 µL (one half of the total amount of extract) was evaporated to dryness under a steady stream of charcoal-filtered nitrogen gas. The residues were then re-dissolved in 10 µL of internal standard solution containing ~1 mg octanoic acid and ~1 mg tetradecane prepared in 4 mL DCM. To this, 10 µL of bis-(trimethylsily) trifluoroacetamide, BSTFA (Sigma-Aldrich, Chemie Gmbh Munich, Germany) was added to derivatise the fatty acids, reducing their polarity and making them easier to separate chromatographically.

Separation of the mandibular secretions was done using an Agilent 6890N Gas Chromatograph in the split-less mode using a methyl silicone coated fused silica column (Zebron-1 MS column, 25 m × 0.2 mm × 0.33 µm). Helium (with a flow rate of 1 mL per minute) was used as a carrier gas. The oven temperature was programmed from 60 °C for 1 min, then ramped up to 100 °C at 50 °C per min and to 220 °C at a rate of 3 °C per minute and maintained at this final temperature for 10 min.

Identification of the pheromone components was based on comparisons of retention time of the isolates against those of known authentic standards while quantification was based relative to the mass ratios of the internal standards octanoic acid and tetradecane.

2.4.1.1. Classification of pheromone profiles into queen-like or worker-like signals. The pheromone signals were classified as either 'queen-like', 'intermediate' or 'worker-like' based on the ratio of the amount of the queen-substance (9-ODA) to that of the worker component (10-HDA) as follows; 9-ODA/(9-ODA + 10-HDA). Ratios below 0.5 were considered 'worker-like', > 0.5 ≤ 0.7 'intermediate', while those above 0.8 considered 'queen-like' (Dietemann et al., 2006; Schäfer et al., 2006).

For the younger bees (day 0 *A. m. capensis* and day 3 *A. m. scutellata*) an analysis of 9-HDA (the precursor of the queen 9-ODA) to that of the worker substance (10-HDA) was performed, using the ratio 9-HDA/(9-HDA + 10-HDA). This ratio was used to assess the predisposition of the workers to become either 'queen-like' or 'worker-like' in their mandibular gland production.

2.4.2. Expression of cytochrome P450 hydroxylation genes

2.4.2.1. Primer design. Target specific primers were designed using Primer3Plus software www.primer3plus.com) and by manual curation (Suppl. Table 1). To test the primers, cDNA from *A. m. capensis* clones and *A. m. scutellata* workers was amplified using conventional PCR and the amplicons were separated on a 1.5% agarose gel with 1 × GoldView Nucleic Acid Gel Stain (Solarbio® Life Sciences, Beijing, China). The gel-resolved amplicons were visualised under a UV transilluminator and target bands excised from the gel using clean scalpels. Gel purification carried out using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). This was followed by Sanger sequencing. Edited sequences were deposited in GenBank under the accession numbers (MK548572-MK548574).

Cyp4g11 and elf3-s8 were included as an endogenous control as they have been shown to be constitutively expressed in the mandibular glands of queens and workers under various social conditions (Malka et al., 2009; Mumoki et al., 2018) and do not seem to be involved in the biosynthesis of mandibular gland fatty acids (Mao et al., 2015).

2.4.2.2. Ribonucleic acid isolation. RNA was isolated using TRIzol® reagent following the manufacturer's instructions, where half-heads were homogenised in 200 µL of TRIzol® reagent and phase separation achieved by adding 60 µL of Chloroform (Merck KGaA, Darmstadt, Germany) after centrifugation at 13000 rpm. The aqueous phase was transferred to a new 1.5 mL Eppendorf Tube® (Hamburg, Germany). To facilitate RNA precipitation, 84 µL of ice-cold isopropanol (Merck KGaA, Darmstadt, Germany) was added to each tube followed by incubation at −20 °C for 16 h. The precipitate was washed twice using 85% molecular grade ethanol (Merck KGaA, Darmstadt, Germany) and re-suspended in 40 µL nuclease-free water.

DNase treatment was carried out using the DNase Kit I (Invitrogen, Carlsbad 92008, USA) following the manufacturer's instructions. RNA quality and quantity were assessed using a nanodrop 2000 (Waltham Massachusetts, USA) and the RNA normalised to 300 ng.

