Queens, Pseudoqueens and Laying Workers; Reproductive Competition in the Cape Honeybee (*Apis mellifera capensis* Eschscholtz)

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ABSTRACT

In honeybees (*Apis mellifera* L.) the queen monopolises reproduction. However, especially after queen loss, workers can lay eggs, but are unable to mate. They produce haploid male offspring (drones) from unfertilised eggs via arrhenotokous parthenogenesis. In contrast, workers of the honeybee subspecies *Apis mellifera capensis* Eschscholtz typically produce diploid female offspring from unfertilised eggs thelytokously. After queen loss and without queen-derived brood *A. m. capensis* colonies can successfully requeen from worker-derived brood. This, however, is a relatively rare event in wild populations. Moreover, worker-derived queens were described to be smaller, more worker-like and reproductively inferior. On the other hand, the fixation of the thelytokous trait relies mainly on sufficient numbers of viable drones produced by worker-derived queens. Small numbers of reproductively inferior worker-derived queens in *A. m. capensis* populations would be clearly counter-intuitive. It is therefore necessary to quantify the significance of worker-dependant queen rearing pathways on the individual (queen) and on population level.

Reproductive inferiority of worker-derived queens could not be confirmed on the individual (queen) level when comparing parameters indicating potential reproductive success of queen- and worker-derived queens. Queen- and worker-derived queens clearly showed a congruent range of reproductive performance.

In queen rearing preference tests, increased acceptance of worker-derived female larvae was exactly counterbalanced by increased mortality, resulting in an equal number of eclosing virgin queens from an equal number of grafts in both test groups. Larval survival and successful eclosion is a prerequisite for a queen's reproductive success. I found no difference in eclosion success for queen- and worker-derived virgin queens, indicating a similar potential for reproductive success in both queen types. Assessments of the developmental patterns of colonies headed by both queen and worker-derived queens in long-term experiments revealed no significant differences in reproductive success. Colonies headed by queen-derived queens and colonies headed by worker-derived queens could not be separated when comparing the different developmental pathways observed or from differences in worker-force. Reproductive dominance in *A. m. capensis* appeared to be determined by a function of relative compositional and absolute quantitative pheromonal patterns, where individuals, which produce compositionally most queen-like blends in highest quantities, occupy top positions. Queen- and worker-derived virgin queens occupied intermediate positions between pseudoqueens and mated queens. However, no significant differences between the pheromonal status of queen- and worker-derived virgin

queens were observed, suggesting a similar range of reproductive dominance for both queen types. In behavioural bioassays queen- and worker-derived virgin queens appeared to be similarly attractive to clustering workers and to drones in a drone congregation area, indicating no differences in potential reproductive success for queens from both origins for those parameters. The significant influence of the queen substance 9-ODA on attractiveness to workers and drones was confirmed.

Rare requeening events from worker-derived female brood in queenless *A.m. capensis* do not satisfactorily explain the fixation of the thelytokous trait at a population level. I observed *A. m. capensis* worker ovipositing into empty artificial queen cell cups in queen-right colonies. The queen was confined behind a queen excluder grid in a separate compartment of the colony, to imitate reduced pheromonal flow, similar to swarming or superseding colonies. Eggs oviposited by workers in artificial queen cell cups were readily accepted for queen rearing and successful eclosion of viable virgin queens was observed.

Consequently I suggested an alternative worker-dependant reproductive pathway in *A. m. capensis*, which was never described before: In swarming or superseding queenright colonies, laying workers may directly compete with the queen for reproductive success by ovipositing (instead of the queen) into natural queen cell cups. At a population level this reproductive tactic may result in large numbers of worker-derived queens of high reproductive quality in natural populations of *A. m. capensis*.

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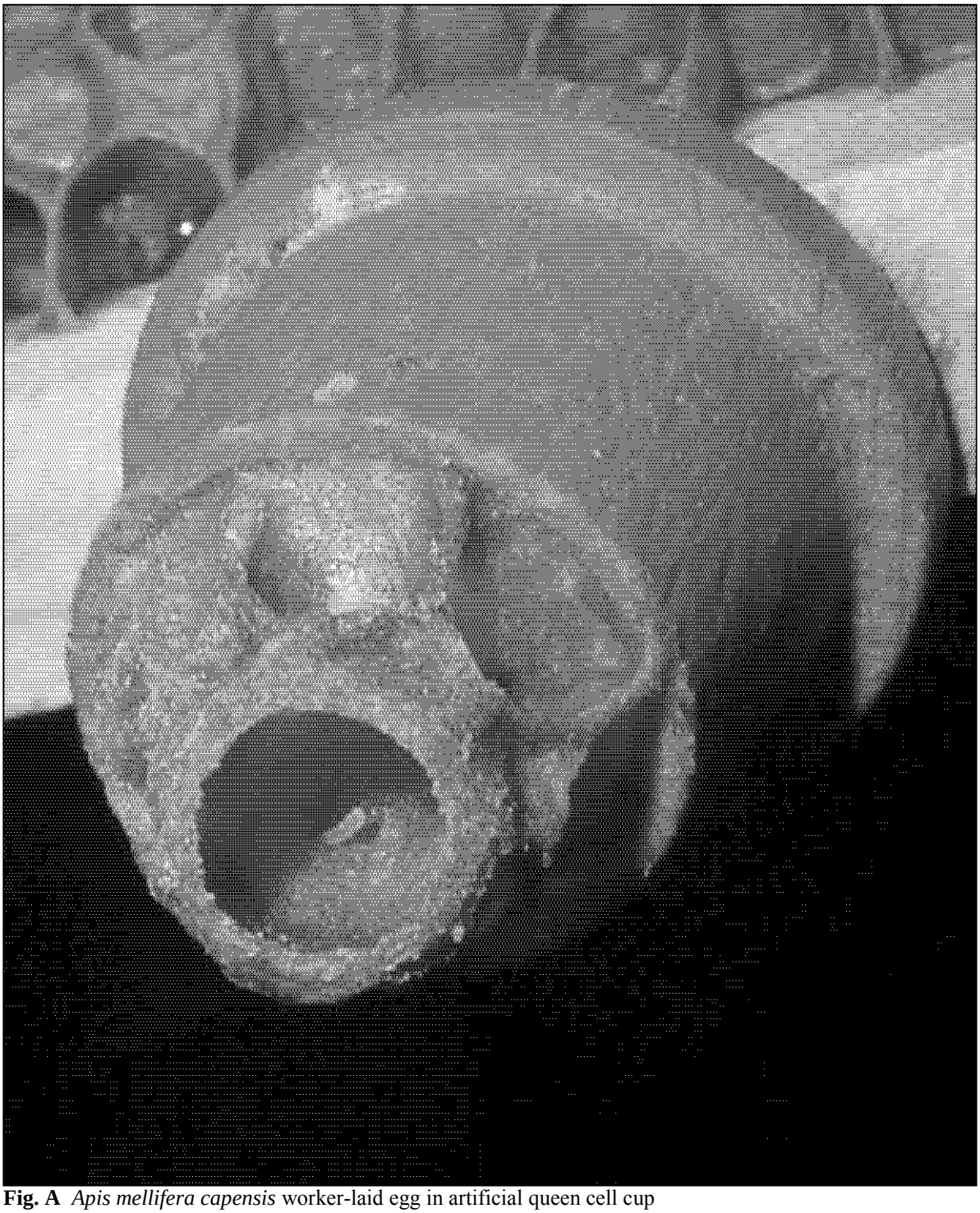

Fig. A *Apis mellifera capensis* worker-laid egg in artificial queen cell cup

This thesis is partly based on the following publications:

CHAPTER 3: Muerrle TM, Neumann P, Hepburn HR, Radloff SE (2007) Increased mortality counterbalances preferential acceptance of worker-derived female larvae in Cape honeybee queen rearing experiments. *Manuscript.*

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CHAPTERS 6 and 7: Muerrle TM, Hepburn HR, Radloff SE, Pirk CWW (2007) Worker and drone attraction to *Apis mellifera capensis* virgin queens. *Manuscript.*

CHAPTER 8: Muerrle TM, Neumann P, Hepburn HR, Radloff SE (2007) Developmental patterns of colonies headed by queen and worker offspring in *Apis mellifera capensis*. *Manuscript.*

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CHAPTER 11: Muerrle TM, (2007) Queenright colonies as starter-finishers for Cape honeybee (*Apis mellifera capensis*) queen-rearing experiments. *South African Bee Journal. Submitted.*

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Section 1

GENERAL INTRODUCTION

"The story of the Cape bee is a kind of coloured flower in most of the books on the biology of honeybees. It is astonishing and amusing and not quite believable. It remains a curious story until one sees it with one's own eyes and becomes convinced it is a reality."

(Friedrich Ruttner 1977a)

CHAPTER 1

Apis mellifera capensis Eschscholtz: a historical review

Introduction

Scientific research is triggered by new discoveries and improved analytical methods as well as by incidences which threaten organisms or biodiversity, or which have negative economical impact. Absence of scientific research may (but not necessarily) indicate a lack of actual problems. The pattern of scientific output by numbers of publications, therefore leaves typical 'footprints' during the course of time, accumulations of scientific knowledge, indicating actual problems or new ideas. The history of scientific research on the African honeybee subspecies *Apis mellifera capensis* Eschscholtz is a good, well documented, example and can be followed from the first description of a specimen and attempts at classification in the early nineteen hundreds up to complex genetical or socio-ecological hypotheses under research today. Here I will concentrate on peaks of interest to outline the broad spectrum of topics, to demonstrate development of ideas and to recognise trends for future research. This approach leaves out smaller 'traces', and is therefore incomplete by intention. It should nevertheless, in all its limitations, allow an understanding of the significance of further studies on *A. m. capensis*.

The honeybee subspecies *A. m. capensis*, endemic to the fynbos biome, a relatively small area along the southern coast of Africa (Hepburn and Crewe 1990, 1991a,b; Hepburn and Radloff 2002), is unique in its ability to produce diploid female offspring from unfertilised worker-laid eggs (Onions 1912, 1914; Kerr and Laidlaw 1956; Kerr and

Portugal Araújo 1958; Anderson 1963) in a process called thelytokous parthenogenesis. All other honeybee subspecies worldwide are, with negligible exceptions (Mackensen 1943; Tucker 1958), only able to produce male offspring (drones) parthenogenetically (arrhenotokous parthenogenesis), in accordance with the hymenopteran haplodiploid sex-determination system. This uniqueness has far reaching consequences and is a starting point for a number of different but strongly interconnected research pathways.

Thelytoky

It is beyond doubt that the merit of being the first to accurately observe and describe the occurrence of female worker brood derived from laying workers in *A. m. capensis* belongs to Onions (1909, 1912). His report was scientifically correct and most of his observations are standard knowledge today. It is, however, necessary to mention Lord De Villiers, who published an article about Cape bees in the journal *Nature* in 1883 (Hepburn 1990a). De Villiers (1883) described in detail the occurrence of eggs, which developed into workers in a Cape bee colony with a caged queen. He repeated the experiment several times but then obviously failed to recognise the true origin of the brood and concluded that "The only explanation of the presence of the eggs in the cells was that they had been laid or passed by the queen through the holes of the cage and taken up and deposited into the cells by some of the workers."

The reactions, however, to Onions' observations were full of adverse criticisms (Attridge 1918). It seemed incredible that South Africa should have a strain of honeybees that differed so radically from all other closely studied races of the honeybee (Lundie 1954). More evidence accumulated with reports of similar observations from different authors (Terrell 1912; Trollip 1912; Mowbray 1916) and when Onions' findings found full appreciation and confirmation by the Government entomologist of Rhodesia (now Zimbabwe), Rupert W. Jack (1916). But despite increasing evidence and Mackensen's (1943) and Tucker's (1958) observations that thelytokous parthenogenesis occurred in European honeybee subspecies as well but to a far lesser degree, it took more than fifty years before Onions' remarkable discovery was accepted on an international scale (Hepburn 1990b). Confidence in the peculiar biology of the Cape bee stemmed largely from Kerr and Portugal Araújo (1958) and Anderson (1963). However, two main issues remained unresolved, namely: the cytological and genetical mechanism for diploidy of unfertilised eggs.

Woyke (1979) observed 44% versus 97% larval survival when comparing worker-derived and queen-derived brood and suggested automictic fusion of two haploid egg nuclei with the same sex alleles, and consequently the production of diploid drone larvae, which are eliminated by the workers. Verma and Ruttner (1983) analysed the cytological mechanisms for thelytoky in honeybees in detail and, confirming Woyke's suggestion, demonstrated the occurrence of a meiotic division and subsequent fusion of the egg pronucleus with one descendant of the first polar body, to form the diploid zygote nucleus.

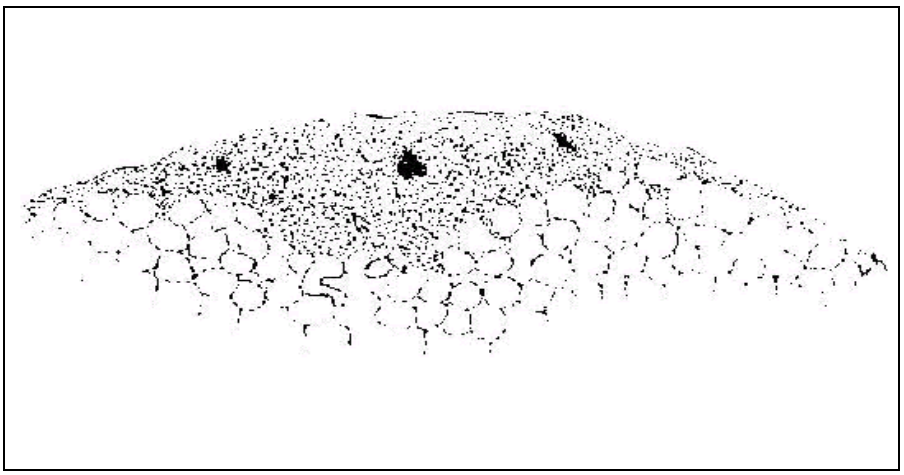

Fig. 1.1. Central fusion of two haploid nuclei during meiosis II in a worker-laid egg of *A. m. capensis* (from Verma and Ruttner 1983)

Because this mechanism does not allow for interchromosomal recombination, thelytoky in honeybee workers was supposed to lead to true clonal offspring. Analysis of worker offspring using DNA fingerprinting, confirmed clonal reproduction of laying workers with strongly reduced crossing over rate (Moritz and Haberl 1994; Baudry et al. 2004). The genetic basis for thelytoky seemed to be surprisingly simple. Ruttner (1988) used backcross experiments with *capensis* queens, inseminated with single drones from hybrid (*carnica* x *capensis*) queens. The inseminated queens were removed from their nuclei prior to the hatching of their brood. The start of worker-ovipositing was recorded and the sex of the worker's offspring was determined after pupation, resulting in a strongly bimodal segregation, close to the expected Mendelian 1:1 ratio (thelytoky : arrhenotoky), suggesting two alleles of one single major recessive gene (Moritz 1990).

The results however did not account for the regular occurrence of a small number of drones in the thelytokous colonies, and therefore included the possibility of some form of amphitoky (intermediate or mixed parthenogenesis) as suggested by Cooke (1985). Lattorff et al. (2005) used a double backcross system and inseminated queens reared from laying

worker offspring with semen of *carnica* drones. From their offspring, queens were reared representing the F1 generation. These heterozygous queens were artificially inseminated with the mixed semen of one *capensis* and one *carnica* drones. The laying worker eggs of those test-crosses were genotyped to determine their paternity and their ploidy level (haploid = arrhenotokous, and diploid = thelytokous). Several eggs of individual workers were analysed, to detect any mixed forms of parthenogenesis. The results confirmed Ruttner's (1988) conclusion, that the type of parthenogenesis in laying honeybee workers is determined by a single major recessive gene on one locus. No mixed form of parthenogenesis was recorded. Workers either exclusively laid female or male eggs (Lattorff et al. 2005) The occurrence of drones in *A. m. capensis* laying worker colonies is therefore unmistakably a result of hybridisation with arrhenotokous subspecies.

Classification and distribution

A first description of *A. m. capensis* dates back to the late eighteen hundreds, published by Carl de Geer (1778) in Volume VII, Memoire X of his 'Mémoires pour servir a l'histoire des insectes': "Abeille noire, à deux bandes transverses jaunes fauves sur le devant du ventre et à ailes vitrees. *Apis (fulvo-cincta) nigra*, (...)." (cited from Crane 1980). The title of De Geer's paper suggests that the specimen he named *Apis (fulvo-cincta) nigra* was found near the Cape of Good Hope, but the type specimen, still held at the Swedish Museum of Natural History, lacks authentic details of its provenance (Crane 1980). Description and presumed origin would match with what currently is known as *Apis mellifera capensis*, a name assigned by Eschscholtz (1821).

The entire 19th and beginning of the 20th century was characterised by different relatively unsuccessful attempts to separate the subspecies *A. m. capensis* from its neighbour *A. m. scutellata* and by classification errors. Buttel-Reepen (1906) grouped all African varieties under the subspecies name *unicolor*, a name used by Latreille (1804) for honeybees collected in Madagascar. The name stuck, especially for the generally darker Cape honeybees. Alpatov (1933) used honeybee samples, which he received from Lundie under the names *A. m. adansonii* collected near Pretoria and *A. m. unicolor* collected near Cape Town, for biometrical investigations. And even much later Lundie (1954) referred to the dark bees from the Cape as *A. m. unicolor* showing that specimens of African honeybees were still named according to their colour with no consideration of their geographical origin.

The specific biology of *A. m. capensis* was first used by Kerr and Portugal Araújo (1958) for subspecies differentiation: The presence of a spermatheca in workers and the ability to produce female offspring from unfertilised worker-laid eggs.

Ruttner (1976, 1981) was the first to use multivariate statistical analysis on a number of morphological characters, but was not able to separate *capensis* from *scutellata* on morphometric criteria alone. He suggested that, despite the lack of clear morphometric markers, three reproductive traits could separate *capensis* from *scutellata*: 1. diploid eggs from laying workers, 2. size of spermatheca and 3. ovariole number.

Initially the distribution of *capensis* was stated to coincide with the winter rainfall region of South Africa (Kerr and Portugal Araujo 1958; Smith 1961; Anderson 1963). Ruttner (1977b) claimed that pure (classical) *capensis* only occurred in a very small area around Cape Point, a very humid zone of the winter rainfall region. Finally the distribution was extended to more or less coincide with the fynbos biome (Tribe 1983; Hepburn and Crewe 1990, 1991a,b; Crewe et al. 1994). Only after the discovery that queenless colonies from the Eastern Cape were reluctant to rear emergency queens and regularly produced female brood from laying workers (Hepburn et al. 1988; Hepburn 1989), extensive sampling of the southern African honeybee populations was initiated to define the limits of distribution of the two subspecies (Hepburn and Crewe 1990). By using a combination of ovariole numbers and the sex ratio of laying worker offspring, the geographic location of the two races could be defined, obviously separated and stabilised by a broad band of hybridisation (Hepburn and Crewe 1991a,b). To verify this largely extended distribution defined by ovarial structure and sex of worker-derived offspring, morphometrical, pheromonal, and genetical markers were subjected to multivariate statistical analyses, revealing a structure of a highly complex, continuous population of honeybees (Hepburn and Radloff 2002) (Fig. 1.2.). Different propensities for or susceptibility to reciprocal invasiveness exhibited by *A. m. capensis*, *A. m. scutellata* and their hybrids pose further questions (Hepburn et al. 1998; Neumann et al. 2001a; Neumann and Hepburn 2002). At present therefore, the only way to use the subspecific names *A. m. scutellata* and *A. m. capensis* in a meaningful way, is in connection with a bee's geographical point of origin (Hepburn and Radloff 2002).

