The effects of genotype and/or environment on the phenotypic expressions of mandibular gland signals in honeybees (*Apis mellifera*)

by

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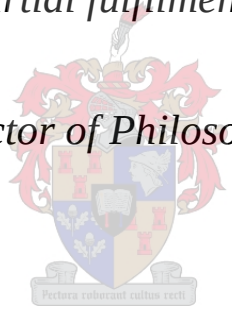

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Abstract

Insect societies utilize advanced chemical communication systems to organize many aspects of their social life, which among others, include reproduction, thus maintaining colony homeostasis. The queen pheromone complex (QMP), dominated by (E)-9-keto-2-decenoic acid (9ODA) is of integral importance in regulating worker reproductive development. Unique characteristics, associated with reproductive dominance, enabled the successful establishment of *Apis mellifera capensis* workers as social parasites (or pseudoqueens) in colonies of the neighbouring *A. m. scutellata*. This suggested that producing a queenlike pheromonal bouquet is one of the proximate factors in their success.

In this study we attempted to address the pheromone communication dilemma by investigating whether the phenotypic expression of mandibular gland signals in honeybee workers are under genetic and/or environmental influence. It was hypothesized that the mandibular gland profiles of queens and workers may be closely correlated to specific genotypes in the colony. However, different ageing and rearing environments (social context) can ultimately influence gene expression with respect to mandibular gland signals, highlighting the fact that environmental influences are not necessarily non-significant. In our experiments, both environmental/social conditions and genotypes of our test individuals were manipulated.

The *capensis* workers used in our experiment from their native range (Western Cape area are refered to as native workers, while *capensis* parasitic workers, from the clonal parasitic lineage, were obtained from the Gauteng area. *A. m. scutellata* workers were obtained from their native range, north of the hybrid zone.

Both native and parasitic workers showed the potential to become reproductively active, but the rapid pheromonal development of parasitic workers placed them at a reproductive advantage. Parasitic workers started producing low levels of 9HDA, the precursor to the queen substance 9ODA, between 12-24 hrs, while native workers only did so after 24 hrs. Despite this, rapid signal development did not culminate in the parasitic clones always pheromonally out-competing native workers. Withinin groups of native workers and a single clonal parasitic worker, the mandibular gland profiles of most workers were dominated by 9ODA and 9HDA (> 80% of extracts) with only 43% of the single parasitic workers producing higher amounts of 9ODA than native workers.

Mandibular gland pheromone profiles converged in groups of workers sharing a greater proportion of genes, providing support for a link to genotypic affects. Workers that were 75 – 99% related diverged significantly from groups with lower levels of relatedness was largely due to the presences of 9ODA (Spearman's rank correlation $r = 0.66$, $p < 0.0001$). Despite the tendency for signal to convergence in groups of closer relatedness a considerable amount of signal variability was also observed under varying social conditions. Workers originating from a single *capensis* queen but aged under queenright and queenless conditions had very distinct mandibular gland profiles (Wilks' lambda $\lambda = 0.118$, $\chi^2 = 331.002$, $p < 0.0001$). This variability was thus a result of the social environment that the workers were exposed to. The physiological traits, namely mandibular gland pheromone production, linked to reproductive potential in honeybee workers seem to be determined by a combination of environmental and genetic factors. Queen mandibular gland pheromone biosynthesis is genetically predisposed in certain workers however the final oxidation step to 9ODA is strongly influenced by the social environment. The signal plasticity

observed in this study is adaptive and assists workers to realize their reproductive potential.

Uittreksel

Insek gemeenskappe gebruik gevorderde chemiese kommunikasie sisteme om verskeie aspekte van sosiale lewe, onder andere reproduksie, te organiseer en sodoende word korf homeostasis handhaaf. Die feromoon kompleks van die koninginby, wat hoofsaaklik uit (E)-9-keto-2-decenoic acid (9ODA) bestaan speel 'n belangrike rol in die regulering van reproduksie in heuningby werkers. Die suksesvolle vestiging van *Apis mellifera capensis* werkers as sosiale parasiete (pseudo koninginne) in die korwe van die naburige *A. m. scutellata,* is bewerkstellig deur hul unieke kenmerke, wat met reproduktiewe oorheersing verband hou. Dit suggereer dat die produksie van 'n tipiese koningin feromoon sein een van verskeie beduidende faktore is in *capensis* werkers se sukses.

In hierdie studie het ons die dilemma van feromoon kommunikasie probeer aanspreek deur te ondersoek of die fenotipiese uitdrukking van seine van die mandibulêre kliere deur genetiese en/of omgewings faktore beïnvloed word. Die hipotese was dat die mandibulêre klier profiele van koninginne en werkers korreleer met spesifieke genotipes in die korf. Die verskillende omgewings waarin werkers groot gemaak word en verouder (sosiale konteks), kan uiteindelik die uitdrukking van gene, raakende mandibulêre kliere, beïnvloed. Dit beklemtoon die feit dat omgewings faktore nie noodwendig onbeduidend is nie. Beide omgewings/sosiale toestande and genotipes van toets individue is in ons eksperimente gemanipuleer.

Die *capensis* werkers afkomstig uit hul natuurlike habitat (Weskaap area) wat in ons eksperimente gebruik is word na verwys as inboorling werkers, terwyl parasitiese *capensis* werkers, van klonies parasitiese afkoms, vanuit die Gauteng area verkry is.

A. m. scutellata werkers was vanuit hul natuurlike habitat, noord van die, hybried sone, verkry.

Beide inboorling en parasitise werkers het die potensiaal getoon om reproduktief aktief te word, maar versnelde feromoon ontwikkeling van parasite werkers het hulle ŉ reproduktiewe voordeel gegee. Parasiet werkers het reeds lae hoeveelhede 9HDA, die voorganger van 9ODA, begin produseer tussen 12 – 24 uur, terwyl inboorling werkers produksie eers na 24 uur begin het. Ten spyte van die versnelde ontwikkeling in parasiet werkers het dit nie gelei daartoe dat kloniese parasiete altyd feromonies die oorhand oor inboorling werkers gekry het nie. In groepe bestaande uit inboorling werkers en ŉ enkele parasite werker, was die mandibulêre klier profiele altyd deur 9ODA en 9HDA (> 80% van ekstrakte) gedomineer. Slegs 43% van parasite werkers het groter hoeveelhede 9ODA as inboorling werkers geproduseer.

In groepe werkers, wat ŉ groter proporsie gene in gemeen gehad het, het mandibulêre klier profiele konvergeer. Dit ondersteun die bestaan van ŉ verband met genotipiese invloed. Werkers van 75 – 99% verwantskap het beduidend verskil van groepe met laer verwantskapsvlakke, hoofsaaklik as gevolg van die teenwoordigheid van 9ODA (Spearman's rank korrelasie $r = 0.66$, $p < 0.0001$). Ten spyte van die konvergerende neiging van profiele, van meer verwante groepe, was aansienlike veranderlikheid onder verskillende sosiale toestande waargeneem. Werkers, afkomstig vanaf ŉ enkele *capensis* koninginby, maar òf in die teenwoordigheid òf afwesigheid van ŉ koningin verouder is, het baie kenmerkende mandibulêre klier profiele getoon (Wilks' lambda $\lambda = 0.118$, $\chi_2 = 331.002$, $p < 0.0001$). Die veranderlikheid was dus ŉ gevolg van die sosiale omgewing waaraan die werkers blootgestel was. Dit blyk asof die fisiologiese kenmerke wat met reproduksie potensiaal in heuningbye verband hou, naamlik mandibulêre klier feromoon produksie, deur ŉ kombinasie van genetiese – en

omgewings faktore beïnvloed word. Sekere werkers is meer geneig tot die biosintese van koningin mandibulêre klier feromoon as gevolg van hul genetika, terwyl die finale oksidasie na 9ODA onder sterk omgewings invloed is. Die plastisiteit in mandibulêre seine waargeneem in hierdie studie, is aanpasbaar en help werkers om hul reproduksie potensiaal te bereik.

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CHAPTER 1

INTRODUCTION

1.1 Background

Eusociality is an extensively studied social system (Michener, 1974; Wilson, 1971) and is found in three main insect orders: Hymenoptera (ants, bees, wasps), Isoptera (termites) and Homoptera (aphids). These insects live in societies that rival that of humans' in complexity and internal cohesion. Eusocial insects are recognized by three main characteristics: 1) the mother is assisted by individuals that may or may not be directly related, to care for the young; 2) a reproductive division of labour exists with the so-called sterile worker caste possessing certain propensities or characteristics associated with helping behavior in which the members must do the work required at the appropriate time; 3) there is an overlapping of generations which allows for the older generations of offspring to help related, younger generations (Wilson, 1971; Fletcher et al., 1985; Hölldobbler et al., 1990). It is of utmost importance that the needs of the society be communicated to the individual members who respond appropriately (behaviourally or physiologically) to achieve success of the society. Thus communication, whether it is visual or chemical, is the glue which bonds these societies. The sum of current evidence indicates that pheromones play the central role in the organization of honeybee societies.

A typical honeybee (*Apis mellifera*) colony contains three adult castes each morphologically specialized to perform certain functions. A single fertile queen, whose primary function is to produce offspring, thousands of functionally sterile workers who do all the work and a few drones that serve a singular but important role

as mates for queens (Winston, 1987; Page et al., 2007). Insect societies utilize advanced chemical communication systems to organize many aspects of their social life, which among others include brood care, defence, foraging and reproduction (Robinson, 1987b; Huang et al., 1994). Pheromones, chemical messengers that convey information from one member of a colony to another, therefore act as the main source of information transmission. Pheromones can be grouped into releaser pheromones with short term effects that change the behaviour of the recipient and primer pheromones with long term effects that change the physiology of the recipient (Slessor et al., 1988; Slessor et al., 2005; Winston et al., 1992; Hoover et al., 2003).

In the honeybee colony the single queen secretes a suite of important pheromones from the cephalic mandibular gland, which is taken up by the workers and passed throughout the colony (Velthuis, 1970; Crewe et al., 1980; Winston et al., 1992a; Pankiw et al., 1996; Slessor et al., 2005). The mandibular gland secretion (MGS) is composed of a large number of compounds, however the major signal of queen presence is conferred by a five compound blend coined the queen mandibular gland pheromone complex (QMP, Slessor et al., 1988). This pheromone complex (QMP) is responsible for controlling and regulating many activities important for maintaining colony homeostasis. QMP consist of (E)-9-keto-2-decenoic acid (9ODA), two enantiomers of (E)-9-hydroxy-2-decenoic acid (9HDA), methyl-*p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA, Slessor et al., 1988). It acts as a releaser pheromone that attracts workers to the queen, resulting in a retinue around the queen (Slessor et al., 1988; Kaminski et al., 1990) and inhibits queen rearing by workers (Pettis et al., 1995; Winston et al., 1989, 1990). It also acts as a primer pheromone, regulating ovarian development of workers, thus regulating worker reproduction (Butler, 1959; Hepburn 1992; Hoover et al. 2003, 2005). Butler (1959)

was the first to show direct inhibition of worker reproduction by the queen mandibular gland in the honeybee. To date *Apis mellifera* is the only case for which this primer function of mandibular gland pheromones has been empirically demonstrated.

QMP is dominated by the queen substance, 9ODA (Barbier et al., 1960; Butler et al., 1961; Pain, 1961) while that of workers are dominated by 10-hydroxy-2-decenoic acid (10HDA) and 10-hydroxydecanoic acid (10HDAA; Winston et al., 1992; Plettner et al., 1993). These biosynthetic pathways are however not fixed and depending on the age and social environment of the worker she can produce the queen substance (Crewe et al., 1980; Page et al., 1988). This implies that workers producing 9ODA are also able to prevent other workers from developing reproductively. Most of the variation in mandibular gland secretions hinge on a quantitative difference in the relative proportions making up the mixture. It has been shown that mandibular gland signal production varies within subspecies but variability also exists between individuals, queens as well as workers (Crewe, 1982; Moritz et al., 2000). To date it remains uncertain whether this is as a consequence of genetic and/or environmental factors.

1.1.1 South African honeybees

South Africa is home to two neighbouring honeybee subspecies. Native to the fynbos biome we find the black Cape honeybee (*Apis mellifera capensis* Escholtz, hereafter *capensis*) (Tribe 1983, Hepburn et al., 1991) while the more yellow African honeybee (*Apis mellifera scutellata* Lepeletier, hereafter *scutellata*) inhabits most of sub-Saharan Africa. The latter is the honeybee on which the majority of commercial beekeeping is based in South Africa. On the basis of the number of ovarioles and sex ratio of laying worker offspring, Hepburn et al. (1991) defined the geographical

location of *capensis* and *scutellata* and a hybrid zone separating these two subspecies. This natural occurring 200km hybrid zone confines *capensis* to the southern and *scutellata* to the northern regions of the country and has apparently remained stable for decades with neither subspecies increasing its range, despite mixing of these subspecies (Hepburn et al., 1990; 2002).

The fact that *capensis* can establish themselves as social parasites of *scutellata*, produce clonal offspring in the host colonies, eventually leading to colony death (Allsopp, 1992; Martin et al., 2002; Neumann et al., 2002) makes the hybrid zone and the stability thereof very interesting. Beekman and colleaugues (2008) hypothesized that the *capensis-scutellata* hybrid zone is really a tension zone formed between the two parental populations, which prevents gene flow between the two populations, resulting in less fit hybrids (Barton et al., 1985). The proposers of this tension zone premised their idea on the basis of the pheromonal imbalances between the two subspecies (Wossler, 2002). Hybrid colonies are less fit due to *capensis* workers having higher levels of 9ODA and ovary activation which is not sustainable and ultimately leads to the death of the colony (Martin et al., 2002b), effectively preventing gene flow between *capensis* and *scutellata* across the hybrid zone.

1.2 The *Capensis* problem in South Africa

The ability of *capensis* workers to successfully establish themselves as social parasites of *scutellata* has led to the death of thousands of *scutellata* colonies (Allsopp, 1992; 1995; Allsopp et al., 1993). Parasitism starts when a *capensis* worker enters a *scutellata* host colony where she develops a so-called pseudo-queen phenotype with both high ovarian development and a queenlike pheromonal bouquet (Ruttner, 1988; Crewe et al., 1980; Velthuis et al., 1990), a key characteristic of *capensis* workers.

Unlike other honeybee subspecies, in which workers produce males by arrhenotokous parthenogenesis (development of males from unfertilized eggs), workers of the Cape honeybee produce female offspring through thelytoky (development of females from unfertilized eggs) (Onions, 1912; Anderson, 1963; Verma et al., 1983). Genetic analysis using microsatellites (Kryger, 2001; Neumann et al., 2002; Baudry et al., 2004; Hartël et al., 2006a, b) has shown that the billions of *capensis* workers now parasitizing South African honeybee colonies are all parthenogenetic descendants of a single worker lineage and can therefore correctly be regarded as a clonal population. Consequently, thelytoky results in the number of *capensis* workers in the host colonies increasing. While *capensis* pseudoqueen brood is preferentially nurtured by host workers (Beekman et al., 2000; Calis et al., 2002), the host queen is eventually lost and the *scutellata* host colony is progressively taken over by the parasite (Hepburn et al., 1998; Martin et al. 2002b). Since the parasitic workers do not participate in normal hive duties such as foraging, brood rearing, etc (Allsopp 1998; Martin et al., 2002), infected colonies become less efficient and dwindle down to a few host workers and eventually die.

The dynamics of colony usurpation is not yet clearly understood, but it would seem that the problem is largely one of communication. *A.m. capensis* are very plastic in their production of pheromones, since they are capable of rapidly switching their biosynthetic pathways from producing workerlike to more queenlike pheromones when placed in queenless *scutellata* colonies (Crewe et al., 1980; 1990; Moritz et al., 2000).

1.3 Mandibular gland pheromones and reproductive dominance

In queens the mandibular gland biosynthetic pathway leads to the so-called "queen substance" (9-oxo-2-(E)-decenoic acid; 9ODA) while the pathway in workers produces a secretion that is dominated by 10HDAA and 10HDA, the "worker substance" (Plettner et al., 1996, 1998). The ratio between queen and worker substances is a highly sensitive indicator of reproductive hierarchy status (Moritz et al., 2000, 2002). Consequently not only the queen's but also the laying worker's pheromonal mandibular gland signals suppress ovary development (Velthuis et al., 1965) and the production of a 9ODA dominated signal in other workers (Velthuis et al., 1965; Velthuis, 1970; Crewe et al., 1980; Crewe, 1984, 1988).

Queenright *capensis* workers are unique in that they are able to produce queenlike mandibular secretions, dominated by 9ODA (Ruttner et al., 1976; Hemmling et al., 1979: Crewe et al., 1980; Plettner, et al., 1993, 1996). Consequently *capensis* workers have a reproductive advantage over other subspecies. The establishment of *capensis* workers as social parasites of *scutellata* colonies in the northern regions of South Africa drew renewed attention to the unique characteristics of *capensis* workers. On a queen-worker continuum, parasitic workers (workers of the invasive clonal lineage) are possibly more queenlike than workers from the native *capensis* populations (workers found in the Western Cape and Southern parts of the Eastern Cape) with regards to characteristics that promote reproductive dominance (Beekman et al. 2000; Calis et al., 2002; Allsopp et al. 2003). The production of typical queen pheromones forms an important basis for the reproductive success of laying *capensis* workers.

Initial studies of signal variation focused on the variation of 9ODA in queens and 10 HDA in workers (Pain et al., 1960, 1967, 1976; Barbier et al., 1960). Boch and Shearer (1982) were the first to investigate whether the relative composition or the

total quality of the mandibular gland secretion varied with the age of the bee. They selected 5 components to correlate mandibular gland secretion composition with the age of the bee. They showed that the quantity of the 5 selected acids increased over time (with age) and reached a plateau around 17 days. Pain et al. (1967) however found great variability in the production of acids by similar aged workers and queens. The literature on age-dependent changes in mandibular gland ontogeny in the various castes of honeybees is extensive (Lensky et al., 1985; Allsopp, 1988; Whiffler et al., 1988; Crewe et al., 1989; Slessor et al., 1990; Engels et al., 1997; Wossler et al., 2006). The parasitic population north of the hybrid zone has been separated from their native *capensis* population for approximately 20 years and almost certainly has experienced different selection pressures. On a queen-worker developmental continuum, *capensis* parasitic workers are possibly more advanced than native workers for characteristics that promote reproductive dominance and social parasitism. In *capensis* workers the expression of queenlike characteristics is strongly affected by larval feeding. Larvae that receive food containing more royal jelly as well as receiving a greater amount of food develop into more queenlike individuals (Allsopp et al., 2003; Beekman et al., 2000; Calis et al., 2002). *A. m. capensis* brood reared by *scutellata* or *capensis*-*scutellata* hybrids receive more and better food than when they are reared by their own sisters (Allsopp et al., 2003; Calis et al., 2002). As a result *capensis* workers reared by *scutellata* nurses have a strong tendency to develop a queenlike phenotype (Allsopp et al., 2003; Beekman et al., 2000).

More recently however, it has been shown that parasitism by the clonal parasitic *capensis* lineage is not unique to *scutellata* colonies since it has been found that native *capensis* workers, expressing the correct suite of characteristics, also parasitize their own colonies in the Western Cape (Härtel et al., 2006a; Jordan et al., 2008). Of

interest however, is how far the parasitic population up north has diverged from their natal sister population in the last 20 years or so? Ultimately, the key to successful parasitism is getting into the host colonies. During a number of field trials, irrespective of method of introduction, we were unable to introduce very young parasitic workers into colonies. The literature suggests that these parasitic workers possess very queenlike pheromone signals, but this begged the question how quickly do they develop these signals and how different are they from that of native *capensis* workers? In this study we thus investigated worker mandibular gland secretions from the time of emergence, to track developmental changes over time (from emergence to 60 hours). Building on this we also investigated whether parasitic workers always win the pheromone arms race when compared to native workers.

A.m. capensis workers in queenright colonies show higher levels of signal and ovary development than workers of other races (Anderson, 1963). It is possible that these dominant workers, who do not follow an age polyethism (age-based division of labour), are waiting for the chance to reproduce, and on queen loss they would have a head start in egg laying (Moritz et al., 1985; Hillesheim et al., 1989). In queenless colonies the question arises: who becomes dominant? Dominance hierarchies in *capensis* have been studied by Moritz et al. (1996), who showed that certain patrilines had a greater probability of becoming reproductively dominant. However, the sample size was small, raising the question is dominance hierarchies really patriline based?

These dominant workers synthesize both qualitatively and quantitatively queenlike amounts of 9ODA, the queen substance, in their mandibular glands (Hemmling et al., 1979; Crewe et al., 1980; Crewe 1982). Within patrilines there is individual competition for dominance since only a few workers develop into laying workers/pseudoqueens (Martin et al., 2004; Robinson et al., 1990; Oldroyd et al.,

1994; Moritz et al., 1996). Reproductive dominant workers suppress the reproductive capacity of subordinate workers, which consequently do not develop their ovaries or produce queenlike signals in the presence of dominant workers (Velthuis et al., 1965; Velthuis, 1970; Crewe et al., 1980; Crewe, 1984, 1988; Moritz et al., 2000). *A.m. capensis* workers placed in pairs compete to produce the strongest queenlike signal and the production of 9ODA, which inhibits further 9ODA production in subordinate workers, and consequently 9ODA may therefore be an important signal in pseudoqueen selection (Moritz et al., 2000). Thus, mandibular gland signal production varies within subspecies but variability also exists between individuals (Moritz et al., 2000).

Owing to the polyandrous nature of honeybee queens (Adams et al., 1977; Koeniger 1987; Koeniger et al., 2000) the colony is characterised by a high intracolonial genotypic variance. It is composed of many subfamilies each sired by a different father (drone). Within a subfamily the workers are related by $r = 0.75$ and termed super-sisters (Page et al., 1988). Workers of two different subfamilies are half-sisters and consequently related by $r = 0.25$ (Ratnieks, 1988; Pirk et al., 2003). In order to resolve the suggested pheromone communication problem, the extent to which the environment and/or genotype affects the mandibular gland signals produced by workers needs to be determined. The parasitic clonal *capensis* population, on account of their very low genetic variance, offers us an opportunity to highlight the environmental influences on mandibular gland signal production. Previous experiments indicated that worker dominance was largely genetically based (Moritz et al., 1985; Hillesheim, 1987, Hillesheim et al., 1989) with the expression of the pseudoqueen phenotype in *capensis* workers particularly well expressed and under strong genetic influence (Moritz et al., 1985). The high genetic variance in natural

colonies and the clonal nature of parasitic workers as well as the use of artificially inseminated queen offspring (to limit the number of patrilines) allowed us to investigate the potential influence of genotype on mandibular signal production. By altering the environmental variables we attempted to ascertain whether the expression of worker mandibular gland signals contain genetic information or whether genetic predispositions are overridden by environmental influences.

In their investigation of reproductive *capensis* workers, Moritz and Hillesheim (1985) found that the production of 9ODA is influenced by genotype with an estimated heritability value of 0.89. This heritability value was estimated by an analysis of variance comparing variation within and between offspring of *capensis* laying workers. Heritability is the proportion of the total phenotypic variance due to the additive genetic effects in a specific population (Falconer, 1981). Heritability values can be used to estimate the relative importance of the genetic effects subtracted from the environmental effects in the regulation of a certain trait's manifestation (Milne, 1985a; 1985b). Using Moritz and Hillesheim's protocol, Wossler (unpublished results) found that mandibular gland secretions were strongly dependent on environmental influences with minimal genetic influences. Heritability estimates for 9ODA production was determined to be approximately 0.18. These contradictory findings highlight the need to establish the extent of the role of genes and environment on the production of mandibular gland signals.

All behaviours are modulated by interactions between genes and the environment. In social organisms, social interactions are a key component of the environment. To understand the link between genotypes and phenotypes, therefore, requires an understanding of how the individual's phenotype is influenced by its own genes (direct genetic effects) and the phenotype expressed in its social partners (indirect

effects) (Moore et al. 1997; Linksvayer et al., 2005; Keller, 2009). The ability of organisms to change their appearances, behaviour or physiology in response to environmental conditions is well known. Such environmentally induced changes in the phenotype of an organism are referred to as phenotypic plasticity. The genetic control of plastic responses on the other hand has not received that much attention. Two classes of genetic effects that influence plastic responses have been proposed: firstly, allelic sensitivity, where some alleles may be expressed in several different environments with varying effects on phenotype and secondly, gene regulation, where regulatory loci cause genes to turn off or on in certain environments (Schlicting et al., 2002; Via et al., 2005). They suggested that regulatory plasticity, with the potential for controlling multiple trait responses, is the likely mechanism for adaptive plasticity. The genetic influences on the production of mandibular, tergal and Dufour's gland pheromones are not well studied; however the same cannot be said for the hydrocarbon profiles produced by wasps, ants and bees. Dani et al. (2004) found that the cuticular hydrocarbon profile of the wasp, *Polistes dominulus*, does contain genetic information, since the composition of the hydrocarbons strongly correlated to the level of relatedness. It was also found that the cuticular hydrocarbon profiles in honeybees are partly genetically based (Page et al., 1991b; Arnold et al., 1996). Page et al. (1991b) found differences in the lipid composition between two worker patrilines in honeybee colonies headed by artificially doubly mated queens. Moreover, workers in a honeybee colony with a single queen, mated 16 times, could be correctly assigned to their patriline on the basis of their cuticular lipid composition, both when the workers were isolated and when they were allowed to remain in their colony (Arnold et al., 1996). It is therefore possible that mandibular gland secretions could

also be genetically derived since, like cuticular hydrocarbons (produced by either modified epidermal cells or tegumentary glands), it is also from exocrine origin.

Since the advent of the strong analytical molecular tools of microsatellites, the genetic influences on physiological and biochemical characters can now be positively determined. Microsatellite markers are a class of DNA markers that involve a variable number (up to 100) of short tandemly repeated simple sequences, 1-6 base pairs (bp). Microsatellites, which are polymorphic and abundant co-dominant markers, are ideal to determine parentage relationships between individuals (Queller et al., 1993; Blouin et al., 1996). Variation at microsatellite loci is readily assessed by polymerase chain reaction (PCR) amplification using primers complementary to the unique sequences flanking specific repetitive arrays (Ashley et al., 1994). For honeybees, a vast array of primers and loci has been described in the literature (Estoup et al., 1994; 1995; Haberl et al., 1999; Solignac et al., 2003). Demonstrating that signal phenotype has a strong correlation to genotype, lays the first steps for establishing a honeybee breeding programme.

1.4 Objectives

Due to the opposing outcomes reached for heritability values of 9ODA production in honeybees (Moritz et al., 1985; Wossler, unpublished) this study aims to determine whether the phenotypic expression of pheromonal signals that honeybee workers express, more particularly the mandibular gland secretions, are more strongly influenced by genes or environment. It is hypothesized that the mandibular gland profiles of queens and workers may be closely correlated to specific genotypes in the colony. However, different ageing and rearing environments can ultimately influence gene expression with respect to mandibular gland signals (Wossler, 2002; Jones,

unpublished PhD thesis), highlighting the fact that environmental influences are not necessarily non-significant. Due to the format of this thesis, some repetition and consequential overlapping within the introductions and in the materials and methods sections of chapters may occur.

In chapter 1 the study organism, *Apis mellifera* (honeybee) was introduced and its biology briefly discussed. It specifically focuses on the Cape honeybee (*capensis*), which through its ability to become facultative social parasites in colonies of conspecifics have caused large scale damage to the apicultural industry in South Africa. Each of the following chapters covers more relevant topics in further depth.

In chapter 2, the mandibular gland signal variation between native *capensis* and parasitic *capensis* workers were compared. The objective was to determine whether mandibular gland secretion development within the first 60 hours differed between natal and parasitic populations, while minimizing environmental effects. Moreover, different aged bees from the two populations were introduced into *capensis* discriminator colonies to determine whether their acceptance rates could be linked to the mandibular gland profiles.

Pseudoqueen development was investigated in chapter 3. The objective was to determine whether the parasitic workers (clone) always pheromonally out-compete the native workers.

In chapter 4 we compared the mandibular gland signal variation between worker groups of varying degrees of relatedness. Consequently, the objective was to determine whether mandibular gland profile variability would increase within worker groups of decreasing levels of relatedness.

The mandibular gland secretions of clones, which are near-identical, were compared to native workers in chapter 5. The objective was to determine whether native

capensis workers showed more signal variability than clones and also how near identical clone signals are. This would hopefully indicate whether mandibular gland secretions had a stronger genetic or environmental component.

