

m. scutellata samples were sourced from *A. m. scutellata* colonies with naturally mated queens. The combs were taken to the laboratory, where they were reared in incubators at 34 °C and 60% relative humidity (Williams et al., 2013).

Sample collection for *A. m. capensis* clones: 10 newly-emerged *A. m. 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 *A. m. 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 *A. m. capensis* clones with *A. m. scutellata* workers in proportions that ensured that there were sufficient non-reproductive (*A. m. scutellata*) workers to feed the reproductively dominant *A. m. 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 *A. m. 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 hours; *A. m. capensis* clones), 3 days (*A. m. scutellata*), and 7 days (*A. m. capensis* clones and *A. m. 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 hours 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 *A. m. 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).

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 CaCl₂, 1.25 mL of 1 M KCl in 1L ddH₂O). 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 μ L of dichloromethane (DCM) HPLC grade (Sigma-Aldrich, Chemie GmbH, Munich, Germany) and pheromones extracted for at least 24 hours.

Assessment of ovary activation and presence of spermatheca

Abdominal dissection was carried out using standard techniques to expose the ovaries and spermatheca (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).

Pheromone analyses and gene expression

Gas Chromatographic analysis of mandibular gland pheromones

Head extracts obtained from above were analysed by Gas Chromatography as described by (Dietemann et al., 2006; Simon et al., 2001; Yusuf et al., 2015) where 100 μ L (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 μ L of internal standard solution containing ~1 mg octanoic acid and ~1 mg tetradecane prepared in 4 mL DCM. To this, 10 μ L of bis-(trimethylsilyl) 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 × 0.2mm × 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 minutes.

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.

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; $9\text{-ODA} / (9\text{-ODA} + 10\text{-HDA})$. Ratios below 0.5 were considered 'worker-like', $0.5 \leq \text{ratio} \leq 0.7$ '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 $9\text{-HDA} / (9\text{-HDA} + 10\text{-HDA})$. This ratio was used to assess the predisposition of the workers to become either 'queen-like' or 'worker-like' in their mandibular gland production.

Expression of Cytochrome P450 hydroxylation genes

Primer Design

Target specific primers for two cytochrome P450 genes *cyp6as8* and *cyp6bd1*, were designed using Primer3Plus software (www.primer3plus.com) and by manual curation (Suppl. Table 1). The two P450s selected been shown to be expressed in a highly-caste specific manner, with *cyp6as8* highly abundant in worker mandibular glands while *cyp6bd1* is abundant in queen mandibular glands (Malka et al., 2014; Wu et al., 2017). 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 1X 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., 2019) and do not seem to be involved in the biosynthesis of mandibular gland fatty acids (Mao et al., 2015).

Ribonucleic acid isolation

RNA was isolated using TRIzol[®] reagent (Invitrogen, Carlsbad 92008, USA) following the manufacturer's instructions, where half-heads were homogenised in 200 μ L of TRIzol[®] reagent and phase separation achieved by adding 60 μ L 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 μ L of ice-cold isopropanol (Merck KGaA, Darmstadt, Germany) was added to each tube followed by incubation at -20 °C for 16 hours. The precipitate was washed twice using 85% molecular grade ethanol (Merck KGaA, Darmstadt, Germany) and re-suspended in 40 μ L 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.

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 μ L of the cDNA template, 1X

PowerUP SYBR mix, 10 pmoles/ μ L of each primer, and water to top up to make 20 μ L reaction volume. The thermocycler regimen used was as followed; 95 °C for 2 min, 55 cycles of 95 °C for 15 seconds, and 60 °C for 30 seconds. The amplification was followed by a standard dissociation program.

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 $2^{-\Delta\Delta C_p}$ 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 $\alpha \leq 0.05$.

Results

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) (Figure 2.2 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; $U=62.00$, $N_{SC}=20$, $N_{CL}=20$, $p < 0.001$, Figure 2.3 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 (Figure 2.3 A-i & ii).

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 \pm 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 *A. m. capensis* clones produced the least amount of pheromone (0.58 ± 0.23 μ g) but this was not significantly different ($z=2.49$, $p>0.05$) from that of the young *A. m. scutellata* workers (2.72 ± 0.58 μ g). Similarly, there was no significant difference in the total amount of pheromones produced between the young and older *A. m. scutellata* (3.70 ± 0.57 μ g; $z = 0.84$, $p>0.05$). However, with 49.74 ± 8.61 μ g, the older *A. m. capensis* clones produced significantly larger pheromone amounts when compared to both young clones ($z=7.49$, $p<0.05$) and their older *A. m. scutellata* counterparts ($z=4.39$, $p<0.05$) (Supplementary Figure 2.1).

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) (Figure 2.2 B).

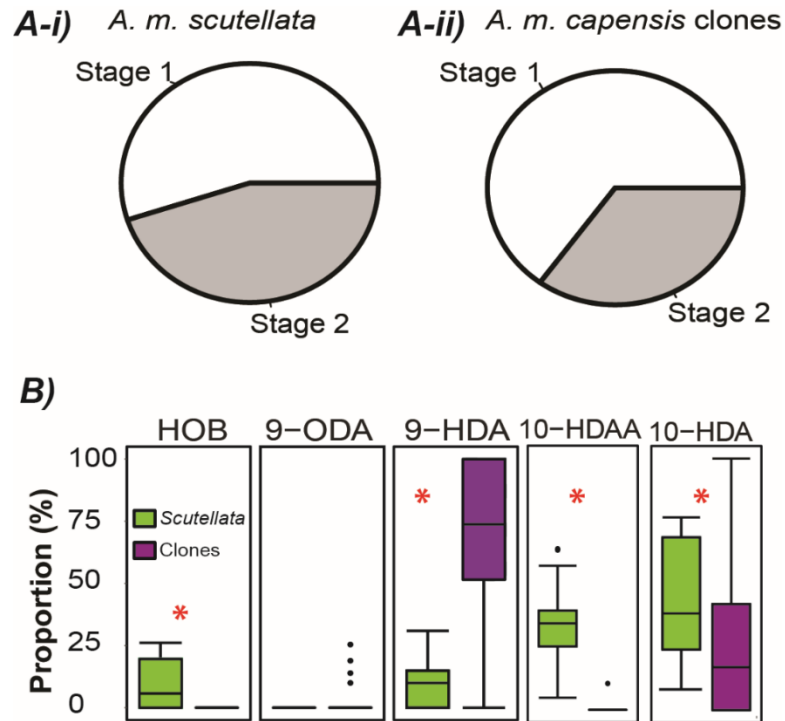


Figure 2.2: Ovarian activation stages in day 3 *A. m. scutellata* (A-i) and day 0 *A. m. capensis* clones (A-ii), where Stages 1 & 2 had threadlike ovarioles. B shows the proportions (percentage of the total amounts) of mandibular gland pheromone from young *A. m. scutellata* (green) and *A. m. capensis* clones (purple) (— = mean, □ = 25-75%, I = min-max, ● = outliers).

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; $H(1, N=47) = 10.83410$ $p < 0.001$) (Figure 2.3B).

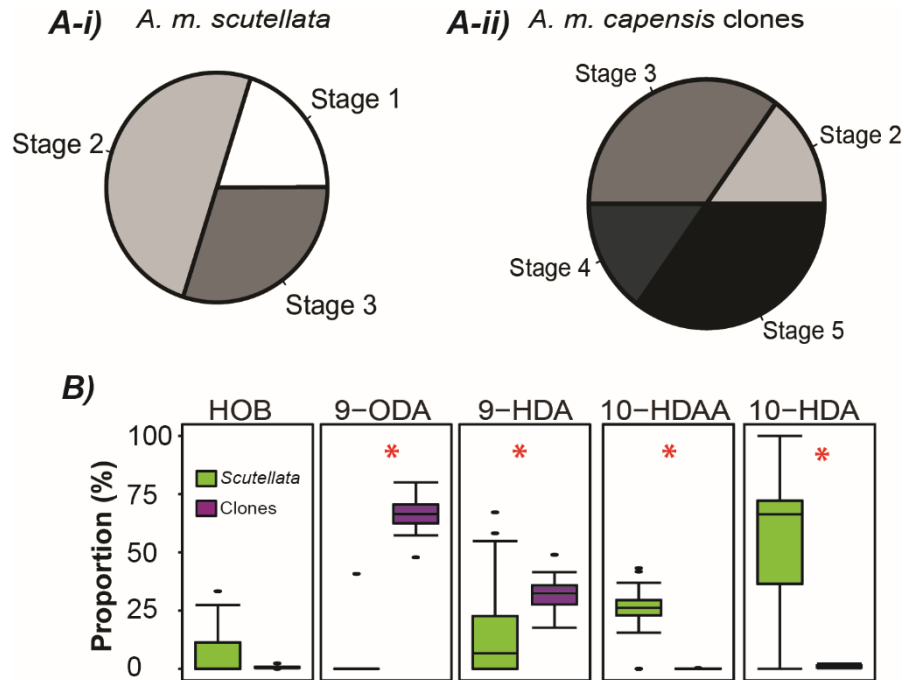


Figure 2.3: Ovarian activation stages in seven-day old *A. m. scutellata* (A-i) and *A. m. capensis* clones (A-ii), where stages I & II had threadlike ovarioles, III = intermediate with early oocyte development, IV & V = clearly developed oocytes. B shows proportions (percentage of the total amounts) of the mandibular gland pheromone from older *A. m. scutellata* (green) and *A. m. capensis* clones (purple) (— = mean, □ = 25-75%, I = min-max, ● = outliers).

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.

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) (Figure 2.4A).

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) (Figure 2.4B). 9-ODA was not found in the older *A. m. 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 (Figure 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. 2.4C).

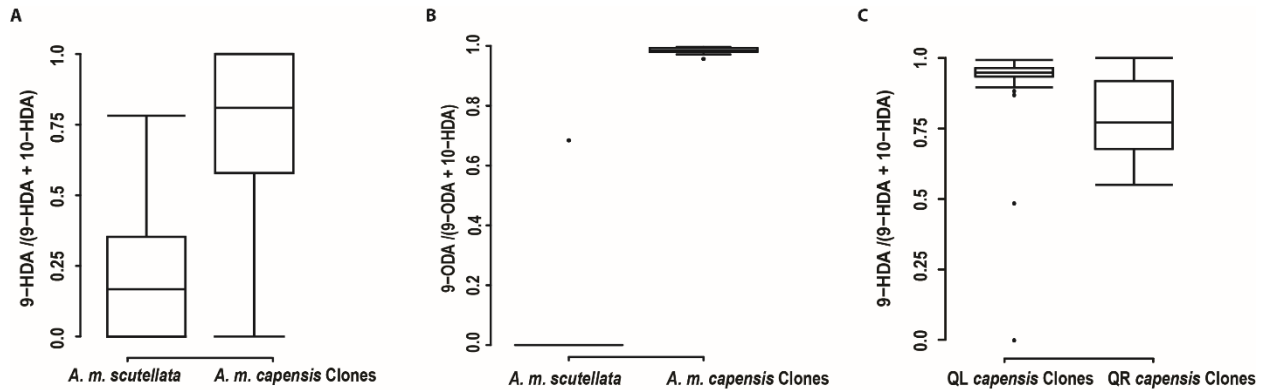


Figure 2.4: The pheromone ratios in (A) young and (B) older *A. m. scutellata* and *A. m. capensis* clones. (C) Shows the pheromone ratios of field-collected adult *A. m. capensis* clones from queenright and queenless colonies. Error bars are SD from the means. A queen-like signal has a ratio of $>0.8-1$, and a worker-like signal has a ratio of < 0.5 .

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* ($\omega-1$ hydroxylation, $t=0.2585$, $df=4$, $p>0.05$) in both groups of young bees Figure 2.5A. However, for the older bees, the relative amounts of *cyp6as8* transcripts were significantly higher in seven-day old *A. m. scutellata* ($t=3.003$, $df = 4$, $p<0.05$) while *cyp6bd1* transcripts significantly higher in their *A. m. capensis* clone counterparts ($t=5.636$, $df = 4$, $p<0.001$) Figure 2.5B. 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 ($t=0.1800$, $df=4$, $p > 0.05$) (Figure 2.5C).

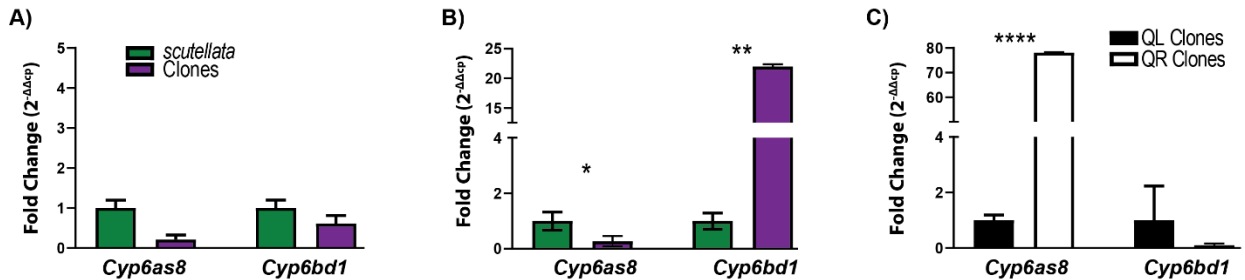


Figure 2.5: Expression of *cyp6as8* and *cyp6bd1* in (A) young *A. m. scutellata* (green) and *A. m. capensis* clones (purple) (B), older Day 7 bees and (C) and queenright (open bars) and queenless (closed bars) field-collected *A. m. capensis* clones (* indicate statistical significance; * $p < 0.05$, ** $p < 0.001$ and **** $p < 0.0001$, error bars= se of fold change)

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 *A. m. 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 *A. m. capensis* clones (Crewe and Velthuis, 1980; Okosun et al., 2017; Yusuf et al., 2015; Yusuf et al., 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 *A. m. 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 proportions of 9-HDA production shows that the *A. m. capensis* clones are indeed predisposed 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 *A. m. 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 *A. m. 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 *A. m. 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 *A. m. 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 *A. m. capensis* clones producing significantly smaller amounts of this compound, even though the amount of 10-HDA was relatively high in young the *A. m. capensis* clones. Indeed, young bees from both *A. m. scutellata* and *A. m. capensis* groups produced large proportions of the worker compound 10-HDA, with the *A. m. 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 *A. m. 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 9-HDA (precursor molecule to 9-ODA) 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 *A. m. 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 Figure 2.2). Zheng et al. (2010) and Yusuf et al. (2015) 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 *A. m. capensis* clones and *A. m. scutellata* bees respectively, leading to the majority of the clones being classified as queen-like, and the *A. m. scutellata* as worker-like.

Finally, analysing the expression of the two P450 genes putatively shown to participate in either ω or $\omega-1$ 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 *A. m. 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 *A. m. 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 *A. m. 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 *A. m. capensis* clones had much higher levels of *cyp6as8* and very little *cyp6bd1* while there was no significant difference in the expression of the two genes in the queenless clones. 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 hours 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.