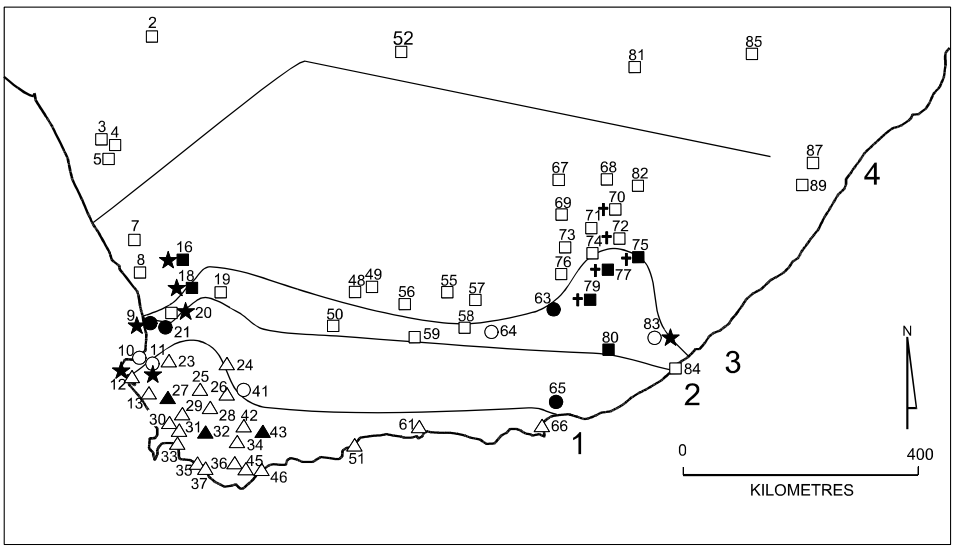

Fig 1.2. Map of southern Africa illustrating distributions of morphometric and non-morphometric features of *capensis*, *scutellata* and zone of introgression. Line 1 = northern limit of *capensis* morphocluster; line 2 = southern limit of *scutellata* morphocluster; line 3 = northern limit for thelytokous parthenogenesis; line 4 = northern limit for 100% frequency of *capensis* haplotype P_oQQa. Open triangles = *capensis* morphocluster, closed triangles = high morphometric variance; open squares = *scutellata* morphoclusters, closed squares = high morphometric variance; open circles = morphometric hybrids, closed circles = high morphometric variance in hybrids; stars = high sting pheromone variance; crosses = area of high mitochondrial and nuclear DNA variance (from Hepburn and Radloff 1998)

Biogeography

Several different but not mutually exclusive attempts have been made, to explain the relatively stable distribution of *A. m. capensis* and the persistence of its thelytokous reproductive trait, which is absent in its neighbouring subspecies *A. m. scutellata*. Does the ability to requeen from laying worker brood after queen loss offer an evolutionary advantage, and if so, why is it confined to the *capensis* territory and to the transitional zone of hybridisation, without spreading into the *scutellata* population?

In a first attempt to answer these questions, Ruttner (1977b) concluded that the possibility to requeen after queen loss from laying worker brood increases (however slightly) the fitness of the colony and that with each thelytokous generation an autoselection occurs directed towards a homozygous *capensis* genome. However, he left the question how the trait could have evolved unanswered. Guy (1976) suggested geographical isolation due to periodical flooding of the Cape flats, in combination with high predation pressure from Alpine swifts and the prevalence of high winds. Moodie (1983) observed the vanishing of 120 *scutellata* colonies introduced into *capensis* territory

within seven years, which eventually were overtaken by *capensis* and suggested bad adaptation of *scutellata* colonies to the harsh climate of the Western Cape. Tribe (1983) finally integrated different theories to account for the evolution of *capensis* and its separation from *scutellata*. The Cape bee, he suggested, evolved along the southern African coast, separated from the highlands by distinct mountain ranges, in adaptation to a unique environment, with strong winds, high rainfall frequency, sudden drastic changes in weather and relatively high predation pressure. Better cold adaptedness of *capensis* together with the ability to invade *scutellata* colonies would further enhance the fitness of the population to withstand pressure from the neighbouring subspecies. Thelytoky evolved as an adaptation to regular incidences of queen loss during mating flights. Moritz (1986) stated that a massive fitness disadvantage for thelytoky outside the autochthonous distribution of *capensis* must be assumed to explain its genetical restriction, and was able to give evidence for this in a population model. He showed that only under a high risk of loosing a queen during mating flights thelytoky should be advantageous, otherwise the production of drones should be favoured by natural selection.

Queens, pseudoqueens and laying workers

Invariably all first observations of thelytokous laying workers in *A. m. capensis* were made by dedicated apiculturists (Onions 1909, 1912, 1914; Terrel 1912; Trollip 1912; Mowbray 1916; Pettey 1921, 1922; Morkel 1946; Camp 1950; Stuart-Findlay 1953; Lundie 1954; Wheeler 1958), especially during apicultural queen-rearing and can be summarised as follows: *A. m. capensis* colonies were reluctant to accept grafted larvae, when standard rearing techniques in queenless breeding colonies were applied. The rapid occurrence of behavioural queen-like laying workers, termed 'pseudoqueens' (Onions 1912; Crewe and Velthuis 1980), was generally preceded by heavy intracolonial conflicts and fighting. Workers were recorded with large spermatheca and conspicuously developed ovaries containing large numbers of ovarioles. Laying worker brood was observed, even in queenright colonies, mainly in the honey supers above the queen excluder.

Only with Kerr's and Portugal Araujo's (1958) classification of African honeybees and after Anderson (1963) confirmed and extended earlier findings, was international acceptance of those observations achieved. Anderson (1963) noted the presence of anatomical laying workers with developed ovaries in queenright colonies, confirmed the presence and large size of a spermatheca in most workers (about half the size of a queen's spermatheca) which is vestigial in all European honeybee subspecies, and mentioned the

large number of ovarioles per ovary (average 19.6) compared to other subspecies (about 4, for *A. m. ligustica*) (Levin and Haydak 1951). Concerning the intracolonial conflicts, following queen loss, Anderson (1963) admitted that the reason for this fighting was unknown but suggested the formation of a new dominance hierarchy due to developing pseudoqueens.

Butler (1954, 1956, 1961) and Pain (1961) found a queen substance to be responsible for repression of worker ovary development and queen cell construction in queenright colonies. Gas-chromatography allowed the identification of the main component of this substance as 9-oxodecenoic acid (9-ODA), produced in the mandibular glands of queen bees. In addition, a worker substance, 10-hydroxy-2-decenoic acid (10-HDA), was identified in worker mandibular gland extracts (Barbier and Lederer 1960; Callow and Johnston 1960). Ruttner et al. (1976) was the first to detect the queen substance 9-ODA in *A. m. capensis* workers, later confirmed as comparable with queen levels in *capensis* pseudoqueens (Hemmling et al. 1979). Crewe (1982) demonstrated the ability of single Cape bee workers to quickly develop into pseudoqueens inducing retinue behaviour, the "queen court", when kept together with small groups of workers of other honeybee subspecies, due to their queen-like mandibular gland secretions. However, when kept with small groups of other Cape bees, their signals remained more worker-like, suggesting mutual pheromonal inhibition. The transition from worker-like to queen-like appeared to be gradual and the 9-ODA/10-HDA ratio was useful to quantify the developmental status. *A. m. capensis* queens had extremely high 9-ODA/10-HDA ratios, much higher than queens of other honeybee subspecies. This almost superqueen-like character of *capensis* may be related to the fact that they have to control workers that can easily turn into pseudoqueens (Crewe 1981, 1982, 1987).

After dequeening *capensis* colonies, the victims of aggression were always workers demonstrating signs of differentiation towards a more queen-like mandibular gland status and with developing ovaries (Crewe 1984), characteristics which co-vary in *capensis* (Hepburn 1992a). The attacks were comparable to those observed against foreign queens, the aggressees behaved submissively and offered food to worker bees in the vicinity or tried to escape or hide in cells (Crewe 1984). Aggression towards differentiating workers therefore may prevent the attacked workers from occupying the dominant position allowing the aggressor to gain competitive advantage (Van der Blom 1991). Societies are often characterised by the presence of a dominance hierarchy (Wilson 1975), in monogynous eusocial insects obviously represented by one queen dominating all workers and

monopolising colony reproduction. But dominance hierarchies within the worker caste also occur (Röseler and Röseler 1977; Simon et al. 2005). In honeybees a worker's dominance can be quantified by trophallactic behaviour and reproductive success. Dominant workers receive food from subordinate ones, and are able to develop their ovaries and lay eggs in queenless colonies (Korst and Velthuis 1982). Moritz and Hillesheim (1985) quantified and compared dominance of worker-offspring groups from eleven unrelated *A. m. capensis* laying worker mothers, and observed relatively low variation within the offspring groups suggesting a strong genetic component. Variation of dominance between the groups, however, was relatively high. In natural populations colonies with higher frequencies of altruists had higher fitness (better hoarding behaviour, higher numbers of produced queens and drones), than those with highly dominant individuals (Wilson 1975; Starr 1979), showing clearly that selection works on the individual and on the colony level in wild honeybee populations resulting in balanced dominance structures (Moritz and Hillesheim 1985; Hillesheim 1987; Hillesheim et al. 1989).

Individual honeybee workers, however, will try to increase their inclusive fitness by producing male sexuals in arrhenotokous subspecies. The polyandrous mating system, where the queen mates with several drones, results in great genetic heterogeneity within honeybee colonies and different patrilines represented by groups of super-sisters and half-sisters with potential reproductive conflict amongst workers. On the other hand, polyandry helps to align the colony's interests towards queen support, because all workers are relatively more related to the queen's male offspring than individual workers are to drones produced by sisters. The value of an own son to a worker, however, is large relative to the component of fitness it gains through helping rear the queen's offspring (Visscher 1989). Reproductive dominance and control by the queen is not absolute in honeybee colonies, and there is a high frequency of active attempted reproduction by workers. The low number of only about 0.12% of worker-derived males produced in queenright colonies, can only be explained by direct and active worker conflict in the form of worker policing, active removal of worker-laid eggs by reproductively passive workers (Visscher 1989).

A completely different situation prevails in the thelytokous honeybee subspecies *A. m. capensis*. Because worker reproduction results in clonal female offspring, absence or strongly reduced worker policing can be predicted due to the lack of relatedness differences between queen-derived and worker-derived female offspring (Greeff 1996a,b; Moritz et al. 1999). The Cape honeybee therefore provided a test case for the worker policing theory and worker-laid eggs should be accepted in queenright colonies. Results supported the

assumption that laying workers regularly produce offspring in queenright *capensis* colonies and provided an explanation for the relatively high number of physiological laying workers observed earlier by Anderson (1963).

In addition to the ability of *capensis* workers to produce the queen substance 9-ODA in their mandibular glands and to shift the 9-ODA/10-HDA ratio towards a more queen-like composition (Hemmling 1979; Hemmling et al. 1979; Crewe and Velthuis 1980; Crewe 1981, 1982, 1987), the presence of queen-like tergal glands was detected in *capensis* workers (Billen et al. 1986), which initially was only described in queens of European honeybee races (Renner and Baumann 1964). Tergal gland secretions elicit retinue behaviour and induce colony cohesion (Velthuis 1970a; Free 1987; Billen et al. 1986). The presence of queen-like tergal glands reinforced the unique nature of the chemical signalling system exhibited by *capensis* workers and indicated why it is possible for these workers to immediately act as pseudoqueens when placed in groups of workers of other subspecies (Crewe et al. 1990).

Within *capensis* colonies aggressive and trophallactic interactions between older workers and pheromonal contests between young honeybee workers start with queen loss. During this process new hierarchical dominance structures are established until one or more workers exceed a certain threshold of queen signals that suppresses further development in others (Moritz et al. 2004). This competition results in eight different developmental pathway options, described by Hepburn (1994), with the main fate-determining variables being the presence of eggs and brood, the occurrence and development of laying workers, newly constructed queen cells and the development of queen-like pheromonal bouquets by workers. Most optional pathways are detrimental to artificial queen-rearing attempts which explains why most queen-rearing techniques failed. However, Anderson (1965) solved the problem of queen-rearing in *A. m. capensis* by maintaining the delicate balance between inhibition of queen cell construction and development of pseudoqueens. The controlling pheromones of the queen had to be present in quantities, to de-inhibit queen cell construction but to inhibit significant restructuring of dominance hierarchies and pseudoqueen development (Hepburn and Radloff 1998). The queen-rearing system was based on separation of the queen in one compartment of the breeding colony using a wire mesh screen, allowing only restricted pheromonal exchange between queen and nurse bees.

Invasiveness

The early nineteenth century was characterised by a lively cosmopolitan trade in plant and animal species for private and economical reasons. In many cases this resulted in the reduction of biodiversity due to competitive advantages of foreign organisms invading their new habitats. Second only to the destruction of natural habitats by human activities, are invasions by alien species, one of the major causes for extinctions, resulting in the recent 'Biodiversity Crisis' with a loss of species rated as much as 50 times higher than at any time during the past 100 000 years (Campbell et al. 1999). Foreign honeybee subspecies were especially prone to trading because individual queen bees can easily be transported over extended periods of time and then successfully used for colony build up using the native worker-bee force. It is therefore not surprising that the first observations of the invasive character of *A. m. capensis* (and of *A. m. scutellata*) were made by apiculturists trying to breed alien honeybee subspecies (mainly *A. m. ligustica*) within the native range of African bees. The fate of introduced temperate stocks into Africa is well documented for South Africa. None of the introduced *ligustica* queens with their own progeny became established anywhere (Hepburn and Radloff 1998). In contrast, the introduction of the African subspecies *A. m. scutellata* into South America caused one of the most striking examples of honeybee invasions worldwide (Johannsmeier 1992; Lear 1992; Allsopp 1992a; Allsopp and Crewe 1993; Hepburn and Allsopp 1994; Neumann and Hepburn 2002; Moritz et al. 2005a).

The invasive character of *A. m. capensis* into a colony of temperate races was first recorded by Onions (1912). He observed a single laying *capensis* worker in a queenless *A. m. ligustica* colony, "which occupied a separate part of the apiary and was well out of the range of flight of young African bees (...). That she was a pseudoqueen was evidenced by her demeanour and by the peculiar attentions of the surrounding bees towards her."

Onions (1914), after moving to Rhodesia (now Zimbabwe), also described invasions of laying workers from one single queenless *capensis* colony into a large number of native *A. m. scutellata* hives, which he kept at the same apiary. His observations suggesting some dispersal mechanisms were fully confirmed by Jack (1916) and Lundie (1954). Guy (1976) noted that *scutellata* guard bees seemed to permit *capensis* workers to enter freely. Johannsmeier (1983) compiled a most detailed report of *capensis* invasions into *scutellata* colonies as a result of the introduction of eight *capensis* colonies into the Government apiary in Pretoria in 1977 for experimental purposes. All eight hives were killed after

termination of the experiments in 1979. Within those two years, however, some 40 to 50 *scutellata* colonies at the same apiary were infested with *capensis* laying workers as well as three further colonies as far away as 3.5 km, suggesting long range dispersal mechanisms. All infested colonies were killed which eradicated the problem. Johannsmeier (1983) concluded that infection of *A. m. scutellata* colonies results in the loss of the host queens, dwindling and finally absconding or annihilation of the infected colonies and stressed that this condition could persist in an apiary for more than ten years, unless drastic measures are taken to eliminate the Cape bees from the apiary. He therefore distinctly warned that it would be "foolish" for beekeepers to move Cape bees out of their endemic range.

Despite these concerns, large-scale transports of *A. m. capensis* colonies into the northern provinces were carried out in the winter of 1990, to take advantage of the Aloe honey flow. Thousand of colonies of *A. m. scutellata* are concentrated there every year by migratory beekeepers both for honey production and to increase their stock by splitting colonies (Allsopp 1992b). Two years later Allsopp (1992b, 1993) concluded that more than 10 000 *scutellata* colonies were infected with a loss of 50-70% of colonies in commercial apiaries with no end in sight. Because pollination requirements often could not be met, the issue transformed from a beekeeping disaster to a national problem: The '*Capensis* Calamity'. The severity and persistence of the problem made concerted actions necessary resulting in a range of practical and scientific approaches. Practical observations and the analysis of earlier invasions strongly suggested that infection was spread and persisted due to beekeeping activities, such as uncontrolled migration, uncontrolled splitting and excessive stress on colonies from pollination services (Allsopp 1993; Allsopp and Crewe 1993). A range of measurements to limit or reduce the spread of the problem was proposed, including the killing of all colonies infested with Cape laying workers, and restricted beekeeping practises. A "Siegfried Line" was drawn across the country, separating *capensis* and *scutellata* zones. Beekeepers would not be permitted to transport their bees across this line (Allsopp 1993; Allsopp and Crewe 1993).

Scientific research concentrated mainly on the mechanisms of the invasions and the question posed 'What makes *capensis* workers predisposed to successfully enter *scutellata* colonies and take over queen functions'. Beekman et al. (2000) showed that *capensis* larvae in European honeybee colonies received 'royal treatment', receiving higher quantities and more queen-like food than the European brood, resulting in *capensis* adult workers expressing transitions towards more queen-like characters. They concluded that *capensis* may therefore be termed a well adapted social parasite of other bee races. These

observations were confirmed by Calis et al. (2002) in *A. m. scutellata* colonies, where *capensis* brood was also preferentially treated and therefore prone to develop into pseudoqueens. Another characteristic behaviour of *capensis* workers, thought to be responsible for facilitating reproduction in host colonies, was shown to be their tendency to evade queen control by staying away from her. Because ovary development is suppressed by queen pheromones (Butler 1954b, 1956, 1957), avoiding the queen's influence may enhance ovary development and reproductive success (Moritz et al. 2001a).

Genetical studies revealed that the social parasite found in all infested colonies now was genetically very similar and possibly the repeated clonal offspring, a "pseudo-clone", of one highly successful and virulent thelytokous *A. m. capensis* worker, maybe even an obligate parasite, because its reproduction seemed to rely completely on host colonies resources (Kryger 2001). Wossler (2002) suggested that this pseudo-clone may be the end product of rapid selection of the most virulent line of *A. m. capensis* workers which had outcompeted the less virulent lines of invaders and was now extremely queen-like. The specific composition of its mandibular gland secretions, the presence of queen-like tergal glands and possibly the ability to mark eggs with a queen-like pheromone to avoid policing proved the queen-mimicking abilities of the parasite. Usurpations of *scutellata* colonies always followed similar patterns: invasion due to beekeeping activities, drifting or active dispersal; ovarial development, due to quick development of queen-like characteristics; loss of host queens, due to worker-neglect or aggression; unsuccessful requeening attempts, with no sexual reproductives observed in all cases; and finally pseudo-clone expansion and collapse, due to the inability of the pseudo-clone to maintain colony functions when lacking host workers (Martin et al. 2002b).

Neumann and Moritz (2002) finally gave some evolutionary evidence for the possibility of the pseudo-clone being on the verge of developing into a new obligate parasitic species capable of reinvading *A. m. capensis* colonies. Extreme selection favoured those laying workers that were able to develop queen-like signals most quickly and therefore suppressed development of their competitors. Clonal lineages of these selected, highly virulent parasites could theoretically be maintained indefinitely without any recombination with the social gene pool. The gene flow from the parasitic pseudo-clone into the social host population was either absent or extremely reduced because no sexual reproductives, neither drones nor queens, were produced. As a consequence, drift and mutation would increase the genetic distance between the highly virulent parasitic lineage

and the sexual reproducing host population, resulting in the development of a social parasitic species in real time (Neumann and Moritz 2002).

In a more moderate approach, Moritz (2002) used a population ecological host-parasite model to investigate the impact of the pseudo-clone in populations of *scutellata* host colonies in apiaries and in the wild. The results showed that infestations were likely to be fatal for apiary populations irrespective of beekeeping practises applied to compensate for losses. Wild populations were less likely to be affected and the pseudo-clone seemed unlikely to cause a threat to conservation of biodiversity. On the other hand, even low frequencies of parasitic *A. m. capensis* workers in wild honeybee populations could cause a permanent threat to beekeeping. Allsopp (2004) warned not to confuse cause and consequence: It was not the pseudo-clone, which initiated the *Capensis* Calamity, but irresponsible beekeeping practise promoting the invasion of *scutellata* hives by simple *capensis* laying workers. Selection resulted in a highly virulent clone with reduced dispersal and survival abilities, a freak result of beekeeping (Martin et al. 2002b) more, than a social parasite. Application of the recommended beekeeping practises now could be more fruitful than ever before.

