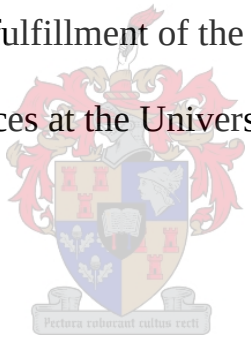
THE EFFECT OF BROOD AND QUEEN PHEROMONES, AS WELL AS THE COLONY ENVIRONMENT, IN THE SUCCESS OF *APIS MELLIFERA CAPENSIS* SOCIAL PARASITES

Thesis presented in partial fulfillment of the requirements for the degree of Master of Sciences at the University of Stellenbosch



March 2007

Candidate: Marc C Hanekom

Supervisor: Dr. Theresa C Wossler

DECLARATION:

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: …….. **Date:** ……..

ABSTRACT

Honeybee queens typically inhibit the reproductive development of workers in the colony. However, African, *Apis mellifera scutellata*, honeybee queens seem to have little effect on neighbouring *A. m. capensis* honeybee workers as is evident in the huge losses of African honeybee colonies due to the invasion by 'social parasitic' Cape honeybees (pseudoclones). Certain factors; such as queen and brood presence, the level of colony defence and food availability may render host colonies more vulnerable to invasion by the Cape worker honeybees. In this study host African colonies were split to determine whether a "window of opportunity" existed for Cape honeybee infiltration and thus critical to the *capensis* problem. Nine African colonies were infected with native and pseudoclone Cape workers over different time periods; before, during and after splitting (treatments). I measured survival rates, as well as reproductive and pheromone development of introduced workers. The effect of brood pheromones on Cape worker reproduction was also examined. Approximately 70% of all workers were removed within 72 hours, a critical period to avoid detection by Cape workers. Queen absence significantly affected the success rate of intrusion and establishment by Cape honeybee workers (GLZ; Wald χ^2 = 4.49, df = 1, P = 0.033). 21% of 21-day old pseudoclones survived African queenless colonies and only 6% queenright colonies. Native Cape workers showed no difference in survival rates between African queenless (12%) and queenright (11%) colonies. Looking at introduction time, considerably more pseudoclone honeybee workers survived in treatment 1 than did native Cape honeybee workers while for treatment 3 the converse was true. These data show no obvious 'window of opportunity' surrounding the swarming process promoting Cape honeybee infiltration and establishment of African honeybee colonies, however the period immediately prior to colony fission represents the best opportunity for

invasion by pseudoclones. As for ovary and mandibular gland secretion development, all surviving pseudoclones, irrespective of *A. m. scutellata* queen presence, fully developed their ovaries and concomitantly produced a mandibular gland secretion dominated by 9-oxo-2-decenoic acid (9ODA). Native Cape workers showed low levels of ovary development in queenright host colonies (8-17%) but this was not true for queenless colonies, with all but one worker developing their ovaries when introduced during and after splitting. Only 40% of native Cape workers introduced before splitting developed their ovaries suggesting that queen pheromones in the three days before splitting retarded ovary development in native Cape workers. These data strengthens the suggestion that the pseudoclone honeybee workers have advanced along the queen-worker developmental continuum. Preliminary studies on brood pheromones, an important factor regulating worker reproduction, indicated that Cape workers reproduce quicker and more eggs when exposed to African brood pheromones, compared to both *A. m. capensis* brood pheromones and no brood pheromones. Pheromones produced by African larvae therefore do not simply inhibit Cape worker reproductive development but accelerate the commencement of egg laying by these workers. On the whole, host African colonies, especially in the absence of their queen, appear vulnerable surrounding colony fission to invasion by both Cape honeybee worker populations even though there are low survival rates. I conclude that these two Cape honeybee worker populations do differ significantly regarding their reproductive capacity and ability in becoming social parasites.

Pectora roborant cultus recti

UITTREKSEL

Heuningby koninginne verbied die reproduktiewe ontwikkeling van werker heuningbye in die kolonie. Die Afrika, *Apis mellifera scutellata*, heuningby koninginne het egter geen of min effek op die Kaapse, *A. m. capensis* heuningby werkers. Dit kan duidelik gesien word in die groot verliese van Afrika heuningby kolonies te danke aan die aanval deur die 'sosiale parasitiese' Kaapse heuningbye (pseudoclones). Sekere faktore, bv. Koningin- en broei teenwoordigheid, die vlak van kolonie verdediging en voedsel beskikbaarheid speel dalk n groot rol in hoe Afrika kolonies die aanval deur die Kaapse heuningby werkers hanteer. In hierdie studie is Afrika kolonies verdeel in koningin-volle en koningin-lose helftes om te bepaal of daar 'n "venster van geleentheid" bestaan ten spyte van die infiltrasies deur die Kaapse heuningby wat dus krities is tot die '*capensis* probleem'. Nege Afrika kolonies was geïnfekteer met aangebore en pseudoclone Kaapse werkers oor verskillende tydperiodes; voor, gedurende en na (behandelings 1, 2 en 3). Ek het oorlewingskoerse gemeet, asook reproduktiewe en pheromone ontwikkeling van die bekendgestelde werkers. Die effek van broei pheromone op Kaapse werkers se herproduksie ontwikkeling was ook ondersoek. Ongeveer 70% van alle werkers is binne 72 uur verwyder met verskillende reaksies tussen individuele Afrika kolonies. Koningin afwesigheid affekteer duidelik die sukses koers van indringing en vestiging deur die Kaap heuningby (GLZ; wald $\chi^2 = 4.49$, df = 1, p = 0.033). Hierdie is waar vir die pseudoclones met 21% oorlewende werkers in Afrika kolonies wat sonder 'n koningin bestaan en 6% wat met 'n koningin bestaan. 21-dag ou aangebore Kaapse werkers toon geen verskil in oorlewings verhouding tussen Afrika koningin-lose (12%) en koningin-volle (11%) kolonies. Dit is duidelik dat meer pseudoclone heuningby werkers oorlewe in behandeling 1 wat heeltemal verskil sodra ons dit met behandeling 3 vergelyk waar meer aangebore

Kaapse heuningby werkers oorlewe as pseudoclones. Hierdie data toon geen vanselfsprekende “venster van geleentheid” rondom die swerm proses van die Kaapse heuningby tydens infiltrasie en lewe in Afrika heuningby kolonies nie. Die periode onmiddellik voor die kolonie verdeel word, is duidelik die beste geleentheid vir aanval deur pseudoclones. Met betrekking tot die eierstok en mandibulêre klierafskeiding ontwikkeling, het alle oorlewende pseudoclone, ongeag die Afrika koningin se teenwoordigheid, hul eierstokke ten volle ontwikkel en mandibulêre klierafskeiding geproduseer, deur 9-oxo-2-decenoic suur (9ODA). Aangebore Kaapse werkers het lae vlakke van eierstok ontwikkeling getoon in koningin-volle kolonies (8-17%) maar hierdie was nie waar van koningin-lose kolonies nie, waar almal behalwe een, eierstokke ontwikkel het toe dit bekendgestel is gedurende en na die kolonies gedeel was. 40% van aangebore Kaapse werkers wat bekendgestel is voor die kolonie gedeel is, ontwikkel eierstokke. Dit wil voorkom of die teenwoordigheid van koningin pheromone in die drie dae voor deling eierstok ontwikkeling vertraag in aangebore Kaapse werkers. Die konsentrasie van 9ODA in Kaapse werker pruduksie lyk verwant tot eierstok ontwikkeling, met eierstokke wat ontwikkel parallel met die produksie van hoë 9ODA konsentrasies. Ten slotte, voorafgaande studies op die broei pheromone, 'n belangrike faktor wat werker reproduktiewe ontwikkeling gehelp het, het getoon dat Kaapse werkers hulle eiers vinniger reproduseer wanneer blootgestel met Afrikaanse broei pheromone, vergeleke tot beide Kaapse broei pheromone en geen broei pheromone. Pheromone wat deur Afrika larwes geproduseer is, affekteer nie net Kaapse werkers se reproduktiewe ontwikkeling nie, maar lyk asof dit die aanvang van die eierlê proses deur hierdie werkers versnel. Dit lyk dus asof die eerste 72 uur periode die kritiese stadium is in die Afrika kolonies se vermoë om die Kaapse bye te ontdek en vernietig. Afrika kolonies, veral in die afwesigheid van hul koningin, is kwesbaar wanneer die kolonie gedeel word met die inval deur beide Kaapse

heuningby werker populasies, sowel as lae oorlewings koerse. Hierdie data versterk die suggestie dat die pseudoclone heuningby werkers ‘sterker’ vertoon, siende dat hulle fisiologies meer gevorderd is as hul aangebore Kaapse heuningby susters. Die rede hiervoor is dat almal behalwe twee van die opgespoorde pseudoclone hoë 9ODA pheromone ontwikkel het in verbinding met eierstok ontwikkeling, terwyl baie aangebore Kaapse werkers hierin misluk het. Ten slotte meen ek dat hierdie twee Kaapse heuningby werker populasies beduidend verskil aangaande hul reproduktiewe kapasiteit en vermoë om maatskaplike parasiete te word.

ACKNOWLEDGEMENTS

I Marc Hanekom, wish to express my thanks to the following people and institutions:

- My supervisor, Theresa Wossler, for all her guidance, patience and expertise, without which, I would not have been able to complete my study. I am grateful for her useful advice and helpful supervision, as well as the necessary financial support.
- Michael Allsopp, (ARC-PPRI Stellenbosch) for showing great interest in my study and spending many hours in the field and especially in showing me how to drop everything and run away from angry African bees, this handy hint has proven most useful on numerous occasions, thanks Mike. Furthermore I'd like to thank Mike for the supply of the native Cape honeybees and the use of their facilities and honeybee equipment.
- Christiaan Fransman, (ARC-PPRI Stellenbosch) who accompanied me on numerous fieldwork outings and who taught me so much about the practical side of dealing with honeybee colonies. Dankie Chris.
- Theins Engelbrecht, (Douglas Bee Farms) for his generosity in accommodating me and in supplying African colonies and showing me around his farm and in what happens in his line of work.
- Theuns van Niekerk, (from Pretoria) who supplied the parasitic Cape clones from the Highveld region.
- Ruan Veldtman, for your invaluable effort in aiding me with statistical analysis and always showing an interest in the data. Thank you very much.
- Rudolf Maleri, Ruhan Slabbert and Martine Jordaan, for the many coffee breaks, late nights out, extra mural activities and for keeping me sane, and, most importantly, for making life enjoyable and interesting. Good luck to all of you.

Pectora roborant cultus recti

- To all the ladies in the office: Lee-Ann Noach-Pienaar, Boipelo Ramongalo, Gayle Pedersen, Nicole Wakeford, Sheena Findley and Natasha Muna, you girls took me in as one of your own, especially Sheena, thanks to all of you and good luck with what ever you ladies do in life.
- The NRF and the University of Stellenbosch, for financial support.
- My parents, for believing in me. Thank you for your continuous encouragement and support throughout my studies.
- To my Uncle Andre and Aunty Carol who took me in for more than two years and made my stay in the Cape most memorable and hospitable. Thanks for everything.
- Finally to the many other people out there who supported me in everything I did, this includes you Hayley.

Pectora roborant cultus recti

This study is a product of the ongoing research done by Dr Theresa Wossler, based at the University of Stellenbosch. It forms part of a long-term project which focuses on the mechanism of potential host colony 'resistance' in decreasing the parasites' transmission rate and ability to develop into reproductives with the aim to acquire information which could be used for management purposes surrounding the persistent *A. m. capensis* Cape clone social parasite's.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

1.1.1 Distribution of honeybees in South Africa

In South Africa there are two honeybee subspecies (*Apis mellifera*) which reside on either side of a natural hybrid belt (Hepburn and Crewe 1990; Hepburn and Radloff 1998). This natural hybrid belt (Figure 1.1) stretches across South Africa from the mid West coastal region (31°43'*S*18°18'*E*) spanning along the provincial border dividing the Northern and Western Cape province to the South coastal region in the Eastern Cape (32°55'*S*28°01'*E*). The African honeybee subspecies, *Apis mellifera scutellata* is found to the north of this hybrid belt and to the south the Cape honeybee subspecies, *Apis mellifera capensis* (Hepburn and Radloff 2002). This hybrid belt has remained stable where naturally mated *A. m. scutellata* and *A. m. capensis* hybrid colonies occur (Hepburn and Crewe 1990).

Pectora roborant cultus recti

1.1.2 Honeybees in general

In both these honeybee subspecies there is a well developed caste system. Under typical conditions the queen lays the colony's eggs and her daughters, the workers, tend to all other duties such as foraging, feeding of the queen, brood rearing, hive cleaning, building and guarding (Butler 1954; Verheijen-Voogd 1959; Free 1987). This division of labour is largely regulated by the queen who produces and secretes an array of pheromones, influencing the workers within the hive (Butler 1957, 1959; Butler and Fairey 1963;

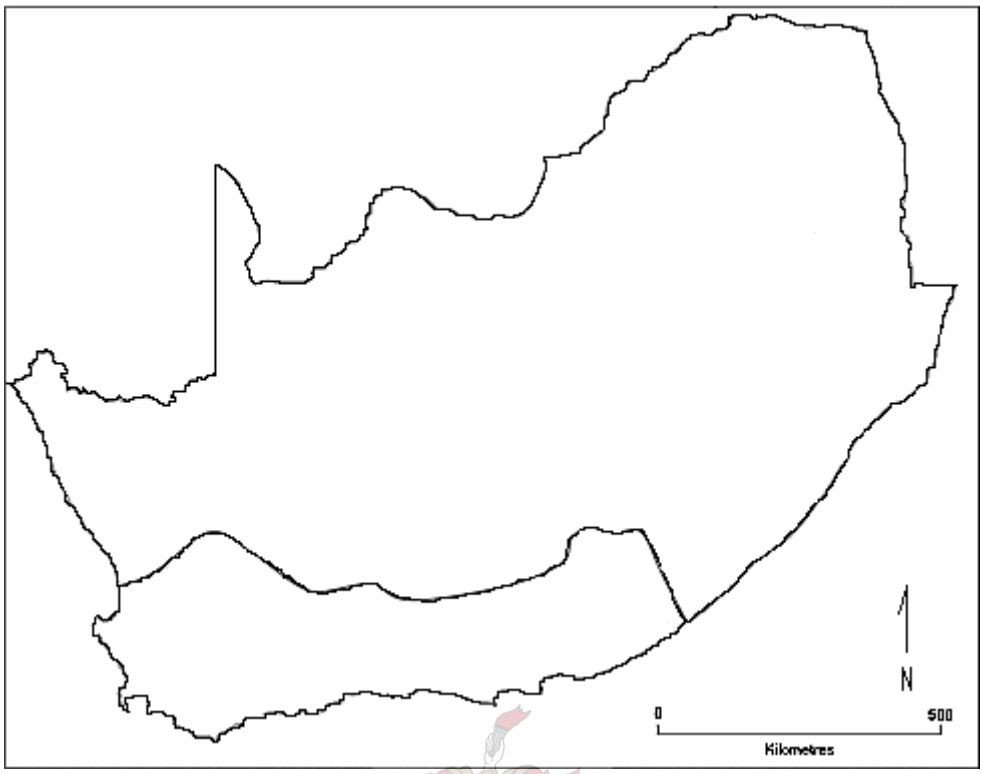


Figure 1.1 Map showing the approximate hybrid belt dividing the two honeybee subspecies (*A. m. capensis* to the south and *A. m. scutellata* to the north) within South Africa. (Map used from Hepburn and Radloff 2002)

Blum 1974; Seeley 1985; Free 1987; Winston 1987; Slessor et al. 1988; Winston and Slessor 1992a, 1992b; Plettner et al. 1993; Wossler and Crewe 1999; Moritz et al. 2002; Hoover et al. 2003). Caste division is maintained by regulating worker reproduction, as the queen is the sole reproducer. The most prominent queen pheromone, which regulates worker ovary development, is secreted by her mandibular glands situated in her head (Velthuis 1970a; Crewe and Velthuis 1980; Winston and Slessor 1992a). Queen mandibular pheromones of mated queens predominantly contain 9-keto-2(E)-decenoic acid (9-ODA), (R,E)-(-) and (S,E)-(+)-9-hydroxy-2-decenoic acid (9-HDA) aliphatic compounds (Crewe and Velthuis 1980; Slessor et al. 1988; Winston and Slessor 1992a; Plettner et al. 1993, 1995). The queen's mandibular gland pheromones alone however,

are not enough to ensure this division of labour (Willis et al. 1990). Other queen produced pheromones are also believed to assist in the regulation of the division of labour (Velthuis 1970b; Wossler and Crewe 1999; Katzav-Gozansky et al. 1997, 2003).

Besides queen pheromones regulating the reproductive division of labour within a colony, brood pheromones compliment the queen in inhibiting workers from activating their ovaries and taking over reproductive tasks (Jay 1968, 1970, 1972; Winston 1987, 1992; Arnold et al. 1994; Le Conte et al. 1995; Mohammedi et al. 1998). Other than the role queen and brood pheromones play in the regulation of the workers' physiological reproductive state, the worker caste is also in an important position to maintain worker sterility through worker policing, which is widespread in honeybees (Ratnieks 1988; Hillesheim et al. 1989; Ratnieks and Visscher 1989; Keller and Nonacs 1993; Barron et al. 2001; Martin et al. 2002). Worker policing is either eating of worker laid eggs or the aggression towards workers with activated ovaries (Visscher and Dukas 1989; Ratnieks 1995; Oldroyd and Ratnieks 2000; Martin et al. 2002). Ultimately the queens' presence plays a major role in the colonies dynamics. Her pheromones are distributed throughout the honeybee colonies via antennation and trophallaxis (allogrooming) amongst worker bees, which ensure this well developed worker caste system (Butler 1967; Seeley 1979, 1985; Velthuis 1972; Winston 1987, Naumann et al. 1993).

Pectora roborant cultus recti

1.1.3 Commercial beekeeping in South Africa

In South Africa, commercial beekeepers on either side of the hybrid zone practice migratory beekeeping following the high pollen and nectar flow of the surrounding flora

within their native boundaries (Swart 2001). A typical migratory route for honey production by the African, *A. m. scutellata*, beekeepers is from Highveld gums, to sunflowers, onto saligna gum, Aloes and then citrus orchards (Johannsmeier et al. 2001). Cape beekeepers, in general, also move their Cape, *A. m. capensis*, colonies within their natural boarders and their migration pattern is governed by the flowering pattern of the unique Fynbos flora within the Western and Eastern Cape regions. African beekeepers annually congregate on the rich Highveld *Aloe greatheadii davyana* regions, where the copious supply of pollen from the Aloes, especially during the winter months, results in extensive brood production which invariably leads to swarming (colony fission) and thus excellent conditions for commercial beekeeping. Commercial beekeepers mimic the swarming process by increasing their hive numbers through splitting of their colonies. This process simply involves splitting the colony into a queenright half and queenless half.

Pectora roborant cultus recti

1.1.4 The *'capensis calamity'*

In 1990, a migratory beekeeper from the Cape with the idea of increasing his honeybee colonies, moved a large number of Cape honeybee colonies from the Western Cape across the natural hybrid belt (Hepburn and Crewe 1990), onto the high Aloe flow in the Highveld region of South Africa (Hepburn and Crewe 1991; Johannsmeier 1992; Allsopp 1993; Hepburn et al. 1998). This unnatural introduction of the Cape honeybee, *A. m. capensis*, into the African, *A. m. scutellata*, honeybee territory has lead to the widespread take over by *A. m. capensis* laying honeybee workers (Allsopp 1992, 1995; Hepburn and Allsopp 1994; Martin et al. 2002). These foreign Cape colonies were placed in congested

African apiaries where beekeepers continuously split their colonies on this high nectar and pollen flow in order to increase their colony numbers. The natural swarming process is when colonies that get too large swarm off as a queenright half colony and where the other half remains queenless until they rear a new queen (Johannsmeier et al. 2001). However, this commercial splitting process is not exactly the same as in nature, during commercial splitting; the queenless half is queenless for a longer period than during a natural swarming event and is thought to aid the Cape honeybee workers in infiltrating undetected into African colonies (Woyke 1995). It is believed that splitting colonies in these congested apiaries place African honeybee colonies under great threat to invasion by Cape honeybee workers, where many flying bees, largely in the absence of guarding, enter neighbouring colonies accidentally or due to the increased probability of robbing, especially when colonies in an apiary are inspected by beekeepers (Moritz 2002). Once in, Cape honeybee workers even in the presence of the African queen, show signs of physiological changes where their reproductive capability alter from that of being worker-like to more queen-like (Hepburn and Allsopp 1994). Within one year of this introduction of Cape honeybee colonies up north, it became apparent that the Cape honeybee workers were parasitizing the African honeybee colonies (Hepburn and Crewe 1991; Allsopp 1993). These invasive Cape honeybee workers have successfully established themselves as social parasites within their African host colonies and this phenomenon has been coined the *'capensis calamity'* (Allsopp 1992, 1993, 1995; Oldroyd 2002; Neumann and Hepburn 2002; Neumann and Moritz 2002).

1.1.5 The *'capensis calamity'* and important factors surrounding this phenomenon

The *'capensis calamity'* is thought to have been triggered primarily by the unnatural migration of Cape honeybee colonies onto the high pollen flows of the Aloes in the Highveld region (Allsopp and Crewe 1993; Hepburn and Allsopp 1994; Moritz 2002). Here the high swarming incidence increases the probability that Cape honeybee workers gain access into African honeybee colonies either through probable drifting, where foreign worker honeybees enter a neighbouring hive by accident, or actively searching for host African colonies (Neumann et al. 2000, 2001). Once these social parasitic Cape honeybee workers gain access into their African host colonies, they almost certainly avoid the queen and her signal (Moritz et al. 2002) and in so doing become reproductively active producing pheromones that mimic those of the queen, specifically their mandibular gland pheromones (Crewe and Velthuis 1980; Crewe 1981, 1988; Hepburn and Crewe 1991; Allsopp 1993; Hepburn and Allsopp 1994; Simon et al. 2001; Moritz et al. 2002; Martin et al. 2002; Neumann and Moritz 2002). These Cape honeybee worker mandibular pheromones under normal Cape queenright conditions consists of 10-hydroxy-2(E)-decenoic acid (10-HDA, a regioisomeric form of 9-ODA) and 10-hydroxydecanoic acid (10-HDAA) (Winston and Slessor 1992b; Plettner et al. 1993). However, Cape honeybee workers, primarily in the absence of any queen, have shown to switch their mandibular gland pheromone production from being worker-like (10-HDA and 10-HDAA) to that of queen-like (9-ODA and 9-HDA) much faster than African honeybee workers (Crewe and Veldthuis 1980; Winston and Slessor 1992b; Plettner et al. 1993; Hepburn and Allsopp 1994; Moritz et al. 2000; Simon et al. 2001).

In addition, Cape honeybee workers have an extremely short period between losing their queen and developing their ovaries (4-6 days) compared to African (5-7 days) honeybee workers (Velthuis et al. 1990). These Cape honeybee workers' reproductive potential is increased further by their large number of ovarioles 12-15 compared to 2-6 ovarioles per ovary in African workers (Velthuis 1970c). Cape honeybee workers are also unique in that they lay diploid female eggs (Onions 1912; Lundie 1954; Anderson 1963) through a process of self fertilization known as thelytoky (Moritz and Haberl 1994; Radloff et al. 2002). All offspring produced by thelytokous parthenogenesis express some level of genetic variability due to low levels of recombination and are therefore referred to as pseudoclones (Kryger 2001a; Baudry et al. 2004; see Moritz and Haberl 1994 for a different interpretation). Cytological analysis suggests that the current social parasitic population was derived from a single worker lineage that out-competed others in the early stages of the infection (Baudry et al. 2004; Dietemann et al. 2006). The current parasitic population was initially selected due to their increased ability of reproducing in queenright host colonies and even after many generations the worker offspring are still as effective (Kryger et al. 2003; Dietemann et al. 2006).

In addition to the African queens' inability to govern foreign Cape workers, it would appear that the regulation of worker reproduction by brood pheromones also seems to breakdown in parasitized African honeybee colonies with African brood not preventing Cape workers' ovary activation. Thus, either the African brood secretes low concentrations of the inhibiting compounds, and/or the parasitic Cape honeybee workers have high pheromone response thresholds, rendering the African brood pheromone along

with the African queens' pheromones as ineffective. Similar behaviour has been witnessed in European honeybees where anarchistic queen-laid brood pheromones do not have the same inhibitory effect as wild type brood (Oldroyd et al. 1999). The ability to escape pheromone regulation and worker policing (Martin et al. 2002; Ratnieks 1988, 1992, 1993, 1995; Miller and Ratnieks 2001; Pirk et al. 2002), as well as being preferentially fed as larvae within the African colonies (Beekmann et al. 2000; Calis et al. 2002; Allsopp et al. 2003) have to some extent shaped their parasitic lifestyle. As the lifecycle progresses, the ratio of functional host African honeybee workers that are laid by the host African queen in relation to parasitic Cape honeybee worker laid offspring soon shifts from the former to the latter where the host's effectiveness as a colony rapidly deteriorates. It is not known how or when, but sometime during the social parasitic infestation of the host African colony, the loss of the host queen occurs and soon there after the death of the host colony (Hepburn and Allsopp 1994). Social parasitism appears widespread in all major groups of social insects (Schmid-Hempel 1998) but the *''capensis calamity''* was the first report of social parasitic behaviour within the honeybees (*Apis mellifera*). Recently however, the suggestion is that social parasitism by Cape workers is not unique to African colonies in South Africa, but rather that parasitism by laying Cape workers is a naturally occurring feature, albeit rare, of Cape honeybees with workers parasitizing (queenless) colonies of their own subspecies from time to time (Härtel et al. 2006).

The success of these Cape parasitic workers to date, would suggest that African colonies are susceptible to Cape worker takeover, however recent experiments (Calis et al. in

preparation) have shown that African colonies are surprisingly resilient to Cape honeybee worker invasion and attempts of take over. Beekman and her co-workers (2002) showed that invasive *A. m. capensis* workers have no special mechanisms to bypass the African guards where 18% African non-nestmates and 15% Cape non-nestmates were accepted in one experiment. Having observed my colleagues fieldwork, it has proved extremely difficult to get Cape workers into African colonies with almost all introduced Cape bees being killed and removed by the African workers within the first few days. However, this invasion still persists and causes huge losses for commercial beekeepers in the interior of South Africa and was thought to show signs of threatening wild colonies as well (Allsopp personal communication) which was previously not considered (Moritz 2002). Most recently it has been found that the wild *A. m. acutellata* populations appear to be un-infected and have safe refuge within wild game farms throughout South Africa (Härtel et al. 2006). Why then does the *'capensis calamity'* persist? It would seem that these workers are somehow capable of entering African colonies, probably during more vulnerable periods in the colony's lifecycle (possibly impacted on by environment), and this together with their unique characteristics gives these Cape honeybee workers an advantage on a successful social parasitic lifestyle. Here we hope to obtain some data in understanding the relationship between host and parasite which may offer solutions in halting the spread of the parasite up north. It is important to conserve the African honeybee since it provides essential pollination services, naturally as well as commercially, playing a key role in sustaining biodiversity.