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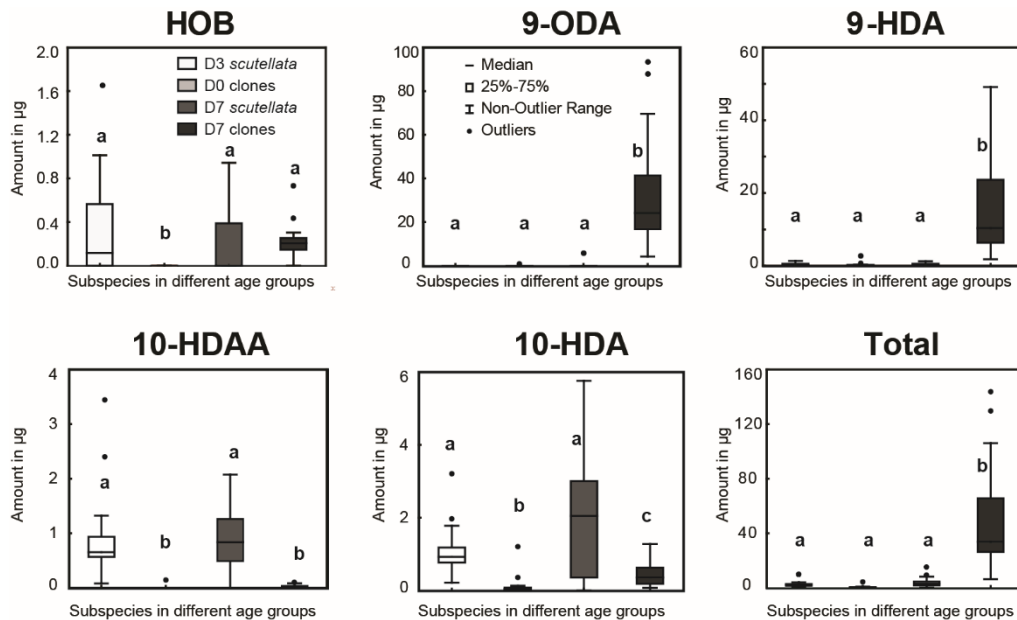
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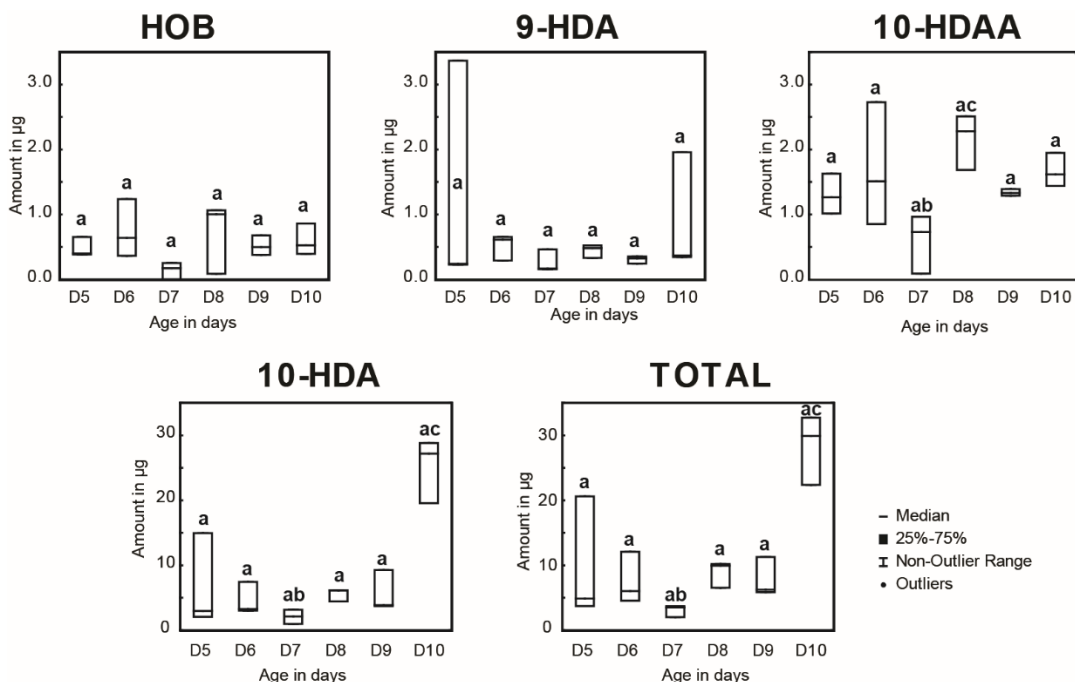
Appendix I

Supplementary Table 2.1: Primers used in qPCR of genes in the mandibular glands responsible for hydroxylation of stearic acid in honey bee mandibular glands

Gene/Primer name	Primers (5' to 3')	Reference
<i>CYP4G11</i>	GGC TGT AAT GAA GAT GTG CGA C GTG CGC TAT TAT CAA TGA TGT TAC G	Mumoki <i>et al.</i> 2018
<i>eIF3-S8</i>	TGA GTG TCT GCT ATG GAT TGC AA TCG CGG CTC GTG GTA AA	Mao <i>et al.</i> 2011
<i>CYP6BD1</i>	CAA ATT CGA AGG TGA TGC ATT GTT G ACA CCT TCA TAA GTC ATT CCA TGT	This study
<i>CYP6AS8</i>	GAC GTA ACA AAT GAA TTC TTG GCA G GGT TTC GTA TCT CCA TTC TCC GTC	This study



Supplementary Figure 2.1: Amounts (µg) of the mandibular gland pheromone components from; day 3 *A. m. scutellata* (open bars), day 0 *A. m. capensis* clones (stip-pled bars), day 7 *A. m. scutellata* workers (grey bars) and day 7 *A. m. capensis* clones (black bars) (— = median, □ = 25%-75%, I = min-max). The letters above the bars denote differences in statistical significance within the groups; KWA H (3, N=78) > 22.57244 p < 0.0001).



Supplementary Figure 2.2: Amounts (in μg) of the mandibular gland pheromone components from queenright *A. m. scutellata* nurse bees from five to ten days old (— = mean, \square = 25-75%, I = min-max, \bullet = outliers). The letters above the bars denote differences in statistical significance within the groups. 9-ODA and HVA were not detected in any of the samples.

CHAPTER 3

Transcriptomic analysis of the mandibular gland genes associated with reproductive dominance in *A. m. capensis* worker facultative parasites[#]

Abstract

Regulation of reproductive labour is generally modulated by the queen's chemical secretions, particularly the mandibular gland (MG) pheromone complex. Workers can, in principle, activate their ovaries and lay unfertilised eggs with some dominant workers being able to produce queen-like mandibular gland signals to become pseudo-queens. In this study, we examined the effect of social context as a regulator of social behaviour, by comparatively investigating differential gene expression in the MG of two subspecies of African honey bees, with differing reproductive potentials and, at two age groups. Transcriptome sequencing of RNA from young and older *Apis mellifera scutellata* (low reproductive potential) and *A. m. capensis* (high reproductive potential) was carried out. Principal Component Analysis of all the genes with a non-zero read count showed that the variation in gene expression was due to the effect of subspecies (PC1; 52.8%) and age group (PC2; 26.6%). A total of 5,646 transcripts were differentially expressed across four libraries, classified into 29 biological processes based on their gene ontology accessions. Further, 48 differentially expressed genes (DEGs) were found to be putatively associated with the caste-specific biosynthesis of MG fatty acids. Of the 48 DEGs, 25 were cytochrome P450s thought to participate in the caste specific hydroxylation of stearic acid, a crucial regulatory point in the biosynthesis pathway. Multiple putative points of regulation were identified starting from the *in situ* biosynthesis and activation of stearic acid, the caste-specific hydroxylation, transport between organelles, uncompleted β -oxidation and finally the oxidation of 9-HDA to 9-ODA. Our data shows that regardless of age, reproductively dominant individuals switch on a very specific set of genes as they transition from worker-like to queen-like mandibular

gland signals. This study provides an insight into molecular-level changes that occur as workers transit from being reproductively inactive to activating their ovaries.

Key words: Reproductive dominance/ Transcriptomics/ pheromone biosynthesis/
Regulation of gene expression/ African honey bees

#This chapter is written as a manuscript for submission to the journal *Insect Molecular Biology*

Introduction

Eusocial insects are characterised by division of reproductive labour between the queens and mostly sterile workers (Winston, 1987). Mediating the distinction between the roles of different female castes are sociochemicals produced by the reproductively dominant queens, producing sterility-inducing signals which have been shown to be highly conserved in the major groups of eusocial insects. Indeed, Van Oystaeyen et al. (2014) showed that reproductive individuals from ants, wasps and certain bees all seem to utilise non-volatile saturated hydrocarbons to advertise fecundity and/or to suppress worker reproduction in their colonies, proposing that these queen signals may have developed from conserved compounds in solitary ancestors. Various components of the sociochemicals produced by the dominant individuals are then combined in multiple species- and caste-specific ways, yielding 'signature mixtures' that elicit specific stereotypic responses in the recipient colony members (Wyatt, 2010).

The *Apis mellifera* queen utilises multi-source secretions to produce both long-term physiological (primer) responses, reviewed by Le Conte and Hefetz (2008) and immediate behavioral (releaser) effects in the workers (Pankiw et al., 1998; Pankiw et al., 1995) and drones (Villar et al., 2015; Villar and Grozinger, 2017) in her colony. Multiple glands are involved in the production of these sociochemicals including the Dufour's gland whose esters are thought to function as egg discriminators and fertility indicators (Katzav-Gozansky et al., 2001). Wossler and Crewe (1999a) showed that secretions of the queen's tergal glands can induce worker sterility and also elicit retinue behavior in caged workers (Wossler and Crewe, 1999b), although

to a lesser extent than that elicited by the mandibular gland pheromones. Most recently, Okosun et al. (2019) demonstrated that tergal secretions of reproductively dominant *A. m. capensis* pseudo-queens can also elicit the same behavioural and physiological effects in subordinate as described for queen tergal glands. The composition, primer and releaser effects of the honey bee queen mandibular gland pheromones (QMP) have indeed been studied widely. QMP has been shown to inhibit ovarian activation (Butler, 1961; Hoover et al., 2003) and the rearing of new queens (Melathopoulos et al., 1996) by workers. In addition, this pheromone complex regulates age-related division of labour among workers (Pankiw et al., 1998) by modulating the expression of critical molecules such as dopamine (Beggs et al., 2007; Beggs and Mercer, 2009) in the workers' brains, thus regulating behaviour (Vergoz et al., 2007).

The *A. mellifera* queen's mandibular gland secretions are mainly composed of five components; the fatty acids 9-oxo-2 (E) - decenoic acid (9-ODA; 'the queen's substance') and its precursor molecules (S, E) + (R, E)-9-hydroxy-2-decenoic acid (9-HDA) and the aromatic compounds methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Crewe and Velthuis, 1980; Plettner et al., 1997; Plettner et al., 1996; Slessor et al., 1990; Yusuf et al., 2015). Worker mandibular gland secretions typically contain trace amounts of 9-HDA and HOB, and copious amounts of the 'worker substance' 10-hydroxy-2-(E)-decenoic acid (10-HDA), and its precursor molecule 10-hydroxydecanoic acid (10-HDAA) (Plettner et al., 1996). However, subspecies-level variations to these pheromone signature mixtures do exist. Workers of African honey bees, for example, are generally known

to produce higher levels of 9-HDA than their European and Asian counterparts (Crewe and Moritz, 1989; Keeling et al., 2001; Yusuf et al., 2015; Zheng et al., 2010).

With the exception of HVA, the aforementioned mandibular gland components have also been identified in other honey bee species, reviewed by Pirk et al. (2011). For instance, the presence of 10-HDAA, 10-HDA, 9-ODA and 9-HDA has been reported in *Apis nigrocincta* and *Apis cerana* queens and workers (Keeling et al., 2001) and the dwarf honey bee *Apis florea* (Keeling et al., 2000). In the latter, 10-HDA (the 'worker substance' in *A. mellifera*) was the main pheromone component in the mandibular glands of mated *A. florea* queens. Very high amounts of 9-ODA have been detected in the open-nesting giant Asiatic honey bee *Apis dorsata*, combined with trace quantities of 9-HDA and 10-HDA (Plettner et al., 1997).

The biosynthesis of the mandibular gland fatty acids takes place in a stepwise process commencing with the acylation of the precursor molecule stearic acid. This is followed by the hydroxylation of the activated stearic acid in a caste-specific bifurcated manner where ω -1 hydroxylation takes place predominantly in queen mandibular glands while ω hydroxylation occurs mostly in worker mandibular glands (Plettner et al., 1996). This hydroxylation step forms a major point of reproductive regulation by the queen as workers have been shown to switch to ω -1 hydroxylation in the absence of the queen (Malka et al., 2009; Malka et al., 2014; Mumoki et al., 2018; Wu et al., 2017). The hydroxylated compounds are then transported to the peroxisome where they undergo uncompleted β -oxidation (Plettner et al., 1998; Reddy and Hashimoto, 2001) leading to formation of 9-HDAA and 9-HDA in queens

and 10-HDAA and 10-HDA in workers. In the queen mandibular glands, 9-HDA is further oxidised to the 'queen substance' 9-ODA, a tightly-regulated step catalysed by the enzyme alcohol dehydrogenase (Malka et al., 2014; Mumoki et al., 2018; Wu et al., 2017).

In this work, we set out to describe the gene expression patterns involved in the biosynthesis of mandibular gland fatty acids in queenless honey bees of two African honey bee subspecies, with differing reproductive potentials; *Apis mellifera capensis* laying parasites (higher reproductive potential) and *A. m. scutellata* workers (lower reproductive potential). The classification based on reproductive potential was founded on the fact that *A. m. capensis* workers are characterised by a high number (>10) of ovarioles per ovary (Hepburn and Crewe, 1991; Ruttner, 1977) and the presence of a well-developed spermatheca (a queen-associated trait) in most (but not all) workers (Phiancharoen et al., 2010; Zheng et al., 2010). In contrast, all *A. m. scutellata* workers lack spermatheca and possess a lower number (<5) of ovarioles per ovary (Hepburn and Crewe, 1991). Further, the typical latency period (time between loss of a queen and start of egg laying by workers) for *A. m. capensis* workers is between 2- 6.5 days (Hemmling et al., 1979; Ruttner and Hesse, 1981), compared to 10-12 days for *A. m. scutellata* (Ruttner and Hesse, 1981). We hypothesise that social environment will have no influence on the pheromone biosynthesis pathways utilised and that regardless of age, *A. m. capensis* social parasites will employ the gene expression pathways typically upregulated in queens, and that the *A. m. scutellata* workers will utilise pathways typically upregulated in workers.

Materials and methods

Colony maintenance and sample collection

Samples of *Apis mellifera scutellata* workers were obtained from colonies with naturally mated queens maintained using standard beekeeping practices at the University of Pretoria apiary. Colonies of *A. m. scutellata* infested with *A. m. capensis* reproductive parasites were donated by beekeepers from Gauteng and Limpopo provinces, South Africa. Infested colonies were kept and maintained in isolation under a restriction tent and terminated at the end of the experiment.

Combs were inspected to locate late-stage (black-eyed) pupae that were ready to emerge. Parts of the comb identified to have these late-stage brood were then cut out and taken to the laboratory, where they were reared in Memmert Incubator Ovens INB200 (Memmert GmbH + Co. KG, 91186 Buchenbach, Germany) at 35°C and 60% relative humidity. Upon emergence, 100 callow *A. m. scutellata* workers (<24 hours old) 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). The cages were then placed in incubators at 34 °C and 60% relative humidity where bees were fed on pollen, 50% (w/v) sucrose solution and water *ad libitum*. Three hoarding cages were set up for each time interval (day 3 and day 10). From each cage, four individuals were randomly selected for mandibular gland and ovarian dissection and RNA isolation. In total 24 individuals (12 per day) were dissected.

For the *A. m. capensis* clone cages, two newly-emerged *A. m. capensis* clones were housed with 50 newly-emerged *A. m. scutellata* workers to form mixed cages. These

combination cages were set up to ensure that the clones had a sufficient number of *A. m. scutellata* workers available to feed them through trophallaxis (Schäfer et al., 2006), ensuring that they receive the protein necessary for oogenesis (Crailsheim, 1991; Korst and Velthuis, 1982). These cages were nourished, incubated and handled in a similar manner to the *A. m. scutellata* cages. Six cages were set up for each time interval. All surviving clones were collected from the cages at the end of 24 hours (day 0 samples) and after ten days (day 10 samples). A total of 22 individuals were dissected (twelve day 0 and ten day 10 samples).

Dissection of mandibular glands and RNA Isolation

After the particular time interval had elapsed, the honey bees were immobilised by freezing and then decapitated. Dissection of mandibular glands was carried out as described by (Mumoki et al., 2018) and guidelines in the Standard methods for *Apis mellifera* anatomy and dissection (Carreck et al., 2013). Upon removal, dissected mandibular glands were quickly put into 1.5 ml Eppendorf tubes and stored at -80°C until used. The thorax and abdomen were immobilised on wax plates containing Insect Ringer pH 7.4 (6.4ml 5M NaCl, 3.75 ml 0.1M CaCl₂, 1.25 ml 1M KCl) for dissection. RNA isolation was carried out using TRIzol[®] Reagent (Invitrogen, Carlsbad 92008, USA) following manufacturer's recommendations and as described by Aumer et al. (2018) and Mumoki et al. (2018). Briefly, 200µl of TRIzol[®] Reagent was used to homogenise pools of 10-12 mandibular glands and 60µl of Chloroform (Merck, Modderfontein, 1645, South Africa) was added and the samples vortexed gently. Samples were then spun using an Eppendorf[™] 5415R micro-centrifuge

(Hamburg, Germany) at 13 000 rpm for 20 minutes at room temperature, creating phase separation. The aqueous phase was removed into a new tube and 84 μ l of ice-cold Isopropanol (Merck, Modderfontein, 1645, South Africa) added. Precipitation was carried out overnight (for about 16 hours). The tubes were again centrifuged at 13 000 rpm for 15 minutes at 4⁰C, washed with 75% ethanol and re-suspended in 40 μ l of ultra-pure water. Samples were treated with DNase 1 (New England Biolabs, Ipswich Massachusetts, USA) to remove genomic DNA contamination.

RNA quantity and quality was measured by determining its absorbance at 260nm and the quality determined using the A260/A280 and A260/A230 ratios, on a NanoDrop™ 1000 (Thermo Scientific Inc. (Wilmington, DE 19810 USA). Samples were stored at -80 degrees before shipping to the Beijing Genomics Institute (BGI) (Hong Kong) for quality control analysis, library construction and transcriptome sequencing.

Quality Control Analysis, Library Construction and Transcriptome Sequencing

QC analyses were carried out using the Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA) and the Qubit™ Fluorometer (Invitrogen, Carlsbad, CA). All samples had quantities of ≥ 0.5 μ g, concentration of ≥ 28 ng/ μ l, RIN (Schroeder et al., 2006) ≥ 7.4 , and 28S/18S ≥ 0.1 (Supplementary materials; Table 1).

cDNA libraries were constructed using the Illumina TRISeq RNA Preparation kit generating 100bp paired ended fragments. Sequencing was carried out using the Illumina HiSeq 4000. Raw reads will be submitted to the sequence read archive.