2.4.2.3. cDNA synthesis and quantitative PCR. cDNA synthesis was carried out as described by (Mumoki et al., 2018) using the Superscript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's instructions.

Quantitative PCR was carried out using the PowerUP qPCR kit (Applied Biosystems, Foster City, California, USA) on a LightCycler® 1.5 Instrument II Real Time PCR thermocycler (Roche, Basel Switzerland). Three µL of the cDNA template, 1 × PowerUP SYBR mix, 10 pmol/µL of each primer, and water to top up to make 20 µL reaction volume. The thermocycler regimen used was as followed; 95 °C for 2 min, 55 cycles of 95 °C for 15 s, and 60 °C for 30 s. The amplification was followed by a standard dissociation program.

2.5. Statistical analyses

Normality was tested using the Shapiro-Wilk test. As the mandibular gland profiles revealed non-normal distribution, non-parametric tests were used.

The Mann Whitney *U* test was used to examine the difference in ovarian activation between the young and older *A. m. capensis* clones and *A. m. scutellata* workers. A Kruskal-Wallis ANOVA with multiple comparisons was carried out to examine the differences in production of each pheromone component in both *A. m. capensis* clones and *A. m. scutellata* workers. In addition, the test was used to assess the differences in pheromone ratios between age groups among *A. m. capensis* clones and *A. m. scutellata* workers and also to examine how the identified ovarian activation groups varied with the expression of the different components of the mandibular gland pheromone. Statistical significance was accepted if $\alpha \leq 0.05$.

The expression of the Cytochrome P450s, homogeneity in the amplification of the genes was analysed by examining the dissociation curves of the amplified genes. Standard curves were constructed by assessing the amplification trends of cDNA of the target and standard genes, covering 100-fold dilutions. The mean normalised expression value of each target gene was calculated by comparing its threshold cycle (C_p) against that of the reference genes, as described for the $2^{-\Delta\Delta Cp}$ method (Livak and Schmittgen, 2001), using the *ddct* algorithm; Bioconductor package *ddct* (Zhang et al., 2010). Statistical significance in the expression of each Cytochrome P450 between the samples was calculated using the unpaired Student t-tests and statistical significance accepted if $\alpha \leq 0.05$.

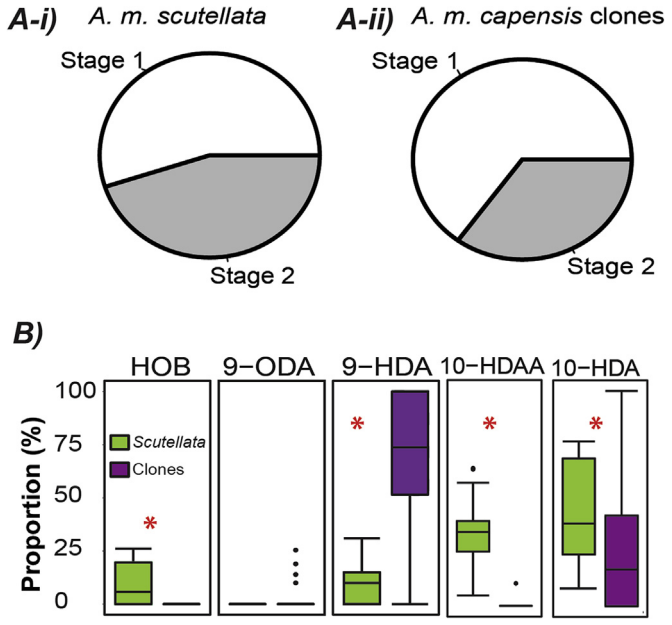

Fig. 2. Ovarian activation stages in day 3 *A. m. scutellata* (A-i) and day 0 *A. m. capensis* clones (A-ii), where Stage 1 & 2 consist of threadlike ovarioles. B shows the proportions (percentage of the total amounts) of mandibular gland pheromone from young *A. m. scutellata* (green) and *A. m. capensis* clones (purple) (▬ = mean, ▯ = 25–75%, ⊥ = min-max, • = outliers). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