1.2 OBJECTIVES

Bearing in mind the difficulty of successfully introducing the Cape honeybee workers into African colonies, with the knowledge that commercial African colonies are indeed infected and lost to the Cape invaders, begged the question whether there was a specific "window of opportunity" for the Cape honeybee workers to successfully infiltrate African colonies. This most likely window is thought to be when colonies swarm, where the colony is left queenless for a number of days after the old queen has left and the young queen yet to emerge. It is suggested that the colony dynamics during such a period is sufficiently disrupted that Cape honeybee workers may more readily infiltrate the now more vulnerable queenless African colony.

Pectora roborant cultus recti

In this study host African colonies were split to determine if a "window of opportunity" does exist. The survival rates of introduced Cape honeybees were recorded since obtaining entry and becoming established in the host colony is critical to the *capensis* problem. African colonies were infected over different time periods (treatments); before, during and after splitting of the colonies to determine whether a specific period rendered African colonies especially vulnerable to Cape honeybee worker invasion. I then investigated the physiological development (degree of ovary development) and chemical profiles (mandibular gland secretions) of all surviving Cape honeybee workers to ascertain whether treatment affected reproductive development. During this study both native Cape honeybee workers (from the Western Cape), as well as the pseudoclones from the Highveld were used. The reason for analysing the two populations within the same species (*A. m. capensis*) was to ascertain how different the social parasitic

pseudoclone honeybee workers differ from their native counterparts. Finally, since African brood appears ineffectual in regulating these Cape workers, the effect of brood pheromones on Cape worker reproduction development was also examined.

Due to the thesis layout (paper format), some repetition and consequential overlapping within the introductions and in the materials and methods may occur. Having said this though, each chapter does not have a separate abstract, but rather one comprehensive abstract encompassing all the main findings precedes the thesis. The composition of this thesis is summarized below:

Chapter 1 introduced the study organism (*Apis mellifera*, honeybees), and discussed the biology of honeybees, focusing on *A. m. capensis* and their role in the *'capensis calamity'*. Commercial aspects of beekeeping are introduced, particularly the splitting of colonies, and the probable impact this has had on the success of *A. m. capensis* workers as social parasites.

Chapter 2 compares the survival rates of the two isolated *A. m. capensis* populations (native Cape and the pseudoclone honeybee worker) over three different infection periods (treatments - before, during and after) surrounding the splitting process of host African honeybee colonies into queenright and queenless halves. The objective was to assess whether the presence and/or absence of the host African queen, surrounding the three treatments, affects Cape worker acceptance by host African colonies. Simply put, are there periods around colony splitting that renders colonies more vulnerable to take-over,

generating a “window of opportunity” that increases the incidence of infection? This chapter is currently being prepared as a manuscript for submission to Behavioural Ecology and Sociobiology.

Wossler TC, Hanekom MC, Allsopp MH. Do parasitic Cape workers make use of a “window of opportunity” to infiltrate African host colonies? In prep: Behav Ecol Sociobiol

Chapter 3 follows on from chapter 2 with all the surviving workers harvested and their reproductive development assessed. The aim was to compare all surviving native Cape workers and pseudoclones with respect to their reproductive development. Mandibular gland secretions and ovary development were compared between the two populations for the three treatments.

Chapter 4 focuses specifically on the effect open host African brood; open native Cape brood and no brood has on regulating ovary development and laying of eggs by native Cape honeybee workers. The data collected during this experiment is unfortunately only preliminary, due to a range of unforeseen problems, but these data still however suggest an interesting phenomenon.

Chapter 5 discusses these findings in relation to commercial beekeeping and the *‘capensis calamity’*.

1.3 REFERENCES

Allsopp MH (1992) The *'capensis calamity'*. S Afr Bee J 64:52-55

Allsopp MH (1993) Summerized overview of the capensis problem. S Afr Bee J 65:127-136

Allsopp MH (1995) The capensis problem 1992-1995. In: Magnuson P (ed) Proceedings of the First International Electronic Conference on the Cape Bee problem in South Africa 5-30 June 1995. PPRI, Pretoria pp 10-31

Allsopp MH, Crewe R (1993) The Cape honeybee as a Trojan horse rather than the hordes of Jenghiz Khan. Am Bee J 133:121-123

Anderson RH (1963) The laying worker in the Cape honeybee, *Apis mellifera capensis*. J Apic Res 2: 85-92

Arnold G, Le Conte Y, Troullier J, Hervet H, Chappe B, Masson C (1994) Inhibition of worker honeybee ovary development by a mixture of fatty acid esters from larvae. C R Acad Sci Paris 317:511-515

Barron AB, Oldroyd BP, Ratnieks FLW (2001) Worker reproduction in honey-bees (*Apis*) and the anarchic syndrome: a review. Behav Ecol Sociobiol 50:199-208

Baudry E, Kryger P, Allsopp M, Koeniger N, Vautrin D, Mougel F, Cornuet J-M, Solignac M (2004) Whole-Genome Scan in Thelytokous-Laying workers of the Cape honeybee (*Apis mellifera capensis*): Central fusion, reduced recombination r ates and centromere mapping using Half-Tetrad analysis. Genetics 167:243-252

Beekman M, Calis JNM, Boot WJ (2000) Parasitic honeybees get royal treatment. Nature 404:723

Beekman M, Wossler TC, Martin SJ, Ratnieks FLW (2002) Acceptance of Cape honey bees (*Apis mellifera capensis*) by African guards (*A. m. scutellata*). Insect Soc 49:216-220

Blum MS (1974) Pheromonal bases of social manifestations in insects. In: Birch MC (ed) Pheromones. North-Holland Publishing Company, Amsterdam, pp 194-199

Butler CG (1954) The world of the honeybee. Collins London, United Kingdom

Butler CG (1957) The control of ovary development in worker honeybees (*Apis mellifera*). Experientia 13:256-257

Butler CG (1959) Queen substance. Bee World 40:256-257

Butler CG (1967) Insect pheromones. Biol Rev 42:42-87

Butler CG, Fairey EM (1963) The role of the queen in preventing oogenisis in worker honey bees. J Apic Res 2:14-18

Calis JNM, Boot WJ, Allsopp MH, Beekman M (2002) Getting more than a fair share: nutrition of worker larvae related to social parasitism in the Cape honey bee *Apis mellifera capensis*. Apidologie 33:193-202

Crewe RM (1981) Queens, false queens and *capensis*. S Afr Bee J 53:18-21

Crewe RM (1988) Natural history of honey-bee mandibular gland secretions: development of analytical techniques and the emergence of complexity. In: Needham GR, Page RE, Delfinado-Baker M, Bowman CE (eds) Africanized honey bees and bee mites, Wiley, New York, USA pp 149–158

Crewe RM, Velthuis HHW (1980) False queens: a consequence of mandibular gland signals in worker honeybees. Naturwissenschaften 67:467-469

Dietemann V, Pflugfelder J, Härtel S, Neumann, Crewe R (2006) Social parasitism by

honeybee workers (Apis mellifera capensis Esch.): evidence for pheromonal resistance to host queen's signals. Behav Ecol Socio 60:785-793

Free JB (1987) Pheromones of social bees. Correll University Press, Ithaca, New York, USA.

Härtel S, Neumann P, Kryger P, von der Heide, Moltzer G, Crewe R, van Praagh J, Moritz F (2006) Infestation levels of *Apis mellifera scutellata* swarms by parasitic Cape honeybee workers (*Apis mellifera capensis*). Apidologie 37:462-470

Hepburn HR, Crewe RM (1990) Defining the Cape Honeybee – Reproductive traits of Queenless workers. S Afr J Sci 86:524-527

Hepburn HR, Crewe RM (1991) Portrait of the Cape honeybee, *Apis mellifera capensis*. Apidologie 22:567-580.

Hepburn HR, Allsopp MH (1994) Reproductive conflict between honeybees: usurpation of *Apis mellifera scutellata* colonies by *Apis mellifera capensis*. S Afr J Sci 90:247-249

Hepburn HR, Radloff SE (1998) Honeybees of Africa. Springer-Verlag, Berlin p 370

Hepburn HR, Radloff SE (2002) *Apis mellifera capensis*: an essay on the subspecific classification of honeybees. Apidologie 33:105-1127

Hepburn HR, Radloff SE, Fuchs S (1998) Population structure and the interface between *Apis mellifera capensis* and *Apis mellifera scutellata*. Apidologie 29:333-346

Hillesheim E, Koeniger N, Moritz RFA (1989) Colony performance in honeybees (*Apis mellifera capensis* Esch.) depends on the proportion of subordinate and dominant workers. Behav Ecol Sociobiol 24:291–296

Hoover SER, Keeling CI, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90:477-480

Jay SC (1968) Factors influencing ovary development of worker honeybees under natural conditions. Can J Zool 46:345-347

Jay SC (1970) The effect of various combinations of immature queen and worker bees on the ovary development of worker honeybees in colonies with and without queens. Can J Zool 48:169-173

Jay SC (1972) Ovary development of worker honeybees when separated from worker brood by various methods. Can J Zool 50:661-664

Johannsmeier MF (1992) The Cape bee: a problem in Transvaal. Plant Protection Research Institute, Pretoria, Leaflet

Johannsmeier MF, Swart DJ, Tribe GD, Kryger P (2001) Diseases and pests of honeybees. In: Johannsmeier MF (ed) Beekeeping in South Africa, 3rd edition, Plant Protection Research Institute Handbook No. 14, Agricultural Research Council of South Africa, Pretoria, South Africa pp 205-206

Katzav-Gozansky T, Soroker V, Hefetz A, Cojocaru M, Erdmann DH, Francke W (1997) Plasticity of caste-specific Dufour's gland secretion in the honey bee (*Apis mellifera* L.). Naturwissenschaften 84:238–241

Katzav-Gozansky T, Soroker V, Francke W, Hefetz A (2003) Honeybee egg-laying workers mimic a queen signal. Insect Soc 50:20-23

Keller L, Nonacs P (1993) The role of queen pheromones in social insects: queen control or queen signals? Anim Behav 45:787-794

Kryger P (2001a) The Capensis pseudo-clone, a social parasite of African honey bees. In: Proceedings of the 2001 Berlin Meeting of the European Section of IUSSI, 25-29 September p 208

Kryger P (2001b) The pseudo-clone of *Apis mellifera capensis* - an obligate social parasite in honeybees. In: Proceedings of the XXXVII International Apicultural Congress, Durban South Africa p 33

Kryger P, Dietemann V, Crewe RM (2003) Have we found a solution to the *capensis* problem? S Afr Bee J 75:123-128

Le Conte Y, Sreng L, Poitout S (1995) Brood pheromone can modulate the feeding behaviour of *Apis mellifera* workers (Hymenoptera: Apidae). J Econ Entomol 88:798-804

Lundie A (1954) Laying worker bees produce worker bees. S Afr Bee J 29:10-11

Martin S, Wossler TC, Kryger P (2002) Usurpation of *Apis millifera scutellata* colonies by *A. m. capensis* workers. Apidologie 33:215-232

Miller DG, Ratnieks FLW (2001) The timing of worker reproduction and breakdown of policing behaviour in queenless honeybee (*Apis mellifera* L.) societies. Insect Soc 48:178-184

Mohammedi A, Paris A, Crauser Y, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera L.*). Naturwissenschaften 85:455-458

Moritz RFA (2002) Population dynamics of the Cape bee phenomenon: The impact of parasitic laying worker clones in apiaries and natural populations. Apidologie 33:233-244

Moritz RFA, Hillesheim E (1985) Inheritance of dominance in honeybees. (*Apis mellifera capensis* Esch.). Behav Ecol Socio 17:87–89

Moritz RFA, Haberl M (1994) Lack of meiotic recombination in thelytokus parthenegenesis of laying workers of *Apis mellifera capensis* (the Cape honeybee). Heredity 73:98-102

Moritz RFA, Simon UE, Crewe RM (2000) Pheromone contest between honeybee workers (*Apis mellifera capensis*). Naturwissenschaften 87:395-397

Moritz RFA, Crewe RM, Hepburn HR (2002) Queen avoidance and mandibular gland secretion of honeybee workers (*Apis mellifera* L.). Insect Soc 49:86-91

Naumann K, Winston ML, Slessor KN (1993) Movement of honey bee (*Apis mellifera* L.) queen mandibular gland pheromone in populous and unpopulous colonies. J Insect Behav 6:211–223

Neumann P, Hepburn HR (2002) Behavioural basis for social parasitism of Cape honeybees (*Apis mellifera capensis* Esch.). Apidologie 33:165-192

Neumann P, Moritz RFA (2002) The Cape honeybee phenomenon: the evolution of a social parasite in real time? Behav Ecol Sociobiol 52:271-281

Neumann P, Moritz RFA, Mautz D (2000) Colony evaluation is not affected by drifting of drone and worker honeybees (*Apis mellifera* L.) at a performance testing apiary. Apidologie 31:67-79

Neumann P, Radloff SE, Moritz RFA, Hepburn HR, Reece SL (2001) Social parasitism by honeybee workers (*Apis mellifera capensis* Escholtz): host finding and resistance of hybrid host colonies. Behav Ecol 12:419–428

Oldroyd BP (2002) The Cape honeybee: an example of a social cancer. Trends

Ecol Evol Vol 17, pp 6

Oldroyd BP, Ratnieks FLW (2000) Evolution of worker sterility in honey-bee (*Apis mallifera*): how anarchistic workers evade policing eggs that have low removal rates. Behav Ecol Sociobiol 47:268-273

Oldroyd BP, Halling L, Rinderer TE (1999) Development and behaviour of anarchistic honey bees. Proc R Soc Lond B 266:1875-1878

Onions GW (1912) South African 'fertile worker bees'. S Afr Agric J 1: 720-728

Plettner E, Slessor KN, Winston ML, Robinson GE, Page RE (1993) Mandibular gland components and ovarian development as measures of caste differentiation in honeybee (*Apis mellifera L*.). Insect Physiol 39:235-240

Plettner E, Sutherland GRJ, Slessor KN, Winston ML (1995) why not be a queen? Regioselectivity in mandibular secretions of honeybee castes. J Chem Ecol 21:1017–1029

Pirk CWW, Neumann P, Hepburn HR (2002) Egg laying and egg removal by workers are positively correlated in queenright Cape honeybee colonies (*Apis mellifera capensis*). Apidologie 33:203–211

Radloff SE, Hepburn R, Neumann P, Moritz RFA, Kryger P (2002) A method for estimating variation in the phenotypic expression of morphological characters by thelytokous parthenogenesis in *Apis mellifera capensis*. Apidologie 33:129-137

Ratnieks FLW (1988) Reproductive harmony via mutual policing by workers in eusocial Hymnoptera. Am Nat 132:217-236

Ratnieks FLW (1992) Evidence for an egg marking pheromone in the honey bee. Am Bee J 132:813-814

Ratnieks FLW (1993) Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. Behav Ecol Sociobiol 32:191-198

Ratnieks FLW (1995) Evidence for queen-produced egg-marking pheromone and its use in worker policing in the honey bee. J Apic Res 34:31-37

Ratnieks FLW, Visscher PK (1989) Worker policing in the honeybee. Nature 342:796-797

Schmid-Hempel P (1998) Parasites in social insects. Princeton University Press, Princeton, NJ

Seeley T (1979) Queen substance dispersal by messenger workers in honeybee colonies. Behav Ecol Sociobiol 5:391-415.

Seeley TD (1985) Honeybee ecology. Princeton University Press, Princeton, NJ

Slessor KN, Kaminski L, King GGS, Borden JH, Winston ML (1988) Semiochemical basis of the retinue response to queen honey bees. Nature 332:354-356

Simon UE, Moritz FA, Crewe RM (2001) The ontogenetic pattern of mandibular gland components in queenless worker bees (*Apis mellifera capensis* Esch.). Insect Physiol 47:735-738.

Swart DJ (2001) Specialized management. In: Johannsmeier MF (ed) Beekeeping in South Africa, 3rd edition, Plant Protection Research Institute Handbook No. 14, Agricultural Research Council of South Africa, Pretoria, South Africa pp 85-94

Velthuis HHW (1970a) Chemical signals and dominance communication in the honeybee *Apis mellifera* (Hymenoptera: Apidae). Entomol Gen 15:83-90

Velthuis HHW (1970b) Queen substance from the abdomen of the honey bee queen. Z Vergl Physiol 70:210-222

Velthuis HHW (1970c) Ovarian development in *Apis mellifera* worker bees. Entomol Exp Appl 13: 343-357

Velthuis HHW (1972) Observations on the transmission of queen substances in the honeybee colony by the attendants of the queen. Behaviour 41:105–129

Velthuis HHW, Ruttner F, Crewe RM (1990) Differentiation in reproductive physiology and behaviour during the evelopment of laying worker honey bees. In: Engels W (ed) Social Insects, Springer-Verlag, Berlin pp 231-243

Verheijen-Voogd C (1959) How worker bees perceive the presence of their queen. Z Vergl Physiol 41:527-582

Visscher PK, Dukas R (1989) Honey bees recognize development of nestmates' ovaries. Anim Behav 49:542-544

Willis LG, Winston ML, Slessor KN (1990) Queen honey bee mandibular pheromone does not affect worker ovary development. Can Entomol 122:1093-1099

Winston ML (1987) The biology of the bee. Harvard University Press, Cambridge, MA

Winston ML (1992) Semichemicals and insect sociality. In: Isman M, Roitberg B (eds) Evolutionary perspectives on insect chemical ecology. Chapman and Hall, New York

Winston ML, Slessor KN (1992a) The essence of royalty: honey bee queen pheromone Am Sci 80:374-385

Winston ML, Slessor KN (1992b) Honey bee primer pheromones and colony organization: gaps in our knowledge. Apidologie 29:81-95

Wossler TC, Crewe RM (1999) Honeybee queen tergal gland secretion affects ovarian development in caged workers. Apidologie 30:311-320

Woyke J (1995) Invasion of Capensis bee. In: Magnuson P (ed) Proceedings of the first International Electronic Conference on the Cape bee problem in South Africa. 5-30 June, Pretoria PPRI, 35

CHAPTER 2

DO SOCIAL PARASITIC CAPE HONEYBEE WORKERS MAKE USE OF A "WINDOW OF OPPORTUNITY" TO INFILTRATE AFRICAN COLONIES?

2.1 INTRODUCTION

Honeybee, *Apis mellifera*, guards exclude non-nest mates and maintain a closed society (Breed et al. 1988). Honeybee guards use recognition cues that are derived exogenously from food or nest materials (Breed et al. 1988, Downs and Ratnieks 1999) and endogenously, or genetically, via the queen and/or workers (Getz 1981; Getz and Smith 1983; Moritz and Crewe 1988; Ratnieks 1991; Breed et al. 1992; Moritz and Neumann 2004). Previous studies suggest that nest mate recognition cues are specific to colonies, rather than individuals (Lacy and Sherman 1983; Downs and Ratnieks 1999) and that the colony's own recognition odour is circulated throughout the hive via antennation and trophallaxis (Naumann et al. 1993). Guards then compare their complex odour templates (gestalt odour hypothesis, also see Lenoir et al. 2001) to odours of other individuals attempting to enter the nest (Breed and Bennett 1987).

Despite this we know that Cape, *A. m. capensis,* honeybee workers still manage to gain access into African, *A. m. scutellata,* honeybee colonies where they behave as facultative social parasites (Hepburn and Crewe 1991; Neumann and Hepburn 2002). Honeybee workers may enter neighbouring colonies through robbing (Moritz and Southwick 1992), a possible route used by Cape honeybee workers to get into African colonies could be, drifting, where foreign worker honeybees enter a neighbouring hive by accident (Neumann et al. 2000) and by actively seeking out African host colonies (Neumann et al. 2001). Once these social parasites bypass guarding they rapidly develop into reproductive workers (Hepburn and Allsopp 1994), lay non policed or accepted eggs (Ratnieks and Visscher 1989; Martin et al. 2002a), produce queen-like pheromones

(Crewe and Velthuis 1980; Crewe 1981; Moritz et al. 2000; Wossler 2002) and get preferentially fed as larvae (Beekman et al. 2000; Calis et al. 2002; Allsopp et al. 2003). This social parasitic behaviour has lead to the spread of Cape laying worker honeybees among African colonies throughout South Africa (Allsopp and Crewe 1993; Martin et al. 2002b) and has been dubbed the *'capensis calamity'* (Allsopp 1992, see chapter 1 on the *'capensis calamity'*).

Commercial beekeeping practices have been identified as the prime mechanism having resulted in the *'capensis calamity'*. In the early 1990's Cape honeybee colonies were interspersed with African honeybee colonies in the same apiaries on the Aloes, where many beekeepers who farm with African colonies converge on these enriched foraging areas (Allsopp 1992; Hepburn and Allsopp 1994; Swart et al. 2001; Moritz 2002, see chapter 1). Firstly, under these congested apiary conditions, especially when pollen and nectar are in abundance, guarding becomes more permissive (Downs and Ratnieks 1999) and drifting of the two subspecies into other colonies is more likely to increase (Martin et al. 2002a). It is believed that during colony inspection in these congested apiaries African honeybee colonies are placed under great threat to invasion by Cape honeybee workers who accidentally enter their African neighbouring colonies (Moritz 2002). Secondly, beekeepers make use of the rapid colony growth on these rich food sources to split their colonies, again rendering these colonies more vulnerable to take-over (Neumann et al. 2001; Neumann and Hepburn 2002). For the past 15 years, under apiary conditions in the formerly *A. m. scutellata* regions of South Africa, Cape bees have been able to infiltrate and persist in *A. m. scutellata* apiaries (Moritz 2002).

Yet recent efforts to elucidate this infiltration have not been particularly successful. Beekman et al. (2002) showed that pseudoclone Cape workers are effectively recognized as non-nestmates and removed, and new attempts to introduce native Cape workers and pseudoclones into African

colonies have proven ineffective, with all or almost all the introduced Cape worker honeybees invariably being detected and eliminated, notwithstanding the varied means of introductions attempted (Calis et al. in preparation). This would suggest that African colonies are not as susceptible to Cape honeybee worker infection as has been suggested and that under normal circumstances colony integrity is retained and infiltration resisted. This does not come as any surprise as African guards are known to be highly efficient as they have been shown to have low response thresholds to alarm behaviour, are more readily recruited and more persistent at guarding tasks (Crewe 1976; Moore et al. 1987; Breed et al. 2004a).

Why then, do pseudoclone honeybee workers still persist in African commercial stock throughout South Africa? As previously mentioned, there may be special circumstances under which African colonies are particularly vulnerable to Cape worker infiltration. We know that commercial beekeepers constantly move their colonies to high pollen and nectar regions and split their colonies (Johannsmeier et al. 2001). Honeybee colonies swarm off naturally when they become too large, thus replicating colonies. Beekeepers use a similar process of colony fission to increase their commercial colony numbers by the same principals surrounding natural swarming by splitting their colonies and capturing both halves before swarming (Swart et al. 2001). Half the colony members swarm off with the queen (swarm) and establish a new queenright hive elsewhere while the other half remains behind as a queenless colony (post-swarm) for a period of time having started re-queening from the brood left behind (Butler 1960; Butler and Simpson 1967). It is believed that the period surrounding the splitting process when the colonies maybe queenless or have a virgin queen for a short period heightens the invasion probability concerning the *'capensis calamity'* (see Woyke 1995; Neumann and Hepburn 2002). Since African colonies are still being infected by the Cape laying pseudoclones found in commercial stock of African beekeepers (Moritz 2002) it certainly suggests that host colonies present a probable increased

window of opportunity for invasion by Cape honeybee workers surrounding the splitting phenomenon. This social parasitic behaviour between the two endemic yet separated southern African honeybee subspecies (*A. m. scutellata* and *A. m. capensis*) provides the ideal opportunity to test acceptance and survival rates of Cape workers in host colonies.

It is hypothesized that this period of queenlessness inherent in the swarming process is the crucial period to the "window of opportunity" necessary for Cape worker infiltration, and hence is critical to the *capensis* Problem. This was investigated by simulating the commercial beekeeping process of splitting strong African colonies into two halves. The half with the queen represents the swarm and the queenless half represents the post-swarm colony. The ability of Cape honeybee workers to infiltrate the two African colony components (swarm and post-swarm) was examined to determine the importance of the swarming event in the *capensis* problem. Two Cape honeybee worker populations; native Cape workers from Stellenbosch and pseudoclone workers from Pretoria, were used to ascertain whether the established parasitic pseudoclones evade detection by queenright and queenless host African colonies more successfully compared to their native Cape honeybee worker relatives. Moreover, host African colonies were infected at three different time periods that surround the swarming process of host colonies (treatments; before, during and after), to determine whether there indeed is a specific "window of opportunity".

2.2 MATERIALS AND METHODS

2.2.1 Experimental host colony setup

In this experiment I used conditions similar to those used by commercial beekeepers when they intend to increase their number of colonies. Nine unrelated and non parasitized African honeybee colonies were obtained from a commercial beekeeper from Douglas who was recognized for

having no Cape worker laying activity in his *A. m. scutellata* colonies. These host African colonies were split into queenright (swarm) and queenless (post-swarm) halves (n = 18, see Section 2.2.3) that were infected by two *A. m. capensis* honeybee worker populations (native Cape and pseudoclones) at various stages surrounding the splitting process (see Section 2.2.2 and Figure 2.1). The approximate number of *A. m. scutellata* workers was approximately 10 000 in each of the free-flying colonies (Allsopp personal communication). Queenless split colonies (post swarm) remained in the original 10 frame hives, whereas the queenright colonies (swarm) were moved into new 10 frame hives (see Figure 2.1). Unfortunately, one of the queenright colonies lost its queen and was therefore excluded from the experiment (Table 2.2). Open and sealed African brood as well as the food were equally shared in each of the splits. Colonies were housed at two separate apiaries approximately 3km apart. Queenright (swarm) colonies were housed at the Western Cape Agricultural experimental farm Kromme Rhee, and the queenless (post swarm) colonies were located at the University of Stellenbosch's experimental farm Mariendahl, this helped prevent drifting.

Pectora roborant cultus recti

2.2.2 Introduced bees

Fifty 1-day old native Cape honeybee workers (obtained from the Plant Protection Research Institute, Stellenbosch) and fifty 1-day old pseudoclones (obtained from a commercial beekeeper, Pretoria) were introduced into each split colony at three specific infection periods surrounding the splitting stage (see Section 2.2.3). All introduced bees were uniquely marked and if in the event of drifting (as all bees were free-flying) they would be easily identified. Both *A. m. capensis* honeybee workers, native Cape and pseudoclones, were collected from sealed brood frames that were incubated separately at 32°C with 60% relative humidity overnight. Emerging 1-day old native Cape and pseudoclone honeybee workers were colour marked on their thoraces with non-toxic coloured paint for easy identification. All introduced workers were newly emerged and thus

of the same age and therefore contained a clean or blank slate, where recognition cues are absent and consequently makes them generically acceptable in honeybee colonies (Breed et al. 2004b).