Assessment of ovary activation and presence of spermatheca

Following the removal of the heads, bodies of the honey bees were pinned on wax molds on Petri dishes and the abdomen ventrally dissected to expose the ovaries and spermatheca. Ovaries were classified into one of 5 stages as described by (Hess, 1942); stage I & II having threadlike ovarioles, III intermediate with early oocyte development, IV & V with clearly developed oocytes (Schäfer et al., 2006; Velthuis, 1970). Presence and absence of spermatheca (a queen-associated trait) was also recorded (Fyg, 1950; Phiancharoen et al., 2010).

Data processing

Quality checks on the raw sequence reads was carried out using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and adaptor sequences trimmed using Trimmomatic version 0.36 (Bolger et al., 2014). Resultant paired reads were mapped on to the most recent honey bee genome annotation Amel_4.5 (Elsik et al., 2014) using the gapped mappers Bowtie2 (Langmead and Salzberg, 2012) and Tophat2 version 2.0.10 (Kim et al., 2013). The resulting bam reads were sorted using SAMtools and the sorted reads counted using HTSeq (Anders et al., 2015). These sorted and counted reads were normalised using the RPKM method (Mortazavi et al., 2008) and analysed for differential expression following the method of Audic and Claverie (1997) and Claverie and Ta (2018), with a False Discovery Rate of ≤ 0.001 . Venn diagrams and heatmaps of the differentially expressed genes were constructed in the R environment (R Core Team, 2017). To analyse the variation within the four libraries, all normalised transcripts with a non-

zero-read count were included in a Principal Component Analysis using the software ClustVis (Metsalu and Vilo, 2015).

Differentially expressed transcripts were used for gene ontology (GO) analysis using DAVID v. 6.8 (Dennis et al., 2003; Huang et al., 2008) and the resultant GO terms classified into the representative molecular, cellular and biological processes using the software CateGORizer (Hu et al., 2008).

Results

Ovary activation and presence of spermatheca

The analysis of ovary activation revealed that all the young bees regardless of subspecies had inactivated ovaries (stage I and II) while more than half of the day 10 *A. m. scutellata* workers also had inactivated ovaries. In contrast, all the clone workers at day 10 had either intermediate (stage III) or activated ovaries (stage IV and V). (Figure 3.1). Overall, there was no significant difference in the ovarian activation stages from the different samples (KWA; $H(4, N=46) = 7.21, p > 0.05$). However, when the samples were grouped into younger (*A. m. scutellata* day 3 and *A. m. capensis* clones day 0) and older honey bees (*A. m. scutellata* and *A. m. capensis* clones day 10), there is a significant difference in the degree of ovarian activation in the two groups; Mann-Whitney M Test (with continuity correction) $U = 31.00, N_{\text{younger}} = 26, N_{\text{older}} = 20, Z\text{-adjusted} = -5.37, p < 0.0001$).

All *A. m. capensis* clones had a spermatheca while none of the *A. m. scutellata* workers did confirming their classification (Phiancharoen et al., 2010).

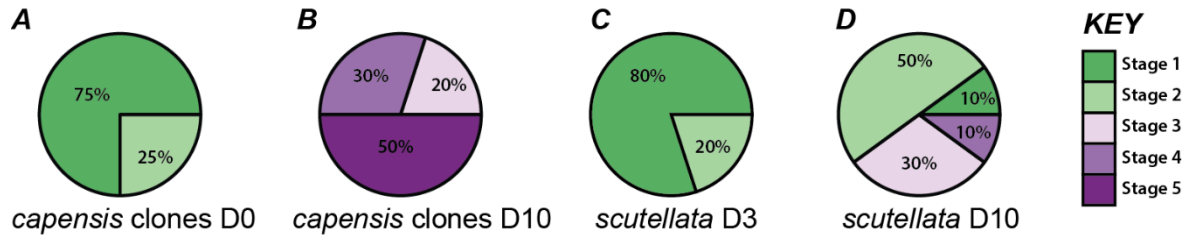


Figure 3.1: Ovarian activation stages in *A. m. capensis* clone workers day 0 (A), *A. m. capensis* clones day 10 (B), queenless *A. m. scutellata* day 3 (C) and *A. m. scutellata* day 10 (D). Ovarian stages are based on the Hess five-point system (Hess, 1942) where Stage I & II = threadlike ovarioles, stage III =intermediate with early oocyte development while stage IV & V = clearly developed oocytes

Transcriptome data output

Read mapping, principal component discrimination and GO classification

The sequenced libraries consisted of on average 57, 372, 204 ($\pm 5, 716, 828$; SD) reads per library. These were cleaned resulting in an average of 53, 320, 707.3 ($\pm 5, 284, 483$; SD) reads which were 92.94 % (± 0.41 ; SD) of the original total number of reads. Of the cleaned reads 44, 377, 669.4 ($\pm 4, 433, 111$; SD) were mapped onto the reference genome forming 83.24% ($\pm 2\%$; SD), and of these 76.65% ($\pm .02\%$; SD) were concordant pairs (Supplementary materials; Table 2). On average, 12, 988 (± 250 ; SD) genes were expressed in the four libraries, representing 84% of the known honey bee genes (Elsik et al., 2014).

All four libraries (*A. m. scutellata* day 3, *A. m. scutellata* day 10, *A. m. capensis* clone day 0, *A. m. capensis* clone day 10) were clearly separated in a principal component

analysis that included all genes from the four libraries with non-zero read counts. The type of subspecies (principal component 1) accounted for 52.8% to the variation in the data set and led to the discrimination of the two subspecies *A. m. scutellata* workers and *A. m. capensis* clones. Age (principal component 2) which accounted for 26.6% of the data variance clearly differentiated the dataset by age into the older (day 10) and younger (day 3 and day 0) groups (Figure 3.2), hence the effect of social environment and physiological development.

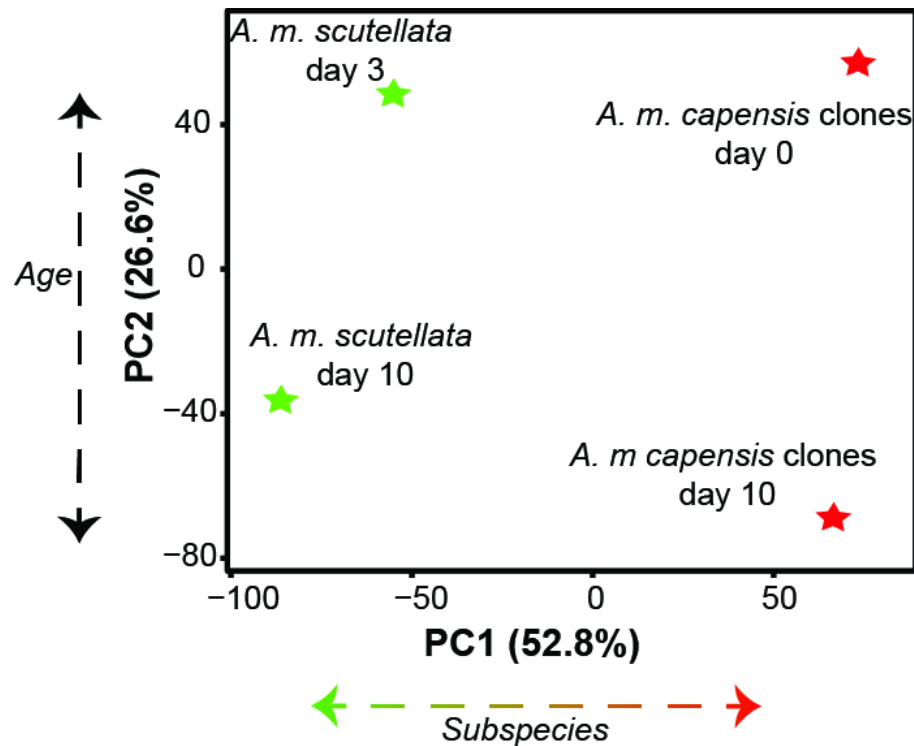


Figure 3.2: Principal component analysis of all mandibular gland transcripts with a non-zero read count were included. PC1 clearly divided the two subspecies of honey bees *Apis mellifera scutellata* and *A. m. capensis* clones while PC2 accounting for 28.3 % of the variation, divided the data into the younger (day 3 and day 0) and older (day 10) samples.

A total of 5,646 transcripts were differentially expressed in the *A. m. capensis* clone day 10 individuals vs *A. m. capensis* clone day 0 individuals, *A. m. capensis* clone day 10 vs *A. m. scutellata* day 10, *A. m. capensis* clone day 0 vs *A. m. scutellata* day 10 and *A. m. capensis* clone day 0 vs *A. m. scutellata* day 0 comparisons. Of these transcripts 83.65% had *Drosophila* orthologues and were thus used in gene ontology (GO) analysis, where 3257 transcripts (68.9%) were utilised. The generated GO accession numbers classified resulted in 29 biological processes that were significantly overrepresented (Table 3.1). The main biological process categories included transporter, binding and catalytic activities.

Table 3.2: Gene Ontology (GO) terms significantly over-represented among all the mandibular gland transcripts from all four libraries ($p < 0.05$)

GO Class ID	GO Term	Number of GO Categories
GO:0008150	Biological process	114
GO:0003674	Molecular function	87
GO:0008152	Metabolism	51
GO:0009987	Cellular process	51
GO:0005215	Transporter activity	35
GO:0005488	Binding	24
GO:0006810	transport	21
GO:0003824	Catalytic activity	21
GO:0043170	macromolecule metabolism	19
GO:0015075	ion transporter activity	17
GO:0050789	regulation of biological process	17
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	15
GO:0009056	catabolism	13
GO:0016787	hydrolase activity	11
GO:0015267	channel or pore class transporter activity	10
GO:0007154	cell communication	9
GO:0050896	response to stimulus	8
GO:0030234	enzyme regulator activity	7
GO:0009058	biosynthesis	5

GO:0016740	transferase activity	5
GO:0016491	oxidoreductase activity	3
GO:0016301	kinase activity	3
GO:0004872	receptor activity	3
GO:0005198	structural molecule activity	2
GO:0016874	ligase activity	1
GO:0004871	signal transducer activity	1
GO:0007610	behaviour	1
GO:0003676	nucleic acid binding	1

A comparative analysis of the significantly expressed genes (DEGs) from the four libraries revealed that there were greater differences in the DEGs when comparing the two subspecies, and fewer genes when the comparison was between age groups in the same subspecies (Figure 3.3 A). This is in agreement with the PCA output outlined earlier. Further, only 11.5% of the DEGs were commonly expressed in all the groups compared (Figure 3.3 B).

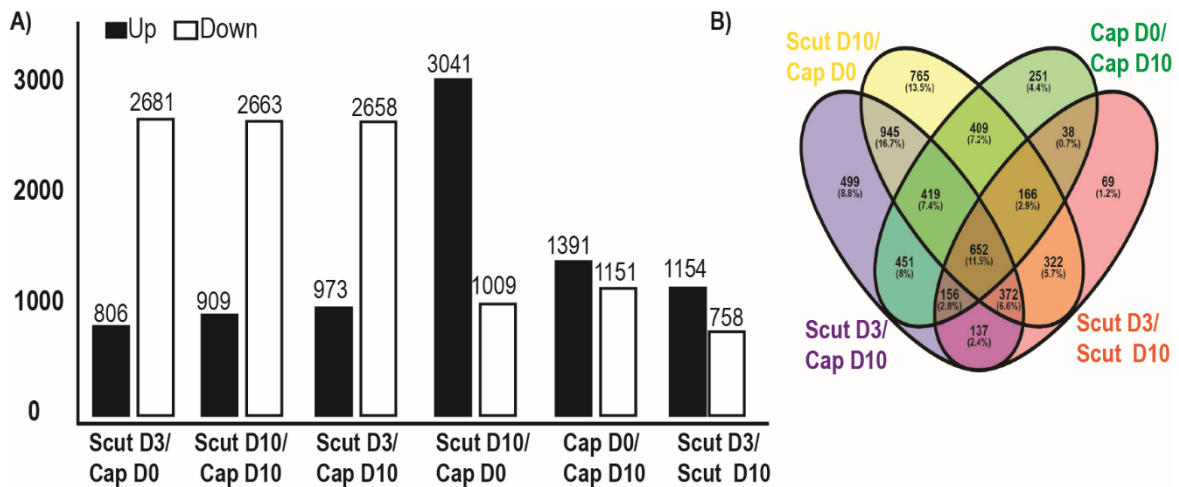


Figure 3.3: Combinations of differentially expressed genes from the four different libraries; *A. m. scutellata* Day 3 (Scut D3), *A. m. scutellata* Day 10 (Scut D10), *A. m. capensis* clone day 0 (Cap D0), *A. m. capensis* clone day 10 (Cap D10) with the up

and down regulated genes (A) and differentially expressed genes are shared among the libraries and ones differentially expressed exclusively within each library (B).

Gene expression patterns

Genes involved in pheromone biosynthesis

Of the differentially expressed genes (Table 3.2), 48 transcripts are involved directly in the biosynthesis of mandibular gland fatty acids. These genes consisted of two Acetyl-CoA synthases (*LOC409624* and *LOC551395*), both relatively upregulated in day 10 clones as compared to *A. m. scutellata* of both age groups and the younger *A. m. capensis* clones. Seven genes putatively associated with the biosynthesis and transport of stearic acid were also identified. Of these, only *LOC551880* was comparatively upregulated in the *A. m. scutellata* workers as compared to five genes in *A. m. capensis* clones. The carrier molecule Acyl CoA binding protein 6 was relatively highly expressed in the older *A. m. capensis* clones as compared to the young *A. m. capensis* clones and both *A. m. scutellata* age groups. This is the protein responsible for transporting the caste-specific hydroxylated molecules into the peroxisome where the uncompleted β -oxidation takes place.

Of the four transcripts encoding enzymes identified as putatively catalysing the uncompleted β -oxidation in the peroxisomes, only the probable peroxisomal acyl-coenzyme A oxidase 1 was comparatively highly expressed in *A. m. scutellata* as compared to *A. m. capensis* clones. Transcripts of the enzyme 3-ketoacyl-CoA thiolase were very relatively highly expressed in both ages of the *A. m. capensis* clones as compared to their *A. m. scutellata* counterparts.

The last step in the biosynthesis of mandibular gland fatty acid is the oxidation of functional groups carried out by various dehydrogenases. Five dehydrogenases were identified as differentially expressed in this study. Of these, only alcohol dehydrogenase class 3 was shown to be preferentially expressed in the mandibular gland tissue of *A. m. scutellata* honey bees. Aldehyde dehydrogenase (*LOC550687*) and 1, 5-anhydro-D-fructose reductase-like were very strongly expressed in the *A. m. capensis* clone tissue as compared to that of the *A. m. scutellata*.

Table 3.2: A summary of identified genes associated with mandibular gland pheromone biosynthesis in the honey bee. The ratios represent log₂ratios (**B/A**) of the RPKM values of the transcripts. Positive ratios mean that **B** is higher than **A** while negative values show that **A** is higher than **B**. Genes encoding Cytochrome P450 enzymes responsible for the caste-selective hydroxylation of acylated stearic acid have not been included.

Step	Gene name	Ortholog	CapD0/ CapD10	ScutD3/ ScutD10	ScutD3/ CapD0	ScutD10/ CapD10
Acetyl CoA biosynthesis	<i>LOC409624</i>	Acetyl-coenzyme A synthetase	1.79	0.84	0.82	1.77
	<i>LOC551395</i>	Acetyl-CoA acetyltransferase	2.92	0.89	0.00	2.36
Stearic acid biosynthesis	<i>LOC412815</i>	Fatty acid synthase	3.36	1.70	0.96	2.62
	<i>ACSF2</i>	Acyl-CoA synthetase family member 2,	0.46	0.00	5.69	6.43
	<i>LOC409515</i>	Long-chain-fatty-acid--CoA ligase 4	0.00	0.30	0.53	0.00
	<i>LOC412541</i>	Long-chain-fatty-acid--CoA ligase 6	-3.82	0.00	2.66	-0.84
	<i>LOC551837</i>	Long-chain-fatty-acid--CoA ligase ACSBG2	-0.39	0.00	0.00	0.00
	<i>LOC551880</i>	Uncharacterized LOC551880	-2.37	0.00	-0.85	-3.13
Carrier molecule	<i>LOC102656914</i>	Acyl CoA binding domain-containing protein 6	1.36	0.00	0.00	1.63
Uncompleted β -oxidation	<i>LOC552757</i>	Probable peroxisomal acyl-coenzyme A oxidase 1	0.00	0.00	-1.51	-1.21
	<i>LOC412020</i>	Peroxisomal acyl-coenzyme A oxidase 3-like	2.07	-0.60	2.99	5.67
	<i>LOC409986</i>	Peroxisomal multifunctional enzyme type 2-like	1.97	0.00	0.92	3.05
	<i>LOC408291</i>	3-ketoacyl-coa thiolase, mitochondrial	0.00	-1.08	4.89	5.97
Oxidation	<i>LOC411202</i>	1,5-anhydro-D-fructose reductase-like	0.00	-1.65	1.81	3.05
	<i>LOC412163</i>	Adose reductase-like	0.00	0.47	2.07	1.49

	<i>LOC551968</i>	Adose reductase	-0.70	-0.69	1.51	1.50
	<i>LOC409773</i>	Alcohol dehydrogenase class-3	0.00	-0.39	-1.32	-1.13
	<i>LOC550687</i>	Aldehyde dehydrogenase	3.36	0.00	3.18	6.58
Related molecules	<i>LOC408564</i>	Long-chain fatty acid transport protein 4-like	0.81	0.58	0.27	0.51
	<i>LOC408920</i>	Long-chain fatty acid transport protein 1	0.00	0.51	-1.83	-2.35
	<i>Fabp</i>	Fatty acid binding protein	1.75	0.00	1.17	2.95
	<i>LOC410034</i>	Fatty acid-binding protein, adipocyte-like	0.65	0.00	0.00	0.61
	<i>Fabp</i>	FABP-like protein (Fabp)	0.00	0.66	-5.30	-5.82

Cytochrome P450s involved in caste-specific hydroxylation of mandibular gland pheromones

Of the 48 transcripts differentially expressed, 25 were orthologues of Cytochrome P450 genes which participate in the caste-specific hydroxylation of the acylated-stearic acid from all the four CYP clades (Figure 3.4).