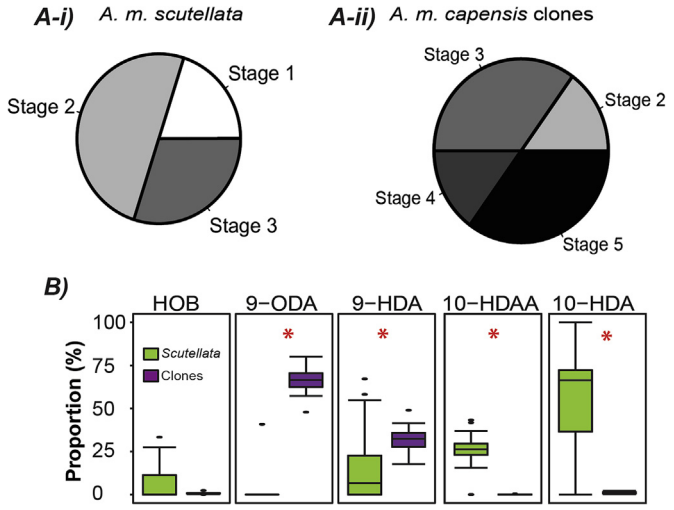

Fig. 3. Ovarian activation stages in seven-day old *A. m. scutellata* (A-i) and *A. m. capensis* clones (A-ii), where stage I & II consist of threadlike ovarioles, III = intermediate with early oocyte development, IV & V = clearly developed oocytes. B shows proportions (percentage of the total amounts) of the mandibular gland pheromone from older *A. m. scutellata* (green) and *A. m. capensis* clones (purple) (▬ = mean, ▯ = 25–75%, ⊥ = min-max, • = outliers). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Ovarian activation

There was no significant difference in ovarian activation between the two groups of the young bees i.e., 3 day old *A. m. scutellata* or day 0 *A. m. capensis* clones (MWU; $U = 172.00$, $N_{SC} = 20$, $N_{CL} = 20$, $p > 0.05$), with bees from both groups having only stages II and I ovaries. Thus, all the young bees were considered to have inactive ovaries consisting of threadlike ovarioles (Hess, 1942) (Fig. 2 A-i and -ii) and were together classified as young bees.

In contrast, there was a significant difference in ovarian activation when comparing the older (day-7) *A. m. capensis* clones and day 7 *A. m. scutellata* workers (MWU; $U = 62.00$, $N_{SC} = 20$, $N_{CL} = 20$, $p < 0.001$, Fig. 3 A i and ii). For the *A. m. scutellata*, 70% of the bees had inactivated ovaries (stage I and II) with only 30% having stage III activated ovaries. For the *A. m. capensis* clones, only a small proportion, 15% of the clones had stage II ovarian activation while the rest, 85% had stage III, IV and V activated ovaries (Fig. 3 A-i & ii).

3.2. Analysis of pheromone expression

The composition of the pheromone components from the younger bees differed qualitatively and quantitatively (both in terms of actual amounts and proportions) when compared between the two sub-species and also against their older counterparts. None of the bees sampled in this work contained any detectible homovanillyl alcohol (HVA).

Based on the actual amounts of pheromone produced (mean ± s.e.m), there were significant differences in the total amount of mandibular gland pheromone produced by the different groups of honey bees (KWA: $H (3, N = 78) = 57.99871$ $p < 0.05$). The young *capensis* clones produced the least amount of pheromone (0.58 ± 0.23 μg) but this was not significantly different ($z = 2.49$, $p > 0.05$) from that of the young *scutellata* workers (2.72 ± 0.58 μg). Similarly, there was no significant difference in the total amount of pheromones produced between the young and older *scutellata* (3.70 ± 0.57 μg; $z = 0.84$, $p > 0.05$). However, with 49.74 ± 8.61 μg, the older *capensis* clones produced significantly larger pheromone amounts when compared to both young clones ($z = 7.49$, $p < 0.05$) and their older *scutellata* counterparts ($z = 4.39$, $p < 0.05$) (Supplementary Fig. 1).