In chapter 6 we investigated the relationship between mandibular gland profiles and genotype, but more specifically the link between profile and patriline. The polyandrous nature of the honeybee queen results in the production of genotypically diverse offspring in monogynous colonies. This facilitates the detection of possible genotypic effects because offspring, of similar age cohort, fathered by different drones (patrilines) within a colony share the same maternal genotype on average, the same maternal effects, and the same environmental rearing conditions and differ only in their paternal genotype. If respective patrilines within a colony express a specific signal phenotype, it would indicate that the expression of the mandibular gland signals contain genetic information. However, if all workers within a colony express a more homogenous signal, it would indicate a stronger environmental influence.

In chapter 7 we investigated whether workers from *capensis* patrilines (sired by *capensis* drones) are more likely to become reproductively active compared to *capensis-scutellata* hybrid workers (sired by *scutellata* drones). If workers of *capensis* paternity are more likely to become reproductively active, it would suggest that genetically mixed colonies may suffer from a breakdown in reproductive division of labour and that the hybrid zone is indeed a tension zone. Under our experimental conditions, similar aged pure *capensis* and hybrid workers shared all environmental influences and the same maternal genotype on average. They therefore only differ in their paternal genotype which allowed us to determine whether the expression of the reproductive traits can be influenced by paternity. This chapter has been submitted as a multi-authored paper to Behavioural Ecology and Sociobiology (Appendix 1).

Chapter 8 summarizes and discusses the main results of this study.

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CHAPTER 2

AGE-DEPENDENT CHANGES IN MANDIBULAR GLAND PROFILES OF NATIVE AND PARASITIC WORKERS OF *A. M. CAPENSIS*

2.1 Introduction

Social insects possess several pheromone producing exocrine glands involved in priming or releasing various biological functions (Hölldobbler et al., 1990). In honeybee colonies the complex social organization is largely regulated by pheromones from the monogynous queen. Honeybee queens and workers produce caste-specific mandibular gland pheromones (Blum, 1992; Plettner et al., 1997). To date the most extensively researched is the queen mandibular gland pheromones (QMP) dominated by (E)-9-keto-2-decenoic acid (9ODA), refered to as the queen substance, and (R,E)-(-) and (S,E)-(+)-9-hydroxy-2-decenoic acid (9HDA; Slessor et al., 1998). Under queenright conditions, workers' mandibular gland secretions are characterized by the dominant "worker substance", 10-hydroxy-2-decenoic acid (10HDA) and 10-hydroxydecanoic acid (10HDAA; Plettner et al., 1996; 1998). However, both castes are capable of producing the other's compounds depending on their social context (Naumann et al., 1991; Plettner et al., 1997) and consequently the biochemical pathways are not mutually exclusive.

Chemical analysis of the mandibular gland extract has demonstrated that the composition of the secretion is affected by age and race of honeybees in both queens (Engels, et al., 1997) and workers (Crewe, 1988; Crewe et al., 1989; Simon et al., 2001). Early studies investigating signal dissimilarity focused on the variation of 9ODA in queens and 10 HDA in workers (Pain et al., 1960, 1967, 1976; Barbier et al., 1960). For example, Pain and his colleagues (1967) found great variability in the

production of acids by individual workers and queens, with the selected acids shown to increase with age (Boch et al., 1982). Queens of several *Apis* species, including *A. m. scutellata* (hereafter refered to as *scutellata*), show a definite ontogenetic pattern in the development of their mandibular gland secretions (Crewe, 1988; Crewe et al., 1989). The 9ODA level in virgin queens increases from trace levels at emergence to nearly one third queen equivalent prior to mating (Slessor et al., 1990; Pankiw et al., 1996) while *A. m. capensis* (hereafter refered to as *capensis*) queens differ in that they produce large quantities of 9ODA at emergence (Crewe, 1982, 1988).

In *Apis mellifera* subspecies, removal or loss of the queen leads to either queen rearing or the development of laying workers (Sakagami, 1954, 1958; Page et al., 1988; Van der Blom 1991). In *capensis* such laying workers act as false queens that produce queenlike mandibular gland secretions. These workers behave queenlike and are treated as such and are even capable of regulating the reproductive development in other workers (Sakagami 1958; Velthuis, 1970 Velthuis et al. 1990; Hemmling et al. 1979; Hepburn et al., 1991; Neumann et al., 2002; Moritz et al., 2000; Dietemann et al., 2007). The mandibular gland signal of *capensis* workers undergo a transition from workerlike to more queenlike under queenless conditions with the increased production of 9ODA and 9HDA (Crewe et al., 1980; Simon et al., 2001; 2005). The production of these queen substances is not unique to queenless workers as detectable amounts are found in queenright workers, of *capensis* and other *A. mellifera* subspecies (Crewe et al., 1989; Plettner et al., 1993, 1997). However, Cape honeybee workers are particularly prone to switching their biochemical pathway from worker- to more queenlike.

The Cape honeybee is native to the southern parts of South Africa and possesses a suite of distinguishing characteristics related to worker reproduction. On queen loss

workers of all subspecies are able to develop reproductively. Cape honeybee workers are unique in that they produce almost clonal female offspring through thelytokous parthenogenesis while worker reproduction in other subspecies results in male offspring (Onions, 1912, 1914; Anderson, 1963, Hepburn et al., 1991; Ruttner, 1992; Moritz et al., 1994; Hepburn et al., 1998; Neumann et al., 2000; Radloff et al; 2002). These workers display high reproductive potentials in that they develop their ovaries more rapidly and possess a larger number of ovarioles than other subspecies (Hess, 1942; Velthuis et al., 1988; Hepburn et al., 1991; Hepburn et al., 1994). Moreover, as mentioned earlier, Cape workers are able to produce queenlike mandibular secretions, dominated by 9ODA, by switching from a worker biosynthetic pathway to a queen's biosynthetic pathway (Ruttner et al., 1976; Hemmling et al., 1979, Crewe et al., 1980; Plettner, et al., 1993, 1996). Consequently these traits put Cape workers at a pheromonal and reproductive advantage over other subspecies.

The success of *capensis* workers as social parasites, following their anthropogenic movement into the range of *scutellata*, emphasized their ability to become dominant reproductives. Since the first recorded invasions, almost two decades ago, it has been found that the parasitic workers currently infecting colonies in the north of South Africa are from a single lineage (Kryger, 2001; Neumann et al., 2002; Baudry et al., 2004; Hartël et al., 2006a, b). On a queen-worker continuum parasitic workers are possibly more queenlike than workers from the native *capensis* populations (workers found in the Western Cape and Southern parts of the Eastern Cape) with regards to characteristics that promote reproductive dominance (Beekman et al., 2000; Calis et al., 2002; Allsopp et al., 2003).

This queenlike phenotype in dominant workers is recognized by other less dominant workers and consequently results in the less dominant workers taking on a

reproductively subordinate role. The pheromone signal produced by dominant workers allows other workers to assess the reproductive status and quality of dominant workers and sets the stage for the evolution of fertility and reproductive-dominance primer pheromones (Malka et al., 2009). Reproductive competition in the honeybee is linked to two major pheromones: the mandibular gland pheromones which was the first primer pheromone demonstrated to inhibit ovarian development in workers (Butler 1959; Butler et al., 1963; Hoover et al., 2003) and also mediates dominance hierarchies in workers (Malka et al., 2008; Moritz et al., 2004), and the Dufour's gland that signals fertility due to its tight correlation with ovarian development (Katzav-Gozansky et al., 2004).

In this study we set out to ascertain whether the mandibular gland profiles of parasitic *capensis* workers develop more rapidly over time compared to native workers. Ageing workers from these two populations in a controlled environment allowed us to follow mandibular signal development across age groups, while minimizing environmental effects. Furthermore we also investigated whether mandibular gland profiles were in any way correlated to acceptance rates of the two populations by introducing both native and parasitic workers of various age cohorts into two discriminator colonies. We had observed exceptionally low acceptance rates for both native and parasitic workers introduced into *A. m scutellata* host colonies during field trials and thus we predicted that their high rejection rates were a consequence of queenlike mandibular gland signal development.

2.2 Materials & Methods

2.2.1 Experimental setup

Frames with sealed parasitic worker brood were obtained from a commercial beekeeper in Gauteng while sealed native *capensis* brood frames were obtained from the Agriculture Research Council in Stellenbosch, South Africa. These frames were incubated at 35°C and 60% relative humidity until adult emergence and the freshly emerged workers of the same age cohort were group-specific labelled (native and parasitic *capensis*) on the thorax with non-toxic paint (Posca Paint Pens, Mitsubishi Pencil Co., Japan). The experiment was conducted over a 60 hour period and 15 newly emerged bees were marked at 6, 12 and 24 hour intervals depending on the numbers that emerged (see table 1 for final sample sizes). This resulted in 4 age groups: 0-6 hrs, 6-12 hrs, 12-24 hrs and 24-60 hrs. Marked workers from each of the two test populations were: 1) placed on a food frame containing honey and pollen in a specially constructed frame box to simulate a natural environment and then placed in an incubator and allowed to age, and 2) introduced into queenright *capensis* field colonies to ascertain acceptance rates of different age groups of native and parasitic workers. The number of workers introduced into each discriminator colony was dependent on emergence rates. For emergence, < 6 hrs and 12-60hrs, 40 workers of each population were introduced into the respective colonies, while only 20 workers from 6-12 hrs of each population were introduced. During field trials we observed exceptionally low acceptance rates of both native and parasitic workers when introduced into *scutellata* host colonies, therefore we used *capensis* colonies as discriminators. We predicted that their high rejection rates were a consequence of queenlike mandibular gland signal development.

On termination of the experiment all bees were decapitated and individually labelled heads placed in 200µl dichloromethane (DCM) for gas chromatographic (GC) analyses. Bodies of each bee were correspondingly labelled and frozen for ovary dissections.

2.2.2 Chemical Analysis

The heads of individual workers were removed from the solvent which was evaporated with a stream of N_2 to dryness. The residue was redissolved in 15µl internal standard solution (tetradecane and octanoic acid, Sigma) and derivitised in 15µl bis-(trimethylsilyl) trifluoroacetamide (BSTFA, Sigma). One microlitre of this solution was injected into a gas chromatograph (Hewlett Packard 6850) equipped with a split-splitless injector and a flame ionization detector. Compound separation was achieved on a cross-linked methyl silicone HP-1 column (25m x 0.32mm) under a temperature programme: 60°C (1 min), 50°C/min to 90°C, 3°C/min to 220°C (10 mins) using helium as the carrier gas. The injection port was set at 230°C and the flame ionization detector at 320°C. Peak areas were determined using HP Chemstation software and the mandibular gland compounds were identified based on the retention times of authentic compounds (Sigma) and were quantified using peak area (Simon et al., 2001).

The compounds quantified in this study included: methyl p-hydroxybenzoate (HOB), 9-keto-(*E*)-2-decenoic acid (9ODA), 4-hydroxy-3-methoxyphenylethanole (HVA), 9-hydroxydecanoic acid (9HDAA), 9-hydroxy-2-decenoic acid (9HDA), 10-hydroxydecanoic acid (10HDAA), 10-hydroxy-2-decenoic acid (10HDA), stearic acid, and some minor aliphatic acids, palmitoleic acid, palmitic acid and oleic acid. Due to the similar levels of abundance of oleic acid in both native and parasitic

workers masking subtle but possibly important differences in other mandibular gland compounds it was excluded from the analyses. Authentic purchased compounds were used to determine the relative mass ratios of all the tested compounds (Gehrke et al., 1971) and tetradecane was used to calculate the absolute amounts. A standard solution was run every day to insure that the relative mass ratios were within the variability range of the series of standard runs (Crewe et al., 1989).

The quantitative ratio of (9ODA + 9HDA)/(9ODA + 9HDA + 10HDA+10HDAA) was calculated to determine how queenlike the mandibular gland pheromone profiles were (Moritz et al., 2000, 2004; Schäfer et al., 2006). A ratio close to one indicates a queenlike blend, whereas a ratio close to zero indicates a workerlike blend.

2.2.3 Statistical Analysis

The chemical data were analysed using PRIMER (Version 5.2.9, Plymouth Marine Laboratory, Clarke et al., 1994). Before analysis the data of absolute amounts were double square root transformed in order to limit the effect of zeros and the contrast of extreme high and low values. Non-metric multidimensional plots (MDS) were generated using Bray-Curtis similarities as distance measures. This allows for complex data to be graphically presented in two dimensions such that the relative distances between all points are in the same rank order as the relative similarities (Clarke et al., 2001). The interpretation of the MDS is in accordance with the similarity matrix: samples that are close together are more similar than those that are far apart. Stress values generated by MDS indicate the degree to which ranks are not preserved. A general rule for the interpretation of stress values is that a value less than 0.15 represent a good ordination with no real chance of misinterpretation (Clarke et al., 1994).

To determine whether mandibular gland profiles differed between native and parasitic workers per age group, Analysis of Similarities (ANOSIM) was performed with Bonferonni correction for multiple pairwise comparisons. ANOSIM is a non-parametric permutation procedure that is applied to the distance matrix underlying the ordination of samples. This procedure is an approximate analogue of the one- and two-way ANOVA (analysis of variance) test, testing only the similarity of samples rather than variation about the mean (Clarke et al., 1994). This implies that fewer assumptions about the data are required. ANOSIM produces a global *R* value that can range between -1 and 1. A value of 0 or lower indicates no differences between test groups while values greater than 0 indicates differences between test groups. Mann-Whitney pairwise comparisons were carried out to determine significant differences in compounds produced per age group between native and parasitic workers. The ratio of queen to worker substances was determined as this has been suggested to be a good indicator of reproductive dominance.

Chi-square tests (with Yates correction) were performed to determine significant differences in acceptance rates between native and parasitic workers. To determine if there was any relationship between signal profile and acceptance rates of workers, a Spearman rank correlation was performed.

2.3 Results

2.3.1 Ontogeny of mandibular gland profiles of A. m. capensis and parasitic workers over time

The mandibular gland extracts of worker samples revealed that three aliphatic compounds, palmitoleic-, palmitic- and stearic acid, were consistently present in all age groups (table 1). From emergence to 12 hrs the differences between native and

parasitic workers were as a result of quantitative differences in the abovementioned compounds. The levels of stearic acid, the entry point to both queen and worker biochemical pathways, was consistently significantly higher in parasitic workers for all age groups. Parasitic workers started producing 9HDA, the precursor to the queen substance 9ODA, between 12-24 hrs, albeit in very low levels (table 1) while native workers only did so after 24 hrs. The mandibular gland profiles of parasitic workers changed markedly after 24hrs with individuals producing the full suite of identified queen compounds while native workers only started producing very low levels of 9HDA. At this age parasitic workers produced significantly more 9ODA (MW: $U_{55,58}$ = 1210, $p < 0.0001$) and 9HDA (MW: $U_{55,58}$ = 1017.5, $p < 0.0001$) than native workers (table 1). No ovary development was detected in any of the sampled workers. The production of the worker compounds, 10HDA and 10HDAA was inconsistent and present in very low levels in both native and parasitic workers.

Pairwise one-way ANOSIM revealed that mandibular gland profiles of native and parasitic workers were significantly different for all age groups (see fig. 1 for global *R* values). From emergence to 24 hrs the separation between the two test populations was consistent with no or little overlap of profiles (fig.1a-c). After 24 hrs there was discernible variability in signal development between individuals from both populations resulting in a greater overlap of profiles between individuals, yet parasitic workers were still distinguishable from native workers (fig.1d). No ovary development was observed in any of the sampled workers.

Table 1: Absolute amounts (mean ± SE, µg) of compounds in the mandibular gland extracts of *A. m. capensis* native and parasitic workers of various age groups.

			Compounds								
Age (hrs)	***A. m. capensis* workers**	**N**	**9ODA**	**HVA**	**9HDAA**	**9HDA**	**10HDAA**	**10HDA**	**PALMOL**	**PALM**	**STEARIC**
0-6	Native	18	-	-	-	-	0.02 ± 0.02	-	0.81 ± 0.04	7.4 ± 0.28	3.88 ± 0.47
	Parasitic	19	-	-	-	-	-	0.03 ± 0.03	0.59 ± 0.04	5.43 ± 0.42	8.14 ± 0.82
6-12	Native	11	-	-	-	-	-	-	0.97 ± 0.09	7.38 ± 0.47	4.88 ± 0.39
	Parasitic	14	-	-	-	-	-	-	0.78 ± 0.06	6.18 ± 0.48	9.83 ± 0.88
12-24	Native	13	-	-	-	-	-	-	0.86 ± 0.05	6.18 ± 0.32	3.1 ± 0.27
	Parasitic	26	-	-	0.01 ± 0.01	0.14 ± 0.12	-	0.09 ± 0.08	0.61 ± 0.04	4.29 ± 0.23	6.87 ± 0.33
24-60	Native	55	-	-	-	0.04 ± 0.02	-	0.01 ± 0.01	0.46 ± 0.06	5.1 ± 0.17	3.12 ± 0.23
	Parasitic	58	4.24 ± 1.92	0.03 ± 0.02	0.03 ± 0.02	2.87 ± 1.2	0.01 ± 0.01	0.04 ± 0.02	0.79 ± 0.13	4.53 ± 0.46	8.06 ± 0.83

Abbreviations for the compounds are as follows: 9ODA = (E)-9-oxodec-2-enoic acid; HVA = 4-hydroxy-3-methoxyphenylethanol; 9HDAA = 9-hydroxydecanoic acid; 9HDA = (E)-9-hydroxydec-2-enoic acid; 10HDAA = 10-hydroxydecanoic acid; 10HDA = (E)-10-hydroxydec-2-enoic acid; PALMOL = palmitoleic acid; PALM = Palmitic acid; STEARIC = Stearic acid.

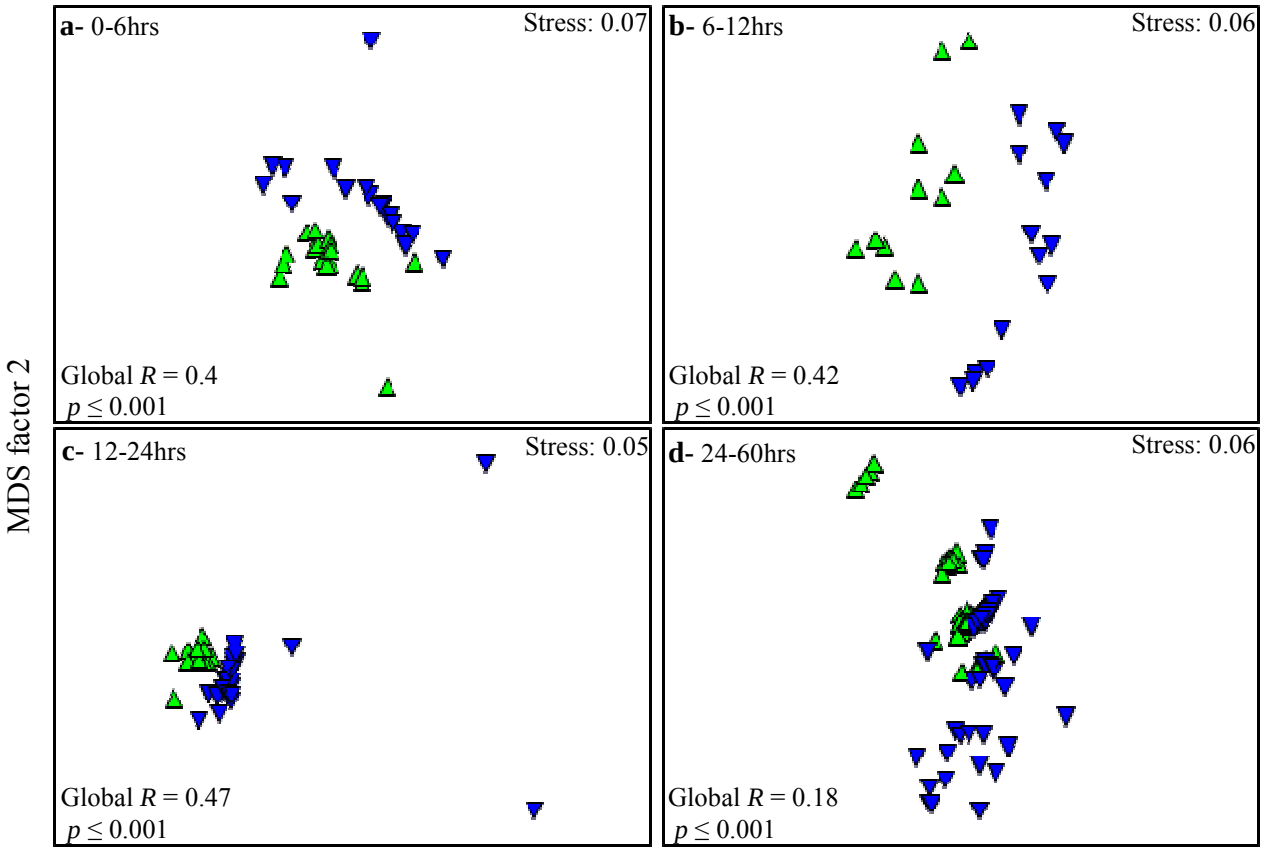

Figure 1: Multidimensional scaling ordination of the mandibular gland secretion profiles of four age groups (a-d) of *A. m. capensis* native (▲) and parasitic workers (▼). Distance between markers indicates similarity between profiles of individuals. The stress value (< 0.15) indicates the plot is suitable to be interpreted in two dimensions.

2.3.2 Acceptance rates of A. m. capensis and parasitic workers

Native and parasitic workers were not differentially accepted by host colonies from 0-12 hrs. Acceptance rates of native *capensis* workers were significantly higher than that of parasitic workers when aged 12-24 hrs (fig.3, $\chi^2 = 35.28$, $df = 1$, $p < 0.0001$) and 24-60hrs ($\chi^2 = 6.84$, $df = 1$, $p \leq 0.01$). The mean ratio of (9ODA+9HDA)/ (9ODA+9HDA+10HDAA+10HDA) was only significantly different between native and parasitic workers aged 24-60 hrs (MW: $U_{55,\ 58} =. 1090.5$, $p < 0.0001$). As mandibular profiles became more queenlike, the acceptance of workers declined (fig.

3). This relationship was significant for both native workers (Spearman: *rho* = -0.21, $p \leq 0.04$) and parasitic workers (Spearman: *rho* = -0.34, $p < 0.0001$).

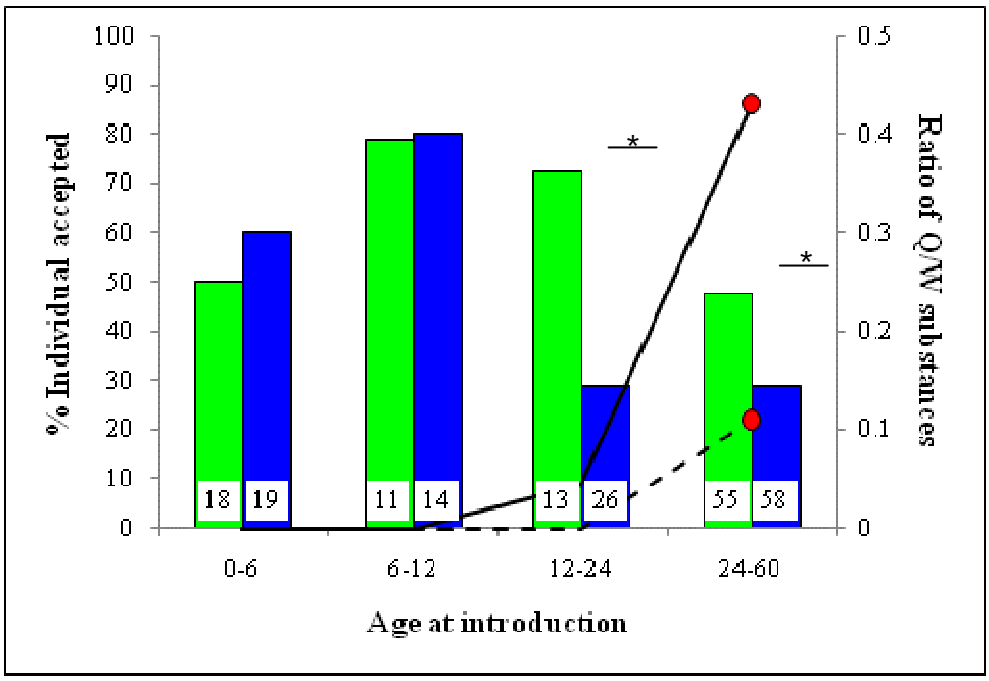

Figure 2: Combination plot of % individuals accepted (bars: native ■ & parasitic ■) & mean ratio (9ODA+9HDA/9ODA+9HDA+10HDAA+10HDA) (native – dashed line & parasitic – solid line) plotted against age at introduction. Acceptance rates of native and parasitic workers were significantly different after 12 hrs (* χ^2), while significant differences in queen/worker compound ratio were only detected after 24hrs (● Mann-Whitney). Numbers in bars indicate the actual number of individuals retrieved. See table 1 for sample sizes used in ratio calculations.

2.4 Discussion

Native and parasitic *capensis* workers produce significantly different mandibular gland signals from emergence to 60 hrs, with parasitic workers showing a faster signal development than native workers. Parasitic workers started producing 9HDA, the 9ODA precursor, after 12 hrs while native workers only did so after 24 hrs. Moreover, parasitic workers produced almost the full range of the QMP after 24 hrs, while native workers did not advance to producing any 9ODA. The absence of 9ODA in native workers suggests that they fail to complete the final oxidation of 9HDA to 9ODA

(Plettner et al., 1996, 1998). These findings support previous studies that illustrated how *capensis* workers swiftly change their mandibular gland signal from workerlike to queenlike under queenless conditions (Crewe et al., 1980; Pankiw et al., 1996). The early onset of 9HDA and 9ODA production we detected in parasitic workers suggests an even more rapid signal development in parasitic workers. This production of typical queen compounds forms an important basis for the reproductive success of these workers and this superior ability of parasitic workers to dominate other *capensis* lineages was also demonstrated by Dietemann et al. (2007). They showed that emergent parasitic workers housed in pairs with either *capensis* queen or worker offspring produced pheromones associated with reproduction at a much faster rate, enabling them to dominate *capensis* worker- as well as queen offspring within four days. Our data suggest that this pheromone arms race start as early as 24 hrs.

An interesting observation was the virtual absence of worker compounds (10HDA, 10HDAA) from the majority of sampled workers. This is similar to what has been observed in the mandibular gland secretions of *A. mellifera* queens (Crewe, 1982). In most *A. mellifera* subspecies, developing queenlike pheromones involves changes in the ratio of queen to worker compounds (Crewe et al., 1980; Crewe, 1988). The virtual absence of worker compounds in our sampled workers suggests that for *capensis* workers to become queenlike the signal development simply requires an increase in queen compound production. In addition, the expression of these queenlike signals was found to be independent of reproductive status as no sampled worker showed any ovary activation. Despite the fact that *capensis* workers have a short latency period of approximately 4 – 6 days (Ruttner et al., 1981) workers in this study (< 3 days old) might still have been too young and phsyiologically underdeveloped to activate their ovaries.

Only 24 % of the 58 parasitic workers, older than 24 hrs, produced queen compounds in highly variable amounts (0.3 - 70µg). The higher observed variability in parasitic profiles, specifically in 9ODA, suggests competition among parasitic workers for access to reproduction in host colonies (Moritz et al., 1996). It is likely that those with a head start in 9ODA production inhibit their sisters from developing pheromonally (Velthuis, 1976; Crewe et al., 1980; Crewe, 1981; Free, 1987; Moritz et al., 2000; Martin et al., 2002).

The rapid production of queen compounds by parasitic workers also has a negative effect in that their acceptance by host colonies decreases significantly. Native workers not yet producing 9ODA were more likely to be accepted than the more queenlike parasitic workers. This supports the findings by Wossler and colleagues (2006) who found that 9ODA was positively correlated to aggression with virgin queens, ≥ 3 day old, eliciting more aggressive behaviour from nestmates than 1 day old virgin queens. Thus lower levels of QMP have been suggested to assist or exaggerate worker acceptance in a colony (Pettis et al., 1998). In our study native workers produced significantly lower quantities of queen compounds and were consequently more readily accepted by host colonies, supporting the view that worker acceptance is partly related to mandibular gland signal profiles. The fact that parasitic workers become established as a result of queen-like signals yet are the individuals most aggressed is paradoxical. This could be explained by the fact that these parasites gain entry into host colonies prior to signal development or another possibility could be that they enter colonies that have suffered queen loss or are preparing to swarm. There are no data to support these claims and therefore further experimentation is necessary.

Our findings show that the mandibular gland profiles of native and parasitic workers are age-dependent and diverge significantly at a very young age. The most marked compositional differences appear right after 24 hrs. Signal ontogeny was found to be more rapid in parasitic workers. Although both native and parasitic workers have the potential to become reproductively active, faster pheromonal development of parasitic workers place them at an advantage, even though ability to infiltrate host colonies is correspondingly decreased. This suggests that there needs to be a balance between signal development and successful infiltration.

