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Robin M Crewe
Social Insects Research Group
Dept. Zoology and Entomology
University of Pretoria
P/Bag X20
Hatfield 0028
South Africa
Phone: +27 82 212 9510
E-mail: robin.crewe@up.ac.za

REVIEW ARTICLE

The biology of the Cape honey bee *Apis mellifera capensis*; a review of thelytoky, and its influence on social parasitism and worker reproduction.

Fiona N. Mumoki, Abdullahi A. Yusuf, Christian W. W. Pirk and Robin M. Crewe

Social Insects Research Group, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, Pretoria, South Africa

Abstract

Cohesion in social insect colonies is maintained by use of chemical signals produced by the queen, workers and brood. In honey bees in particular, signals from the queen and brood are crucial for the regulation of reproductive division of labour, ensuring that the only reproductive female individual in the colony is the queen, while the workers remain reproductively sterile. However, even given this strict level of control, workers can, in principle, activate their ovaries and lay eggs. While much is known about the behavioural and physiological traits that accompany the switch from worker sterility to being reproductively active, much less is known regarding the molecular changes that accompany this switch. This review will examine what is currently known about the genes and molecular pathways involved in the making of laying workers / false queens in the Cape honey bee *Apis mellifera capensis* Eschscholtz, through an analysis of the basis for thelotoky in this subspecies, the exocrine glandular chemistry of reproductively dominant workers and what is known about the biosynthesis of their pheromone components. This work will contribute to our understanding of the genetic regulation of thelotoky and the molecular mechanisms that govern reproductive division of labour in honey bees and provide generalisations that may be applicable to other social hymenoptera using this evolutionary fascinating example of worker reproduction.

Key words: Thelytoky, reproductive dominance, pheromone profiles, gene regulation, laying workers

Introduction

In a colony, the main role of reproduction is undertaken by the honey bee queen, as the workers normally possess inactive ovaries and are thus reproductively sterile. However, in the event of queen loss, workers can activate their ovaries and lay unfertilised (and thus haploid) eggs which through arrhenotokous parthenogenesis will result in drones (Crozier 1975). While this is the case in most honey bee subspecies, workers of the Cape honey bee *Apis mellifera capensis* have evolved the exceptional ability to produce female offspring from unfertilised eggs (Onions 1912) -thelytokous parthenogenesis- where the unfertilised eggs become diploid as a result of the central fusion of meiotic products in anaphase II (Verma and Ruttner 1983); although some workers from this subspecies reproduce arrhenotokously (Hepburn and Crewe 1991). The contribution of worker reproduction to drone production is rather small in European subspecies of the honey bee (Visscher 1996). However, the proportion of worker-laid eggs in African honey bee colonies is quite significant (Moritz et al. 1998) with most subspecies producing drones while the Cape honey bee produces females via thelotoky. *A. m. capensis* is one of two subspecies of *A. mellifera* found in South Africa and is native to the Western Cape region of the country in the fynbos biome (Hepburn and Crewe 1991, Hepburn et al. 1998), while the Savannah honey bee *A. m. scutellata* on the other hand is widely distributed across South Africa, and northwards into various parts of East Africa (Hepburn and Radloff 1998, Radloff and Hepburn 2000) (Figure 1A). Separating the two subspecies is a stable natural introgression zone, restricting the naturogenic spread of *A. m. capensis* beyond its native region. Indeed, examining the honey bee colonies surrounding this zone, Neumann et al. (2001) showed that workers from *A. m. capensis* colonies utilised dispersal behaviour (workers drifting over long distances) as a host-seeking mechanism but these dispersing

A. m. capensis workers were strongly rejected by colonies in the hybrid zone. Further, workers of the hybrid colonies also dispersed less than both *A. m. scutellata* and *A. m. capensis* into the surrounding colonies. These two mechanisms (rejection of dispersers and lack of dispersal by host hybrid workers) are thought to contribute greatly to the maintenance of the stability of the hybrid zone (Figure 1B). Through short-sighted evolution, a virulent lineage of *A. m. capensis* workers developed into a facultative reproductive parasite (Moritz et al. 2008) of *A. m. scutellata* (Allsopp and Crewe 1993, Pirk et al. 2014) colonies in South Africa, with the spread of the *A. m. capensis* reproductive parasite mainly facilitated by anthropogenic means (Dietemann et al. 2006a) and maintained through parasite-infested colonies (Figure 1B).

Reproductive parasitism by *A. m. capensis* workers begins when these workers through active drifting and dispersal seek out and gain entry into susceptible hosts such as queenless colonies (Neumann et al. 2001, Hepburn and Radloff 2002, Reece 2002), produce queen-like pheromones (Crewe and Velthuis 1980, Dietemann et al. 2006b, Dietemann et al. 2007, Zheng et al. 2010, Okosun et al. 2017), activate their ovaries and lay eggs, therefore becoming so called false queens (Sakagami 1958). The laying workers' eggs are preferentially nursed by host workers (Beekman et al. 2000, Allsopp et al. 2003) and will eventually emerge as reproductive parasites, continuing the cycle of infestation (Neumann and Moritz 2002). In South Africa, infestation of host colonies by the *A. m. capensis* clonal laying workers continues to result in heavy colony losses to South African apiculture (>40% colony loss annually) in what has come to be known as '*the capensis* calamity' (Allsopp 1993, Pirk et al. 2014).

While relatively more is known regarding the pheromones produced by the laying Cape honey bees, much less is known about the molecular mechanisms that govern the production of these chemical signals. In this review, we will bring together the current body of knowledge regarding the various genetic models for the origins of thelytokous parthenogenesis, describe the composition of the multi-sourced pheromones associated with reproductive dominance and explore some of the genes and molecular pathways involved in the biosynthesis of some of the pheromone components. We will shed light on what is known regarding the basis of reproductive dominance in the Cape honey bee, and hence provide a richer understanding of the evolution of reproductive division of labour in hymenopteran social insects.