Examining the proportions of the different pheromone components, there were significant differences in the expression of various components of the mandibular gland secretions of the young bees HOB (KWA; $H (1, N = 31) = 12.30829$ $p < 0.001$), 9-HDA (KWA; $H (1, N = 31) = 15.14261$ $p < 0.001$), 10-HDAA (KWA; $H (1, N = 31) = 25.42472$ $p < 0.001$) and 10-HDA (KWA; $H (1, N = 31) = 4.647952$ $p < 0.05$) (Fig. 2B).

Day 7 *A. m. scutellata* workers had significantly higher levels of the worker acids 10-HDAA (KWA; $H (1, N = 47) = 25.38229$ $p < 0.001$) and 10-HDA (KWA; $H (1, N = 47) = 24.49216$ $p < 0.001$), while *A. m. capensis* clones had significantly elevated levels of the queen acids 9-ODA (KWA; $H (1, N = 47) = 40.61930$ $p < 0.001$) and its precursor molecule 9-HDA (KWA; $H (1, N = 47) = 10.83410$ $p < 0.001$) (Fig. 3B).

The expression of the queen substance 9-ODA was significantly higher in honey bees with activated ovaries as compared to those with inactive ovaries [KWA; $H (4, N = 78) = 38.10744$; $p < 0.001$). There were no significant differences in the expression of any of the other pheromone components when compared to ovarian activation.

3.2.1. Classification of the pheromone signals

Evaluating the predisposition of the young bees to queen or worker-like mandibular secretions revealed that the young *A. m. scutellata* workers were significantly more likely to produce worker-like signals as compared to *A. m. capensis* clones (MWU, $U = 30$, $N_{SC} = 14$, $N_{CL} = 17$, $p < 0.001$) (Fig. 4A).

Assessing the nature of queen-like to worker-like signals produced by the older bees (at Day 7) using the ratio of the queen-substance 9-ODA to the total of the queen substance and the worker substance (10-HDA) revealed a similar pattern to that of the young bees where *A. m. scutellata* produced significantly more worker-like mixture of acids as compared to their clone counterparts (MWU, $U = 361$, $N_{SC} = 27$, $N_{CL} = 20$, $p < 0.001$) (Fig. 4B). 9-ODA was not found in the older

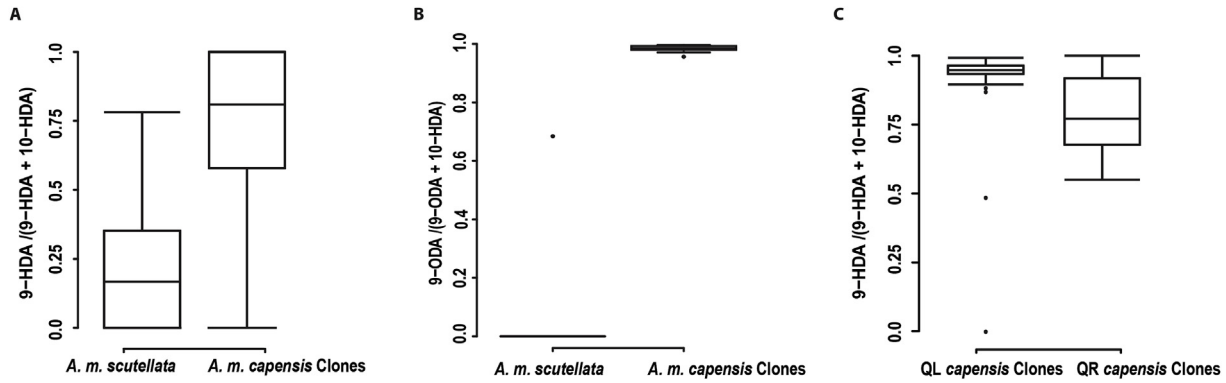

Fig. 4. The pheromone ratios in (A) young and (B) older *A. m. scutellata* and *A. m. capensis* clones. (C) Shows the pheromone ratios of field-collected adult *A. m. capensis* clones from queenright and queenless colonies. Error bars are SD from the means. A queen-like signal has a ratio of > 0.8–1, and a worker-like signal has a ratio of > 0.5.

scutellata workers.