Diseases, pests and parasites

The international apicultural literature is dominated by disease-related research. Recent invasions, for example by the parasitic mite *Varroa destructor* or the Nitidulid beetle *Aethina tumida* threaten apiculture worldwide. Bacterial, viral or fungal infections increase in populations of the European derived subspecies. The relatively low level of disease-related research on South African subspecies indicate that diseases and pests have little or no impact on wild or commercially used colonies. The relatively scarce literature allows for some positive perspectives.

Godlonton (1913) and Cooper (1914) mentioned the occurrence of bee pirate wasps (*Palarus latifrons*) being a nuisance for honeybees when the summers are dry and hot, causing high losses in foragers. Alpine swifts were recorded all year round to prey on Cape bees with incalculable impact (Cooper 1914; Clifton 1917; Moodie 1983). Lounsbury (1918) recorded the occurrence of European Foulbrood (*Melissococcus pluton*, *Streptococcus pluton*) in *A. m. capensis*. In 1940 Lundie published a full account of the life history of the Small Hive Beetle (*Aethina tumida*), a minor beetle pest of *A. m. capensis* and *A. m. scutellata*.

Moritz and Hänel (1984) predicted restricted development of the parasitic mite *Varroa destructor* due to shorter post-capping intervals in *A. m. capensis*. Laboratory and field observations indicated that the course of Varroatosis, which caused the death of millions of colonies world-wide, would not be as dangerous as in European honeybees. Because the post capping stage has high heritability, Moritz (1985) suggested the possibility for this trait to be bred into *A. m. carnica* and other European derived subspecies to increase their *Varroa*-tolerance, a possibility also considered by Woyke (1989). Further experiments confirmed the high tolerance of *A. m. capensis* against the mite (Moritz and Mautz 1990).

Hepburn (1991) noted the occurrence of high infestations of *capensis* workers with larvae of the parasitic Tachinid bee fly *Rondanioestrus apivorus* with incalculable impact. Fork-tailed Drongos were termed 'honeyguides of the Eastern Cape' by Hepburn (1992c). These birds are attracted to wild and hived honeybee colonies where they prey in small flocks on honeybees leaving or entering the colonies. Fork-tailed Drongos are used by traditional ethnic honey hunters in detecting honeybee colonies in apiaries or in the wild, no matter how well protected or remote the location. In South Africa theft and vandalism of hives is considered the most serious problem by many beekeepers, which may eventually disappear with the improvement of economic conditions observed currently (Swart et al. 2001b).

The obvious lack of, or the high tolerance and resistance against diseases and pests, increases the value of the African honeybee subspecies. International research increasingly not only tries to control pests and diseases but to find the reasons for resistance. The wild populations of honeybees in Southern Africa and especially those of *A. m. capensis* may reveal the answers.

CHAPTER 2

The significance of worker dependent queen-rearing pathways in *Apis mellifera capensis* Eschscholtz

Queen-derived versus laying worker-derived queens

In the hymenopteran haplodiploid sex determination system, unfertilised eggs with a haploid set of chromosomes usually develop into male individuals (arrhenotokous parthenogenesis), whereas fertilised eggs with a diploid chromosomal set develop into females (Crozier 1975). In honeybees (*Apis mellifera* L.), the system relies on a range of different sex alleles, whereby heterogeneity of sex alleles (in diploid individuals) results in the female phenotype and homogeneity of sex alleles (in diploid individuals) or a single sex allele (in haploid individuals) results in the male phenotype (Woyke 1969; Zander and Böttcher 1982). Diploid drones (with matching sex alleles) are removed by worker bees in the early larval stage (Woyke 1973) but are viable and can be artificially reared when grafted into specially prepared queen cups (Woyke 1969).

Ovarial development in honeybee workers is inhibited by queen pheromones in queenright colonies, but is de-repressed after queen loss or due to otherwise reduced pheromonal signals (Butler 1956). Reproductively more dominant workers receive protein-rich food from submissive workers and can develop into laying workers (Wilson 1971; Korst and Velthuis 1982; Seeley 1985). Laying workers are unable to mate (Wilson 1971) and their unfertilised haploid eggs (Bourke 1988; Ratnieks 1988; Bourke and Franks 1995) develop into drones (Crozier 1975; Ruttner 1992; Crozier and Pamilo 1996; but see Mackensen 1943 and Tucker 1958 for rare exceptions). In contrast, laying workers of the honeybee subspecies *A. m. capensis* are able to produce diploid eggs resulting in female offspring in a process called thelytokous parthenogenesis (Crozier and Pamilo 1996; but see Mackensen 1943 and Tucker 1958 for rare cases of thelytoky in other subspecies). During oogenesis, products of the meiotic division, the egg pronucleus (containing one form of the sex allele) and one of the descendants of the first polar body (containing the other form of the sex allele) fuse to produce the diploid zygote nucleus (Verma and Ruttner

1983). The mismatch in sex alleles is continuous and diploid female individuals are produced. Recombination does not contribute to any detectable variability during this form of automictic parthenogenesis (Slobodchikoff and Daly 1971; Moritz and Haberl 1994; Solignac et al. 2001), resulting in almost true clonal offspring (Kryger 2001).

In honeybee subspecies with arrhenotokous worker reproduction, female sexual reproductives (queens) can only be produced from fertilised queen brood. After queen loss, a colony without queen-derived eggs or larvae of the appropriate age is doomed. Laying workers produce a last batch of drones before the colony dies (Ruttner and Hesse 1981; Hastings 1989). Male contribution to colony fitness is often underestimated (Kraus et al. 2003; Königer et al. 2005), and worker-derived drones may significantly contribute to the gene-pool especially during periods where queen-derived drones are lacking in a population (Ruttner and Hesse 1981). In *A. m. capensis*, however, where laying workers produce female offspring thelytokously, female sexual reproductives can be produced from fertilised queen brood as well as from clonal worker offspring, offering an additional, alternative way for queen production in this subspecies (Ruttner 1977a,b; Hepburn 1992b, 1994). Caste determination in the female sex, besides the use of different cell types for both castes, depends entirely on nutrition in honeybees. Differences in the quantity and quality of larval food provided by the nurse bees are the decisive factors for queen or worker development (Weiss 1983a). Successful requeening of colonies lacking queen-derived brood was often observed and is well documented for *A. m. capensis* (Onions 1909, 1912; Pullinger 1922; Gough 1928; Lundie 1929, 1954; Morkel 1946; Ormsby 1958; Anderson 1961, 1963, 1965). In addition several successful attempts have been made, to artificially rear queens from laying worker larvae (Onions 1912; Jack 1916; Lundie 1954; Kerr and Portugal Araújo 1958; Tribe 1981, 1983; Moritz and Crewe 1988; Hartmann and Hartmann 1991; Woyke 1995; Lattorff et al. 2005).

It was, therefore, assumed that queen production from laying worker brood is a viable alternative route to re-establish a queenright condition after accidental queen loss, thereby rendering a colony of *A. m. capensis* theoretically immortal (Ruttner 1977b). Ruttner (1977a,b) suggested that this may be an evolutionary advantage resulting in a slight increase in colony fitness due to this specific reproductive trait. Moritz (1986), on the other hand, demonstrated in a population model that thelytoky was advantageous only under conditions of high queen loss probability, as observed in *A. m. capensis*. This confirmed earlier suggestions that thelytoky might have evolved to counteract high queen losses during mating flights due to strong winds and the harsh climate observed in the

geographical range of *A. m. capensis* (Tribe 1983). Allsopp and Hepburn (1997) demonstrated, that queen replacement based on the eggs of laying workers may arise following queen loss during mating or supersedure, but is relatively rare at 7%. Nonetheless it was shown, using genetic models of the trait, that even though only a small percentage of colonies produce queens in this way, the gene frequency for thelytoky may be remarkably high, and the trait would remain entrenched in the gene pool (Moritz 1986, 1989; Greeff 1996a,b; Moritz et al. 1998). Neumann et al. (2000a) observed that pure queenless *A. m. capensis* colonies tried to requeen more often from laying worker brood than queenless *capensis/scutellata* hybrid colonies, which would support the idea that the primary function of thelytoky is to replace lost queens. All models, however, were based on the assumption of similar reproductive success of laying worker- and queen-derived queens.

Onions (1912) noted that queens from workers' eggs were comparatively small and inferior looking, possibly due to poor feeding and that they were quickly superseded. Mowbray (1916) described queens from laying worker brood to appear smaller and darker than usual queens. In a correspondence between Dr. Lundie and A. R. Walter, from 1939, the latter reported a mated queen from laying worker offspring, which did not build up a colony very well (Hepburn 1989). Lundie (1954) reared queens from laying worker brood, and allowed them to head strong colonies in two standard ten-frame Langstroth brood chambers and described them as "in every way normal queens". Ormsby (1958) recorded a queenless swarm, which eventually successfully restored a queenright condition. The queen from a laying worker egg was described as appearing to function normally. Hartmann and Hartmann (1991) reported weak development of colonies headed by queens derived from laying worker offspring, due to a high degree of anti-social interactions between workers.

In general opinions tend to lean towards the assumption that queens derived from laying worker brood are somewhat lower in reproductive quality than queens derived from queen brood. They generally occurred after several failed requeening attempts in weakened laying worker colonies, where brood care and hygienic behaviour are greatly reduced, resulting in small, worker-like queens, which quickly were superseded (Muerrle, Neumann, Hepburn 2006 unpublished observations).

Quantitative data concerning the contribution of laying workers to population fitness were provided by Moritz et al. (1998), who used the *capensis/scutellata* interface as an experimental setting to evaluate the reproductive significance of laying workers. *Capensis* laying workers produce female offspring from which they are able to rear female

sexual reproductives. Therefore they pass on both mitochondrial (mt) and nuclear (nuc) genes to future generations. Because the *capensis* mitochondrial genome is propagated by queenright as well as queenless colonies, the cline for introgression of (nuc) markers should be different to (mt) markers in the *scutellata* population. And indeed, the (nuc) hybrid zone was shown to begin 200 km south of the (mt) hybrid zone, indicating significant contribution of worker reproduction, measurable at population level (Moritz et al. 1998).

However, the model does not allow any statements concerning the fitness of single colonies headed by queens derived from laying worker offspring or at the individual queen level. Woyke (1995) presented data, indicating reduced survival of larvae derived from laying worker brood. In rearing experiments the mortality of these larvae until eclosion was significantly increased, with a hatching rate of queen-derived workers at 70.3% compared to only 39.6% in the laying worker-derived group. He concluded that one of the causes for the dwindling of Cape colonies headed by laying workers, may be the low survival of larvae originating from workers. Because caste determination in the female sex is nutrient dependent (Weiss 1983a) and no difference exists between eggs destined to be workers or queens, those observations would also indicate reduced survival of laying worker-derived queen larvae. Allsopp (1995), however, argued that acceptance of larvae in queen-rearing experiments was equal for both groups, but data for subsequent survival until eclosion were lacking.

Measuring fitness

The aim of this study is to quantify differences in the fitness of *A. m. capensis* honeybee queens from clonal worker-derived brood in comparison to queens derived from fertilised queen-derived brood. The hypothesis can be summarised in the question: Are queens (and their respective colonies) derived from thelytokous laying worker brood reproductively inferior and less fit when compared to queens from queen-derived brood in *A. m. capensis*? Measuring fitness in a population of social insects is a difficult task, because selection operates at least at three distinct levels: Direct individual selection (classical Darwinian selection); indirect individual selection (through kin selection, Hamilton 1964); and directly at the colony level (Kraus et al. 2003). Colonies produce both male and female reproductives. The reproductive success at colony level depends on the number of sexual reproductives produced and the individual reproductive success of each of those queens or drones (Kraus et al. 2003). An individual queen's reproductive quality is a function of her

mating success, fecundity and offspring viability (Futuyama 1998; Gilley et al. 2003) and is determined in social insects with colony fissioning through the number of surviving reproductive colonies (swarms) produced (Kraus et al. 2003). It is, therefore, impossible to determine the reproductive quality of an individual queen without taking into account the fitness of the colony she is heading. It is also impossible to exactly quantify all variables because swarms depart from a colony to an unknown fate and a drone's mating success is barely observable (Kraus et al. 2003). Statements concerning the fitness of honeybee queens will therefore be estimates, based on a few selected measurable parameters which are linked to reproductive success and which indicate fitness.

Parameters linked to reproductive success indicating fitness

To avoid the difficulties outlined above in assessing a queen's fitness, I used a number of measurable parameters which are linked to reproductive success and which indicate fitness. These parameters were used to quantify the relative potential reproductive success of *A. m. capensis* queens from fertilised queen-derived and from clonal worker-derived offspring. In combination they allowed the comparison of the range of reproductive performance and hence general statements about differences in the reproductive quality between queens from queen and worker offspring.

CHAPTER 3: Worker response to grafted larvae: Worker choice tests were used to determine differences in attractiveness between queen- and worker-derived female *A. m. capensis* larvae in artificial emergency queen-rearing experiments. Acceptance of female larvae and their survival until eclosion as virgin queens clearly are a prerequisite for further development and for reproductive success. One-day-old larvae were used for grafting. During emergency queen replacement the nurse bees' interests appear to shift completely towards cooperation, with the aim to produce a future queen of highest reproductive quality (Breed et al. 1984; Visscher 1986; Tarpy and Fletcher 1998). Differences in rates of acceptance and survival until queen eclosion, of alternating groups of larvae grafted from queen-derived and from laying worker-derived brood were used to determine the differences in potential reproductive success of larval and pupal queen stages.

CHAPTER 4: Worker ovipositing in artificial queen cell cups (cf. p. 27):

CHAPTER 5: Pheromonal status of virgin queens: Reproductive success in honeybees is linked to dominance hierarchies, which are mainly pheromonally modulated. A correlation can be assumed between the position within the dominance hierarchy and the pheromonal status of an individual honeybee. I used the mean amounts and percentage

composition of five essential mandibular gland pheromone compounds of laying workers and mated queens to establish pheromonal reference patterns for low and high reproductive success. This allowed the ranking of queen- and laying worker-derived virgin queens according to their pheromonal status and a comparison of their relative position within the reproductive dominance hierarchy.

CHAPTER 6: Worker clustering: During reproductive swarming a number of potential future queens are reared within one colony (Hatch et al. 1999; Gilley and Tarpy 2005). Eclosing virgin queens engage in deadly fights for the inheritance of the colony's resources or depending on the strength of the colony, leave with a number of workers as after-swarms (Gilley et al. 2003; Gilley and Tarpy 2005). Virgin queens must, therefore, be able to attract worker bees and to induce worker clustering as soon as they leave the colony. Larger swarms have survival advantages over smaller ones (Hepburn and Radloff 1998). Queens which are able to attract more workers and to induce stronger worker clustering than other queens can be assumed to be reproductively more successful. Choice tests were carried out in confinement, using caged virgin queens derived from queen- and from laying worker-brood simultaneously to compare worker response. A queen's potential reproductive success was determined by the relative number of workers a queen was able to attract into her swarm cluster.

CHAPTER 7: Drone response to virgin queens: Drones and virgin queens meet at Drone Congregation Areas (DCA) where mating takes place during flight (Jean-Prost 1957; Tribe 1982; Königer and Königer 1991). Drones gather there in large numbers, and as soon as a virgin queen enters the DCA they compete for mating success, following her in a typical 'comet' formation continually entering and leaving the comet, vying for prime position (Königer et al. 2005). When the queen leaves the DCA the 'comet' dissolves, but as soon as she returns it regroups (Gerig 1985; but compare Tribe 1982 for differences in African honeybee subspecies). A queen mates with several drones during one or a couple of mating flights (Moritz et al. 1995, 1996; Oldroyd et al. 1997; Neumann and Moritz 2000; Palmer and Oldroyd 2000). After completion, the mixed sperm stored in her spermatheca suffices for her entire life and no further mating flights are carried out during later life stages (Ruttner 1992; Palmer and Oldroyd 2000). Drones are attracted to the queens in the DCA by visual and pheromonal cues (Gerig 1985). The queen's tergal gland secretions in synergy with those of the mandibular glands act as an attractant (Butler 1971; Vierling and Renner 1977; Winston and Slessor 1998). When given a choice, drones may compete for the queen with the strongest signals, to increase their reproductive success by

mating with the queen of the highest reproductive quality. To compare the reproductive success of queens from queen-derived and from laying worker-derived brood, choice tests were carried out, using caged virgin queens of both groups simultaneously as lures exposed at approximately 5 - 7 m in a DCA. Differences in the number of copulation attempts with the caged queens and differences in the size of forming comets were used to determine differences in potential reproductive success between individual virgin queens.

CHAPTER 8: Long term-development of colonies: The ability to build a strong and healthy colony indicates reproductive success of a queen (Swart 2001a). A larger colony will be able to accumulate more resources, produce more sexual reproductives and generate more and larger reproductive swarms with an increased survival rate (Liebig 1998). Colony performance over time therefore is a useful criterion to determine a queen's reproductive output and success. Equally strong colonies were used to produce two splits each; one headed by a queen reared from queen-derived brood, the other headed by a queen reared from laying worker-derived brood. Acceptance of the virgin queen, mating success or queen-status (e.g. queenless) were recorded after three weeks. Differences in colony performance were assessed after 12 months of undisturbed development, until spring development and before the departure of swarms, using standard methodology. The comparison of the observed developmental pathways was used to determine differences in reproductive success of queen- versus worker-derived queens.

The accumulated data of all experiments described above, in combination, were used to describe and compare the range of reproductive performance for both queen- and worker-derived *A. m. capensis* queens.

The adaptive value of thelytoky in *A. m. capensis*

The unique ability to produce diploid female offspring from unfertilised worker-laid eggs in the honeybee subspecies *A. m. capensis*, in a process called thelytokous parthenogenesis, was suggested several times to have evolved as an adaptive trait to high queen losses during mating flights, mainly due to adverse weather conditions in the subspecies' geographic range (Guy 1976; Moodie 1983; Tribe 1983). Thelytokous parthenogenesis also occurs in other honeybee subspecies but at far lesser frequencies (Mackensen 1943; Tucker 1958). As a rule, arrhenotokous worker reproduction results in haploid male offspring from laying workers (Crozier 1975; Crozier and Pamilo 1996). Moritz (1986) showed in a population model that the production of drones from laying worker brood is favoured by natural selection if queen loss during mating flights is not increased. The adaptive value of

thelytoky can only get established in the gene-pool of a population when sexual reproductives (queens) are produced from worker offspring, and when these queens are reproductively successful (Moritz 1986, 1989; Greeff 1996a,b; Moritz et al. 1998).

A thelytokous colony headed by laying workers, without rearing a new queen, is able to survive for some time, but eventually dwindles and dies without any contribution to the gene-pool (Hepburn 1994; Hepburn et al. 1988). Successful requeening from laying worker-derived brood in queenless *A. m. capensis* colonies occurs, but at relatively low frequencies (Allsopp and Hepburn 1997). Queens produced from laying worker colonies under natural conditions are often described as inferior to queens from queenright colonies and their reproductive quality may be lower, due to reduced brood care and hygienic behaviour in established laying worker colonies (Onions 1912; Mowbray 1916; Hepburn 1989; Hartmann and Hartmann 1991; Muerrle, Neumann, Hepburn 2006 unpublished observations). However, in genetic models it was shown that the thelytoky gene persists even though only a small percentage of colonies produce queens from laying worker brood, and the gene frequency for thelytoky may be remarkably high supported by unusually high multiple matings such as occur in *capensis* queens (Moritz 1986, 1989; Greeff 1996a,b; Moritz et al. 1996, 1998). Therefore it was suggested and generally accepted, that the main adaptive value of thelytoky is the ability to requeen after queen loss, as well as the ability of queenless swarms, which are observed regularly in *A. m. capensis*, to establish queenright colonies from laying worker brood (Neumann et al. 2000a).