Genetic models governing thelytoky

Thelytokous parthenogenesis has been reported in workers of a number of hymenopteran species including about 11 ant species such as *Cataglyphis cursor*, *Cerapachys biroi*, *Mycocepurus smithii*, *Platythyrea punctata* and *Pristomyrmex punctatus* reviewed by Crozier and Pamilo (1996) and Rabeling and Kronauer (2013). At the cytological level, thelytokous parthenogenesis can result from either mitotic (apomictic) or meiotic (automictic) parthenogenesis. For *A. m. capensis* workers, thelytokous parthenogenesis has been shown to take place during the Anaphase II stage of meiosis, with diploidy restored by the fusion of the two central polar nuclei, followed by the disintegration of the other two terminal nuclei (Verma and Ruttner 1983). While this process allows *A. m. capensis* workers to produce female offspring without the need for fertilisation, Hepburn and Crewe (1991) found that some workers from this subspecies still reproduced

arrhenotokously. The genetic switch that allows for arrhenotokous and thelytokous reproduction in *A. m. capensis* has been the subject of a variety of investigations (Ruttner 1988, Lattorff et al. 2005, Lattorff et al. 2007, Jarosch et al. 2011, Chapman et al. 2015, Aumer et al. 2017, Aumer et al. 2019, Christmas et al. 2019, Yagound et al. 2020).

In perhaps one of the earliest assessments of the inheritance of thelytoky in *A. m. capensis*, Ruttner (1988) carried out a cross between *A. m. capensis* and *A. m. carnica* to produce F1 hybrid queens. The sperm from drones that emerged from the hybrid queens (thus the F2 gametes) were then used to inseminate native *A. m. capensis* queens, with each queen inseminated with the sperm of a single drone only. After the emergence of a large enough worker population from each of these queens, the queens were removed in order to induce egg-laying from the workers, with the ratio of female: male offspring from worker-laid eggs recorded. The results generally showed a bimodal distribution of male and female offspring leading, Ruttner (1988) to conclude that thelytoky in *A. m. capensis* is controlled by a single gene. He was however unable to account for the high numbers of offspring that were amphitokous (mixed parthenogenesis).

Using classic backcross experiments and in a follow up to the work of Ruttner (1988), Lattorff et al. (2005) reported that thelytoky indeed was controlled by a single major gene *th,* which segregates in a classic Mendelian manner. The *th* allele was identified as recessive, with the wildtype (*+/+)* and heterozygous dominant *(+/ th)* being arrhenotokous while the homozygous recessive *(th/th)* being thelytokous. Using microsatelite quantitative trait loci analyses, the *th* gene was later mapped to the honey bee Chromosome *13* and a locus on that chromosome, *thelytoky* identified as influencing not only the switch into diploid egg production, but also the full ‘thelytoky syndrome’ which

includes production of queen-like pheromones and rapid ovary activation (Lattorff et al. 2007). Screening candidate genes at the thelytoky locus revealed that alternative splicing of *gemini* (a transcription factor in the CP2 family) regulated sterility in workers by influencing the rate of ovarian activation in the usually sterile bees (Jarosch et al. 2011), and also influenced the production of queen-like mandibular gland pheromone components (Jarosch-Perlow et al. 2018). That *gemini* is the genetic switch controlling thelytoky was however challenged, first by Chapman et al. (2015) who suggested that polymorphism within *gemini* is unlikely to be the sole switch into thelytokous reproduction in the Cape honey bee and suggested instead that a recessive gene tightly linked to three markers within *th* may instead play this role. This multiple-loci model was refuted by Aumer et al. (2017) upon reanalysis of Chapman et al. (2015) genotype data sets and also by examining the segregation of various modes of parthenogenesis in workers of a new mapping population drawn from a single naturally-mated *A. m. capensis* queen. In Chapman et al. (2015), the queen was inseminated with the semen of a single drone, while *A. m. capensis* queens normally mates with up to 56 males (Kraus et al. 2004). Aumer et al. (2017) did however also conclude that while *gemini* plays a significant role in the regulation of reproduction in female honey bees, it is highly unlikely to be the genetic switch to diploid egg production.

Using a population genomics approach and a time-course abundance dynamics analysis, Aumer et al. (2019) showed that this shift in worker reproduction is caused by a single non-synonymous single nucleotide polymorphism (SNP) in the heterozygous dominant thelytoky locus (*Th*) located on Chromosome *1*. The thelytoky allele (Th_{Th}) together with a complementing arrhenotoky allele (Th_{ar}) results in thelytokous workers (Th_{Th}/Th_{ar}), with

158 *(Th_{Th}/Th_{+})* being possibly non-functional and *(Th_{al}/Th_{+})* being fertile arrhenotokous
159 workers. In addition, Aumer et al. (2019) report that the *Th* locus forms a linkage group
160 (*Th-Ethr*), with the *Ecdysis triggering hormone receptor* (*Ethr*). *Ethr* is known to regulate
161 ecdysis (Roller et al. 2010) and the synthesis of Juvenile hormone in insects (Areiza et
162 al. 2014) and in this case it possibly helps in the full expression of the thelytoky syndrome
163 which includes the development of spermatheca, ovary activation and production of
164 queenlike pheromones during larval development of the false queen.

165 The Aumer et al. (2019) model of thelytoky has recently been challenged, initially by
166 Christmas et al. (2019) who through the analysis of a data set of *A. mellifera* subspecies
167 (but without mapping populations) argued that there were populations where the
168 suggested SNP associated with thelytoky is present but the thelytoky syndrome is absent,
169 and that in queens produced through thelytoky, the proposed SNP is absent. Most
170 recently, Yagound et al. (2020) generated backcrosses between thelytokous *A. m.*
171 *capensis* queens and non thelytokous *A. m. scutellata,* looking for markers that co-
172 segregated with the thelytokous phenotype. Yagound et al. (2020) identified the gene
173 GB45239 (LOC100576557) located on Chromosome *11* that encodes a protein putatively
174 involved in chromosomal segregation. The gene GB45239 is expressed in the honey bee
175 ovaries where it is downregulated in thelytokous bees, possibly as a result of
176 polymorphisms in the promoter region found upstream of this gene. Yagound et al. (2020)
177 showed that GB45239 consistently co-segregated with thelytoky and was absent from the
178 genomes of all other honey bee subspecies they sequenced, thus concluding that this
179 must be the gene responsible for the thelytoky syndrome in *A. m. capensis*.