The ratio 9-HDA/(9-HDA + 10-HDA) was used to measure the predisposition of queenright and queenless field-collected *A. m. capensis* clones to produce queen-like secretions (Fig. 4C). Both QL and QR *A. m. capensis* clones showed a strong predisposition to producing queen-like pheromonal secretions, although the queenless clones showed a significantly stronger predisposition as compared to their queenright counterparts (MWU, $U = 269.00$, $N_{QL} = 36$, $N_{QR} = 28$, $p < 0.05$; Fig. 4c).

3.3. Expression levels of genes involved in hydroxylation of mandibular gland pheromones

There was no significant difference in the relative expression levels of both *cyp6as8* (thought to participate in ω hydroxylation; $t = 2.240$, $df = 4$, $p > 0.05$) and cyp6bd1 (ω-1 hydroxylation, $t = 0.2585$, $df = 4$, $p > 0.05$) in both groups of young bees Fig. 5A. However, for the older bees, the relative amounts of *cyp6as8* transcripts were significantly higher in seven-day old *scutellata* ($t = 3.003$, $df = 4$, $p < 0.05$) while *cyp6bd1* transcripts significantly higher in their *capensis* clone counterparts ($t = 5.636$, $df = 4$, $p < 0.001$) Fig. 5B. Finally, when examining the expression of the two cytochrome P450s in queenright and queenless field-collected *A. m. capensis* clones, we see that there were significantly higher *cyp6as8* transcripts in the queenright social parasites ($t = 22.81$, $df = 4$, $p < 0.0001$), and no significant difference in the expression of *cyp6bd1* in these field-collected clones ($t = 0.1800$, $df = 4$, $p > 0.05$) (Fig. 5C).

4. Discussion

To date, there are about 46 CYPs identified in the honey bee genome (Claudianos et al., 2006) with about 34 of those thought to play a role in the hydroxylation step of the biosynthesis of mandibular gland pheromone components (Wu et al., 2017). We examined the expression of two such P450 genes in the mandibular gland tissues of young and old *A. m. scutellata* and *A. m. capensis* clones. Both Malka et al. (2014) and Wu et al. (2017) showed that the two candidate genes *cyp6as8* and *cyp6bd1* were strongly differentially expressed in a caste-specific manner, with the former highly expressed in workers while the latter highly expressed in the mandibular glands of queens. Our work shows that indeed in accordance with our predictions, social condition with regard to both age and presence or absence of queen, influences the production of the mandibular gland fatty acids predominant in queens and workers, by influencing the expression of these two cytochrome P450 enzymes responsible for caste specific hydroxylation.

While there were no significant differences in the ovarian activation of young bees, there was a significant difference in the activation of ovaries in the older bees, with the *capensis* clones that have a higher reproductive capacity having only 15% of individuals with inactive ovaries. The only pheromone component that was produced at significantly higher levels in both groups of bees with activated ovaries was the queen substance 9-ODA. None of the samples had HVA present, a pheromone component expressed in highest quantities in mated queens (Plettner et al., 1997; Strauss et al., 2008) and thought to work with the other components in eliciting retinue around the queen. Previous studies have indeed shown that this pheromone component is expressed in very low quantities in African honey bees and very rarely in the reproductively-dominant *capensis* clones (Crewe and Velthuis, 1980; Okosun et al., 2017; Yusuf et al., 2015, 2018).

As expected, the expression of the queen-like signals dominated the mandibular glands of both the younger and older parasitic bees. Although they had the least total amount of pheromone, the newly emerged *capensis* clones produced predominantly 9-HDA, the precursor of the queen substance 9-ODA, with the second most highly produced component being the worker component 10-HDA. The high level of 9-HDA production shows that the *capensis* clones are indeed predisposed