Laying workers as social parasites

The invasion of thousands of *A. m. scutellata* colonies in the northern provinces by parasitic *A. m. capensis* laying workers caused the dwindling colony syndrome resulting in the near collapse of commercial apiculture in South Africa. Neumann and Hepburn (2002) reviewed and summarised the behavioural basis rendering *capensis* workers predisposed for social parasitism in the neighbouring honeybee subspecies *A. m. scutellata*. The '*Capensis* Calamity' was triggered by large-scale transportation of *A. m. capensis* colonies into the native range of the neighbouring subspecies *A. m. scutellata* in 1990 (Allsopp 1992b). In successive years selection among the initial invaders (Wossler 2002) resulted in a widespread population of genetically distinct and very similar social parasites, the 'pseudo-clone' (Kryger 2001), obviously the progeny of one single, highly successful and virulent *A. m. capensis* worker (Wossler 2002).

Social parasitism by worker honeybees could have potentially evolved in all honeybee subspecies, because in principle they all are able to reproduce parthenogenetically either via arrhenotoky (the production of drones, Ruttner 1992; Crozier and Pamilo 1996) or thelytoky (the production of diploid females; Onions 1912; Crozier and Pamilo 1996). However, thelytoky appears more predisposed to aggressive worker reproduction (Greeff 1996a,b, 1997) in host colonies of other honeybee subspecies, where self-replicating thelytokous laying worker offspring can immediately infest new host colonies without an intervening sexual generation (Neumann and Hepburn 2002). Moreover, worker egg-laying and even successful worker reproduction is common in queenright colonies of *A. m. capensis* (Pettey 1922; Moritz et al. 1999). This indicates that effects of brood and queen pheromones on worker ovary inhibition is reduced (Wossler 2002) and that *A. m. capensis* laying workers are able to evade the removal of worker-laid eggs in queenright honeybee colonies (Martin et al. 2002a; Pirk et al. 2002). Both ovary activation and escape of worker policing are essential features explaining the successful reproduction of laying *A. m. capensis* workers in queenright colonies of their own and of other subspecies (Neumann and Hepburn 2002).

In addition, Cape honeybee workers show a unique series of traits that reflect important physiological and genetic pre-adaptations for intraspecific social parasitism: high fecundity (Hepburn and Crewe 1990; Velthuis et al. 1990; Ruttner and Hesse 1981; Hepburn and Radloff 1998), longevity (Velthuis et al. 1990; Tribe and Allsopp 2001) and high and fast queen-like pheromonal development (Hepburn 1994; Simon et al. 2001; Wossler 2002). Inside the host colonies *A. m. capensis* workers tend to avoid the host queen (Moritz et al. 2001a,b; 2002) and gain trophallactic dominance over host workers (Velthuis et al. 1990), which provide them with protein-rich high quality nutrients necessary for further ovarial development and oviposition (Crailsheim 1991, 1992; Schäfer et al. 2006). They evade worker-worker aggression and successfully establish themselves as pseudoqueens (Tribe 1981; Allsopp 1995). If several pseudoqueens are present in one host colony it can be assumed that they keep spatially separated as observed in queenless *A. m. capensis* colonies to maintain reproductive dominance (Moritz et al. 2001a).

Ovipositing behaviour changes in the course of the infestation. Initially single eggs are laid per cell by parasitic workers, but at later stages, especially after the loss of the host queen, when pheromonal suppression vanishes and parasitic offspring are reared in great numbers, multiple eggs can be found in the cells (Allsopp 1995; Martin et al. 2002b). In any case, queen cell cups are highly attractive to laying workers and especially in and

around these queen cells, high numbers of multiple eggs can be found regularly (Onions 1912; Johannsmeier 1983; Swart et al. 2001b; Martin et al. 2002b). Nevertheless, the successful production of sexual reproductives in host colonies was never observed, apparently due to the pheromonal dominance of the pseudoqueens, which represses queen cell construction in the host colonies (Swart et al. 2001b; Martin et al. 2002b), resulting in a genetically distinct population of social parasites (Kryger 2001).

Laying workers as direct competitors with the queen for the production of new queens

Social insects, ants in particular, exhibit many different examples of alternative reproductive tactics in females. Apart from socially parasitic queens that enter foreign colonies and exploit the work force to gain a head-start in reproduction (Buschinger 1986), and flightless queens that mate near their natal nest and return to it (Buschinger and Heinze 1992; Heinze and Tsuji 1995), some species have lost the queen caste altogether (Peeters 1991), whilst others reproduce by thelytokous parthenogenesis (Itow et al. 1984). In the following I will try to outline a scenario where worker competition with the queen for the production of sexual reproductives is a logical deduction of all observations above. This scenario is hypothetical and no experimental evidence can yet be given for the full behavioural range. Results from preliminary observations, however, indicate a high probability for their occurrence in wild populations of *A. m. capensis*.

All characteristics predisposing *A. m. capensis* laying workers as social parasites can be assumed to work in a similar, slightly weaker form in queenright colonies of *A. m. capensis*. The pheromonal control of *A. m. capensis* queens is significantly higher in comparison to queens from other honeybee subspecies, possibly as an adaptation to the quick developmental capabilities of laying workers into pseudoqueens (Crewe 1982, 1984, 1987, 1988). Nevertheless, physiological and/or active laying workers with fully developed ovaries and ripe eggs are common in queenright *A. m. capensis* colonies (Onions 1912; Pettey 1922; Moritz et al. 1999; Pirk et al. 2002). This may be due to a "selection for readiness" (Visscher 1989, for arrhenotokous honeybee subspecies), in the case of queen loss. More developed workers would then be in a predisposed position to achieve dominance as pseudoqueens and for monopolising reproduction in queenless colonies (Moritz et al. 2000). Reduction of suppression of ovarial development however, is not only observed after queen loss, but also before and during preparations for supersedure and reproductive colony fission (Butler 1957; Taranov 1961).

In late spring, with increasing strength of a colony after massive pollen and nectar influx, the ovipositing capacity of a queen reaches its peak and ovipositing sites become scarce (Winston et al. 1980; Crane 1990). With the daily eclosion of hundreds of young worker bees the queen pheromone transfer is reduced and ovary development of the laying workers 'in preparation' is enhanced, presumably resulting in ovipositing of single laying worker eggs (compare laying worker ovipositing pattern in queenright invaded colonies of A. *m. scutellata*). With the advance of the swarm preparations, food provisioning of the queen by the workers is reduced, the queen looses weight, a precondition for her ability to fly and to leave the colony in the prime swarm (Liebig 1998; Swart 2001b). Reduced queen feeding presumably results in further reduction of queen pheromone levels within the colony, and queen cell construction becomes de-repressed. Queen cell cups are produced in great numbers in the periphery of the brood nest and mainly at the lower margins of the combs (Liebig 1998; Swart 2001b). In the initial phase of queen cell construction the queen is reluctant to oviposit in these cells, a situation well known to beekeepers, because swarm preventing techniques are successful during this phase (Zander and Böttcher 1982; Crane 1990; Swart 2001b). On the other hand, the colony should already be prepared to rear new queens.

The attraction of laying workers to queen cells (Onions 1912; Johannsmeier 1983; Swart et al. 2001b; Martin et al. 2002b), their advanced ovary development and the overlapping spatial distribution of laying workers evading the queen and queen cells in the periphery of the brood nest, are highly indicative of laying worker ovipositing into queen cells prior to the queen. Ovipositing into queen cell cups and clonal production of sexual reproductives (future queens) offers the highest possible gain in direct fitness that an individual *capensis* worker can achieve and should be strongly selected for at the individual level. Due to a lack of relatedness differences between queen-derived versus clonal worker-derive female offspring, worker policing is reduced in *A. m. capensis* (Greeff 1996a,b; Moritz et al. 1999), which increases the chance of survival of worker-laid eggs in queen cell cups. The assumed presence of several laying workers would further reduce the probability for a queen ovipositing in queen cell cups. Worker-produced queen brood would therefore prevail and precede queen-produced queen brood in swarming colonies of *A. m. capensis*. This would increase the probability for worker-derived queens to eclose earlier, to eliminate later produced queen-derived queen cells, to win queen-duels, or to depart in after-swarms successfully (Gilley and Tarpy 2005). This hypothetical scenario would result in high numbers of thelytokously produced queens in wild populations of *A.*

m. capensis, with far-reaching consequences for current genetical, behavioural, evolutionary and biogeographical explanations.

However, some aspects are contradictory to this assumption. Despite the strong attraction of laying workers to queen cell cups in queenright *A. m. scutellata* colonies and the oviposition of a large number of eggs into those cells (Onions 1912; Johannsmeier 1983; Swart et al. 2001b; Martin et al. 2002b), which possibly originate from several laying workers, the successful production of sexual reproductives was never observed in invaded *A. m. scutellata* host colonies (Swart et al. 2001b; Martin et al. 2002b). Similarly, multiple worker-laid eggs in queen cups in queenless *A. m. capensis* colonies never hatched (Onions 1912). Moreover, there seemed to be a clear preference to initiate queen-rearing from worker-derived larvae in the form of emergency cells and queen cell cups containing worker-derived eggs were neglected (Onions 1912). In addition, despite the presence of physiological laying workers in queenright *A. m. capensis* hives, the queen may be able to suppress successful ovipositing despite advanced swarming preparations, confirming Kropáčová and Haslbachová (1970), who recorded the absence of increased ovary development prior to swarming in colonies of European honeybee subspecies. On the other hand, no data are available for *capensis* laying worker ovipositing preferences and for the viability of worker-derived eggs in queen cell cups in queenright *A. m. capensis* colonies.

The aim of this study, therefore, is to establish whether laying workers in queenright *A. m. capensis* colonies prefer queen cell cups for ovipositing and whether queen-rearing is initiated from these cells, when queen pheromone levels are reduced.

CHAPTER 4: Worker ovipositing in artificial queen cell cups: To assess whether laying workers in queenright colonies of *A. m. capensis* preferentially oviposit in queen cell cups, the queens of test colonies were confined on three combs together with workers behind a wire mesh grid allowing only reduced pheromonal exchange between the confined queen and free-roaming bees, to imitate a failing queen or conditions during reproductive swarming. In addition, the confinement of the queen ensured that only laying workers can participate in the ovipositing experiment. Empty artificial wax queen cell cups were offered on standard breeding frames. This frame with empty cells was positioned at the periphery of the brood nest and at some distance from the frame containing the confined queen. The acceptance of those empty cell cups, patterns of laying worker ovipositing, preferences for certain cell types, as well as egg viability and queen-rearing attempts from the offered cups were recorded. Positive results would indicate similar ovipositing patterns and possibilities

for successful production of sexual reproductives from worker-laid eggs under natural conditions in queenright *A. m. capensis* colonies.

Section 2
REPRODUCTIVE SUCCESS AND REPRODUCTIVE COMPETITION

CHAPTER 3

Increased mortality counterbalances preferential acceptance of worker-derived female larvae in Cape honeybee queen rearing experiments

SUMMARY

In honeybee colonies, *Apis mellifera* L., the queen is the dominant female and typically monopolises reproduction. Queen larvae are non-randomly reared by the workers from the large number of subfamilies coexisting in the colony. Laying workers of the Cape honeybee, *A. m. capensis*, produce clonal female offspring and one or a few subfamilies dominate reproduction after queen loss. I observed that female *A. m. capensis* laying worker-derived larvae were preferentially accepted for queen-rearing over queen-derived larvae. Thus, my data indicate that larvae are preferentially reared from these subfamilies, which are able to dominate reproduction after queen loss. This suggests that early larval stages from dominant patrilines may be able to solicit brood care from workers more efficiently than other larvae from less dominant patrilines thereby influencing worker decisions for acceptance and brood care.

However, preferential acceptance of grafted laying worker-derived over queen-derived female *Apis mellifera capensis* larvae did not translate into eclosion advantages for laying worker-derived virgin queens. Significantly reduced survival of laying worker-derived compared to queen-derived queen stages, post-acceptance until eclosion, counterbalanced initially biased worker decisions, resulting in equal numbers of successfully eclosing virgin queens from equal number of grafts for both queen groups.

INTRODUCTION

The queen is the dominant female in the honeybee, *Apis mellifera* L., colony and usually monopolises reproduction. The queen's mandibular gland secretions (Winston et al. 1989),

brood pheromones (Le Conte et al. 1990) and other queen signals act in synergy to suppress ovary activation in workers (Moritz et al. 2000). These control mechanisms sometimes fail, and in queenright colonies few laying workers can be found (Visscher 1989). Workers, however, cannot mate, and haploid male offspring (drones) are produced from unfertilised eggs. These laying workers often are not only successful in producing male offspring, but may also mimic queens by secreting mandibular gland signals rich in the queen substance 9-ODA (Crewe and Velthuis 1980). Those 'pseudoqueens' (Onions 1912; Velthuis et al. 1990) can suppress ovary activation in other workers, and release retinue behaviour (= attraction of a court of tending workers; Velthuis et al. 1990).

Pseudoqueen development is particularly common in colonies of the Cape honeybee, *A. m. capensis* Esch. (Onions 1912; Anderson 1963; Neumann and Moritz 2002). *A. m. capensis* workers have the unique ability to produce diploid clonal female offspring from unfertilised eggs via thelytokous parthenogenesis (Onions 1912; Anderson 1963; Moritz and Haberl 1994). Because the female worker offspring genetically represent their mothers (Moritz and Haberl 1994), worker policing is reduced and active laying workers are common even in queenright *A. m. capensis* colonies (Moritz et al. 1999). Especially after queen loss *A. m. capensis* workers swiftly develop a queen-like mandibular gland pheromonal bouquet, dominated by the queen substance 9-ODA (Crewe and Velthuis 1980).

The thelytokous trait, generally more queen-like pheromonal signals (Hepburn and Radloff 1998) and the ability to quickly develop into pseudoqueens, enables *A. m. capensis* workers to act as social parasites in colonies of their own (Härtel et al. 2006) and other honeybee subspecies (Onions 1912; Velthuis et al. 1990; Martin et al. 2002b; Neumann and Hepburn 2002; Dietemann et al. 2006). Social parasitism by invading *A. m. capensis* workers was regularly observed when colonies of *A. m. capensis* were introduced into the geographical range of its neighbouring subspecies *A. m. scutellata* (Neumann and Hepburn 2002).

Not only adult *A. m. capensis* workers are able to communicate reproductive dominance, but apparently also *A. m. capensis* worker brood. Beekman et al. (2000) observed 'royal treatment' with increased, royal-jelly-like food provision of queen-derived *A. m. capensis* worker larvae when reared in colonies of European honeybees, resulting in *A. m. capensis* workers with queen-like characteristics. Calis et al. (2002) and Allsopp et al. (2003) confirmed these findings for worker-derived *A. m. capensis* brood reared in colonies

of the African honeybee *A. m. scutellata*, indicating the ability of *A. m. capensis* brood to manipulate their host's feeding behaviour (Beekman et al. 2000).

A honeybee queen mates with a large number of males resulting in as many as 45 subfamilies coexisting in the colony (Palmer and Oldroyd 2000). After accidental loss of the queen, during emergency queen replacement, queen cells are constructed over a number of eggs and larvae (Fell and Morse 1984), but only one of the potential future queens finally inherits the colony resources (Tarpy and Fletcher 1998). Therefore, kin selection theory (Hamilton 1964) predicts nepotistic conflict between workers in favour of a new queen from their own subfamily. However, most attempts to demonstrate nepotism during queen replacement yielded either negative evidence (Breed et al. 1984; Woyciechowski 1990; Tilley and Oldroyd 1997) or were not supported statistically (Page et al. 1989; Carlin and Frumhoff 1990; Oldroyd et al. 1990). Nevertheless, honeybee queens are not reared at random and some subfamilies obviously are over-represented in the worker's choice of future queens (Moritz et al. 1996; Tilley and Oldroyd 1997; Moritz et al. 2005b). Other factors than nepotistic interactions may therefore be decisive during emergency queen replacement. Heritable brood signals indicating 'royalty' were suggested to play a crucial role, resulting in biased subfamily representation during queen replacement (Moritz et al. 2005b).

Artificial honeybee queen-rearing methods exploit emergency queen replacement impulses in honeybee colonies (Ruttner 1983) and therefore present an ideal opportunity to test for worker preferences among different selectively offered larvae. Acceptance and larval survival until eclosion clearly are prerequisites for reproductive success as an adult queen. In this study I carried out choice tests, by simultaneously introducing queen-derived female larvae alternately with *A. m. capensis* pseudoqueen-derived female larvae into a number of different (*A. m. capensis* and *A. m. scutellata*) recipient colonies for queen-rearing. Preferential acceptance and feeding of female *A. m. capensis* worker-derived larvae would indicate the ability to elicit brood care more efficiently than queen-derived larvae (Calis et al. 2002; Allsopp et al. 2003). If clonal laying worker-derived female larvae from reproductively dominant patrilines are significantly more attractive for queen-rearing than queen-derived larvae, it can be assumed that early brood-signals may be indicative of later dominance signals in adults.

It is often assumed that once accepted, grafted female larvae are reared until eclosion (Zander and Böttcher 1982). Young laying worker-derived larval queen stages therefore would have eclosion advantages over queen-derived brood due to bias in worker

preferences during queen production. However, the assumption of undisturbed survival of initially accepted queen larvae until eclosion is often challenged during their development. The neglect or abortion of grafted larvae (post-acceptance), are frequently described events in commercial queen-rearing and mainly ascribed to adverse environmental conditions (Weiss 1983b). Lack of (pollen) forage, for example, as well as heavy nectar flows or extreme external temperatures are major reasons for failures in queen-rearing success, often despite the initial acceptance of grafts (Weiss 1983b). Moreover, larval infections (Shimanuki and Knox 2000) may cause mortality and negatively influence the number of successfully eclosing queens. Selective queen cell destruction by nurse bees seems to affect the outcome of queen-rearing as well (Gary and Morse 1962; Fletcher 1978). It seems highly plausible that colonies have evolved elaborate mechanisms to recognise quality differences prior to emergence of the queens (Tarpy et al. 2004). Queen cells started over older brood, for example, are destroyed significantly more often than cells initiated over eggs during emergency queen replacement (Hatch et al. 1999). The number of successfully eclosing adult virgin queens therefore may differ significantly from the number of initially accepted larvae. The mortality of larval or pupal queen stages must therefore be taken into consideration when stating differences in potential reproductive success of queens.

In this context I compared the mortality of queen- versus laying worker-derived female *A. m. capensis* larvae, from introduction of the grafts into the recipient colonies until eclosion as adult virgin queens. If genetic dominance of clonal laying worker-derived larvae would translate into preferential acceptance and brood care, a higher number of successfully eclosing laying worker-derived than queen-derived adult virgin queens can be assumed.

MATERIALS AND METHODS

Honeybee colonies

In January 2005, a honeybee colony pool was stocked at three apiaries in Grahamstown (Eastern Cape, South Africa) with unrelated, queenright colonies of: 1) *A. m. capensis* from the Eastern and Western Cape (N = 51), and 2) *A. m. scutellata* from the Pretoria area (N = 7), (see Hepburn and Radloff (1998) for a detailed review on the distribution and biology of these honeybee populations). The *A. m. scutellata* colonies were kept at a separate apiary to minimise intersubspecific drifting, dispersing, naturally occurring swarm mergers and infestations by socially parasitic workers (Neumann and Hepburn 2002). All

colonies were kept in standard Langstroth hives (one breeding chamber and one shallow honey chamber) and had sufficient food (honey and pollen).

Donor colonies

All donor colonies (N = 6 *A. m. capensis* colonies; and N = 1 *A. m. scutellata* colony) intended to provide queen-derived female larvae for grafting were selected from the colony pool 24 h prior to grafting. All donor colonies (N = 3 *A. m. capensis* colonies) intended to provide laying worker-derived female larvae for grafting were selected from the colony pool at least three weeks prior to grafting and de-queened. All emergency queen cells were removed from these colonies and worker ovipositing was monitored at weekly intervals until sufficient larvae of the appropriate age were available.