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CHAPTER 3

PSEUDOQUEEN ESTABLISHMENT IN GROUPS OF *A. M. CAPENSIS* WORKERS

3.1 Introduction

Social insect societies are often characterized by extreme reproductive skew where reproduction is monopolized by one or a few females. Non-reproductive females fulfil worker roles (Butler, 1957) and are consequently the main contributor to colony success. In many species, the sterility of workers is only functional as they are able to start reproducing at certain stages of colony development or when orphaned. The social milieu (i.e. queen presence and quality) affects the reproductive development of workers (Keller et al., 1993; Strauss et al., 2008). Reproductive plasticity is thus highly context dependent (Bourke et al., 1995; Crozier et al., 1996, Katzav-Gozansky et al., 2004). In addition, workers can enhance their inclusive fitness under certain conditions of kinship by reducing the reproduction of other workers through worker policing (Ratnieks, 1988; Crozier et al., 1996).

Due to the inability of workers to mate, as in many social insect species, honeybee workers produce males, arrhenotokously. However it has been unambiguously demonstrated that reproduction by workers of some ant species (Heinze et al., 1995; Schilder et al, 1999; Grasso et al, 2000; Fournier et al, 2005) and also *capensis* honeybees (Onions, 1912; Anderson, 1963), give rise to female offspring through a process of thelytokous parthenogenesis. *A. m capensis* workers exploit this ability to reproductively parasitize colonies of both other (Allsopp 1993; Neumann et al. 2001; Baudry et al. 2004; Dietemann et al. 2006) and their own subspecies (Härtel et al., 2006a; Jordan et al., 2008).

The population of *capensis* workers parasitizing *scutellata* colonies are clonal (Kryger, 2001a,b) and have been separated from their native population for close to 20 years, and are thus different from more recent demonstrations of *capensis* workers parasitizing *capensis* colonies (Härtel et al., 2006a; Jordan et al., 2008). In both the latter studies, the parasitic *capensis* workers are still part of the native population while our reference to clonal parasitic workers points to those *capensis* workers infesting *scutellata* colonies in Gauteng. An important difference between these two groups of *capensis* workers would be their feeding regimes. Cape larvae reared in *scutellata* host colonies develop into worker-queen intermediates with large numbers of ovarioles and enlarged spermathecae (Beekman et al., 2000; Calis et al., 2002) and their pheromone bouquet is expected to be even more queen-like, which will further enhance parasitism (Calis et al., 2002).

It is believed that reproductive specialization in social insects is mediated by caste-specific pheromones, particularly queen pheromones. Research has shown that queens regulate worker ovary activation through mandibular gland pheromones (QMP, Slessor et al., 2005, Hoover et al., 2003). QMP consists of five compounds namely 9-keto-2(E)-decenoic acid (9ODA), (R,E)-(−) and (S,E)-(+)-9-hydroxy-2-decenoic acid (9-HDA), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA, Slessor et al, 1988; Winston et al., 1989, 1992). Although queens preferentially synthesize 9ODA (queen substance) and workers do so for 10HDA, these biosynthetic pathways are plastic, and each caste can produce the dominant compound of the other (Crewe, 1982; Naumann et al., 1991; Plettner et al., 1995). Virgin queens of most *Apis mellifera* sp. typically produce a mandibular gland signal that is more worker-like with lower amounts of 9ODA and higher amounts of 10HDA, but ageing and mating ultimately induces changes to the signal (Crewe,

1982; Slessor et al., 1990; De Grandi-Hoffman et al., 1993; Plettner et al., 1995; Pankiw et al., 1996; Wossler et al, 2006).

Under queen- and broodless conditions workers can change their mandibular gland signal to a more queen-like signal by producing 9ODA. This switch in biosynthetic pathways is, in particular, strongly expressed in *capensis* workers (Hemmling et al., 1979; Crewe et al., 1980). Strong reproductive competition among workers ultimately results in pseudoqueen establishment of only a very few workers (Moritz et al., 1985, 1996; Hillesheim et al., 1989). Although workers of other honeybee races are also able to produce 9ODA, production in *capensis* workers is greater and under certain social conditions they can change their signal very rapidly (Crewe et al., 1980, Crewe, 1988, Velthuis et al., 1990, Plettner et al., 1993; Ruttner et al., 1981, Simon et al., 2001). These unique characteristics put *capensis* workers at a reproductive advantage over workers from other subspecies (Crewe et al., 1980; Allsopp, 1988; Hepburn et al., 1994; Wossler, 2002). This possibly aided their establishment as social parasites in colonies of *scutellata* and also their own subspecies (Allsopp, 1992; Allsopp et al., 1993; Hepburn et al., 1994; Martin et al., 2002; Härtel et al., 2006a, b).

The movement of *capensis* colonies to the northern parts of South Africa, the native range of *scutellata*, led to their establishment as social parasites of *scutellata*, resulting in the losses of thousands of colonies (Hepburn, et al., 1991; Allsopp, 1993, 1995; Allsopp et al., 1993). Since then research has shown that a single clonal lineage are infesting the colonies in the north (Kryger, 2001a, b; Baudry et al., 2004; Härtel et al., 2006b). Similar to native *capensis* workers, parasitic workers rapidly develop a queenlike mandibular gland secretion assisting them in acquiring reproductive status and suppressing reproductive development in other workers (Crewe et al., 1980; Velthuis et al., 1988; Hepburn, 1992; Moritz et al., 2000, 2004; Simon et al., 2005).

Native *capensis* workers and clonal parasitic workers share characteristics that place them at a reproductive advantage over workers from other subspecies and both groups of *capensis* workers behave as parasites. We therefore wanted to determine if clonal parasitic workers have developed (during 20 years of isolation) more queenlike mandibular gland signals allowing them to out-compete native parasites. To achieve this we assessed how queenlike the mandibular gland profiles of native and clonal parasitic workers are. In this study a single parasitic worker was placed in a Liebefeld cage together with a group of native workers. We tested whether the single *capensis* parasitic worker always produced greater amounts of queen substance than the native workers. If parasitic workers consistently outcompete native workers pheromonally, this may suggest that on the queen-worker development continuum parasitic workers are even more queenlike, thus aiding their parasitic lifestyle.

3.2 Materials & Methods

3.2.1 Experimental setup

Sealed *capensis* parasitic brood frames were obtained from a commercial beekeeper in the Gauteng province of South Africa and native *capensis* brood frames from the Agriculture Research Councils's Plant Protection and Research institute (ARC-PPRI, Stellenbosch). Frames were incubated overnight at 34°C and 60% relative humidity until adult emergence. One hundred Liebefeld cages were set up with a single clonal parasitic worker (< 24 hrs old) together with a cohort of 50 native workers (< 24 hrs old). Cages were set up in a temperature controlled room at the PPRI of the Agriculture Research Council in Stellenbosch. Bees were fed pollen enriched candy and water *ad libitum*. Dead bees were removed from cages daily and water and food replenished when necessary. The experiment was terminated at 10 days since this is

sufficient time to develop mandibular gland signals (supported by Dietemann et al., 2007) and all surviving bees were collected, decapitated and individually labelled heads placed in 200µL dichloromethane (DCM, Sigma) for gas chromatographic (GC) analyses. Bodies of each bee were correspondingly labelled and frozen for ovary dissections.

3.2.2 Ovary Dissections

In order to analyse the reproductive status of the sampled workers, the abdomens were dissected and the developmental stage of the ovaries was assessed using standard criteria (adapted from Velthuis, 1970): 1 = no development; 2 = round or bean shaped eggs visible (early stage of activation), 3 = fully developed ovarioles with mature eggs.

3.2.3 Chemical Analysis

Individual heads were removed from the solvent and the extract evaporated under a stream of nitrogen to dryness and then redissolved in 15µl internal standard (tetradecane and octanoic acid in DCM) and derivatised in 15µl (bis-trimethylsilyl) trifluoroacetamide (BSTFA, Sigma). One microlitre of this solution was injected into a gas chromatograph (Hewlett Packard 6850) equipped with a split-splitless injector and a flame ionization detector. Compound separation was achieved on a cross-linked methyl silicone HP-1 column (25m x 0.32mm) under a temperature programme: 60°C (1 min), 50°C/min to 90°C, 3°C/min to 220°C (10mins) using helium as the carrier gas. The injection port was set at 230°C and the flame ionization detector at 320°C. Peak areas were determined using HP Chemstation software and the mandibular gland compounds were identified based on the retention times of authentic compounds

(Sigma) and were quantified using peak area and the relative mass ratios (for methodological details see Simon et al. 2001).

Since we were interested in a worker's ability to develop a queenlike mandibular gland signal, only compounds of the QMP (methyl p-hydroxybenzoate (HOB), 9-keto-(*E*)-2-decenoic acid (9ODA), 4-hydroxy-3-methoxyphenylethanole (HVA), 9-hydroxydecanoic acid (9HDAA), 9-hydroxy-2-decenoic acid (9HDA) and worker compounds (10-hydroxydecanoic acid (10HDAA), 10-hydroxy-2-decenoic acid (10HDA)) were quantified. Tetradecane was used to determine the relative mass ratios of all the tested compounds (Gehrke et al., 1971) and the absolute amounts calculated. A standard solution was run every day to insure that the relative mass ratios were within the variability range of the series of standard runs (Crewe et al., 1989).

The quantitative ratios of (9ODA + 9HDA)/ (9ODA + 9HDA + 10HDA+10HDAA) were calculated to assess how queenlike mandibular gland pheromone bouquets were (Moritz et al., 2000, 2004; Schäfer et al., 2006). A ratio close to one indicates a queenlike blend, whereas a ratio close to zero indicates a workerlike blend.

3.2.4 Statistical Analysis

Results are reported as absolute amounts, percentages, ratios for individual workers or means (± sd) for groups.

3.3 Results

The ratio of queen to worker substance in all clonal parasitic workers was typically queenlike, > 0.9 (fig.1a). This also held true for the majority of native workers, but more variation was observed between individuals from the native range with ratios

ranging from approximately 0.6 – 0.99 (Crewe, 1982; Strauss et al., 2008). In addition there were three native workers with typical workerlike mandibular gland profiles.

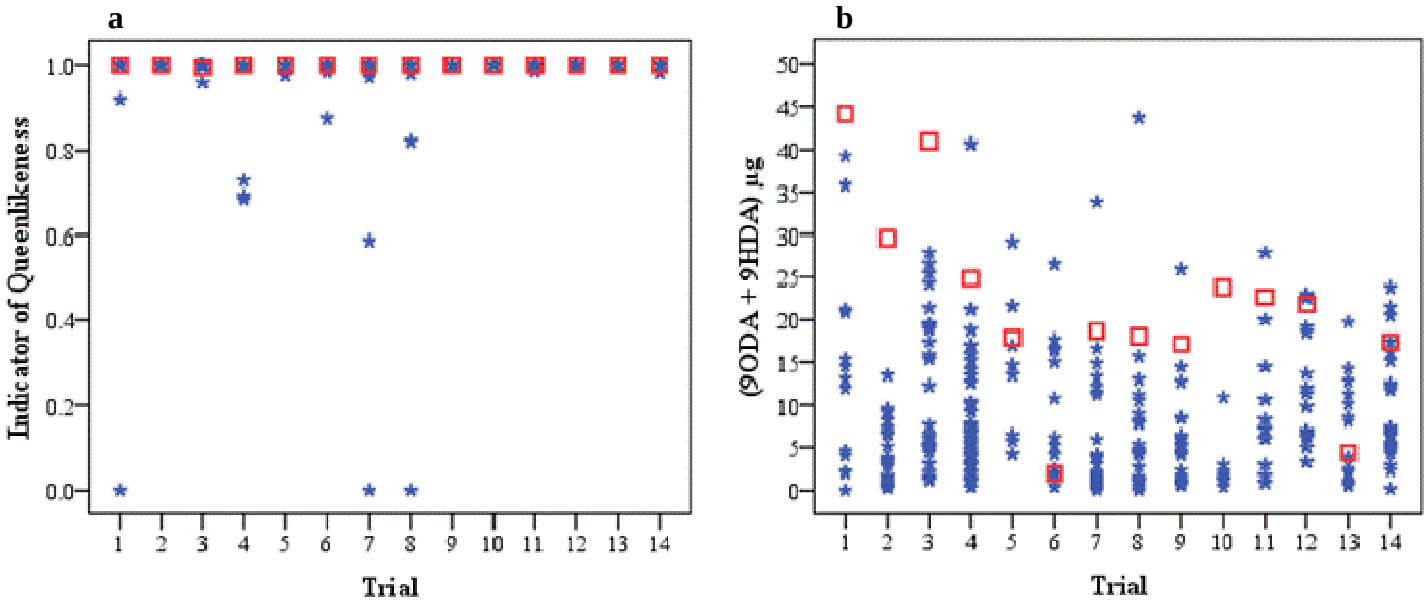

Figure 1: a) Ratio of (9ODA + 9HDA)/(9ODA + 9HDA + 10HDA+10HDAA) of native(*) and parasitic (□) *A. m. capensis* workers. Parasitic workers of only 14 trials (cages) survived to day 10 therefore only workers from these surviving trials were used in the analyses; b) Scatterplot of the absolute amounts of queen substances in the mandibular gland secretions of caged native (*) and parasitic (□) workers. Sample sizes of native *capensis* workers per trial: 1 = 13; 2 = 19; 3 = 25; 4 = 34; 5 = 8; 6 = 14; 7 = 29; 8 = 20; 9 = 18; 10 = 7; 11 = 10; 12 = 15; 13 = 15; 14 = 23

In all trials the mandibular gland profile of groups of native workers and the single clonal parasitic worker was similar in composition, containing almost the full suite of compounds of the queen pheromone complex (fig. 2). The two queen substances, 9ODA and 9HDA, were found to be the dominant compounds in the secretions of both worker groups, contributing > 80% to the total secretion of individuals. The worker compounds 10HDA and 10HDAA were virtually absent in the majority of sampled workers.

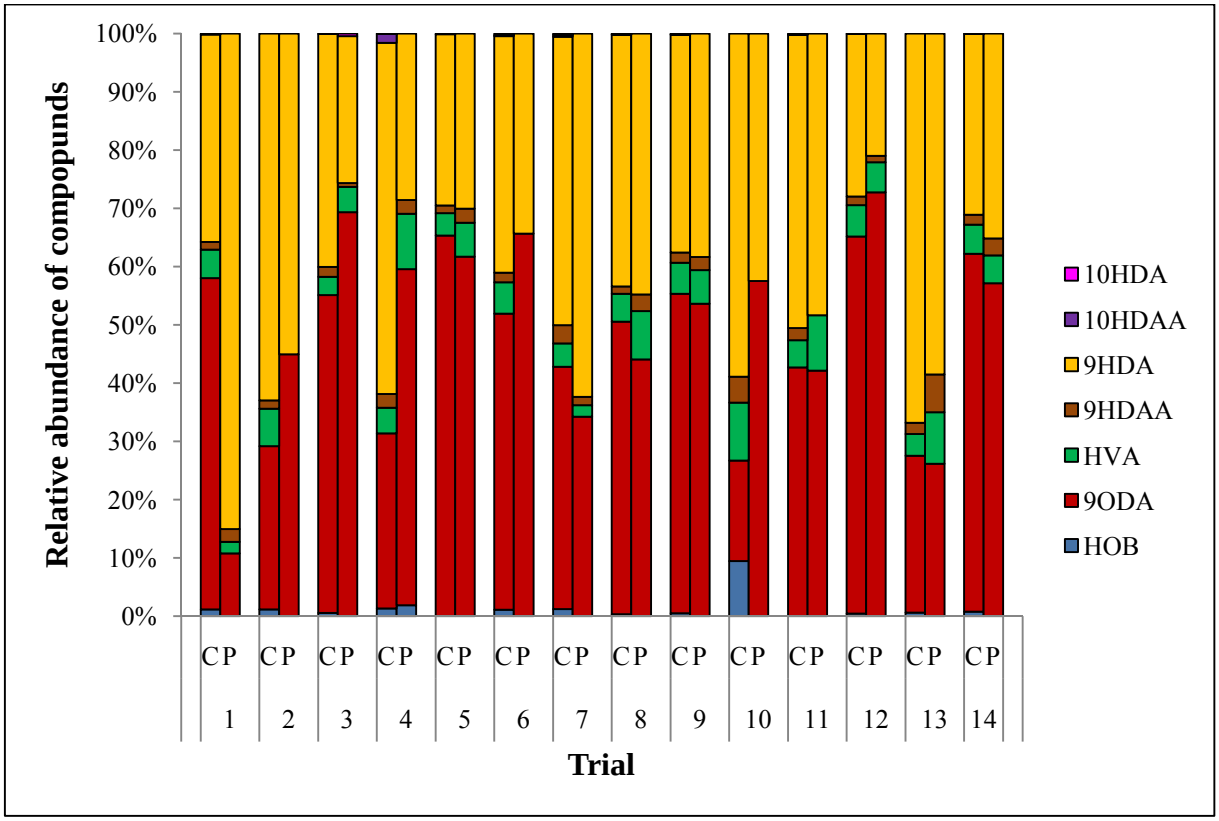

Figure 2: Relative abundance of mandibular gland compounds in the secretions of groups of native (C) and single parasitic (P) *A. m. capensis.* Data for native workers represents the group average per trial.

In 6 out of the 14 trials the single parasitic workers produced higher amounts of 9ODA. The production of 9ODA in the clonal parasitic lineage (n = 14) ranged from 1.29 – 30.01µg and in natives (n = 250) from 0 – 26.69µg. Combining the two queen compounds (9ODA + 9HDA) we found that parasitic workers only outcompeted native workers in 4 trials (trials 1, 2, 3 and 10, see fig. 3). . From figure 1b it is clear that native workers are very variable in their production of queen compounds. Similarly, the parasitic workers also produce variable amounts of queen pheromone across the 14 trials and in many instances the native workers often produced higher levels of these queen compounds compared to the clonal parasitic worker as well as the remaining native workers. No ovary activation was detected in any of the test groups.

3.4 Discussion

Even though the mandibular gland secretions of clonal parasitic workers are very queenlike, these workers are not always pheromonally superior to native workers with regard to the production of queen mandibular gland substances. Similar to the findings of Hepburn (1992) we found the worker compounds, 10HDA and 10HDAA, were virtually absent from native and parasitic workers. These compounds are the major components of most workers of *Apis mellifera* subspecies and their absence in *capensis* workers, both native and parasitic, suggests that these workers readily utilize the queen- rather than the worker biochemical pathway (Plettner et al., 1996; 1997; Moritz et al., 2000). The absolute amounts of 9ODA and 9HDA showed high variation in groups of native workers, yet still overlapped with that of the introduced parasitic worker in most trials. This variability in queen substance production is probably an expression of genetic variation in the native *capensis* population since 9ODA is reported as having a high degree of heritability (Moritz et al., 1985). Since the level of queen compound production is a reliable indicator of worker reproductive success it supports previous reports of high genetic variance for worker reproduction (Moritz et al., 1985; Hillesheim, 1987; Hillesheim et al., 1989; Moritz et al., 1996), suggesting that *capensis* workers do not all have the same potential to develop into successful laying workers. In fact most workers remain sterile. This variation in dominance can be explained by certain patrilines expressing greater reproductive dominance and outcompeting less dominant patrilines. Moritz et al. (1996) studied the genotypic composition of four queenless splits of each of two *capensis* colonies over a 9 week period using single locus DNA fingerprinting and found that the workers of only a few patrilines were able to produce offspring. Moreover, the same patrilines of a colony appeared to become reproductively dominant in all splits. Similarly in their

study on reproductive dominance in groups of queenless *capensis* workers, Simon and colleagues (2005) found that worker reproduction had a high repeatability among subfamilies with 3 out of 30 patrilines producing > 50% of the offspring.

Considering the clonal nature of parasitic workers, less variability is expected due to reduced genetic variance. We however observed high variation in compound production between trials. The resulting hierarchies observed in each trial are likely the result of self-organised mechanisms of worker-worker interactions in response to varying levels of queen compounds (Moritz et al., 2005). The process ultimately results in a reproductive division of labour between reproductive and non-reproductive workers. Although both native and parasitic workers seem to utilize the queen biochemical pathway, as suggested by the absence of worker compounds, the oxidation of 9HDA to 9ODA is inhibited in some workers, who ultimately become subordinate workers.

The ratio of queen- to worker substances failed to show that parasitic workers are pheromonally superior with respect to the mandibular gland secretions since a number of native workers showed comparable proportions of queen substances to that of parasitic workers. This is in contrast with the findings of Dietemann et al. (2007) who established that offspring of parasitic *capensis* pheromonally dominates offspring of *capensis* queens and laying workers within four days after emergence. They evaluated pheromonal dominance between pairs of individuals (i.e. queen offspring *vs* parasitic offspring; native laying worker offspring *vs* parasitic offspring; etc.) under standard laboratory conditions. In our study groups of native workers were caged with a single parasitic worker. Therefore our results may reflect worker-worker competition for access to reproduction in the absence of a queen (Moritz et al., 1996). Workers are thus inhibiting each other from developing as pseudoqueens (Velthuis, 1976; Crewe et

al., 1980; Crewe, 1981; Free, 1987; Moritz et al., 2000). Although Dietemann et al. (2007) found that queen offspring were dominated by parasitic workers in most test pairs; there were instances where they were not. This is in line with our observations where the production of (9ODA+9HDA) was higher in parasitic workers in only some of our trials supporting the view that the reproductive success of certain patrilines (in this instance the single clone) in one colony may not be duplicated in another when other potentially more dominant patrilines are present (Dietemann et al., 2007; Martin et al., 2004; Moritz et al., 2000). In support of earlier analyses of Cape worker pheromones (Hemmling et al., 1979; Velthuis et al., 1990; Hepburn 1992) these findings corroborate the existence of a queen-worker development continuum along which pseudoqueens are intermediates, possessing a queenlike pheromonal bouquet but remain reproductively workerlike.

The ratio of (9ODA + 9HDA) / (9ODA+ 9HDA + 10HDAA+10HDA) has been used as an indicator of reproductive dominance (Moritz et al., 2004). However, the secretion of a queenlike mandibular gland pheromone in this study was not associated with concomitant ovary activation in workers. The reason for the lack of ovary development is not clear but it could be attributed to numerous factors. Proteins are essential for oogenesis whereas a lack of proteins will limit it (Wheeler, 1996). In this study workers were fed on candy patties mixed with pollen however it is possible that insufficient pollen was added to the food source. The ability of workers to develop their ovaries and lay eggs is dependent on the amount and quality of nourishment they receive both as adults and larvae. Young workers are thus dependent on older workers for food resources. Lin et al (1998) observed that when caged workers were fed a protein rich diet, ovary development increased correspondingly. Although the effect of age on ovary development is not clear, Hepburn et al. (1994) found that *capensis*

workers underwent some ovary development within 14 days of losing their queen. Since workers in this study were only aged under queenless conditions for 10 days, this could also be a contributing factor for the lack of ovary development. However it is largely the complex blend of mandibular pheromones that inhibit the development of workers' ovaries in the presence of a dominant individual (Moritz et al. 1996; 2000). It is thus also possible that mutual inhibition between workers in this caged experiment could account for the lack of ovary development.

Previous studies investigating pheromone production in pseudoqueens (Velthuis, 1970; Crewe et al., 1980; Plettner et al., 1993) showed that the production of a queenlike pheromonal bouquet may precede ovary development. Thus pseudoqueens do not necessarily have to have developed ovaries to switch their mandibular gland signal from workerlike to queenlike. Malka et al. (2008) suggested that queen absence triggers the onset of queen pheromone production in workers with compound production being independent of ovarian development. The continued production of these pheromones is however dependent on ovarian development. Studies focussing specifically on Cape honeybees have demonstrated a relationship between ovary activation and a more queenlike mandibular gland substance production (Crewe et al., 1980; Moritz et al., 1985; Crewe, 1987; Allsopp, 1988; Velthuis et al., 1990) while others failed to do so (Hemmling et al., 1979; Hepburn et al., 1988). In our study we found no link between ovary activation and queen pheromone production.

In summary therefore, genetic variance among workers facilitates QMP production and it is thus inevitable that those workers which are genetically predisposed to become pseudoqueens will become dominant over others. The fact that native *capensis* workers parasitize colonies of their own subspecies can be explained by the presence of dominant patrilines which are likely to express the right combination of

traits to become local-based parasites. Consequently some dominant native workers are capable of pheromonally outcompeting clonal parasitic workers. So although clonal parasitic workers rapidly develop their mandibular gland signal in the first 2-3 days, post-emergence, this does not equate them to winning the pheromonal dominance contest over native workers over time.

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CHAPTER 4

DO THE MANDIBULAR GLAND PROFILES OF *APIS MELLIFERA CAPENSIS* WORKERS VARY WITH LEVELS OF RELATEDNESS?

4.1 Introduction

In the darkness of the honeybee colony an intricate chemical communication system exists that maintains the cooperation between colony members and the organization of various colony tasks. The achieved colony cohesion is largely maintained through pheromone emissions from the resident queen. The honeybee queen produces pheromones that prompt workers to feed and groom her, attract drones on mating flights (Gary, 1962; Kaminski et al., 1990) as well as regulating worker reproductive development (Hepburn 1992; Wossler et al., 1999a, 1999b, Katzav-Gozansky et al. 2001; Hoover et al. 2003, 2005). These pheromones are dispersed through the colony via worker-worker transmissions (Naumann et al., 1992; Winston et al., 1992; Pankiw et al., 1994; Slessor et al., 2005). Thus, workers who normally refrain from reproducing will often on queen loss start to develop reproductively.

Workers of *Apis mellifera capensis* (hereafter *capensis*) are unique in that they produce unfertilized eggs that results in clonal female offspring, through a process of thelytokous parthenogenesis (Verma et al., 1983; Baudry et al. 2004; Oldroyd et al. 2008). This is in contrast to other subspecies in which worker reproduction gives rise to male offspring (arrhenotoky; Ruttner, 1992; Crozier et al., 1996). Thelytoky is determined by a single gene which also affects egg-laying and pheromone production (Lattorf et al., 2005, 2007). In addition, c*apensis* workers have increased reproductive potential due to the large numbers of ovarioles (10-20) compared to other subspecies

(3-5) (Ruttner, 1977; Allsopp et al., 2003) and reproductively dominant individuals produce a very queenlike mandibular gland signal, dominated by the queen substances 9ODA and 9HDA (Crewe et al., 1980; Moritz et al., 2000).

Average relatedness levels used in this study were based on the assumption of the haploid-diploid sex determination mechanism present in honeybees (and generally in *Hymenoptera*). Queens and workers have two sets of chromosomes making them diploid while drones only have one set, and are thus haploid (Harbo et al., 2002). Consequently, due to the polyandrous nature of honeybee queens (Adams et al., 1977; Koeniger, 1987; Koeniger et al., 2000; Palmer et al., 2000), natural colonies consist of a mixture of super-sisters and half sisters (Page et al., 1988). As a result of thelytoky, workers are related to their offspring by unity ($r \approx 1$) and on average, equally related to the female offspring of their sister workers ($r \approx 0.3$, assuming an effective paternity of 10, Greeff, 1996; Ratnieks, 2002) as they are to the queen's offspring (Ratnieks, 1988; Pirk et al., 2003). According to Ratnieks (2002) honey bee queens mate with approximately 10 males so that the workers in a colony are mostly half sisters, with an average relatedness of 0.30. If the queen only mated with one male then the workers would all be super-sisters and be related by 0.75, whereas if every worker had a different father then workers would all be half sisters and be related by 0.25 (Pirk et al., 2003)

In this study our aim was to determine whether groups, characterized by different levels of relatedness (0-99% average relatedness) could be delineated on the basis of their mandibular gland profiles. In essence this is not a novel idea, with a study by Dani et al. (2004) demonstrating that the cuticular hydrocarbon profile of the wasp, *Polistes dominulus*, contains genetic information, due to the strong correlation found between hydrocarbon composition and the level of relatedness. Moreover, it was also

found that the cuticular hydrocarbon profiles in honeybees are partly genetically based (Page et al., 1991b; Arnold et al., 1996). Page et al. (1991b) found differences in the lipid composition between two worker patrilines in honeybee colonies headed by artificially doubly mated queens. In addition, workers in a honeybee colony with a single queen, mated 16 times, could be correctly assigned to their patriline on the basis of their cuticular lipid composition (Arnold et al., 1996). Consequently mandibular gland secretions, also of exocrine origin, are probably also genetically determined. This was indeed established by Moritz et al. (1985) in their investigation on reproductive dominance in *capensis* workers, and they showed that queenlike mandibular pheromone secretions in honeybee workers were genetically determined (Moritz et al., 1985; Lattorff et al., 2007). More recently however, Härtel and his co-workers demonstrated that the regulation of the final synthesis from the precursor 9HDA to the end product 9ODA is strongly influenced by the social environment (Härtel et al., in review).