Eight CYP genes were expressed in an intraspecific manner, discriminating between the *A. m. capensis* clones and the *A. m. scutellata* workers. Three of these were significantly specifically up-regulated only in *A. m. capensis* clones; *LOC724946* (*Cyp6as11*), *LOC551560* (*Cyp6bd1*) and *LOC551626* (*Cyp6s17*) while the following five CYP; P450s *Cyp6as1*, *CYP6as2*, *LOC410492* (*CYP9Q1*), *LOC550965* (*CYP6ar1*) and *LOC 551028* (*Cyp6as13*) were relatively up-regulated in *A. m. scutellata* workers.

Although no transcripts were found to be relatively upregulated in an age-related pattern in both subspecies, *LOC727598* was comparatively highly expressed only in the mandibular gland of young *A. m. scutellata* workers (as compared to their *A. m. capensis* counterparts) while *LOC724175* and *LOC551197* (*Cyp6as15*) were found to be comparatively highly expressed in the mandibular gland tissue of only day 10

A. m. capensis clones. The expression patterns of *LOC724175* and *LOC551197* (*Cyp 6as15*) signify the possibility that these genes are specifically upregulated only in reproductively dominant individuals.

Curiously, two P450s *Cyp6aq1* and *LOC552418* (*Cyp6be1*) were only relatively upregulated in the mandibular glands of young *A. m. scutellata* and old *A. m. capensis* clones.

The dendrogram (Figure 3.4) overlaying the expression patterns revealed that the CYP expression pattern from analysis of the young bees from the two subspecies (ScutD3/Cap D0) closely mirrored that of the combination of the younger and older *A. m. scutellata* workers, while, the expression pattern from the older bees from both sub-species more closely resembles that from the parasitic *A. m. capensis* combination (Cap D0/CapD10).

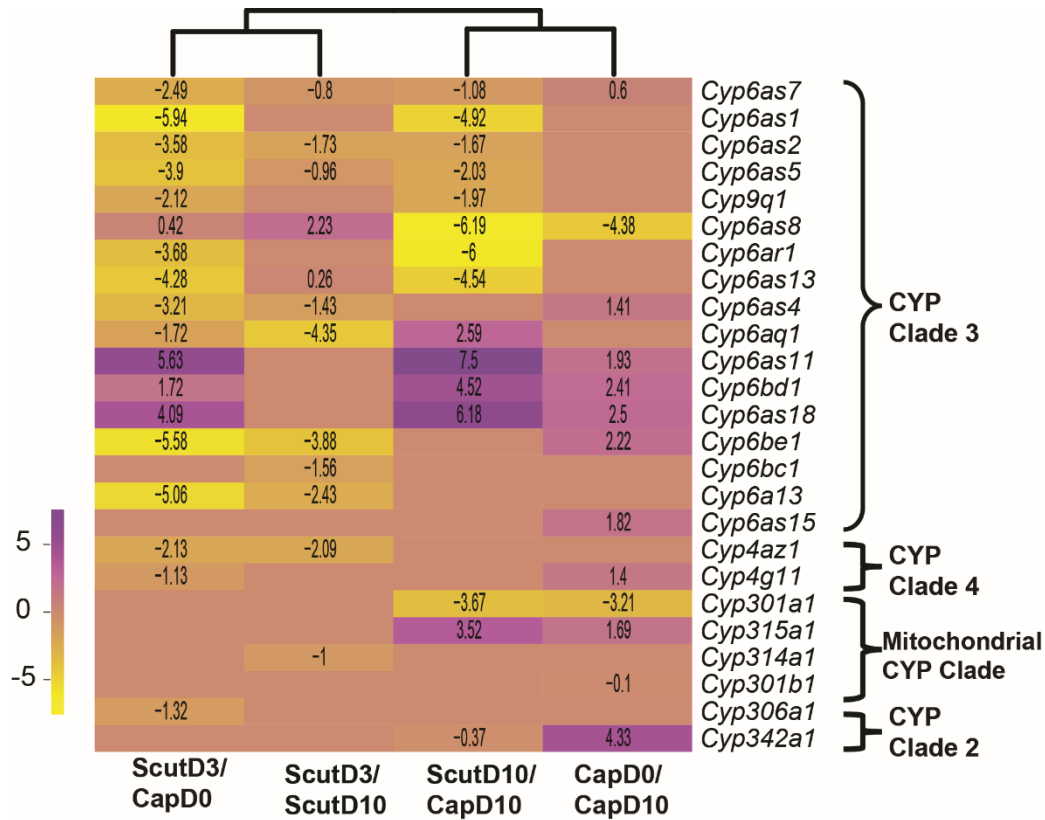


Figure 3.4: Heat Map showing expression patterns of Cytochrome P450 genes. The Log₂Ratios (B/A) were calculated from RPKM values. Positive values show that B was higher than A while negative values show that A was higher than B. Cells with no value given had a Log₂Ratio of 0. The experimental groups are shown below; ScutD3 = *A. m. scutellata* day 3, ScutD10= *A. m. scutellata* day 10, CapD0= *A. m. capensis* clones less than 24 hours old, while CapD10= *A. m. capensis* clones aged 10 days.

Discussion

Pheromone production in social insects is highly plastic and varies with the social environment and age-dependent physiological conditions in which the individuals find themselves. Further still, any changes that individuals go through as a result of alterations in their social environments or physiological states in turn affect the social environment and physiological states of other individuals that interact with them

(Robinson et al., 2008). While the honey bee queen is generally able to regulate reproductive dominance in her workers, this is not always the case. Workers of the Cape honey bee *A. m. capensis* have evolved an exceptional ability to give rise to diploid female offspring through thelytokous parthenogenesis (Hepburn and Crewe, 1991; Ruttner, 1977; Verma and Ruttner, 1983). Thelytoky in the Cape bee was recently shown to be controlled by a single heterozygous dominant locus (*Th*) and that a single SNP at this locus is sufficient to cause *A. m. capensis* to switch from social worker to facultative reproductive parasite (Aumer et al., 2019). The *Th* locus has previously been shown to be responsible for the rapid development of queen-like phenotypes in *A. m. capensis* clones, such as rapid ovarian activation and production of queen-like mandibular gland secretions (Jarosch-Perlow et al., 2018; Jarosch et al., 2011; Lattorff et al., 2007).

We analysed the gene expression patterns of the mandibular glands of *A. m. scutellata* workers and *A. m. capensis* reproductive parasites, at two time points for each subspecies; day 10 (older bees) and day 0 (*A. m. capensis* clones) and 3 (*A. m. scutellata*). Our analyses focused on genes encoding enzymes involved in the biosynthesis of various components of the honey bee mandibular gland pheromone. While the mandibular gland pheromone is a complex made up of fatty acids (9-ODA, 9-HDA, 10-HDAA and 10-HDA) and aromatic compounds (HOB and HVA), our focus was limited to the regulation of the biosynthesis of the fatty acid components.

The biosynthesis of honey bee mandibular gland fatty acids begins with the *in situ* biosynthesis of the precursor molecule stearic acid (Plettner et al., 1998). We identified various genes that may be associated with this synthetic step including

fatty acid synthase, acyl CoA synthases and Long-chain fatty acid CoA ligases (synthases). As these genes were differentially expressed between the clone and *A. m. scutellata* libraries, this might be a key regulatory point in the biosynthetic pathway that the clones are differentially able to express, in their progression to becoming pseudo-queens.

The activated stearic acid is then hydroxylated in a caste-specific manner using monooxygenase Cytochrome P450 enzymes (Plettner et al., 1998; Plettner et al., 1996). Aligned using the Cytochrome P450 Log2 ratios, the clone mandibular gland profile closely resembled the profile of older queenless bees, while that of the *A. m. scutellata* workers closely resembled the profile of young bees. This is evident from the select cytochrome genes that are specifically upregulated in *A. m. scutellata*. The Cytochromes *Cyp6as1*, *Cyp6as2* and *Cyp6as5* have previously been described as upregulated in the mandibular glands of queenright workers of various species of *A. mellifera* (Malka et al., 2009; Malka et al., 2014; Wu et al., 2017). These genes were comparatively upregulated in the mandibular gland tissue of both young and old *A. m. scutellata* workers as compared to their *A. m. capensis* counterparts, possibly catalysing the ω hydroxylation of stearyl-CoA, which typically occurs predominantly in workers. Malka et al. (2014) showed that *Cyp6as2* and *Cyp6as13* were highly expressed in the mandibular glands of virgin queens and queenless workers with undeveloped ovaries with *Cyp9q1* highly expressed in the mandibular glands of queenright workers (Malka et al., 2014; Wu et al., 2017) as compared to those of virgin queens. *Cyp6as4* has been shown to be highly expressed in virgin queen mandibular glands (Malka et al., 2014). In this study, we found this enzyme also

comparatively highly expressed in the mandibular glands of day 10 *A. m. capensis* clones, though to a much lesser extent than in *A. m. scutellata*. No comparative studies have reported significant expression of *Cyp6ar1*. In contrast, *Cyp6as1* was shown to be comparatively highly expressed in the mandibular glands of mated queens (Wu et al., 2017) as compared to those of workers.

Three particular P450s; *Cyp 6as11*, *Cyp6bd1* and *Cyp6s17* were uniquely expressed in *A. m. capensis* clones and not in *A. m. scutellata*. *Cyp 6as11* has been shown to be comparatively highly expressed in the mandibular glands of queenless workers with developed ovaries (Malka et al., 2014) as contrasted to workers with undeveloped ovaries. Further, all three genes have been shown to be relatively highly expressed in the mandibular glands of both virgin queens (Malka et al., 2014) and freely mated 1-year old queens (Wu et al., 2017) as compared to worker mandibular glands. As the transcripts of these enzymes have been shown to be upregulated in virgin queens and queenless workers with activated ovaries (Malka et al., 2014), the presence of these genes may explain the plasticity seen in the synthesis of mandibular gland pheromones, particularly, the ability of queenless workers to synthesise queen-like mandibular gland pheromones. The protein products of these transcripts will enable the reproductive parasites to utilise the ω -1 hydroxylation pathway characteristic of queens.

Expression of *Cyp 304a1* and *Cyp 6as15* is clearly dependent on both age and the reproductive status of the honey bees as these two transcripts were comparatively highly expressed only in the pseudo-queens with activated ovaries as is seen in the day 10 *A. m. capensis* clones. While no comparative data exists for *Cyp 304a1*,

cyp6as15 has been shown to be relatively highly expressed in mandibular gland tissue from mated queens (Wu et al., 2017) as compared to worker mandibular glands. Previous studies have shown that the *A. m. capensis* clones at this age produced a queen-like pheromonal repertoire consisting mainly of the queen-substance 9-ODA and its precursor compound 9-HDA (Mumoki et al., 2019). This strongly suggests that these two transcripts play a significant role in the formation of 9-HDA.

Cyp6aq1 and *LOC552418 (Cyp6be1)* were comparatively upregulated only in the mandibular glands of young *A. m. scutellata* and old *A. m. capensis* clones. The former has been reported to be upregulated in the mandibular tissue of workers (Malka et al., 2014; Wu et al., 2017) while the latter has been reported to be upregulated in the mandibular tissue of mated queens (Wu et al., 2017). The presence of these transcripts in older *A. m. capensis* clones may explain the presence of worker-typical acids such as 10-HDA found in relatively high quantities in the mandibular glands of queenright clones. However, due to the fact that the *A. m. capensis* clones in this work are queenless, and seeing that the amount of worker acids in typical day 10 clones is negligible (Mumoki et al., 2019), this might signify the possibility that the protein product of these genes participate in other enzymatic cascades that contribute to the formation of queen acids. Alternatively, regulation of the synthesis of these enzymes may take place post-transcriptionally.

While no transcripts were found to be upregulated in an age-related pattern in both subspecies, *Cyp6as8* was shown to be relatively upregulated in both young and old *A. m. scutellata* and also in young *A. m. capensis*. This cytochrome gene has been

reported to be significantly upregulated in workers (Malka et al., 2014), specifically queenright workers (Wu et al., 2017), reinforcing the possibility that the protein product of this gene participates in the formation of worker-like mandibular gland fatty acids such as 10-HDAA and 10-HDA and may be under very stringent queen-regulation.

The expression of the carrier molecule Acyl CoA binding domain containing protein 6 was comparatively highly expressed in the mandibular glands of only day 10 *A. m. capensis* clones, suggesting this is a rate-determining step in the biosynthesis of fatty acids.

The differentially hydroxylated molecules are transported into the peroxisome for uncompleted β -oxidation carried out by Acyl CoA oxidase, peroxisomal multifunctional enzymes and 3-ketoacyl-CoA thiolase. While the probable peroxisomal acyl-co enzyme A oxidase was comparatively highly expressed in *A. m. scutellata*, all the other aforementioned enzymes were strongly upregulated in the mandibular glands of *A. m. capensis* clones. Taken together, the strong upregulation of genes controlling rate-limiting steps may explain why the *A. m. capensis* clone produces fatty acid components faster and in quantities similar to those of European honey bee queens.

The final regulatory step in the biosynthesis of mandibular gland fatty acids is the reductive oxidation of the products of uncompleted β -oxidation, such as 10-HDAA to 10-HDA (worker-characteristic) and 9-HDA to the queen substance 9-ODA (queen-characteristic) (Plettner et al., 1998; Plettner et al., 1996), carried out by the multi-

functional ubiquitous alcohol dehydrogenases. This regulation is evidenced by the fact that *alcohol dehydrogenase class 3* was comparatively highly expressed in the mandibular glands of *A. m. scutellata* while 1, 5 -anhydro-D-fructose reductase-like dehydrogenase was highly expressed in the *A. m. capensis* clone mandibular glands, suggesting a possible sub-species-specific regulation of the biosynthetic pathway. Previous studies have also shown that 1,5-anhydro-D-fructose reductase-like enzymes are specifically upregulated in the mandibular glands of queens, with transcripts rarely found in worker glands (Malka et al., 2014). These results clearly show that the *A. m. capensis* clones are utilising biosynthetic pathways characteristic of European honey bee queens.

Conclusion

This study examined the gene expression patterns of enzymes involved in pheromone synthesis of two honey bee subspecies with differing reproductive potentials found in South Africa (Hepburn and Crewe, 1991; Ruttner, 1977). We have identified multiple regulatory points in the mandibular gland fatty acid biosynthetic pathway, suggesting that regulation starts from the biosynthesis of the precursor molecule stearic acid, down to its acylation, hydroxylation and the uncompleted β -oxidation of the hydroxylation products. We have shown that the *A. m. capensis* clones are able to utilise the queen's pathway by increasing the expression of transcripts encoding enzymes characteristic of the queen mandibular glands. Further, we have shown that the relative expression of key queen enzymes is indeed age-dependant in these workers, as exemplified by the unique expression of the

queen-associated transcripts *LOC724175* and *LOC551197*. This study highlights the molecular basis of pheromonal plasticity in honey bee workers and provides an insight into differential gene expression that underlies this plasticity.

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CHAPTER 4

Reproductive parasitism by worker honey bees suppressed by queens through regulation of worker mandibular secretions[#]

Abstract

Social cohesion in social insect colonies can be achieved through the use of chemical signals whose production is caste-specific and regulated by social contexts. In honey bees, queen mandibular gland pheromones (QMP) maintain reproductive dominance by inhibiting ovary activation and production of queen-like mandibular gland signals in workers. We investigated whether honey bee queens can control reproductively active workers of the intraspecific social parasite *Apis mellifera capensis*, parasitising *A. m. scutellata* host colonies. Our results show that the queen's QMP suppresses ovarian activation and inhibits the production of QMP pheromone signals by the parasitic workers, achieved through differential expression of enzymes involved in the biosynthesis of these pheromones at two points in the biosynthetic pathway. This is the first report showing that honey bee queens can regulate reproduction in intraspecific social parasites and deepens our understanding of the molecular mechanisms involved in the regulation of worker reproduction in social insects.