Queen-rearing

Queen-rearing in queenright recipient colonies (= recipient colonies; N = 13 *A. m. capensis* colonies; N = 2 *A. m. scutellata* colonies) was carried out during the reproductive swarming season of *A. m. capensis* and *A. m. scutellata* (September - December 2005), according to standard protocols, after nine days of restricted ovipositing by the queen (Zander and Böttcher 1982). On the day of grafting, one hour prior to the introduction of the breeding frame containing the grafted larvae, the open brood was removed and the queen was restricted to a peripheral part of the colony.

Grafting of young female larvae (12 - 24 h old) followed routine procedures (Laidlaw 1979). The queen-cell cups were attached to labelled, standard wooden cell bases indicating the donor colony of each larva. Each breeding frame contained alternating groups of individually labelled queen-cell cups with queen-derived and laying worker-derived larvae which were offered simultaneously (a total number of 540 female *A. m capensis* larvae were tested in 15 recipient colonies: 15 breeding frames with N = 36 larvae on each frame; 18 larvae queen-derived, 18 larvae laying worker-derived).

Assessment of acceptance of grafted larvae for queen-rearing

The breeding frames were assessed for acceptance 48 h after introduction of the grafts into the recipient colonies. The presence of three-day-old larvae in elongated cells was verified and individual cells were recorded.

Assessment of mortality of queen larvae and pupae post-acceptance until eclosion

Initially rejected larvae were recorded as 'larvae not accepted' (1) (Tab. 3.2.). Eight days after grafting (about three days prior to virgin queen emergence), the breeding frames were assessed for capped queen cells, and their individual numbers recorded. Initially accepted cells, which were not capped, empty and/or torn down, were recorded as 'aborted' (2) queen cells (Tab. 3.2.). Each capped queen cell was secured in a queen emergence cell cage with five workers. The breeding frames with caged cells were then reintroduced into the recipient colonies to allow further pupal development. About one day after queen emergence, the breeding frames were assessed for successfully eclosed queens. The individual numbers of live virgin queens in the cages, as well as the individual numbers of 'dead pupae in capped cells' (3) were recorded. The 'total mortality' (4) of queen stages during queen-rearing until eclosion was calculated as the sum of mortality caused by (1, 2 and 3) (Tab. 3.2.).

Statistical analysis

Pearson χ^2 tests were used to test for significant differences in the acceptance rates of queen-derived female *A. m. capensis* larvae versus laying worker-derived female *A. m. capensis* larvae, and queen- and laying worker-derived *A. m. capensis* larvae nursed by *A. m. scutellata* versus *A. m. capensis* recipient colonies.

Pearson χ^2 tests were used to test for significant differences in mortality of queen- and laying worker-derived queen stages until pupation and until successful eclosion.

Log-linear G-test analysis was used to test for homogeneity of acceptance rates among the recipient colonies (Sokal and Rohlf 1995). The power of the tests, which is the probability of not detecting significant differences in acceptance rates when in actuality differences occurred, were also determined for each test (Zar 1996). All tests were performed using Statistica© (StatSoft 2004).

RESULTS

Acceptance of grafted larvae for queen-rearing

Acknowledging the low power of the test due to sample size imbalances, no significant differences in acceptance rates were generally observed when comparing different subspecific combinations of grafts and recipient colonies: there were no significant

differences in acceptance rates observed when comparing queen-derived female *A. m. capensis* larvae grafted into *A. m. scutellata* (50.0 ± 7.8%) versus *A. m. capensis* recipient colonies (59.8 ± 13.6%; $\chi^2 = 1.24$, 1df, $p = 0.27$; power of the test = 0.51); similarly no significant differences in acceptance rates were observed when comparing worker-derived female *A. m. capensis* larvae grafted into *A. m. scutellata* (66.7 ± 7.9%) versus *A. m. capensis* (71.4 ± 11.1%; $\chi^2 = 0.33$, 1df, $p = 0.56$; power of the test = 0.52) recipient colonies.

Data from *A. m. capensis* and *A. m. scutellata* recipient colonies were pooled, explicitly assuming lack of significant differences due to different subspecific combinations of the samples. From this, however, highly significant differences in acceptance rates were found between queen-derived female larvae versus laying worker-derived female larvae, when grafted into different recipient colonies (Tab. 3.1.). The acceptance rates for laying worker-derived female larvae (70.7 ± 10.6%; 191 out of 270) were significantly higher compared to queen-derived larvae (58.5 ± 13.3%; 158 out of 270; $\chi^2 = 8.82$, 1df, $p = 0.003$), with similar results when testing only *A. m. capensis* samples (worker-derived: 71.4 ± 11.1%; queen-derived: 59.8 ± 13.6%; $\chi^2 = 6.90$, 1df, $p = 0.008$). The acceptance rates were homogeneous among the different colonies (G-test: $\chi^2 = 4.18$, 14df, $p = 0.99$) (Tab. 3.1.).

Mortality of queen larvae and pupae post-acceptance until eclosion

From a total number of 540 grafted larvae, 286 larvae (52.96%) did not survive until eclosion (= 'total mortality' (4)). Reasons for this mortality were most frequently rejection of grafted larvae by nurse bees (191 larvae 'not accepted' (1) = 66.78%), followed by pupal death (62 'dead pupae in capped cell' (3) = 21.68%) and finally larval death prior to pupation (33 'aborted' cells (2) = 11.54%) (Tab. 3.2..; Fig. 3.1.). Queen-derived and laying worker-derived queen stages differed significantly in frequencies of the events (1, 2 and 3) where mortality occurred during their development: Significantly more queen-derived (41.5%) than laying worker-derived (29.3%) grafted larvae were initially 'not accepted' (1) ($\chi^2_1 = 8.8$, P=0.0029). In contrast, significantly more laying worker-derived (9.3%) than queen-derived (3.0%) larvae were 'aborted' (2) prior to pupation ($\chi^2_1 = 9.3$, P=0.0023). Similarly significantly more laying worker-derived (14.4%) than queen-derived (8.5%) pupae were found 'dead in capped cells' (3) ($\chi^2_1 = 4.7$, P=0.0308). However, there was no difference in 'total mortality' (4) of all queen stages between queen-derived (53.0%) and laying worker-derived queen stages (53.0%) (Tab. 3.2.; Fig. 3.1.).

DISCUSSION

Acceptance of grafted larvae for queen-rearing

Our data clearly show a significantly higher acceptance of laying worker-derived larvae compared to queen-derived larvae during emergency queen-rearing in honeybees. Since Cape honeybee laying worker-derived brood represents genotypes of reproductively dominant subfamilies, clonal worker-derived larval stages seem to be able to elicit worker brood care more efficiently than queen-derived larvae, thereby influencing the choices of nurse bees as to which larvae to accept or reject as future queens.

Our data confirm earlier studies, that the rearing of honeybee queens is not at random (Estoup et al. 1994; Moritz et al. 1996; Tilley and Oldroyd 1997; Schneider and DeGrandi-Hoffmann 2003). Moreover, an individual from a subfamily which dominated reproduction after queen loss appears to have a higher probability of becoming a queen than individuals from others. The observed significantly increased acceptance rate of laying worker-derived brood may well be due to heritable cues signalling dominance. Brood care is greatly pheromonally mediated in honeybees (Free 1987) and 'royal treatment' of laying worker-derived brood when introduced into innately less dominant honeybee subspecies was repeatedly observed (Calis et al. 2002; Allsopp et al. 2003). These larvae received more royal jelly-like nutrition and eclosed from worker cells morphologically as workers but with more queen-like characteristics, reduced pollen combs, enlarged spermathecae and higher numbers of ovarioles (Calis et al. 2002). Clonal larvae with genotypes from dominant laying workers may be pheromonally more attractive and therefore able to positively influence worker decisions during queen replacement.

Mortality of queen larvae and pupae post-acceptance until eclosion

Significantly increased initial acceptance of laying worker- over queen-derived female *A. m. capensis* larvae did not translate into eclosion advantages for laying worker-derived queens. Their survival, following acceptance until eclosion, was significantly reduced compared to queen-derived queen stages. Despite significant differences in acceptance of grafted larvae after 48 h, an equal number of queen-derived (127) and laying worker-derived (127) adult virgin queens eclosed successfully from an equal number of grafted larvae (270 each), suggesting equality of potential reproductive success of queen- and laying worker-derived virgin queens (Fig. 3.1.). However, significant differences in the events, where mortality occurred, were observed between both groups. Nurse bee decisions

whether to accept or reject a grafted larva were most frequently the reason for larval mortality during the initial queen-rearing phase.

On the other hand, after initial acceptance significantly more queen cells containing laying worker-derived larvae were aborted. Similarly significantly more laying worker-derived pupae did not eclose from their cells but died during pupation.

Increased larval and pupal mortality of laying worker-derived offspring was repeatedly observed in dwindling queenless *A. m. capensis* colonies and generally ascribed to reduced hygienic behaviour and brood neglect due to lack of nurse bees (Allsopp 1995; Hepburn and Radloff 1998). I similarly observed larval and pupal mortality with diffuse disease symptoms in all queenless donor colonies for worker-derived larvae. Laying worker-derived brood often dried out or degenerated into brown slime with symptoms resembling European Foulbrood. Woyke (1995) reported, as one of three reasons for 'dwindling' in queenless laying worker colonies of *A. m. capensis*, that the survival of brood produced by laying workers was reduced compared to queen-derived brood. He introduced pieces of combs containing queen-derived and laying worker-derived eggs into strong colonies of *A. m. capensis* and observed a high increase in the mortality of laying worker-derived brood, which peaked shortly after the larvae hatched from the egg. His observations, however, were lacking descriptions of the symptoms and no suggestions as to what could have caused this mortality were made. However, significantly reduced survival of laying worker-derived queen stages following preferential acceptance, counterbalanced their initial advantages resulting in equal numbers of successfully eclosed queen-derived and laying worker-derived virgin queens from an equal number of grafted larvae in *A. m. capensis*. No difference in potential reproductive success for queens from both origins therefore was observed.

Tab. 3.1. Acceptance rates of queen- and laying worker-derived female larvae (pooled data from *A. m. capensis* and *A. m. scutellata* recipient colonies)

Recipient colony	**Acceptance rates of female *A. m. capensis* larvae**	
	queen-derived larvae (out of 18 per colony)	**worker-derived larvae (out of 18 per colony)**
1	9	15
2	12	12
3	8	11
4	10	12
5	9	12
6	10	17
7	12	12
8	13	10
9	14	14
10	9	12
11	10	14
12	8	11
13	16	15
14	10	13
15	8	11
Total	**158**	**191**
Mean	**58.5 ± 13.3%**	**70.7 ± 10.6%**

Tab. 3.2. Mortality of 270 queen- and 270 worker-derived grafted female larvae until eclosion

mortality	**queen-derived grafts (N=270)**	**worker-derived grafts (N=270)**	**total grafts (N=540)**
(1) larvae not accepted	112	79	191
(2) cells aborted	8	25	33
(3) dead pupa in cell	23	39	62
(4) total mortality	**143**	**143**	**286**

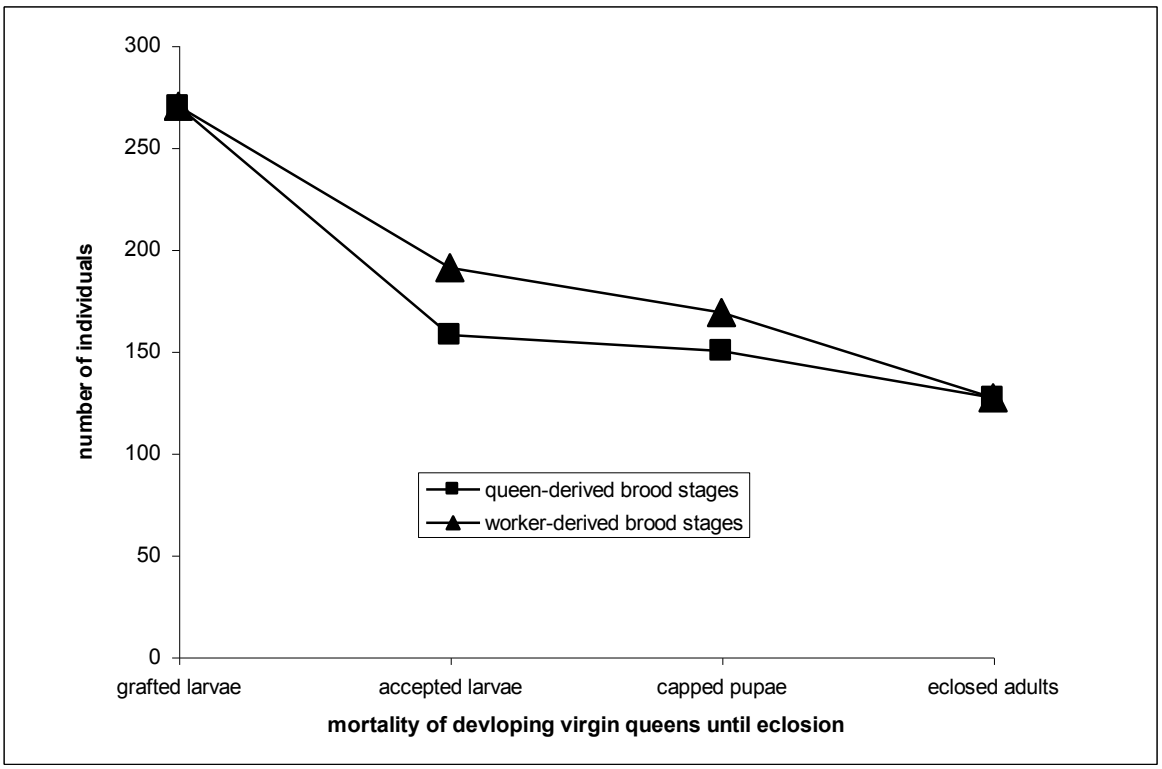

Fig. 3.1. Mortality of 270 queen- and 270 worker-derived grafted female larvae until eclosion

CHAPTER 4

Laying workers oviposit in artificial queen cell cups in *Apis mellifera capensis* Eschscholtz

SUMMARY

Honeybee (*Apis mellifera* L.) workers may develop their ovaries and lay eggs. However, they cannot mate and generally produce haploid male offspring (drones) from unfertilised eggs arrhenotokously. In strong contrast, laying *A. m. capensis* workers produce diploid female offspring from unfertilised eggs thelytokously, from which queens can be reared. Laying worker ovipositing into old queen cell cups was frequently observed in queenless colonies of *A. m. capensis*, but successful requeening of orphaned colonies is mostly achieved from emergency cells constructed over brood in worker comb. Laying workers are relatively common even in queenright *A. m. capensis* colonies. I observed worker ovipositing in artificial handmade wax queen cell cups in queen-deprived compartments of otherwise queenright *A. m. capensis* colonies, which were successfully reared until eclosion of the virgin queens. I therefore posit that similar worker behaviour occurs in wild swarming or superseding *A. m. capensis* colonies, where the reduced supply of queen-pheromone triggers construction of natural queen cell cups and queen-rearing. Queen production in queen cells from worker-derived eggs in queenright swarming or superseding wild colonies would suggest direct competition between queen and laying workers for reproductive success in *A. m. capensis*.

INTRODUCTION

In honeybee (*Apis mellifera* L.) colonies, usually one single queen monopolises reproduction by pheromonally suppressing worker ovarial development (Butler 1954b; Slessor et al. 1988; Winston et al. 1989; Winston and Slessor 1992; Naumann et al. 1991). However, in the case of queen loss and in the absence of brood, honeybee workers

generally develop ovaries and start ovipositing (Bourke 1988). These unfertilised eggs are haploid and give rise to male offspring (drones) via arrhenotokous parthenogenesis (Crozier 1975; Crozier and Pamilo 1996). Usually, diploid female offspring only develop from fertilised queen-derived eggs. An orphaned honeybee colony without queen-derived female brood from which to rear a new queen therefore is doomed (Hepburn and Radloff 1998). In strong contrast, workers of the honeybee subspecies *A. m. capensis* typically produce diploid female offspring via thelytokous parthenogenesis (Onions 1912; Anderson 1963). This offers *A. m. capensis* colonies an additional pathway to restore a queenright condition from worker-derived female brood after accidental queen loss (Ruttner 1977a,b). Indeed, successful requeening from worker-derived brood in queenless and broodless colonies is well documented in this honeybee subspecies (Moritz et al. 1996; Hepburn and Radloff 1998).

A small number of laying workers occur even in queenright *A. mellifera* colonies (Visscher 1989). Their reproductive success, however, is reduced because worker-laid eggs, which would develop into drones, are removed by policing workers (Ratnieks and Visscher 1989; Visscher 1996, 1998). In contrast, laying workers are relatively common in queenright *A. m. capensis* colonies (Onions 1912; Anderson 1963; Neumann and Moritz 2002). These laying workers are reproductively more successful, because the removal of worker-laid eggs, which develop into females, is reduced (Greeff 1996a,b; Moritz et al. 1999).

In this study I demonstrate, that the rearing of worker-derived queens may not be restricted to queenless and broodless *A. m. capensis* colonies, but may also at higher frequencies occur possibly in queenright colonies preparing to swarm or to supersede the old queen. Swarming or superseding honeybee colonies generally attempt to exchange the old queen by producing a number of queen cell cups (natural queen cell primordials). Queen cell cup production is pheromonally triggered (Butler 1954b, 1957; Caron 1979) and probably due to a reduced supply of queen-substance from overcrowding or a failing queen. Usually the queen must oviposit into those queen cell cups in order to successfully produce new queens. Butler (1954a) noted that arrhenotokously reproducing laying workers were sometimes observed laying eggs in queen cell cups, but that such unfertilised eggs gave rise to males, except in very rare cases when a female larvae is produced, which may develop into a queen. *A. m. capensis* laying workers, on the other hand, were regularly observed laying multiple (up to 20 or more) eggs in old queen cell cups in queenless colonies (Onions 1912; Swart et al. 2001b; Martin et al. 2002b). Observations as to whether

these eggs hatch and the larvae are reared into adult queens had not been undertaken. It was generally assumed that successful requeening was achieved from emergency cells constructed over worker-derived brood (Hepburn and Radloff 1998). It can, however, be assumed that laying workers in queenright swarming or superseding *A. m. capensis* colonies may as well lay eggs in newly produced queen cell cups. These eggs, if viable, could be reared into adult worker-derived queens.

I simulated swarming or superseding conditions by confining the queen behind a wire mesh screen in a separate compartment of the test colonies, thereby reducing pheromone distribution in the queen-deprived compartments, simulating overcrowding conditions or failing queens. I introduced breeding frames with artificial wax queen cell cups, similar to queen-rearing procedures (Laidlaw 1979), but without any grafted larvae, into the queenless compartment of the test-colonies. If laying workers oviposit in artificial queen cell cups and viable adult queens eclose from these cells, it can be assumed that laying worker-derived queens may be produced in queenright swarming or superseding natural *A. m. capensis* colonies. Worker ovipositing in queen cell cups would constitute direct competition between queen and laying workers for reproductive success in wild *A. m. capensis* colonies, an alternative reproductive tactic never before described in honeybees.