2.2.3 Treatment periods

The three treatments; before, during and after splitting of African, *A. m. scutellata,* colonies were used to identify a 'window of opportunity' for successful invasion (Figure 2.1). For each treatment (1 to 3) three replicates were run independently and simultaneously to minimize environmental influences. 1-day old native Cape and pseudoclone honeybee workers were introduced three days prior to splitting the hives in treatment 1. On splitting of the African colonies, marked bees were equalised between the two splits. In treatment 2, native Cape and pseudoclone honeybee workers were introduced during the splitting process of the African

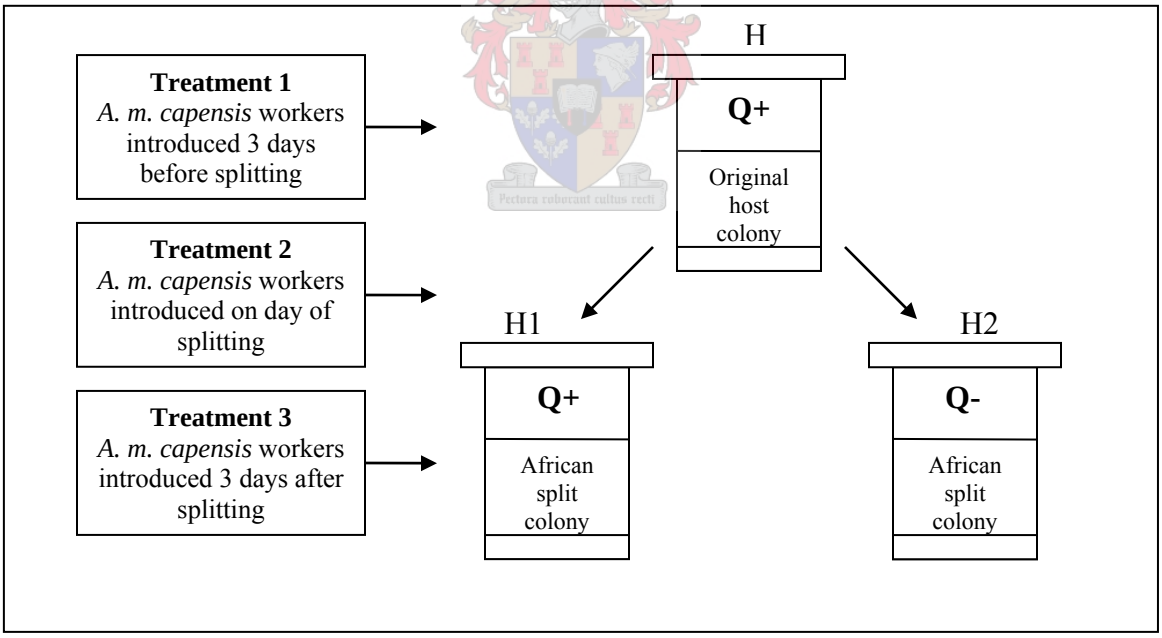


Figure 2.1 Experimental design of host African colonies (H) split equally into swarming queenright halves (H1) and post-swarming queenless halves (H2). The three different infection periods (Treatments 1 to 3) by Cape honeybee workers are also indicated and were run independently; Treatment 1 introduced Cape workers (into H) three days before splitting host colonies; Treatment 2 introduced Cape workers during the splitting process; Treatment 3 represents a period of introduction of Cape workers three days after the splitting process

colonies. Finally the African hives were initially split and only three days later were they infected with both the native Cape and pseudoclone honeybee workers (Treatment 3). These three treatments investigate the three most likely infection periods surrounding the splitting period.

2.2.4 Statistical analysis

All introduced and marked native Cape and pseudoclone honeybee workers were counted every third day from introduction to ascertain the survival rates of the introduced *A. m. capensis* workers. Counts were conducted early morning ensuring consistency. The duration of the experiment ran over 21 days. Days 3, 12 and 21 were selected for analysis. The data was analysed using STATISTICA, version 7; 2004. A generalized linear model (GLZ) was constructed using a Poisson distribution to analyse the full queen and treatment effects as well as the between effects, on the survival rate of both the native Cape and pseudoclone worker populations within host African colonies across the three treatments. During the construction of the model non significant interactions were eliminated and the model rerun until the best fit model was achieved. The GLZ model detected significant interactions at day 21. The percentages explained by the best fit GLZ model was relatively low and not in total accordance with the absolute count data (see Table 2.2) suggesting that there maybe differences not detected by running a multivariate model such as a GLZ. The difference in survival rates between African queenright and queenless conditions for the 3 levels of treatments and further cross interactions between queen and treatment were compared using Chi square (χ^2) tests with Yates's correction (Zar 1999) . Probability values (P) smaller than 0.05 were considered significant for all statistical analyses performed.

2.3 RESULTS

The number of native Cape and number of pseudoclones found was recorded every 3 days for both split African colonies (Table 2.1). At first glance the initial survival rate trend indicates that the primary discriminatory period lies within the first three days after the introduction of the Cape honeybees (for both native Cape and pseudoclones). Within the first three days an immediate loss of approximately 70% of all introduced bees was observed (Figure 2.2). Thereafter until day 21 there is a gradual decline in the numbers of Cape workers for both introduced populations remaining in the colonies (see Figure 2.2). It must be noted that these workers were 1-day old when being introduced and all shared a blank slate (Breed et al. 2004b). Moreover, drifting was also monitored and controlled for as each host African split colony contained uniquely marked Cape honeybee workers from both populations and only one instance of a single drifting marked worker honeybee was observed.

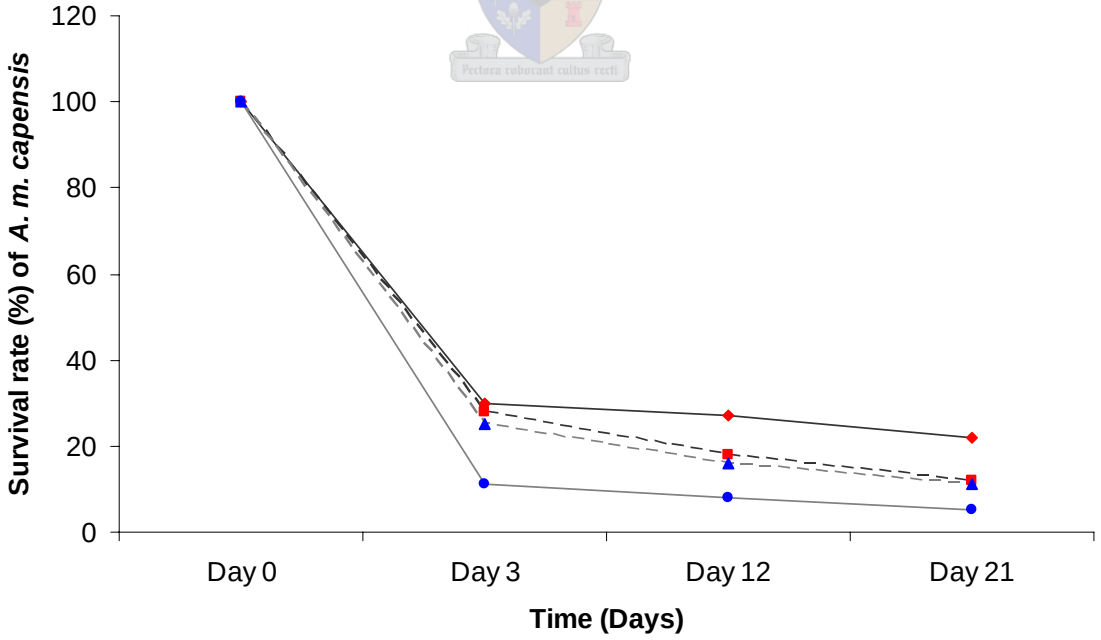


Figure 2.2 Percentage survival rate of introduced native Cape and pseudoclone honeybee workers in African queenright (n = 8) and queenless (n = 9) colonies over 21 days. Native Cape workers, in queenright (--▲--) and queenless (--■--) colonies; pseudoclone workers, in queenright (—●—) and queenless (—♦—) colonies

The second immediately evident feature is the vast colony variation with respect to the survival rates observed for these introduced Cape honeybees, both in African queenright (swarm) and queenless (post-swarm) colonies (refer to Table 2.1). In colonies A and probably D, all introduced Cape honeybees had been removed by the third day, for both African queenright and queenless splits. In other colonies (E, F, G) all introduced bees were removed from the queenright (swarm) split colonies by the third day, but some introduced Cape honeybee workers remained in the queenless (post-swarm) half until at least 21 days. In yet other colonies (B, C, H, I), introduced Cape honeybee workers were not entirely removed from either queenright or queenless colonies (Table 2.1). These data strongly suggests that there is a variable response to Cape honeybee worker infection among African colonies.

A best fit Generalized Linear Model (GLZ), using a stepwise backward removal system, was constructed to examine the survival rate of both *A. m. capensis* worker populations. Both population type (native Cape workers or pseudoclones; Wald $\chi^2 = 0.34$, $df = 1$, $P = 0.557$) and treatment (3 days before queen removal, the day of queen removal, 3 days after queen removal; Wald $\chi^2 = 1.01$, $df = 2$, $P = 0.601$) were non-significant main effects and removed from the best fit model. Subsequently the best fit GLZ model (Table 2.2) indicated queen presence as a significant factor in the success rate of intrusion and establishment by all Cape honeybee individuals. Significantly more Cape honeybee workers from both populations survived in queenless African colonies (Table 2.2; Wald $\chi^2 = 4.49$, $df = 1$, $P = 0.033$). This clearly shows that the absence of a queen does facilitate the establishment of Cape workers in African colonies.

Table 2.1 Absolute count data over 21 days of the number of surviving marked *A. m. capensis* workers from both introduced populations observed for each of the 18 *A. m. scutellata* (African) colonies across the various infection treatment periods (T) (also see section 2.2.3). Cape honeybee populations; native Cape honeybee worker from the Western Cape = Cape and pseudoclone honeybee workers from the Highveld = Clones. NA = data not applicable due to original queen loss and colony re-queening during observation period

Queen status	T	Colony ID	Honeybee population	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
Queenright	1	A	Cape	50	0	0	0	0	0	0	0
			Clone	50	0	0	0	0	0	0	0
		B	Cape	50	27	25	14	14	5	4	4
			Clone	50	24	22	21	16	14	9	11
		C	Cape	50	21	18	18	15	12	11	8
			Clone	50	19	15	13	15	12	11	8
	2	D	Cape	50	NA	NA	NA	NA	NA	NA	NA
			Clone	50	NA	NA	NA	NA	NA	NA	NA
		E	Cape	50	0	0	0	0	0	0	0
			Clone	50	0	0	0	0	0	0	0
		F	Cape	50	0	0	0	0	0	0	0
			Clone	50	0	0	0	0	0	0	0
	3	G	Cape	50	0	0	0	0	0	0	0
			Clone	50	0	0	0	0	0	0	0
		H	Cape	50	39	38	32	32	30	30	30
			Clone	50	3	11	5	3	5	2	3
		I	Cape	50	33	24	23	15	10	6	0
			Clone	50	2	1	0	0	0	0	0
Queenless	1	A	Cape	50	0	0	0	0	0	0	0
			Clone	50	0	0	0	0	0	0	0
		B	Cape	50	22	10	19	11	9	3	3
			Clone	50	50	48	50	46	38	44	41
		C	Cape	50	16	10	11	7	5	3	2
			Clone	50	24	22	20	19	17	16	10
	2	D	Cape	50	0	0	0	0	0	0	0
			Clone	50	0	0	0	0	0	0	0
		E	Cape	50	24	24	15	16	15	11	8
			Clone	50	28	27	25	25	21	18	23
		F	Cape	50	23	27	17	17	15	14	20
			Clone	50	28	31	24	24	22	22	20
	3	G	Cape	50	15	12	12	10	8	10	14
			Clone	50	0	0	0	0	0	0	0
		H	Cape	50	16	14	12	3	2	1	0
			Clone	50	4	6	3	6	0	4	0
		I	Cape	50	12	16	16	9	8	7	5
			Clone	50	3	0	0	0	0	1	2

In addition, the best fit GLZ model also generates a queen*treatment interaction (see Figure 2.3 and Table 2.2 for statistical results), as well as a significant treatment*population interaction (see Figure 2.5 and Table 2.2 for statistical results). It's important to note that the queen*treatment interaction is largely driven by treatment 2 where all native Cape and pseudoclone workers were killed in the African queenright colonies (Figure 2.3).

Table 2.2 Significant interactions (best fit GLZ model) of Cape honeybee worker survival rates within African colonies. Significance is indicated at $P < 0.05$

Interactions (effect) tested	*df*	Scaled deviance (Stat/*df*)	Deviance explained (%)	Log-Likelihood	Wald χ^2	*P* value
Queen	1	1.067	34.8	-404.19	4.49	0.033
Queen*Treatment	2	1.067	34.8	-404.99	6.09	0.047
Treatment*Population	2	1.067	34.8	-406.27	8.68	0.013

These above mentioned results have largely combined both Cape honeybee worker populations as one variable during the analysis. The GLZ results would suggest that the two populations are being treated similarly in African colonies (population: native Cape honeybees or pseudoclones; (Wald $\chi^2 = 0.34$, $df = 1$, $P = 0.557$) however the count data suggests otherwise (Table 2.1). Therefore, the question remains whether native Cape workers survived differentially to pseudoclone workers in both African queenright and queenless colonies, and whether this was influenced by the different treatments?

The two different populations of Cape honeybees introduced into the African colonies, do show significantly different survival rates on most days in both queenright (swarm) and queenless (post-swarm) colonies (Figure 2.4 and see Appendix Table I for results). On day 3, 12 and 21 significantly more native Cape compared to pseudoclones workers survived in host African

queenright swarm colonies while on day 12 and 21 more pseudoclones survived in host African queenless post-swarm colonies (Figure 2.4). Clearly, a greater percentage of pseudoclones survive and establish in the queenless post-swarm colonies and a greater percentage of native Cape bees in the queenright swarm colonies for the three observational days. Moreover the decline in survival rate for native Cape workers over time occurs concomitantly in both

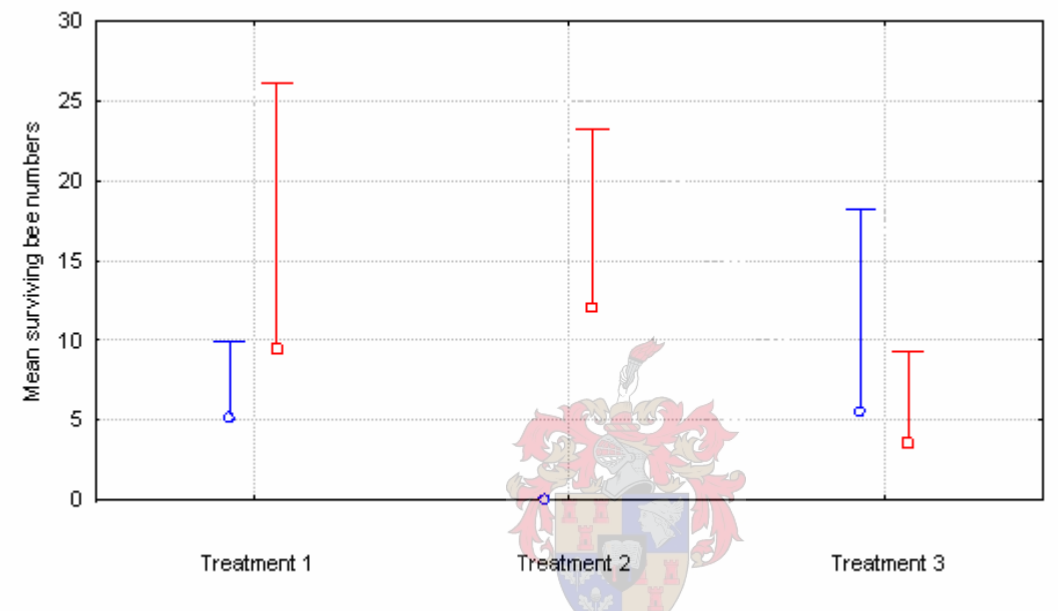


Figure 2.3 The effect of African queenright (—○—) and queenless (--□--) host colonies on the survival rate (mean and 95% confidence interval) of all 21 day-old Cape workers for all treatments. Treatment 2 is responsible for the significance of queen*treatment interaction

queenright and queenless colonies, consequently there is no difference in survival rate for Cape workers over time between queenright and queenless colonies (χ^2 with Yates's correction = 0.17, $df = 1$, $P > 0.05$ for day 21 but similar results were obtained for all days observed, see Figures 2.2 and 2.4). This is not true for pseudoclone workers though, with significantly more surviving within host queenless colonies than queenright colonies over time (χ^2 with Yates's correction = 10.7, $df = 1$, $P < 0.05$ for day 21 but similar results were obtained for all days observed, see Figures 2.2, 2.4 and see Appendix Table I for statistical values).

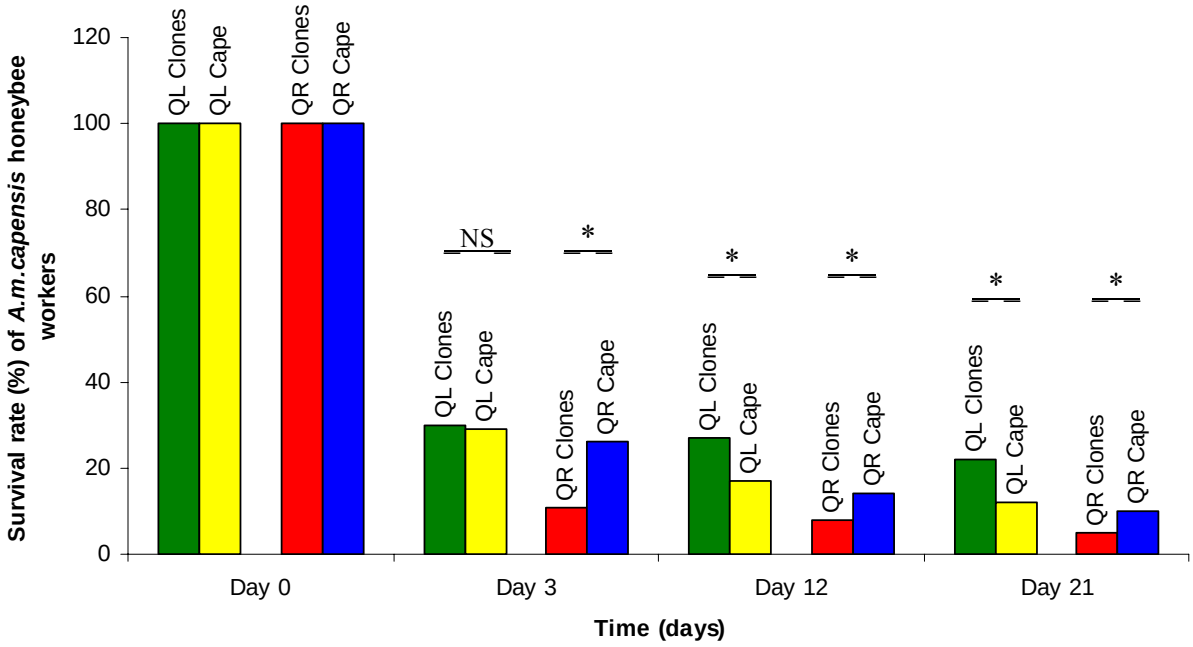


Figure 2.4 Percentage survival rate comparing the number of surviving pseudoclone honeybee workers within queenless (■) and queenright (■) African colonies to native Cape honeybee workers within queenless (■) and queenright (■) African colonies across all three treatments over time. Significant differences in survival rates between pseudoclones and native Cape workers are denoted by "*" where $P < 0.05$, using χ^2 tests with Yates's correction (see Appendix Table I for statistical values)

The next important question being asked was whether the timing of infiltration (treatment effect) of the Cape honeybees (both native Cape and pseudoclones) into African colonies was important in their establishment. That is, in an effort to determine more accurately a possible "window of opportunity" surrounding the swarming event, Cape honeybees were introduced 3 days prior to colony fission, on the day of colony fission, and three days after colony fission. Looking at introduction time alone, there is no difference in the survival of all introduced Cape honeybees between queenright (swarm) colonies and queenless (post-swarm) colonies for either treatment 1 (3 days before fission) or treatment 3 (3 days after fission), while more introduced workers from treatment 2 survived in the queenless colonies (Figure 2.3). This is simply because all Cape bees introduced on the day of colony fission were removed from the queenright colonies (Table 2.2 and see Figure 2.3).

Taken alone, the data suggests that while the swarming period presents an opportunity for Cape honeybees to successfully get established in African honeybee colonies, there is no clear window of opportunity for this infiltration (Figure 2.5). However, an interaction effect between the types of Cape worker populations used across the three treatments for all colonies was evident in the best fit GLZ model (Table 2.2; Wald $\chi^2 = 8.68$, $df = 2$, $P = 0.013$) with notable differential survival rates for the two Cape honeybee worker populations for treatment 1 and treatment 3 (Figure 2.5). Considerably more pseudoclone honeybee workers survived in treatment 1 than did native Cape honeybee workers while for treatment 3 the converse occurred with more native Cape honeybee workers surviving than pseudoclones (Figure 2.5 and see Table 2.1 for count data).

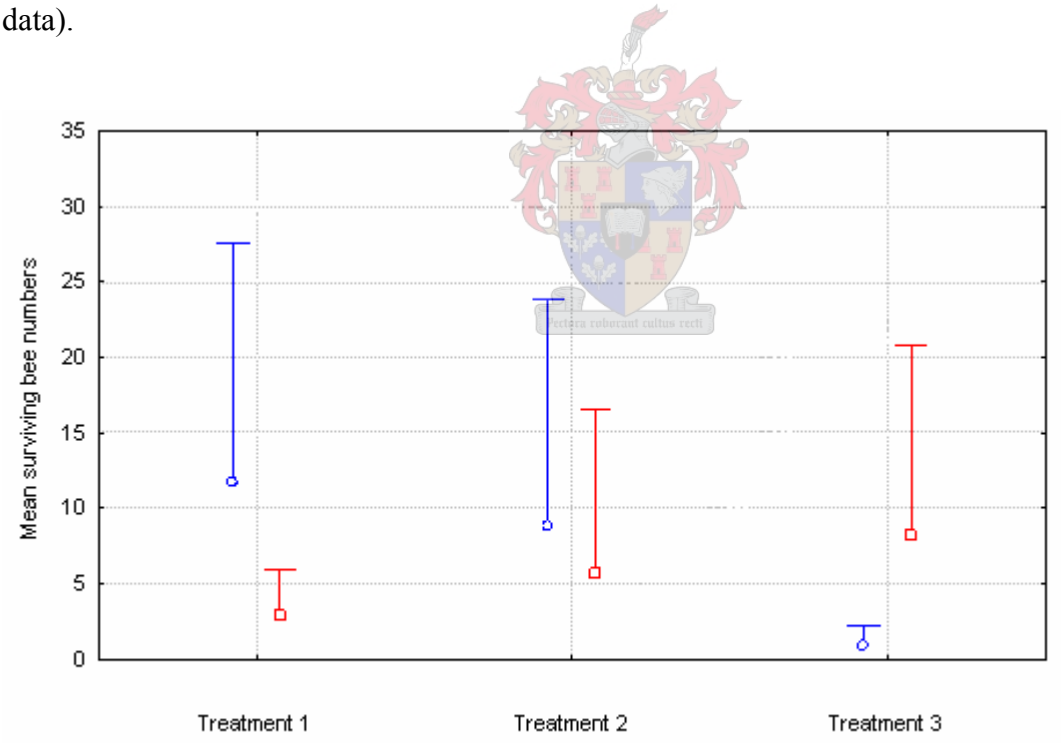


Figure 2.5 Mean survival rates (mean and 95% confidence interval) of 21 day-old native Cape workers (--□--) and pseudoclone workers (—○—) within all host African colonies (n = 17)

It is perhaps significant that there is no survival difference between pseudoclones and native Cape honeybees in treatment 1 and treatment 2 in the queenright colonies, but that more native

Cape honeybees survive than do pseudoclones in the post fission treatment 3 (Table 2.3). The same is found for the post fission treatment (treatment 3) in the queenless colonies; that is, more native Cape honeybees survive than pseudoclones (Table 2.3). In contrast however, more pseudoclones survive than do native Cape honeybees for both treatments 1 and 2 in similar post fission queenless colonies (Table 2.3).

Table 2.3 Comparative survival rates for 21-day old native Cape (Cape) and pseudoclone (Clone) worker populations across the three treatment levels (T1, T2, T3) in both queenright and queenless host African colonies. Differences between populations for a given treatment were evaluated by χ^2 tests with Yates's correction (Zar 1999). All significant interactions are denoted with "*". Significance indicated at $P < 0.05$. Direction of the significance is indicated in the population column (see Table 2.1 for sample sizes)

African colony state	Treatment level	Population favoured	χ^2	*df*	*P* values
Queenright	T1	Clone ≈ Cape	1.67	1	0.250
	T2	Clone ≈ Cape	0.00	1	0.999
	T3	Clone < Cape	30.4	1	0.001*
Queenless	T1	Clone > Cape	44.0	1	0.001*
	T2	Clone > Cape	4.42	1	0.030*
	T3	Clone < Cape	13.9	1	0.001*

Pectora roborant cultus recti

Besides the two worker populations surviving differentially within most experimental colonies (Table 2.3), each of the worker populations independently, survive differentially across treatments (Figure 2.6). Pseudoclones always survive significantly more in treatment 1 than treatment 3; however, the opposite is true for native Cape workers, where significantly fewer survive treatment 1 than treatment 3. Survival rates for treatment 2 for both populations are variable with neither surviving in queenright colonies while a large proportion survives in queenless colonies Figure 2.6; see Appendix Table II for statistic values).