The findings of Jordan et al. (2008) suggested that the expression of the queen phenotype in workers is under both genetic and environmental control. They investigated traits (number of ovarioles, number of basitarsal hairs, and size of spermatheca) advocated to be associated with reproductive potential in *capensis* workers. These traits were found to be influenced by the genotype and rearing environment of the individual except for the number of ovarioles which was less affected by the rearing environment. In chapter 7 we studied the effect of genotype on the reproductive potential of workers of the pure *capensis* and hybrids of *capensis* and *scutellata*. Our findings support the view that the worker genotype affects the reproductive potential of workers in queenless colonies, particularly the production of typical queen compounds but realizing this in the context of the social environment.

In light of this we expected that mandibular gland profiles of workers sharing a greater proportion of genes would converge, and those sharing less would show more profile variability when we controlled for environmental variables.

4.2 Materials & Methods

4.2.1 *Experimental setup*

Single *capensis* workers (< 24 hrs) of various origins and estimated levels of relatedness (proxy for the proportion of shared genes on average) were distinctly marked on the thorax with non-toxic paint (Posca Paint Pens, Mitsubishi Pencil Co., Japan) and introduced into respective Liebefeld cages containing 50 1-day old *A. m. scutellata* (hereafter *scutellata*) workers. The single introduced *capensis* workers originated from: i) a parasitized *scutellata* colony (Clones; r ≈ 0.99 - group 1); ii) a colony of which the queen was artificially inseminated with the semen of a single drone (r ≈ 0.75, group 2); iii) a colony of which the queen was artificially inseminated with the semen of five drones, however a functional paternity of two (r ≈ 0.5, group 3); iv) workers were collected from a single, naturally mated *capensis* native colony (r ≈ 0.3 - group 4, assuming an effective mating of 10); v) a single worker from 50 geographically separated *capensis* native colonies (r = 0, group 5). The intention was that workers from group 3 had a functional paternity of five, however microsatellites analysis (see chapter 6) revealed that three of the five drones were related and the other two were also related. Therefore for the purpose of our analysis, group 3 consisted of two patrilines with average relatedness of workers in this group ≈ 0.5.

Fifty Liebefeld cages per group were set up. *A. m. scutellata* (< 24 hrs) were collected from emerging brood frames sourced from the same donor colony to eliminate host worker effects. Workers for group 5 were collected from their respective natal

colonies as they were emerging. Bees were fed a pollen enriched candy pattie and water *ad libitum*. Dead bees were removed daily and cages cleaned. Dietemann et al. (2007) established that *capensis* queen and worker offspring pheromonally dominated *scutellata* workers within 4-7 days, thus all bees were aged for 10 days. All surviving individuals (*capensis* and *scutellata*) were harvested from the cages and placed in a fridge until immobile. Heads of individual workers were removed and placed in distinctly labeled vials with 200µl of dichloromethane (DCM, Merck) for gas chromatographic analyses. Abdomens of individuals were correspondingly labeled and frozen for ovary dissections.

4.2.2 Ovary Dissections

In order to analyse the reproductive status of the sampled workers, the abdomens were dissected and the developmental stage of the ovaries was assessed using standard criteria (Velthuis, 1970): 1 = no development; 2 = round or bean shaped eggs visible (early stage of activation), 3 = fully developed ovarioles with mature eggs.

4.2.3 Chemical analysis

The heads were removed from the vials and the DCM evaporated under a stream of nitrogen just to dryness. The residue was redissolved in 15µl internal standard (octanoic acid and tetradecane in 4 ml dichloromethane) and 15 µl bis-(trimethylsilyl) triflouroacetamide (BSTFA, Merck). One microlitre was injected into a gas chromatograph (HP 6850) with a split-splitless inlet and a 25mm x 0.32 mm methyl silicone coated fused silica capillary column. Helium was used as carrier gas at a flow rate of 1.9ml/min. The oven temperature was programmed as follows: the temperature was kept for 1 min at 60 °C; followed by a heating phase of 50°/min to

110°C, and subsequently another of 3°C/min to 220°C. Finally the temperature was held at 220°C for 10 min. Peak areas were determined using HP Chemstation software. The mandibular gland compounds were identified based on the retention times of synthetic compounds or their retention time relative to the internal standards, and were quantified using peak area.

The compounds quantified in this study and used in the subsequent analyses included: methyl p-hydroxybenzoate (HOB), 9-keto-(*E*)-2-decenoic acid (9ODA), 4-hydroxy-3-methoxyphenylethanole (HVA), 9-hydroxydecanoic acid (9HDAA), 9-hydroxy-2-decenoic acid (9HDA), 10-hydroxydecanoic acid (10HDAA), 10-hydroxy-2-decenoic acid (10HDA), stearic acid, and some minor aliphatic acids, palmitoleic acid and palmitic acid. Oleic acid was excluded from the analysis as it was the most abundant compound in all sampled individuals and its inclusion masked subtle but important differences.

4.2.4 Statistical Analysis

The total peak area for all quantified compounds per sample was standardized to 100% and a multivariate analysis (using SPSS 17.0) was performed to assess the divergence/convergence of the chemical profiles of the various family groups. Peak areas represent compositional data and were thus transformed to logcontrasts (Aitchinson, 1986) prior to the analysis. The original transformation procedure makes it necessary to exclude compounds that do not occur in all samples. We therefore modified the transformation ($\log_{10}$ (relative peak area/ (geometric mean of all peak areas) + 1)) to avoid undefined values for peaks with an area of zero.

The transformed peak areas were subjected to a discriminant analysis (DA) to assess whether groups with different relatedness levels could be separated on the basis of

their mandibular gland pheromone profiles. The DA also produces a classification matrix which reports how well the cases (individuals) have been placed in their known groups based on the discriminant functions.

4.3 Results

The discriminant analysis revealed a significant separation of the individuals according to their levels of relatedness (Wilk's $\lambda = 0.117$ $\chi^2 = 273.44$ $df = 40$ $p < 0.0001$. fig. 1) with 76.5% of all individuals correctly classified to their respective groups.

Discriminant function 1 explained 51.9% of the variation and largely separated groups 1 and 2 from 3. Although some overlap was observed, it also separated groups 4 and 5 from group 3. This function was highly correlated with the queen substance 9ODA (Spearman's rank correlation $r = 0.66$, $p < 0.0001$). Function 2 explained 29% of the variation and separated groups 1, 2 and 3 from groups 4 and 5.This function was associated with stearic acid and palmitic acid.

The profiles of groups 1 and 2 showed a high tendency to cluster with the lowest degree of variation in their profiles and this is also reflected in the classification results (table 1). Ninety six and 92% of groups 1 and 2, respectively, were correctly classified. The variability in profiles increased in group 3 and 4 individuals with 79% and 78% of group members assigned to their correct groups. The profiles of group 5's individuals were the most variable with only 44% correctly classified. (table 1).

The mandibular gland profiles of the *scutellata* host workers used in this study were distinctly workerlike, with no 9HDA or 9ODA present in their extracts. We observed no ovary development in any of the sampled individuals.

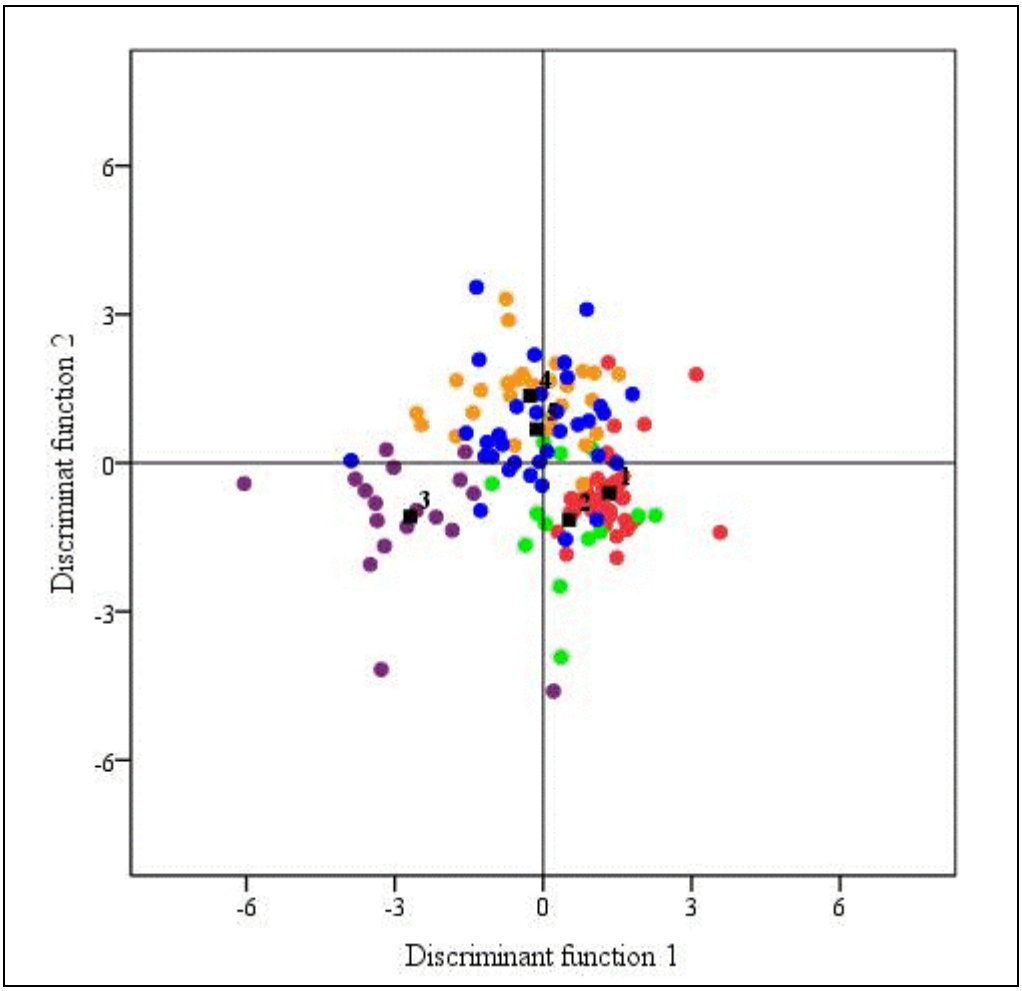

Figure 1: Discriminant analysis of the five test groups of *A. m. capensis* workers. Despite some overlap, the groups were significantly separated on the basis of the relative areas of ten pheromones peaks: ● group 1, n= 42; ● group 2, n = 13; ● group 3, n = 17; ● group 4, n = 28; ● group 5, n = 34, ■ group centroids.

Table 1: Classification results of the discriminant analysis for the five test groups.

	Predicted group				
Actual group	1	2	3	4	5
1	96	2	0	2	0
2	0	92	0	0	8
3	5	0	79	5	11
4	4	4	7	78	7
5	6	12	6	32	44

Data are the proportions of classifications to the different test groups (%).
See Materials and Methods for levels of relatedness for each group

4.4 Discussion

Our data supports the existence of a link between the level of relatedness and mandibular gland profile. The results show that that mandibular gland profiles became more variable as relatedness decreased. The assumption that parasitic workers' (group 1) profiles would show a high degree of signal convergence, due to their clonal nature, was definitively confirmed. Under queenless conditions, the mandibular gland secretions of parasitic individuals become increasingly queenlike, with the production of 9HDA and 9ODA (Dietemann et al., 2007; Moritz et al., 1996). This is true for our data and was supported by the fact that the first discrimant function which was associated with the queen substance, 9ODA, and was responsible for ≈ 52% of the observed variation. Although parasitic individuals might acquire a queenlike pheromone phenotype more rapidly and frequently than native *capensis* workers (see chapter 2), not all parasitic individuals fully progress to becoming pseudoqueens (see chapter 3), thus a range of intermediates between worker and queen phenotypes are possible (Beekman et al., 2008; Calis et al., 2002).

The lack of ovary development observed in this study could be attributed to the potentially low protein diet that sampled workers consumed (Wheeler, 1996), their age (± 10 days old, Hepburn et al., 1994) or the inhibition by more dominant individuals (Moritz et al., 1996; 2000).

Since individuals shared all maternal genes on average as well as ageing environment, the variability observed can be ascribed to patriline differences. Within honeybee colonies members of certain patrilines are more likely to develop into pseudoqueens (Moritz et al., 1996; Martin et al., 2004; Härtel et al., 2006a; Makert et al., 2006), suggesting high genetic variance for reproductive dominance among worker subfamilies. Reproductive dominance hierarchies are largely mediated by workers' mandibular secretions, and the more queenlike the secretions, the higher the worker's position in the hierarchy (Moritz et al., 2000, 2004; Simon et al., 2005; Dietemann et al., 2006, 2007). Unfortunately not all individuals were genotyped and therefore we cannot assign dominance to a given patriline.

Our experimental setup also presented us with a social environment (queenless with *scutellata* workers) ideal for pseudoqueen development. This is in contrast to ageing clones or *capensis* with their own subspecies in queenless colonies. In these social environments, reciprocal suppression is evident with a large proportion of these workers failing to complete the final oxidation step from 9HDA to 9ODA (see chapter 2). This supports the recent work by Härtel and his co-workers (in review) who found that the final synthesis from the precursor 9HDA to the end product 9ODA is strongly influenced by the social environment.

While this may be true in field-based colonies, when clones and *capensis* workers aged together in small Liebefeld cages all but a few advanced to producing 9ODA (see chapter 2). Thus, in this confined space workers are more likely to interact,

creating the opportunity for worker-worker competition for pheromonal dominance. Simon et al. (2001; 2005) found that the mandibular gland amounts in paired bees were three times higher than in isolated workers. Also the composition of the secretion differed, with isolated bees producing an average of 7.9% of 9ODA whereas bees kept in groups produce 44.2% 9ODA. Therefore the production and composition of the mandibular gland components certainly are affected by the social environment but laboratory and field-based trials may not always render similar trends. Isolated or spatially separated workers are not enforced into pheromone competition with other workers and as a result their pheromone signals will remain workerlike.

The results provide support for our hypothesis that workers having a higher proportion of genes in common have a more similar mandibular gland profile than workers sharing a lower proportion of genes. We observed that pheromone composition is more similar within than among family groups. Breed et al. (1988) showed that workers aged in lab (from pupae) were able to discriminate sisters from non-sisters, using hydrocarbon cues. However, these workers were rejected when introduced into parental colonies, so hydrocarbons did not provide the entire explanation for honeybee nestmate recognition. Bees use secretions from mandibles containing fatty acids which are used to modify comb wax. Nestmate recognition bioassays with fatty acids yielded significant results, providing information to differentiate kin and are thus active in a recognition pheromone bioassay (Breed, 1998). Thus being a suggested means of nestmate recognition in honeybees our results demonstrate that mandibular gland secretions possibly possess the necessary prerequisites of sufficient variation and genetic determinism for use as labels in patriline recognition.

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CHAPTER 5

MANDIBULAR GLAND SIGNAL VARIABILITY WITHIN AND BETWEEN NATIVE AND PARASITIC *A. M. CAPENSIS* WORKERS AGED IN QUEENLESS *A M. SCUTELLATA* COLONY SPLITS

5.1 Introduction

In most social Hymenoptera (ants, bees and wasps) individuals are morphologically specialized for the performance of reproductive and helper roles: queens produce eggs while functionally sterile workers perform a range of activities (Wilson, 1971; Hölldobler et al., 1990). Honeybee queens exhibit extreme levels of polyandry (Adams et al., 1977; Koeniger 1987, Koeniger et al., 2000; Palmer et al., 2000) which drastically reduce the average relatedness between nestmate workers. Within the monogynous honeybee colony workers sired by the same drone are on average 75% related while those sired from different drones are approximately 30% related (Pamilo et al., 1982; Page et al., 1988, Breed et al., 1994; Estoup et al., 1994).

Insect societies require a highly developed communication system due to their complex organization. The organization of group-level behaviour in social insects is mediated by pheromone signals that regulate social behaviour (Wilson, 1971). Pheromones are synthesized and stored in exocrine glands and behave either as primer or releaser pheromones (Free, 1987). The mandibular gland secretion (MGS) is composed of a large number of compounds, however the major signal of queen presence is conferred by a five compound blend, namely (E)-9-keto-2-decenoic acid (9ODA – queen substance), two enantiomers of (E)-9-hydroxy-2-decenoic acid (9HDA), methyl-*p*-hydroxybenzoate (HOB) and 4-hydroxy-3-ethoxyphenylethanol (HVA) (Slessor et al., 1988).

The mandibular gland signals of queens are dominated by 9ODA while that of workers are dominated by 10-hydroxy-2-decenoic acid (10HDA), and 10-hydroxydecanoic acid (10HDAA) (Plettner et al. 1996, 1998). As a primer pheromone, queen mandibular pheromone (QMP) regulates worker reproduction (Butler, 1959; Hepburn 1992; Hoover et al. 2003, 2005) and as a releaser pheromone, it elicits behaviours such as retinue formation and attracts drones during queen mating flights (Gary, 1962; Kaminski et al., 1990).

Through pheromone signalling the queen exerts her influence over colony members, thus maintaining harmonious and functional cohesion of the unit. However, this system is not flawless, as the presence of reproductively active workers in queenright colonies suggest (Ratnieks et al., 1989). The mandibular gland secretions of these workers have a similar composition to that of queens and as a result elicit behaviours from workers as would a queen (Crewe et al., 1980; Velthuis et al.; 1990). These pseudoqueens inhibit other workers from activating their ovaries and developing a queenlike mandibular gland signal (Moritz et al., 2000). Worker reproduction is generally tightly regulated, whether by control or honest signalling, through queen pheromones (Keller et al., 1993). The mechanisms underpinning this process are not fully understood and several lines of evidence have been proposed (Mohammedi et al., 1998; Wossler et al., 1999b; Hoover et al. 2003). In spite of the existing evidence, according to Katzav-Gozansky et al. (2006) the data on pheromone regulation of worker exocrine expressions are limited.

Apis mellifera capensis (Cape honeybee, hereafter *capensis*) are native to the Western Cape region of South Africa. Workers of this subspecies have the ability to establish themselves as social parasites in colonies of other subspecies as well as their own (Allsopp, 1992; Allsopp et al., 1993; Hepburn et al., 1994; Martin et al., 2002; Härtel

et al., 2006a, b, Jordan et al., 2008). These workers have a unique set of traits that possibly aided their establishment as social parasites among which include the capacity to produce a queenlike pheromone signal (Hemmling et al., 1979; Crewe et al., 1980). Through their unique thelytokous mode of reproduction they produce females that are almost clonal (pseudoclones) in nature (Moritz et al., 1994; Baudry et al., 2004). These distinctive traits have contributed to *capensis* workers successfully establishing themselves as social parasites in *Apis mellifera scutellata* (hereafter *scutellata*) colonies after their anthropogenic movement into the native range of the latter (Allsopp, 1992; Allsopp et al., 1993; Greeff, 1997; Neumann et al., 2002). Genetic analyses suggest that the current parasitic population is derived from a single worker lineage (Kryger 2001a, b; Baudry et al., 2004; Dietemann et al., 2006; Härtel et al., 2006a).

In this study we investigated the mandibular gland signal variability within and between *capensis* native and parasitic populations in queenless colonies of *scutellata* so as to optimize signal development. The native population is a natural population that grows through polyandrous reproduction leading to high genetic variability (Crozier et al., 1985; Keller et al., 1994; Oldroyd et al. 1998; Cole et al., 1999; Palmer et al., 2000) among colony members, while on the other hand the parasitic population originated from one (or a few) workers parthenogenetically and are as a result almost clonal thus displaying low genetic variability.

Mandibular gland signal variability exists not only between subspecies but also between individuals (Moritz et al., 2000). It has been suggested that the qualitative rather than quantitative differences are responsible for the variation in the relative proportions making up the mandibular gland extracts (Crewe, 1988, Velthuis, 1985; Velthuis et al., 1990; Pankiw et al., 1996). In their investigation of reproductive

capensis workers (clonal offspring) Moritz et al. (1985) found a high heritability estimate for the production of 9ODA (h = 0.82). In contrast Wossler (unpublished data) found that 9ODA was strongly dependent on environmental influences with minimal genetic influence. Recently however, Härtel et al (in review) proposed that the mandibular gland biosynthetic pathway chosen by workers are genetically determined but the regulation of the final synthesis from the precursor 9HDA to the end product 9ODA is strongly influenced by the social environment.

Our results from chapter 4 suggest that the social environment of workers has a substantial influence on mandibular gland signal phenotype. We also observed lower variability in the profiles of clonal parasitic workers. We expect that similar trends will prevail in this field-based experiment owing to the clonal nature of these parasitic workers.

5.2 Materials & Methods

5.2.1 Experimental setup

Brood frames were collected from a native *capensis* colony in the Stellenbosch area and clonal *capensis* brood frames were obtained from a commercial beekeeper in Gauteng. Frames were placed in an incubator at 34ºC and 60% relative humidity and workers allowed to emerge. The resident queens were removed from four queenright *scutellata* colonies (1-4) which were then split into two queenless halves (A, B). During the course of the experiment one colony split (4B) swarmed, therefore only the data from the three remaining colonies were included in the final analysis. For each discriminator colony the two halves (A & B) were genotypically the same therefore removing any genetic bias. Colonies were housed in 5 frame standard Langstroth boxes containing two brood frames and food frames in an apiary at ARC-

PPRI (Stellenbosch, South Africa 33º56´S, 18º52´E). Three hundred emerging workers (<24 hrs old) of both test groups were distinctly marked with a non-toxic marker (Posca Paint Pens, Mitsubishi Pencil Co., Japan) on the thorax and introduced into each split of the experimental colonies. One split of a colony received only native workers (A) and the other split only parasitic workers (B). These workers were allowed to age undisturbed for ten days, the experiment was then terminated and samples harvested (Dietemann et al., 2007). Heads of individual workers were removed and placed in labelled vials with 200µl of dichloromethane (DCM, Merck) for gas chromatography. Bodies were stored and frozen in correspondingly labelled vials for ovary dissections to score stage of development.

5.2.2 Ovary Dissections

In order to analyse the reproductive status of the sampled workers, the abdomens were dissected and the developmental stage of the ovaries was assessed using standard criteria (Velthuis, 1970): 1 = no development; 2 = round or bean shaped eggs visible (early stage of activation), 3 = fully developed ovarioles with mature eggs.

5.2.3 Chemical analysis

The heads of individual workers were removed from the solvent and evaporated with a stream of N_2 to dryness. The residue was redissolved in 15µl internal standard solution (tetradecane and octanoic acid, Sigma) and derivitised in 15µl bis-(trimethylsilyl) trifluoroacetamide (BSTFA, Merck). One microlitre of this solution was injected into a gas chromatograph (Hewlett Packard 6850) equipped with a split-splitless injector and a flame ionization detector. Compound separation was achieved on a cross-linked methyl silicone HP-1 column (25m x 0.32mm) under a temperature

programme: 60°C (1 min), 50°C/min to 90°C, 3°C/min to 220°C (10 mins) using helium as the carrier gas. The injection port was set at 230°C and the flame ionization detector at 320°C. Peak areas were determined using HP Chemstation software and the mandibular gland compounds identified based on retention times relative to authentic standard compounds (Sigma, Wossler et al., 1999).

The compounds identified and quantified in this study included: methyl-*p*-hydroxybenzoate (HOB), 4-hydroxybenzoic acid (4HBA), 9-keto-(*E*)-2-decenoic acid (9ODA), 4-hydroxy-3-methoxyphenylethanole (HVA), 9-hydroxydecanoic acid (9HDAA), 9-hydroxy-2-decenoic acid (9HDA), 10-hydroxydecanoic acid (10HDAA), 10-hydroxy-2-decenoic acid (10HDA). Tetradecane was used to determine the relative mass ratios of all the quantified compounds (Gehrke et al., 1971) and the absolute amounts calculated. A standard solution was run every day to ensure that the relative mass ratios were within the variability range of the series of standard runs (Crewe et al., 1989).

5.2.4 Statistical Analysis

The variance in the quantity of the 8 selected compounds between native and parasitic workers was analysed using Mann–Whitney U tests, to determine whether differences in variation of compounds between worker types exist. The same procedure was used to examine the variance of the relative proportions (transformed to log contrasts, Aitchinson, 1986) of the measured compounds. A discriminant analysis was performed to determine whether the native and parasitic workers could be separated on the basis of their mandibular gland profiles. Similar trends were observed in compound production, for the respective worker groups in all three test colonies, therefore data for all native workers were pooled as was for parasitic workers. The

ratio of queen to worker substances was determined as this has been suggested to be a good indicator of reproductive dominance (Moritz et al., 2000; Strauss et al., 2008).

5.3 Results

5.3.1 Absolute amounts of mandibular gland compounds

The combined total amounts of all eight identified mandibular gland compounds (HOB, 4HBA, 9ODA, HVA, 9HDAA, 9HDA, 10HDAA, 10HDA) showed a significant difference between native and parasitic workers (MW: $U_{121, 212} = 19651.5$; $p < 0.0001$). Native workers produced 6.92 ± 0.81µg while parasitic workers produced 23.56 ± 1.53µg of the 8 selected compounds of the mandibular gland secretions (mean ± SE).

The absolute amounts of the individual compounds revealed specific differences between native and parasitic workers for all compounds except for 10HDA (table 1, fig.1, MW: $U_{121, 212} = 13985.5$, $p = 0.06$). This is however not very clear from figure 1 due to the scale used necessary to accommodate the extreme values. Native and parasitic workers were significantly different with respect to the worker compound 10HDAA (MW: $U_{121, 212} = 11589.5$, $p = 0.001$), with native workers producing higher levels and showing more variability. A similar trend was also observed for 4HBA. The significant differences between native and parasitic workers in HOB, 4HVA and 9HDAA were largely due to their absence in native workers.

The most marked differences observed were in the typical queen compounds, 9ODA and 9HDA. Parasitic workers produced significantly more 9ODA than native worker (MW: $U_{121, 212} = 20006.5$, $p < 0.0001$, fig. 1) and in addition parasitic workers showed more variability ($sd = 20.32$) in 9ODA production than native workers ($sd = 8.27$). The same trend was observed in 9HDA production with parasitic workers producing

significantly higher amounts (MW: $U_{121,\ 212}$ = 19470.5, $p < 0.0001$) and showing slightly more variability (*sd*: native workers = 1.88, parasitic worker = 2.54). No ovary development was detected in any of the individuals sampled.

Table 1: Absolute amounts (µg; mean ± SE) of compounds present in the mandibular gland extracts of native and parasitic workers of *A. m. capensis* aged in queenless splits of *A. m. scutellata* colonies.

		Compounds							
A.m capensis workers	N	HOB	4HBA	9ODA	HVA	9HDAA	9HDA	10HDAA	10HDA
Native	121	-*	0.14 ± 0.03*	5.47 ± 0.75*	-*	-*	0.87 ± 0.17*	0.15 ± 0.04*	0.32 ± 0.12*
Parasitic	212	0.07 ± 0.01*	0.01 ± 0.005*	20.87 ± 1.4*	0.01 ± 0.003*	0.02 ± 0.004*	2.42 ± 0.17*	0.02 ± 0.01*	0.13 ± 0.06*

Abbreviations for the compounds are as follows: HOB = methyl-*p*-hydroxybenzoate; 9ODA = (E)-9-oxodec-2-enoic acid; HVA = 4-hydroxy-3-methoxyphenylethanol;
9HDAA = 9-hydroxydecanoic acid; 9HDA = (E)-9-hydroxydec-2-enoic acid; 10HDAA = 10-hydroxydecanoic acid; 10HDA = (E)-10-hydroxydec-2-enoic acid.
* Denotes significant differences at $p < 0.05$ (Mann-Whitney).