MATERIALS AND METHODS

Four queenright *A. m. capensis* colonies (W, X, Y, Z) were prepared for queen-rearing as described by Anderson (1965), at an experimental apiary in Stones Hill, Grahamstown, Eastern Province, South Africa, in November 2006, during the natural reproductive (swarming) season in *A. m. capensis* (Hepburn and Radloff 1998). All four colonies had settled as wild swarms in empty trap boxes during August and September 2006. At the start of the experiment, the colonies were moderately strong, occupying nine Langstroth frames in one brood chamber and had sufficient food (2 frames of honey and 1 frame with pollen). To avoid any emergency queen-rearing from queen-derived brood during the course of the experiment, the queens were confined to one comb behind a queen excluder for seven days, where they were able to continue ovipositing. Those combs, containing eggs and young open brood, were removed from the colonies one day prior to the start of the experiment, leaving exclusively larvae 4 days and older in the remaining combs. Three of the colonies (treatment colonies W, X, Y) were divided into two separate compartments by a vertical wire screen of 3.2 mm mesh (Fig. 4.1.). The bees were prevented from exchanging

compartments except through the entrances (Figs. 4.1.; 4.2.). One colony (Z) was not divided and received its old queen back with no restriction of movement within the colony. This colony (Z) was used as a control. In all treatment-colonies (W, X, Y) the queen was confined to the smaller compartment containing one comb of honey and two frames of emerging brood. The entrance to this compartment was secured with a queen excluder grid, to prevent the queen from moving between the compartments via the entrance (Fig. 4.2.). The larger compartment comprised of three frames of older brood, one frame of honey, one frame with pollen and one Doolittle type feeder (the size of a Langstroth frame), leaving space for one breeding frame per colony. All colonies received 150 g of food patty (icing sugar : pollen : honey; by volume 5 : 3 : 2). The experiment started 24 h after separation into queenless and queenright compartments, when one breeding frame was introduced between two frames of older brood within the queenless compartment of each treatment colony (W, X, Y). The control colony (Z) received one breeding frame placed in the centre of the brood. Each breeding frame contained 18 empty handmade artificial wax queen cell cups (∅ 8 mm, depth 6 mm). The queen cell cups were attached to standard wooden bases and fixed 5 cm apart in groups of 9 on two horizontal bars of the breeding frame (Fig. 4.7.). The combs and breeding frames were checked for worker ovipositing after 11 and 17 days, and the actual rearing of queens was controlled on days 21, 23, 25, 27 and 31 after introduction of the breeding frames (Tab. 4.1.). On day 23 after start of the experiment all sealed or nearly sealed cells from the breeding frames of all colonies were combined next to each other on the top bar of one breeding frame (Fig. 4.10.) and introduced into the queen-deprived compartment of the strongest treatment colony (W) to finish the queen-rearing process To avoid interference with other queen cells or uncontrolled eclosing of virgin queens from natural queen cells, all natural queen cell cups or queen cells were destroyed and all other artificial queen cell cups were removed from this colony (W). In colonies X and Y I terminated the experiment, removed the breeding frames and the separation screen between the two compartments, allowing the queen to roam freely again. On day 27 after start of the experiments all capped cells were secured in cylindrical queen cages (∅ 2 cm, height 6 cm, with 180 holes: 3x3 mm^2). The queen cages allowed nurse bee feeding from outside and contained five workers to facilitate queen eclosion and their initial food provision. Successful eclosion was assessed 31 days after the start of the experiment.

RESULTS

Eleven days after introduction of the breeding frames into the colonies, the first worker-derived eggs were detected in the queen-deprived compartments of the treatment colonies (W) and (Y). In these colonies eggs were detected on worker comb as well as in artificial queen cell cups. Some artificial queen cell cups contained single eggs and others multiple eggs. No worker-derived eggs were detected in treatment colony (X). Worker larvae were not detected in any of the treatment colonies (Tab. 4.1.).

Seventeen days after introduction of the breeding frames, laying worker ovipositing was observed in all treatment colonies. In treatment colony (W) worker eggs and some larvae were found on the worker comb, and single or multiple eggs in some artificial queen cell cups. In treatment colony (X) some worker eggs, but no larvae, were found on the worker comb, and some artificial queen cell cups contained multiple worker-derived eggs. In treatment colony (Y) worker eggs and some larvae were found on the worker comb, and single or multiple eggs in some artificial queen cell cups (Tab. 4.1.).

Twenty-one days after introduction of the breeding frames, worker eggs and larvae were observed in all treatment colonies on the worker comb. In addition, queen-rearing was initiated in all treatment colonies with single larvae on royal jelly in elongated natural (Fig. 4.4.) and/or artificial queen cells (Figs. 4.7.; 4.8.; 4.9.). In colony (W) some natural queen cell cups at the margins of the worker comb contained single or multiple eggs, larvae (queen-rearing initiated) and some artificial queen cell cups contained multiple eggs. Four artificial queen cells in colony (W) contained single larvae (queen-rearing initiated) (Tab. 4.1.; Fig. 4.7.). In colony (X) some natural queen cell cups at the margins of the worker comb contained single or multiple eggs, larvae (queen-rearing initiated) and some artificial queen cell cups contained multiple eggs. One artificial queen cell in colony (X) contained a larva (queen-rearing initiated) (Tab. 4.1.; Fig. 4.8.). In colony (Y) some natural queen cell cups at the margins of the worker comb contained single or multiple eggs, larvae (queen-rearing initiated) and some artificial queen cell cups contained multiple eggs. One artificial queen cell in colony (Y) contained a larva (queen-rearing initiated) (Tab. 4.1.; Fig. 4.9.).

Twenty-three days after introduction of the breeding frames, the situation in all treatment colonies was similar to that on day twenty-one. However, some of the natural queen cells at the margins of the worker combs were sealed in colonies (W) and (Y) and the artificial queen cells on the breeding frames were sealed or about to be sealed in colonies (W) and (Y). (W): 2 queen cells sealed, 2 cells about to be sealed; (Y): 1 cell

about to be sealed. The artificial queen cell on the breeding frame in colony (X) was aborted (Tab. 4.1.).

We observed a relative reduction in the worker force and an increased shift of workers via entrances into the queenright compartment in all treatment colonies. Therefore all five sealed or nearly sealed queen cells from colonies (W) and (Y) containing larvae were combined on one breeding frame and introduced into the strongest treatment colony (W), to finish the queen-rearing process until eclosion (Fig. 4.10.). To avoid interference with other queen cells or uncontrolled eclosing of virgin queens from natural queen cells, all natural queen cell cups or queen cells were destroyed and all other artificial queen cell cups were removed from colony (W).

Twenty-five days after the start of the experiment, all five queen cells (combined) on the breeding frame in colony (W) were sealed (Tab. 4.1.; Fig. 4.10.).

Twenty-seven days after the start of the experiment, two of those sealed queen cells (one initially from colony (Y) and the other from colony (W) were aborted, chewed open and removed. The remaining three sealed cells were secured in queen cages (Tab. 4.1.).

Thirty-one days after the start of the experiment, two live virgin queens eclosed successfully within the queen cages (Fig. 4.11.). One caged sealed queen cell contained a dead pupa (Tab. 4.1.).

In the control colony (Z) no ovipositing in artificial queen cell cups was observed during the entire duration of the experiment and no natural queen cells were constructed (queen-rearing not initiated) (Tab. 4.1.). However, queen-derived worker brood was constantly observed on the worker comb in the control colony (Z). In the treatment colonies (W, X and Y) queen-derived brood was present in the queenright compartments during the entire duration of the experiment.

DISCUSSION

Laying worker ovipositing in empty, artificial handmade wax queen cell cups was observed in the queen-deprived compartments of all three *A. m. capensis* treatment colonies (W, X and Y), 17 days after the start of the experiment. In all three treatment colonies, successful hatching of worker-derived eggs in artificial queen cell cups was observed and queen-rearing was initiated with elongated queen cells containing a single larva on royal jelly. From six initiated queen cells on breeding frames in the three treatment colonies, five cells from two colonies (W and Y) were later sealed. Two of these cells were subsequently aborted. Of the three remaining sealed cells, two virgin queens successfully eclosed, the

third cell contained a dead pupa. In the control colony (Z), where the queen was allowed to roam freely, no ovipositing in empty queen cell cups was observed, queen-rearing was not initiated and queen brood in worker cells was observed throughout the entire experiment.

Separation of the queen into a peripheral compartment of the colony obviously resulted in de-repression of worker ovipositing, but on the other hand still allowed initiation of queen-rearing, suggesting a reduced supply of queen pheromone which triggered both behaviours. Worker-derived eggs, when oviposited in artificial or natural new queen cell cups, proved to be potentially viable in queen-deprived *A. m. capensis* colonies and, in low frequencies, were accepted for queen-rearing (Figs. 4.4.; 4.7.; 4.8.; 4.9.). However most (single or multiple) eggs in artificial queen cells did not seem viable and obviously did not hatch and were removed by workers or exchanged for newly laid eggs. Single or multiple eggs in artificial queen cell cups were often observed (Figs. 4.5.; 4.6.). The abortion of a queen cell prior to sealing of an initially accepted artificial queen cell in colony X, was possibly due to a reduced nurse bee presence from worker migration towards the queenright compartment of the colony.

This migration was observed in all colonies and was mainly triggered by cool temperatures over a period of rainy weather, after initiation of queen-rearing. Worker bees were observed shifting from one compartment to the other via the separated entrances. Weaker colonies obviously suffered more severely from the losses of nurse bees in the queen-deprived compartment, with more abandoned frames, than stronger colonies. Therefore, all five remaining artificial queen cells (sealed or about to be sealed) were combined next to each other on the top bar of one breeding frame and introduced into the strongest treatment colony (W) to continue the rearing process. All five cells were sealed and tended by clustering workers two days after combination on the breeding frame. Two of these cells were subsequently aborted and only three sealed cells remained to be secured in queen cages prior to virgin queen eclosion. From two of these sealed queen cells, virgin queens eclosed successfully 31 d after start of the experiment.

Classical apicultural queen-rearing approaches usually rely on the removal of the queen and subsequent emergency queen-rearing from artificially grafted selected larvae in queenless breeding colonies (Ruttner 1983). Apicultural queen-rearing in *A. m. capensis* is a difficult task. The removal of the queen quickly results in strong worker competition for reproductive dominance, the occurrence of active laying workers and the neglect of grafted queen cells (Hepburn and Radloff 1998). The problem of queen cell neglect or abortion during apicultural queen-rearing in *A. m. capensis* can only be solved by using queen-

deprived breeding colonies, which provide a finely tuned reduced supply of queen pheromone to suppress worker ovipositing and at the same time triggering emergency queen-rearing (Anderson 1965). Queen-rearing becomes even more difficult in *A. m. capensis* when relying on worker-ovipositing in empty artificial queen cell cups, without offering grafted larvae, as demonstrated in my experiment. Here, the queen pheromone supply must be strongly reduced over an extended period of time to initiate worker reproduction and ovipositing into artificial queen cell cups on the breeding frames.

On the other hand, enough queen pheromone must still be present to prohibit the neglect of queen cells after initiation of queen-rearing from these cells. Pheromone levels can be assumed to fluctuate strongly in the queen-deprived compartments of the treatment colonies due to the variable presence of worker-derived eggs, larvae or queen cells (Hepburn and Radloff 1998), and due to fluctuations in the worker force from worker migrations to and from the queenright compartments (via entrances). The low survival rate (33.3%) of a very low number of initiated queen-rearings from artificial queen cell cups (6 queen cells containing worker-derived larvae out of 54 offered empty cups in all treatment colonies) in my experiment is therefore not surprising. It is, however, surprising that *A. m. capensis* laying workers obviously regularly oviposit single or multiple eggs in artificial queen cell cups on breeding frames, as was observed in all treatment colonies. This behaviour was never observed in mated queens. Weiss (1983b) reported that mated European honeybee queens were hardly ever observed to oviposit into artificial queen cell cups, even when the queen cups were pressed into a comb, similar to natural queen cell cups, in colonies preparing to swarm. Laying workers in queen-deprived *A. m. capensis* colonies therefore seem to be highly attracted to queen cups and readily accept artificial substitutes.

Furthermore, the successful eclosion of virgin queens from those cells, as demonstrated in my experiment, shows that worker-derived eggs in queen cell cups are potentially viable and may be accepted by nurse bees as potential future queens if the pheromonal composition in the colony favours the initiation of queen-rearing. In the treatment colonies I tried to simulate the queen pheromone supply as that which may be present during swarming or superseding in wild colonies. My simulation, however, using queen separation and artificial queen cups was possibly only a weak substitute for natural cues in swarming or superseding colonies. Successful worker ovipositing in natural queen cell cups and queen-rearing from worker-derived brood may therefore be a more frequent event with a higher success rate in wild swarming or superseding *A. m. capensis* colonies.

Moreover, swarming and supersedure are frequent reproductive events in natural populations of *A. m. capensis* (Allsopp and Hepburn 1997). The number of worker-derived queens produced in queenright swarming or superseding colonies may therefore be high in natural populations of *A. m. capensis*.

The queen is generally the dominant female in a honeybee colony. Worker ovipositing in queen cell cups instead of the queen therefore would constitute direct reproductive competition between dominant and subordinate females within one colony. If laying workers are able to outcompete the queen in her attempt to oviposit in queen cell cups by 'sneaking' their own eggs into these cups prior to the queen, and if these eggs are successfully reared until eclosion, a new alternative female reproductive tactic could be assumed in *A. m. capensis*. The results of my experiments strongly suggest worker competition with the queen for reproductive success in wild swarming or superseding *A. m. capensis* colonies. However, only exact assessments of the occurrences and frequencies of worker-derived queens produced in queenright swarming or superseding colonies in wild populations of *A. m. capensis* can resolve the issues in question.

Tab. 4.1. Events during queen production from eggs oviposited by laying workers into artificial queen cell cups in queen-deprived *A. m. capensis* colonies

colony	**day 0** receive 'empty' breeding frame	**day 11** eggs in artificial queen cell cups detected	**day 17** eggs in artificial queen cell cups detected	**day 21** queen-rearing initiated (# queen cells)	**day 23** cells sealed or about to be sealed (# queen cells)	**day 25** cells sealed combined in (W) (# queen cells)	**day 27** cells caged (# queen cells)	**day 31** virgin queens eclosed (# virgin queens
W	yes	yes	yes	4	4	5 (+1 (Y))	3 (2 abort.)	2 (1 dead)
X	yes	no	yes	1	0 (aborted)	terminated	terminated	terminated
Y	yes	yes	yes	1	1	terminated	terminated	terminated
Z (contr.)	yes	no	no	0	0	0	0	0

Fig. 4.1. Wire mesh screen to separate queenright and queen-deprived compartments

Fig. 4.2. Treatment colony with separate entrances into queen-deprived and queenright compartments

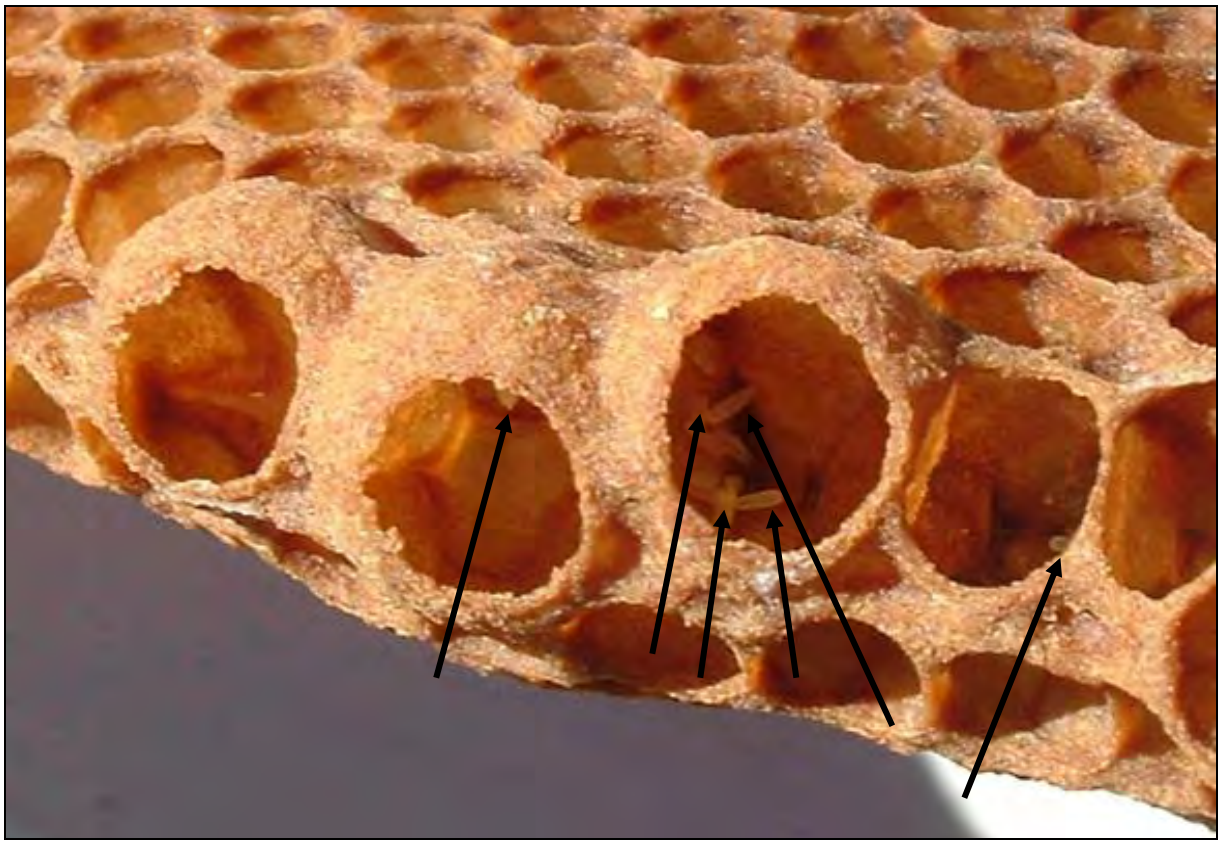

Fig. 4.3. Single and multiple worker-laid eggs in newly constructed natural queen cell cups

Fig. 4.4. Worker-laid worker brood, single egg in cell cup and queen-larva in elongated natural queen cell