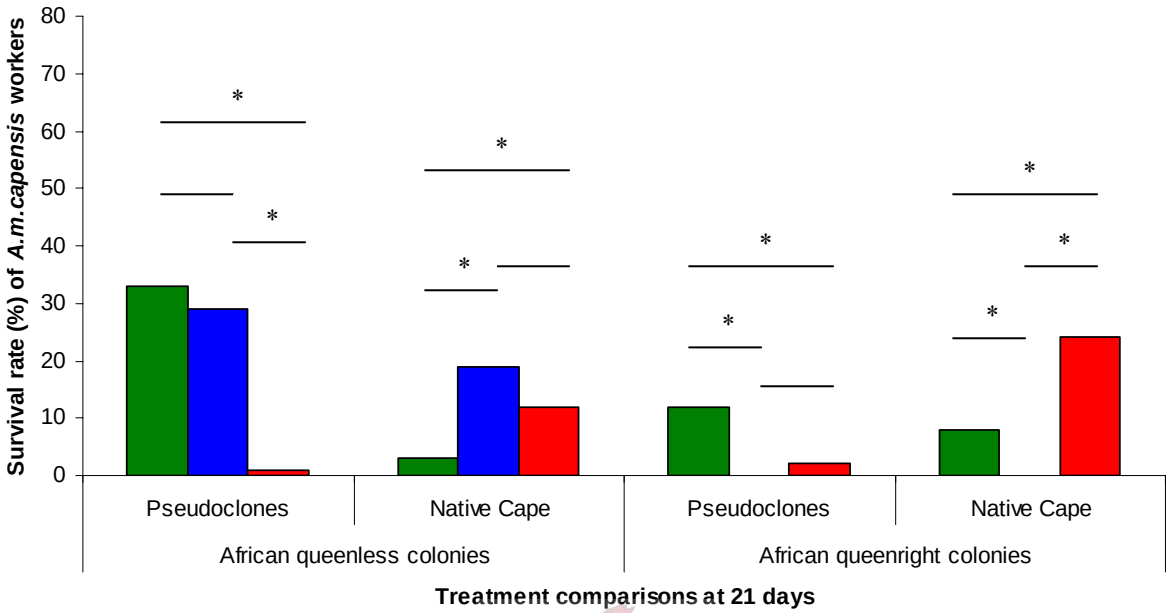


Figure 2.6 Comparative survival rates for 21 day-old pseudoclones and native Cape honeybee workers within host African queenless and queenright colonies across the three treatments. Treatment 1 (■), Treatment 2 ([■]) and Treatment 3 ([■]).Differences between the treatment effects were evaluated with χ^2 tests with Yates's correction (Zar 1999). Significant differences in survival rates between the treatment effects are denoted by "*" where $P < 0.05$ (see Appendix Table II for statistical values)

2.4 DISCUSSION

Previous studies have shown that African colonies' guards are efficient guards and are known to be more readily recruited and persistent guards (Moore et al. 1987; Beekman et al. 2002; Breed et al. 2004a). The data in this experiment support these previous studies which show a high mortality rate within the first three days of infection for all treatments tested, for both queenright and queenless colonies. This was true for both introduced Cape honeybee populations, those native to the Western Cape and those established as pseudoclones in the Highveld region. Even though we used a non conventional guarding bioassay, we introduced newly emerged (less than 24hrs old) *A. m. capensis* honeybee workers which hypothetically contain a clean or blank slate (odour) increasing their chance of surviving in non-natal colonies (Breed et al. 2004b). This

however does not appear to be the case with 1-day old Cape honeybee workers, where 70% were detected and killed within 3 days by African guards, showing very little support for the blank slate hypothesis for Cape honeybee workers.

Beekman et al. (2002) showed that invasive *A. m. capensis* foraging pseudoclone workers also have no special mechanisms to bypass the African guards where 18% African non-nest mates and 15% Cape (pseudoclone) non-nest mates were accepted in one experiment. These data were pooled across both queenright and queenless colonies with no significant difference between queenright and queenless host colonies in their rejection of non-nestmates. Beekman and her co-workers' (2002) experiment were similar to our treatment 3 design where host African colonies were initially split into African queenright and queenless colonies prior to infection by pseudoclone workers. Our results for treatment three, regarding the survival rate of pseudoclones between African queenright and queenless colonies, support their findings with no significant difference in acceptance for *A. m. capensis* pseudoclone non-nest mates between African queenright (6%) and queenless (10%) colonies. These low acceptance rates by these queenless colonies can best be explained by the host colony becoming over-sensitive due to the internal colony disruption and evict all foreign non-nest mate workers, as demonstrated by Beekman et al. (2002).

During a controlled colony split the queenless half split occupies a relatively brief period of a colony's life, approximately seven days, where normal colony activities are altered due to the absence of a mated queen's pheromones (Gilley and Tarpy 2005). This queenless period therefore renders a colony pheromonally deficient and more vulnerable to possible invasion. Our data clearly supports this supposition with significantly more pseudoclone workers surviving in queenless host colonies. However, a few pseudoclones survived in some queenright colonies

which does suggest that the African queen offers some protection to invasion but that she does not appear to be completely effective. We know that she produces a less powerful mandibular gland signal compared to Cape queens who secrete the highest proportion of 9-ODA compared to queens of other races (Crewe 1982, 1988). Consequently African queens do not have these so-called "super" signals of Cape queens (Crewe 1988), concomitantly Cape workers also have high response thresholds to queen signals (Crewe 1982, 1988; Magnuson 1995), leading to rapid development of laying Cape workers in host African colonies. This factor has proven to be critical in the usurpation of African colonies by Cape workers. These mandibular gland secretions of African queens are lacking and in some way may play a role in a small percentage of pseudoclones surviving the initial three days of heightened aggression by host workers in some queenright colonies.

Notably the data collected in this study strongly suggest that there is a variable response to Cape honeybees among African colonies and that some colonies are more capable of mounting a successful defence than others, and that the "window of opportunity" for Cape honeybees to penetrate African colonies defences might also be variable. Some colonies removed all Cape workers while a variable proportion of Cape workers survived in others. Why some workers survive in African queenright colonies is probably firstly due to individual queen variability in signal production which plays a likely role in the differential survival and acceptance of workers. Such queen variability is believed to be due to a number of factors such as queen age and her pheromone bouquet (Butler 1959; Free 1987). Secondly, the behaviour of some workers in avoiding the queen and possibly the host workers (Moritz et al. 2001; Martin et al. 2002b) may offer a survival advantage to these workers. Early in the usurpation of African colonies, there's a high level of aggression directed towards Cape honeybee individuals (Cooke 1992) because their signals need time to develop. Only when they reach the end point of development do they

produce sufficient 9-ODA (Crewe 1984) to elicit 'pseudoqueen' treatment from the resident African workers. While their signal is developing it is likely that those invaders that succeed profit by avoiding contact with "messenger workers", and consequently escape queen repression factors, a common trait for workers with a high reproductive potential like pseudoclones (Moritz et al. 2001). This is in contrast to what usually happens in queenless host colonies of other races, where Cape workers show tropallactic dominance, frequently receiving food from the host workers (Velthuis et al. 1990). Thirdly, colonies vary genetically due to unrelated queens heading neighbouring colonies as well as due to the polyandrous nature of honeybee queens which generates genetic variability among subfamilies (Moritz and Fuchs 1998; Fuchs and Moritz 1999; Palmer and Oldroyd 2000; Martin et al. 2004). This can result in certain colonies consisting of subfamilies predisposed to colony defence while others may show genetic predispositions to other colony activities (Robinson et al. 1990; Page and Robinson 1994; Guzman-Novoa and Page 1994; Page et al. 1995).

As mentioned above, given that the African colonies were obviously variable in their response and effectiveness, it might be expected that an effective social parasite would move from the highly defensive African colonies to the less defensive African colonies; but clearly this does not happen. As colonies were monitored for 21 days after introduction and yet only one "drifted" Cape honeybee was found, it brings into question the directed parasitism ascribed to Cape honeybees in general and the pseudoclone in particular (Neumann et al. 2000, 2001).

The nature of the chemical signalling system within the *A. m. capensis* workers seems to promote the establishment of pseudoqueens (Plettner et al. 1993; 1996; 1997). *A. m. capensis* workers are capable of shifting along the worker-queen developmental continuum exhibiting a mosaic of worker and queen traits (Crewe et al. 1990). It would however appear that native Cape worker's

when in the presence of an African queen slow down their development more then their pseudoclone counterparts and remain quite worker-like and in so doing, probably avoid detection. Native Cape workers do differ physiologically with respect to their pseudoclone relatives in the presence of an African queen (see chapter 3 for further details). The pseudoclone invaders have probably moved further along the developmental continuum, expressing more and/or more advanced queen characteristics (see chapter 3 on this topic), the majority being rapidly detected by queenright host workers and removed. This begs the question that those pseudoclones that survive may actually be those workers that refrain from rapid signal development within the first 72 hours, reducing their chances of detection?

Previous Cape invasions, which were documented in 1928 (Lundie 1954) and in 1977 took place when African colonies swarmed, were superseded or were being manipulated (Johannsmeier 1983). These previous invasions showed similar symptoms as the *'capensis calamity'* of today but differed as the invasion then only persisted for 4 years. What is different today is that there is now an established social parasitic *A. m. capensis* pseudoclone population that has supposedly derived from a single worker (Kryger 2001). Even though many of the pseudoclone's characteristics are common to native Cape workers, some traits do however appear to be more advanced in the pseudoclone (Neumann and Moritz 2002; Dietemann et al. 2006). Here we show that the pseudoclones are accepted and survive in queenless host colonies significantly more than their native counterparts (see chapter 1 for explanation on the origin of the parasitic worker lineage). Those workers that lead the race in signal development and simultaneously avoid detection during this period of development will adopt the reproductive roles within the colony over time while those failing will be rapidly removed.

We know reproduction by colony fission is widespread in honeybees (Winston 1987) and common practice at a commercial level (Swart et al. 2001). By manipulating the commercial splitting process of honeybee hives and infecting them at various periods, we could ascertain whether there was in actual fact a "window of opportunity" for colony take-over. No introduced *A. m. capensis* workers from both isolated populations survived in African queenright colonies when introduced on the day of splitting. Queenright colonies that have just been split appear to be at their most alert yet at the same time at their most disorientated for the queenless half split. The queenright half evicted all introduced *A. m. capensis* workers within the first 3 days. This suggests the queenright half colonies at this stage during colony splitting could be at their most resistant to invasion and infection. On the contrary, in the queenless halves both *A. m. capensis* worker populations had a 20% or greater survival rate at 21days. Queen pheromones play a critical role in colony organization/behaviour, regulating and coordinating the activities of workers (Ferguson and Free 1980; Free 1987; Prince and Gunson 1992) and when these pheromones are removed normal colony functioning breaks down. Workers who have just lost their queen probably exhibit reduced guarding capacity, with invaders gaining access, developing and becoming established.

Pseudoclones appear to be well suited in gaining access to African honeybee colonies in the period immediately before swarming (treatment 1). That does not seem to be the case in the period immediately following colony fission (treatment 3). This would suggest that pseudoclones infiltrating colonies that have been queenless for 3 days or more may have a low success rate of survival. The colonies at this specific stage are entering a queen rearing phase with a likely period of heightened defence and sensitivity in these colonies, which might be leading to the more rapidly developing pseudoclones being recognized and targeted. This may explain why some of the native Cape honeybees survive in treatment 3 because if we look along a queen-worker

continuum, a number of the workers will fall on the worker-like end of this continuum, rendering them more likely as non-threatening worker-like bees, within these sensitised colonies. On the whole, it would appear that the first 72 hour period is critical in the host African colonies' ability to detect and eradicate Cape non-colony nest mates. Host African colonies, especially in the absence of their queen, appear vulnerable surrounding colony fission to invasion by both Cape honeybee worker populations even though there are low survival rates.

It's important to remember that in this study one variable, that being the splitting and infection period, was investigated but it alone is not responsible for host colony susceptibility. It becomes apparent that there are numerous factors which come into effect such as; pollen/nectar flows (Downs and Ratnieks 1999), congested apiaries (Moritz 2002), queen status and pheromones (Ferguson and Free 1980; Free 1987) and the effects of worker genotypic diversity on honeybee colony development and behaviour (Page et al. 1995). When a number of 'uncontrollable' factors co-occur then the host colony's vulnerability maybe heightened. These factors having been identified should be considered in further field experiments where many uncontrollable variables are present.

In conclusion, there is no obvious 'window of opportunity' around the swarming process for Cape honeybees to infiltrate and become established in African honeybee colonies. The whole colony fission period is found to be vulnerable to infiltration. For the pseudoclones however, the period immediately prior to colony fission appears to represent the best opportunity for invasion.

2.5 REFERENCES

Allsopp MH (1992) The *'capensis calamity'*. S Afr Bee J 64:52–55

Allsopp MH, Crewe R (1993) The Cape honeybee as a Trojan horse rather than the hordes of

Jenghiz Khan. Am Bee J 133:121–123

Allsopp MH, Calis JNM, Boot WJ (2003) Different feeding of worker larvae affects caste characters in Cape honeybee, *Apis mellifera capensis*. Behav Eco Sociobiol 54:555-561

Beekman M, Calis JNM, Boot WJ (2000) Parasitic honeybees get royal treatment. Nature 404:723

Beekman M, Wossler TC, Martin SJ, Ratnieks FLW (2002) Acceptance of Cape honey bees (*Apis mellifera capensis*) by African guards (*A. m. scutellata*). Insectes Soc 49:216-220

Breed MD, Bennett B (1987) Kin recognition in highly eusocial insects. In: Fletcher DJC and Michener CD (ed) Kin Recognition in Animals. J Wiley, New York pp 243-285

Breed MD, Stiller TM, Moor MJ (1988) The ontogeny of kin discrimination cues in the honey bee *Apis mellifera*. Behav Gen 18:439-448

Breed MD, Stiller TM, Torres A (1992) Guarding honey bees: role in nestmate recognition and replacement. Ann Entomol Soc Am 85:633-637

Breed MD, Guzman-Novoa E, Hunt GJ (2004a) Defensive behaviour of honey bees: Organization, genetics and comparisons with other bees. Annu Rev Entomol 49:271-298

Breed MD, Perry S, Bjostad LB (2004b) Testing the blank slate hypothesis: why honey bee colonies accept young bees. Insect. Soc 51:12-16

Butler CG (1959) Queen substance. Bee World 40:256-257

Butler CG (1960) The significance of queen substance in swarming and supersedure in honey-bee (*Apis mellifera L.*) colonies. Proc R Soc Lond A 35:129-132

Butler CG, Simpson J (1967) Pheromones of the queen honey bee (*Apis mellifera* L.) which enable her workers to follow her when swarming. Proc R Soc Lond A 42:149-154

Calis JNM, Boot WJ, Allsopp MH, Beekman M (2002) Getting more than a fair share: nutrition of worker larvae related to social parasitism in the Cape honey bee *Apis mellifera capensis*. Apidologie 33:193-202

Cooke MJ (1992) Turnabout is fair play – Cape bee invades African bee territory. Am Bee J 132:519–521

Crewe RM (1976) Aggressiveness of honeybees and their pheromone production. S Afr J Sci 72:209-212

Crewe RM (1981) Queens, false queens and *capensis*. S Afr Bee J 53(6):18-19, 21

Crewe RM (1982) Compositional variability:The key to the social signals produced by honeybee mandibular glands. In: Breed MD, Michener CD, Evans HE (eds) The Biology of Social Insects, Westview Press/Boulder, Colorado. pp 318–322

Crewe RM (1984) Differences in behaviour and morphology between *capensis* and *adansonii*. S Afr Bee J 56:16–21

Crewe RM (1988) Natural history of honey-bee mandibular gland secretions: development of analytical techniques and the emergence of complexity. In: Needham GR, Page RE, Definado-Baker M, Bowman CE (eds) Africanized honey bees and bee mites. Ellis Horwood Limited, England. pp 149–158

Crewe RM, Velthuis HHW (1980) False queens: a consequence of mandibular gland signals in worker honeybees. Naturwissenschaften 67:467-469

Crewe RM, Wossler T, Allsopp MH (1990) Workers in queens clothing: why *capensis*workers become pseudoqueens. In: Anderson RW, Buys B (eds) Bees and beekeeping in Southern Africa, Apimondia, University Press, Stellenbosch, pp 83–89

Downs G, Ratnieks FLW (1999) Recognition of conspecifics by honeybee guards uses nonheritable cues acquired in the adult stage. Animal Behaviour 58:643-648

Free JB (1987) Pheromones of social bees. Correll University Press, Ithaca, New York, USA.

Ferguson AW, Free JB (1980) Queen pheromone transfer within honeybee colonies. Physiol Entomol 5:539–366

Fuchs S, Moritz RF (1999) Evolution of extreme polyandry in the honeybee *Apis mellifera* L. Behav Ecol Sociobiol 45:269–275

Getz WM (1981) Genetically based kin recognition system. J of Theo Biol 92:209-226

Getz WM, Smith KB (1983) Genetic kin recognition: honey bees discrimination between full and half sisters. Nature 302:147-148

Gilley DC, Tarpy DR (2005) Three mechanisms of queen elimination in swarming honey bee colonies. Apidologie 36:461-474

Guzman-Novoa E, Page RE (1994) Genetic dominance and worker interactions affect honeybee colony defence. Behav Eco 5(1):91-97

Hays WL (1988) Statistics. Holt, Rinehart and Winston, Fort Worth

Hepburn HR, Crewe RM (1991) Defining the Cape honeybee: reproductive traits of queenless workers. S Afr J Sci 86:524–527

Hepburn HR, Allsopp MH (1994) Reproductive conflict between honeybees: usurpation of *Apis mellifera scutellata* colonies by *Apis mellifera capensis*. S Afr J Sci 90:247-249

Hepburn HR, Radloff SE (1998) Honeybees of Africa. Springer-Verlag, Berlin

Johannsmeier MF (1983) Experiencices with the cape bee in the Transvaal. S Afr Bee J 55:130-138

Johannsmeier MF, Swart DJ, Tribe GD, Kryger P (2001) Diseases and pests of honeybees. In: Johannsmeier MF (ed) Beekeeping in South Africa, 3rd edition, revised, Plant Protection Research Institute Handbook No. 14, Agricultural Research Council of South Africa, Pretoria, South Africa, pp 205–206

Kryger P (2001) An obligate social parasite in honey bees: the pseudo-clone of *Apis mellifera capensis*. Proc 13th Entomol Congr S Afr p 38

Lacy RC, Sherman PW (1983) Kin recognition by phenotype matching. American Naturalist 121:489-512

Lenoir A, D'Ettorre P, Errard C, Hefetz A (2001) Chemical ecology and social parasitism in ants Ann R Entomol 46:573-599

Lundie AE (1954) Laying worker bees produce worker bees. S Afr Bee J 29:10–11

Magnuson P (1995) The Cape honeybee problem – understanding honeybee biology offers possible solution. S Afr Bee J 67:134–136

Martin S, Wossler TC, Kryger P (2002a) Usurpation of *Apis millifera scutellata* colonies by *A. m. capensis* workers. Apidologie 33:215-232

Martin S, Beekman M, Wossler TC, Ratnieks FLW (2002b) Parasitic honeybee workers, *Apis mellifera capensis*, evade worker policing. Nature 415:163–165

Martin CG, Oldroyd BP, Beekman M (2004) Differential reproductive success among subfamilies in queenless honeybee (*Apis mellifera* L.) colonies. Behav Ecol Sociobiol 56:42–49

Moore AM, Breed MD, Moor MJ (1987) The guarding honey bee; ontogeny and behavioural variability of workers performing a specialized task. Anim Behav 35:1159-1167

Moritz RFA (2002) Population dynamics of the Cape bee phenomenon: The impact of parasitic laying worker clones in apiaries and natural populations. Apidologie 33:233–244

Moritz RFA, Crewe RM (1988) Chemical signals of queens in kin recognition in honeybees, *Apis mellifera* L. Journal of Comparative Physiology A 164:83-89

Moritz RFA, Southwick EE (1992) Bees as superorganisms. An evolutionary reality. Springer, Berlin Heidelberg, New York

Moritz RFA, Neumann P (2004) Differences in nestmate recognition for drone and workers in honeybee, (*Apis mellifera* L.). Anim Behav 67:681-688

Moritz RFA, Crewe RM, Hepburn HR (2001) Attraction and repellence of workers by the honeybee queen (*Apis mellifera* L.). Ethology 107:465-477

Moritz RFA, Simon UE, Crewe RM (2000) Pheromone contest between honeybee workers (*Apis*

mellifera capensis). Naturwissenschaften 87: 395-397

Naumann K, Winston ML, Slessor KN (1993) Movement of honey bee (*Apis mellifera* L.) queen mandibular gland pheromone in populous and unpopulous colonies. J Insect Behav 6:211–223

Neumann P, Hepburn HR (2002) Behavioural basis for social parasitism of Cape honeybees (*Apis mellifera capensis* Esch.). Apidologie 33:165-192

Neumann P, Moritz RFA (2002) The Cape honeybee phenomenon: the evolution of a social parasite in real time? Behav Ecol Sociobiol 52:271-281

Neumann P, Moritz RFA, Mautz D (2000) Colony evaluation is not affected by the drifting of drone and worker honeybees (*Apis mellifera* L.) at a performance testing apiary. Apidologie 31:67–79

Neumann P, Radloff SE, Moritz RFA, Hepburn HR, Reece SL (2001) Social parasitism by honeybee workers (*Apis mellifera capensis* Escholtz): host finding and resistance of hybrid colonies. Behav Ecol 12:419-428

Page RE, Robinson GE (1994) Reproductive competition in queenless honey bee colonies (*Apis mellifera* L.). Behav Ecol Sociobiol 35:99–107

Page RE, Robinson GE, Fondrk MK, Nasr ME (1995) Effects of worker genotype diversity on honey-bee colony development and behavior (Apis mellifera L). Behav Ecol Sociobiol 36(6):387–396

Palmer KA, Oldroyd BP (2000) Evolution of multiple mating in the genus *Apis.* Apidologie 31:235–248

Plettner E, Slessor KN, Winston ML, Robinson GE, Page RE (1993) Mandibular gland components and ovarian development as measures of caste differentiation in honeybee (*Apis mellifera L.*). Insect Physiol 39:235-240

Plettner E, Slessor KN, Winston ML, Oliver JE (1996) Caste-selective pheromone biosynthesis

in honeybees. Science 271:1851–1853

Plettner E, Otis GW, Wimalaratne PDC, Winston ML, Slessor KN, Pankiw T, Punchiheewa PWK (1997) Species- and caste-determined mandibular gland signals in honeybees (*Apis*). J Chem Ecol 23:363–377

Prince RC, Gunson DE (1992) Anarchie in the feminine monarchie: the case of the Cape bee. T R E E 7:398–399

Ratnieks FLW (1991) The evolution of genetic cue diversity in social Hymenoptera. American Naturalist 137:202-226

Ratnieks FLW, Visscher PK (1989) Worker policing in the honeybee. Nature 342:796-797

Robinson GE, Page RE, Fondrk MK (1990) Intracolonial behavioral variation in worker oviposition, oophagy, and larval care in queenless honey bee colonies. Behav Ecol Sociobiol 26:315–323

Swart JD, Johannsmeier MF, Tribe GD, Kryger P (2001) Diseases and pests of Honeybees. In: Johannsmeier MF (ed) Beekeeping in South Africa, 3rd edition revised, Plant Protection Research Institute Handbook No. 14, Agricultural Research Council of South Africa, Pretoria, South Africa. pp 198–222

Velthuis HHW, Ruttner F, Crewe RM (1990) Differentiation in reproductive physiology and behaviour during the development of laying worker honey bees. In: Engels W (ed) Social Insects, Springer, Berlin. pp 231–243

Winston ML (1987) The biology of the bee. Harvard University Press, Cambridge, MA

Wossler TC (2002) Pheromone mimicry by *Apis mellifera capensis* social parasites leads to reproductive anarchy in host *Apis mellifera scutellata* colonies. Apidologie 33:139–163

Woyke J (1995) Invasion of Capensis bee. In: Magnuson P (ed) Proceedings of the first International Electronic Conference on the Cape bee problem in South Africa. 5-30 June, Pretoria PPRI, 35

Zar JH (1999) Biostatistical analysis (3rd edition). Prentice Hall, Upper Saddle River, NJ

CHAPTER 3

HAS THE REPRODUCTIVE POTENTIAL OF PARASITIC *A. M. CAPENSIS* PSEUDOCLONES DIVERGED FROM THEIR NATIVE CAPE HONEYBEE WORKER COUNTERPARTS?

3.1 INTRODUCTION

The queen's primary purpose is to reproduce and her daughters, the workers, to tend to brood and other colony activities (Butler 1959). The removal of a queen bee from her hive has a profound effect on the colony dynamics. A queen must advertise her presence in the colony constantly to ensure normal colony activities. The queen honeybee secretes a complex blend of pheromones that affects the behaviour and physiological state of members of the worker caste (Free 1987; Seeley 1985; Winston 1987; Hoover et al. 2003). Many previous studies have revealed the composition of various queen compounds secreted by a number of glands, namely the Dufour's glands (Katzav-Gozansky et al. 1997), tergal glands (Wossler and Crewe 1999a) and mandibular glands (Velthuis 1970a; Crewe and Velthuis 1980; Crewe 1988; Slessor et al. 1988; Winston and Slessor 1992; Plettner et al. 1993, 1997; Keeling et al. 1999, 2003; Hoover et al. 2003) of which the mandibular gland secretions are instrumental in worker ovary regulation (Crewe and Velthuis 1980; Plettner et al. 1993; Keeling et al. 1999; Hoover et al. 2003). Her pheromones are distributed continuously throughout the colony via antennation, trophallaxis and allogrooming by the worker force (Butler 1967; Seeley 1979; Korst and Velthuis 1982; Naumann et al. 1993). We however still do not fully understand the nature and mode of action of queen signals on ovary development but her presence strongly influences worker reproduction.

Worker honeybee reproduction clearly depends on queen state (absence or presence) within the colony. Generally, colonies are queenright with queen pheromones suppressing the reproductive development of workers of their own honeybee subspecies. However in the absence of the queen, some worker honeybees are able to develop their ovaries and start producing more queen-like mandibular gland signals (Butler 1959; Butler and Fairey 1963; Velthuis 1970a, 1970b; Seeley 1985; Winston 1987, Slessor et al. 1988; Winston and Slessor 1992; Moritz and Southwick 1992; Plettner et al. 1993; Wossler and Crewe 1999a, Moritz et al. 2002; Hoover et al. 2003). Previous studies have revealed that Cape and other (*Apis mellifera*) honeybee workers are able to change their worker mandibular gland secretions from worker-like (10-hydroxy-2(*E*)-decenoic acid, 10-HDA) to queen-like (9-keto-2(*E*)-decenoic acid, 9-ODA) under queenless, and sometimes queenright, conditions (Butler et al. 1962; Crewe and Velthuis 1980; Hemmling et al. 1979; Crewe 1982; Hepburn 1992; Plettner et al. 1996; Moritz et al. 2000; Simon et al. 2001). Switching from a worker biosynthetic pathway to that of a queen's is relatively easy for Cape, *A. m. capensis,* honeybee workers who readily secrete queen-like mandibular gland secretions, dominated by 9-ODA (Ruttner et al. 1976; Hemmling et al. 1979; Crewe and Velthuis 1980; Plettner et al. 1993). Cape honeybee workers are not unique in producing 9-ODA since workers of other races can but what is distinctive, is that Cape honeybee workers produce greater proportions of 9-ODA compared to 10-HDA which is not the case for other workers. Moreover their signal development is rapid (Hepburn 1994; Simon et al. 2001; Wossler 2002) which automatically gives Cape honeybee workers a pheromone advantage over other races

possibly leading to a reproductive advantage. At the same time whilst their mandibular glands are developing so too do their ovaries and Cape honeybee workers have been shown to have an extremely short latency period of 4–6 days between queen loss and oviposition by workers (Velthuis 1970a; Ruttner and Hesse 1981; Velthuis et al. 1990). Not only do Cape honeybee workers develop their mandibular glands faster than African workers but their tergal glands are also better developed than African workers appearing more queen-like which are thought to aid in their pseudoqueen status (Wossler 2002; Wossler and Crewe 1999a, 1999b; Wossler et al. 2000).