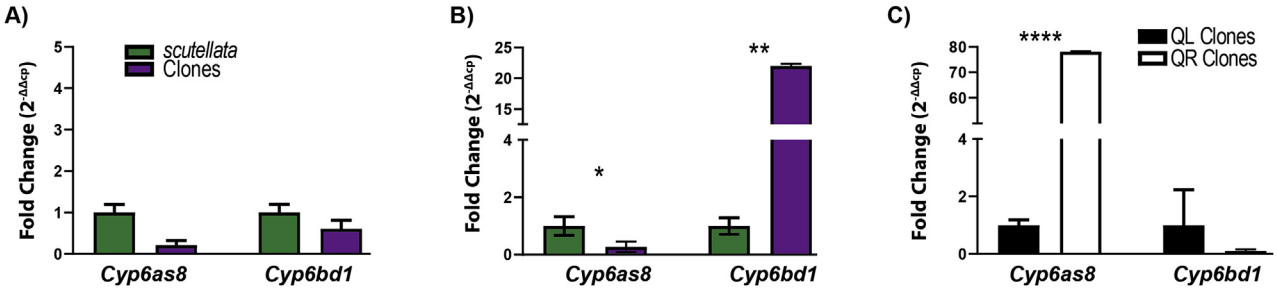

Fig. 5. Expression of *cyp6as8* and *cyp6bd1* in (A) young *A. m. scutellata* (green) and *A. m. capensis* clones (purple) (B), older Day 7 bees and (C) and queenright (open bars) and queenless (closed bars) field-collected *A. m. capensis* clones (* indicate statistical significance; *$p < 0.05$, **$p < 0.001$ and ****$p < 0.0001$, error bars = se of fold change). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to parasitism from the very start, and can be considered to be 'incipient false queens' producing queen-associated pheromone signals but without activated ovaries (Okosun et al., 2017). Simon et al. (2001) examined the mandibular gland pheromonal components of (non-parasitic) *A. m. capensis* workers from queenright colonies and showed that the young workers (up to day 4) produced predominantly worker-like mandibular gland products. This result is not surprising in view of the discovery that *capensis* colonies have a diversity of workers only some of whom carry the locus *Th*, causing them to become false queens (Aumer et al., 2019). Our results show that *capensis* clones which clearly possess the *Th* locus, are primed from their emergence from their brood cells, to pheromonally dominate their hosts. As the clones get older, the precursor 9-HDA is converted to 9-ODA, a substance that dominated in the mandibular glands of the seven-day old parasitic bees. The adult field-collected *capensis* clones analysed in this study were obtained from clone-infested *A. m. scutellata* colonies. In the process of clone infestation, a pheromonal contest between the host queen and the invading reproductive parasites typically ensues (Moritz et al., 2000) and during this process the host *A. m. scutellata* queen may be lost (Martin et al., 2002). Previous studies have shown that the *A. m. scutellata* queens are able to inhibit reproductive dominance by inhibiting ovarian activation and the production of the queen substance, with the latter done through the prevention of the oxidation of 9-HDA to 9-ODA in the mandibular glands of queenright field-collected *capensis* clones (Mumoki et al., 2018). Mandibular gland pheromone signals of the queenright and queenless field collected *A. m. capensis* clones were reported earlier (Mumoki et al., 2018) where the QR clones produced significantly high amounts of 9-HDA and 10-HDA as compared to their queenless counterparts. The queenless clones, however, were able to produce significantly higher amounts of the queen substance 9-ODA, which queenright bees could not produce. In this study, we show that production of the high amounts of 10-HDA by the QR clones may have been aided by the significantly higher amounts of the enzyme CYP6AS8, inferred through the significantly high abundance of transcripts encoding this enzyme.

There was a subspecies-specific expression of the pheromone component 10-HDAA, the precursor molecule to the worker substance 10-HDA. Both young and older *A. m. scutellata* workers produced large amounts of 10-HDAA, while the *capensis* clones producing significantly smaller amounts of this compound, even though the amount of 10-HDA was relatively high in young the *capensis* clones. Indeed, young bees from both *scutellata* and *capensis* groups produced significant amounts of the worker compound 10-HDA, with the *scutellata* bees producing larger amounts of the precursor molecule 10-HDAA. The worker compound 10-HDA has been shown to be secreted by the mandibular glands of workers, and is included in brood food (Winston, 1987) and also in the royal jelly, where it lowers the pH of royal jelly, making it more viscous and hence able to retain eggs layed in the vertically-orientated queen cells (Buttstedt et al., 2018; Pirk, 2018). The large amounts of 10-HDA in the *capensis* clone mandibular glands early in their development shows that the switch to queen-like pheromone production is a function of age.