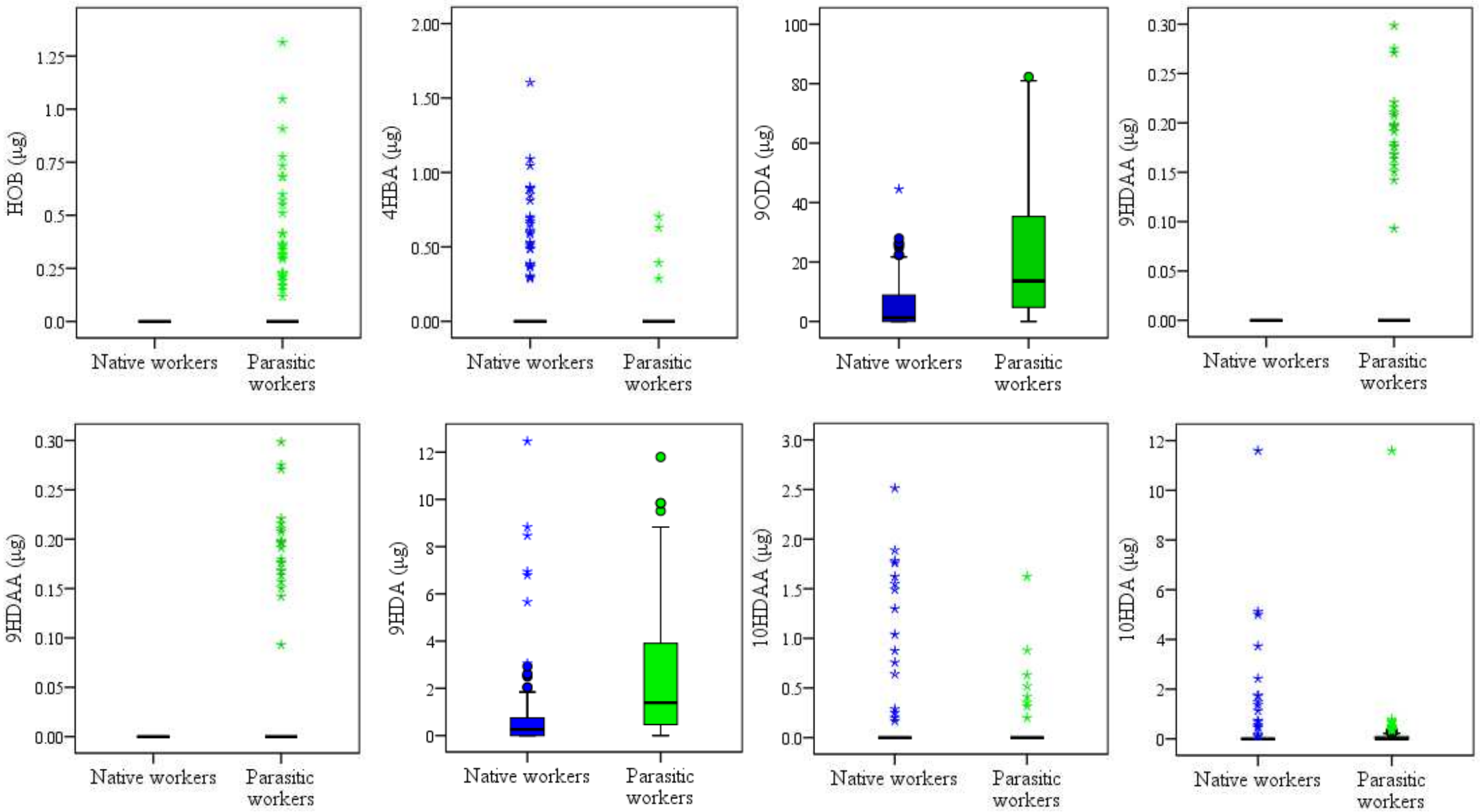

Figure 1: Absolute amounts of eight mandibular gland compounds extracted from the secretions of native (■) and parasitic (■) *capensis* workers. The black line inside each box marks the median of the distribution. The lower and upper hinges, or box boundaries, mark the 25th and 75th percentiles of each distribution, respectively. Whiskers appear above and below the hinges. Whiskers are vertical lines ending in horizontal lines at the largest and smallest observed values that are not statistical outliers. Outliers are identified with a circle (○) and extreme values are marked with an asterisk (*). N: native workers = 121, parasitic workers = 212.

5.3.2 Relative proportions of mandibular gland compounds

In an attempt to ascertain whether the two groups showed qualitative differences, we compared their profiles using the relative proportions of mandibular gland compounds (Crewe 1982; Strauss et al., 2008). 9ODA was the major constituent in the secretions of both native and parasitic workers, but the relative proportions were higher in parasitic workers (MW: $U_{121,\ 212}$ = 16661.5, p < 0.0001, fig. 2). The relative proportions were however more variable in native workers (*sd* = 43.48) compared to the profiles of parasitic workers (*sd* = 26.74). Statistically the presence of outliers tends to abnormally inflate values of standard deviations.

Although the mean relative proportion of 9HDA was similar in both the native and parasitic worker groups, the variation between groups was significantly different (MW: $U_{121,\ 212}$ = 16049.5 p < 0.0001). Figure 2 shows that the 9HDA levels of at least 50% of native workers were more variable (the box: interquartile range = 14.53%) compared to the tighter clustering observed in parasitic workers (interquartile range = 4.97%).

The relative proportion of 10HDA was similar in both worker groups (MW: $U_{121,\ 212}$ = 13780 p = 0.12) but native workers displayed more variability (fig.2). This trend was also observed for HVA. More than 86% of native and 96% of parasitic workers had no 10HDAA present in their extracts. Those individuals with 10HDAA present are seen as extreme outliers in figure 2.

Our analysis of the ratio of queen to worker compounds yielded significant results (MW: $U_{121,\ 212}$ = 14986.5, p = 0.002). Although both groups produced what is considered to be secretions that indicate queen specificity (≥ 0.64, Crewe, 1982; Moritz et al., 2004), parasitic workers were found to be more queenlike (0.95 ± 0.01, *sd* = 0.21) than native workers (0.64 ± 0.04, *sd*=0.47).

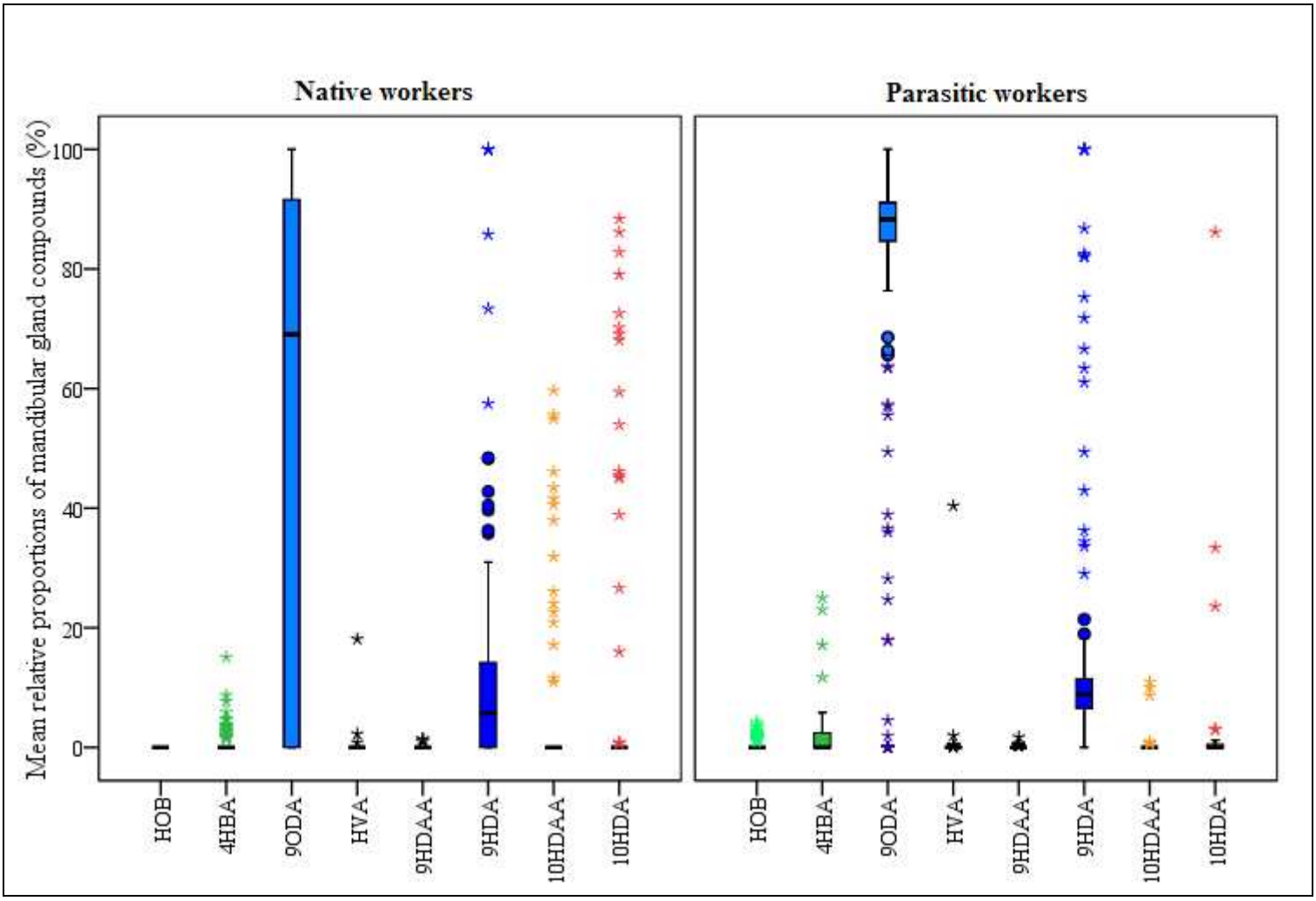

Figure 2: Relative proportions of mandibular gland compounds extracted from the secretions of native and parasitic *capensis* workers. The black line inside each box marks the median of the distribution. The box marks the interquartile range of each distribution. Whiskers appear above and below the box hinges and are vertical lines ending in horizontal lines at the largest and smallest observed values that are not statistical outliers. Outliers are identified with circle (○) and extreme values are marked with an asterisk (*).N: native workers = 121, parasitic workers = 212.

5.3.3 Discriminant Analysis

A discriminant analysis was conducted as a multivariate approach to include the relative proportions of all eight mandibular gland compounds in a single test (fig. 3). This analysis produced one discriminant function that explained 100% of the variance and distinguished native from parasitic workers (Wilk's $\lambda = 0.764$ $\chi^2 = 87.98$ $df = 8$ $p < 0.0001$), despite considerable overlap of individual profiles (fig. 3).

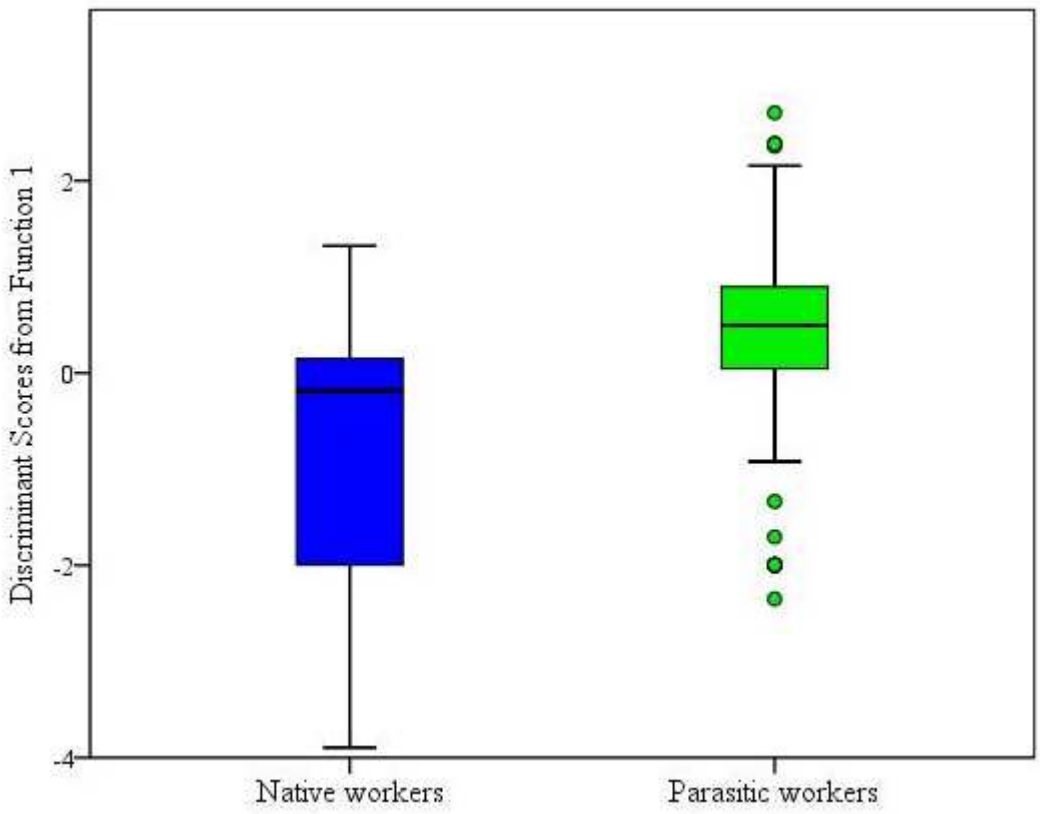

Figure 3: Box plots of the discriminant scores for native and parasitic workers. Discriminant scores are the scores for each case (individual) on the discriminant function. The single discriminant function produced by the discriminant analysis was able to distinguish groups on the basis of the profiles, despite the great deal of overlap observed in the profiles of the two groups. It also shows that variability in native workers is higher than in parasitic workers. The black line inside each box marks the median of the distribution. The lower and upper hinges, or box boundaries, mark the 25th and 75th percentiles of each distribution, respectively. Whiskers appear above and below the hinges. Whiskers are vertical lines ending in horizontal lines at the largest and smallest observed values that are not statistical outliers. Outliers are identified with a circle (○). N: native workers = 121, parasitic workers = 212.

Among the measured compounds, 9ODA showed the highest correlation with the single discriminant function. Overall 73.9% of workers were correctly classified. Misclassifications were highest for native workers with 58.7% classified as parasitic workers while only 7.5% of parasitic workers were misclassified as native.

5.4 Discussion

Our results showed that the cumulative variation of all compounds in the mandibular gland extracts of workers resulted in native workers having a more variable profile than that of parasitic workers. The most marked difference was found in the queen compounds 9ODA and 9HDA. Although the extracts of native and parasitic workers were very queenlike with a high ratio of queen to worker compounds, and dominated by 9ODA, parasitic workers produced significantly higher amounts (µg) of both queen substances while the relative proportions were more variable in native workers. Our finding in this study corroborates that in chapter 4, where we demonstrated that workers produced more similar mandibular gland profiles when sharing a a higher proportion of genes; however here we show that social context ultimately affected the production of queen specific compounds probably due to reciprocal pheromone competition, with greater variability in mandibular profiles evident.

In queenless colonies *capensis* workers are able to produce a pheromonal bouquet that is very similar to that of the queen (Crewe et al., 1980). Queenless workers change their typical worker signal dominated by 10HDA and 10HDAA to a queenlike signal with higher 9ODA content within a few days (see chapter 3). Like queens, workers with queenlike pheromones can inhibit the development of other workers' ovaries (Hepburn et al., 1991), and the queen mandibular pheromone seems to be associated with reproductive dominance (Hillesheim et al., 1989). There is evidence for genotypic variance in worker reproduction under queenless conditions but the knowledge on pheromonal reproductive competition between workers with identical genotypes is sparse. Differential queen pheromone production is normally facilitated through genetic variance (Moritz et al., 1996; Martin et al., 2004; Härtel et al., 2006a; Makert et al., 2006). As a result workers that are genetically predisposed to become

reproductive active/pseudoqueens through the production of queenlike pheromones will dominate other (subordinate) workers (Moritz et al., 2000, 2004; Simon et al., 2005; Dietemann et al., 2006, 2007).

Moritz and colleagues (1985) showed that the ability to become reproductively dominant in populations of *capensis* is subject to a strong genetic variance. Dominant workers synthesize both qualitatively and quantitatively queenlike amounts of the queen substance, 9ODA (Hemmling et al. 1979; Crewe et al., 1980; Crewe 1982). Since only a few workers develop into pseudoqueens (Martin et al., 2004; Robinson et al., 1990; Oldroyd et al., 1994; Moritz et al., 1996), individual competition for dominance exists. These dominant workers inhibit the reproductive capacity of subordinate workers (Velthuis et al., 1965; Velthuis, 1970; Crewe et al., 1980; Crewe, 1984, 1988). Pairs of emerging *capensis* workers placed together in the absence of a queen or synthetic 9ODA were found to gradually develop their mandibular gland signal (Moritz et al., 2000). Instead of just producing a pheromone signal independent of each other, workers were found to compete for the strongest signal, leading to distinct dominance hierarchies within 4 days. Subordinate workers produced a stronger signal when paired with a dominant worker.

Although our results suggested that worker genotype does predispose them developing a queenlike phenotypic signal, the variability in queen substances, observed specifically in parasitic workers, suggest their production also has a substantial social component. The regulation of the final synthesis from the precursor 9HDA to the end product 9ODA seems to be strongly influenced by the presence of established dominant reproductive workers. So workers that show rapid development of 9ODA quickly become the dominant individuals and consequently inhibit their sisters from developing reproductively, and as a result this worker-worker competition

leads to the development of dominance hierarchies in colonies. These findings are congruent with those of Härtel and his colleagues who also advocate a strong social environmental component in production of a queenlike signal (Härtel et al., in review).

The effect of age, environment and genes was studied by Velthuis and colleagues (1988) and they stated that "the mandibular gland's secretions express the progress an individual has made in the differentiation process leading to reproduction". Although the impact of genes on the expression of the queen phenotype is strong (Moritz et al., 1995), environmental effects clearly contribute to this expression. Similarly, the lack of ovary development was marked in this study. It is clear that for workers to achieve a typical queenlike signal and activate their ovaries they have to overcome the inhibition imposed by other, more dominant workers. In addition nutrition, age and developmental stage will also affect their mandibular gland secretions (Crewe, 1988; Crewe et al., 1989; Simon et al., 2001) and ovary development/activation (Wheeler, 1996, Hepburn et al. 1994).

Prudence is required with the interpretation of mandibular gland pheromone data since it depends on whether either the absolute or relative amounts of the tested components are being considered since the interpretation of results might lead to different conclusions. This is due to the fact that relative amounts are based on the total amount of secretions, of those compounds included in the analysis thus the proportions of each compound is relative to all others selected. Two individuals might have the same absolute amounts of a specific compound but depending on the total amount of secretions the representation of the compound might differ between said individuals. Crewe et al. (1980) found that the percentage of the content of 9ODA in queenless workers was similar to that found in laying workers, but the absolute

amount was just half of it. I would suggest that both ways of interpretation should be taken into account depending on the objective of the experiments.

Although queen pheromone biosynthesis seems to be genetically determined, with both native and parasitic workers being predisposed to utilizing the queen biochemical pathway, the expression of the queen phenotype depends on the social context of the workers. The data supports this view since we found that the expression of a queenlike mandibular gland signal is strongly influenced by the presence of dominant workers (social environment). In parasitic workers rapid signal development (chapter 2) resulted in dominant workers inhibiting their sisters before they could develop their signals, while in native workers, the same behaviour results from polyandry which facilitates reproductive variance. The lower production of queen compounds in native workers and higher levels in parasitic workers also suggests that the latter might have lower sensitivity/high response threshold (Crewe, 1982, 1988; Naumann et al., 1993; Magnuson, 1995) to the pheromone levels of other workers. As a result they are less inhibited by other workers. This could allow them a head start in becoming reproductively active. This mimicry of queen pheromones which leads to the establishment of pseudoqueens demonstrates the existence of the proposed ongoing worker-worker pheromonal arms race (Katzav-Gozansky et al, 2006, Malka et al., 2008).

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CHAPTER 6

SIGNAL VARIATION IN MANDIBULAR GLAND PROFILES ACCORDING TO PATRILINE AND SOCIAL ENVIRONMENT

6.1 Introduction

The honeybee queen exhibits the most extreme degree of polyandry among the social insect genera (Koeniger et al., 2000; Palmer et al., 2000). Moritz and colleagues (1996) reported up to 44 matings per queen for *Apis mellifera*. Members of the same patriline, super sisters (Page et al., 1988), share genes from both the queen mother and a drone father and in the absence of inbreeding have a coefficient of genetic relatedness of 0.75 (Pamilo et al., 1982). Half-sisters belong to different patrilines and only share on average 50% of their genes from the mother, and have a genetic relationship of 0.3 (effective paternity of 10 males, Ratnieks, 1988; Pirk et al., 2003). Due to this high genetic variability in honeybee colonies the potential for conflict over reproduction is high (Ratnieks, 1988; Greef, 1996). If members of a patriline are able to recognize each other, they could increase their reproductive success by making sure that one of their full sisters becomes the next queen (Getz, 1981; Moritz et al., 1992; Oldroyd et al., 1994). Previous efforts attempting to prove such nepotism have however failed (Chaline et al., 2005). It has been shown that cuticular hydrocarbons are more variable among patrilines than within (Arnold et al., 1996; 2000). The question remains whether mandibular gland secretions show the same trend and could it thus be used for subfamily recognition? A recent study by Fan and colleaugues (2010) found that the queen pheromone complex (QMP) significantly changes the cuticular hydrocarbons of workers treated/exposed to QMP. QMP-treated bees in

their study accumulated traces of the pheromone on their bodies which was rapidly internalized as the half-life on the cuticle surface is ≈13 min (Naumann et al., 1991). It thus indirectly affects nestmate recognition as treated workers are no longer recognized as nestmates. Colonies are generally harmoniously functioning units. This is achieved through social regulation via chemical signals of the mandibular gland signals of the queen (Slessor et al., 1988). Caste specificity and biosynthetic plasticity are known properties of mandibular gland secretions in the honeybee (Crewe, 1982, Plettner et al., 1993, 1997). Under queenright conditions the pheromonal bouquet of workers are typically queenright, with the dominant presence of 10-hydroxy-2-decenoic acid (10HDA) and 10-hydroxydecanoic acid (10HDAA), while under queenless conditions certain workers start producing the queen substances (E)-9-keto-2-decenoic acid (9ODA) and (E)-9-hydroxy-2-decenoic acid (9HDA). Chemical analyses of the mandibular gland secretions of both female castes have been shown to regulate a variety of key functions acting both as primer and releaser pheromones (Free, 1987). Initial studies of signal variation focused on the variation of 9ODA in queens and 10HDA in workers (Pain et al., 1960; 1967; 1976; Barbier et al., 1960).

The composition of the mandibular gland secretions in both queens (Engels, et al., 1997; Wossler et al., 2006) and workers (Crewe, 1988; Crewe et al., 1989; Simon et al., 2001) are affected by age, caste, race and social context. *A. m. capensis* workers are unique in that they swiftly change their signal from workerlike to more queenlike under queenless conditions (Crewe et al., 1980; Simon et al., 2001; 2005). Furthermore it has been shown that mandibular gland signal production varies between subspecies but variability also exists between individuals (Moritz et al., 2000).

The phenotype that an individual displays is influenced by both its genotype and environment. Moritz and Hillesheim (1985) found that the production of 9ODA is largely influenced by genotype. This study investigates the relationship between mandibular gland profiles and genotype, but more specifically the link between profile and patriline. The polyandrous nature of the honeybee queen results in the production of genotypically diverse offspring in monogynous colonies. This facilitates the detection of possible genotypic effects because offspring, of a similar age cohort fathered by different drones (patrilines) within a colony, share the same maternal genotype on average, the same maternal effects, and the same environmental rearing conditions and differ only in their paternal genotype. If respective patrilines within a colony express a specific signal phenotype, it would indicate that the expression of the mandibular gland signals contain genetic information. However, if all workers within a colony express a more homogenous signal, it would indicate a stronger environmental influence.

6.2 Materials & Methods

6.2.1 Queen rearing and artificial inseminations

A large natural *capensis* colony, at the Agriculture Research Unit, Stellenbosch (33°56′ S, 18°51′E), was dequeened and the queenless workers were allowed to naturally initiate queen rearing. . Two capped queen cells were collected from the rearing colony and placed into an incubator (35°C, 60% relative humidity) to emerge. The two *capensis* virgin queens (4-6 days old) were artificially inseminated with the semen of 5 *capensis* drones (Laidlaw, 1978) and respectively introduced into two queenless 5-frame splits of the original rearing colony. The following day, queens were recaptured and anaesthetized with CO_2 to stimulate egg-laying (Mackensen,

1947) and placed back into the colony. Drones used for inseminations were kept for genetic analysis to determine paternity of harvested workers. Colonies were inspected weekly for laying activity and removal of queen cells. Unfortunately only one queen survived. Genetic analysis revealed that three of the drones used were related and the other two were also related. The molecular markers used in the analysis were not informative enough to distinguish between brothers, and therefore based on the relatedness between drones our analysis was in affect based on two patrilines (3 brothers and 2 brothers).

6.2.2 Experimental set up

Once the surviving queen was established, a frame of her emerging brood was removed from the colony and placed in an incubator and allowed to emerge. Approximately 300 emerged bees were colour marked on the thorax with a non-toxic paint (Posca paint pens, Mitsubishi pencil Co., Japan) and returned to the natal colony. All marked bees were harvested 10 days after introduction. Heads were removed for mandibular gland GC analysis and individually labelled and stored in 200µl dichloromethane (DCM, Merck) and bodies frozen for dissections to determine ovary development after which they were stored in alcohol until genetic analysis was done.

Data for mandibular gland extracts of workers (with the same parental origins as workers in the abovementioned experiment) aged singly with a cohort of *scutellata* workers (chapter 4) was used in the analysis of this study. Individual *capensis* workers (< 24 hrs), distinctly marked on the thorax with paint, were introduced into 50 respective Liebefeld cages containing 50 1-day old *scutellata* workers. The single introduced *capensis* workers originated from the same queen as for this experiment

(see chapter 4). The s*cutellata* workers were collected from emerging brood frames sourced from the same donor colony to eliminate host worker effects. Bees were fed a pollen enriched candy pattie and water *ad libitum*. Dead bees were removed daily and cages cleaned.

Thus, workers aged in a queenright *capensis* colony were compared to their sisters aged individually with queenless *scutellata* workers. The aim was to determine the affect of the social environment on mandibular gland profiles.

6.2.3 Chemical analysis

The heads of dissected workers were removed from the vials and the DCM evaporated under a stream of nitrogen just to dryness. The residue was redissolved in 15µl internal standard (octanoic acid and tetradecane in 4 ml DCM) and 15µl bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, Merck). One microlitre was injected into a gas chromatograph (HP-6850) with a split-splitless inlet and a 25mm x 0.32 mm methyl-silicone coated fused silica capillary column. Helium was used as carrier gas at a flow rate of 1.9ml/min. The oven temperature was controlled as follows: 1 min at 60 °C; followed by a heating phase of 50°/min to 110°C, and subsequently another of 3°C/min to 220°C. Finally the temperature was held at 220°C for 10 min. Peak areas were determined using HP Chemstation software and the mandibular gland compounds were identified based on the retention times of authentic compounds and were quantified using peak area and the relative mass ratios (for methodological details see Simon et al. 2001). Ten compounds were identified but only nine were included in the analysis (table 1). Oleic acid was excluded from the analysis as it was the most abundant compound in all sampled individuals and its inclusion masked subtle differences.

6.2.4 Genetic Analysis

6.2.4.1 DNA Extraction

DNA was obtained from the fathering drones and workers using an adaptation of a salt extraction method (Aljanabi et al., 1997). For drones and workers, 2 legs were homogenized in 500µL digestion buffer (0.1mg/mL Proteinase K (Roche), 10mM NaCl, 10mM Tris pH 8.0, 10mM EDTA pH 8.0, 0.5% sodium dodecyl sulphate) in a 96 well 1ml plate, each well containing a 3mm stainless steel ball bearing. Tissue was homogenized for 10min at 25Hz using a TissueLyser (Qiagen). Samples were incubated overnight at 55°C. After incubation, 20µL volume of 5M NaCl was added to each sample. Samples were mixed by inversion then incubated for 30-45min at -20°C until cloudy. Samples were then centrifuged for one hour at 4300rpm at 4°C. Following centrifugation, 200µL of the supernatant was added to 400µL 99.7% ethanol, mixed by inversion and then incubated at -20°C overnight. Samples were again centrifuged at 4300rpm and 4°C for one hour before discarding the supernatant and rinsing once with 70% ethanol. Samples were air-dried and then resuspended in 50µL 1 x Tris-EDTA buffer pH 8.

6.2.4.2 Amplification of Genomic DNA

The fathering drones and workers were genotyped at HB-SEX-01, HB-SEX-03, HB-THE-01, HB-THE-03, HB-THE-04 (Shaibi et al, 2008). DNA was amplified in 5µL multiplex PCR reactions consisting of 0.2µM of each primer (reverse primers 5' labelled); 0.3µM of dATP, dTTP, dCTP and dGTP; 2.5mM $MgCl_2$; 1x TAQ-Ti Polymerase reaction buffer (Fisher Biotec); 0.2U TAQ-Ti DNA Polymerase (Fisher Biotec); 2.5%(w/v) glycerol and 1µL genomic DNA. PCR conditions were:

denaturation at 94°C for 10 min; 35 cycles of 94°C for 30s, 56°C for 30s, 72°C for 30s and a final extension period at 72°C for 10 min.

6.2.4.3 Capillary Electrophoresis of PCR Products

PCR products were run on a 3130*xl* Genetic Analyser (Applied Biosystems) with capillary length 36cm and injection time of 15s at 1200V, for 41 minutes. Results were analysed using Genemapper software (Applied Biosystems) and the genotypes of drones and workers were determined.