These conflicting outcomes of the experimental exploration of the genetics of thelotoky in the Cape honey bee are due to the fact that some of the studies are based on very small sample sizes, use of few microsatellite markers and also, a lack of consensus on the most appropriate mapping populations for use. Resolving these conflicts will require the functional characterisation of the various genetic variants that govern the switch from social worker to social parasite, and the factors involved in the full expression of the thelytoky syndrome. Further, there is a need for more extensive sampling of this population to generate larger mapping populations in order to establish a better consensus on the divergent results and come to a better understanding of genetic mechanisms underlying thelotoky.

Pheromone signatures of laying *A. m. capensis* workers and their associated pheromone biosynthetic pathways

The phenotype of an organism is influenced by both its genotype and the environment and for social insects, this prevailing environment is further influenced by the genotypes and phenotypes of other conspecific individuals with which it interacts, leading to a complex communication system mediated by chemical signals. In the honey bee colony, the queen is reproductively dominant and will produce chemical signals that inhibit reproductive activity in workers. In the absence of the queen, however, workers can activate their ovaries and, in some subspecies, start producing queen-like chemical signals that will be received by other workers, some of whom will also attempt reproductive behaviour (Dietemann et al. 2007). On the other hand, in the presence of the queen, workers that produce queen-associated signals would be identified and killed by other workers and eggs laid by these workers removed through the process of worker

204 policing (Pirk et al. 2003). For *A. mellifera*, the primary channel for communication is via
205 pheromones. The pheromones and their sources of production have been reviewed by
206 (Slessor et al. 1990, Pankiw 2004, Slessor et al. 2005, Pirk et al. 2011) and include
207 cuticular hydrocarbons (Page et al. 1991), and compounds produced from multiple
208 exocrine glands (Winston 1987), including the mandibular (Crewe and Velthuis 1980,
209 Slessor et al. 1988, Plettner et al. 1997), Dufour's (Katzav-Gozansky et al. 1997b, Sole
210 et al. 2002) and tergal glands (Wossler and Crewe 1999a, Okosun et al. 2015, 2019).
211 The production and composition of pheromones in honey bees is highly plastic, with
212 phenotypic variation mainly caused by the physiological state of the organism (e.g. age
213 or mating status of a queen) and the social environment (e.g., presence or absence of
214 the queen) in which the organism finds itself. Pankiw et al. (1996) showed that mated *A.*
215 *m. ligustica* queens produce higher amounts of the aromatic components 4-hydroxy-3-
216 methoxyphenylethanol (HVA) and methyl *p*-hydroxybenzoate (HOB) (as compared to
217 virgin and drone laying queens), higher amounts of the queen substance 9-oxo-2 (E)-
218 decenoic acid (9-ODA) and its precursor compound (*R,S*)-9-hydroxy-2-decenoic acid (9-
219 HDA). In a display of intraspecific variation, Strauss et al. (2008) showed that while *A. m.*
220 *carnica* mated queens produced higher amounts of 9-HDA and HVA, there was no
221 significant difference in the production of 9-ODA or HOB in the mandibular glands of
222 mated, virgin or drone laying *A. m. carnica* queens.

223 The social environment in which workers find themselves plays a crucial role in
224 determining composition of worker pheromones. It has been shown that the composition
225 of mandibular glands of queen-right workers are generally dominated by the worker acids
226 10-hydroxy-2 (E)-decenoic acid (10-HDA) and its precursor 10-hydroxydecanoic acid (10-

HDAA) (Zheng et al. 2010, Yusuf et al. 2015). However, in the event of queen loss, some workers can switch to production of queen-associated compounds 9-ODA and 9-HDA, and activate their ovaries (Plettner et al. 1993, Mumoki et al. 2018). This plasticity has also been demonstrated in the pheromone composition of the Dufour's (Katzav-Gozansky et al. 1997b, Katzav-Gozansky et al. 2000, Sole et al. 2002) and the tergal glands (Okosun et al. 2015, Okosun et al. 2017). These false queens are able to change their pheromonal composition in response to the loss of a queen, which in turn further alters the social environment in the colony. The rest of the workers in the queenless colony respond by either increasing their own production of queen-associated signals in a pheromonal arms race or having the production of queen signals inhibited (Moritz et al. 2004, Yusuf et al. 2018).

In examining the complete pheromonal bouquet of *A. m. capensis* we see certain signature mixtures displayed. Described by Wyatt (2010) as "*a distinctive mix of molecules",* in this case, pheromone components used in recognition or identification of certain individuals. In the case of *A. m. capensis* we see that workers from queenright Cape honey bee colonies and worker social parasites possess different signature mixtures, and that these signatures are indeed different from those of the arrhenotokous workers of the sister subspecies *A. m. scutellata*.

Mandibular gland signals

The laying *A. m. capensis* worker phenotype initially described by Onions (1912) was subsequently shown by Crewe and Velthuis (1980) to produce pheromone components predominant in the mandibular glands of queens such as the 'queen substance' 9-ODA

and its precursor compound 9-HDA. Further, an examination of the mandibular gland profiles of queenright (non-reproductive) *A. m. capensis* workers from different parts of South Africa showed that the workers from the Western Cape regions of Heidelberg, George and Stellenbosch produced a more queenlike mixture containing high amounts of 9-ODA and 9-HDA, and very little of the worker components 10-HDA and 10-HDAA. In contrast, workers from Grahamstown which is found in the zone of introgression (Figure 1B) were found to have mandibular gland pheromone profiles very similar to those of workers of other subspecies (Zheng et al. 2010). The large amounts of 9-HDA in these workers exposed the fact that *A. m. capensis* workers are indeed predisposed to parasitism, by synthesising the precursor compound (9-HDA) used to produce the queen substance (9-ODA) (Zheng et al. 2010, Mumoki et al. 2018). In the event of queen loss, the workers are then able to easily convert 9-HDA to 9-ODA.