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Introduction

Division of labour is one of the key characteristics of social insects, where the reproductive role is carried out by the queen who mates with males and lays the overwhelming majority of eggs, while workers carry out the routine tasks in the colony. This strict colony organisation, of which honey bees are an example, is maintained by use of chemical communication, with caste-related tasks and reproductive development mostly controlled by means of pheromones produced by the queen and brood (Pirk et al., 2013; Winston, 1987). While the reproductive role of the queen in most honey bee colonies is firmly maintained, especially in European subspecies of the Western honey bee (*Apis mellifera* L.), it is more complex in others, such as the African subspecies where the majority of the colonies are not kept in apiaries (Pirk et al., 2017). Workers are, in principle, able to activate their ovaries and lay unfertilised eggs that normally become drones (Crozier, 1975). The contribution of worker reproduction is rather small in the European honey bee (Visscher, 1996) but quite significant in African honey bees (Moritz et al., 1998), where many colony members evade the queen-induced sterility, activate their ovaries and lay unfertilised eggs that become drones (Crozier, 1975).

At the extreme end of this evasion is the Cape honey bee (*Apis mellifera capensis* Eschscholtz 1822). Workers of this subspecies have evolved an exceptional ability to give rise to female offspring through the process of thelytokous parthenogenesis (Hepburn and Crewe, 1991; Onions, 1912; Ruttner, 1977), a trait shown to be under the control of a single recessive locus (*thelytoky*, *th*) which segregates in a Mendelian fashion (Aumer et al., 2017; Lattorff et al., 2005). This locus (*th*) has also

been shown to influence the development of certain queen-like phenotypes in the *A. m. capensis* laying workers including rapid ovary activation and queen pheromone synthesis (Hemmling et al., 1979; Jarosch et al., 2011; Lattorff et al., 2007; Ruttner and Hesse, 1981), enabling these (morphologically) worker honey bees to develop into false queens. Through a short-sighted evolutionary selection process (Moritz et al., 2008), a specific invasive lineage of *A. m. capensis* workers has developed into a facultative reproductive parasite (Dietemann et al., 2007; Dietemann et al., 2006; Hepburn and Allsopp, 1994; Lundie, 1954) of *A. m. scutellata* (Lepelletier 1836) colonies (Härtel et al., 2006; Onions, 1912; Pirk et al., 2014; Woyke, 1995). As facultative parasites, *A. m. capensis* workers do not reproduce while in the presence of their own queen (Härtel et al., 2006), but they actively seek out and gain entry into susceptible host colonies (e.g., queenless hosts)(Neumann et al., 2001), where they produce a queen-like pheromonal bouquet (Moritz et al., 2004; Okosun et al., 2015; Sole et al., 2002; Zheng et al., 2010) and activate their ovaries (therefore establishing themselves as false queens) (Hepburn, 1992; Martin et al., 2002; Neumann and Hepburn, 2002). In host colonies of honey bee subspecies other than *A. m. capensis*, a pheromonal contest between the host queen and the false queens ensues, usually leading to the death of the queen (Moritz et al., 2000). The clonal workers (=clones) then proceed to take over the reproductive role in the colony, leading to its eventual collapse. This is what has come to be known as the ‘*capensis* problem’ (Allsopp, 1993; Baudry et al., 2004; Dietemann et al., 2007; Dietemann et al., 2006; Dietemann et al., 2009; Zheng et al., 2010). While we know that some host queens survive clone infestation, the specific conditions that favour host queen survival are still under investigation. In this work, we examine the effect that the

presence of a host queen has on the development of dominance in the infesting *A. m. capensis* workers.

The queen's pheromones are produced in various glands, with the mandibular glands producing one of the best described insect pheromone complexes; the queen mandibular gland pheromone (QMP) complex. QMP is part of a nine-compound pheromone blend that attracts workers to attend the queen (Butler, 1961; Crewe and Velthuis, 1980; Keeling et al., 2003; Slessor et al., 2005) and is composed of: methyl *p*-hydroxybenzoate (HOB), 9-oxo-2 (E)-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), (*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). Functions of the QMP in the social organisation of the colony include: inhibiting worker ovary activation (Butler, 1959; Hoover et al., 2003), preventing rearing of new queens (Butler, 1961; Melathopoulos et al., 1996), influencing age-related division of labour in worker bees by delaying honey bee behavioural maturation (Pankiw et al., 1998), and controlling development of various physiological systems of young bees (Morgan et al., 1998). These functions have been well reviewed (Le Conte and Hefetz, 2008; Slessor et al., 2005; Winston and Slessor, 1998).

Biosynthesis of the main components of the mandibular gland pheromones takes place in a caste-specific manner where stearic acid (octadecanoic acid), the principal starting material for queen substance, is converted into the main mandibular gland pheromone component through a bifurcated three-step process (Plettner et al.,

1996) (Figure 4.1). The first step is the hydroxylation (functionalisation) of stearic acid in either the ω or $\omega-1$ positions (caste biased), followed by the chain shortening of the 18- and 17-hydroxystearic acids through β -oxidation. The final step is the oxidation of the ω and the $\omega-1$ hydroxy group to give diacids and keto acids. The mandibular gland pheromones of honey bee workers have high amounts of 10-HDA and its precursor 10-HDAA, followed by HOB, 9-ODA and 9-HDA (Plettner et al., 1997; Yusuf et al., 2015). In contrast, queen mandibular gland pheromones have high amounts of 9-ODA, R and S 9-HDA, HOB and HVA, in this order, with 9-ODA being the predominant component (Slessor et al., 1990) (Figure 4.1). This caste specific bifurcated pathway is achieved by the differential expression of various genes thought to participate in the biosynthesis of honey bee mandibular gland pheromones (Le Conte and Hefetz, 2008; Malka et al., 2009; Malka et al., 2014).

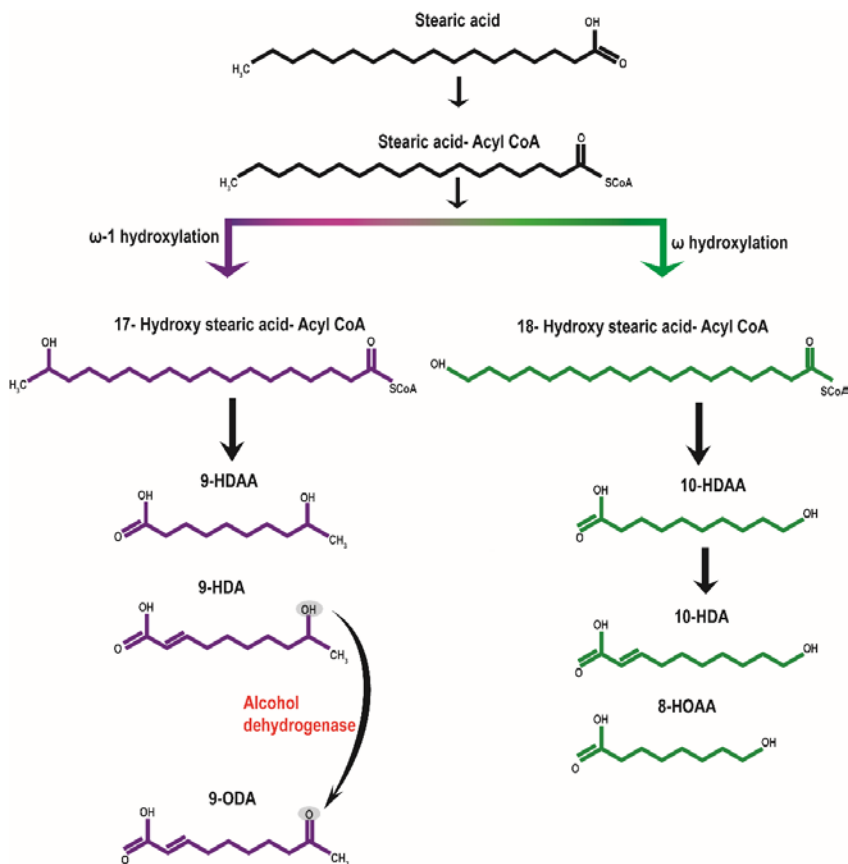


Figure 4.1: A schematic representation of the biosynthetic pathway of the main mandibular gland fatty acids in honey bee queens (ω -1 hydroxylation; purple) and queen right workers (ω hydroxylation; green). The oxidative reduction of 9-HDA to 9-ODA is catalysed by the enzyme alcohol dehydrogenase.

There is great plasticity in the production and expression of pheromones in various groups of social insects (Le Conte and Hefetz, 2008; Slessor et al., 2005; Vander Meer et al., 1998). The variations are based on social environment; presence of brood, presence or absence of the queen (Katzav-Gozansky et al., 2004; Katzav-Gozansky et al., 1997; Winston, 1987) and physiological development; age (Okosun et al., 2015) and mating (Slessor et al., 1990). In honey bees for instance, the presence or absence of the queen (queenright or queenless colonies, respectively)

has been shown to greatly affect pheromone expression in the worker bees from these hives (workers from queenright colonies = QR workers and those from queenless colonies = QL workers) (Katzav-Gozansky et al., 2004; Katzav-Gozansky et al., 1997; Winston, 1987). The mandibular glands of typical queenright *A. mellifera* workers tend to have high amounts of 10-HDA and 10-HDAA (Crewe and Velthuis, 1980; Hepburn and Radloff, 1996; Yusuf et al., 2015; Zheng et al., 2010). In the absence of the queen, their queenless counterparts employ the molecular pathways ordinarily utilised by queens in production of 9-ODA through the conversion of its precursor 9-HDA into 9-ODA (Jarosch et al., 2011; Malka et al., 2009; Malka et al., 2014). This reductive-oxidation of 9-HDA to 9-ODA is catalysed by the enzyme alcohol dehydrogenase (ADH) (Malka et al., 2014; Wu et al., 2017). This group of enzymes (Alcohol dehydrogenases) are ubiquitous to almost all groups of living organisms, from Archaea (Vitale et al., 2010), yeast and bacteria (Reid and Fewson, 1994) to almost all eukaryotes (Duester et al., 1999). ADHs perform a wide range of functions including biosynthesis and degradation of beneficial insect metabolites such as pheromones (Vogt, 2005; Zhang et al., 2014) to the breakdown of toxins such as the oxidative metabolism of ethanol in the mammalian liver (Lands, 1998). *Adh* transcripts have been identified in the honey bee mandibular glands where the enzyme is thought to catalyse pheromone biosynthetic reactions (Malka et al., 2014; Wu et al., 2017) in the gut microbiome where it participates in fermentation (Lee et al., 2015) and fat bodies where it participates in various cellular metabolism processes (Chan et al., 2011). In the mandibular glands, the expression of ADH has been shown to be higher in queens as compared to workers from both queenright and queenless colonies (Malka et al., 2014; Wu et al., 2017). Production of

pheromone components typically found in queen mandibular glands allows workers to become egg-layers and regulate the reproductive capacity of their nest mates.

Here, we examined the effect that social environment has on the expression of reproductive dominance in *A. m. capensis* parasitic workers by recording the pheromone profiles and ovarian activation of field-collected parasitic workers obtained from queenright and queenless colonies. Further, we examined the levels of transcription of ADH, the enzyme responsible for the reductive-oxidation of 9-HDA to the 'queen substance' 9-ODA. Bearing in mind the high colony losses experienced annually by South African beekeepers due to the '*capensis* problem' (Pirk et al., 2014), we hypothesised that, the development of reproductive parasitism by the *A. m. capensis* parasitic workers would be independent of the social environment of the host colonies, specifically presence or absence of *A. m. scutellata* queens. We predicted that that all parasitic workers, regardless of the social environment of the host colony, would produce queen-like mandibular gland pheromones.

Materials and Methods

Experimental Procedures

Honey bee samples

Colonies of *A. m. scutellata* honey bees infested by *A. m. capensis* clones were donated by local beekeepers from Gauteng and Limpopo provinces of South Africa. These were kept in quarantine in a restriction tent to stop them from flying freely and potentially infesting other colonies. They were then managed throughout the experimental period using standard beekeeping procedures (Williams et al., 2013).

Colonies were inspected for the presence or absence of a queen; either by visually searching and locating the queen or by looking for recent queen-laid eggs in unsealed brood cells which signifies the presence of a queen. Adult *A. m. capensis* clone workers (=clones) (tentatively identified as black bees among the typical yellow-black *A. m. scutellata* workers) were aspirated into collection jars from the frames of the infested colonies. Clones collected from queenright *A. m. scutellata* colonies were referred to as QR clones while those from queenless colonies were termed QL clones. The collected clones were then frozen and the heads removed for both pheromone analysis using gas chromatography and gene expression studies, while the abdomens were dissected for assessment of ovary activation and presence or absence of spermatheca. Presence of the spermatheca (a queen-associated trait) was used as a distinguishing feature between the *A. m. capensis* clones and *A. m. scutellata* bees (Hepburn and Crewe, 1991; Okosun et al., 2015). A total of 112 clones were examined in this study, 60 from *A. m. scutellata* colonies without queens and 52 from queen-right colonies. Of these, 48 individuals (24 from each group) were used for the gene expression analysis while a total of 64 individuals (28 QR and 36 QL) were used for the pheromone analysis.

Dissection and extraction of mandibular glands

The honey bees were immobilised by freezing at -20 °C. The heads were then removed and put on ice while the thorax and abdomen placed on wax plates containing Insect Ringer pH 7.4 (6.4 mL 5 M NaCl, 3.75 mL 0.1 M CaCl₂, 1.25 mL 1 M KCL and 1L ddH₂O) to facilitate abdominal dissections. For gene expression analysis, mandibular gland dissection was done as described by (Carreck et al.,

2013) and the removed glands placed in an Eppendorf Tube[®] (Hamburg, Germany) containing 200 μ L of TRIzol[®] Reagent (Invitrogen, Carlsbad 92008, USA) and stored at -80 °C awaiting RNA isolation. For GC analyses heads were placed in a glass vial containing 200 μ L of dichloromethane, DCM (HPLC grade, Sigma-Aldrich, Chemie GmbH München, Germany) and extracted for at least 24 hours.

Assessment of ovary activation and presence of spermatheca

Abdominal dissection was carried out using standard techniques to expose the ovaries and spermatheca (Carreck et al., 2013). Ovaries were classified into one of five 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 (Okosun et al., 2015; Schäfer et al., 2006; Velthuis, 1970; Zheng et al., 2010). Presence of spermathecae (a queen-associated trait) was also recorded (Phiancharoen et al., 2010).

Gas Chromatographic analysis of mandibular gland pheromones

Heads were extracted in 200 μ L of DCM at -20 °C for at least 24 hours. Before the start of the chromatographic analysis, the 200 μ L head extract was divided into two, with 100 μ L stored as backup, should there be need for further confirmation or analyses. The other 100 μ L was evaporated to dryness under a steady stream of charcoal-filtered nitrogen and GC analysis carried out using the procedures reported in (Dietemann et al., 2006; Simon et al., 2001; Yusuf et al., 2015) with slight modifications as follows: the residues were re-dissolved in 10 μ L of internal standard solution (~1 mg octanoic acid and ~1 mg tetradecane in 4 mL DCM). To this, 10 μ L of bis-(trimethylsilyl) trifluoroacetamide, BSTFA (Sigma-Aldrich, Chemie GmbH

München, Germany) was added to derivatise the fatty acids. Separation of the mandibular gland pheromones was carried out using an Agilent 6890N Gas Chromatograph (GC), in the split-less mode on a methyl silicone coated fused silica column (HP-1MS, 25 m × 0.20 mm × 0.33 µm). Helium with a constant flow rate of 1 mL per minute was used as a carrier gas. The temperature of the oven was programmed at 60 °C for 1 min, then increased to 100 °C at 50 °C per min and to 220 °C at a rate of 3 °C minute. This final temperature was maintained for 10 minutes. Identification of the constituent pheromones was based on comparisons of the retention times of the analytes with those of known synthetic mandibular gland pheromone standards. Whilst quantification was achieved relative to the mass ratios of the internal standard mixture.

Classification of pheromone profiles into queen or worker pheromone signals

To assess how queen-like or worker-like mandibular gland profiles from the QL and QR clones were, ratios of the amount of the queen substance (9-ODA) to those of the worker component (10-HDA) were computed as follows: $9\text{-ODA} / (9\text{-ODA} + 10\text{-HDA})$. Where a ratio of <0.5 was classified as worker-like, $>0.5 \leq 0.7$ considered to be intermediate and $>0.8 - 1.0$ queen-like (Dietemann et al., 2006; Schäfer et al., 2006)

Expression of the alcohol dehydrogenase (Adh) gene

Primer synthesis

The honey bee alcohol dehydrogenase (*Adh*) coding sequence was obtained from GenBank (accession number GB44209 or BeeBase accession number GB15375) and primers flanking the coding region designed using Primer3 Plus Software (www.primer3plus.com) and by manual curation. The sequence of the primers designed were: F; 5` GCT TCC TGC TGT AGG AAA TAG AGC 3` and R; 5` CTT GTT TCT CCA TTT CGG CCC 3`. The *Adh* mRNA is made up of 4 exons and the primer set designed here spans the end of exon 2 and extends to half of exon 3, amplifying a fragment that is 218 bp long. To test the designed primers, cDNA from *A. m. capensis* was used in PCR amplification using the gene-specific primers and the amplicons purified and then sequenced. The identity of the gene region amplified was confirmed through homology searching against the GenBank repository where it was subsequently deposited with the accession number MF144184.