Ever since the *'capensis calamity'* (see chapter 1 and Allsopp 1993) it has become clear that Cape honeybee workers develop fully functional ovaries (Ruttner and Hesse 1981) as well as a more queen-like mandibular gland secretion even in the presence of the African queen (Allsopp and Crewe 1993; Hepburn 1994; Hepburn and Allsopp 1994; Simon et al. 2001; Moritz 2002; Moritz et al. 2002; Martin et al. 2002a, 2002b; Neumann and Hepburn 2002). On the whole, it seems that African queen regulation of ovary activation in Cape honeybee workers is not effective (Dietemann et al. 2006). This phenomenon is not new, with African colonies having previously been lost to invasions by Cape workers not belonging to the current social parasitic population (Lundie 1954; Guy 1976; Johannsmeier 1983). This clearly indicates that the pseudoclone's characteristics are common to the Cape honeybee workers as well, but some may be more advanced in the pseudoclone, complementing the parasitic lifestyle (see chapter 1 for explanation on the origin of the parasitic worker lineage).

This chapter takes a closer look at the physiological changes, namely pheromone development (production of queenlike mandibular gland sectretions) and ovary development within the 21-day old Cape honeybee workers harvested during the previous experiment (see chapter 2). Splitting the colonies into queenright and queenless halves made it possible to test how both Cape honeybee populations; those native to the Western Cape compared to the established pseudoclones from the Highveld, behave under such host African queenright and queenless conditions. The previous chapter demonstrated African colony vulnerability to invasion during the period surrounding colony fission. In this chapter we investigate whether worker reproductive development is also strongly associated with host colony state and whether these two populations differ with respect to each other.

Pectora roborant cultus recti

3.2 MATERIALS AND METHODS

In chapter 2 the survival rate of two separate Cape honeybee worker populations during three stages of infection surrounding colony fission were observed. In this chapter ovary development and mandibular gland profiles were analysed from the remaining 21-day old Cape honeybee workers which were collected from the experiment mentioned in the previous chapter. The experimental design for this is thus exactly the same as in chapter 2 and due to the thesis format, parts of the methods are repeated.

3.2.1 Experimental host colony setup

In this experiment I used conditions similar to those used by commercial beekeepers when they intend to increase their number of colonies. Nine unrelated and non parasitized African honeybee colonies were obtained from a commercial beekeeper from Douglas who was recognized for having no Cape worker laying activity in his *A. m. scutellata* colonies. These host African colonies were split into queenright (swarm) and queenless (post-swarm) halves (n = 18, see Section 3.2.3) that were infected by two *A. m. capensis* honeybee worker populations (native Cape and pseudoclones) at various stages surrounding the splitting or swarming process (see Section 3.2.2 and chapter 2 Figure 2.1). The approximate number of *A. m. scutellata* workers was approximately 10 000 in each colony (Allsopp personal communication). Queenless split colonies (post swarm) remained in the original 10 frame hives, whereas the queenright colonies (swarm) were moved into new 10 frame hives (see chapter 2 Figure 2.1). Unfortunately, one of the queenright colonies lost its queen and was therefore excluded from the experiment (see chapter 2 Table 2.2). Open and sealed African brood as well as the food were equally shared in each of the splits. Colonies were housed at two separate apiaries approximately 3km apart. Queenright (swarm) colonies were housed at the Western Cape Agricultural experimental farm Kromme Rhee, and the queenless (post swarm) colonies were located at the University of Stellenbosch's experimental farm Mariendahl, this helped prevent drifting.

3.2.2 Introduced bees

Fifty 1-day old native Cape honeybee workers (obtained from the Plant Protection Research Institute, Stellenbosch) and fifty 1-day old pseudoclones (obtained from a commercial beekeeper, Pretoria) were introduced into each split colony at three specific infection periods surrounding the splitting stage (see Section 3.2.3). All introduced bees were uniquely marked and if in the event of drifting (as all bees were free-flying) they would be easily identified. Both *A. m. capensis* honeybee workers, native Cape and pseudoclones, were collected from sealed brood frames that were incubated separately at 32°C with 60% relative humidity overnight. Emerging 1-day old native Cape and pseudoclone honeybee workers were colour marked on their thoraces with non-toxic coloured paint for easy identification. All introduced workers were thus of the same age and contained a clean or blank slate (Breed et al. 2004) giving them an equal chance of surviving.

Pectora roborant cultus recti

3.2.3 Treatment periods

The three treatments; before, during and after the splitting of African, *A. m. scutellata* colonies were used to identify a 'window of opportunity' for successful invasion (see chapter 2 Figure 2.1). 1-day old native Cape and pseudoclone honeybee workers were introduced three days prior to splitting the hives (Treatment 1). On splitting, marked bees were equalised between the two splits. Native Cape and pseudoclone honeybee workers were introduced during the splitting process (Treatment 2). Finally the hives were initially split and only three days later infected with both the native Cape and

pseudoclone honeybee workers (Treatment 3). These three treatments investigate the three most likely infection periods surrounding the splitting period.

3.2.4 Sampling of bees

The surviving 21-day old colour marked Cape honeybee workers from both populations were collected, their heads individually stored in glass vials containing 100µl dichloromethane (DCM; Merck, Uvasol) for mandibular gland analysis. Their bodies were also individually frozen to preserve their ovaries for ovary development analysis.

3.2.5 Gas chromatography

The heads were removed from the DCM which was evaporated under a flow of nitrogen gas till dryness. The residue was redissolved in 15µl IS (internal standard; about 1 mg of each octanoic acid and tetradecane in 4 ml dichloromethane, purchased from Sigma) and 15µl BSTFA (bistrimethylsilyl trifluoroacet-amide, Sigma) was added to the sample. 20µl of the redissolved IS and BSTFA solution was placed in a vial and 1 µl of this solution was injected into a Hewlett Packard gas chromatograph (HP 6850) fitted with a split–splitless inlet and a 25mm×0.32mm methyl silicone coated fused silica capillary column. Helium was used as the carrier gas with a flow rate of 1.4 ml/min. The oven temperature was automated as follows; 60°C for 1 min, ramped at 50°C/min to 110°C, then 3°C/min from 110°C to 220°C and held at 220°C for 10 min.

Peak areas were determined using HP Chemstation software and the mandibular gland compounds were identified based on retention times relative to authentic standard compounds (Sigma, Wossler and Crewe 1999b). The following eight mandibular gland

components were quantified due to their consistency in the various test groups; 9-keto-2(E)-decenoic acid (9-ODA), 9 hydroxydecanoic acid (9-HDAA), 9-hydroxy-2(E)-decenoic acid (9-HDA), the so-called queen pheromones,10 hydroxydecanoic acid (10-HDAA), 10-hydroxy-2(E)-decenoic acid (10-HDA), the so-called worker pheromones, palmitic acid (PALM), palmitoleic acid (PAL) and finally stearic acid (STEARIC), the latter which is an important precursor to queen and worker pheromones (Plettner et al. 1995). The proportion of each individual compound was calculated as the ratio of that compound in relation to the other 7 compounds in the mandibular gland extract. A standard mandibular gland solution containing; 9-ODA, 9-HDA, 10-HDAA, 10-HDA, PALM and PAL compounds were run every third day to ensure that the retention times did not drift.

3.2.6 Mandibular gland analysis

Multivariate analyses were performed using PRIMER 5 (Plymouth Routines in Multivariate Ecological Research) developed by the Plymouth Marine Laboratory, UK. The composite signals (incorporating the eight compounds mentioned above) were transformed using double root transformation to give an equal weighting to compounds with high or low representation in the profiles. The similarity matrix was calculated using Bray-Curtis coefficients (Bray and Curtis 1957). MDS (multi-dimensional-scaling plots) was used to provide visual representations which have inter-point distances that match the rank order of dissimilarities among the samples within the matrix (Kruskal and Wish 1978). MDS was rerun and matched three times to ensure that the stress value was at the global minimum. Deviations from these matches are expressed in terms of stress, with

stress values < 0.15 indicating good MDS plots (Clarke and Warwick 2001). ANOSIM (analysis of similarity) was used to test for differences in mandibular gland profiles between the two Cape honeybee worker populations; native Cape and pseudoclones in African queenright and queenless colonies (Clarke and Green 1988). FDR (false discovery rate) were used where necessary to correct for any type 1 error across numerous pair wise analysis (Garcia 2004). Further analyses were conducted using Chi square tests (χ^2) with Yates's correction to assess specific effects between and across related conditions (Zar 1999).

3.2.7 Ovary analysis

The ovaries of both Cape honeybee worker populations; native Cape and pseudoclone were dissected under a dissecting microscope and scored in two different categories. 1 = undeveloped (ovaries with no oocytes); 2 = fully developed (ovaries with distinct ova). This scoring procedure applied criteria similar to those used by Velthuis (1970b). Multivariate analysis tests compared ovary development to the mandibular gland profiles. Chi square tests compared ovary development between native Cape and pesudoclone workers for all treatments and across both African queenright and queenless conditions.

3.3 RESULTS

In this study two important physiological factors were examined for both the native Cape and pseudoclone honeybee workers; firstly their ovary development and secondly their mandibular gland signals. To reiterate, no native Cape or pseudoclone honeybee workers introduced on the day of splitting (treatment 2) were accepted by the African queenright

half colonies (see Table 2.2 chapter 2 for survival results). Consequently, ovary and mandibular gland signal comparative analysis can only be compared across treatment 1 and treatment 3 for the African queenright colonies but can be compared across all three treatments for the African queenless colonies (see Appendix Table III).

Firstly, levels of ovary development (developed vs. non-developed) were compared between African queenright and queenless colonies. For all colonies (n = 9, see chapter 2 Table 2.1 for those colonies in which workers survived 21 days), irrespective of treatment, significantly more *A. m. capensis* workers (of both populations) developed fully activated ovaries in queenless host African colonies compared to queenright colonies. Focusing only on the native Cape honeybee workers, significantly more developed fully functional ovaries in African queenless halves (78.4%) compared to queenright halves (13.3%; χ^2 with Yates's correction = 140.9, $df = 1$, $P < 0.001$). This however is not true for the pseudoclones, with all retrieved pseudoclones fully developing their ovaries in both queenright and queenless colonies (see table 3.1). Since all 21-day old retrieved pseudoclones possessed fully developed ovaries across all treatments in both African queenright and queenless conditions (Table 3.1), it suggests that the timing of infection (treatment effect) has no subsequent effect on ovary development in pseudoclones. Native Cape honeybees on the other hand, showed a great variation in their ability to develop activated ovaries across treatments (Table 3.1). In African queenright colonies 8-17% native Cape workers developed their ovaries while in queenless colonies most workers (over 90%) developed their ovaries except for treatment 1 where only 40% showed ovary development.

Even though some of the sample sizes for the pseudoclones retrieved under different treatments are not high (see Table 3.1 for sample sizes), these results indicate a 100% success rate in ovary development by pseudoclones compared to their native relatives (Table 3.1). To recap, significantly more pseudoclone honeybee workers develop activated ovaries compared to native Cape in queenright colonies for both treatment 1 (χ^2 with Yates's correction = 134.7, df = 1, $P < 0.001$) and treatment 3 (χ^2 with Yates's correction = 161.6, df = 1, $P < 0.001$). Pseudoclone honeybees also develop their ovaries significantly more compared to native Cape workers for treatment 1 in queenless colonies (χ^2 with Yates's correction = 81.3, df = 1, $P < 0.001$). Interestingly however, both populations develop equally in queenless colonies for both treatment 2 (χ^2 with Yates's correction = 0.96 df = 1, $P > 0.05$) and 3 (χ^2 with Yates's correction = 0.00, df = 1, $P > 0.05$; see Table 3.1). This suggests, be it only tentatively, that pseudoclones have a competitive reproductive advantage over native Cape workers in queenright host colonies, as well as queenless host colonies for treatment 1.

Turning now to the Cape honeybee workers ability to produce a more queenlike mandubular gland secretion profile (with 9ODA contributing more than 40% overall), the presence of the queen has a significant effect on the mandibular gland secretion for all workers (ANOSIM Global R = 0.473, $P < 0.001$, permutations = 999, random sample from total possible number of permutations; n = 64 queenright workers and n = 149 queenless workers; see Appendix Table III for ovary and signal development for each 21-day old *A. m. capensis* retrieved Table 3.1 worker). Mandibular gland profiles for both native Cape and pseudoclone honeybee workers collected from African queenless

colonies are more queen-like than worker-like (see Table 3.1; note the high 9-ODA values, Crewe and Velthuis 1980). The mandibular gland secretions for all harvested workers largely diverge with respect to queen state in African colonies (Figure 3.1).

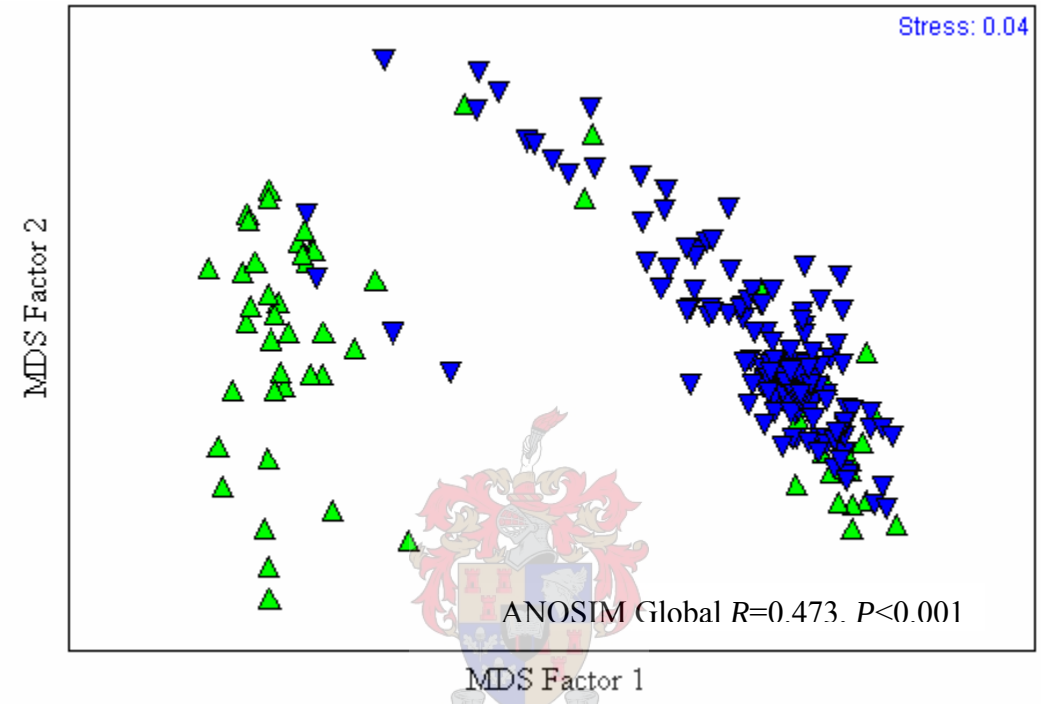


Figure 3.1 Multidimensional scaling plot (MDS) for the mandibular gland secretion profiles of the eight major compounds known to have some biological activity, showing the effect of queen state on the production of queenlike mandibular gland components within the Cape and pseudoclone honeybee workers. A clear queen effect on mandibular gland development for both *A. m. capensis* populations from queenright (▲) and queenless (▼) colonies is evident. Mandibular gland secretions from individuals retrieved from queenright colonies that cluster with those individuals retrieved from queenless colonies were for queenright pseudoclones that produced queen-like signals (see Table 3.1 and Figure 3.2; see Clarke and Warwick 2001 for MDS methods)

However there is some overlap between signals produced in queenright and queenless colonies. On further inspection though (analysing the two population's separately) pseudoclone honeybees retrieved from African queenright colonies possessed mandibular gland profiles which are similar to, and overlap those from queenless samples (Figure

3.1). Pseudoclone workers from both queenright and queenless conditions had signals dominated by 9-ODA (see Table 3.1 for compound proportions).

From this it appears that the presence of an African queen honeybee does not affect the production of queen-like mandibular gland secretions by pseudoclones (Figure 3.1, 3.2 and see Table 3.1). On the other hand, native Cape honeybee workers produced mandibular gland secretions that differed significantly between African queenright and queenless colonies (ANOSIM pair wise $R = 0.661$, $P < 0.001$, permutations = 999, random sample from total possible number of permutations; see Figure 3.2), producing a much more queen-like mandibular gland secretion when retrieved from African queenless colonies (see Appendix Table III). What's more, queenless pseudoclones expressed mandibular gland profiles that differed from queenless native Cape workers (ANOSIM pair wise $R = 0.272$, $P < 0.001$, permutations = 999, random sample from total possible number of permutations; also see Figure 3.2 and Table 3.2). This difference is probably due to the large variability in signal production by individual native Cape workers which is not evident for pseudoclone signals, where there is little variation in their mandibular signals (see tight clustering of pseudoclones on Figure 3.2, also see Appendix Table III for individual profiles).

Table 3.1 Mean proportions (+ standard deviation) of the eight major mandibular gland secreted compounds, from native Cape worker honeybees (Cape) and pseudoclone worker honeybees (Clones) under queenright and queenless conditions collected (day 21) from the various treatment periods (T). Ovary data are presented as percentage of bees with fully developed ovaries (%)

Queen state	Queenright				Queenless					
Treatment	T 1		T 3		T 1		T 2		T 3	
Bee population	Cape	Clone	Cape	Clone	Cape	Clone	Cape	Clone	Cape	Clone
(n)	(n=12)	(n=19)	(n=30)	(n=3)	(n=5)	(n=51)	(n=28)	(n=43)	(n=19)	(n=2)
Mandibular extracts										
9-ODA	14.7±26.8	57.7±11.4	1.78±6.75	56.9±4.08	10.2±16.5	52.9±9.47	31.5±18.9	54.5±7.63	42.4±15.5	59.4±11.7
9-HDAA	NT	NT	0.04±0.27	NT	NT	NT	NT	NT	NT	NT
9-HDA	4.72±2.48	10.1±3.43	8.90±6.55	5.37±0.40	5.17±4.01	7.70±2.85	4.90±3.82	8.29±2.28	4.33±2.29	9.52±5.12
10-HDAA	0.84±1.87	NT	4.99±7.00	NT	NT	NT	NT	NT	NT	NT
10-HDA	0.10±0.36	0.20±0.24	0.27±0.61	NT	NT	0.01±0.05	0.02±0.09	0.06±0.13	0.11±0.22	0.41±0.23
PAL	2.66±2.45	3.62±1.75	2.31±0.61	4.24±0.48	7.50±8.35	5.37±1.81	7.94±5.13	5.11±1.42	9.62±2.72	4.38±1.33
PALM	18.9±5.40	10.5±4.45	20.2±3.38	14.1±2.16	22.6±3.26	12.6±3.26	17.3±3.59	12.7±3.13	16.2±3.74	12.0±2.93
STEARIC	57.9±23.0	17.7±7.21	61.4±11.6	19.2±2.77	54.3±19.6	21.3±6.32	24.8±8.62	16.4±3.67	27.2±9.88	14.2±2.62
Ovary development										
% fully developed	17	100	8	100	40	100	98	100	100	100

9-ODA = 9-keto-2(*E*)-decanoic acid; 9-HDAA = 9 hydroxydecanoic acid; 9-HDA = 9-hydroxy-2(*E*)-decenoic acid; 10-HDAA = 10 hydroxydecanoic acid; 10-HDA = 10-hydroxy-2(*E*)-decenoic acid; PAL = Palmitoleic acid; PALM = Palmitic acid; STEARIC = Stearic acid. Compounds not detected are denoted by 'NT'

Table 3.2 Mandibular gland comparisons for both *A. m. capensis* honeybee worker populations (pseudoclones = Clone and native Cape = Cape) across the specific treatments (1, 2 or 3) between African queenright and queenless colonies. Differences between populations within a treatment were evaluated with ANOSIM. All significant interactions are denoted with "*" where $P < 0.05$. 9-ODA contributes >50% proportion to the mandibular gland secretions of pseudoclones, for all treatment levels

Queen status	Treatment	Bee population	Pair-wise statistic (*R*)	*P* value
Queenright	1	Clone > Cape	0.727	0.001*
	3	Clone > Cape	0.983	0.001*
Queenless	1	Clone > Cape	0.880	0.001*
	2	Clone > Cape	0.606	0.001*
	3	Clone > Cape	0.184	0.004*

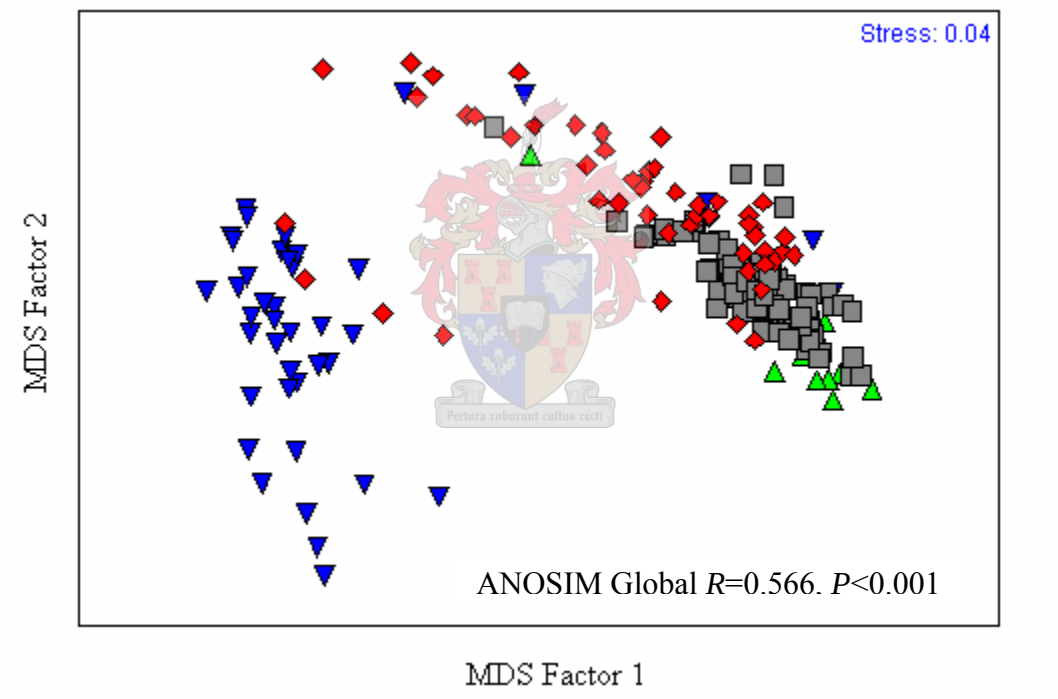


Figure 3.2 MDS plot of mandibular gland profiles for native Cape (▼) and pseudoclone (▲) honeybee workers collected from African queenright colonies as well as native Cape (◆) and pseudoclone (■) honeybee workers that were retrieved from African queenless colonies. The clustered groups to the right contained significantly higher proportions of 9-ODA than the group to the left (see Table 3.1; see Clarke and Warwick 2001 for MDS methods)

Thus far I have focused on the differences between the two populations with respect to queen state and treatments (Table 3.2) but how different are the signals produced by the

pseudoclones, as well as native Cape workers, across treatments? There were no differences in mandibular gland secretions for pseudoclones and Cape native workers collected from African queenright colonies (Table 3.3). This was also true for pseudoclones that were collected from African queenless colonies (Table 3.3). Differences were however evident within the native Cape workers, with more queen-like signals being produced by native Cape honeybee workers that were introduced during (treatment 2) and after (treatment 3) colony splitting compared to those which were introduced before colony splitting (treatment 1; Table 3.3 and see Appendix Table III for individual ovary status and 9-ODA values). Again, these data suggest that the timing of infection (treatment effect) has no subsequent effect on signal development in pseudoclones. Pseudoclone workers that survive in queenright and queenless colonies, irrespective of treatment, produce more queen-like signals than their native honeybee worker relatives (Tables 3.1, 3.2 and 3.3; Figure 3.2 and see Appendix Table III).

Table 3.3 Mandibular gland comparisons across treatments for each specific *A. m. capensis* population for both African queenright and queenless colonies. Differences between treatments for each population were evaluated with ANOSIM. FDR (false discovery rate) analysis indicated no borderline deviance where $P < 0.05$. All significant interactions are denoted with "*". Workers from those treatments containing a significantly higher proportion of 9-ODA are denoted by "<", most however show no treatment effect

Queen status	Bee population	Treatment	Pair-wise statistic (*R*)	*P* value
Queenright	Cape	1 = 3	0.088	0.152
	Clone	1 = 3	0.202	0.121
Queenless	Cape	1 < 2	0.333	0.026*
		2 = 3	0.024	0.364
		1 < 3	0.449	0.001*
	Clone	1 = 2	-0.035	0.757
		2 = 3	0.047	0.056
		1 = 3	0.005	0.457

Finally, do mandibular gland secretions and ovary development occur concomitantly? There's largely a relationship between ovary and mandibular gland secretion development with most *A. m. capensis* workers from both populations developing queen-like mandibular gland secretions concurrently with ovary development (Figure 3.3). This link however is not always predictable.