On the other hand, an examination of the mandibular gland pheromone profiles of *A. m. capensis* worker social parasites collected from host *A. m. scutellata* colonies reveals a queenlike pheromone bouquet dominated by the four compounds HOB, 9-ODA, 9-HDA and some 10-HDA (Schäfer et al. 2006, Dietemann et al. 2007, Okosun et al. 2017, Mumoki et al. 2018, Yusuf et al. 2018) with the queenlike signals increasing in quantity and age of the parasitic workers. The queen-associated aromatic compound HVA is rarely identified in the mandibular glands of these laying workers. Indeed, examining the mandibular gland pheromone composition of non-parasitic A*. m. capensis* workers in their native region, Simon et al. (2001) showed that while the secretions of the queenless *A. m. capensis* were dominated by the queen substance 9-ODA at day 4, the secretion profiles for the younger (day 1) bees were very worker-like, dominated by 10-HDAA and

10-HDA. The mandibular gland pheromone profiles from newly emerged (less than 24 hours post-emergence) *A. m. capensis* parasitic bees collected from infested *A. m. scutellata* colonies contain predominantly 9-HDA, the precursor component to 9-ODA demonstrating a clear predisposition to social parasitism (Mumoki et al. 2019).

The fatty acid components of the *A. mellifera* mandibular gland pheromones are synthesised in a caste-dependent stepwise manner, starting with the acylation and activation of stearic acid which is the precursor molecule (Figure 2). The activated stearic acid then undergoes hydroxylation in a caste-selective manner leading to a bifurcation in the biosynthetic pathway where hydroxylation in the ω position predominates in workers while that in the ω-1 position predominates in queens (Plettner et al. 1996). In *A. mellifera*, the hydroxylation of acylated stearic acid is catalysed by Cytochrome P450 enzymes (Plettner et al. 1996, Malka et al. 2014, Wu et al. 2017), with different sets of genes responsible for *ω* and ω-1 hydroxylation and thus forming a crucial point of regulation of pheromonal dominance in honey bees.

Reproductively dominant *A. m. capensis* workers have however shown the ability to switch from worker specific hydroxylation gene sets to gene sets known to be upregulated in queens, as they transition to producing queen-associated fatty acid molecules such as 9-ODA and 9-HDA (Mumoki et al. 2019). This tendency has also been shown by queenless workers with activated ovaries in *A. mellifera* from European populations. For instance, Malka et al. (2009), Malka et al. (2014) and Wu et al. (2017) demonstrated that queenless workers mainly upregulated the same CYP sets of genes expressed at higher levels in queens, while their queenright counterparts generally upregulated worker-

296 associated genes. Eventually, this upregulation of queen-associated cytochrome P450
297 gene sets could lead to the production of queen pheromone components, in the
298 mandibular glands of the queen-less workers. The hydroxylated components are
299 transported to the peroxisome for uncompleted β-oxidation (Reddy and Hashimoto 2001),
300 catalysed by various oxidases, thiolases and hydrolases (Plettner et al. 1998, Malka et
301 al. 2014, Wu et al. 2017) leading to the formation of 9-HDAA, 9-HDA 10-HDAA and 10-
302 HDA. In queens and reproductive workers, the 9-HDA is catalysed by alcohol
303 dehydrogenases into 9-ODA (Malka et al. 2014, Wu et al. 2017, Mumoki et al. 2018). In
304 queen-right colonies of *A. m. scutellata* infested by *A. m. capensis* reproductive parasites,
305 this oxidation of 9-HDA to 9-ODA has been shown to be much reduced as compared to
306 *A. m. capensis* parasites in queenless colonies (Mumoki et al. 2018) an indication of
307 queen-regulation in the expression of alcohol dehydrogenases in the honey bee
308 mandibular glands.
309

310 **Tergal gland signals**

311 Tergal glands of *A. mellifera* are located on the edges of abdominal tergites II-V and have
312 been shown to consist of sub-epidermal unicellular and bicellular complexes of glandular
313 cells opening into the intersegmental membrane of the cuticle (Renner and Baumann
314 1964, Billen et al. 1986, Wossler et al. 2000, Azevedo et al. 2007). There exists
315 morphological differences in the development of the tergal glands of various *A. mellifera*
316 subspecies. For instance, while the glands have been shown to be well developed in
317 virgin and mated *A. m. melifera* queens, the tergal glands of workers from this subspecies
318 are either poorly developed or completely absent (Billen et al. 1986). In contrast, both

queens and workers of *A. m. scutellata* and *A. m. capensis* posess well developed tergal glands, although the structure of the tergal glands in the workers of these two subspecies is different. Wossler et al. (2000) showed that both *A. m. capensis* and *A. m. scutellata* virgin queens possess Type B tergal glands which are located in the posterior edges of tergites II-V, are bicellular in nature, mostly consisting of secretory cells and secretory vessicles. In contrast, Type A cells are found on the anterior edge of tergite II-V, and consist of single cells with numerous rough endoplasmic reticula and are closely associated with fat cells and oenocytes. While *A. m. scutellata* workers had predominantly Type A tergal cells and few or no Type B tergal cells (Wossler et al. 2000), their *A. m. capensis* counterparts contained Type A cells that were larger in size than those found in *A. m. scutellata* workers and also large numbers of the queen-associated Type B cells (Billen et al. 1986, Wossler et al. 2000). These differences in the structure of tergal glands no doubt has implications for the composition of the chemicals secreted from these glands.