6.2.5 Statistical Analysis

To overcome statistical problems associated with multicolinearity and non-normality of GC-derived data, we applied a standard procedure of normalisation of peak areas. Peak areas were transformed according to Aitchison (1986) and Reyment (1989). The original transformation procedure makes it necessary to exclude compounds that do not occur in all samples. We therefore modified the transformation ($\log_{10}$ (relative peak area/ (geometric mean of all peak areas) + 1)) to avoid undefined values for peaks with an area of zero.

The transformed peak areas were subjected to a discriminant analysis (DA) to assess whether the two patrilines were separated on the basis of their mandibular gland pheromone profiles. The DA also produces a classification matrix which reports how well the cases (individuals) have been placed in their known groups based on the discriminant functions.

In addition, we applied an alternative method to test for significant differences between patrilines. Using relative peak areas we computed an analysis of similarity (ANOSIM) based on between groups and within group comparisons. To identify

compounds that contributed most to the differences across patrilines we compared the proportional peak areas with non-parametric Mann–Whitney-*U* pairwise comparisons of groups.

In order to ascertain the affect of social environment on mandibular gland profiles, workers aged in a queenright *capensis* colony were compared to their sisters aged individually with queenless *scutellata* workers. Consequently this analysis allowed us to investigate differences in mandibular gland profiles between two groups sharing both maternal and paternal origins but differing in their social environment. The initial experiment plan was to age workers in their natal queenright *capensis* colony since mandibular gland signals could potentially be used as patriline signals and we wanted to ascertain if the affect was apparent in normal colonies. This was to be followed by removing the queen and ageing another cohort of her workers under queenless conditions and then comparing worker signals from the two environments. Unfortunately not enough brood could be obtained from the queen to continue with the queenless part of the experiment.

6.3 Results

Discriminant analysis was not able to distinguish workers from patriline 1 from those of patriline 2 (Wilks' lambda $\lambda = 0.961$, $\chi^2 = 5.515$, $p = 0.701$) even though the classification results correctly classified 74.5% of all workers to their respective patrilines. The less conservative ANOSIM also resulted in overall non significant differences between patrilines (Global $R = 0.005$, $p = 0.44$; permutations = 999, random sample from a large number). This is supported by the MDS (fig. 1) which shows overlap in profiles between workers from the two patrilines. Pairwise

comparisons only revealed significantly higher relative amounts of 9HDA (Mann-Whitney: $U_{37, 108} = 2452.5$, $p = 0.039$) in workers from patriline 2 (table 1).

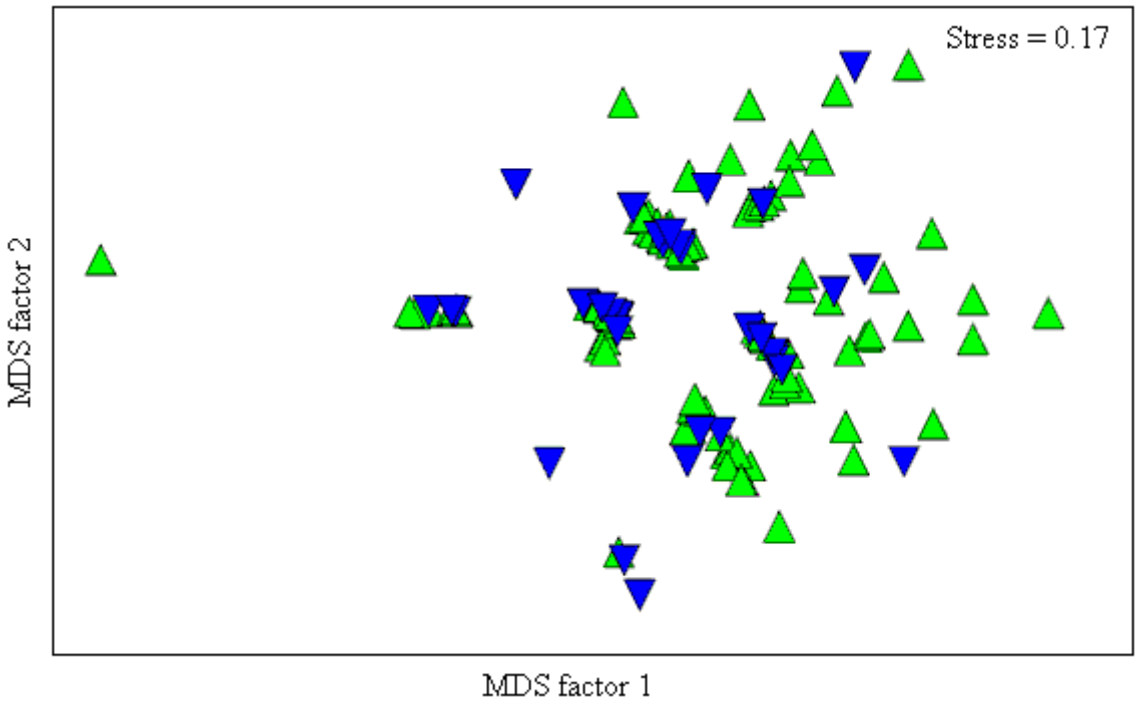

Figure 1: MDS ordination based on the mandibular gland profiles of patriline 1 (▲, n = 37) and 2 (▼, n = 108) from queenright *capensis* colony. No clear separation between the two patrilines can be observed.

However when comparing the groups of workers aged in different social environments a single discriminant function was responsible for the complete separation between the two groups (explaining 100% of the variation between groups, Wilks' lambda $\lambda = 0.118$, $\chi^2 = 331.002$, $p < 0.0001$). The discriminant function was associated with the typical queen compounds, 9HDAA, 9HDA and 9ODA. Classification results showed that all individuals were correctly (100%) assigned to their respective groups.

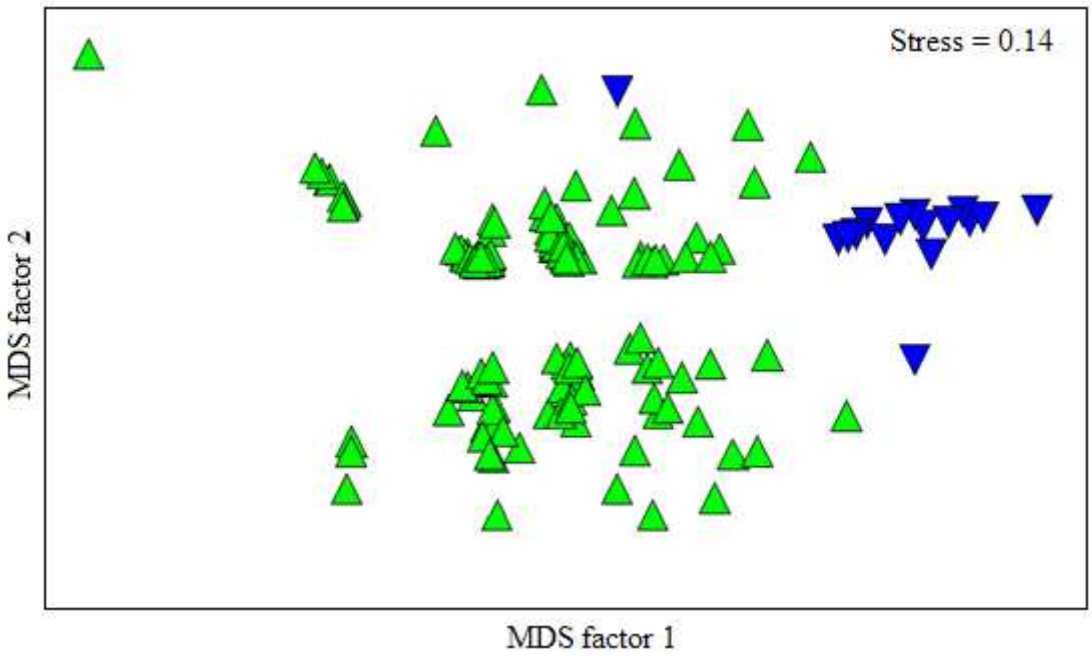

Figure 2: MDS ordination of the mandibular gland profiles of two groups of workers aged under different social conditions, originating from a single *capensis* queen inseminated with the semen of 5 *capensis* drones in: queenright natal *capensis* colony = ▲ (n = 145), and Liebefeld cages in presence of queenless *scutellata* workers = ▼ (n = 16) shows a distinct separation between groups.

The MDS ordination (fig. 2) shows a distinct separation between workers (irrespective of patriline) aged under queenless conditions in the presence of *scutella* workers and those aged under natural queenright conditions in the presence of *capensis* workers (ANOSIM Global R = 0.808, p = 0.001; permutations = 999, random sample from a large number). Pairwise comparisons revealed a significant difference in the relative amounts of all compounds, except 10HDAA, between workers aged in different environments. No ovary development was found in any of the sampled workers.

Table 1: Comparison of the relative amounts of the identified mandibular gland compounds extracted from the test groups (mean proportion ± se). Significant differences in specific compounds between groups are denoted by ∗ (Mann Whitney, $p < 0.05$)

	N	9ODA	HVA	9HDAA	9HDA	10HDAA	10HDA	PALMOL	PALM	STEARIC
Level 1 analysis:										
Patriline										
Patriline 1	37	0.02 ± 0.02	0.02 ± 0.02	0.17 ± 0.1	5.83 ± 1*	0.18 ± 0.1	2.28 ± 0.73	0.67 ± 0.13	21.93 ± 0.44	68.9 ± 1.28
Patriline 2	108	0.14 ± 0.05	0.06 ± 0.02	0.27 ± 0.05	8.42 ± 0.77*	0.12 ± 0.08	2.7 ± 0.48	0.74 ± 0.09	21.12 ± 0.42	66.43 ± 1
Level 2 analysis:										
Social environment										
Queenright	145	0.11 ± 0.04*	0.05 ± 0.02*	0.24 ± 0.04*	7.76 ± 0.63*	0.13 ± 0.07	2.6 ± 0.4*	0.72 ± 0.07*	21.33 ± 0.33*	67. 06 ± 0.77*
Queenless	16	8.94 ± 2.63 *	1.45 ± 0.31*	3.19 ± 0.32*	46.87 ± 4.42*	-	0.09 ± 0.09*	4.05 ± 0.55*	13.17 ± 1.55*	22.14 ± 2.8 *

Abbreviations for the compounds are as follows: 9ODA = (E)-9-oxodec-2-enoic acid; HVA = 4-hydroxy-3-methoxyphenylethanol; 9HDAA = 9-hydroxydecanoic acid; 9HDA = (E)-9-hydroxydec-2-enoic acid; 10HDAA = 10-hydroxydecanoic acid; 10HDA = (E)-10-hydroxydec-2-enoic acid; PALMOL = palmitoleic acid; PALM = Palmitic acid; STEARIC = Stearic acid.

6.4 Discussion

Our results clearly show that the social environment plays a major role in the production of mandibular gland compounds in honeybee workers which are not unexpected (Crewe et al., 1980; Härtel et al., 2006a, 2006b). Despite the presence of similar compounds in the mandibular gland extracts of queenright and queenless workers, the latter group had a distinctly different profile. It was not possible however to distinguish the mandibular gland profiles of workers on the basis of their patrilines in this study. Our results only showed a significant difference in the 9ODA precursor, 9HDA. This does suggest that workers from certain patrilines are predisposed to following the queen mandibular pheromone biosynthetic pathway (Martin et al., 2004; Robinson et al., 1990; Oldroyd et al., 1994; Moritz et al., 1996) yet the final oxidation to 9ODA is dependent on the social environment (Hartel et al., in review; Moritz et al 2000). We therefore tentatively suggest that the production of queen substance, 9ODA, may initially hinge on paternal effects but the end point is very much dependent on the social environment of the workers.

Investigating patriline differences in pheromone signal development can be important in the context of kin recognition. This is because the identification of relatives is required for organisms to favour close relatives in social interactions (Hamilton, 1964). Recognition of true relatives requires among others a strong association between phenotype and genotype (Breed, 1998; Blaustein, 1983; Pfennig et al., 1995). Patriline differences in signal phenotype would allow workers to treat their sisters preferentially thus increasing their own reproductive capacity. However, if all subfamilies treated their full-sisters preferentially, colony production would decrease and those colonies would probably die out. Workers may therefore be selected not to show preferential treatment even though Arnold and his colleagues (1996, 2004) have

shown that hydrocarbon profiles are patriline specific. Our data however did not show distinct separation between the two patrilines which could either mean that mandibular gland secretions are not patriline specific or the subtle differences between patrilines were not detectable using our methods of analyses.

Effective discrimination of kin and non-kin in birds and social insects can be accomplished through learning characteristics of individuals or groups and this recognition is dependent on phenotype matching, which enables the individual to form a template for discerning relatives (Lacy et al., 1983). A drawback of these recognition or discrimination methods is that it creates the opportunity for parasitic individuals to exploit the system. This appears to be the case for *capensis* workers which are very plastic in their production of pheromones, since they are capable of rapidly switching their biosynthetic pathways from producing workerlike to a more queenlike pheromone signal when placed in queenless *scutellata* colonies (Crewe et al., 1980; 1990; Moritz et al., 2000) as was evident in this study. This mimicry of queenlike pheromones enables *capensis* workers to establish themselves as pseudoqueens, that prime and release very similar reactions in sterile workers to those of true queens (e.g. suppress ovary activation; release retinue behavior; Wossler 2002).

The queen substance, 9ODA, is known to play an important role in worker reproductive hierarchies (Moritz et al., 1985; Hillesheim et al., 1989). The more queenlike the mandibular gland signal of a worker, the more that worker could dominate other workers, leading to the suppression of pheromone signal development in the subordinate workers as well as ovary inhibition (Hillesheim et al., 1989). It would appear that all *capensis* workers have a predisposition to selecting queen biosynthetic mandibular secretion biosynthetic pathways, but what may be under

strong selection is the speed of signal development which would give some patrilines a head start in the race to produce a more queenlike signal. The existence of reproductively dominant and subordinate patrilines in colonies (Martin et al., 2004; Robinson et al., 1990; Oldroyd et al., 1994; Moritz et al., 1996) suggests patriline differences in signal production is an important mechanism in establishing reproductive dominance hierarchies in colonies.

It is apparent that there is a high degree of social influence on the production and composition of queen-specific mandibular gland compounds. Our results clearly reflect this with queenless workers producing distinctly different signals to queenright workers, despite similar genetic origins. In the presence of *scutellata* workers as well as the absence of a queen, *capensis* workers experienced no suppression, thus developing their signal. Although *capensis* workers have a genetic predisposition for developing a queenlike phenotype it is also well known that changes in mandibular gland secretions in both workers and queens (Crewe et al., 1989, Engels et al., 1997; Simon et al, 2001) are age and race dependent. Ontogenetic patterns also changes post-emergence, thus in queenright colonies the composition of the mandibular gland secretions coincide with the task that the honeybee worker is involved in at a given stage in its life (Free, 1965; Wilson, 1971, Seeley, 1982; Robinson et al., 1989). Thus both the physical environment and social context that the workers experience also impact on the mandibular gland secretions.

In this study we demonstrated that mandibular gland profiles are substantially affected by the social context of workers. This supports our findings from chapters 3 and 4 where we found that althought *capensis* workers are genetically predisposed to developing a queenlike pheromone signal; it is their social context which ultimately determines the development.

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CHAPTER 7

GENOTYPE AFFECTS *A. M. CAPENSIS* AND HYBRID HONEYBEE WORKERS' REPRODUCTIVE POTENTIAL

7.1 Introduction

Reproductive division of labour is a defining characteristic of insect societies. The ability of colony members to become reproductively active varies from species to species. In many social bees, wasps and ants, reproductive division of labour is determined by extrinsic (e.g. mating opportunities) or intrinsic (e.g. relative fighting ability) factors. Differences in reproductive ability among adults are typically established during larval development, but all adult females can, in theory, become breeders (O'Donnell, 1998). In other Hymenopteran species the differentiation between worker and breeder caste is rigidly determined by metamorphosis, by either genetic factors (Anderson et al., 2006), larval feeding (Wheeler, 1986), or a combination of both (Kerr et al., 1966; reviewed in Schwander et al., 2010). In these species there is a loss of totipotency early in the larval stage, if not before, that results in discrete reproductive (queen) and helper (worker) castes. Here, the queen is the only individual capable of sexual reproduction, while all other colony members are specialised workers.

The loss of totipotency of colony members decreases the potential for reproductive conflicts within colonies as it results in divergent selection pressures such that helpers are selected to increase their inclusive fitness (Hamilton, 1964a, b) via becoming better workers, and breeders by increasing their direct fitness by massively increasing their reproductive output (Beekman et al., 2006). However, as insect societies rarely

consist of clones, the reproductive interests of colony members do not completely overlap (Beekman et al., 2003). Therefore, though the potential for conflict remains, the reproductive options for workers are severely limited because workers cannot mate; the best they can do is to produce a few males via arrhenotokous parthenogenesis if they reproduce at all (Bourke, 1988).

When workers produce diploid female offspring without mating (thelytokous parthenogenesis), conflicts within the colony are expected to increase because the workers' reproductive potential are greater since they are related to their female-producing eggs by unity (Greeff, 1996). In the Cape honeybee, *Apis mellifera capensis* (hereafter *capensis*) the workers are capable of thelytokous parthenogenesis (Onions, 1914) and this leads to overt competition among workers and between the queen and the workers over reproduction (Hepburn, 1994; Jordan et al., 2008b; Moritz et al., 1996; Moritz et al., 2004). Selection for increased reproductive capacity in workers has resulted in *capensis* workers exhibiting morphological traits not found in workers of any other (sub)species of *Apis*. *A. m. capensis* workers have higher numbers of ovarioles compared with workers of other subspecies (Hepburn 2001; Hepburn et al., 1991) and often have a spermatheca, a sperm storage organ usually only present in queens (Hepburn 2001; Hepburn et al., 1991; Jordan et al., 2008a).

Caste determination in honeybees is determined by larval feeding, with queen-destined larvae receiving a greater amount of food that is nutritionally different to that received by worker-destined larvae ('royal' versus 'worker' jelly) (de Wilde et al., 1982). The expression of queenlike characteristics in *capensis* workers is strongly influenced by larval feeding. Larvae that are fed more and better develop into more queenlike individuals relative to those fed a more frugal diet (Allsopp et al., 2003; Beekman et al., 2000; Calis et al., 2002).

The Cape honeybee is not the only honeybee present in South Africa. While *capensis* is confined to the southern part of the Western and Eastern Cape, the African honeybee *A. m. scutellata* (hereafter *scutellata*), is found throughout the rest of South Africa and in countries to its north (Ruttner, 1988). The two subspecies interact within a hybrid zone situated in the semi-arid areas of the Karoo ecotone (Hepburn et al., 1991). This hybrid zone is particularly interesting because *capensis* can be a lethal social parasite of *scutellata* (Allsopp, 1992). *Capensis* workers are known to enter *scutellata* colonies and parasitise them with their eggs thus producing more clonal, parasitic workers (Martin et al., 2002a; Neumann et al., 2002). The end result of such invasion is the death of the host colony (Martin et al., 2002b). Despite its parasitic potential, *capensis* has not been found outside its natural range without artificial movement by humans (reviewed in Beekman et al., 2008). The stability of the honeybee hybrid zone is even more intriguing given that *scutellata* also has traits that make it highly invasive. Since its introduction into Brazil in 1956, *scutellata* has largely displaced all European *A. mellifera* subspecies in the Americas (Schneider et al., 2004).

One hypothesis for the stability of the *capensis-scutellata* hybrid zone is that it is in fact a tension zone (Beekman et al., 2008). Tension zones arise when hybrids are less fit than either parental genotype (Barton et al., 1985). Due to the reduced fitness of hybrids, gene flow between the parental populations is curtailed or prevented (Barton et al., 1985). Within the honeybee hybrid zone of South Africa, queens of both *capensis* and *scutellata* may mate with drones of both subspecies, resulting in hybrid or 'mixed' colonies consisting of pure workers of the queen's genotype and *capensis-scutellata* hybrids (Beekman et al., 2008). Beekman et al. (2008) suggested that

mixed colonies may be less fit than pure colonies of either *capensis* or *scutellata* due to pheromonal imbalances that lead to a breakdown in reproductive division of labour. The tension zone hypothesis (Beekman et al., 2008) is based on the pheromonal polymorphism between *capensis* and *scutellata* (Wossler, 2002). Pheromones are essential in regulating reproductive division of labour in honeybee colonies. For example, workers use the pheromones emitted by the queen and her brood to assess her fecundity (Mohammedi et al., 1998). Pheromones produced by larvae affect the amount and quality of food larvae receive and there is a strong interaction between the genotype (*capensis* or *scutellata*) of the larva and the genotype of the nurse workers (Allsopp et al., 2003; Beekman et al., 2000). This interaction is particularly important in colonies of mixed genotypes (Jordan et al., 2008a).

A. m. capensis brood reared by *scutellata* or *capensis-scutellata* hybrids receive more and better food than when they are reared by their own sisters (Allsopp et al., 2003; Calis et al., 2002). Thus *capensis* workers reared by *scutellata* nurses have a strong tendency to develop queen-like characteristics (Allsopp et al., 2003; Beekman et al., 2000). When these workers become reproductively active, colony productivity is likely to decline because reproductive workers work less than their sterile sisters (Hillesheim et al., 1989; Martin et al., 2002b). Thus, Beekman et al. (2008) hypothesised that colonies in which a large proportion of workers are reproductively active suffer a reduction in colony-level fitness relative to colonies of either *capensis* or *scutellata* genotype (in which most or all workers are functionally sterile). This suggests that genetically mixed colonies in the natural hybrid zone may have significantly lower fitness than 'pure' colonies, thus selecting against hybrids and hybridisation.

Here we investigate whether workers from *capensis* patrilines (sired by *capensis* drones) have a higher propensity in becoming reproductively active compared to *capensis-scutellata* hybrid workers (sired by *scutellata* drones). If we find that workers of *capensis* paternity are more likely to become reproductively active, it would suggest that genetically mixed colonies may suffer from a breakdown in reproductive division of labour and that the hybrid zone is indeed a tension zone. Under our experimental conditions, similar aged pure *capensis* and hybrid workers shared all environmental influences and the same maternal genotype on average. They therefore only differ in their paternal genotype which allowed us to determine whether the expression of the reproductive traits can be influenced by paternity.

7.2 Materials & Methods

7.2.1 Queen-rearing

Capensis queens were reared in the Stellenbosch area (33°56' S, 18°51'E) in late September and early October 2008. Queen-cells were harvested from the rearing colonies nine days after grafting and emerged in an incubator at 35°C. Upon emergence, the queens' wings were clipped and individually stored for genetic analysis. Newly emerged queens were placed with 20-30 young attendant bees in cages and stored in an incubator for 5-6 days until insemination.

7.2.2. Artificial insemination of queens

Two *capensis* queens were artificially inseminated with semen from five *capensis* (originating from a native *capensis* colony) and five *scutellata* drones (for details see Holmes et al., 2010). The queens therefore produced workers that were either pure *capensis* (sired by a *capensis* drone) or *capensis-scutellata* hybrids (sired by a

scutellata drone). As there are no diagnostic markers with which *capensis* and *scutellata* genotypes may be distinguished (Franck et al., 2001), drones used were kept for genetic analysis to allow us to determine the father of the workers sampled (see below).

Immediately after insemination queens were introduced into a 5-frame hive with *scutellata* workers and brood only. The day after insemination, queens were retrieved from their colony, anaesthetized with CO2 to initiate egg-laying (Mackensen, 1947), and released into the colony. Colonies were subsequently checked every two to three days. Colonies with laying queens were checked weekly by removing each frame and checking the state of the brood. Queen-cells, if any, were removed to prevent supersedure.

7.2.3 Test environment

Newly emerged bees were collected by placing a frame of emerging brood in an incubator at 35°C. Frames were inspected daily. All bees that had emerged overnight were marked on the thorax with non-toxic paint (Posca Paint Pens, Mitsubishi Pencil Co., Japan), using a different colour for each colony. After we marked 500 bees per colony, we checked each frame every hour and collected 100 emerged bees to determine their weight at emergence. These weighed bees were retained for genotyping to determine if paternity affects emergence weight. We wanted to maximise the probability that *capensis* and *scutellata* patriline workers would become reproductively active. Therefore we introduced the marked workers into the same queenless *scutellata* colony for both trials. In both trials the marked bees (n = 500) from both source colonies were placed in the same *scutellata* colony to control for host colony effects. All marked bees were harvested 12 days after introduction. Heads

were removed for mandibular gland gas chromatographic (GC) analysis and individually labelled and stored in 200µl dichloromethane (DCM, Merck). The abdomen and thorax (also individually labeled) were frozen for dissections and genetic analysis. This experiment was repeated with the same two queens two months later (January 2009 – trial 2).

7.2.4 Ovary dissections

We pinned each worker onto a wax plate through the thorax and separated the fifth and sixth dorsal tergites using fine forceps to expose the reproductive organs, under irrigation with water. In workers, the section of the ovary containing ovarioles is positioned above the hind gut, and the spermatheca below the hind gut (Dade, 1977). Spermathecae were scored by lifting the hind gut aside and recording whether a spermatheca was present or absent. We assessed the developmental stage of the ovaries using standard criteria (Velthuis, 1970): 1 = no development; 2 = slightly thickened ovarioles; 3 = round or bean shaped eggs visible (early stage of activation); 4 = fully developed ovarioles with eggs greater than 50% of full size. To determine the father's subspecies, the dissected bees were stored in alcohol in microcentrifuge tubes prior to genetic analysis.

7.2.5 Chemical analysis

The heads of dissected workers were removed from the vials and the DCM evaporated under a stream of nitrogen just to dryness. The residue was redissolved in 15µl internal standard (octanoic acid and tetradecane in 4 ml DCM) and 15µl bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, Merck). One microlitre was injected into a gas chromatograph (HP-6850) with a split-splitless inlet and a 25mm x 0.32 mm

methyl-silicone coated fused silica capillary column. Helium was used as carrier gas at a flow rate of 1.9ml/min. The oven temperature was controlled as follows: 1 min at 60 °C; followed by a heating phase of 50°/min to 110°C, and subsequently another of 3°C/min to 220°C. Finally the temperature was held at 220°C for 10 min. Peak areas were determined using HP Chemstation software and the mandibular gland compounds were identified based on the retention times of authentic compounds and were quantified using peak area and the relative mass ratios (for methodological details see Simon et al. (2001)). Of the 12 mandibular gland compounds identified, only the amount of 9ODA was quantified since this compound is a potential indicator of reproductive dominance in honeybee workers (Moritz et al., 2000; Simon et al., 2005).

7.2.6 Genetic Analysis

DNA was obtained from the queen (wingtips), fathering drones and workers (2-3 legs) from each colony using a high salt extraction method (Aljanabi et al., 1997). The fathering drones were screened with seven *A. mellifera* microsatellite markers used in previous parentage studies: Am005, Am006, Am008, Am046, Am052, Am059 and Am061 (Solignac et al., 2003). For one colony one microsatellite locus (Am061) was sufficient to distinguish workers sired by *capensis* drones from those sired by *scutellata* drones. For the second colony two microsatellite loci (Am061 and Am008) were required.

PCR products (0.4µl) from each reaction were added to 10µl formamide and 100nl LIZ DNA size standard (Applied Biosystems). Samples were run on a 3130*xl* Genetic Analyser (Applied Biosystems) with capillary length 36cm and injection time of 15s at 1200V, for 41 minutes. Results were analysed using Genemapper software

(Applied Biosystems) and the sire of the workers (*capensis* or *scutellata*) was determined. Microsatellite allele sizes were distinguishable due to a unique combination of dye colour and amplicon size range.

7.2.7 Statistical Analysis

We used a univariate Analysis of Variance to compare mean weight at emergence between *capensis* and *capensis-scutellata* hybrids. We also used a univariate Analysis of Variance to test if the absolute amount of 9ODA differed between *capensis* and *capensis-scutellata* hybrids. We tested the hypothesis that ovary activation (active or not active) and presence/absence of a spermatheca was independent of worker paternity with 2x2 contingency tables using G-tests (Sokal et al., 1995). Ovary activation was classified as 'non-active' (developmental stages 1 and 2) or 'active' (developmental stages 3 and 4). Differences between source colony and trial were tested using G-tests of heterogeneity.

7.3 Results

7.3.1 Emergence weight

Workers of *capensis* paternity had significantly higher emergence weights than workers of *scutellata* paternity ($F_{1,352}$ = 5.303, $p < 0.001$, fig. 1). There was no significant interaction of source colony ($F_{1,352}$ = 2.827, p = 0.094) but workers in the second trial were significantly heavier than in the first trial ($F_{1,352}$ = 4.730, $p < 0.03$).