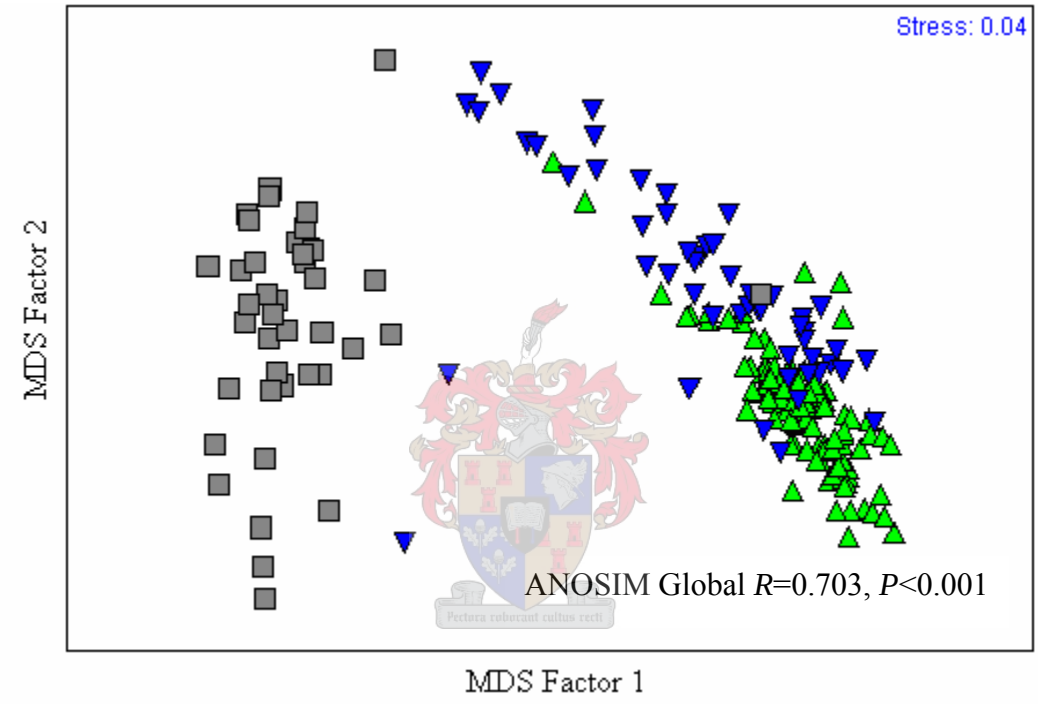


Figure 3.3 The relationship between ovary development and mandibular gland secretion development within the two *A. m. capensis* honeybee populations reveal pseudoclones (▲) and native Cape (▼) workers with developed ovaries secreted largely queen-like mandibular gland signals and native Cape (■) workers which possessed non-developed ovaries, secreted mostly worker-like signals. All pseudoclones developed their ovaries (see Clarke and Warwick 2001 for MDS methods)

There were some *A. m. capensis* individual honeybee workers which showed some degree of variation between ovary development and the production of queen-like mandibular gland secretions, this discrepancy in ovary and signal development was more

Table 3.4 The relationship between ovary development and 9-ODA production, for native Cape worker honeybees (Cape) and pseudoclone worker honeybees (Clones) under queenright and queenless conditions across the various treatment periods (T). Results taken from Appendix Table III

Ovary and 9-ODA development	Queenright				Queenless					
	T 1		T 3		T 1		T 2		T 3	
	Cape (n=12)	Clone (n=19)	Cape (n=30)	Clone (n=3)	Cape (n=5)	Clone (n=51)	Cape (n=28)	Clone (n=43)	Cape (n=19)	Clone (n=2)
Developed ovaries and high 9-ODA (> 40 %) quantity	2	18	0	3	1	50	15	43	13	2
Undeveloped ovaries and high 9-ODA (> 40 %) quantity	1	0	0	0	0	0	0	0	0	0
Developed ovaries and low 9-ODA (< 40 %) quantity	0	1	3	0	1	1	12	0	6	0
Undeveloped ovaries and low 9-ODA (< 40 %) quantity	9	0	27	0	3	0	1	0	0	0

pronounced for the native Cape workers (Table 3.4 and see Appendix Table III for individual ovary and mandibular gland development).

3.4 DISCUSSION

It has been suggested that queenless Cape honeybee workers appear more queen-like than worker-like on the worker-queen continuum across all *Apis mellifera* honeybee subspecies (Moritz et al. 2000; Martin et al. 2002b). These data in this study support this observation and clearly shows that once Cape workers from both populations successfully gain access into African colonies they posses the ability to develop both their ovaries as well as considerably high 9-ODA mandibular gland secretions, in accordance with a number of previous studies (Ruttner et al. 1976; Hemmling et al. 1979; Crewe and Velthuis 1980; Saiovici 1983; Crewe 1984, 1988; Free 1987; Velthuis and van der Kerk 1988; Crewe et al. 1990; Hepburn 1992). In this study I found that Cape honeybee workers are able to become reproductively dominant in both African queenright and queenless colonies but more so when the African queen is absent. This phenomenon is well documented for queenless conditions (Hepburn et al. 1988; Velthuis et al. 1990) and for queenright conditions (Crewe and Velthuis 1980; Velthuis et al. 1990; Hepburn 1992). Hepburn et al. (1991) demonstrated that mated Cape queens inhibit ovary development with only approximately 1% of the workers showing activated ovaries, while colonies headed by virgin queens have 2-5% of the workers reproductively active. These data collected here indicated that mated African queens also inhibit worker development (8-17% developed) but not as successfully as mated Cape queens. This is

due to Cape queens having extremely high concentrations of 9-ODA which allow the Cape queen to regulate Cape worker reproduction (Crewe 1982, 1988).

What is most interesting from these data collected in this experiment is that it is obvious that the established Highveld pseudoclones differ physiologically (and express more queen-like characteristics) when compared to their native Cape worker relatives placed under similar treatments for both African queenright or queenless conditions. It would seem the African queen's presence has little effect on inhibiting the reproductive development of these pseudoclones but, as previously mentioned, does to some extent inhibit native Cape workers. Besides the fact that the African queen does not regulate the reproductive development of the pseudoclones, the timing of their introduction, before, during or after African colony splitting, also did not affect their capacity to develop their ovaries and queen-like signals. In chapter 2 I found that treatment may be responsible for limiting the success of infiltration but (as indicated in this chapter) has no effect on the reproductive development of pseudoclones. The challenge seems to be getting into the colonies and avoiding detection (see chapter 2) but once that barrier has been crossed, reproductive development continues unhindered.

In contrast to the pseudoclones, the presence of the African queen has an effect on reproductive development in the native Cape workers. Not only was this evident in the queenright colonies but also in the queenless colonies where workers were functionally queenright for three days before splitting (treatment 1), with only 40% developing their ovaries. These data would suggest that the presence of the African queen even if only for

a few days prior to swarming seems to certainly influence the native Cape workers' potential to develop their ovaries and produce queen-like mandibular gland secretions. It has been shown that younger workers have a greater chance of developing their ovaries than older bees (Delaplane and Harbo 1987) and since these workers were older on queen loss, may have had a reduced capacity for reproductive development. This however was not apparent for the pseudoclones who spent three days in the queen's presence before splitting, with 100% developing their ovaries.

The absence of the African queen seems to allow for a rapid reproductive acceleration in the native Cape honeybee workers. The fact that Cape workers are capable of doing this is not new (Crewe and Velthuis 1980), but what is interesting, is that pseudoclones develop greater concentrations of the queen-like compound 9-ODA and all managed to develop their ovaries which was not the case for the native Cape honeybee workers under similar African queenright and queenless conditions. On a developmental pheromone continuum, the biosynthetic capabilities of the mandibular glands begins with mated queens, followed by virgin queens together with pseudoclone pseudoqueens, native Cape pseudoqueens, pseudoqueens of other races, then laying workers, dominant workers and finally subordinate workers (see Plettner et al. 1993).

Reproductively dominant individuals frequently produce 9-ODA which seems to precede ovary activation (Crewe and Velthuis 1980, Sasaki et al. 1989), however this relationship between signal development and ovary activation is inconsistent with some workers with activated ovaries secreting worker-like signals (Hemmling et al. 1979; Hepburn et al.

1988; Hepburn and Allsopp 1994). More often than not however, a positive relationship between pheromone bouquet and ovary development is evident in Cape workers competing for reproduction (Crewe and Velthuis 1980; Moritz and Hillesheim 1985, Crewe 1987; Allsopp 1988; Velthuis and van der Kerk 1988; Velthuis et al. 1990; Hepburn 1992). The majority of the workers in this experiment, with the exception of a few native Cape workers, did show a positive link between ovary activation and the production of a more queen-like mandibular bouquet, with a high ratio of queen substance to worker substance evident. It has been shown that the first differentiation in ovary development coincides with the production of 8-hydroxyoctanoic acid (8-HOA) and 9-HDA with further ovary development linked to 9-ODA (Velthuis and van der Kerk 1988), but this is not a strict rule (Hemmling et al. 1979; Saiovici 1983; Velthuis 1985; Hepburn et al. 1988). These data do show an increase in 9-HDA and 9-ODA in the majority of the developed workers but we did not detect appreciable quantities of 8-HOA. I would argue that pheromone production and ovary development are genetically independent traits which can give rise to various combinations of ovary development and mandibular gland pheromone production (Hepburn 1992), with these two traits simultaneously and more frequently co-induced in Cape workers, especially in the pseudoclones, than other honeybee workers.

Noteworthy is the variability in mandibular gland secretions of the native Cape workers which is not as pronounced in the pseudoclone signals. This variability in reproductive dominance (in terms of ovary and signal development) is a byproduct of the polyandrous nature of honeybee queens giving rise to variability among subfamilies (Fuchs and

Moritz 1999; Palmer and Oldroyd 2000). Recently this reproductive dominance by way of ovary and signal development as a function of patrilines has been shown to occur (Simon et al. 2005). This is a consequence of the genetic variability introduced by polyandry, resulting in certain patrilines being predisposed to reproductive dominance, producing very queen-like signals while other patrilines are not genetically predisposed to reproductive dominance (Moritz and Hillesheim 1985; Robinson et al. 1990; Page and Robinson 1994; Moritz et al. 1996, 2000; Simon et al. 2001). Previously it has been shown that only a few *A. m. capensis* workers establish themselves as dominant laying workers (Moritz et al. 1996; Simon et al. 2005) and this is supported by these data with not all Cape laying workers having the potential to develop into laying workers. It would appear some native Cape workers are better suited to a parasitic life style than others. The pseudoclones however, which are near identical genetically (Kryger 2001a, 2001b), express a suite of traits conferring a reproductive advantage in relation to their native Cape sisters and represents a single worker lineage despite the initial introduction of millions of Cape workers in 1990 (see chapter 1 for explanation on the origin of the parasitic worker lineage).

Pectora roborant cultus recti

These data strengthens the suggestion that the pseudoclone honeybee workers do differ from their native Cape honeybee workers with respect to their reproductive potential on a reproductive developmental continuum. Since all but two of the retrieved pseudoclones developed queen-like pheromones in conjunction with ovary development, irrespective of treatment, while numerous native Cape workers failed this feat, I conclude that these two

Cape honeybee worker populations do differ significantly regarding their reproductive capacity and ability in becoming social parasites.

3.5 REFERENCES

Allsopp MH (1993) Summerized overview of the capensis problem. S Afr Bee J 65:127-136

Allsopp MH (1988) Mandibular gland acids and laying workers in African honey bees, in: Needham G.R., Page R.E., Definado-Baker M, Bowman CE (eds) Africanized honey bees and bee mites. Ellis Horwood Limited, England pp 72–79

Allsopp MH, Crewe R (1993) The Cape honeybee as a Trojan horse rather than the hordes of Jenghiz Khan. Am Bee J 133:121-123

Breed MD, Perry S, Bjostad LB (2004) Testing the blank slate hypothesis: why honey bee colonies accept young bees. Insect Soc 51:12-16

Butler CG (1959) Queen substance. Bee World 40:256-257

Butler CG (1967) Insect pheromones. Biol Rev 42:42-87

Butler CG, Fairey EM (1963) The role of the queen in preventing oogenesis in worker honey bees. J Apic Res 2:14-18

Butler CG, Callow RK, Johnston FRS, Johnston NC (1962) The isolation and synthesis of queen substance, 9-oxodec-trans-2-enoic acid, a honeybee pheromone. Proc R Soc Lond 155:417-432

Bray JR, Curtis JT (1957) An ordination ao the upland forest communities of Southern Wisconsin. Ecol Monogr 27:325-349

Clarke KR, Green RH (1988) Statistical design and analysis for a ‘biological effects’

study. Mar Ecol Prog Ser 46:213-226

Clarke KR, Warwick RM (2001) Change in marine communities: an approach to statistical analysis and interpretation, 2nd edition. PRIMER-E, Plymouth

Crewe RM (1982) Compositional variability: the key to the social signals produced by honeybee mandibular glands. In: Breed MD, Michener CD, Evans HE (eds) The biology of social insects. Proceedings of the 9th congress of the International Union for the Study of Social Insects, Boulder, Colorado, pp 318-322

Crewe RM (1984) Differences in behaviour and morphology between *capensis* and *adansonii*. S Afr Bee J 56:16–21

Crewe RM (1987) Lability of the mandibular gland signal of three races of African honey bees, in: Eder J, Rembold H (eds) Chemistry and Biology of Social Insects. Verlag J Peperny, Munich pp 433–434

Crewe RM (1988) Natural history of honey-bee mandibular gland secretions: development of analytical techniques and the emergence of complexity. In: Needham GR, Page RE, Definado-Baker M, Bowman CE (eds) Africanized honey bees and bee mites. Ellis Horwood Limited, England. pp 149–158

Crewe RM, Velthuis HHW (1980) False queens: a consequence of mandibular gland signals in worker honeybees. Naturwissenschaften 67:467-469

Delaplane KS, Harbo JR (1987) Drone production by young versus old worker honeybees in queenless colonies. Apidologie 18:115–120

Free JB (1987) Pheromones of social bees. Chapman and Hall, London

Fuchs S, Moritz RF (1999) Evolution of extreme polyandry in the honeybee *Apis mellifera* L. Behav Ecol Sociobiol 45:269–275

Garcia LV (2004) Escaping the boneferroni iron claw in ecological studies. Oikos 105:657-663

Guy RD (1976) Whence the Cape bee? S Afr Bee J 48:7–8

Hemmling C, Koeniger K, Ruttner F (1979) Quantitative Bestimmung der 9-oxodecensäure im Lebenszyklus der Kapbiene (*Apis mellifera capensis* Escholtz). Apidologie 10:227–240

Hepburn HR (1992) Pheromonal and ovarial development covary in Cape worker honeybees, *Apis mellifera capensis*. Naturwissenschaften 79:523-524

Hepburn HR, Allsopp MH (1994) Reproductive conflict between honeybees: usurpation of *Apis mellifera scutellata* colonies by *Apis mellifera capensis*. S Afr J Sci 90:247–249

Hepburn HR, Nefdt RJC, Whiffler LA (1988) Queen loss in the Cape honeybee: the interactions of brood, laying workers (false queens?) and queen cells. S Afr J Sci 84:778-780

Hepburn HR, Magnuson, Herbert PL,Whiffler LA (1991) The development of laying workers in field colonies of the Cape honey bee. J Apic Res 30:107–112

Hoover SER, Keeling CI, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90:477-480

Johannsmeier MF (1983) Experiences with the cape bee in the Transvaal. S Afr Bee J 55:130-138

Katzav-Gozansky T, Soroker V, Hefetz A, Cojocaru M, Erdmann DH, Francke W (1997)

Plasticity of caste-specific Dufour's gland secretion in the honey bee (*Apis mellifera* L.). Naturwissenschaften 84:238–241

Keeling CI, Slessor KN, Winston ML (1999) The essence of royalty, the honey bee queen's pheromone arsenal. In: Proc. XXXVI Apimondia Congr. Vancouver, British Columbia, Canada p 309

Keeling CI, Slessor KN, Higo HA, Winston ML (2003) New components of the honey bee (*Apis mellifera L.*) queen retinue pheromone. Proc Natl Acad Sci 100:14486-14491

Korst PJAM, Velthuis HHW (1982) The nature of trophallaxis in honeybees. Insect Soc 29:209–221

Kruskal JB, Wish M (1978) Multidimensional scaling. Sage Publications, Beverley Hills, California

Kryger P (2001a) The Capensis pseudo-clone, a social parasite of African honey bees. In: Proceedings of the 2001 Berlin Meeting of the European Section of IUSSI, 25-29 September p 208

Kryger P (2001b) The pseudo-clone of *Apis mellifera capensis* - an obligate social parasite in honeybees. In: Proceedings of the XXXVII International Apicultural Congress, Durban South Africa p 33

Lundie A (1954) Laying worker bees produce worker bees. S Afr Bee J 29:10-11

Martin SJ, Beekman M, Wossler TC, Ratnieks FLW (2002a) Parasitic Cape honeybee workers, *Apis mellifera capensis*, evade policing. Nature 415:163–165

Martin S, Wossler TC, Kryger P (2002b) Usurpation of *Apis millifera scutellata* colonies by *A. m. capensis* workers. Apidologie 33:215-232

Moritz RFA (2002) Population dynamics of the Cape bee phenomenon: The impact of parasitic laying worker clones in apiaries and natural populations. Apidologie 33:233-244

Moritz RFA, Hillesheim E (1985) Inheritance of dominance in honeybees (*Apis mellifera capensis*). Behav Ecol Sociobiol 17:87–89

Moritz RFA, Southwick EE (1992) Bees as superorganisms. An evolutionary reality. Springer, Berlin Heidelberg, New York

Moritz RFA, Kryger P, Allsopp MH (1996) Competition for royalty in bees. Nature 384:31-32

Moritz RFA, Simon UE, Crewe RM (2000) Pheromonal contest between honeybee workers. Naturwissenschaften 87:395–397

Moritz RFA, Crewe RM, Hepburn HR (2002) Queen avoidance and mandibular gland secretion of honeybee workers (*Apis mellifera* L.). Insect Soc 49:86–91

Naumann K, Winston ML, Slessor KN (1993) Movement of honey bee (*Apis mellifera* L.) queen mandibular gland pheromone in populous and unpopulous colonies. J Insect Behav 6:211–223

Neumann P, Hepburn HR (2002) Behavioural basis for social parasitism of Cape honeybees (*Apis mellifera capensis* Esch.). Apidologie 33:165-192

Page RE, Robinson GE (1994) Reproductive competition in queenless honey bee colonies (*Apis mellifera* L.). Behav Ecol Sociobiol 35:99–107

Palmer KA, Oldroyd BP (2000) Evolution of multiple mating in the genus *Apis*. Apidologie 31:235–248

Plettner E, Slessor KN, Winston ML, Robinson GE, Page RE (1993) Mandibular gland

components and ovarian development as measures of caste differentiation in honeybee (*Apis mellifera L.*). Insect Physiol 39:235-240

Plettner E, Sutherland GRJ, Slessor KN, Winston ML (1995) Why not be a queen? Regioselectivity in mandibular secretions of honeybee castes. J Chem Ecol 21:1017–1029

Plettner E, Slessor KN, Winston ML, Oliver JE (1996) Caste-selective pheromone biosynthesis in honeybees. Science 271:1851–1853

Plettner E, Otis GW, Wimalaratne PDC, Winston ML, Slessor KN, Pankiw T, Punchiheewa PWK (1997) Species- and caste-determined mandibular gland signals in honeybees (*Apis*). J Chem Ecol 23:363–377

Robinson GE, Page RE, Fondrk MK (1990) Intracolonial behavioral variation in worker oviposition, oophagy, and larval care in queenless honey bee colonies. Behav Ecol Sociobiol 26:315–323

Ruttner F, Hesse B (1981) Rassenspezifische Unterschiede in Ovarentwicklung und Eiablage von weisellosen Arbeiterinnen der Honigbiene *Apis mellifera* L. Apidologie 12:159–183

Ruttner F, Koeniger N, Veith HJ (1976) Queen substance bei eierlegenden Arbeiterinnen der Honigbiene, *Apis mellifica* L. Naturwissenschaften 63:434

Saiovici M (1983) 9-Oxodecenoic acid and dominance in honeybees. J Apic Res 22:27–32

Sasaki M, Takuro I, Sato M (1989) Humoral control of the queen pheromone (9ODA)

biosynthesis in honeybees: Induction in queenless worker and in worker mandibular gland implanted into queen. Bull Fac Agric, Tamagawa Univ 29:11–21

Seeley T (1979) Queen substance dispersal by messenger workers in honeybee colonies. Behav Ecol Sociobiol 5:391-415.

Seeley TD (1985) Honeybee ecology. Princeton University Press, Princeton, NJ

Simon UE, Moritz RFA, Crewe RM (2001) The ontogenetic pattern of mandibular gland components in queenless worker bees (*Apis mellifera capensis* Esch.). J Insect Physiol 47:735–738

Simon UE, Moritz RFA, Crewe RM (2005) Reproductive dominance among honeybee workers in experimental groups of *Apis mellifera capensis*. Apidologie 36:1-7

Slessor KN, Kaminski L, King GGS, Borden JH, Winston ML (1988) Semiochemical basis of the retinue response to queen honey bees. Nature 332:354-356

Velthuis HHW (1970a) Chemical signals and dominance communication in the honeybee *Apis mellifera* (Hymenoptera: Apidae). Entomol Gen 15:83-90

Velthuis HHW (1970b) Ovarian development in *Apis mellifera* worker bees. Entomol Exp Appl 13:343-357

Velthuis HHW (1985) The honeybee queen and the social organization of her colony, in: Hölldobler B, Lindauer M (eds) Experimental behavioural ecology and sociobiology, G Fischer Verlag, New York pp 343–357

Velthuis HHW, van der Kerk A (1988) Age, environment, and genes in relation to the

mandibular gland secretion of pure and hybrid *Apis mellifera capensis* worker bees, in: Needham GR, Page RE, Delfinado-Baker M, Bowman CE (eds) Africanized honey bees and bee mites. Ellis Horwood Limited, England pp 80–86

Velthuis HHW, Ruttner F, Crewe RM (1990) Differentiation in reproductive physiology and behaviour during the development of laying worker honey bees. In: Engels W (ed) Social Insects, Springer-Verlag, Berlin pp 231-243

Winston ML (1987) The biology of the bee. Harvard University Press, Cambridge, MA

Winston ML, Slessor KN (1992) Honey bee primer pheromones and colony organization: gaps in our knowledge. Apidologie 29:81-95

Wossler TC (2002) Pheromone mimicry by *Apis mellifera capensis* social parasites leads to reproductive anarchy in host *Apis mellifera scutellata* colonies. Apidologie 33:139–163

Wossler TC, Crewe RM (1999a) Honeybee queen tergal gland secretion affects ovarian development in caged workers. Apidologie 30:311-320

Wossler TC, Crewe RM (1999b) Mass spectral identification of the tergal gland secretions of female castes of two African honey bee races (*Apis mellifera*). J Apic Res 38:137–148

Wossler TC, Veale RB, Crewe RM (2000) How queen-like are the tergal glands in workers of *Apis mellifera capensis* and *Apis mellifera scutellata*? Apidologie 31:55–66

Zar JH (1999) Biostatistical analysis (3rd edition). Prentice Hall, Upper Saddle River, NJ

CHAPTER 4

THE EFFECT OF BROOD PHEROMONES ON *A. M. CAPENSIS* WORKER LAYING ACTIVITY

4.1 INTRODUCTION

Within a honeybee colony not only does the queen presence have a strong influence in governing worker reproduction but brood presence and their pheromones also play a major role in regulating worker reproduction (Jay 1968, 1970; Arnold et al. 1994; Mohammedi et al. 1998; Le Conte et al. 2001). These brood pheromones consist of an array of esters and hydrocarbons and it is the fifth-instar larval stage pheromones that aid in worker ovary inhibition (Mohammedi et al. 1998). These findings have however focused on European honeybee larvae and their effects on worker reproduction within the same subspecies. Nevertheless the role of brood pheromones in regulating worker reproduction is not always effective. There is a breakdown in brood pheromone suppression in anarchistic workers with anarchistic brood not having the same inhibitory effect as wild type brood (Barron and Oldroyd, 2001). The effectiveness of brood pheromones also seems to breakdown in parasitized African honeybee colonies, where large amounts of African, *A. m. scutellata,* brood does not suppress pseudoclone reproductive development. Either African brood secretes low concentrations of the brood compounds and/or the Cape honeybee workers have high pheromone response thresholds (Naumann et al. 1993), rendering the brood pheromone ineffectual. *A. m. capensis* workers probably do have higher response thresholds to brood pheromones since

previous studies have shown that queenless Cape workers are not, or only partly, restricted by the presence of relatively large amounts of young Cape brood (Anderson 1963, 1981; Hepburn 1992, 1994; Hepburn et al. 1988, 1991). This does not offset the suggestion that African larvae produce a signal that is qualitatively and/or quantitatively less functional. This decreased effectiveness of African brood pheromones may aid these social parasitic Cape honeybee workers in establishing themselves as successful parasites within African colonies (Wossler 2002; Boot et al. 2002).

Even though there is no quantitative or qualitative chemical data on Cape and African brood pheromones, there is data showing differential feeding of larvae from these two subspecies, suggesting that larvae from different subspecies may produce different signals (or the nurse bees possess different response thresholds to pheromones). Whatever the mechanism, African workers feed Cape larvae more (Beekmann et al. 2000) while Cape workers feed African larvae less (Allsopp et al. 2003). Those larvae that produce stronger signals will be fed more often and higher quality food, giving them a reproductive head start at an early age.

The aim of this study was to test the effect of open brood (brood pheromones) on inhibiting Cape worker reproduction. Native Cape honeybee workers were exposed to open African brood, open Cape brood as well as no brood within queenless African colonies, to establish whether there is a brood effect on the egg laying capabilities of the native Cape honeybee workers. These data, be they preliminary, do indicate differential reproductive suppression by African and Cape brood.

4.2 MATERIALS AND METHODS

Field experiments were conducted in an isolated apiary on the Stellenbosch Plant Protection Research Institute (PPRI), Vredenburg research grounds. Twelve three frame honeybee boxes (15cm x 25cm x 15cm) were used during the brood experiment. All twelve experimental colonies had an equal proportion of African workers introduced and remained queenless throughout the experiment. Customized frames were manufactured with each hive containing one food frame (pollen and honey) and one capped or sealed African brood frame (to prevent absconding). The third frame contained open brood (larvae) or no brood. The three treatments were as follows; the control had no open brood introduced (n = 4 hives); experiment 1 had a frame of open African brood introduced (African brood effect, n = 4 hives); experiment 2 had a frame of open Cape brood introduced (Cape brood effect, n = 4 hives). On the fourth day a new frame of open African and Cape brood was reintroduced to ensure a continual production of pheromones by uncapped larvae.

Pectora roborant cultus recti

Into each of the 12 hives, 25 one-day old (incubated overnight at 32°C with 60% relative humidity) uniquely marked (non toxic paint) native Cape workers were introduced. All introduced workers were thus of the same age and contained a clean or blank slate (Breed et al. 2004) giving them an equal chance on surviving and developing their ovaries and laying eggs (Velthuis et al. 1990).