Cyp4g11 was used as the endogenous control as it was found to be constitutively expressed in the mandibular glands of queens and also of workers from queen-less and queen-right colonies (Malka et al., 2009). The primer sequence for this fragment was: F; 5` GGC TGT AAT GAA GAT GTG CGA C 3` and R; 5` GTG CGC TAT TAT CAA TGA TGT TAC G 3`.

RNA isolation and purification

RNA isolation from the mandibular glands was done as follows; pairs of glands were homogenised in 200 µL of TRIzol® Reagent (Invitrogen, Carlsbad 92008, USA).

Chloroform and Isoamyl Alcohol (Merck KGaA, Darmstadt, Germany) were used to achieve phase separation, after which the aqueous phase was removed and to it 180 μ L of ice-cold isopropanol added to precipitate the RNA at -80 °C overnight. Washes were carried out using 85% molecular grade Ethanol (Merck KGaA Darmstadt, Germany) and RNA re-suspended in 40 μ L of nuclease-free water.

Degradation of any co-precipitated DNA was carried out using the DNase I kit (Invitrogen, Carlsbad 92008, USA) following manufacturer's instructions. The quality and quantity of the resultant DNA-free RNA was checked using a Nanodrop 2000 (Waltham Massachusetts, USA). Sets (n = 3; biological replicates) of mandibular gland RNA pooled from eight individuals were made and the RNA for cDNA synthesis normalised to 300 ng.

cDNA synthesis and qPCR

cDNA synthesis was carried out using the Superscript IV cDNA synthesis kit (Invitrogen, Carlsbad 92008, USA), using 18-mer oligo dT primers (Thermo Scientific). The following protocol was used; 1x SSIV Buffer, 2.5 μ M Oligo dT, 0.5 mM dNTP mix, 5 mM dTT, 2 U/ μ L RNaseOUT™ Rnase Inhibitor, 2 U/ μ L of SSIV reverse transcriptase and water to top up to 20 μ L. The thermocycler regimen consisted of 65 °C for 5 min (primer-template mix) then incubated on ice for 1 min after which the dNTPs, Oligo dT, Rnase Inhibitor and reverse transcriptase were added. The thermocycler program used for the cDNA synthesis was 23 °C for 10 min, 52 °C for 10 min, 80 °C for 10 min and 4 °C to hold.

Quantitative PCR was done using the PowerUP qPCR kit (Applied Biosystems, Foster City, California, USA) using the LightCycler® 1.5 Instrument II Real Time PCR thermocycler (Roche, Basel Switzerland) in a 20 µL reaction volume with 1X PowerUP SYBR mix, 10 pmoles/µL of each primer, 3 µL of the cDNA template and water to top up to 20 µL. The thermocycler regimen used was as followed; 95 °C for 2 min for enzyme denaturation, 55 cycles of 95 °C for 15 seconds, and 60 °C for 30 seconds (fluorescence collected), followed by a standard dissociation program.

Statistical and data analyses

Normality was tested using the Shapiro-Wilk test. Due to non-normal distribution of the honey bee mandibular gland profiles non-parametric tests were used for all downstream statistical analyses (Pirk et al., 2013).

Mann Whitney U test was used to assess the difference in ovary activation and expression of each of the constituents of the mandibular gland pheromones. Mann Whitney U was also used to carry out pairwise comparisons of the pheromone expression levels for each of the six components in both QR (in the presence of the queen) and QL (absence of the queen) social conditions, and to assess whether there are any overall significant differences in the pheromone ratios between QR and QL clones. Kruskal-Wallis test was carried out to assess the differences in total amounts for each of the MG pheromone components in QR and QL clones. Statistical significance was set at $\alpha < 0.05$.

For the gene expression analyses, homogeneity in the amplification of the genes was analysed by examining the melt curves of the amplified genes. Standard curves were constructed by assessing the amplification trends of cDNA for the target and standard genes, covering 100-fold dilution concentrations. The mean normalised expression values of each target gene were calculated by comparing its threshold cycle (C_p) against those of the reference genes, as described for the $2^{-\Delta C_p}$ method (Livak and Schmittgen, 2001), where $\Delta C_p = \text{the } C_p \text{ of } Adh - C_p \text{ of } Cyp4g11$ and fold change in the expression of *Adh* in clones from QL and QR *A. m. scutellata* colonies calculated as $2^{-\Delta C_p} (\text{QL Clones}) / 2^{-\Delta C_p} (\text{QR Clones})$. Differences in gene expression were inferred from non-overlapping standard error.

All statistical analyses were carried out in R Environment version 3.4.0 (Team, 2015).

Results

Ovarian activation status and presence of spermatheca

All (100%) *A. m. capensis* laying workers collected from both queenright (QR) and queenless (QL) colonies showed the presence of a spermatheca.

Ovarian activation differed (MWU, $U=1.50$, $N_{QR}=28$, $N_{QL}=36$, $p<0.0001$) between clones collected from the QR and QL colonies (Figure 4.2). Clones from QR colonies only had stage I, II and III ovaries (Figure 4.2 a). As only stage III, IV and V are considered to be activated ovaries, only 10.71% of the QR clones had activated ovaries. In contrast, clones collected from QL colonies displayed stage III, IV and V

ovaries (Figure 4.2 b). Therefore, all the clones from QL colonies had activated ovaries with 86.11% having fully developed oocytes in their ovarioles.

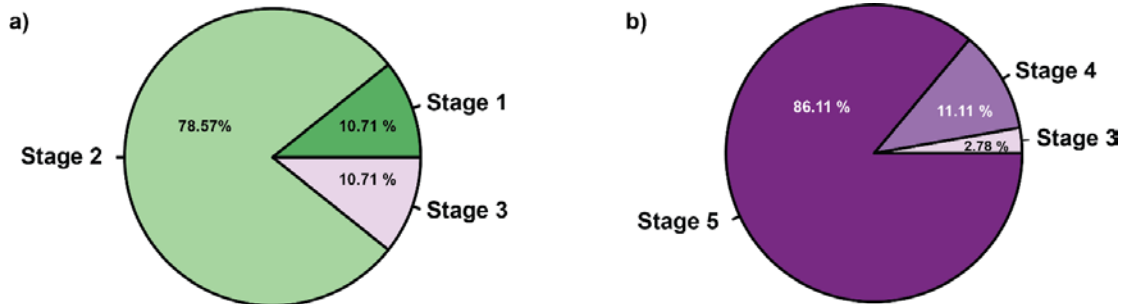


Figure 4.2: Ovary activation status for field-collected *Apis mellifera capensis* parasitic workers from queen-right (QR) (a) and queen-less (QL) (b) *A. m. scutellata* colonies. Stage I & II = threadlike ovarioles, III = intermediate with early oocyte development, IV & V = clearly developed oocytes.

Variation in pheromone profiles of A. m. capensis parasitic workers from QL and QR A. m. scutellata colonies

Mandibular gland pheromone profiles of clones from queen-right (QR) and queen-less (QL) colonies of *A. m. scutellata* differed significantly. In QL clones, the queen substance 9-ODA was the most abundant fatty acid component making up 66.18 ± 1.64 % of the mandibular gland contents, followed by its precursor 9-HDA (25.46 ± 1.77 %). This was in contrast to the profile of QR clones where the most abundant components were 9-HDA (63.64 ± 3.00 %), 10-HDA (15.34 ± 2.11 %) and 9-ODA (8.08 ± 2.26 %), respectively (Figure 4.3).

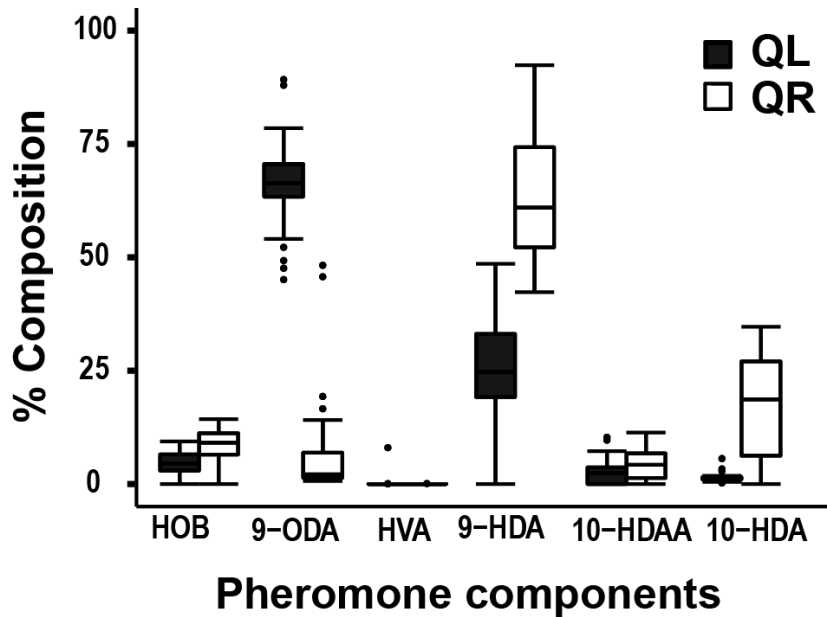


Figure 4.3: Percentage composition of the six mandibular gland components (HOB, 9-ODA, HVA, 9-HDA, 10-HDAA and 10-HDA) of field-collected *Apis mellifera capensis* clones from queenless (closed bars) and queenright (open bars) *A. m. scutellata* colonies (— = mean, \square = 25-75%, I = min-max, \bullet = outliers).

Comparative analyses of the quantities of the six different components in the QR and QL social condition revealed that there were significant differences in the expression patterns of the pheromones given different social conditions (Figure 4.4). The mean total amount of fatty acids produced were significantly higher in QL clones ($163.53 \pm 17.71 \mu\text{g}$) as compared to QR clones ($91.71 \pm 11.89 \mu\text{g}$), (Mann-Whitney U Test (w/ continuity correction; Z-adjusted=-2.686 $N_{QR}=28$; $N_{QL}=36$, $p=0.006$). The mean amount of 9-ODA was significantly higher (MWU; Z-adjusted=-6.38, $N_{QR}=28$, $N_{QL}=36$, $p<0.0001$) in the QL clones than in the QR clones, while both the amounts of 9-HDA (MWU: $U=358.00$, Z-adjusted=1.969, $N_{QR}=28$, $N_{QL}=36$, $p=0.048$) and 10-HDA (MWU: Z-adjusted=3.98, $N_{QR}=28$, $N_{QL}=36$, $p=0.0004$) were significantly lower in the QL Clones as compared to QR clones. There was no significant difference in

the mean amounts of HOB (MWU: Z-adjusted=3.98, $N_{QR}=28$, $N_{QL}=36$, $p=0.3853$), HVA (MWU: Z-adjusted=-0.311, $N_{QR}=28$, $N_{QL}=36$, $p=0.7521$) and 10-HDAA (MWU: Z-adjusted=0.237, $N_{QR}=28$, $N_{QL}=36$, $p=0.8141$) in workers from the two different social conditions.

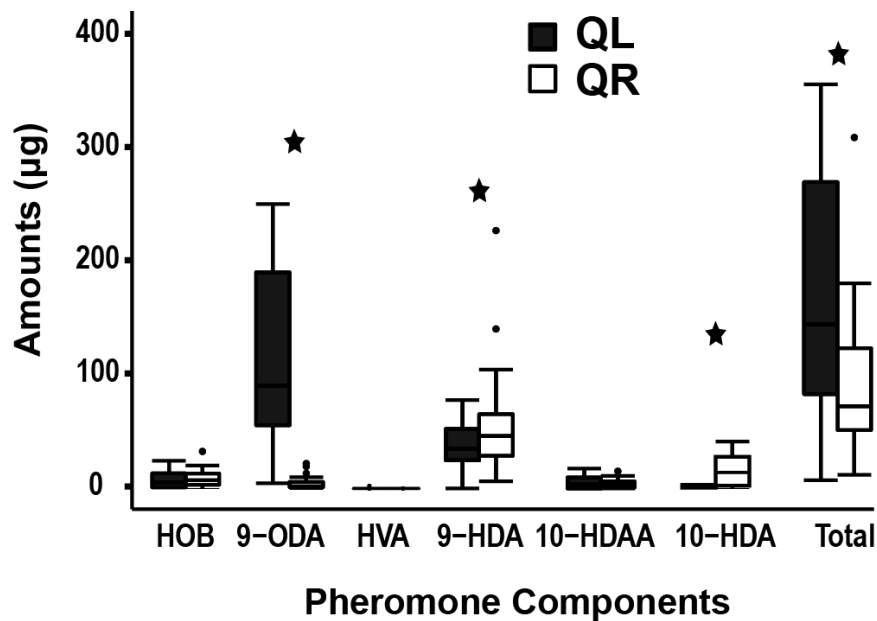


Figure 4.4: Amounts of six mandibular gland components (HOB, 9-ODA, HVA, 9-HDA, 10-HDAA and 10-HDA) of field-collected *Apis mellifera capensis* clones from queenless (closed bars) and queenright (open bars) *A. m. scutellata* colonies (— = mean, □ = 25-75%, I = min-max, • = outliers, * $p \leq 0.05$ indicates statistical significance).

Regardless of whether the clones were from QR or QL colonies, there were significant differences between individuals with activated (AO) and inactivated ovaries (IO), for the expression of 9-ODA (MWU, $U=60.5$, $N_{AO}=25$, $N_{IO}=39$ $P < 0.0001$), 9-HDA (MWU, $U=282$, $N_{AO}=25$, $N_{IO}=39$, $P=0.004$) and 10-HDA (MWU, $U=135$, $N_{AO}=25$, $N_{IO}=39$, $p < 0.0001$) but no significant differences in the expression of HOB, HVA, 10-HDAA or the total pheromone amounts for these honey bees.

Classification of pheromone profiles into queen or worker signals

Pheromone profiles produced by individuals were classified based on the ratio of the major components. The ratio 9-ODA / (9-ODA+10-HDA) revealed that pheromone profiles of the QR parasitic *A. m. capensis* workers were mostly worker-like (0.17 ± 0.23) while their QL counterparts (0.98 ± 0.02) had only queen-like profiles (Figure 4.5). The variation in pheromone ratios was significantly different (MWU, $U=44.00$, $N_{QR}=28$, $N_{QL}=36$, $p<0.0001$).

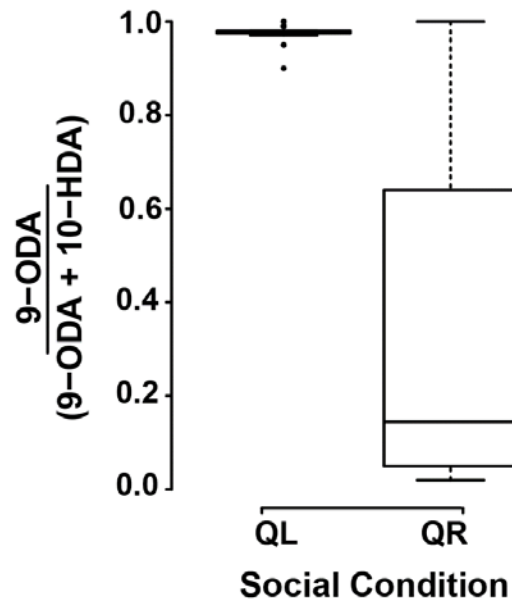


Figure 4.5: The pheromone component ratios in parasitic *A. m. capensis* workers collected from queen-less (QL) and queen-right (QR) colonies of *A.m. scutellata*. Error bars are SD from the means. A queen-like signal has a ratio of $>0.8-1$, and a worker-like signal has a ratio of < 0.5 .

Expression of alcohol dehydrogenase (ADH) transcripts

There were approximately five times as many transcripts of alcohol dehydrogenase in QL *A. m. capensis* parasitic workers as compared to those of the QR workers, showing that the higher levels of alcohol dehydrogenase in dominant workers occurred in the absence of the influence of regulatory pheromones from a queen. There was no significant difference in the *Cyp4g11* (endogenous control) in either QL or QR workers (Figure 4.6).