Tergal gland secretions of queens and workers of *A. mellifera* mainly consist of long chain esters, long chain fatty acids and linear unsaturated hydrocarbons, together with linear saturated hydrocarbons with carbon lengths between 23 and 31 (Espelie et al. 1990, Wossler and Crewe 1999b, Okosun et al. 2015). However variations in the composition of these secretions do exist, enabling discrimination in a caste-specific manner. In fact, the tergal secretions of queens have been shown to work in concert with the queen's mandibular gland secretions in exerting reproductive domiance in workers (Velthuis 1970, Moritz and Crewe 1991) eliciting both short-term (Wossler and Crewe 1999c) and long-term effects (Wossler and Crewe 1999a). Indeed, components of the tergal gland signals

342 are thought to have formed part of the ancestral form of the queen's fertility signals (Moritz
343 and Crewe 2018).
344 The tergal gland secretions of *A. m. capensis* reproductive workers have been shown to
345 be different from those of both non-reproductive *A. m. capensis* and *A. m. scutellata*
346 workers (regardless of the reproductive state) (Wossler and Crewe 1999b, Okosun et al.
347 2015). Okosun et al. (2015) showed that laying *A. m. capensis* parasites produced three
348 specific compounds palmitic acid, n-heneicosene and n-nonacosene that were not
349 produced by *A. m. scutellata* workers. Further, significantly larger quantities of ethyl
350 stearate, ethyl oleate and ethyl palmitate were reported for laying *A. m. capensis* workers.
351 These ethyl esters have also been shown to be part of the *A. mellifera* brood pheromone,
352 where they aid in the supression of ovarion activation in workers (Mohammedi et al. 1998,
353 Maisonnasse et al. 2009, Maisonnasse et al. 2010). These compounds possibly work in
354 tandem with other tergal gland components to supress ovarian activation in non-
355 reproductive workers after a colony infestation by *A. m. capensis* parasites. Okosun et al.
356 (2019) showed that a blend of the three esters; ethyl palmitate, ethyl oleate and ethyl
357 stearate were as attractive to subordinate workers and as effective in supressing ovary
358 activation as the blend of major tergal gland components.
359
360 **Dufour's gland signals**
361 Similar to the mandibular and tergal gland secretions, the signals of the Dufour's glands
362 in *A. mellifera* are caste specific and in workers the composition of these secretions is
363 regulated by the queen's pheromones (Katzav-Gozansky et al. 2006). The queen's
364 Dufour's gland pheromones are part of the multiply-sourced queen's signal (Katzav-
365 Gozansky et al. 2001). Although the roles of the Dufour's gland signals are still not fully

366 understood, they are thought to have a variety of functions in different species of social
367 insects, such as fertility indication (Dor et al. 2005), trail marking and recruitment (Le
368 Conte and Hefetz 2008) in social insects.
369 While the secretions of the Dufour's glands of both honey bee workers and queens consist
370 of hydrocarbons, queen Dufour's gland secretions are further fortified with wax-type
371 esters such us tetradecyl hexadecanoate, tetradecyl tetradecanoate, hexadecyl
372 hexadecanoate (Katzav-Gozansky et al. 1997a, Katzav-Gozansky et al. 1997b) that
373 mediate attraction to nestmates (Katzav-Gozansky et al. 2003).
374 In addition to caste-specific differences, the secretions of the Dufour's gland in workers
375 have been shown to be influenced by the queen's pheromones. Examining *A. m. ligustica*,
376 Katzav-Gozansky et al. (2004) showed that queenleess workers with activated ovaries
377 produced significantly higher amounts of Dufour's gland secretions specifically esters
378 associated with queens secretions in comparison to their queen-right counterparts.
379 Similarly, Sole et al. (2002) showed that laying workers of both *A. m. capensis* and *A. m.*
380 *scutellata* subspecies produced queen-like waxtype esters, although the laying *A. m.*
381 *capensis* parasites produced significantly higher quantities of the esters, amounts similar
382 to the *A. m. scutellata* queen. It is this tight correlation between ovarian activation and
383 queen-like Dufour's gland secretions that suggests the Dufour's gland may be a fertility
384 indicator. Interestingly, non-laying *A. m. capensis* workers produced many of the queen-
385 associated esters, which is in contrast to the long-chain hydrocarbons that dominated the
386 secretions of non-laying queen-right *A. m. scutellata*. This finding further supports the
387 notion that *A. m. capensis* workers are indeed predisposed to parasitism, facilitating their
388 becoming false-queens (Zheng et al. 2010).

While not much is known regarding the biosynthesis of the secretions of the Dufour's gland, Katzav-Gozansky et al. (2000) and Katzav-Gozansky et al. (1997a) have shown that the glands of both queens and workers are metabolically active possibly thorughout the life of the honey bee. Using [1-^{14}C] sodium acetate as a precursor compound in *in vitro* and *in vivo* experiments, Katzav-Gozansky et al. (1997a) showed that while the long chain esters are produced in the Dufour's glands, the long chain hydrocarbons are most probably produced elsewhere and are later sequestered by the Dufour's gland, a phenomenon common for hydrocarbons produced in social insects (Soroker et al. 1994, Schal et al. 2015).

Conclusion

The *A. m. capensis* subspecies reveals the wide reproductive spectrum into which workers in social insect colonies can be categorised. Perhaps the easiest way to make this is through the use of categories based on signals from different exocrine glands and physiological status such as ovarian activation and laying status as suggested by Okosun et al. (2017). Such a classification can be expanded to include signals from other exocrine glands like the Dufour's (Figure 3). This spectrum ranges from the false queen, with activated ovaries and a complete queen-like pheromone repertoire with 9-ODA and 9-HDA dominating the mandibular gland signals, high quantities of long chain esters dominating the Dufour's gland secretions and a queen-like bouquet of tergal gland secretions (Figure 3A). These individuals are characteristic of colonies where laying workers have become established and where attempts at re-queening have been abandoned. The second category in the spectrum would be individuals with inactive ovaries, queen-like Dufour's gland secretions, and queen-like mandibular gland and