Each day the frames were inspected for egg laying activity where the numbers of worker laid eggs were recorded. The duration of the test period was seven days. This would be enough time for Cape workers to fully develop their ovaries (Velthuis 1970). All seven-day old marked bees were collected for ovary dissection. A random selection of *A. m. scutellata* workers (n = 25 per treatment) were also retrieved for ovary analysis, to verify that eggs laid were by Cape honeybee workers and not the resident African honeybee workers. The ovaries were dissected under a dissecting microscope (Olympus TL2) and scored in two different categories: 1 = undeveloped (ovaries with no oocytes); 2 = fully developed (large ovaries with distinct ova), adapted from Velthuis (1970). The χ^2 square test with Yates's correction was used to compare the degree of ovary development in the resident *A. m. scutellata* workers to the introduced *A. m. capensis* workers (Zar 1999). In order to compare the number of eggs laid on the sixth day for each of the experimental brood conditions (i.e. no brood, open African brood and open Cape brood) we performed the one-way analysis of variance (ANOVA) followed by Tukey HSD (Zar 1999). Probability values (P) smaller than 0.05 were considered significant for all statistical analyses performed.

Pectora roborant cultus recti

4.3 RESULTS

The queenless African honeybee workers (n = 75) showed no sign of developing their ovaries (Table 4.1). In comparison however, when exposed to similar African queenless conditions, the seven-day old Cape honeybee workers harvested (n = 186) all had fully activated ovaries (Table 4.1; χ^2 with Yates's correction = 53.4, $df = 1$, $P < 0.001$). The first eggs were observed in some colonies on day 5 (Table 4.1).

Table 4.1 Effect of African and Cape open brood as well as no brood on ovary development and the egg laying activity of Cape worker honeybees within queenless African colonies. Numbers of counted eggs; values are given as (mean number of eggs laid per brood type ± se)

Brood type	No brood	Open African	Open Cape
Colonies (n)	4	4	4
Retrieved Cape bees (n)	56	61	69
Retrieved African bees (n)	25	25	25
Ovary development (%)			
Cape workers	100	100	100
African workers	0	0	0
Egg count (Days)			
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0.25±0.70	6.00±1.87	0
6	9.8±4.30	23.2±6.79	4.94±2.67

The type of brood the introduced Cape honeybee workers were exposed to had an effect on the rate at which the eggs were being laid (Table 4.1). Cape workers exposed to open Cape brood, commenced egg laying later and laid the fewest eggs while those workers exposed to open African brood, had eggs present on day 5 and laid the most eggs (see Table 4.1 and Figure 4.1). Workers not exposed to any brood had some eggs present on day 5, nonetheless the numbers of eggs laid were still less than those laid in colonies with open African brood (Table 4.1). These results suggest that open African brood acts as a catalyst, speeding up egg laying in the Cape workers.

Table 4.2 The effect of brood type on the egg laying activity of six-day old Cape honeybee workers

	df	Sum of squares	*F*	*P*
Effect of brood type	2	268.3	20.16	0.011
Open African vs no brood*	1	294.0	22.05	0.009
Open African vs open Cape*	1	486.7	39.41	0.003

*Pairwise comparison using Tukey posthoc test was used to indicate which brood types differed significantly

Significantly more eggs are laid by Cape workers exposed to open African brood (Table 4.2). There was however no significant difference in the number of eggs laid by Cape workers exposed to no brood or open Cape brood. These results indicate that open Cape brood and to a lesser extent no brood, in contrast to open African brood, inhibit the egg laying ability of the Cape honeybee workers housed in queenless African colonies (see Figure 4.1 and Table 4.1).

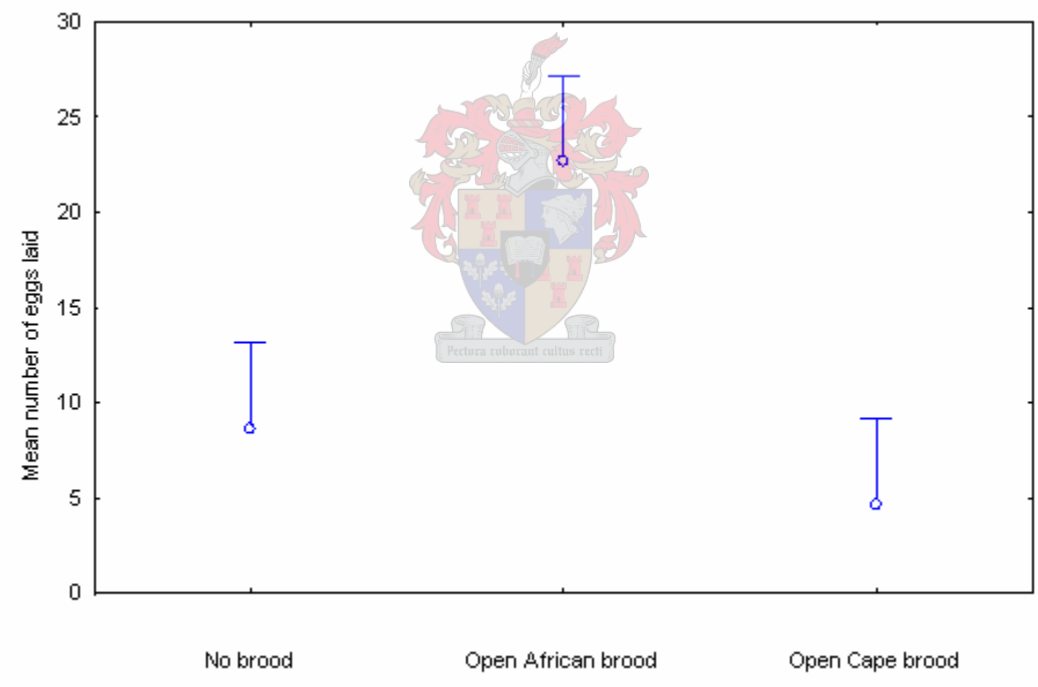


Figure 4.1 Mean number of eggs laid (mean and 95% confidence interval) on day 6 by Cape honeybee workers in African queenless colonies exposed to different brood treatments. Significantly more eggs were laid in hives which contained African open brood compared to those with open Cape brood and no brood, both of which showed similar inhibitory effects

4.5 DISCUSSION

The first eggs were observed at 5 days supporting previous findings that Cape honeybee workers have a short latency to developing their ovaries when conditions are favourable (Ruttner and Hesse 1979; Velthuis et al. 1990). In this experiment, open African brood does not appear to inhibit egg production by Cape workers. In fact colonies with open African brood result in eggs being laid statistically more rapidly than colonies with no open African brood suggesting a stimulatory component to their pheromone in speeding up reproductive development in Cape workers. It has been shown that brood pheromones lose their effectiveness under heavy feeding conditions, with workers rapidly developing reproductively (Anderson 1963; Hepburn et al. 1988). This has lead to the suggestion that the high quality pollen and nectar flows of *Aloe greatheadii davyana* promotes the reproductive development of the pseudoclones, with a two-fold effect; high nutritional values and reduced brood pheromone effectiveness. However this experiment was done under normal feeding conditions (where the food frames contained approximately 30% pollen) and still the African brood was ineffective in slowing down reproductive development. On the other hand, open Cape brood did appear to retard egg production, albeit only for one day. These data are in accordance with Hepburn's data which indicated that Cape workers are only partially prevented from developing reproductively by Cape open brood, and then only in the presence of large amounts of open brood (Hepburn 1992, 1994; Hepburn et al. 1988, 1991). Surprisingly, one would expect that workers exposed to no brood pheromones would develop most rapidly but this was not the case here with African larvae apparently priming Cape workers in developing reproductively. How or why this functions remains an enigma?

The fact that worker policing was not controlled for in this experiment does not bias the results in any way. Even though worker policing is common practice in most queenright colonies of honeybee species (Ratnieks 1993; Ratnieks and Visscher 1989; Pirk et al. 2002) it breaks down in queenless colonies, with worker eggs not being policed (Ratnieks 1992, 1995; Miller and Ratnieks 2001). Consequently the variation in egg numbers is due to laying activity rather than differential worker policing by resident *A. m. scutellata* workers across the brood treatments. Even queenright *A. m. scutellata* workers do not remove eggs laid by parasitic *A. m. capensis* workers (Martin et al. 2002; Neumann et al. 2003)

These data are preliminary and more repetitions need to be conducted but it does suggest that African larvae induce reproductive development in Cape honeybee workers with them laying more rapidly and a greater number of eggs, compared to Cape larvae.

Pectora roborant cultus recti

4.6 REFERENCES

Allsopp MH, Calis JNM, Boot WJ (2003) Different feeding of worker larvae affects caste characters in Cape honeybee, *Apis mellifera capensis*. Behav Eco Sociobiol 54:555-561

Anderson RH (1963) The laying worker in the Cape honeybee, *Apis mellifera capensis*. J Apic Res 2:85-92

Anderson RH (1981) Queens and queen rearing. S Afr Bee J 53:3–12

Arnold G, Le Conte Y, Troullier J, Hervet H, Chappe B, Masson C (1994) Inhibition of

worker honeybee ovary development by a mixture of fatty acid esters from larvae. C R Acad Sci Paris 317:511-515

Barron AB, Oldroyd BP (2001) Social regulation of ovary activation in 'anarchistic' honey-bees (*Apis mellifera*). Behav Ecol Sociobiol 49:214–219

Beekman M, Calis JNM, Boot WJ (2000) Parasitic honeybees get royal treatment. Nature 404:723

Boot WJ, Calis JNM, Allsopp MH (2002) Social parasitism by the Cape honey bee. Proc Exp Appl Entomol NEV (Amsterdam) 13:103-107

Breed MD, Perry S, Bjostad LB (2004) Testing the blank slate hypothesis: why honey bee colonies accept young bees. Insect Soc 51:12-16

Hepburn HR (1992) Pheromonal and ovarial development covary in Cape worker honeybees, *Apis mellifera capensis*. Naturwissenschaften 79:523-524

Hepburn HR (1994) Reproductive cycling and hierarchical competition in Cape honeybees, *Apis mellifera capensis* Esch. Apidologie 25:38–48

Hepburn HR, Nefdt RJC, Whiffler LA (1988) Queen loss in the Cape honeybee: the interactions of brood, laying workers (false queens?) and queen cells. S Afr J Sci 84:778-780

Hepburn HR, Magnuson P,HerbertL, Whiffler LA (1991) The development of laying workers in field colonies of the Cape honey bee. J Apic Res 30:107–112

Jay SC (1968) Factors influencing ovary development of worker honeybees under natural conditions. Can J Zool 46:345-347

Jay SC (1970) The effect of various combinations of immature queen and worker bees on

the ovary development of worker honeybees in colonies with and without queens. Can J Zool 48:169-173

Le Conte Y, Mohammedi A, Robinson GE (2001) Primer effects of a brood pheromone on honeybee behaviour development. Proc R Soc Lond 268:163-168

Martin S, Beekman M, Wossler TC, Ratnieks FLW (2002) Parasitic honeybee workers, *Apis mellifera capensis*, evade worker policing. Nature 415:163–165

Miller DG, Ratnieks FLW (2001) The timing of worker reproduction and breakdown of policing behaviour in queenless honeybee (*Apis mellifera* L.) societies. Insect Soc 48:178-184

Mohammedi A, Paris A, Crauser Y, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera L.*). Naturwissenschaften 85:455-458

Moritz RFA, Kryger P, Allsopp MH (1999) Lack of worker policing in the Cape honeybee (*Apis mellifera capensis*). Behaviour 136:1079–1092

Naumann K, Winston ML, Slessor KN (1993) Movement of honey bee (*Apis mellifera* L.) queen mandibular gland pheromone in populous and unpopulous colonies. J Insect Behav 6:211–223

Neumann P, Pirk CWW, Hepburn HR, Moritz RFA (2003) Spatial differences in working policing facilitate social parasitism of Cape honeybee workers (*Apis mellifera capensis* Esch.) in queenright host colonies. Insect Soc 50:109-112

Pirk CWW, Neumann P, Hepburn HR (2002) Egg laying and egg removal by workers are positively correlated in queenright Cape honeybee colonies (*Apis mellifera capensis*). Apidologie 33:203–211

Ratnieks FLW (1992) Evidence for an egg marking pheromone in the honey bee. Am Bee J 132:813-814

Ratnieks FLW (1993) Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. Behav Ecol Sociobiol 32:191–198

Ratnieks FLW (1995) Evidence for a queen-produced egg-marking pheromone and its use in worker policing in the honey bee. J Apic Res 34:31–37

Ratnieks FLW, Visscher PK (1989) Worker policing in the honeybee. Nature 342:796–797

Ruttner F, Hesse B (1979) Rassenspezifische Unterschiede in Ovarentwicklung und Eiablage von weisellosen Arbeiterinnen der Honigbiene *Apis mellifera* L. Apidologie 12:159–183

Velthuis HHW (1970) Ovarian development in *Apis mellifera* worker bees. Entomol Exp Appl 13: 343-357

Velthuis HHW, Ruttner F, Crewe RM (1990) Differentiation in reproductive physiology and behaviour during the evelopment of laying worker honey bees. In: Engels W (ed) Social Insects, Springer-Verlag, Berlin pp 231-243

Visscher PK (1996) Reproductive conflict in honey bees: a stalemate of worker egg-laying and policing. Behav Ecol Sociobiol 39:237–242

Visscher PK, Dukas R (1995) Honey bees recognize development of nestmates' ovaries. Anim Behav 49:542–544

Wossler TC (2002) Pheromone mimicry by *Apis mellifera capensis* social parasites leads to reproductive anarchy in host *Apis mellifera scutellata* colonies. Apidologie 33:139–163

Zar JH (1999) Biostatistical analysis (3rd edition). Prentice Hall, Upper Saddle River, NJ

CHAPTER 5

GENERAL DISCUSSION

5.1 DISCUSSION

Social parasitism by honeybee workers was first highlighted more than a decade ago when large numbers of commercial *A. m. scutellata* colonies were lost to Cape worker invasions. Cape honeybee workers, from Cape colonies brought into African honeybee territory, infiltrated, established themselves, took over reproductive roles in *A. m. scutellata* host colonies, and destroyed the colony giving rise to what is referred to as the '*capensis calamity*' (Allsopp 1992, 1993, 1995; Allsopp and Crewe 1993; Hepburn and Allsopp 1994; Moritz 2002; Martin et al. 2002). More recently however, with the routine use of microsatellites in assigning relatedness (Solignac et al. 2003; 2004), social parasitism by worker bees may be more widespread then originally thought (see Lopez-Vaamonde et al. 2004; Nanork et al. 2005; Härtel et al. 2006). The one criterion however common to all examples of social parasitism is that the parasite must be able to infect the host. This is a crucial period surrounding the parasite's lifecycle. However previous, and continued, attempts to infect African colonies with Cape honeybee workers has proved extremely difficult and often lead to the removal of all introduced Cape honeybee workers (Calis et al. unpublished; Allsopp personal communication). This prompted the investigation on whether African host colonies were more vulnerable to invasion during periods surrounding colony fission.

Interestingly, in the past before the '*capensis calamity*' was recognized, there were reports of Cape, *A. m. capensis*, colonies exposed to African colonies (Lundie 1954; Johannsmeier 1983). So why then was there no *capensis* problem previously? Why then did the infections

not persist previously? One explanation may be that far fewer Cape honeybee colonies were moved by beekeepers, compared to the hundreds moved more recently (in 1990), into African honeybee territories, reducing the likelihood of infection. A second explanation, and probably highly plausible, is that the recent movement of Cape colonies northwards coincided with the *Aloe greatheadii davyana* flowering periods, where many commercial beekeepers gather with their *A. m. scutellata* colonies (Swart 2001). These aloes are rich in pollen and nectar stimulating colony growth and reproduction (fission; natural, as well as induced through colony splitting by beekeepers), resulting in disruption of colony organization during this swarming/fission phase (Hepburn and Crewe 1991; Allsopp 1993). In addition, guards are more permissive when resource availability is high and this probably facilitates the infiltration of drifted workers into host colonies (Neumann et al. 2001). Furthermore, high pollen flows enhance ovary development in laying workers (Velthuis 1970; Velthuis et al. 1990), assisting workers in the switch over to reproduction. A third explanation for the success of this recent invasion is that the parasitic worker population consists of near-identical individuals (due to parthenogenesis) referred to as pseudoclones (Kryger 2001a, 2001b, Baudry et al. 2004). This single "clonal" worker invasive lineage must have originated from a Cape worker that simultaneously expressed more queenlike traits compared to other native worker lineages promoting their successful parasitic lifestyle.

Strong, uninfected *A. m. scutellata* colonies were obtained from the Northern Cape and split into queenright and queenless halves, imitating a common beekeeping practice. Cape one-day old workers from both the native and parasitic population were introduced before, during and after each splitting process. Their survival and reproductive dominance were recorded.

Pseudoclones show highest acceptance and survival rates when entering a queenright, functional colony where the host workers have not been disrupted due to colony splitting which often culminates in worker fighting. 23% of pseudoclones introduced into queenright functional host colonies survived (treatment 1; includes both splits), the highest survival rate attained for pseudoclones, indicating that these workers evaded detection in that 72 hour critical period (when 70% of all introduced bees get evicted; see chapter 2 for results), a feat native Cape workers could not attain with only 6% surviving. Those workers finding themselves together with a queen for an extended period (queenright split) suffer further increased mortality suggesting that the *A. m. scutellata* honeybee queen, even though she does not secrete a mandibular signal with 9ODA making up 80% or more of the secretion as in Cape queens (Crewe 1982, Crewe et al. 1990; see also Dietemann et al. 2006a for conflicting results), does influence the long term acceptance rates of the pseudoclones (probably through her own workers; see following paragraph for explanation). However her influence does not extend to regulating reproductive development, with all pseudoclones fully developing their ovaries and secreting mandibular gland signals dominated by 9ODA (more than 50% of the selected compounds in this study consisted of 9ODA for the pseudoclones, while 9ODA made up on average 37% of developed Cape worker mandibular signals). The same cannot be said for native Cape workers, with only 9% developing reproductively in queenright colonies. I suggest that this could be a miscommunication between the *A. m. scutellata* queen and the pseudoclones with her pheromone signals not having the right properties to physiologically suppress the parasites' reproduction (see Dietemann et al. 2006a for explanation). There seems to be less of a mismatch between host queen signal and native Cape workers, with the queen pheromones inhibiting the reproductive development of a large proportion of these workers.

The acceptance/survival rates possibly function on another communication level, with the host queen signalling her presence and efficiency to her resident workers who in turn ultimately control colony functioning and integrity. The worker force in the queen's presence plays a role in maintaining colony membership, evicting those individuals who are foreign and disregarding those capable of "masking" their presence. The native Cape workers are not successful in remaining undetected in the queenright host colony while a percentage of the pseudoclones "mask" their presence successfully, probably through pheromone mimicry, but that still needs to be ascertained. Of course those pseudoclone workers surviving may actually be those workers who do not rapidly produce queenlike pheromones and in so doing keep a low profile until they become ensconced. This unfortunately does not account for why native Cape workers are removed since one would expect this population to show greater variation in worker-queen characteristics with the more worker-like individuals surviving, but this does not seem to be the case.

Pectora roborant cultus recti

It comes as no surprise that queenless pseudoclones also develop into pseudoqueens, but surprisingly not at the expense of native Cape workers who under queenless conditions also develop their ovaries completely. This is however not true for treatment 1 where only 40% develop fully, probably because of the exposure to queen pheromone for those 72 hours prior the split which initially may retard development which is further inhibited by the contact with pseudoclone pseudoqueens secreting very queen-like mandibular secretions. Native Cape workers introduced directly into queenless colonies however (treatment 2 and 3), all develop their ovaries (except one out of a total of 47) suggesting that the developed pseudoclones alone are not effective in inhibiting development in the native Cape bees, as has previously been thought (Moritz 2002). Instead it would appear that the presence of queen pheromones in

the three day critical period retards the initial worker development with the pseudoclone pheromones only capable of maintaining that regulation (but not initiating it).

The splitting of colonies disrupts normal colony functioning, with variable responses from host workers depending on when they are split and for how long they have been queenless. Strangely, all workers introduced on the day of splitting were evicted by queenright halves, while 20 and 30% of native Cape and pseudoclones survived respectively in the queenless halves. As previously mentioned, the presence of a functional queen in the queenright splits possibly regulates the host workers' responses to foreign bees through effective colony defence. The queenless halves will enter a period of colony disturbance and fighting and consequently a number of native Cape and pseudoclone workers will not survive these early stages of queenlessness after colony splitting. After the initial period following queen loss (treatment 3), the indiscriminate fighting among workers ceases and the levels of guarding increase once again, with most pseudoclones being evicted (1% survive) if introduced after splitting. In comparison to the low survival rate of queenless native Cape workers, initially introduced into queenright colonies prior splitting (3% survival rate), native Cape workers introduced post-splitting exhibit higher survival rates (13%), this is in contrast to the percentage of pseudoclones being accepted. Why these two worker populations show different patterns of survival is not clear cut. Why a percentage of pseudoclones remain undetected in queenright colonies but not post-splitting queenless colonies is a mystery?

A small percentage of workers remain undetected in the host colonies but the majority of these Cape workers in both African queenright and queenless colonies get detected somehow which means they must have some kind of branding or labelling which enables the African workers

to identify and evict them at such a young stage (Noach-Pienaar et al. 2006). However, even though the majority are killed there are those which survive suggesting there might be a possible evasion technique which is critical to the '*capensis calamity*'? Even though survival rates are low, it only takes one Cape honeybee worker to invade and become established as a parasite perpetuating the problem. Survival rates are variable and depend largely on individual host colony susceptibility (some colonies are more successful at mounting a defence than others), queen state and whether the introduced Cape worker originate from the native or parasitic population (see chapter 2 for details). This variability in successful establishment does not point to a specific window of opportunity for all Cape bees but does indicate that the period prior to colony splitting (highest survival rates for pseudoclones across all treatments) may be the most opportunistic for pseudoclone invasion with 13% and 34% surviving in queenright and queenless colonies respectively. It is important to remember since this is not a natural swarming process I am unable to simulate the pheromone changes taking place in the colony which could have an added effect, with colonies tolerating a greater proportion of intruders. The fact that this experiment could not be conducted on the enriched Aloes of the Highveld may be partly responsible for the low survival rates of Cape workers. I realise that I have isolated one variable, out of a number of factors probably co-occurring, that may render a host colony vulnerable but disentangling the components responsible for the success of the social parasites is important with each finding expanding our understanding.

Another factor which I unfortunately only have preliminary results for, is the effectiveness of *A. m. scutellata* brood pheromones on the development of social parasites. Brood pheromones, together with queen pheromones, play a pivotal role in regulating worker reproduction (Jay 1968, 1970, 1972; Winston 1987, 1992; Keller and Nonacs 1993; Arnold et al. 1994;

Mohammedi et al. 1998). Pheromones produced by African larvae do not simply inhibit Cape worker reproductive development but appear to accelerate the commencement of egg laying by these workers. Brood pheromone analyses has not been undertaken for African larvae (only for European honeybees see, Arnold et al. 1994) and so changes in their signal compared to other subspecies is unknown and so how these pheromones may prime workers reproductively, as my preliminary results suggests, is still unknown.

Ultimately these data suggest that Cape honeybee workers, from both the native and parasitic populations, are capable of gaining access to African host colonies albeit in low numbers. Workers from the pseudoclone population, in comparison to the native Cape workers, produce queen-like signals, dominated by 9ODA, all develop their ovaries and more become established in host colonies. The timing of the worker introductions does have an effect on survival rates but it is not the same for the native Cape and pseudoclone workers. Moreover, individual colonies respond variably to introduced workers with some African colonies more resilient than others which suggests a genetic component to the response by African colonies to social parasites. If this is the case, then a selective queen rearing programme may offer beekeepers a limited solution to the problem. Since the problem is one perpetuated by beekeeping practices an investigation at the commercial scale may shed more light on the continuance of these social parasites (Dietemann et al. 2006b).