In order to assess the predisposition to parasitism, we developed a ratio 9-HDA/(9-HDA + 10-HDA), an additional indicator to the previously used 9-ODA/(9-ODA + 10-HDA), with the former ratio examining the expression of the precursor molecules of the queen substance to that of the worker substance 10-HDA. We examined the ratio of the precursor molecule in order to determine the ability of the young bees to produce either queen-like or worker-like final products. The ratio showed that the young clones were indeed predisposed to queen-likeness while their *scutellata* counterparts were predisposed to worker-like mandibular gland acids. Examining the same ratio in field collected parasitic bees (Mumoki et al., 2018) revealed that queenright clones were predisposed to parasitism due to the very large amounts of 9-HDA produced. Therefore, given the right conditions, these bees would have very easily attained pheromonal dominance over their hosts. While European honey bees produce very little 9-HDA (Plettner et al., 1997) the profile of African honey bees is different, even under queenright conditions (Supplementary Fig. 2). Zheng et al. (2010) and (Yusuf et al., 2018) showed that African honey bees naturally produce larger amounts of 9-HDA, even in the presence of the queen. This ratio takes into account the uniqueness of the mandibular gland pheromone composition of African honey bees. At day seven there were large amounts of queen substance (9-ODA) and worker substance (10-HDA) in the mandibular glands of *capensis* clones and *scutellata* bees respectively, leading to the majority of the clones being classified as queen-like, and the *scutellata* as worker-like.

Finally, analysing the expression of the two P450 genes identified as participating in either ω or ω-1 hydroxylation in either worker or queen mandibular glands respectively, we find that older *A. m. scutellata* workers had a higher abundance of *cyp6as8* transcripts (characteristic of workers), as compared to their *capensis* clone counterparts. The higher transcript numbers of this enzyme may play a role in the formation of 10-HDA produced in abundance in the mandibular glands of the older workers. There was very high transcript abundance for *cyp6bd1* (characteristic of queens) in the mandibular glands of the older *capensis* clones. This P450 enzyme is thought to participate in ω-1 hydroxylation in queens and its abundance may explain the large amounts of 9-HDA and 9-ODA in the mandibular glands of day 7 *capensis* clones. This pattern of transcript abundance in older bees is different from their younger counterparts where neither *cyp6as8* nor *cyp6bd1* were differentially expressed. Queenright *capensis* clones had much higher levels of *cyp6as8* and very little *cyp6bd1* while the reverse was true for their queenless counterparts. This pattern of gene expression, shows that the P450 enzymes are differentially expressed in these honey bees reinforcing the results of earlier studies in European bees (Malka et al., 2014; Wu et al., 2017). Though chosen because they have been seen to be among the key P450s involved in caste-specific hydroxylation of mandibular gland pheromones, these are only two of many genes that may be involved in this crucial role.

This study explores the development and regulation of reproductive dominance in workers from two sub-species of African honey bees with different reproductive potential. We examined the ovarian activation, pheromone expression and cytochrome P450 gene expression in young and older bees and report that young *A. m. capensis* clones show a strong predisposition to pheromonal parasitism from emergence, as seen by the high proportion of 9-HDA in individuals less than 24 h old. Earlier studies of the non-parasitic bees from this same subspecies, have shown that the *A. m. capensis* in their native region, under the regulation of their own queen, express worker mandibular gland components, even up to day four (Simon et al., 2001), which is clearly not the case with the parasitic clones. We provide an insight into the genetic mechanisms underlying the control of reproductive dominance, showing that indeed the hydroxylation step forms a crucial regulatory point in the biosynthesis of mandibular gland pheromones and that this regulation takes place at the transcriptional level.

Author contributions

Conceived and designed the research: FNM, AAY, CWWP, RMC. Performed the experiments and analysed the data; FNM. All authors wrote and commented on all versions of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103230.

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