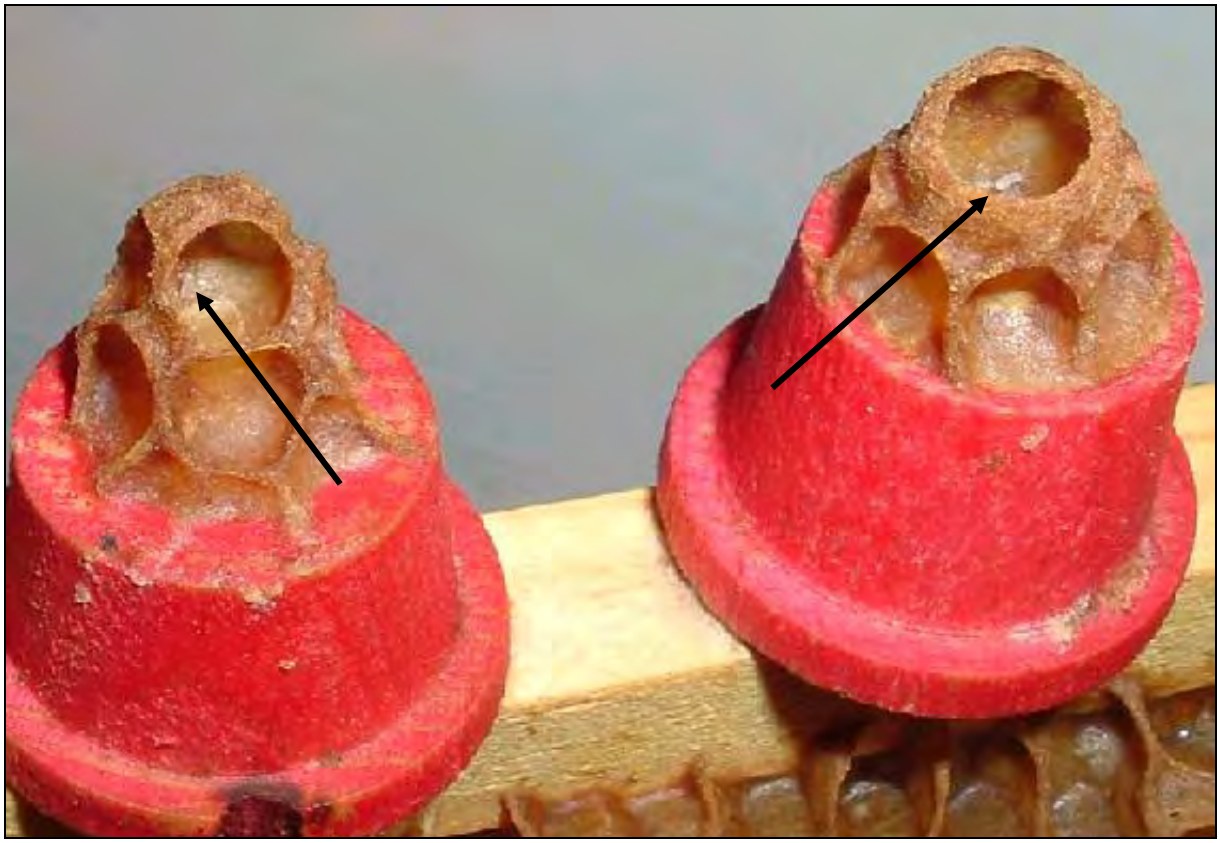

Fig. 4.5. Single worker-laid eggs in artificial queen cell cups

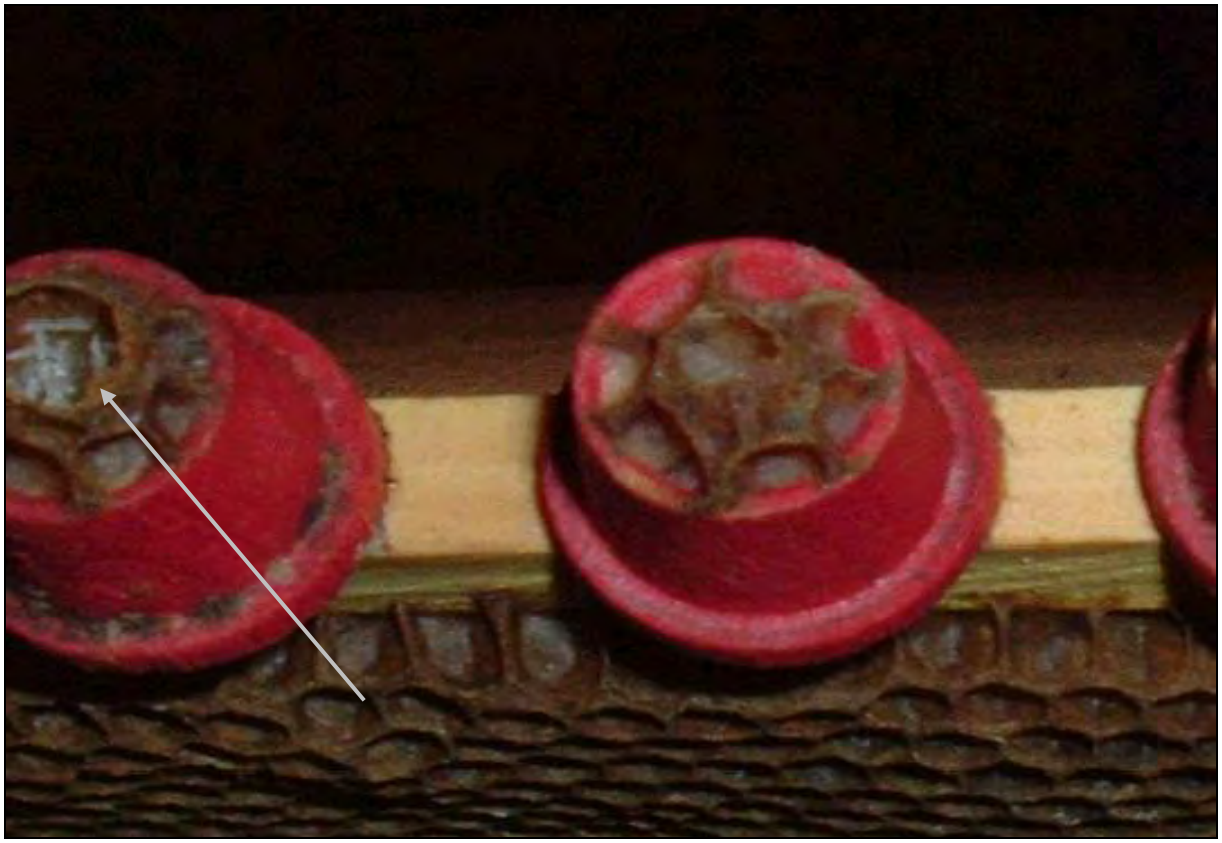

Fig. 4.6. Multiple worker-laid eggs in artificial queen cell cup

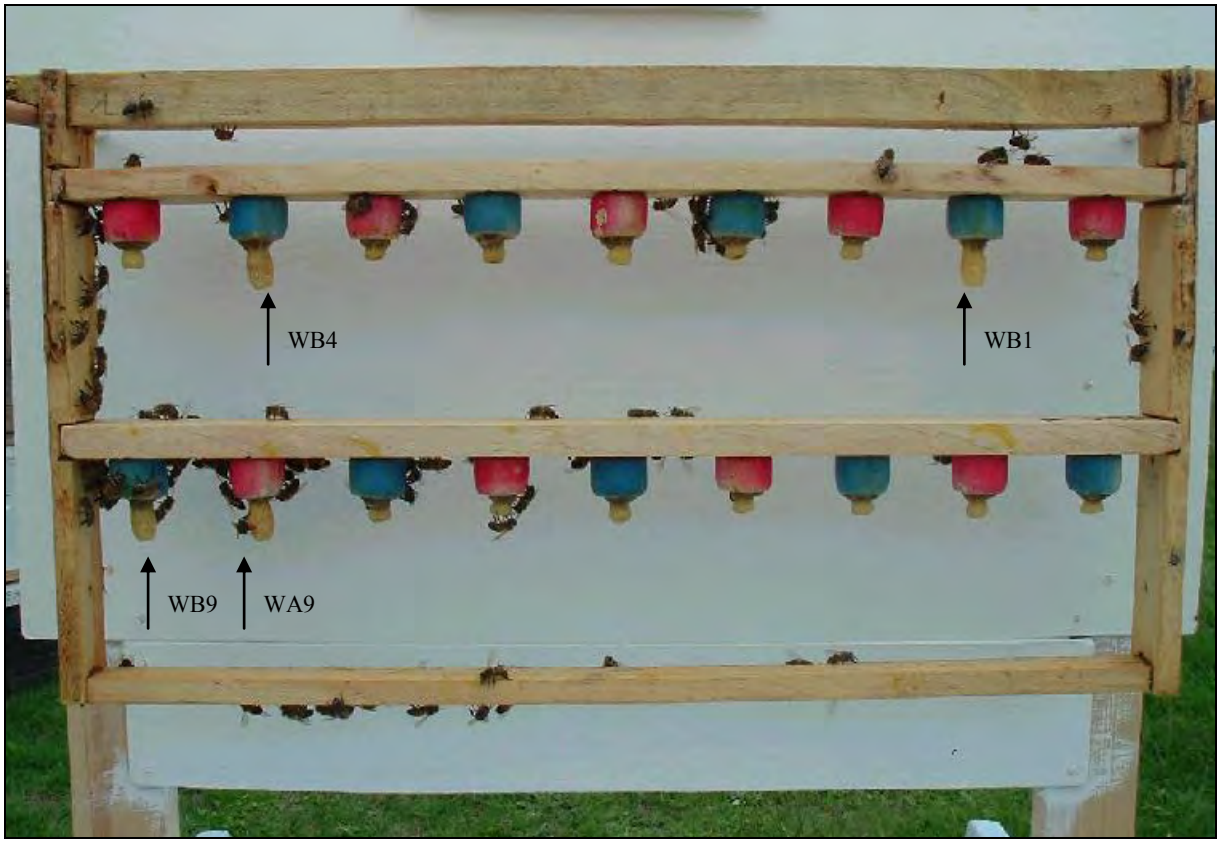

Fig. 4.7. Breeding frame colony (W), with four cells containing worker-derived larvae (day 21)

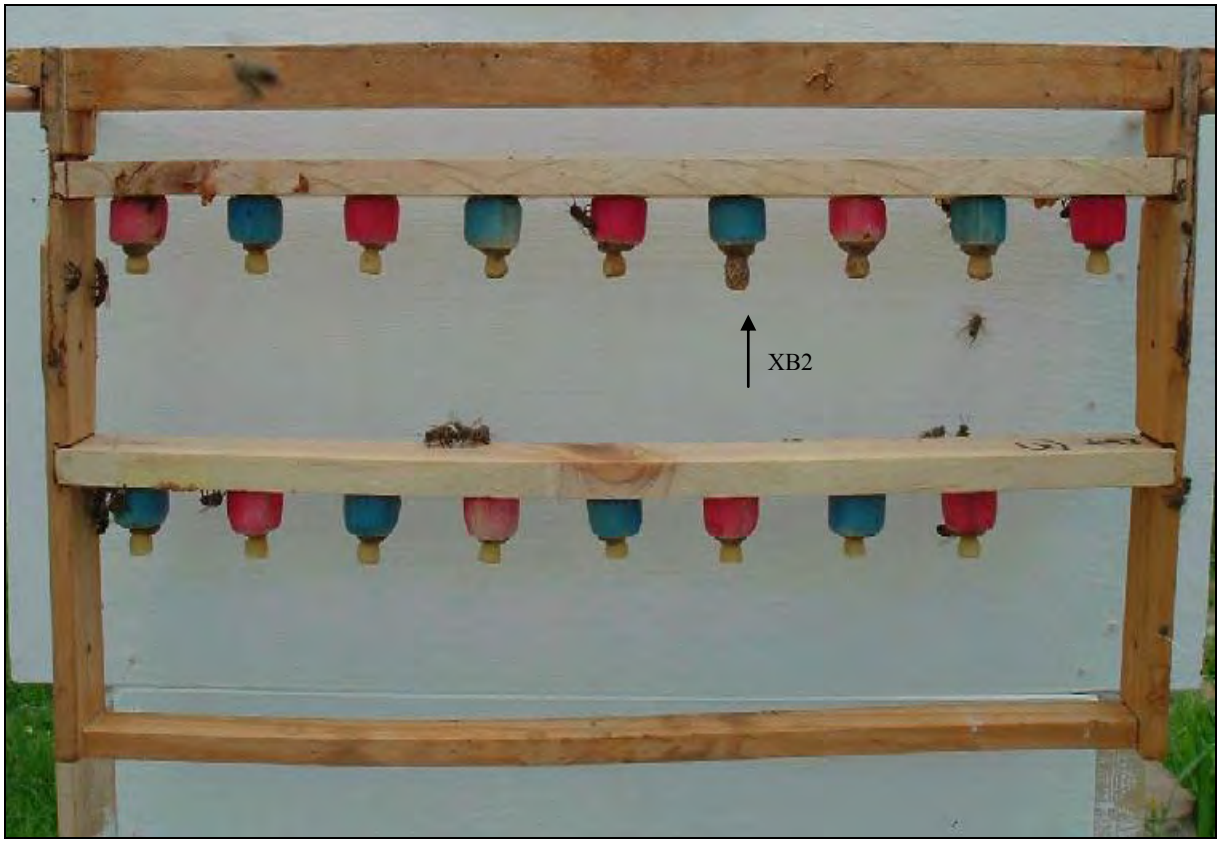

Fig. 4.8. Breeding frame colony (X), with one cell containing worker-derived larva (day 21)

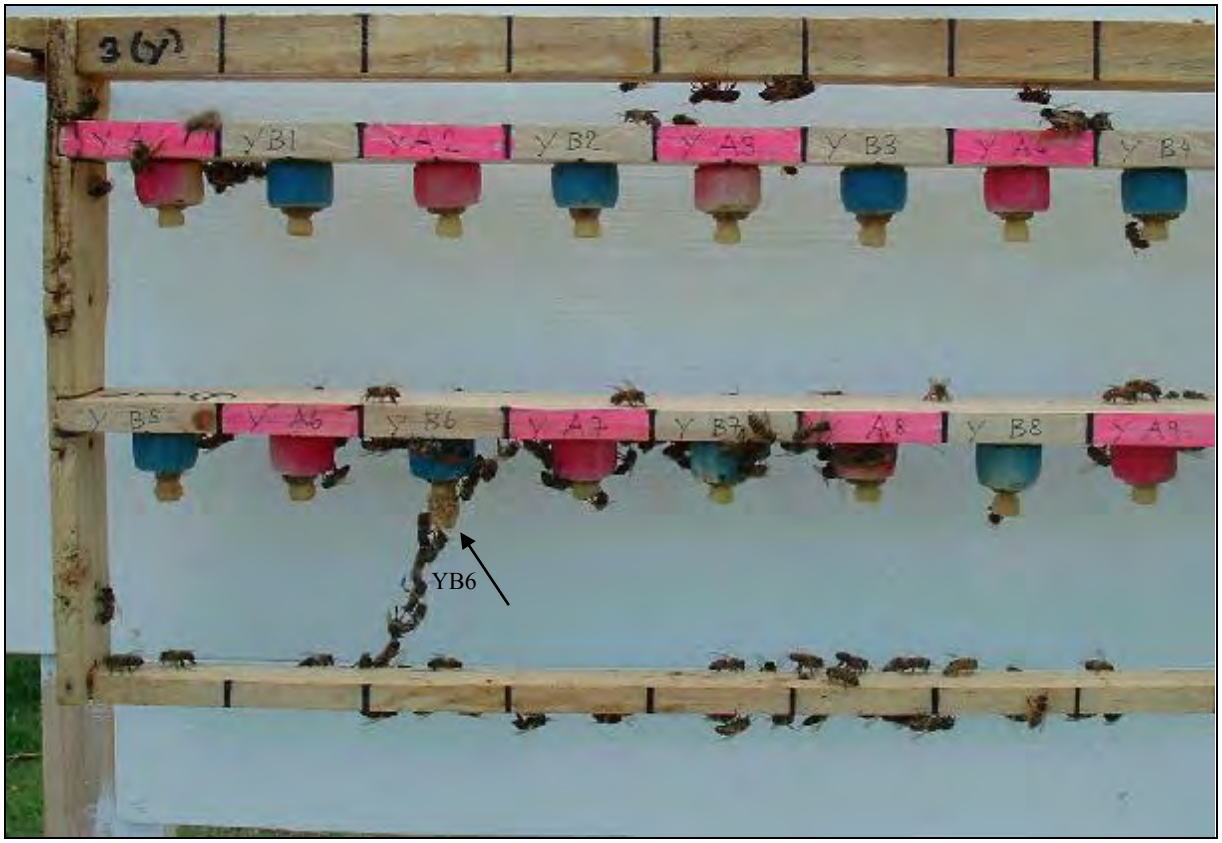

Fig. 4.9. Breeding frame colony (Y), with one cell containing worker-derived larva (day 21)

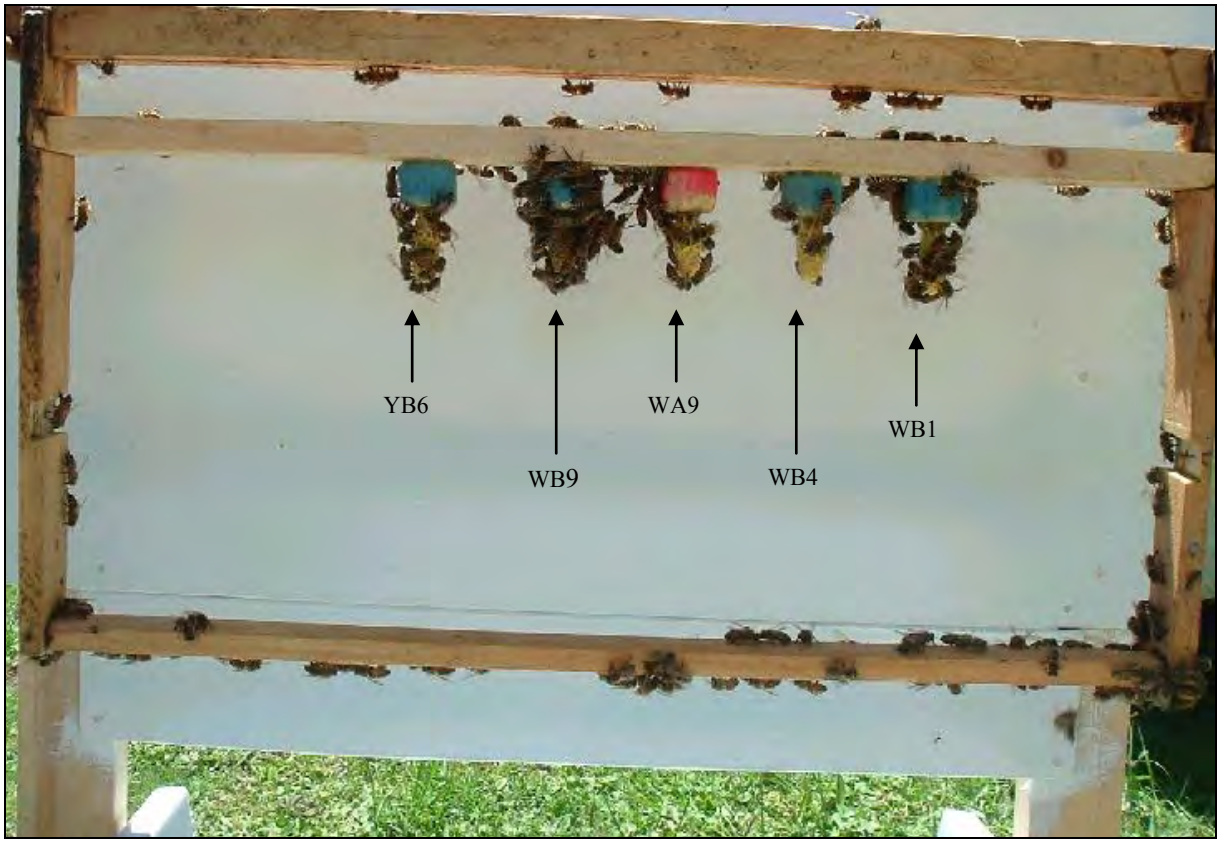

Fig. 4.10. Sealed cells from colonies (W) and (Y) combined on one breeding frame (day 25)

Fig. 4.11. Two successfully eclosed queen cells from colony (W) (day 31)

CHAPTER 5

Ranking pheromonal status in honeybee (*Apis mellifera capensis* Eschscholtz) gynes

SUMMARY

In the Cape honeybee (*Apis mellifera capensis*), laying workers produce clonal, diploid, female offspring from unfertilised eggs via thelytokous parthenogenesis. This unique feature in honeybees allows colonies of the Cape honeybee to produce queens, not only from queen-derived brood but also from worker-derived brood. Due to their different developmental and genetic origins, differences in reproductive success for queen- and worker-derived queens were suggested. Reproductive success in honeybee queens is linked to dominance hierarchies, which are mainly pheromonally modulated. A correlation can be assumed between the position within the dominance hierarchy and the pheromonal status of an individual honeybee. I used the mean amounts and percentage composition of five essential mandibular gland pheromone compounds of laying workers and mated queens to establish pheromonal reference patterns for low and high reproductive success. This

allowed ranking of queen- and laying worker-derived virgin queens according to their pheromonal status. Queen- and laying worker-derived virgin queens could not, however, be separated by differences in their pheromonal blend, suggesting equal dominance position and potential reproductive success. Reproductive dominance appeared to be determined by a function of relative compositional and absolute quantitative pheromonal patterns, where individuals, which produce compositionally most queen-like blends in highest quantities, occupy top positions.

INTRODUCTION

The evolution of sociality requires communication systems (Engels et al. 1993). In social insects the inter-individual exchange of information is largely mediated by semiochemicals (Free 1987). The integration of the large number of individuals into eusocial colonies is accomplished by dominance hierarchies, which are executed mainly by pheromones (Brian 1980; Fletcher and Ross 1985). In honeybees (*Apis mellifera* L.), the queen's reproductive dominance is maintained mainly by suppression of worker reproduction (Free 1987; Winston 1987). This passive control is accomplished by a combination of brood (Le Conte et al. 1990) and queen pheromones (Winston et al. 1989). Queen mandibular gland secretions are particularly important signals for queen-worker interactions, both as releaser and primer pheromones (Pankiw et al. 1996; Winston and Slessor 1998). The diverse actions of queen mandibular gland secretions as primer pheromones to maintain the monogynous homeostasis (Engels 1986) include in part the inhibition of queen-rearing (Butler and Callow 1968; Winston et al. 1991), the suppression of ovary development in workers (Willis et al. 1990; Plettner et al. 1993) and the delay of reproductive swarming events (Winston et al. 1991).