5.2 REFERENCES

Allsopp MH (1992) The '*capensis calamity*'. S Afr Bee J 64:52-55

Allsopp MH (1993) Summerized overview of the capensis problem. S Afr Bee J 65:127-

Allsopp MH (1995) The capensis problem 1992-1995. In: Magnuson P (ed) Proceedings of the First International Electronic Conference on the Cape Bee problem in South Africa 5-30 June 1995. PPRI, Pretoria pp 10-31

Allsopp MH, Crewe R (1993) The Cape honeybee as a Trojan horse rather than the hordes of Jenghiz Khan. Am Bee J 133:121-123

Arnold G, Le Conte Y, Troullier J, Hervet H, Chappe B, Masson C (1994) Inhibition of worker honeybee ovary development by a mixture of fatty acid esters from larvae. C R Acad Sci Paris 317:511-515

Baudry E, Kryger P, Allsopp M, Koeniger N, Vautrin D, Mougel F, Cornuet J-M, Solignac M (2004) Whole-Genome Scan in Thelytokous-Laying workers of the Cape honeybee (*Apis mellifera capensis*): Central fusion, reduced recombination r ates and centromere mapping using Half-Tetrad analysis. Genetics 167:243-252

Crewe RM (1982) Compositional variability:The key to the social signals produced by honeybee mandibular glands. In: Breed MD, Michener CD, Evans HE (eds) The Biology of Social Insects, Westview Press/Boulder, Colorado. pp 318–322

Crewe RM, Wossler T, Allsopp MH (1990) Workers in queens clothing: why *capensis* workers become pseudoqueens. In: Anderson RW, Buys B (eds) Bees and beekeeping in Southern Africa, Apimondia, University Press, Stellenbosch, pp 83–89

Dietemann V, Pflugfelder J, Härtel S, Neumann, Crewe R (2006a) Social parasitism by honeybee workers (*Apis mellifera capensis* Esch.): evidence for pheromonal resistance to host queen's signals. Behav Ecol Socio 60:785-793

Dietemann V, Lubbe A, Crewe RM (2006b) Human factors facilitating the spread of a parasitic honeybee in South Africa. J Econo Entomol 99:7-13

Härtel S, Neumann P, Kryger P, von der Heide, Moltzer G, Crewe R, van Praagh J, Moritz F

(2006) Infestation levels of *Apis mellifera scutellata* swarms by parasitic Cape honeybee workers (*Apis mellifera capensis*). Apidologie 37:462-470

Hepburn HR, Crewe RM (1991) Portrait of the Cape honeybee, *Apis mellifera capensis*. Apidologie 22: 567-580

Hepburn HR, Allsopp MH (1994) Reproductive conflict between honeybees: usurpation of *Apis mellifera scutellata* colonies by *Apis mellifera capensis*. S Afr J Sci 90:247-249

Jay SC (1968) Factors influencing ovary development of worker honeybees under natural conditions. Can J Zool 46:345-347

Jay SC (1970) The effect of various combinations of immature queen and worker bees on the ovary development of worker honeybees in colonies with and without queens. Can J Zool 48:169-173

Jay SC (1972) Ovary development of worker honeybees when separated from worker brood by various methods. Can J Zool 50:661-664

Johannsmeier MF (1983) Experiencices with the cape bee in the Transvaal. S Afr Bee J 55:130-138

Keller L, Nonacs P (1993) The role of queen pheromones in social insects: queen control or queen signals? Anim Behav 45:787-794

Kryger P (2001a) The Capensis pseudo-clone, a social parasite of African honey bees. In: Proceedings of the 2001 Berlin Meeting of the European Section of IUSSI, 25-29 September p 208

Kryger P (2001b) The pseudo-clone of *Apis mellifera capensis* - an obligate social parasite in honeybees. In: Proceedings of the XXXVII International Apicultural Congress, Durban South Africa p 33

Lopez-Vaamonde C, Koning JW, Jordan WC, Bourke AFG (2004) A test of information use

by reproductive bumblebee workers. Animal Bahavior 68:811-818

Lundie A (1954) Laying worker bees produce worker bees. S Afr Bee J 29:10-11

Martin S, Wossler T, Kryger P (2002) Usurption of African *Apis mellifera scutellata* colonies by parasitic *Apis mellifera capensis* workers. Apidologie 33 p 215-216

Mohammedi A, Paris A, Crauser Y, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera L.*). Naturwissenschaften 85:455-458

Moritz RFA (2002) Population dynamics of the Cape bee phenomenon: The impact of parasitic laying worker clones in apiaries and natural populations. Apidologie 33:233-244

Nanork P, Paar J, Chapman NC (2005) Asian honeybees parasitize the future dead. Nature 437:829-829

Neumann P, Radloff SE, Moritz RFA, Hepburn HR, Reece SL (2001) Social parasitism by honeybee workers (*Apis mellifera capensis* Escholtz): host finding and resistance of hybrid colonies. Behav Ecol 12:419-428

Noach-Pienaar L, Wossler TC, Allsopp MH (2006) Are parasitic honeybee workers, *Apis mellifera capensis*, winning the pheromone contest? In: Proceedings of XV congress of the 2006 IUSSI Meeting, Washington DC, 30 July - 04 August p 228

Solignac MD, Vautrin A, Loiseau F, Mogel E, Baudry E (2003) Five hundred and fifty microsatellite markers for the study of honeybee (*Apis mellifera* L.) genome. Mol Ecol Notes 3:307-311

Solignac MD, Vautrin A, Baudry E, Mogel E, Loiseau F (2004) A microsatellite-based linkage map of the honeybee *Apis mellifera* L. Genetics 167:253-262

Swart DJ (2001) Specialized management. In: Johannsmeier MF (ed) Beekeeping in

South Africa, 3rd edition, Plant Protection Research Institute Handbook No. 14, Agricultural Research Council of South Africa, Pretoria, South Africa pp 85-94

Velthuis HHW (1970) Ovarian development in *Apis mellifera* worker bees. Entomol Exp Appl 13: 343-357

Velthuis HHW, Ruttner F, Crewe RM (1990) Differentiation in reproductive physiology and behaviour during the evelopment of laying worker honey bees. In: Engels W (ed) Social Insects, Springer-Verlag, Berlin pp 231-243

Winston ML (1987) The biology of the bee. Harvard University Press, Cambridge, MA

Winston ML (1992) Semichemicals and insect sociality. In: Isman M, Roitberg B (eds) Evolutionary perspectives on insect chemical ecology. Chapman and Hall, New York

APPENDIX

Table I Comparisons of the survival rates for the two *A. m. capensis* honeybee populations (Clone = pseudoclones and Cape = native Cape) within queenright and queenless host *A. m. scutellata* colonies over time, no treatment effect tested. Differences between the two populations were evaluated using χ^2 tests with Yates's correction (Zar 1999). Significance denoted with "*" where $P < 0.05$

Colony status	Time of count	χ^2	*df*	*P* value
Queenless	Day 3	0.409	1	>0.05
	Day 12	13.20	1	<0.05*
	Day 21	15.46	1	<0.05*
Queenright	Day 3	37.08	1	<0.05*
	Day 12	14.91	1	<0.05*
	Day 21	10.05	1	<0.05*

Table II Survival rate comparisons within native Cape and pseudoclone worker populations across treatment levels (T1vsT2, T2vsT3 and T1vsT3 respectfully) in both queenright and queenless host African colonies at 21 days. Differences were evaluated by χ^2 tests with Yates's correction (Zar 1999) and using FDR (false discovery rate) analysis that tested for type 1 errors (Garcia 2004). All interactions still significant after Yates's and the FDR correction are denoted with "*". Significance detected where $P < 0.05$. See Table 2.2 for sample size

Bee population	African colony state	Treatment comparison	χ^2	*df*	*P* values
Cape	Queenright	Treatment 1 vs Treatment 2	11.8	1	0.001*
		Treatment 2 vs Treatment 3	73.1	1	0.001*
		Treatment 3 vs Treatment 1	13.5	1	0.001*
	Queenless	Treatment 1 vs Treatment 2	17.1	1	0.001*
		Treatment 2 vs Treatment 3	1.94	1	0.150
		Treatment 3 vs Treatment 1	8.39	1	0.005*
Pseudoclone	Queenright	Treatment 1 vs Treatment 2	19.2	1	0.001*
		Treatment 2 vs Treatment 3	2.87	1	0.070
		Treatment 3 vs Treatment 1	11.8	1	0.001*
	Queenless	Treatment 1 vs Treatment 2	0.68	1	0.400
		Treatment 2 vs Treatment 3	42.9	1	0.001*
		Treatment 3 vs Treatment 1	52.3	1	0.001*

Table III Ovary and mandibular gland signal data for all 21-day old individual *A. m. capensis* worker bees collected over twenty one days since inception. Hive type (Q+ = Queenright; Q- = Queenless), infection and splitting period (T = Treatment 1, 2 and 3), hive identification (A to I) and honeybee populations (Clone = pseudoclone; Cape = native Cape workers) are included. Ovary status; fully functional and developed ovaries indicated by 2 and no ovary development by 1. The proportion of each individual compound was calculated as the ratio of that compound in relation to the other seven compounds in the mandibular gland extract. 9-keto-2(E)-decenoic acid (9-ODA), 9 hydroxydecanoic acid (9-HDAA), 9-hydroxy-2(E)-decenoic acid (9-HDA) all major queen pheromones, 10 hydroxydecanoic acid (10-HDAA), 10-hydroxy-2(E)-decenoic acid (10-HDA), all major worker pheromones, palmitoleic acid (PAL), palmitic acid (PAM) and stearic acid (STE) are included

Hive type	T	Hive ID	Bee type	Ovary status	9-ODA	9-HDAA	9-HDA	10-HDAA	10-HDA	PAL	PAM	STE
Q+	1	B	Clone	2	62.48	0	10.74	0	0.24	3.41	8.77	14.33
Q+	1	B	Clone	2	64.53	0	11.22	0	0.84	3.09	7.66	12.63
Q+	1	B	Clone	2	58.86	0	12.71	0	0.16	3.30	8.80	16.14
Q+	1	B	Clone	2	61.67	0	8.75	0	0.39	3.53	9.99	15.67
Q+	1	B	Clone	2	66.09	0	7.06	0	0	2.53	8.58	15.69
Q+	1	B	Clone	2	55.96	0	9.11	0	0	4.82	12.14	17.94
Q+	1	B	Clone	2	52.41	0	9.24	0	0	5.26	11.97	21.09
Q+	1	B	Clone	2	60.07	0	17.35	0	0.28	2.20	6.97	13.11
Q+	1	B	Clone	2	51.66	0	17.39	0	0.21	3.13	11.11	16.48
Q+	1	B	Clone	2	62.79	0	10.06	0	0.16	2.93	8.63	15.40
Q+	1	B	Clone	2	72.05	0	8.94	0	0.37	1.75	5.54	11.32
Q+	1	B	Cape	2	63.86	0	2.08	0	0	5.00	10.86	18.22
Q+	1	B	Cape	1	0	0	5.94	1.37	1.25	1.56	22.13	67.72
Q+	1	B	Cape	2	66.69	0	6.19	0	0	4.43	7.76	14.89
Q+	1	B	Cape	1	1.00	0	6.70	0	0	1.67	26.25	64.35
Q+	1	C	Clone	2	54.24	0	6.88	0	0	4.62	13.51	20.72
Q+	1	C	Clone	2	15.52	0	2.78	0	0	9.40	26.81	45.47
Q+	1	C	Clone	2	58.98	0	11.52	0	0	3.06	9.86	16.56
Q+	1	C	Clone	2	63.85	0	8.65	0	0.25	2.99	8.93	15.30
Q+	1	C	Clone	2	54.48	0	6.96	0	0	5.31	12.69	20.53
Q+	1	C	Clone	2	63.90	0	11.23	0	0.71	2.30	8.52	13.31
Q+	1	C	Clone	2	62.39	0	12.17	0	0.31	1.75	8.12	15.24
Q+	1	C	Clone	2	55.43	0	9.63	0	0	3.48	11.78	19.68
Q+	1	C	Cape	1	44.95	0	3.20	0	0	9.34	14.30	28.18
Q+	1	C	Cape	1	0	0	4	0	0	1.67	20.58	73.74
Q+	1	C	Cape	1	0.44	0	10.92	0	0	1.30	18.33	68.98
Q+	1	C	Cape	1	0	0	3.52	6.24	0	1.62	19.94	68.65
Q+	1	C	Cape	1	0	0	3.67	0	0	1.20	20.62	74.48
Q+	1	C	Cape	1	0	0	5.06	0	0	1.13	20.43	73.36
Q+	1	C	Cape	1	0	0	3.21	0	0	1.70	24.59	70.48
Q+	1	C	Cape	1	0	0	2.09	2.47	0	1.33	21.23	72.85
Q+	3	H	Clone	2	52.31	0	4.92	0	0	3.70	16.63	22.45
Q+	3	H	Clone	2	58.62	0	5.69	0	0	4.44	13.32	17.91

Q+	3	H	Clone	2	59.96	0	5.49	0	0	4.60	12.57	17.36
Q+	3	H	Cape	1	0	0	8.77	10.16	1.39	1.88	21.14	56.63
Q+	3	H	Cape	1	0	0	7.73	27.03	1.34	1.34	15.19	47.34
Q+	3	H	Cape	1	0	0	14.51	0	0	1.486	17.56	66.43
Q+	3	H	Cape	1	0	0	15.35	0	0	1.05	19.35	64.23
Q+	3	H	Cape	1	0	0	6.60	0.84	0.66	1.69	23.62	66.56
Q+	3	H	Cape	2	5.15	0	26.79	5.31	0	1.12	14.28	47.31
Q+	3	H	Cape	2	2.45	0	1.44	0	0	16.33	26.07	53.68
Q+	3	H	Cape	2	12.94	0	1.86	0	0	18.10	24.52	42.56
Q+	3	H	Cape	1	0	0	3.85	0	0	0	22.75	73.38
Q+	3	H	Cape	1	0	0	9.90	3.94	0	1.55	20.27	64.31
Q+	3	H	Cape	1	0	0	2.40	1.64	0	1.42	23.19	71.33
Q+	3	H	Cape	1	0	0	10.89	0	0	1.43	20.42	67.24
Q+	3	H	Cape	1	0	0	1.03	0	0	1.26	23.72	73.98
Q+	3	H	Cape	1	0	0	4.45	0	0	1.67	24.19	69.67
Q+	3	H	Cape	1	0	0	10.94	2.02	0	0	18.94	68.08
Q+	3	H	Cape	1	0	0	4.92	2.06	0	1.57	23.42	68.01
Q+	3	H	Cape	1	0	0	3.47	1.55	0	0	21.27	73.69
Q+	3	H	Cape	1	0.90	0	24.02	3.05	1.04	1.45	17.15	52.36
Q+	3	H	Cape	1	0	0	4.14	0	0	0	21.13	74.71
Q+	3	H	Cape	1	0	0	14.87	16.28	0	0.87	14.20	53.75
Q+	3	H	Cape	1	0	0	1.40	0	0	0.99	21.80	75.80
Q+	3	H	Cape	1	0	0	3.96	16.89	0.87	1.11	18.87	58.28
Q+	3	H	Cape	1	0	0	16.48	7.31	0	0	17.46	58.73
Q+	3	H	Cape	1	0	0	5.28	2.61	0	1.42	20.63	70.04
Q+	3	H	Cape	1	0	0	4.08	2.43	0	1.55	22.78	69.13
Q+	3	H	Cape	1	0	0	21.44	15.91	1.21	0.72	13.73	46.96
Q+	3	H	Cape	1	0	0	4.47	0	0	1.16	21.12	73.23
Q+	3	H	Cape	1	0	0	9.96	2.33	0	1.12	26.94	59.61
Q+	3	H	Cape	1	0	0	9.91	3.97	0	1.04	19.24	65.81
Q+	3	H	Cape	1	0	0	3.35	3.95	0	1.46	19.62	71.60
Q-	1	B	Clone	2	48.34	0	4.83	0	0	7.09	14.91	24.81
Q-	1	B	Clone	2	52.17	0	7.24	0	0	5.75	12.30	22.51
Q-	1	B	Clone	2	43.52	0	5.33	0	0	8.01	16.13	26.98
Q-	1	B	Clone	2	65.17	0	12.83	0	0.30	3.13	6.62	11.92
Q-	1	B	Clone	2	48.30	0	8.77	0	0	5.85	12.41	24.64
Q-	1	B	Clone	2	51.61	0	10.05	0	0	6.56	11.84	19.92
Q-	1	B	Clone	2	49.38	0	6.66	0	0	4.94	13.32	25.68
Q-	1	B	Clone	2	45.60	0	8.06	0	0	6.18	14.33	25.80
Q-	1	B	Clone	2	62.20	0	10.64	0	0.18	3.16	8.31	15.48
Q-	1	B	Clone	2	57.30	0	7.60	0	0	5.46	11.04	18.57
Q-	1	B	Clone	2	52.55	0	6.34	0	0	4.15	12.06	24.88
Q-	1	B	Clone	2	57.10	0	6.08	0	0	5.64	11.53	19.63
Q-	1	B	Clone	2	52.94	0	9.52	0	0	5.34	11.56	20.61
Q-	1	B	Clone	2	61.02	0	10.58	0	0.13	4.25	9.25	14.75
Q-	1	B	Clone	2	63.25	0	8.13	0	0	3.35	8.86	16.38

Q-	1	B	Clone	2	54.06	0	12.37	0	0	3.51	10.32	19.77
Q-	1	B	Clone	2	53.29	0	6.93	0	0	5.54	13.12	21.10
Q-	1	B	Clone	2	49.07	0	5.66	0	0	7.12	14.04	24.09
Q-	1	B	Clone	2	53.42	0	5.63	0	0	5.65	13.02	22.25
Q-	1	B	Clone	2	60.60	0	12.31	0	0	3.03	8.581	15.46
Q-	1	B	Clone	2	39.14	0	5.04	0	0	6.56	16.55	32.69
Q-	1	B	Clone	2	50.77	0	10.40	0	0	5.50	12.17	21.14
Q-	1	B	Clone	2	49.94	0	7.72	0	0	4.15	12.71	25.46
Q-	1	B	Clone	2	53.02	0	11.10	0	0	4.73	11.20	19.92
Q-	1	B	Clone	2	49.08	0	7.33	0	0	6.16	13.67	23.74
Q-	1	B	Clone	2	49.82	0	0.62	0	0	7.80	15.24	26.50
Q-	1	B	Clone	2	43.98	0	4.01	0	0	7.67	15.61	28.74
Q-	1	B	Clone	2	43.98	0	4.01	0	0	7.63	15.61	28.74
Q-	1	B	Clone	2	69.90	0	10.49	0	0	1.85	5.687	12.06
Q-	1	B	Clone	2	52.64	0	8.74	0	0	5.71	11.27	21.63
Q-	1	B	Clone	2	51.59	0	8.88	0	0	6.11	12.53	20.86
Q-	1	B	Clone	2	50.66	0	9.25	0	0	4.44	12.71	22.94
Q-	1	B	Clone	2	53.56	0	5.87	0	0	6.09	12.75	21.70
Q-	1	B	Clone	2	53.98	0	8.14	0	0	5.09	12.07	20.69
Q-	1	B	Clone	2	53.84	0	10.07	0	0	4.82	11.05	20.21
Q-	1	B	Clone	2	9.99	0	2.58	0	0	12.48	27.63	47.30
Q-	1	B	Clone	2	59.46	0	9.54	0	0	3.27	8.91	18.79
Q-	1	B	Clone	2	60.52	0	13.80	0	0	3.64	7.94	14.07
Q-	1	B	Clone	2	41.08	0	5.61	0	0	7.99	17.13	28.17
Q-	1	B	Clone	2	49.78	0	8.30	0	0	5.50	12.83	23.56
Q-	1	B	Clone	2	54.07	0	0.27	0	0	8.08	14.83	22.72
Q-	1	B	Cape	1	0	0	6.37	0	0	1.34	22.73	69.54
Q-	1	B	Cape	1	0	0	2.21	0	0	2.27	22.07	73.42
Q-	1	B	Cape	1	1.45	0	11.54	0	0	1.86	24.13	61.00
Q-	1	C	Clone	2	66.67	0	5.58	0	0	2.90	11.32	13.49
Q-	1	C	Clone	2	65.75	0	8.15	0	0	4.52	10.21	11.35
Q-	1	C	Clone	2	52.52	0	7.88	0	0	4.98	15.20	19.39
Q-	1	C	Clone	2	62.85	0	6.85	0	0	4.43	11.83	14.02
Q-	1	C	Clone	2	63.30	0	7.22	0	0	4.78	11.31	13.36
Q-	1	C	Clone	2	54.83	0	5.97	0	0	4.87	14.20	20.14
Q-	1	C	Clone	2	35.70	0	5.40	0	0	6.22	22.57	30.09
Q-	1	C	Clone	2	60.06	0	9.87	0	0	3.38	11.36	15.31
Q-	1	C	Clone	2	58.96	0	7.11	0	0	5.12	12.96	15.86
Q-	1	C	Clone	2	59.17	0	11.23	0	0	3.70	11.38	14.50
Q-	1	C	Cape	2	38.69	0	4.06	0	0	11.73	17.78	27.71
Q-	1	C	Cape	2	11.10	0	1.65	0	0	20.31	26.69	40.22
Q-	2	E	Clone	2	40.84	0	3.75	0	0	5.54	19.25	30.59
Q-	2	E	Clone	2	48.89	0	5.47	0	0	3.72	15.24	26.66
Q-	2	E	Clone	2	54.26	0	5.74	0	0	5.01	14.59	20.39
Q-	2	E	Clone	2	51.20	0	7.04	0	0	4.13	14.69	22.92
Q-	2	E	Clone	2	54.49	0	11.52	0	0	2.92	10.52	20.52

Q-	2	E	Clone	2	52.98	0	9.68	0	0	4.57	13.71	19.04
Q-	2	E	Clone	2	62.54	0	10.38	0	0	2.82	8.99	15.24
Q-	2	E	Clone	2	52.45	0	9.51	0	0	3.12	12.39	22.54
Q-	2	E	Clone	2	54.88	0	6.03	0	0	5.20	14.00	19.88
Q-	2	E	Clone	2	56.74	0	4.92	0	0	4.09	14.85	19.37
Q-	2	E	Clone	2	58.20	0	10.25	0	0	2.47	10.95	18.08
Q-	2	E	Clone	2	52.87	0	7.96	0	0	4.34	14.98	19.83
Q-	2	E	Clone	2	60.69	0	10.28	0	0	3.80	10.61	14.60
Q-	2	E	Clone	2	48.26	0	8.39	0	0	4.04	14.72	24.57
Q-	2	E	Clone	2	47.68	0.18	9.77	0	0	4.37	15.60	22.37
Q-	2	E	Clone	2	61.34	0	7.52	0	0.36	4.11	11.29	15.35
Q-	2	E	Clone	2	50.21	0	6.83	0	0	6.13	15.94	20.87
Q-	2	E	Clone	2	55.69	0	5.66	0	0	4.32	11.90	22.40
Q-	2	E	Clone	2	46.77	0	5.22	0	0	6.75	17.29	23.94
Q-	2	E	Clone	2	58.79	0	5.93	0	0	4.54	12.71	18.01
Q-	2	E	Clone	2	57.91	0	10.62	0	0.32	3.43	11.37	16.32
Q-	2	E	Clone	2	56.11	0.14	8.14	0	0	4.50	12.90	18.19
Q-	2	E	Clone	2	57.90	0	6.47	0	0.37	4.07	11.62	19.55
Q-	2	E	Cape	2	40.38	0	6.64	0	0	10.65	13.49	28.83
Q-	2	E	Cape	2	31.12	0	3.51	0	0	8.07	17.86	39.42
Q-	2	E	Cape	2	6.87	0	2.45	0	0	15.36	26.08	49.22
Q-	2	E	Cape	2	53.21	0	2.13	0	0	7.08	13.27	24.28
Q-	2	E	Cape	2	2.22	0	2.59	0	0	16.71	29.33	49.12
Q-	2	E	Cape	2	56.57	0	5.14	0	0.37	6.66	11.73	19.50
Q-	2	E	Cape	2	50.97	0	6.04	0	0.32	6.49	13.11	23.03
Q-	2	E	Cape	2	34.58	0	15.73	0	0	7.18	14.24	28.25
Q-	2	F	Clone	2	55.34	0	8.39	0	0	5.28	12.79	18.18
Q-	2	F	Clone	2	55.20	0	10.23	0	0	5.02	11.94	17.58
Q-	2	F	Clone	2	28.17	0	7.13	0	0	9.33	21.75	33.59
Q-	2	F	Clone	2	68.28	0	10.55	0	0	2.70	6.83	11.61
Q-	2	F	Clone	2	51.61	0	7.44	0	0	5.31	13.97	21.64
Q-	2	F	Clone	2	57.57	0	5.40	0	0	5.38	13.40	18.23
Q-	2	F	Clone	2	48.67	0	8.95	0	0	6.71	15.03	20.62
Q-	2	F	Clone	2	49.41	0	9.58	0	0	4.96	13.92	22.09
Q-	2	F	Clone	2	51.73	0	8.28	0	0	5.88	13.84	20.25
Q-	2	F	Clone	2	58.21	0	5.25	0	0	4.51	12.15	19.86
Q-	2	F	Clone	2	56.50	0	10.17	0	0	4.48	10.76	18.08
Q-	2	F	Clone	2	59.54	0	13.95	0	0.21	4.10	9.07	13.13
Q-	2	F	Clone	2	52.87	0	9.33	0	0	5.88	12.65	19.25
Q-	2	F	Clone	2	53.08	0	9.20	0	0.29	4.85	12.32	20.25
Q-	2	F	Clone	2	66.47	0	6.52	0	0.38	4.45	9.28	12.86
Q-	2	F	Clone	2	61.48	0	8.99	0	0	5.16	10.13	14.22
Q-	2	F	Clone	2	33.93	0	6.78	0	0	7.42	20.68	31.15
Q-	2	F	Clone	2	53.75	0	9.65	0	0	5.85	12.72	18.00
Q-	2	F	Clone	2	60.69	0	9.69	0	0	4.44	10.08	15.07
Q-	2	F	Clone	2	56.12	0	7.29	0	0	5.73	13.16	17.68

Q-	2	F	Cape	2	24.4	0	2.62	0	0	16.87	20.22	35.81
Q-	2	F	Cape	1	0	0	0.83	0	0	20.28	26.36	52.50
Q-	2	F	Cape	2	53.25	0	3.75	0	0	8.90	13.76	20.32
Q-	2	F	Cape	2	33.18	0	3.92	0	0	12.27	19.66	30.94
Q-	2	F	Cape	2	35.19	0	2.33	0	0	10.34	18.59	33.53
Q-	2	F	Cape	2	33.01	0	4.10	0	0	11.26	19.90	31.71
Q-	2	F	Cape	2	34.71	0	3.10	0	0	13.09	19.17	29.91
Q-	2	F	Cape	2	44.78	0	4.75	0	0	9.98	16.59	23.88
Q-	2	F	Cape	2	54.69	0	7.22	0	0	8.23	11.54	18.30
Q-	2	F	Cape	2	6.77	0.57	13.17	0	0	1.87	22.81	54.79
Q-	2	F	Cape	2	20.93	0	2.22	0	0	15.30	23.16	38.36
Q-	2	F	Cape	2	31.51	0	3.76	0	0	11.70	19.64	33.37
Q-	2	F	Cape	2	24.24	0	4.02	0	0	17.04	19.22	35.46
Q-	2	F	Cape	2	54.16	0	3.81	0	0	7.79	12.23	21.98
Q-	2	F	Cape	2	7.56	0	2.56	0	0	14.87	26.89	48.10
Q-	2	F	Cape	2	42.85	0	4.82	0	0	9.81	15.65	26.84
Q-	2	F	Cape	2	47.76	0	14.22	0	0	4.43	12.66	20.90
Q-	2	F	Cape	2	1.82	0	1.73	0	0	19.62	30.84	45.97
Q-	2	F	Cape	2	4.27	0	1.12	0	0	16.90	27.59	50.15
Q-	2	F	Cape	2	53.37	0	9.02	0	0	6.39	10.61	20.58
Q-	3	G	Cape	2	56.52	0	4.52	0	0	6.63	13.12	19.19
Q-	3	G	Cape	2	13.06	0	2.15	0	0	13.30	22.12	49.34
Q-	3	G	Cape	2	53.53	0	2.82	0	0	7.56	14.25	21.83
Q-	3	G	Cape	2	15.52	0	2.84	0	0.76	13.34	23.23	44.28
Q-	3	G	Cape	2	41.47	0	5.65	0	0.33	10.18	17.52	24.81
Q-	3	G	Cape	2	23.18	0	3.83	0	0	13.32	20.62	39.02
Q-	3	G	Cape	2	22.80	0	9.80	0	0	14.36	20.10	32.90
Q-	3	G	Cape	2	44.17	0	4.98	0	0	10.58	13.85	26.38
Q-	3	G	Cape	2	24.46	0	2.62	0	0	16.87	20.22	35.81
Q-	3	G	Cape	2	38.88	0	5.68	0	0	7.28	19.32	28.82
Q-	3	G	Cape	2	36.05	0	0.91	0	0	11.58	19.17	32.25
Q-	3	G	Cape	2	56.71	0	4.97	0	0	5.02	11.58	21.69
Q-	3	G	Cape	2	34.56	0	5.21	0	0	8.79	17.66	33.77
Q-	3	G	Cape	2	51.53	0	7.84	0	0	7.08	12.14	21.38
Q-	3	I	Clone	2	67.77	0	5.90	0	0.57	3.44	9.94	12.34
Q-	3	I	Clone	2	57.94	0	1.75	0	0	7.93	15.11	17.24
Q-	3	I	Cape	2	51.12	0	13.15	0	0.24	5.32	14.09	16.05
Q-	3	I	Cape	2	54.39	0	1.73	0	0	8.64	15.41	19.79
Q-	3	I	Cape	2	56.64	0	6.27	0	0.35	7.90	11.71	17.09
Q-	3	I	Cape	2	46.08	0	3.79	0	0.46	8.50	15.32	25.83
Q-	3	I	Cape	2	57.68	0	4.54	0	0	8.87	12.58	16.31