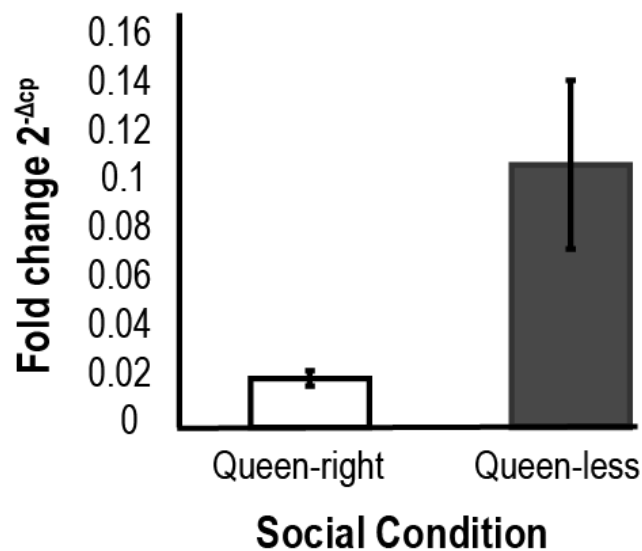


Figure 4.6: Change in levels of gene expression of the alcohol dehydrogenase (normalised to the reference gene *Cyp4g11*) in queen-right (QR; open bar) and queen-less (QL; closed bar) clones. Error bars represent standard error of fold change ($2^{-\Delta C_p}$).

Discussion

The *A. m. capensis* laying workers that act as social parasites, gain entry into host colonies, produce queen-like multi-glandular pheromone secretions (Okosun et al., 2017) and take over the role of reproduction from host queens (Hepburn, 1992;

Martin et al., 2002; Neumann and Hepburn, 2002), eventually leading to the collapse of infested colonies. Here, we show that the presence or absence of a queen in the infested colonies plays a critical role in determining whether the *A. m. capensis* workers (which are already predisposed to social parasitism) develop into fully fledged reproductive parasites. The regulation of pheromonal biosynthetic pathways in these workers by the queen pheromone is shown to regulate worker reproduction in these honey bee colonies.

The presence of a spermatheca (a queen-associated trait) was confirmed in all the *A. m. capensis* parasitic workers from both the QR and QL colonies, although visually it was noted that the size of the spermatheca differed in different individuals. While spermathecae were not measured for this study, previous studies recorded the size of the spermatheca in *A. m. capensis* workers to range between 0.37 mm and 0.55 mm (Phiancharoen et al., 2010). As these were field-collected bees of varying ages, the differences in the size of the spermatheca may be as a result of age or nutritional/feeding differences experienced by the *A. m. capensis* parasites during their developmental stages (Allsopp et al., 2003) and varied geographically (Phiancharoen et al., 2010). The spermatheca has been used before as a key trait in the identification of the parasitic laying *A. m. capensis* worker (Hepburn and Crewe, 1991; Okosun et al., 2015; Phiancharoen et al., 2010).

Ovarian activation clearly differed in the QL and QR clones. While all the QL clones had activated ovaries; stage III, IV and V (with majority of the clones having stage V), only 10% of QR colonies had activated ovaries (at stage III), showing that the presence of the *A. m. scutellata* queen indeed influenced the reproductive condition

of the *A. m. capensis* clones. Various factors influence ovarian activation in honey bees, including environmental factors such as nutrition (Schäfer et al., 2006) and the individual's physiological status such as age (Lin et al., 1999). The greatest influence on suppression of ovarian activation is the influence of pheromones from the queen and brood (Butler, 1959; Hoover et al., 2003; Mohammedi et al., 1998). The queen employs pheromones from various organs to suppress worker reproduction (Hoover et al., 2003; Okosun et al., 2017; Wossler and Crewe, 1999b), with the mandibular gland pheromones playing a crucial role as a 'suppressive agent' effectively inhibiting worker reproduction (Strauss et al., 2008).

The role of QMP in the inhibition of ovarian activation has been well documented (Butler, 1959; Hoover et al., 2003), for queens inhibiting ovarian activation within their own colonies. QMP components 9-ODA and 9-HDA have been shown to inhibit ovarian activation in both *Apis mellifera* and *Apis cerana* (Tan et al., 2010). Other glands contributing to the queen's overall pheromone bouquet such as the tergal glands (Okosun et al., 2017; Okosun et al., 2015; Wossler and Crewe, 1999a) also contribute to the suppression of reproductive dominance in workers through inhibition of both ovarian activation and production of queen-like pheromone signals. The components 9-ODA, 9-HDA and 10-HDA were seen to vary significantly with ovarian activation. These pheromone signals have been seen to covary with ovarian activation in *A. m. capensis* (Hepburn, 1992; Schäfer et al., 2006) and are indeed associated with reproductive dominance. Schafer et al. in 2006 showed that pheromone dominance influenced the food consumed by (dominant) *A. m. capensis* workers who were fed through trophallaxis by the host workers. It is this protein-rich

pre-processed 'jelly-like' pollen meal that enables the reproductively dominant *A. m. capensis* workers to dedicate most of their energies to activating their ovaries and laying eggs (Moritz and Hillesheim, 1985; Schäfer et al., 2006). A component of honey bee brood pheromones, (E)- β ocimene, has also been shown to play a role in suppression of ovarian activation in honey bee workers (Traynor et al., 2014). The effect that the presence of brood may have played in suppression of ovarian activation in these field-collected parasitic workers was not examined in this study but since there was brood in both sets of colonies, we expect that brood on its own did not inhibit ovarian activation of the false queens in the queen right colonies. This premise is further supported by studies on caged *A. m. scutellata* which showed that exposure to brood pheromone had no effect on ovarian activation in the caged bees (Démares et al., 2017).

The pheromone profiles of the QR and QL parasitic *A. m. capensis* workers revealed that the presence of the queen had a significant influence on the pheromone profile of the parasites. Pheromone profiles of the QL *A. m. capensis* laying workers were dominated by fatty acid components typical of queens, such as 9-ODA and 9-HDA while those of QR clones were dominated by 9-HDA and 10-HDA. This QR (worker-like) profile has been described before by (Zheng et al., 2010) when assessing QR *A. m. capensis* (non-laying) workers under the control of an *A. m. capensis* queen, in the Cape region (endemic region of the *A. m. capensis* subspecies). The profiles produced by the QR Clones reported here are more similar to those produced by the QR *A. m. capensis* (non-laying) workers from the Stellenbosch region as reported by Zheng et al (2010). This clearly demonstrates that *A. m. scutellata* queens have

the ability to control reproductive dominance in *A. m. capensis* parasites to the same extent that *A. m. capensis* queens control reproduction in non-laying *A. m. capensis* workers. Since *A. m. capensis* social parasites can takeover colonies of other subspecies (reviewed in (Neumann and Hepburn, 2002) and (Neumann and Moritz, 2002), the ability of the host queen to exercise a level of control suggests that other factors play a role in determining whether social parasites are able to take over a colony. These factors may include stress factors (such as pests and diseases), nutrition, various physiological states of the queen, colony structure (ratio of older to younger bees) and the amount and age of brood. Further, evidence shows that the social environment from which drifting (and host-seeking) *A. m. capensis* workers come, may have an influence on the ability of these workers to develop into reproductive individuals. *A. m. capensis* workers drifting from queenless colonies were better able to develop into reproductives, with queen-like pheromonal ratios and activated ovaries (Reece, 2002). This suggests that as *A. m. capensis* workers from queenright colonies were already under the influence of the queen from their own original colonies (possibly with near worker-like pheromone ratios and inactive ovaries) it was much easier for host queens in the infested host colonies to control these drifting parasitic workers.

The fatty acid 10-HDA was produced in larger quantities in the QR clones as compared to clones from QL colonies (Figure 4.4). Although produced by both queens and workers, this fatty acid is produced in the highest proportions in worker mandibular glands (Plettner et al., 1996) and is a key constituent of royal jelly (Genç and Aslan, 1999). The ratio of 9-ODA:10-HDA for queenright *A. m. capensis* workers

has been found to range between 0.3 and 0.1 (Reece, 2002; Zheng et al., 2010). Only 67% (n=19) of our QR parasitic clones fell within this range (0.17 ± 0.23). A second ratio (9-ODA/9-ODA+10-HDA) widely used to assess the “queen-likeness” of honey bee mandibular gland signals (Dietemann et al., 2006; Schäfer et al., 2006), also showed that only 75% (n=21) of the QR worker signals were “worker-like”. This means that the rest of the QR workers and (100%) of the QL workers had activated the ‘queen-specific’ biosynthetic pathway, enabling them to produce higher quantities of the queen pheromone 9-ODA relative to the worker-like component 10-HDA (Plettner et al., 1996).

As expected, the proportion of 9-ODA produced was significantly higher in QL clones than in their QR counterparts as the queen is not present to suppress production of ‘queen substance’ in the QL colonies (Moritz et al., 2000). However, the amount of the 9-HDA, which is the precursor compound to 9-ODA was significantly higher in the QR workers as compared to the QL bees. This suggests that the queen’s influence on fatty acid biosynthesis in the clone workers’ mandibular glands is exercised at the level of halting the oxidation of 9-HDA into 9-ODA, rather than being able to prevent the clones from using the ω -1 hydroxylation route in the biosynthesis of mandibular gland pheromones (this is the pathway normally upregulated in queens and reproductively dominant workers) (Malka et al., 2009; Malka et al., 2014). Queens prevent the formation of 9-ODA by inhibiting the production of the enzyme alcohol dehydrogenase (Malka et al., 2014; Wu et al., 2017). The production of this enzyme was significantly higher in QL clones than in QR clones, indicating inhibition or suppression of the production of 9-ODA, by the *A. m. scutellata* queen.

As the two arms of the bifurcated biosynthetic pathway are independent of each other (Plettner et al., 1996), the higher amounts of 9-ODA in QL clones as opposed to the QR clones could only come from the enzymatic oxidative-reduction of 9-HDA to 9-ODA a reaction that is inhibited in the worker caste. The low amounts of alcohol dehydrogenase in QR workers suggests that one mechanism employed by the queen to regulate the production of 9-ODA is suppression of the synthesis of this enzyme, resulting in an accumulation of the precursor 9-HDA. Alcohol dehydrogenase has indeed been shown to be highly expressed in the mandibular glands of queens as opposed to those of workers (Malka et al., 2014; Wu et al., 2017). As the amount of 9-ODA has been shown to increase with age in both virgin and mated queens (Slessor et al., 1990), it follows that the amount of alcohol dehydrogenase converting 9-HDA to 9-ODA would increase with the age of the queens as well. Due to the fact that we measured the transcripts of ADH in field collected samples of workers whose ages were undetermined, we cannot rule out the possible effect that age of the *A. m. capensis* parasites had on the expression of ADH. Further, alcohol dehydrogenase has been shown to participate in various other key functions in insect communication, such as pheromone degradation as in the antennae of the moth *Maduca sexta* (Robertson et al., 1999; Vogt, 2005). Even though our analyses were highly targeted to the honey bee mandibular glands, we cannot rule out the effect that other key metabolic functions may have on the expression of this enzyme.

All these pieces of evidence are indicative of intraspecific control of reproductive dominance where the *A. m. scutellata* queen controls dominance by inhibiting both

ovarian activation and the production of queen-like pheromone signals. This is the first report documenting intraspecific control of reproductive dominance between subspecies through suppression of ovarian activation, as we have shown that the *A. m. scutellata* queen was able to prevent ovarian activation in *A. m. capensis* workers. Our work provides an insight into the pheromonal interplay between invasive *A. m. capensis* social parasites and the queens of host colonies in their quest for reproductive dominance. We have shown that the presence of host queens of *A. m. scutellata* inhibits both ovarian activation and oxidation of 9-HDA to 9-ODA in parasitic clone workers. In queenright colonies of most honey bee subspecies, the QMP of the queen prevents the workers from expressing the part of the biosynthetic pathway (Figure 4.1) that results in the ω -1 hydroxylation of stearic acid. If this inhibition fails, then it appears from our work that the QMP can still inhibit the production of the 'queen substance' in these workers through blocking the production of alcohol dehydrogenase that would result in the conversion of 9HDA to 9ODA, thus revealing an ongoing battle for reproductive dominance that takes place in clone-infested colonies.

There is evidence from laboratory based experiments showing that *A. m. capensis* queens are better able to regulate production of dominance signals from the invasive clones than *A. m. scutellata* queens are (Dietemann et al., 2006), possibly owing to the higher amounts of 'queen substance' produced by the *A. m. capensis* queens (Crewe, 1982). The fact that South African beekeepers lose more *A. m. scutellata* colonies than *A. m. capensis* colonies due to the '*capensis* problem' regardless of colony management practices (Pirk et al., 2014), suggests that the results from the

laboratory experiments are reflected in the field. Our results show that given the right colony conditions the *A. m. scutellata* queens can prevent a clone reproductive takeover. The right colony conditions could include sufficient amounts of host colony brood as brood pheromones potentially act as ‘honest signals’ or queen fertility indicators (Pettis et al., 1997; Strauss et al., 2008), in addition to suppression of ovarian activation achieved by having the queen present in the colony (Traynor et al., 2014).

This work demonstrates that reproduction by invasive parasitic lineages is facilitated by the absence of the queen, and that in the presence of the queen there is a pheromonal contest between the queen and the parasitic workers that is dependent on the queen remaining in place. On queen loss, the parasitic workers are able to monopolise reproduction in the colony at the expense of the queen’s offspring.

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CHAPTER 5

Synthesis and General Conclusions

Reproductive division of labour is a key characteristic of social insect societies, where the queen mates with multiple drones and lays the overwhelming majority of eggs. Workers are reproductively sterile (Winston, 1987). Governed by age polytheism and modulated mainly by the queen's pheromones (produced by multiple glands), these non-reproductive workers carry out the day-to-day colony maintenance tasks. Younger bees perform in-hive tasks such as feeding the queen and brood, cleaning the hive and building wax combs while, the older bees perform the riskier out-of-hive tasks such as guarding and foraging for water, nectar and pollen. In the absence of the queen, workers can, in principle, activate their ovaries and lay haploid eggs that become drones (Crozier, 1975). Through thelytokous parthenogenesis induced by the fusion of the two central nuclei during meiosis (Verma and Ruttner, 1983), workers of the *A. m. capensis* sub-species have evolved the exceptional ability to lay diploid eggs resulting in female offspring (Hepburn and Crewe, 1991; Onions, 1912; Ruttner, 1977). In addition, a lineage of this subspecies has evolved into a reproductive parasite (Aumer et al., 2019; Moritz et al., 2008), capable of seeking out susceptible host colonies (Neumann et al., 2001), producing queen-like mandibular gland pheromones (Crewe and Velthuis, 1980; Okosun et al., 2017; Zheng et al., 2010), rapidly activating their ovaries and laying eggs (Neumann and Hepburn, 2002; Sakagami, 1958). In this way, these morphologically-worker bees become pseudo-queens. While the *A. m. capensis* clones principally utilise the mandibular gland signals to achieve reproductive dominance, these pseudo-queens also produce queen-like chemical signals from other glands such as the Dufour's

(Sole et al., 2002) and the Tergal (Okosun et al., 2017; Okosun et al., 2015, 2019) glands. Using a population genomics approach, Aumer et al. (2019) mapped the thelytoky locus (*Th*) associated with the thelytoky syndrome (production of queen-like secretions, rapid ovary activation and laying diploid eggs) and showed that a single SNP (single nucleotide polymorphism) in this locus acts as the genetic switch responsible for turning *A. m. capensis* workers into reproductive parasites.

Dominant *A. mellifera* workers of all subspecies produce various queen-like signatures that enable them to influence the behaviour of subordinate workers. Chapter 1 of this thesis reviews some of the genes and biosynthetic pathways used by dominant workers in their quest to become queens. The data chapters (2, 3 and 4) explore the mandibular gland biosynthetic pathways utilised by *A. m. capensis* clones and how these pathways differ from those of their *A. m. scutellata* counterparts. Chapter 2 examines the expression of reproductive dominance in *A. m. scutellata* workers and *A. m. capensis* clones, by focusing on the tightly-regulated hydroxylation step of the mandibular gland fatty acid pheromone biosynthesis. The expression of two cytochrome P450 genes thought to participate in the hydroxylation of stearyl-CoA, in young and older *A. m. capensis* and *A. m. scutellata* workers were examined. Chapter 3 follows up on the gene expression studies by investigating further the complete set of genes involved in the biosynthesis of mandibular gland fatty acids through transcriptome profiling by *RNASeq*. Finally, in Chapter 4, the effect that social environment may have on the expression of reproductive dominance in field-collected *A. m. capensis* clones was examined through the description of the pheromone profile of these reproductive parasites, delving deeper

into the expression of one of the key enzymes responsible for the conversion of 9-HDA to the 'queen-substance' 9-ODA. This chapter (Chapter 5) summarises the key findings from the thesis as outlined above and explores areas for future research.

The biosynthesis of the fatty acid components of mandibular gland pheromones is a multi-step process, starting with the thioesterification of stearic acid followed by the caste-specific hydroxylation of the newly formed stearyl-CoA. Catalysed by Cytochrome P450 enzymes, ω hydroxylation occurs mainly in worker mandibular glands and results in formation of worker-typical acids including 10-HDAA and 10-HDA while $\omega-1$ hydroxylation takes place predominantly in queen mandibular glands and results in the formation of queen acids such as 9-HDA which is further oxidised to the 'queen substance' 9-ODA (Plettner et al., 1998; Plettner et al., 1996). While a lot more has been documented on the mandibular gland pheromone signatures of queen and worker honey bees from African honey bee subspecies (Crewe, 1982; Crewe and Moritz, 1989; Hepburn and Crewe, 1991; Simon et al., 2001; Yusuf et al., 2015; Zheng et al., 2010), much less is known with regard to the biosynthesis of the mandibular gland pheromone components, and how the plasticity in pheromone production in the different subspecies is affected by the social environments that the bees find themselves in.