tergal gland secretions, that could be called incipient false queens (Okosun et al. 2017) (Figure 3B). These individuals are likely to be newly emerged reproductive parasites in queen-less host colonies, producing queen-associated mandibular, tergal and Dufour's secretions but are yet to activate their ovaries. The production of queen-associated pheromone components in *A. m. capensis* parasites takes place more rapidly than the activation of ovaries, hence the initial disjunction between the two traits (Okosun et al. 2015, Aumer et al. 2018, Mumoki et al. 2019). The third category in this spectrum would consist of reproductive workers with activated ovaries and possibly worker-like Dufour's gland signals containing large amounts of long chain hydrocarbons (Sole et al. 2002), and with worker-like tergal and mandibular gland pheromone composition (Figure 3C). These laying individuals are likely be found in colonies transitioning from a queenright condition to a queen-less condition or in large *A. mellifera scutellata* colonies that have just been infested with *A. m. capensis* parasites whose cryptic pheromonal signals avoid detection by host workers through not secreting queen-associated esters (such as those of the Dufour's gland). The final category consists of workers characterised by inactive ovaries, worker-like pheromone composition, comprising of mandibular gland signals dominated by 10-HDA and 10-HDAA and relatively low amounts of 9-HDA (such as the queenright workers from Grahamstown South Africa in the work by Zheng et al. (2010)), worker-like composition of tergal gland signals and Dufour's gland secretions dominated by long chain hydrocarbons. These individuals comprise the majority of workers in a queenright colony and are characterised as subordinate workers (Figure 3D).

435 Intermediates within the four categories do exist. For instance, individuals with activated
436 ovaries and producing queen-like pheromones either from the mandibular, Dufour's or
437 tergal glands secretions would be a type of false queen, even though not producing the
438 full range of queen like signals. Similarly, individuals with inactive ovaries but producing
439 various combinations of any of the queen-like pheromone secretions would be variations
440 of the incipient false queen (Figure 4).
441
442 This complexity has been revealed through the different social situations that arise in
443 colonies infested with *A. m. capensis* parasites. This provides the conditions that allow
444 for the exploration of plasticity in the biosynthesis and expression of pheromones in adult
445 honey bee workers and provides an insight into the nature of reproductive dominance
446 and its evolution in social insects.
447
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454

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730 LIST OF FIGURES

731 **Figure 1:** Maps showing the distribution of *Apis mellifera scutellata* (green) in Africa (A)
732 and in South Africa (B) where *A. m. scutellata* is predominant in the north of the country
733 (green) while *A. m. capensis* is native to the western cape region (dark purple). The two
734 subspecies are separated by a stable natural hybrid zone (pink) which forms a buffer area
735 restricting the naturogenic movement of the Cape honey bee outside of its native region.
736 *The A. m. capensis* reproductive parasites are maintained in infested colonies (brown
737 hives) throughout the northern part of the country in the *A. m. scutellata* zone.

738

739 **Figure 2**: Biosynthetic pathway of the fatty acid components of the *Apis mellifera*
740 mandibular gland fatty acids. The numbers 1-5 indicate major points of regulation in the
741 biosynthesis pathway.

742

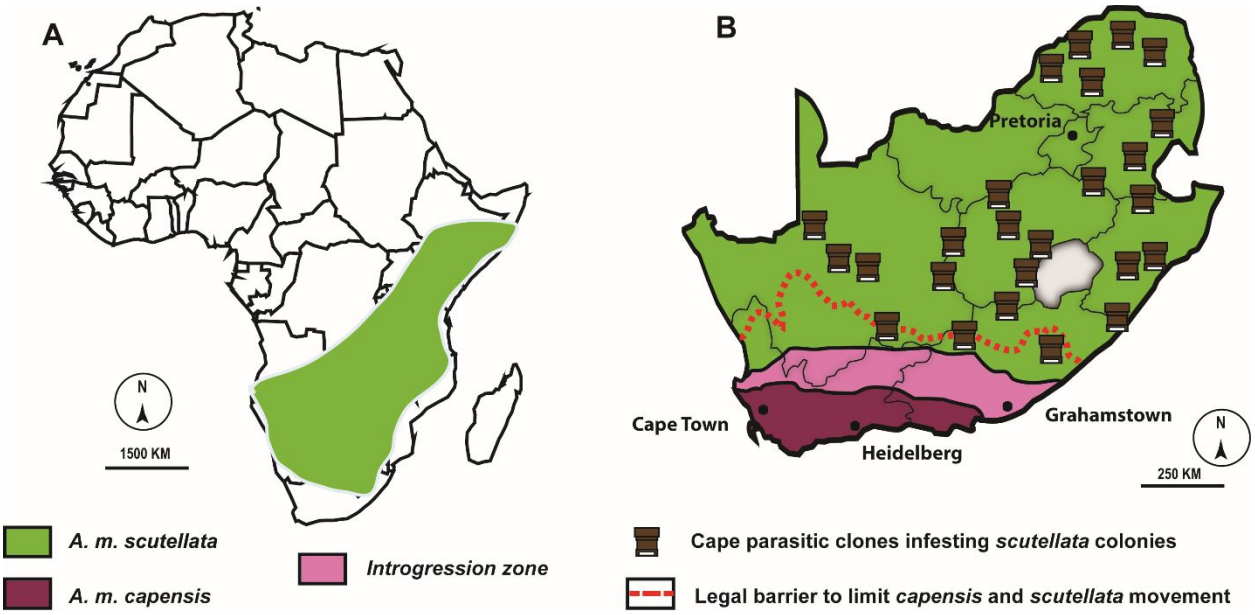
743 **Figure 3**: Phenotypes of *A m. capensis* workers classified into four different categories,
744 based on the extent of ovary activation and the state of mandibular, tergal and Dufour's
745 gland pheromone signals, as either queen-like (purple) or worker-like (green) signals.
746 This classification is expanded from Okosun et al 2017.