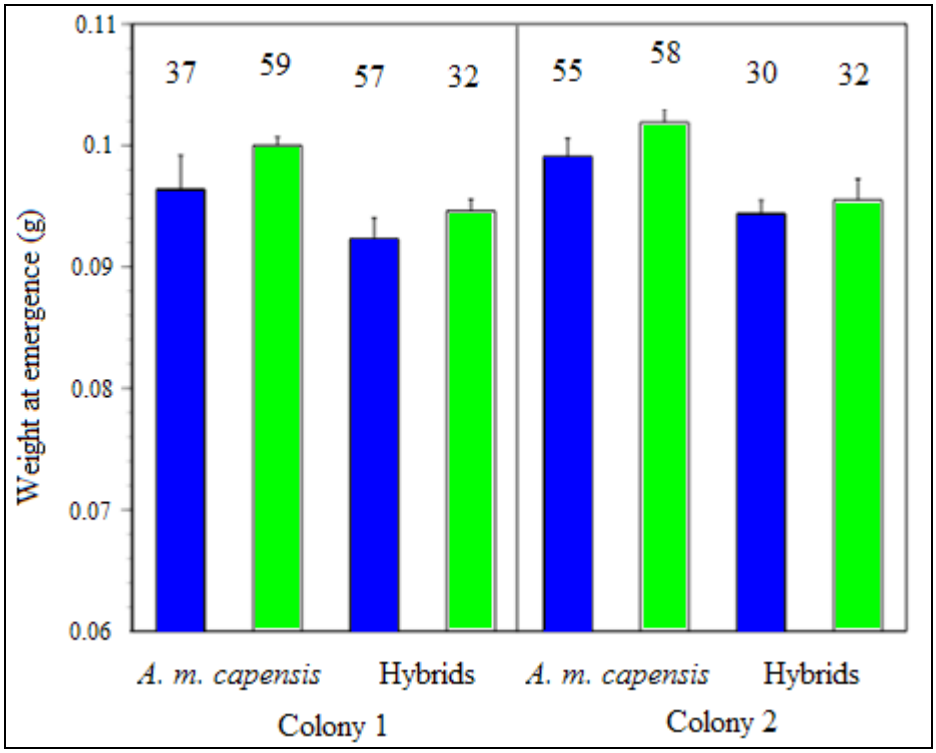

Figure 1: Mean emergence weight of bees collected less than 1 hour after emergence per colony, per trial (trial 1 = ■; trial 2 = ■). '*A. m. capensis*': workers sired by a *capensis* father; 'hybrid': workers sired by a *scutellata* father. Error bars are the standard errors of the means. Workers of *capensis* paternity had significantly higher emergence weights than workers of *scutellata* paternity ($F_{1,352}$ = 5.303, p < 0.001). There was no significant interaction of source colony ($F_{1,352}$ = 2.827, p = 0.094) but workers of both genotypes were significantly heavier in the second trail than in the first trial ($F_{1,352}$ = 4.730, p < 0.03). Numbers denote number of bees successfully genotyped.

7.3.2 Ovary activation

Workers of *capensis* paternity were significantly more likely to have active ovaries than workers of *scutellata* paternity in both trials (table 1, fig. 2). A heterogeneity G-test showed that 'trial' had a significant effect in both colonies (table 1) whereas 'colony' only had a significant effect in trial 2 (table 2).

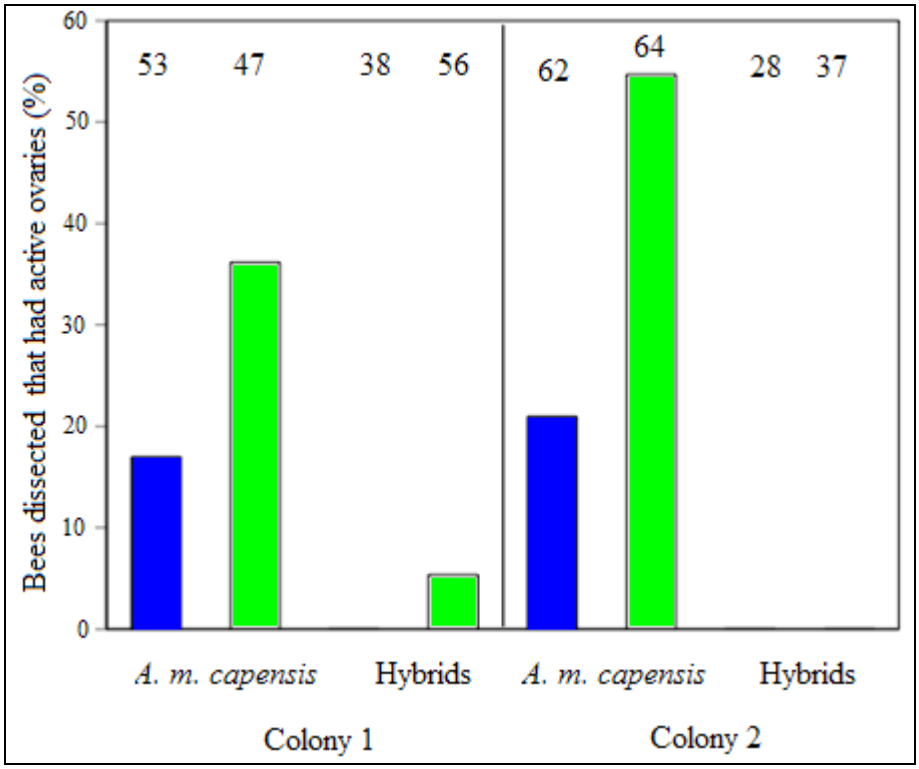

Figure 2: Percentage of *capensis* and *scutellata* patriline individuals with active ovaries per colony, per trial (trial 1 = ■; trial 2 = ■). '*A. m. capensis*': workers sired by a capensis father; 'hybrid': workers sired by a *scutellata* father. Workers of *capensis* paternity were significantly more likely to have active ovaries than workers of *scutellata* paternity in both trials (Table 1 and 2). Numbers denote number of bees successfully genotyped.

Table 1: G tests for the association between ovary activation and presence of a spermatheca with worker paternity. Heterogeneity tests were calculated by taking the absolute value of the difference of 'G of Total' and the 'Total G'. Here we test for the effect of 'trial'.

	Colony 1			Colony 2		
	G	df	*p*	*G*	df	*P*
Trial 1	10.432	1	0.001	10.653	1	0.001
Trial 2	16.488	1	<0.001	18.151	1	<0.001
G of Total	22.833	1	<0.001	22.573	1	<0.001
Total *G*	26.920	2	<0.001	28.804	2	<0.001
Heterogeneity	4.087	1	0.043	6.231	1	0.013
Spermathecae						
Trial 1	23.022	1	<0.001	23.774	1	<0.001
Trail 2	13.361	1	<0.001	21.025	1	<0.001
G of Total	32.030	1	<0.001	44.450	1	<0.001
Total *G*	36.383	2	<0.001	44.799	2	<0.001
Heterogeneity	4.353	1	0.037	0.349	1	0.555

Table 2. G tests for the association between ovary activation and presence of a spermatheca with worker paternity. Heterogeneity tests were calculated by taking the absolute value of the difference of 'G of Total' and the 'Total G'. Here we test for the effect of 'colony'.

	Trial 1			Trial 2		
	G	df	*p*	*G*	df	*P*
Colony 1	10.432	1	0.001	16.488	1	<0.001
Colony 2	10.653	1	0.001	18.151	1	<0.001
G of Total	21.674	1	<0.001	39.187	1	<0.001
Total *G*	21.085	2	<0.001	34.639	2	<0.001
Heterogeneity	0.589	1	0.442	4.548	1	0.033
Spermathecae						
Colony 1	23.022	1	<0.001	13.361	1	<0.001
Colony 2	23.774	1	<0.001	2 1.025	1	<0.001
G of Total	151.717	1	<0.001	281.214	1	<0.001
Total *G*	46.796	2	<0.001	34.386	2	<0.001
Heterogeneity	104.921	1	<0.001	246.828	1	<0.001

7.3.3. Presence of spermatheca

Workers of *capensis* paternity were more likely than those of *scutellata* paternity to have a spermatheca in both trials (table 1; fig. 3). A heterogeneity G-test showed that 'trial' had a significant effect in colony 1 only (table 1) whereas 'colony' had a significant effect in both trials (table 2).

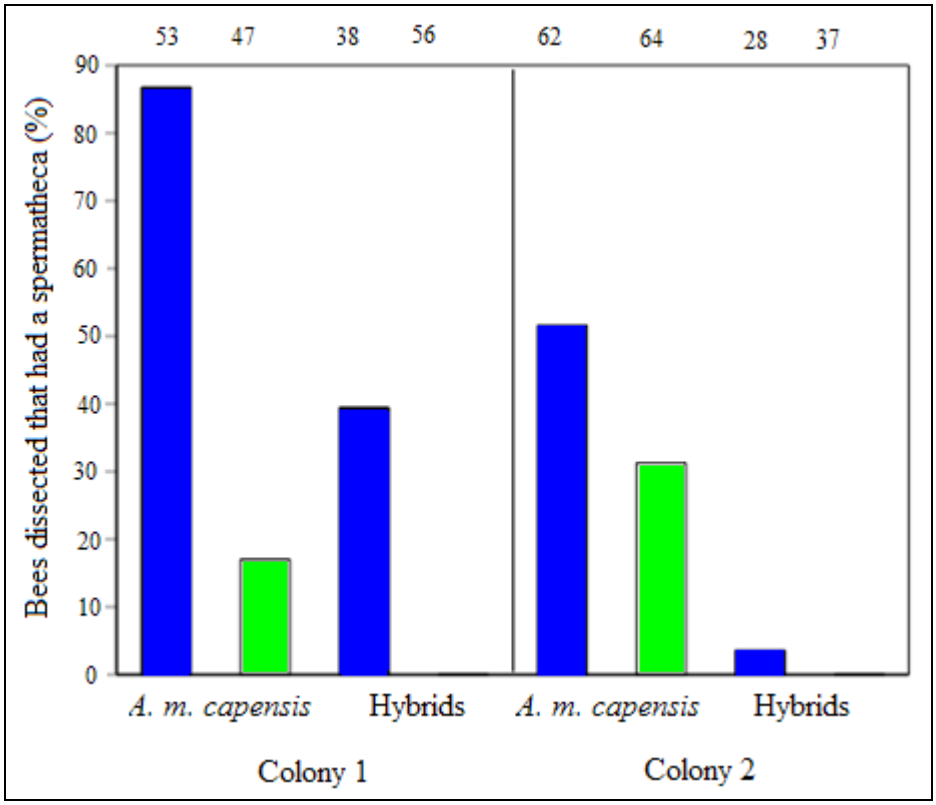

Figure 3: Percentage of *capensis* and *scutellata* patriline individuals that possessed a spermatheca per colony, per trial (trial 1 = ■; trial 2 = ■). '*A. m. capensis*': workers sired by a *capensis father*; 'hybrid': workers sired by a *scutellata* father. Workers of *capensis* paternity were more likely than those of *scutellata* paternity to have a spermatheca in both trials (Table 1 and 2). Numbers denote number of bees successfully genotyped.

7.3.4 Mandibular gland extracts

The 9ODA in the mandibular gland extracts of workers sired by *capensis* was significantly higher compared to those sired by *scutellata* ($F_{1,309} = 13.500$, $p < 0.001$, fig. 4). There was a significant effect of source colony ($F_{1,309} = 18.263$, $p < 0.001$) but not of trial ($F_{1,309} = 1,722$, $p = 0.190$). We found a positive correlation between ovary activation and the amounts of 9ODA (Spearman: $r = 0.329$, $p < 0.001$).

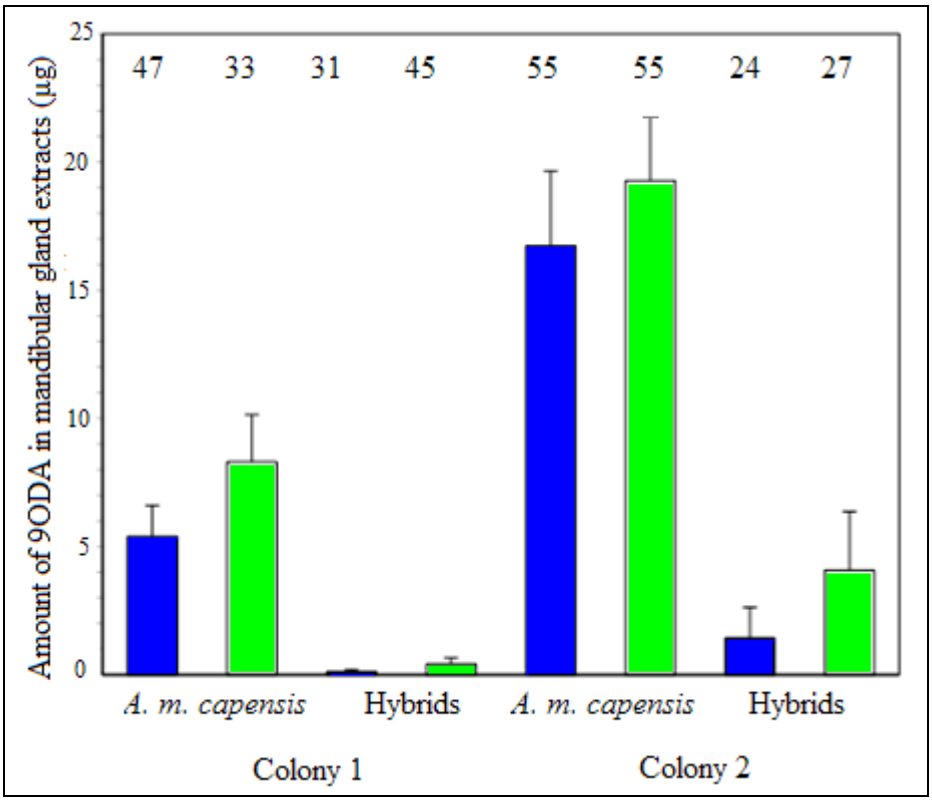

Figure 4: Mean levels of 9ODA secreted by 12 days old workers per colony, per trial (trial 1 = ■; trial 2 = ■). '*A. m. capensis*': workers sired by a *capensis* father; 'hybrid': workers sired by a *scutellata* father. Error bars are the standard errors of the means. Workers of *capensis* paternity had significantly higher levels of 9ODA than workers of *scutellata* paternity ($F_{1,309} = 13.500$, $p < 0.001$). There was a significant effect of source colony ($F_{1,309} = 18.263$, $p < 0.001$) but not of trial ($F_{1,309} = 1.722$, $p = 0.190$). Numbers denote number of bees successfully genotyped.

7.4 Discussion

Pure *capensis* workers were heavier at emergence and showed higher rates of ovary activation and presence of spermatheca than hybrid workers. In addition their mandibular gland secretions were also more queenlike. Our results thus support our hypothesis that in colonies containing both pure *capensis* workers and *capensis* x *scutellata* hybrids, pure *capensis* workers receive more larval food (reflected in a higher weight at emergence) and as a result are more likely to become reproductively active than hybrid workers when reared in the same colony.

Because differential feeding of larvae is responsible for caste determination in honeybees (de Wilde et al., 1982), the amount and quality of larval food received has profound effects on a worker's reproductive potential. In general, increased larval feeding leads to adult workers developing a more queenlike morphology. When *capensis* brood is reared by nurse workers of other subspecies, *capensis* larvae receive more nutritious and greater amounts of food resulting in bees with intermediate traits between those of workers and queens in that they develop faster and have more ovarioles (Allsopp et al., 2003; Beekman et al., 2000; Calis et al., 2002).

Larvae produce pheromones that regulate the quantity of food they receive (Le Conte et al. 1995). Jordan et al. (2008a) postulated the existence of a single locus, *Larva*, which, when homozygous recessive, results in increased expression of reproductive traits via increased larval feeding. The recessive allele, *l*, is presumed to be present in high frequency in the *capensis* population and the dominant allele, *L*, in the *scutellata* population. An individual homozygous for *l* will receive more food than an individual homozygous for *L* or heterozygous and as a result becomes morphologically more queen-like. Our results are consistent with this hypothesis. Judging from the emergence weight data, it would appear that larvae from *capensis* patrilines (of putative genotype *ll*) received more food and as a result were more likely to activate their ovaries than hybrid larvae (putative genotype *Ll*), as well as more likely to have a spermatheca. Based on the latter assumption, increased larval feeding could also have led to the higher levels of queen pheromones as pure *capensis* workers produced higher amounts of 9ODA compared with hybrid workers.

This study simulated conditions under which *capensis* queens mate with both *capensis* and *scutellata* males. Such colonies contain a mixture of pure *capensis* and *capensis-scutellata* hybrid workers. The increased emergence weight observed in

capensis workers suggested increased feeding of these larvae supporting their proposed 'feed-me' signal that results from the existence of a single recessive allele (*l*) that influences the amount of food larvae receives (Jordan et al., 2008a). The lower emergence weights of hybrid workers suggest that they are heterozygous (*Ll*) and the "feed-me" signal they emit is therefore less pronounced. Due to this genetic predisposition to increased feeding, pure *capensis* have higher levels of 9ODA and higher rates of ovary activation. Levels of 9ODA production are a good indicator of reproductive potential (Simon et al., 2005). Therefore in hybrid colonies *capensis* workers are more likely to become reproductively active, producing clonal offspring which in turn can produce their own clonal offspring (Martin et al, 2002a). Such worker reproduction is not sustainable and ultimately leads to the death of the colony (Martin et al., 2002b), effectively preventing gene flow between *capensis* and *scutellata* across the hybrid zone.

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CHAPTER 8

CONCLUSION

8.1 Discussion

One of the proximate factors for native and clonal parasitic *capensis* workers becoming reproductively active is the ability to produce queenlike mandibular gland pheromones. The switch to producing queen pheromone biosynthetic pathways is most likely genetically controlled while the conversion to a full queen signal is controlled by the social environment. Our initial objective was to determine if the mandibular gland profile of native and parasitic workers were influenced by genes and/or the social environment.

Both native and parasitic workers showed a predisposition to produce queenlike mandibular gland compounds. An interesting observation was the fact that the onset of developing a queenlike pheromonal phenotype appears to be genetic, but the continued development to producing the end compound, 9ODA, seems to be influenced by the workers' social context. When native and parasitic workers are aged with members of their own subspecies, only a few individuals are able to continue development by converting 9HDA to 9ODA. This suggests the presence of reciprocal suppression of workers on each other. It is clear from our data that under queenless conditions workers use queenlike mandibular gland pheromones to establish dominance hierarchies. Both the native and parastic *capensis* worker populations share characteristics that place them at a reproductive advantage over workers from other subspecies, despite the fact that these two populations have been separated for over 20 years. Workers from both populations are thus able to behave as social

parasites. Therefore in an appropriate social context, i.e. when workers of these two groups are aged together, the clonal parasitic workers do not necessarily pheromonally out-compete native workers. However, we have established that the speed of mandibular gland signal development is more rapid in parasitic workers than in native workers. This might be one trait in parasitic workers that has been selected for. Early and rapid production of queenlike mandibular compounds in parasitic workers confers a reproductive headstart on them (Dietemann et al., 2007). As a result these parasites can spread faster in the host population. It also enables them to suppress the onset of reproductive development in other workers (Velthuis et al., 1990; Moritz et al., 2000, 2004; Simon et al., 2005). Production of queenlike mandibular secretions also allows workers to obtain food by trophallaxis with increased probability (Hillesheim et al., 1989) and this provides the necessary protein for their oogenesis (Schäfer et al., 2006).

Moritz et al. (2005) proposed the existence of self-organised mechanisms of mutual worker interactions facilitated by differential response to 9ODA concentrations that is controlled by at least two feedback loops. If workers are exposed to a level of queen pheromone (9ODA) lower than their suppression threshold, said workers will not be suppressed. The latter will now increase their queen pheromone production up to their genotypical physiological limit. Being exposed to queen pheromone levels higher than their own will however result in 9ODA production not being initiated or decreased, thereby altering the suppression threshold of the workers. The suppression threshold and 9ODA production are closely related, since it is important that workers do not inhibit themselves. So the production of 9ODA by workers raises their individual thresholds above the level at which they can commence production. Dominant workers thus enhance their pheromone production by suppressing other workers, and

through this they raise both their own suppression thresholds and the 9ODA exposure levels of other workers.

Both native and parasitic workers utilize the queen biochemical pathway but the oxidation of 9HDA to 9ODA is inhibited. This results in workers remaining non-reproductive and the reproductive division of labour is maintained within the colony. The production of 9ODA in *capensis* workers, like in *capensis* queens, has been found to be much higher than in other subspecies (Crewe, 1988; Velthuis et al., 1990; Plettner et al., 1993). This is particularly evident in the presence of *scutellata* workers. This might be linked to the fact that *scutellata* workers do not emit strong enough signals necessary to suppress the pheromonal development of *capensis* workers who have a high response threshold to queenlike signals (Crewe, 1982; 1988; Magnuson, 1995).

Unlike parasitic workers and pure *capensis* workers (offspring from *capensis* queen and *capensis* drone), hybrid workers (offspring from *capensis* queen and *scutellata* drone) do not show a strong queenlike predisposition to mandibular gland signals. It has been suggested that the production of 9ODA is pleiotropically linked to the gene that regulates the mode of reproduction in workers (Lattorff et al., 2007). Workers that are homozygous recessive for the *Thelytoky* (*th/th*) allele are thelytokous, produce high levels of 9ODA and initiate reproduction earlier compared with workers that are either heterozygous (*th*/+) or homozygous wild-type (+/+). Workers of the *th*/+ or +/+ genotype reproduce arrhenotokously. If we assume that Lattorff and colleagues' (2007) genetic model is correct, our pure *capensis* workers are of *th*/*th* genotype whereas our hybrids would be *th*/+. Our results from these crosses suggest that paternity may have an effect on signal production. However to confirm this we would have needed to inseminate *scutellata* queens with the semen of *capensis* drones to

establish whether the resulting offspring also produces queenlike signals, but these signals may just as well be maternally inherited.

Matching genotype to phenotype has been the standard approach to elucidate the genetic foundations of social insect phenotypes (Lynch et al., 1998; Robinson et al. 2005; Hunt et al. 2007; Oldroyd et al., 2007). A flaw in this method is that it does not consider how interacting phenotypes are influenced by or are directly involved in social interactions (Moore et al., 1997). Interacting phenotypes are determined by the interplay of genes and the social environment. As such an individual's phenotype is directly affected by its own genes (direct genetic effects), and indirectly affected by genes expressed in social partners (indirect genetic effects; Moore et al. 1997; 2003). Chemical and physical interactions with brood, other workers and the queen form the basis of the social regulatory network of the environment that individuals experience (Hölldobbler, 1992; Seeley, 1995; Slessor et al., 2005) and determine the developmental trajectories and expressed phenotypes. The social environment becomes critical in determining the reproductive success of individuals within the group due to social competition, age structure of the colony and the genetic relatedness among nestmates which affects the expression of phenotypic traits (West-Eberhard, 1979; Wcsilo, 2000).

A.m. capensis workers have a genetic predisposition to initiating queen biosynthetic pathways but the final product is strongly influenced by the social environment of the workers. Consequently, the physiological traits linked to reproductive potential (i.e. queen pheromone development, ovary activation) in honey bees seems to be determined by a combination of environmental and genetic factors that shift physiological parameters during development and result in altered physiological and behavioural responses (Arthur, 2000).

The advances in molecular biology have enabled the generation of accurate information on the genetics of insect social behaviour and much emphasis has been placed on kinship studies (Robinson et al., 1997). These advances may outpace our understanding of the ecological and behavioural contexts in which genes are expressed and hence in which phenotypes develop. Research must pay attention to the genetic control of developmental strategies to gain insight into how variation in gene expression produce canalized phenotypes yet simultaneously can be modified in response to environmental cues to influence the same character. The signal plasticity assists workers to realize their reproductive potential under changing social contexts. The genetic pathways underlying pheromone signalling may eventually reveal the evolving basis of sociality.

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Appendix A:

GENOTYPE AFFECTS *A. M. CAPENSIS* AND HYBRID HONEYBEE WORKERS' REPRODUCTIVE POTENTIAL

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Abstract We studied the effect of genotype on the reproductive potential of workers of the Cape honeybee (*Apis mellifera capensis*) and hybrids of *A. m. capensis* x *A. m. scutellata.* We produced colonies in which workers were either sired by *A. m. capensis* or *A. m. scutellata* males by artificially inseminating *A. m. capensis* queens. This mimics the situation found in the natural hybrid zone in which both subspecies interbreed. We measured emergence weight and determined rates of ovary activation and the presence/absence of a spermatheca in workers sired by *A. m. capensis* and *A. m scutellata* males. We also calculated the quantity of the major component of queen madibular gland pheromone (E)-9-keto-2-decenoic acid (9ODA), a potential indicator of reproductive dominance in honeybee workers. Workers sired by *A. m. capensis* drones weighed more and were more likely to have active ovaries than those sired by *A. m. scutellata* drones. Similarly *A. m. capensis* sired-workers were more likely to have a spermatheca than *A. m. scutellata* sired-workers and had a more queen-like chemical profile. We discuss the implications of our findings on the stability of the hybrid zone in South Africa.

Keywords: *Apis mellifera capensis*, *Apis mellifera scutellata*, hybrid zone, larval feeding, worker reproduction

Introduction

Reproductive division of labour is a defining characteristic of insect societies. The ability of colony members to become reproductively active varies from species to species. In termites, helper and breeder castes arise from separate developmental trajectories, with the breeder caste being morphologically specialised for aerial dispersal and the foundation of new colonies (Noirot and Pasteels 1987; Roisin 2000). In many social bees, wasps and ants, reproductive division of labour is not determined by morphology but by extrinsic (e.g. mating opportunities) or intrinsic (e.g. relative fighting ability) factors. Differences in reproductive ability among adults are typically established during larval development, but all adult females can, in theory, become breeders (O'Donnell 1998). In other Hymenopteran species the differentiation between worker and breeder caste is rigidly determined by metamorphosis, by either genetic factors (Anderson et al. 2006), larval feeding (Wheeler 1986), or a combination of both (Kerr and Nielsen 1966) (reviewed in Schwander et al. (2010)). In these species there is a loss of totipotency early in the larval stage, if not before, that results in discrete reproductive (queen) and helper (worker) castes. Here, the queen is the only individual capable of sexual reproduction, while all other colony members are specialised workers.

The loss of totipotency of colony members decreases the potential for reproductive conflicts within colonies as it results in divergent selection pressures such that helpers are selected to increase their inclusive fitness (Hamilton 1964a, b) via becoming better workers, and breeders by increasing their direct fitness by massively increasing their reproductive output (Beekman et al. 2006). However, as insect societies rarely consist of clones, the reproductive interests of colony members do not completely overlap (Beekman and Ratnieks 2003). Therefore, though the potential for conflict remains, the reproductive options for workers are severely limited because workers cannot mate; the best they can do is to produce a few males via arrhenotokous parthenogenesis if they reproduce at all (Bourke 1988).

When workers produce diploid female offspring without mating (thelytokous parthenogenesis), conflicts within the colony are expected to increase because the workers' reproductive potential are greater since they are related to their female-producing eggs by unity (Greeff 1996). In the Cape honeybee *Apis mellifera capensis* (hereafter '*capensis*') the

workers are capable of thelytokous parthenogenesis (Onions 1914) and this leads to overt competition among workers and between the queen and the workers over reproduction (Hepburn 1994; Jordan et al. 2008b; Moritz et al. 1996; Moritz et al. 2004). Selection for increased reproductive capacity in workers has resulted in *capensis* workers exhibiting morphological traits not found in workers of any other (sub)species of *Apis*. *Capensis* workers have higher numbers of ovarioles compared with workers of other subspecies (Hepburn 2001; Hepburn and Crewe 1991) and often have a spermatheca, a sperm storage organ usually only present in queens (Hepburn 2001; Hepburn and Crewe 1991; Jordan et al. 2008a).

Caste determination in honeybees is determined by larval feeding, with queen-destined larvae receiving a greater amount of food that is nutritionally different to that received by worker-destined larvae ('royal' versus 'worker' jelly) (de Wilde and Beetsma 1982). The expression of queen-like characteristics in *capensis* workers is strongly influenced by larval feeding. Larvae that are fed more and better develop into more queen-like individuals relative to those fed a more frugal diet (Allsopp et al. 2003; Beekman et al. 2000; Calis et al. 2002).