Chapter 2 focuses on the development of reproductive dominance in *A. m. capensis* clones and *A. m. scutellata* workers, given the higher reproductive potential in the former as compared to the latter. Here, the mandibular gland profiles, ovarian activation and gene expression of two cytochrome P450s thought to participate in the caste-specific hydroxylation of acylated stearic acid, were investigated. The

results showed that although both young *A. m. scutellata* workers and *A. m. capensis* clones had inactive ovaries, their pheromonal profiles were very different. While the young *A. m. scutellata* produced worker-typical acids such as 10-HDAA and 10-HDA, their *A. m. capensis* clone counterparts predominantly produce queen-typical acids, particularly 9-HDA, the precursor compound to the queen substance 9-ODA. This crucial finding points to the fact that, even at emergence, the *A. m. capensis* reproductive parasites are already primed for parasitism. Should these newly emerged parasites identify susceptible host colonies (e.g., queenless colonies), they would then readily convert the 9-HDA into 9-ODA, activate their ovaries and start laying eggs, and therefore becoming functional reproductives.

The pheromone profiles of the older clones were also different from those of the younger ones. While the younger *A. m. capensis* clones produced high amounts of 9-HDA, by day 7 much of the 9-HDA being produced is readily converted to the queen substance 9-ODA. Coupling the queen-like pheromone composition with activated ovaries, seen by day seven, the majority of the clones are indeed reproductively dominant. This is in contrast to the *A. m. scutellata* workers where even by day 7, very little 9-ODA was produced and the bees had still not activated their ovaries in preparation for egg laying.

Exploring the expression of *cyp6as8* and *cyp6bd1* (involved in ω hydroxylation in workers and ω -1 hydroxylation in queens, respectively), revealed that there was no significant difference in the expression of either genes in the young bees. Seeing that the young *A. m. capensis* clones were producing high amounts of 9-HDA (achieved through ω -1 hydroxylation) the low levels of *cyp6bd1* may imply that the

young clones may have upregulated different sets of genes as opposed to the ones investigated here. Indeed, Malka et al. (2014) and Wu et al. (2017) highlighted a total of 24 cytochrome P450s thought to participate in the caste-specific hydroxylation step in the biosynthesis of honey bee mandibular gland fatty acids, and showed that for both workers and queens, multiple P450s participate in hydroxylation. The gene expression profiles of the older bees showed that the *A. m. scutellata* produced significantly higher amounts of *cyp6as8* while the *A. m. capensis* clones produced significantly higher amounts of *cyp6bd1*, possibly implying that when it comes to hydroxylation, honey bee workers possibly employ different sets of P450s when younger and possibly switch to an entirely different set as they get older.

Examining the composition of mandibular gland fatty acids in queen-right *A. m. capensis* workers in their native region, Simon et al. (2001) showed that even at four days old, *A. m. capensis* workers from queenright colonies produced worker-type acids, dominated by 10-HDA and 10-HDAA. Therefore, the finding that after less than 24 hours post-emergence, the parasitic clones are already producing queen typical acids suggests that there are various molecular-level mechanisms involved in the control of reproductive dominance, modulated by the host queen's pheromones. Further, the range in mandibular gland products spanning worker-like acids (non-parasitic *A. m. capensis*) to queen-like products (*A. m. capensis* reproductive parasites) is expected given the genetic diversity found in *A. m. capensis* colonies, where some workers will possess the thelytoky-associated SNP responsible for the expression of the full thelytoky syndrome, while others do not (Aumer et al., 2019).

Future studies on this theme should explore the expression of various sets of genes participating in the biosynthesis of mandibular gland fatty acids, comparing the expression of these genes in queenright and queenless *A. m. capensis* workers in their native region to their parasitic counterparts infesting susceptible host colonies. In addition, deeper understanding of the development and expression of dominance in the earlier life stages (egg, larvae and pupae) should be carried out in order to generate a more complete picture on this question of regulation of reproductive dominance in *A. m. capensis* clones. This could involve silencing key genes associated with the development of the parasitic phenotype in *A. m. capensis* clone larvae and also introducing these genes in non-parasitic larvae.

The transcriptomic analyses carried out in Chapter 3 provided a detailed picture of the sets of genes differentially expressed in the mandibular glands of young and older *A. m. scutellata* and *A. m. capensis* clones. While the previous chapter (Chapter 2) focused primarily on the hydroxylation step, here, gene expression assessment from the *in situ* biosynthesis of stearic acid to oxidation of hydroxylation products leading to the formation of queen- and worker-typical acids was carried out. Indeed, 48 of the differentially expressed genes were directly involved in the biosynthesis of mandibular gland fatty acids and using these expression patterns, in comparison to similar work reported by Malka et al. (2014) and Wu et al. (2017) this study identified the multiple points of regulation in the mandibular gland biosynthesis pathways and how the *A. m. capensis* clone circumvents these queen-imposed checkpoints in her quest to become a false-queen.

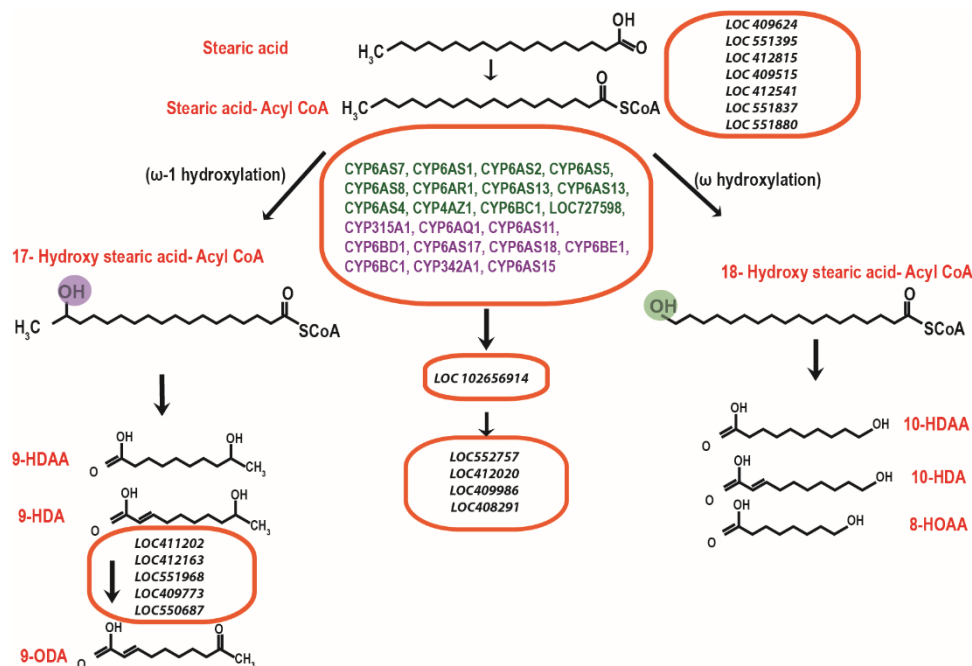


Figure 5.1: A diagram showing the biosynthetic pathway of honey bee mandibular gland fatty acids, also showing the transcripts shown to be differentially expressed in the mandibular glands of queenless *A. m. capensis* reproductive parasites and *A. m. scutellata* workers. Cytochrome P450s in green were differentially expressed in *A. m. scutellata* mandibular glands while those in purple were highly expressed in *A. m. capensis* clones.

The first step of regulation is the synthesis and activation of stearic acid where differential expression of genes encoding various enzymes including fatty acid synthases, acyl CoA synthases and long chain fatty acid CoA synthases were identified. Genes encoding acetyl CoA synthase (LOC409624) and fatty acid synthase (LOC412815) were most abundant in older *A. m. capensis* clones implying that the first way in which the reproductive parasites increased their pheromone production was through increasing the amount or rate of production of the starting raw materials.

The resultant activated stearic acid is then hydroxylated either at the ω (worker-typical) or $\omega-1$ (queen-typical) position (Plettner et al., 1998; Plettner et al., 1996). The results showed that *A. m. scutellata* workers generally upregulated genes encoding enzymes previously shown to be upregulated in queen-right workers of other *A. mellifera* subspecies while *A. m. capensis* clones overexpressed genes shown to be upregulated in queens and queenless workers with activated ovaries. Of the 25 cytochrome P450s differentially expressed, three *LOC724946* (*Cyp6as11*), *LOC551560* (*Cyp6bd1*) and *LOC551626* (*Cyp6as17*) were upregulated only in *A. m. capensis* clones. These transcripts had been shown to be upregulated in the mandibular glands of European virgin queens (Malka et al., 2014; Wu et al., 2017). Further, five genes; *Cyp6as1*, *CYP6as2*, *LOC410492* (*CYP9Q1*), *LOC550965* (*CYP6ar1*) and *LOC 551028* (*Cyp6as13*) upregulated only in the *A. m. scutellata* workers. *CYP6as2*, *CYP9Q1*, *Cyp6as13* were previously shown to be upregulated in the mandibular glands of workers with undeveloped ovaries (Malka et al., 2014) while *Cyp6as1* was highly expressed in queens (Wu et al., 2017). The mandibular gland profiles of 1-year old virgin *A. mellifera* queens were previously shown to contain about $27.3 \pm 2 \mu\text{g}$ of 10-HDA (Plettner et al., 1997) and it is thus possible that *Cyp6as1* plays a significant role in ω -hydroxylation in that case. The pheromone profiles presented in the Chapter 2 revealed the fact that *A. m. capensis* clones commence production of 9-HDA at day 0 (<24 hours) while their day 7 counterparts mainly produced queen-like pheromone. Therefore the three CYP P450 genes upregulated in the mandibular glands of both day 0 and day 10 clones possibly participate in the production of enzymes involved in $\omega-1$ hydroxylation.

Interestingly, the expression of two genes *LOC724175* and *LOC551197* (*Cyp 6as15*) were high only in day 10 *A. m. capensis* clones and while no comparative data exists for *LOC724175*, *Cyp 6as15* has been shown to be highly expressed in the mandibular gland tissue of mated queens (Wu et al., 2017). Taken together, the patterns of cytochrome P450 seen in the *A. m. capensis* clones were similar to those reported as highly expressed in the mandibular glands of queens as opposed to the patterns seen in *A. m. scutellata* workers which closely mirrored those reported as typical for workers with undeveloped ovaries.

Hydroxylated fatty acids are then transported by the Acyl-CoA binding protein (ACBP) from the endoplasmic reticulum to peroxisome where uncompleted β -oxidation takes place. The expression of this carrier molecule (*LOC102656914*) showed high transcript abundance only in day 10 *A. m. capensis* clones, most probably leading to an increase in the rate at which hydroxylated products in the clone mandibular glands are oxidised to the final products. In the peroxisomes, uncompleted β -oxidation takes place, aided by acyl CoA oxidases, peroxisomal multifunctional enzymes and thiolases. Here, *LOC55275* (Probable peroxisomal acyl-coenzyme A oxidase 1) was in high abundance in *A. m. scutellata* of both ages. Transcripts of this enzyme were shown to be highly expressed in worker mandibular glands as compared to those of queens (Malka et al., 2014; Wu et al., 2017). *LOC412020* (Peroxisomal acyl-coenzyme A oxidase 3-like) and *LOC409986* (Peroxisomal multifunctional enzyme type 2-like) were highly abundant in the *A. m. capensis* clone mandibular glands, as was *LOC408291* (3-ketoacyl-CoA thiolase). Indeed Wu et al. (2017) showed that transcripts of *LOC409986* and *LOC408291*

were highly abundant in queen mandibular glands. The products of the uncompleted β -oxidation include 9-HDAA, 9-HDA (queen-typical acids) and 10-HDAA and 10HDA (worker-typical acids).

A final regulation point in this biosynthetic pathway is seen in the oxidation of 9-HDA to form the 'queen substance' 9-ODA, catalysed by the ubiquitous enzyme alcohol dehydrogenase. Here, transcripts of 1, 5 -anhydro-D-fructose reductase-like dehydrogenase were highly abundant in *A. m. capensis* clone mandibular glands. The transcripts of this enzyme were shown to be highly expressed in the mandibular glands of virgin (Malka et al., 2014) and mated (Wu et al., 2017) queens.

The findings here suggest that in order to achieve dominance, *A. m. capensis* clones upregulate the biosynthetic pathways utilised by virgin and mated queens. The switch from the worker to queen biosynthetic pathway does not depend on a single step of the fatty acid pheromone biosynthetic pathway but that this shift is seen from the in situ biosynthesis of stearic acid to the oxidation of 9-HDA to 9-ODA. Finally, the high transcript abundance of genes encoding enzymes related to transport of fatty acids (such as Acyl -CoA binding protein transporting the ω / ω -1 hydroxylated products from the endoplasmic reticulum to the peroxisome for the uncompleted β -oxidation) shows that the *A. m. capensis* clones are not only focused on the production of the queen-like biomolecules, but also on increasing the rate at which these queen like acids are produced.

Future studies in this area should focus on validating some of the differentially expressed genes identified, through gene silencing techniques. This will enable us

to fully understand which genes are critical for the development of pheromone dominance and whether there will be any type of genetic compensation in the individuals with silenced genes. Further, there is a need to go a step beyond the transcriptomic analyses to see which of the differentially expressed cytochrome P450s actually get translated into proteins and whether the proteins formed are functional.

In Chapter 4, the effect that social environment may have on the expression of reproductive dominance in parasitic *A. m. capensis* laying workers was examined. Given the high colony losses currently attributed to ‘the *capensis* calamity’ the working hypothesis was, that the development of reproductive dominance in these laying workers would be independent of the social environment in which the clones found themselves. This implied that, even in the presence of the host queen, the clones would develop into fully fledged pseudo-queens with activated ovaries and queen like mandibular gland pheromones.

The results showed that some *A. m. scutellata* queens were able to regulate the development of reproductive dominance in the infesting clones, as adult laying workers collected from colonies whose host queen was present at the time of sampling (=QR clones) had a very different ovarian and pheromonal profile as compared to adult clones collected from colonies whose host queen was absent (=QL clones). To start with, only 10 % of QR clones had activated ovaries (stage III) while 100% of the QL clones had activated ovaries (86% of those having stage V ovarian activation). Clearly, the host queen had interfered with the ability of the

clones to activate their ovaries in order to lay eggs, which would have given rise to the next stage of parasitism.

Secondly, while QL clones were able to produce copious amounts of the 'queen substance' 9-ODA (>75%), QR clones produced very high amounts of 9-HDA which is the precursor molecule to 9-ODA. This showed that indeed the queen was blocking the QR clones from converting 9-HDA to 9-ODA. Gene expression analysis of two enzymes (*cyp6as8* and *cyp6bd1*) involved in the hydroxylation of activated stearic acid revealed that QR clones produced significantly higher amounts of *cyp6as8* as compared to their QL counterparts. As shown above, this enzyme is thought to be involved in ω -1 hydroxylation in worker mandibular glands. There was no significant difference in the relative expression of *cyp6bd1* between the two groups, showing that perhaps to produce the high amounts of 9-ODA, QL clones were switching on a different set of cytochrome P450s than were examined here. Finally, an examination of the expression of the enzyme Alcohol dehydrogenase (*Adh*) showed that QL clones expressed significantly higher amounts of this enzyme as opposed to their QR counterparts, which would explain why the QL clones were able to oxidise 9-HDA to 9-ODA, while their QR counterparts did so at a much slower rate.

This work has shown that in their quest to become reproductive parasites, *A. m. capensis* clones activate their ovaries and produce queen-like mandibular gland signals. This was shown from the onset (<24 hours old) that newly emerged *A. m. capensis* clones are primed for parasitism as seen by their ability to produce high amounts of the precursor molecule 9-HDA, although this is not readily converted to

9-ODA at this age. However, as the clones get older, they are fully able to express reproductive parasitism, seen in this work in the production of the queen substance 9-ODA and complete ovarian activation. The transcriptomic studies revealed that differential gene expression in the old and young *A. m. scutellata* and *A. m. capensis* clones is not just restricted to the hydroxylation level. DEGs were seen at all levels of the biosynthesis of mandibular gland fatty acids, starting from the *in situ* biosynthesis and activation of stearic acid. Lastly, not all colonies succumb to destruction due to infestation by *A. m. capensis* laying workers as some queens have the ability to inhibit the development of reproductive parasitism in the clones, by inhibiting ovarian activation and the production of queen-like pheromones.

Future work should explore the specific colony-level conditions that enable some queens to resist *A. m. capensis* clone infestation while others succumb. These factors could include the size of the colony, proportion of adult workers to brood, amount and age of brood, age of the queen among others. In addition, more information regarding the communication signals produced by the other members of the colony such as the brood and drones is needed. Understanding what these signals are, their biosynthesis, regulation and how they interact and influence each other will contribute immensely to our understanding of the evolution of social insects.

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