747

748 **Figure 4**: Variations in phenotypes of *A m. capensis* workers classified into four different
749 categories, based on the extent of ovary activation (OA) and the state of mandibular (MG),
750 tergal (TG) and Dufour's (DG) gland pheromone signals, as either queen-like (purple) or
751 worker-like (green) signals.

752

753 **Figure 1**



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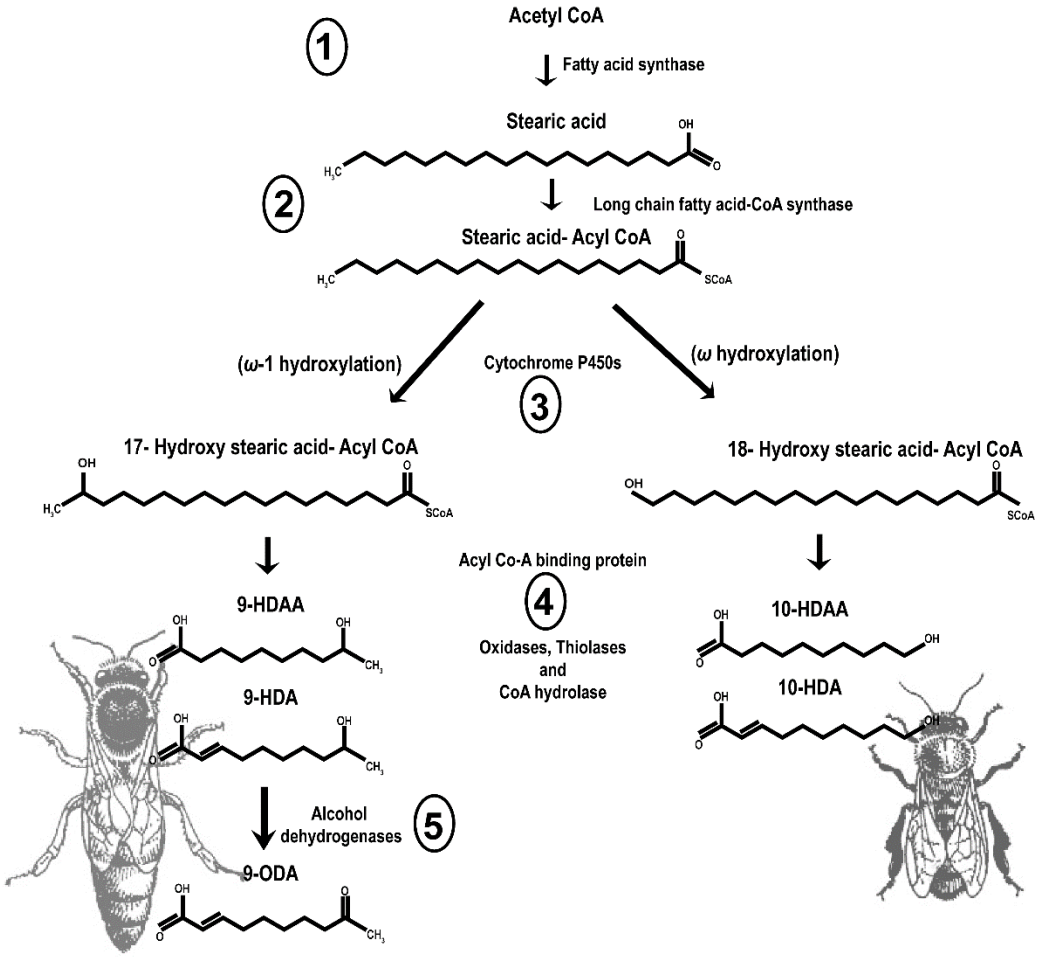
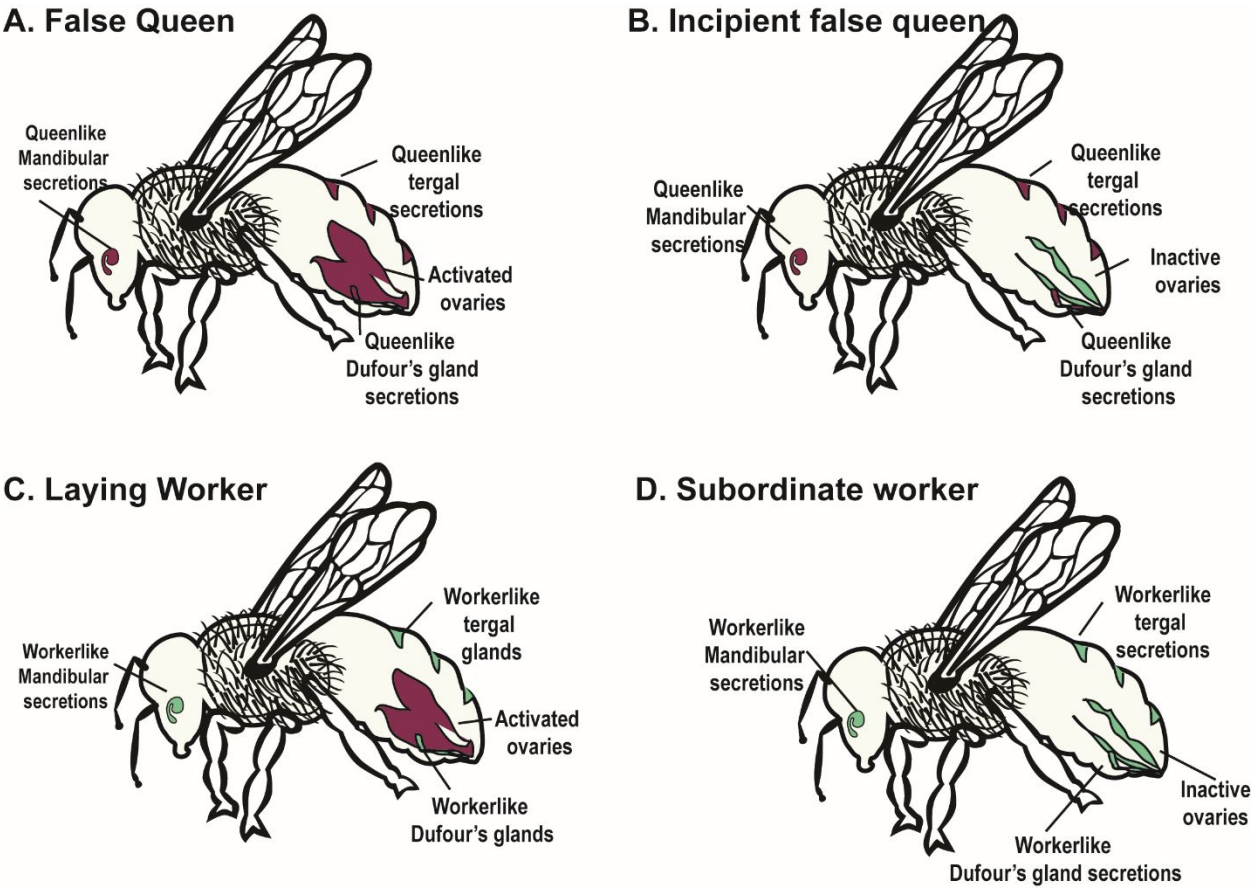
756