The Cape honeybee is not the only honeybee present in South Africa. While *capensis* is confined to the southern part of the Western and Eastern Cape, the African honeybee *A. m. scutellata* (hereafter *scutellata*), is found throughout the rest of South Africa and in countries to its north (Ruttner 1988). The two subspecies interact within a hybrid zone situated in the semi-arid areas of the Karoo ecotone (Hepburn and Crewe 1991). This hybrid zone is particularly interesting because *capensis* can be a lethal social parasite of *scutellata* (Allsopp 1992). *Capensis* workers are known to enter *scutellata* colonies and parasitise them with their eggs thus producing more clonal, parasitic workers (Martin et al. 2002a; Neumann and Hepburn 2002). The end result of such invasion is the death of the host colony (Martin et al. 2002b). Despite its parasitic potential, *capensis* has not been found outside its natural range without artificial movement by humans (reviewed in Beekman et al. (2008)). The stability of the honeybee hybrid zone is even more intriguing given that *scutellata* also has traits that make it highly invasive. Since its introduction into Brazil in 1956, *scutellata* has largely displaced all European *A. mellifera* subspecies the Americas (Schneider et al. 2004).

One hypothesis for the stability of the *capensis-scutellata* hybrid zone is that it is in fact a tension zone (Beekman et al. 2008). Tension zones arise when hybrids are less fit than either

parental genotype (Barton and Hewitt 1985). Due to the reduced fitness of hybrids, gene flow between the parental populations is curtailed or prevented (Barton and Hewitt 1985). Within the honeybee hybrid zone of South Africa, queens of both *capensis* and *scutellata* may mate with drones of both subspecies, resulting in hybrid or 'mixed' colonies consisting of pure workers of the queen's genotype and *capensis-scutellata* hybrids (Beekman et al. 2008). Beekman et al. (2008) suggested that mixed colonies may be less fit than pure colonies of either *capensis* or *scutellata* due to pheromonal imbalances that lead to a breakdown in reproductive division of labour.

The tension zone hypothesis (Beekman et al. 2008) is based on the pheromonal polymorphism between *capensis* and *scutellata* (Wossler 2002). Pheromones are essential in regulating reproductive division of labour in honeybee colonies. For example, workers use the pheromones emitted by the queen and her brood to assess her fecundity (Mohammedi et al. 1998). Pheromones produced by larvae affect the amount and quality of food larvae receive and there is a strong interaction between the genotype (*capensis* or *scutellata*) of the larva and the genotype of the nurse workers (Allsopp et al. 2003; Beekman et al. 2000). This interaction is particularly important in colonies of mixed genotypes (Jordan et al. 2008a).

Capensis brood reared by *scutellata* or *capensis-scutellata* hybrids receive more and better food than when they are reared by their own sisters (Allsopp et al. 2003; Calis et al. 2002). Thus *capensis* workers reared by *scutellata* nurses have a strong tendency to develop queen-like characteristics (Allsopp et al. 2003; Beekman et al. 2000). When these workers become reproductively active, colony productivity is likely to decline because reproductive workers work less than their sterile sisters (Hillesheim et al. 1989; Martin et al. 2002b). Thus, Beekman et al. (2008) hypothesised that colonies in which a large proportion of workers are reproductively active suffer a reduction in colony-level fitness relative to colonies of either *capensis* or *scutellata* genotype (in which most or all workers are functionally sterile). This suggests that genetically mixed colonies in the natural hybrid zone may have significantly lower fitness than 'pure' colonies, thus selecting against hybrids and hybridisation.

Here we investigate whether workers from *capensis* patrilines (sired by *capensis* drones) have a higher propensity in becoming reproductively active compared to *capensis-scutellata* hybrid workers (sired by *scutellata* drones). If we find that workers of *capensis* paternity are

more likely to become reproductively active, it would suggest that genetically mixed colonies may suffer from a breakdown in reproductive division of labour and that the hybrid zone is indeed a tension zone. Under our experimental conditions, similar aged pure *capensis* and hybrid workers shared all environmental influences and the same maternal genotype on average. They therefore only differ in their paternal genotype which allowed us to determine whether the expression of the reproductive traits can be influenced by paternity.

Materials & Methods

Queen-rearing

Capensis queens were reared in the Stellenbosch area (33°56' S, 18°51'E) in late September and early October 2008. Queen-cells were harvested from the rearing colonies nine days after grafting and emerged in an incubator at 35°C. Upon emergence, the queens' wings were clipped and individually stored for genetic analysis. Newly emerged queens were placed with 20-30 young attendant bees in cages and stored in an incubator for 5-6 days until insemination.

Instrumental insemination of queens

Capensis queens were artificially inseminated with semen from five *capensis* and five *scutellata* drones (for details see Holmes et al. (2010)). The queens therefore produced workers that were either pure *capensis* (sired by a *capensis* drone) or *capensis-scutellata* hybrids (sired by a *scutellata* drone). As there are no diagnostic markers with which *capensis* and *scutellata* genotypes may be distinguished (Franck et al. 2001), drones used were kept for genetic analysis to allow us to determine the father of the workers sampled (see below).

Immediately after insemination queens were introduced into a 5-frame hive with *scutellata* workers and brood only. The day after insemination, queens were retrieved from their colony, anaesthetized with CO_2 to initiate egg-laying (Mackensen 1947), and released into the colony. Colonies were subsequently checked every two to three days. Colonies with laying queens were checked weekly by removing each frame and checking the state of the brood. Queen-cells, if any, were removed to prevent supersedure.

Test environment

Newly emerged bees were collected by placing a frame of emerging brood in an incubator at 35°C. Frames were inspected daily. All bees that had emerged overnight were marked on the thorax with non-toxic paint (Posca Paint Pens, Mitsubishi Pencil Co., Japan), using a different colour for each colony. After we marked 500 bees per colony, we checked each frame every hour and collected 100 emerged bees to determine their weight at emergence. These weighed bees were retained for genotyping to determine if paternity affects emergence weight.

We wanted to maximise the probability that *capensis* and *scutellata* patriline workers would become reproductively active. Therefore we introduced the marked workers into a freshly dequeened *scutellata* colony. Young bees from both source colonies were placed in the same *scutellata* colony to control for host colony effects.

All marked bees were harvested 12 days after introduction. Heads were removed for mandibular gland gas chromatography (GC) analysis and individually labelled and stored in 200µl dichloromethane (DCM, Merck). The abdomen and thorax (also individually labeled) were frozen for dissections and genetic analysis. This experiment was repeated with the same two queens two months later (January 2009 – trial 2).

Dissections

We pinned each worker onto a wax plate through the thorax and separated the fifth and sixth dorsal tergites using fine forceps to expose the reproductive organs, under irrigation with water. In workers, the section of the ovary containing ovarioles is positioned above the hind gut, and the spermatheca below the hind gut (Dade 1977). Spermathecae were scored by lifting the hind gut aside and recording whether a spermatheca was present or absent. We assessed the developmental stage of the ovaries using standard criteria (Velthuis 1970): 1=no development; 2=slightly thickened ovarioles; 3=round or bean shaped eggs visible (early stage of activation); 4=fully developed ovarioles with eggs greater than 50% of full size. To determine the father's subspecies, the dissected bees were stored in alcohol in microcentrifuge tubes prior to genetic analysis.

Gas Chromatography

The heads of dissected workers were removed from the vials and the DCM evaporated under a stream of nitrogen just to dryness. The residue was redissolved in 15µl internal standard (octanoic acid and tetradecane in 4 ml DCM) and 15µl bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). One microlitre was injected into a gas chromatograph (HP-6850) with a split-splitless inlet and a 25mm x 0.32 mm methyl-silicone coated fused silica capillary column. Helium was used as carrier gas at a flow rate of 1.9ml/min. The oven temperature was controlled as follows: 1 min at 60 ℃; followed by a heating phase of 50℃/min to 110℃, and subsequently another of 3℃/min to 220℃. Finally the temperature was held at 220℃ for 10 min. Peak areas were determined using HP Chemstation software and the mandibular gland compounds were identified based on the retention times of authentic compounds and were quantified using peak area and the relative mass ratios (for methodological details see Simon et al. (2001)). Of the 12 mandibular gland compounds identified, only the amount of 9ODA was quantified since this compound is a potential indicator of reproductive dominance in honeybee workers (Moritz et al. 2000; Simon et al. 2005).

Genetic Analysis

DNA was obtained from the queen (wingtips), fathering drones and workers (2-3 legs) from each colony using a high salt extraction method (Aljanabi and Martinez 1997). The fathering drones were screened with seven *A. mellifera* microsatellite markers used in previous parentage studies: Am005, Am006, Am008, Am046, Am052, Am059 and Am061 (Solignac et al. 2003). For one colony one microsatellite locus (Am061) was sufficient to distinguish workers sired by *capensis* drones from those sired by *scutellata* drones. For the second colony two microsatellite loci (Am061 and Am008) were required.

PCR products (0.4µl) from each reaction were added to 10µl formamide and 100nl LIZ DNA size standard (Applied Biosystems). Samples were run on a 3130*xl* Genetic Analyser (Applied Biosystems) with capillary length 36cm and injection time of 15s at 1200V, for 41 minutes. Results were analysed using Genemapper software (Applied Biosystems) and the sire of the workers (*capensis* or *scutellata*) was determined. Microsatellite allele sizes were distinguishable due to a unique combination of dye colour and amplicon size range.

Statistical Analysis

We used a univariate Analysis of Variance to compare mean weight at emergence between *capensis* and *capensis-scutellata* hybrids. We also used a univariate Analysis of Variance to test if the absolute amount of 9ODA differed between *capensis* and *capensis-scutellata* hybrids. We tested the hypothesis that ovary activation (active or not active) and presence/absence of a spermatheca was independent of worker paternity with 2x2 contingency tables using *G*-tests (Sokal and Rohlf 1995). Ovary activation was classified as 'non-active' (developmental stages 1 and 2) or 'active' (developmental stages 3 and 4). Differences between source colony and trial were tested using *G*-tests of heterogeneity.

Results

Emergence weight

Workers of *capensis* paternity had significantly higher emergence weights than workers of *scutellata* paternity ($F_{1,352}$ = 5.303, p < 0.001) (Fig. 1). There was no significant interaction of source colony ($F_{1,352}$ = 2.827, p = 0.094) but workers in the second trial were significantly heavier than in the first trial ($F_{1,352}$ = 4.730, p < 0.03).

Ovary activation

Workers of *capensis* paternity were significantly more likely to have active ovaries than workers of *scutellata* paternity in both trials (Table 1, Fig. 2). A heterogeneity *G*-test showed that 'trial' had a significant effect in both colonies (Table 1) whereas 'colony' only had a significant effect in trial 2 (Table 2).

Presence of spermatheca

Workers of *capensis* paternity were more likely than those of *scutellata* paternity to have a spermatheca in both trials (Table 1; Fig. 3). A heterogeneity *G*-test showed that 'trial' had a significant effect in colony 1 only (Table 1) whereas 'colony' had a significant effect in both trials (Table 2).

Mandibular gland secretions

The 9ODA production by workers sired by *capensis* was significantly higher compared to those sired by *scutellata* ($F_{1.309}$ = 13.500, p < 0.001) (Figure 4). There was a significant effect of source colony ($F_{1.309}$ = 18.263, p < 0.001) but not of trial ($F_{1.309}$ = 1.722, p = 0.190). We found a positive correlation between ovary activation and 9ODA production (Spearman: r = 0.329, p < 0.001).

Discussion

Pure *capensis* workers were heavier at emergence and showed higher rates of ovary activation and presence of spermatheca than hybrid workers. In addition their mandibular gland secretions were also more queenlike. Our results thus support our hypothesis that in colonies containing both pure *capensis* workers and *capensis* x *scutellata* hybrids pure *capensis* workers receive more larval food (reflected in a higher weight at emergence) and as a result are more likely to become reproductively active than hybrid workers when reared in the same colony.

Because differential feeding of larvae is responsible for caste determination in honeybees (de Wilde and Beetsma 1982), the amount and quality of larval food received has profound effects on a worker's reproductive potential. In general, increased larval feeding results in adult workers developing a more queen-like morphology. When *capensis* brood is reared by nurse workers of other subspecies, *capensis* larvae receive greater amounts of food as well as more nutritious food resulting in bees with intermediate traits between those of workers and queens in that they develop faster and have more ovarioles (Allsopp et al. 2003; Beekman et al. 2000; Calis et al. 2002).

Larvae produce pheromones that regulate the quantity of food they receive (Le Conte et al. 1995). Jordan et al. (2008a) postulated the existence of a single locus, *Larva*, which, when homozygous recessive, results in increased expression of reproductive traits via increased larval feeding. The recessive allele, *l*, is presumed to be present in high frequency in the *capensis* population and the dominant allele, *L*, in the *scutellata* population. An individual homozygous for *l* will receive more food than an individual homozygous for *L* or heterozygous and as a result becomes morphologically more queen-like. Our results are consistent with this

hypothesis. Judging from the emergence weight data, larvae from *capensis* patrilines (of putative genotype *ll*) received more food and as a result were more likely to activate their ovaries than hybrid larvae (putative genotype *Ll*), as well as more likely to have a spermatheca. Increased larval feeding also led to higher levels of queen pheromones as pure *capensis* workers produced higher amounts of 9ODA compared with hybrid workers.

Levels of 9ODA production are a good indicator of reproductive potential (Simon et al 2005). Lattorff et al. (2007) suggested that the production of 9ODA is pleiotropically linked to the gene that regulates the mode of reproduction in workers. Workers that are homozygous recessive for the *Thelytoky* (*th*/*th*) allele are thelytokous, produce high levels of 9ODA and initiate reproduction earlier compared with workers that are either heterozygous (*th*/+) or homozygous wild-type (+/+). Workers of the *th*/+ or +/+ genotype reproduce arrhenotokously. Although we did not ascertain whether our workers reproduced thelytokously or arrhenotokously, if we assume that Lattorff and colleagues' (2007) genetic model is correct, our pure *capensis* workers are of *th*/*th* genotype whereas our hybrids would be *th*/+. Our results on emergence weight clearly show that workers of *th*/*th* genotype are fed more than bees of genotype *th*/+. This suggests that the traits of thelytokous reproduction and excessive larval feeding are most likely genetically correlated.

This study simulated conditions under which *capensis* queens mate with both *capensis* and *scutellata* males. Such colonies contain a mixture of pure *capensis* and *capensis-scutellata* hybrid workers. We observed increased feeding of *capensis* larvae supporting their proposed 'feed-me' signal that results from the existence of a single recessive allele (*l*) that influences the amount of food larvae receives (Jordan et al. 2008a). The lower emergence weights of hybrid workers suggest that they are heterozygous (*Ll*) and the "feed-me" signal they emit is therefore less pronounced. Due to this genetic predisposition to increased feeding, pure *capensis* have higher levels of 9ODA and higher rates of ovary activation. Therefore in hybrid colonies they are more likely to become reproductively active, producing clonal offspring which in turn can produce their own clonal offspring (Martin et al. 2002a). Such worker reproduction is not sustainable and ultimately leads to the death of the colony (Martin et al. 2002b), effectively preventing gene flow between *capensis* and *scutellata* across the hybrid zone.

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Table 1. *G* tests for the association between ovary activation and presence of a spermatheca with worker paternity. Heterogeneity tests were calculated by taking the absolute value of the difference of '*G* of Total' and the 'Total *G*'. Here we test for the effect of 'trial'.

Active Ovaries						
	Colony 1			Colony 2		
	G	df	*p*	*G*	df	*p*
Trial 1	10.432	1	0.001	10.653	1	0.001
Trial 2	16.488	1	<0.001	18.151	1	<0.001
G of Total	22.833	1	<0.001	22.573	1	<0.001
Total *G*	26.920	2	<0.001	28.804	2	<0.001
Heterogeneity	4.087	1	0.043	6.231	1	0.013
Spermathecae						
Trial 1	23.022	1	<0.001	23.774	1	<0.001
Trail 2	13.361	1	<0.001	21.025	1	<0.001
G of Total	32.030	1	<0.001	44.450	1	<0.001
Total *G*	36.383	2	<0.001	44.799	2	<0.001
Heterogeneity	4.353	1	0.037	0.349	1	0.555

Table 2. *G* tests for the association between ovary activation and presence of a spermatheca with worker paternity. Heterogeneity tests were calculated by taking the absolute value of the difference of '*G* of Total' and the 'Total *G*'. Here we test for the effect of 'colony'.

Active Ovaries						
	Trial 1			Trial 2		
	G	df	*p*	*G*	df	*p*
Colony 1	10.432	1	0.001	16.488	1	<0.001
Colony 2	10.653	1	0.001	18.151	1	<0.001
G of Total	21.674	1	<0.001	39.187	1	<0.001
Total *G*	21.085	2	<0.001	34.639	2	<0.001
Heterogeneity	0.589	1	0.442	4.548	1	0.033
Spermathecae						
Colony 1	23.022	1	<0.001	13.361	1	<0.001
Colony 2	23.774	1	<0.001	2 1.025	1	<0.001
G of Total	151.717	1	<0.001	281.214	1	<0.001
Total *G*	46.796	2	<0.001	34.386	2	<0.001
Heterogeneity	104.921	1	<0.001	246.828	1	<0.001

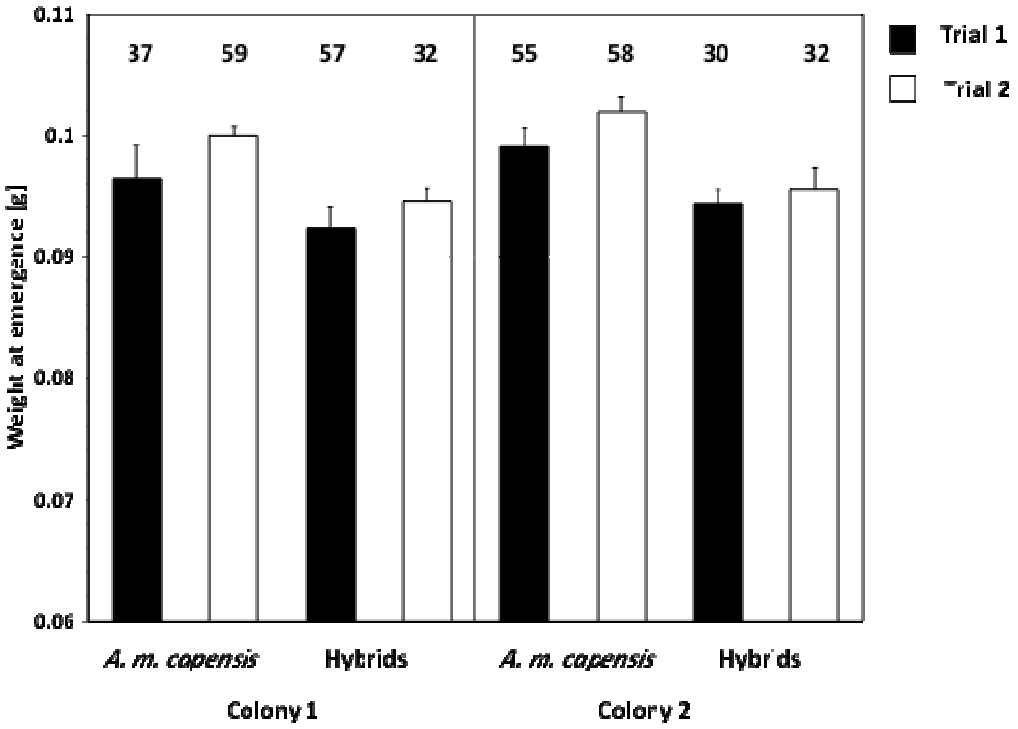

Figure 1. Mean emergence weight of bees collected less than 1 hour after emergence per colony, per trial. '*A. m. capensis'*: workers sired by a *capensis* father; 'hybrid': workers sired by a *scutellata* father. Error bars are the standard errors of the means. Workers of *capensis* paternity had significantly higher emergence weights than workers of *scutellata* paternity ($F_{1,352}$ = 5.303, p < 0.001). There was no significant interaction of source colony ($F_{1,352}$ = 2.827, p = 0.094) but workers of both genotypes were significantly heavier in the second trail than in the first trial ($F_{1,352}$ = 4.730, p < 0.03). Numbers denote number of bees successfully genotyped.

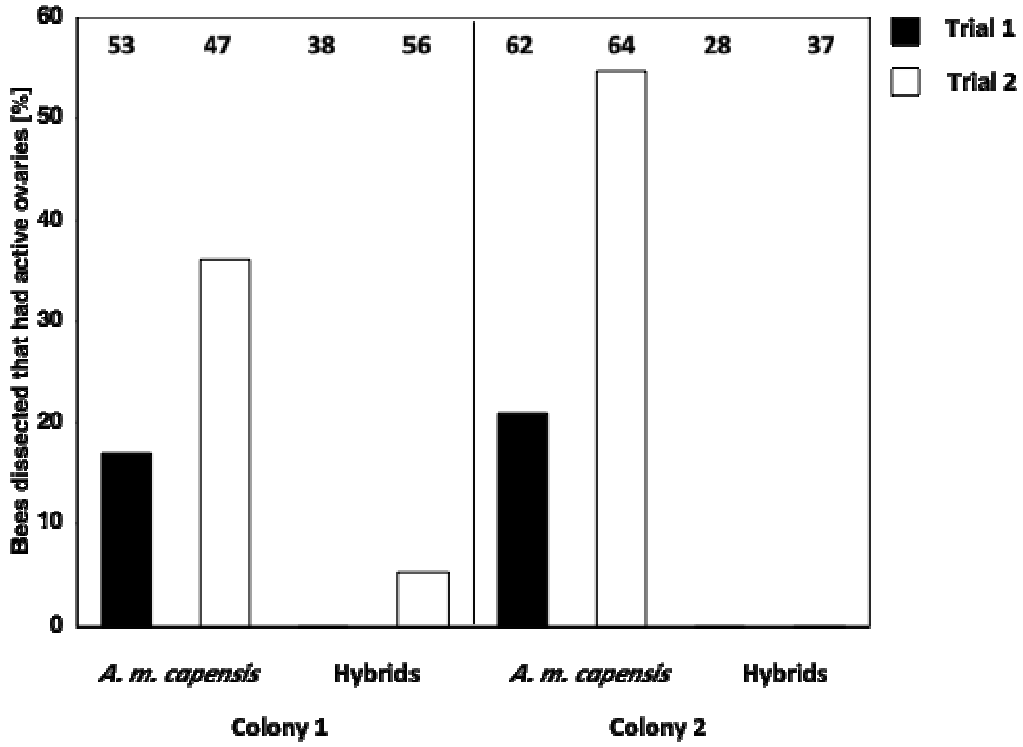

Figure 2. Percentage of *capensis* and *scutellata* patriline individuals with active ovaries per colony, per trial. '*A. m. capensis'*: workers sired by a *capensis* father; 'hybrid': workers sired by a *scutellata* father. Workers of *capensis* paternity were significantly more likely to have active ovaries than workers of *scutellata* paternity in both trials (Table 1 and 2). Numbers denote number of bees successfully genotyped.

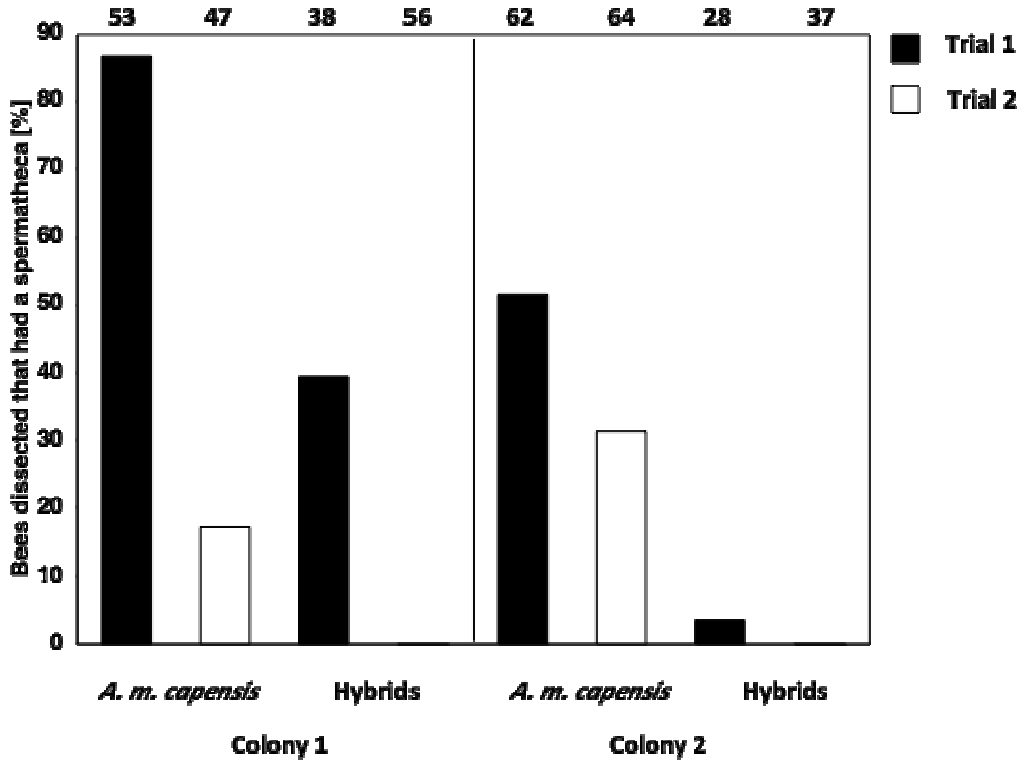

Figure 3. Percentage of *capensis* and *scutellata* patriline individuals that possessed a spermatheca per colony, per trial. '*A. m. capensis'*: workers sired by a *capensis* father; 'hybrid': workers sired by a *scutellata* father. Workers of *capensis* paternity were more likely than those of *scutellata* paternity to have a spermatheca in both trials (Table 1 and 2). Numbers denote number of bees successfully genotyped.

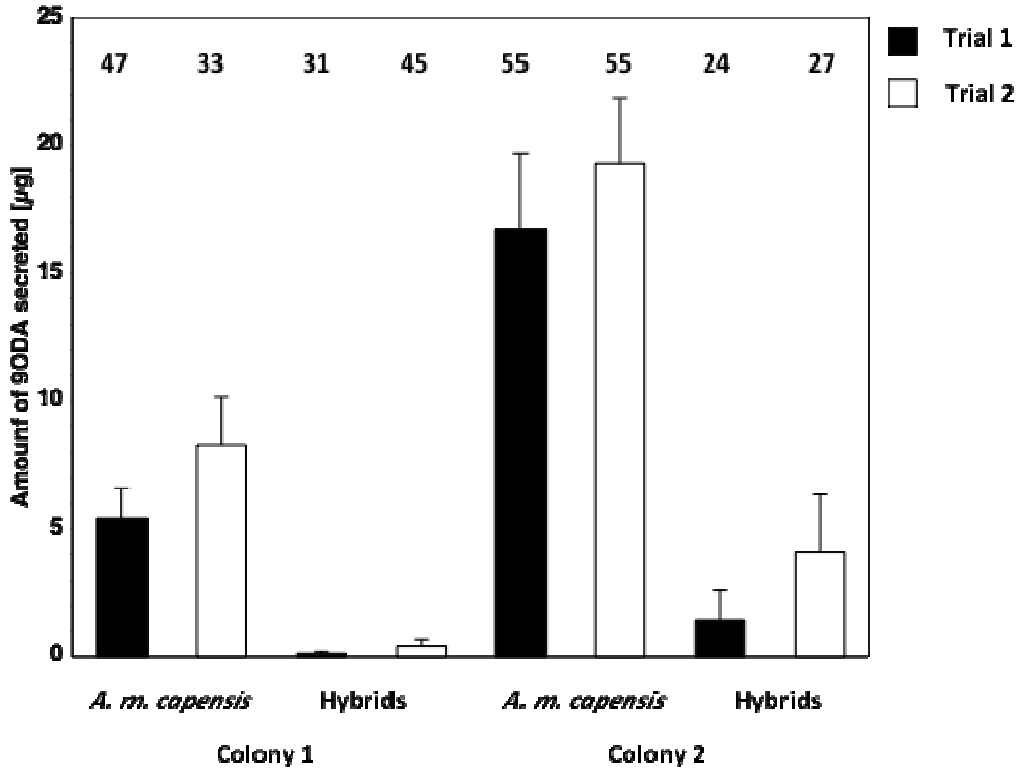

Figure 4. Mean levels of 9ODA secreted by 12 days old workers per colony, per trial. '*A. m. capensis'*: workers sired by a *capensis* father; 'hybrid': workers sired by a *scutellata* father. Error bars are the standard errors of the means. Workers of *capensis* paternity had significantly higher levels of 9ODA than workers of *scutellata* paternity ($F_{1.309}$ = 13.500, p < 0.001). There was a significant effect of source colony ($F_{1.309}$ = 18.263, p < 0.001) but not of trial ($F_{1.309}$ = 1.722, p = 0.190). Numbers denote number of bees successfully genotyped.