Figure 2


Acetyl CoA
1
Fatty acid synthase
Stearic acid
OH
H3C
2
Long chain fatty acid-CoA synthase
Stearic acid- Acyl CoA
O
H3C
SCoA
(ω-1 hydroxylation)
Cytochrome P450s
3
(ω hydroxylation)
17- Hydroxy stearic acid- Acyl CoA
OH
H3C
O
SCoA
18- Hydroxy stearic acid- Acyl CoA
OH
O
SCoA
Acyl Co-A binding protein
4
Oxidases, Thiolases
and
CoA hydrolase
9-HDAA
OH
OH
O
CH3
9-HDA
OH
OH
O
CH3
Alcohol
dehydrogenases
5
9-ODA
OH
O
O
CH3
10-HDAA
OH
OH
O
10-HDA
OH
OH
O

760 **Figure 3**

761

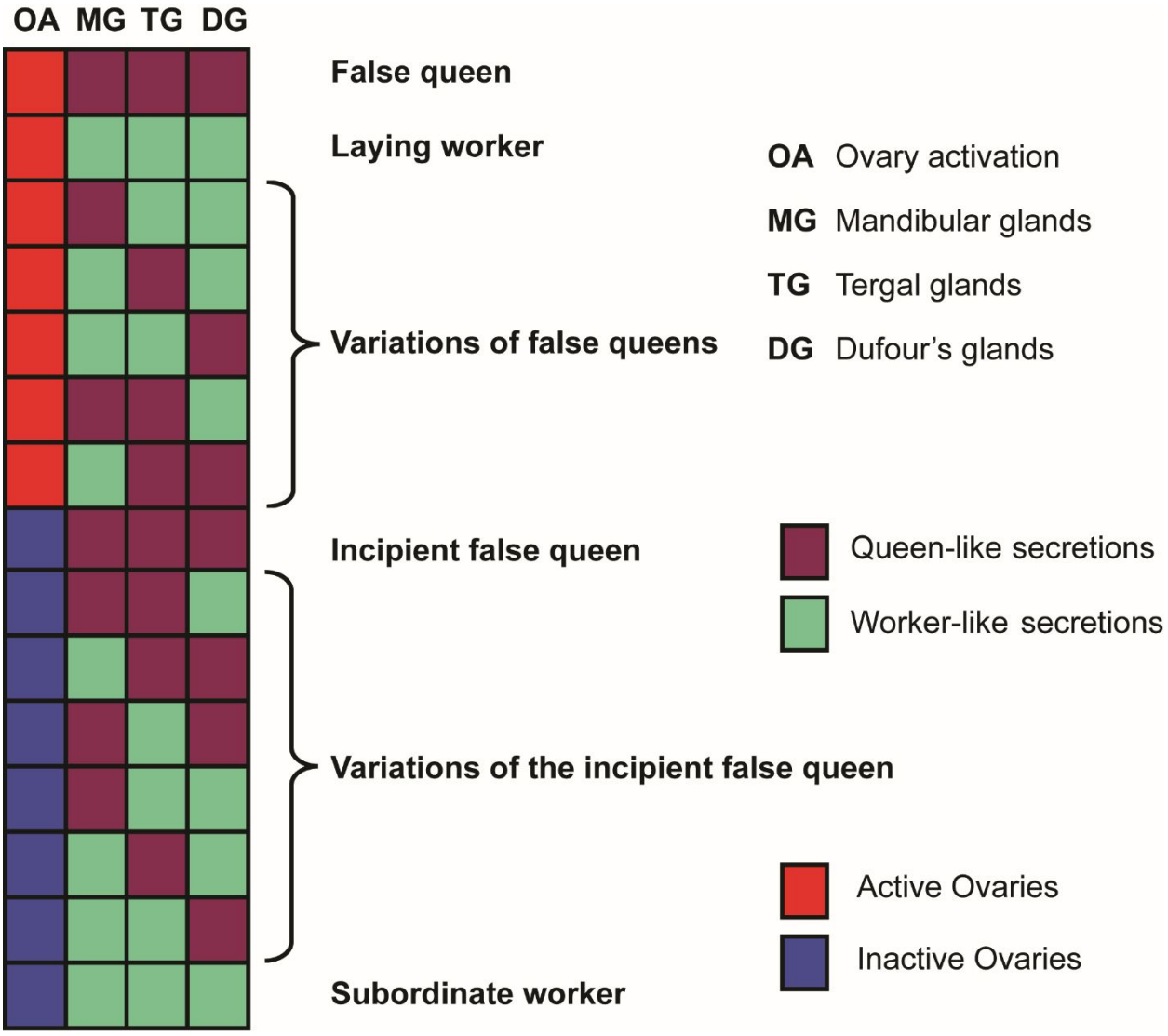


762

763

764 **Figure 4**

765



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