Queen mandibular gland secretions are a blend of more than 50 compounds (Engels et al. 1997), with five main components commonly referred to as the queen's mandibular gland pheromone (QMP) (Winston and Slessor 1998). QMP consists of (*E*)-9-keto-2-decenoic acid (9-ODA), (*R,E*)-(-)- and (*S,E*)-(+)-9-hydroxy-2-decenoic acid (9-HDA), methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Winston and Slessor 1998). All five components are necessary to elicit the full range of worker responses to QMP (Winston et al. 1989, 1990, 1991), but may vary in quantity and proportions of the individual compounds (Slessor et al. 1990; Pankiw et al. 1996). In contrast, the secretions of the workers' mandibular glands mainly consist of (*E*)-10-hydroxy-2-decenoic acid (10-HDA), which is a regioisomeric form of 9-ODA and 10-

hydroxydecanoic acid (10-HDAA) (Winston and Slessor 1992; Plettner et al. 1993). These compounds are also part of the mandibular secretions of queens, but only in minor quantities (Crewe and Velthuis 1980).

However, the colonies' dominance structures as well as the physiological differences between the two female castes, show some plasticity (Crewe and Velthuis 1980). The pheromonal control of worker reproduction sometimes fails and a small number of workers are able to develop their ovaries and to produce offspring even in queenright colonies (Visscher 1989). But especially after queen loss and in the absence of queen-derived brood, when dominance hierarchies collapse, the occurrence of laying workers becomes the rule in honeybee colonies (Bourke 1988). Their unfertilised offspring develop into drones, the worker force dwindles and the colony eventually dies (Hepburn and Radloff 1998). Some laying workers not only regain the ability to lay eggs, but also develop queen-like mandibular gland secretions (Crewe and Velthuis 1980). These 'pseudoqueens' (Onions 1912; Velthuis et al. 1990) are able to elicit retinue behaviour, suppress reproduction of other workers and inhibit emergency queen-rearing, thus mimicking real queens (Velthuis et al. 1990).

Pseudoqueen development is particularly common in queenless colonies of the Cape honeybee, *A. m. capensis* (Onions 1912; Anderson 1963; Neumann and Moritz 2002). *A. m. capensis* workers have the unique ability to produce diploid clonal female offspring from unfertilised eggs via thelytokous parthenogenesis (Onions 1912; Anderson 1963; Moritz and Haberl 1994). This trait allows for the maintenance of a reasonably sized worker force derived from laying worker offspring, enabling the colony to persist queenless for prolonged periods (Hepburn and Radloff 1998). If these colonies are queenless for long enough, they develop an entirely new range of stimulatory and inhibitory properties and new dominance hierarchies represented by normal workers, laying workers, pseudoqueens and intermediates (Hepburn and Radloff 1998). Pseudoqueens are therefore considered to occupy an intermediate position between workers and queens in terms of their ability to produce queen-like signals (Crewe and Velthuis 1980). Mated queens in general produce larger quantities of mandibular secretions which are dominated by 9-ODA with only minor 10-HDA components, and workers produce smaller amounts of mandibular gland secretions which are dominated by 10-HDA with negligible 9-ODA components (Crewe and Velthuis 1980). Clearly the distinction between mandibular gland secretions of queens and workers is more complex with particular quantities (Crewe 1988) and proportions (Hemmling et al. 1979) of the different components dependent on caste differentiation,

social position between more dominant and more subordinate individuals and age (Crewe and Velthuis 1980; Hillesheim et al. 1989).

Thelytokous parthenogenesis in *A. m. capensis* workers results in clonal female offspring, representing the unaltered genotype of their mothers (Moritz and Haberl 1994). Moreover, the dominance structures in honeybees appear to be widely genetically determined (Moritz and Hillesheim 1985). Subsequently the genotypes of clonal female offspring can be classified as superior, because their mothers were able to occupy a superior social status among workers as recipients rather than as donors in trophallactic interactions (Korst and Velthuis 1982; Schäfer et al. 2006), and because they were able to dominate reproduction after queen loss (Moritz et al. 1996). The thelytokous trait in *A. m. capensis* therefore allows the comparison of differences in the composition of mandibular gland secretions between different groups of female reproductives of clearly defined caste, social position, genotype and age. Thus it is possible to deduce patterns of reproductive dominance from mandibular gland pheromone composition.

Here I compare the five essential constituents of the pheromonal blend (HOB, 9-ODA, 9-HDA, 10-HDA, 10-HDAA) of *A. m. capensis* virgin queens reared from randomly selected queen-derived brood and laying worker-derived brood, using the pheromonal composition of pseudoqueens and of mated queens as reference for phenotypes with inferior or superior reproductive dominance status. I hypothesise that virgin queens should pheromonally occupy an intermediate position, virgin queens from worker-derived offspring tending towards the higher, and virgin queens from queen-derived offspring tending towards the lower margins of the dominance potential of virgin queens.

MATERIALS AND METHODS

Honeybee colonies

In January 2005, a honeybee colony pool was stocked at three apiaries in Grahamstown (Eastern Cape, South Africa) with unrelated, queenright colonies of: 1) *A. m. capensis* from the Eastern and Western Cape (N = 51) and 2) *A. m. scutellata* from the Pretoria area (N = 7); (see Hepburn and Radloff (1998) for a detailed review on the distribution and biology of these honeybee populations). The *A. m. scutellata* colonies were kept at a separate apiary to minimise intersubspecific drifting, dispersing, naturally occurring swarm mergers and infestations by socially parasitic workers (Neumann and Hepburn 2002). All colonies were kept in standard Langstroth hives (one breeding chamber and one shallow honey chamber) and had sufficient food (honey and pollen).

Production of 'pseudoqueens'

Freshly emerged *A. m. capensis* workers (N=60) were individually marked and singly introduced into small hoarding cages (10 x 12 x 10 cm^3) containing a piece of comb (6 x 5 cm^2) and 70 young *A. m. scutellata* nurse bees. *A. m. capensis* workers differentiate quickly into pheromonally, trophallactically and reproductively dominant laying workers when kept singly within groups of nurse bees from innately less dominant honeybee subspecies (e.g. *A. m. carnica*, Lattorff et al. 2005; e.g. *A. m. scutellata*, Schäfer et al. 2006). Food was supplied *ad libitum* (sugar candy, pollen and water). The cages were kept in a dark room at 28^o C and a relative humidity of 70-75%. The cages were checked every four days until day 12 for behavioural ratings: two-minute observations were carried out for each hoarding cage to detect and evaluate the marked *A. m. capensis* workers. If a clear court of *A. m. scutellata* nurse bees surrounding the *A. m. capensis* worker was observed (retinue behaviour), the tested worker received one rating point. The evaluated *A. m. capensis* workers therefore could accumulate up to three points for inducing retinue behaviour until day 12. Successful ovipositing was recorded from day 12 until the end of the experiment on day 19. All inspections were carried out under red light conditions to keep the bees undisturbed. On day 20, all live *A. m. capensis* workers were decapitated and their heads placed in 200 µl dichloromethane in order to extract mandibular gland secretions. The extractions lasted for at least 24 h after which gas-chromatographic (GC) analyses were performed (Simon et al. 2001). The workers' abdomens were dissected to determine their degree of ovarial development and to record pollen consumption. Ovarial development was categorised according to Velthuis (1970b): undeveloped, without vitellus or visible oocytes; intermediate, corresponding to the early stages in which the developing oocytes are bean-shaped; and developed, with chorionated oocytes. Pollen consumption, in form of accumulated undigested pollen grain-walls in the rectum, was categorised according to Schäfer et al. (2006): no pollen; small amount of pollen; large amount of pollen (rectum fully distended by pollen). Trophallactically dominant laying workers (Schäfer et al. 2006), able to induce retinue behaviour and to dominate reproduction among queenless workers with queen-like mandibular gland secretions, are termed 'pseudoqueens' (Velthuis et al. 1990). They receive predominantly nutrient jelly from subordinate workers, which leaves no visible sign of digestion in the gut (Schäfer et al. 2006). I therefore selectively determined as 'pseudoqueens' only those tested ovipositing *A. m. capensis* workers with a behavioural retinue-rating >2 points, fully developed ovaries, and no detectable pollen in

the rectum. In this study I used only these selected laying workers (N=11 'pseudoqueens') for comparisons of compositional patterns in mandibular gland secretions.

Donor colonies for virgin queen production

All donor colonies (N = 6 *A. m. capensis* colonies) intended to provide queen-derived female larvae for grafting were selected from the colony pool, 24 h prior to grafting. All donor colonies (N = 3 *A. m. capensis* colonies) intended to provide laying worker-derived female larvae for grafting were selected from the colony pool, at least three weeks prior to grafting and de-queened. All emergency queen cells were removed from these colonies and worker ovipositing was monitored at weekly intervals until sufficient larvae of the appropriate age were available.

Production of virgin queens

Queen-rearing from queen-derived and laying worker-derived 12-24 h old *A. m. capensis* female larvae in queenright breeding colonies (N = 20 *A. m. capensis* breeding colonies), was carried out in October 2006, according to standard protocols (Zander and Böttcher 1982). On the day of grafting, one hour prior to the introduction of the breeding frame with the grafted larvae, the open brood was removed and the old mated queen was confined to a peripheral part of the colony (Anderson 1965). Grafting followed routine procedures (Laidlaw 1979). The queen-cell cups were attached to standard wooden cell bases. Each breeding frame contained alternating groups of individually labelled queen-cell cups with queen-derived and laying worker-derived female larvae (N = 36 larvae on each frame; 18 queen-derived larvae, 18 laying worker-derived larvae).

Eight days after grafting (about three days prior to virgin queen emergence), all capped queen cells were secured in a queen cell cage together with five workers. About one day after queen emergence, successfully eclosed virgin queens were individually marked and transferred into standard plastic 'introduction' queen cages (8.0 x 3.4 x 1.0 cm^3; with 30 holes 3.0 x 3.0 mm^2), containing 9 young worker bees and queen candy (castor sugar and honey), as required for the cages. The old mated queens were removed from the breeding colonies for dissections and gas-chromatography (GC) analyses. All caged virgin queens were stored (banked) within their breeding colonies for at least eight days to reach pheromonal maturity for mating and no longer than 13 days (Wossler et al. 2006) as required for the experiments. The queen cages, allowed optimal nutritional provision by the breeding colonies' nurse bees. After termination of the experiments, all available virgin

queens (N=77 laying worker-derived virgin queens and N=93 queen-derived virgin queens) were live decapitated for GC analyses and dissections as described above.

Samples of mated queens

Samples of mated *A. m. capensis* queens (N=17), more than 100 days old and heading strong colonies, were obtained from dequeening the breeding colonies after termination of the queen-rearing process. They were live decapitated for GC analyses and dissected as described above.

Gas-chromatographic analysis

For each extract, the dichloromethane was evaporated to dryness under a stream of nitrogen and the residue was re-dissolved in 20 µl internal standard (0.38 mg octanoic acid and tetradecane in 0.25 ml dichloromethane) and 20 µl bis-trimethylsilyl-trifluoroacet-amid were added (Simon et al. 2001). One µl of this solution was injected into a gas-chromatograph (Hewlett Packard© 5890), using the analytical conditions described by Simon et al. (2001).

Statistical analysis

The absolute masses (µg) and relative percentage compositions of five essential mandibular gland compounds (HOB, 9-ODA, 9-HDA, 10-HDAA, 10-HDA) were determined for samples of the following four groups of *A. m. capensis* female reproductives: Group 1: actively ovipositing 'pseudoqueens' (20 d old); Group 2: aged queen-derived virgin queens (8-13 d old); Group 3: aged worker-derived virgin queens (8-13 d old); and Group 4: mature mated queens (>100 d old). ANOVA procedures were used to test for differences in absolute masses and relative percentage compositions of the compounds between the four groups (Zar 1996). Prior to analysis, homogeneity of variances of data was examined using Levene's test. Heterogeneity was stabilised after logarithmic transformation of absolute masses and arcsin transformation of relative percentages. Scheffé post-hoc multiple comparisons tests were used for significant group effects. Correlation analyses were used to investigate the relationship between age, absolute masses and relative percentage compositions of the mandibular gland compounds within groups 2 and 3. All tests were performed using Statistica© (StatSoft 2004).

RESULTS

Masses

We found no significant correlation between age and absolute masses (µg) of five essential mandibular gland compounds within groups 2 and 3 of age 8-13 d (Pearson correlation: $-0.047 < r < 0.255$; $P>0.05$). I found significant mass-differences of all assessed pheromonal compounds among the four groups of *A. m. capensis* female reproductives (ANOVA: HOB: $F_{3,197} = 24.3$; 9-ODA: $F_{3,197} = 6.3$; 9-HDA: $F_{3,197} = 12.1$; 10-HDAA: $F_{3,197} = 5.8$; 10-HDA: $F_{3,197} = 11.7$; $P < 0.001$). Scheffé post-hoc tests revealed no significant differences in all mean pheromonal masses between queen-derived and worker-derived virgin queens (groups 2 and 3; $P> 0.947$) of different age (8-13 d; Tab. 5.1.; Fig. 5.1.).

However, highly significant differences in the mean masses of all pheromonal compounds were observed between 'pseudoqueens' (group 1) and mated queens (group 4; Scheffé: $P< 0.006$). The 'queen substance' 9-ODA, was the major compound for all test groups and the only one without significant differences in mean masses between virgin queens (groups 2 and 3) and mated queens (group 4; Scheffé: 9-ODA: $P>0.05$), but with significant differences between virgin queens (groups 2 and 3) and 'pseudoqueens' (group 1; Scheffé: 9-ODA: $P<0.05$). For all other compounds (HOB, 9-HDA, 10-HDAA, 10-HDA) this pattern was reversed with highly significant differences between virgin queens (groups 2 and 3) and mated queens (group 4; Scheffé: 9-ODA: $P<0.0001$) and no significant differences between virgin queens and 'pseudoqueens' (group 1; Scheffé: 9-ODA: $P>0.434$; Tab. 5.1.; Fig 5.1.). The mean masses of all assessed compounds increased from 'pseudoqueens' (group 1) to mated queens (group 4). The virgin queens (groups 2 and 3) held intermediate positions between 'pseudoqueens' (group 1) and mated queens (group 4) for mean masses of all compounds, with the exception of the worker substance 10-HDA, which was insignificantly higher in 'pseudoqueens' (group 1; Tab. 5.1.; Fig. 5.1.).

Percentages

Weak correlations were found between age and relative percentage compositions of five essential mandibular gland compounds within groups 2 and 3 of age 8-13 d (Pearson correlation: $-0.101 < r < 0.312$). The mean differences in percentage composition among the four groups were statistically significant for four pheromonal compounds, namely HOB, 9-ODA, 9-HDA and 10-HDA (ANOVA: HOB: $F_{3,197} = 35.2$; 9-ODA: $F_{3,197} = 14.4$; 9-HDA: $F_{3,197} = 11.0$; 10-HDA: $F_{3,197} = 5.0$; $P < 0.001$). There was no significant difference in the mean percentage of 10-HDAA among the groups (10-HDAA: $F_{3,197} = 0.82$,

P=0.487). Moreover, Scheffé post-hoc tests showed no significant differences in the mean percentages of all assessed compounds between queen-derived and worker-derived virgin queens (groups 2 and 3; Scheffé: P>0.635) of different age (8-13 d). Furthermore, there were no significant differences in the mean percentages of all pheromonal compounds between 'pseudoqueens' (group 1) and mated queens (group 4; Scheffé: P>0.05; Tab. 5.2.; Fig. 5.2.).

9-ODA, the queen substance, was the major component of the assessed pheromonal spectra for all test groups. However, together with HOB highly significant differences in mean percentages between the virgin queens (groups 2 and 3) and 'pseudoqueens' (group 1; Scheffé: P<0.0001) as well as mated queens (group 4; Scheffé: P<0.002) were observed: Mean 9-ODA percentages were significantly higher, and mean HOB percentages significantly lower in the virgin queens (groups 2 and 3) when compared to 'pseudoqueens' and mated queens (groups 1 and 4). Virgin queens (groups 2 and 3) were significantly different to mated queens (group 4; Scheffé: P<0.0001) in mean percentages of 9-HDA and to pseudoqueens (group 1; Scheffé: P<0.004) in mean percentages of 10-HDA (Tab. 5.2.; Fig. 5.2.).

DISCUSSION

When comparing five essential mandibular gland pheromonal compounds (HOB, 9-ODA, 9-HDA, 10-HDAA, 10-HDA), *A. m. capensis* queen-derived and worker-derived virgin queens of different ages (8-13 d) could not be separated using relative compositional or quantitative data of the assessed pheromonal blend. Pseudoqueens differed significantly from mated queens in the quantities of pheromonal compounds, but not in the relative composition of the assessed pheromonal spectrum. Virgin queens quantitatively held a pheromonally intermediate position between pseudoqueens and mated queens, but compositionally top position for 9-ODA and lowest position for HOB.

A. m. capensis colonies can be headed and maintained by thelytokously reproducing laying workers ('pseudoqueens') over extended periods of time (Hepburn and Radloff 1998). However, 'pseudoqueens' are morphologically workers, unable to mate and physically limited, with highly reduced reproductive success (Hepburn and Allsopp 1994). Colonies headed by laying workers decrease in strength and eventually die, if requeening from worker-derived brood fails (Hepburn and Radloff 1998). Laying workers therefore can be considered lowest ranking within the reproductive hierarchy of *A. m. capensis* colonies. Clearly the highest position is occupied by successfully mated queens, which are

able to build up colonies of increasing strength and with high reproductive success (number of male and female reproductives produced and number of swarms issued) (Moritz and Southwick 1992; Kraus et al. 2003).

Between both extremes intermediates occur, with queens of reduced reproductive quality, increasingly more worker-like in appearance (Weiss 1983a) and laying workers with more queen-like characteristics, behaviourally 'pseudoqueens' (Allsopp et al. 2003). Dominance hierarchies are mainly pheromonally executed in honeybees, with a range of biologically active pheromonal compounds produced in the mandibular glands of workers and queens (Hemmling et al. 1979; Pankiw et al. 1996; Winston and Slessor 1998). A correlation can therefore be assumed for dominance position and differences in the expression of a complex pheromonal blend. And indeed, *A. m. capensis* 'pseudoqueens' can, in the absence of a queen, express a compositionally queen-like mandibular gland bouquet and pheromonally dominate and control reproduction in other workers (Crewe and Velthuis 1980). However, after successful emergency queen-rearing, they are in turn controlled and reproductively re-repressed by a new virgin queen or later by a successfully mated queen of higher social status (Hepburn and Radloff 1998).

We used the mean pheromonal masses and percentage composition of pseudoqueens and mated queens to establish pheromonal reference patterns for low and high reproductive status. Virgin queens, morphologically queen-like, but unmated and therefore immature, can be assumed to hold an intermediate dominance position between these two groups. The two different groups of virgin queens (queen-derived and laying worker-derived) could then be ranked according to their pheromonal status, in order to positively determine higher or lower dominance positions as an indicator for potential differences in reproductive quality.

However, virgin queens of different ages (8-13 d) and both different virgin queen types could not be separated by their pheromonal spectra, suggesting equal dominance status and potentially equal reproductive success of queen-derived and laying worker-derived virgin queens. When comparing absolute masses of pheromonal compounds, both types of virgin queens were indistinguishable from pseudoqueens for all compounds, with the exception of the queen substance 9-ODA, they did not differ significantly from mated queens. When comparing relative pheromonal composition, no significant differences between pseudoqueens and mated queens were observed, both types of virgin queens were either indistinguishable from pseudoqueens or mated queens in the substances 10-HDAA, 10-HDA, and 9-HDA, therefore holding intermediate positions. They differed, however,

significantly from both mated queens and pseudoqueens with a pheromonal bouquet highly dominated by 9-ODA and very low HOB levels.

Differences between pheromonal bouquets of pseudoqueens and mated queens were largely quantitative, clearly indicating an increasing trend with increasing reproductive quality for absolute pheromonal masses, once a compositionally queen-like pheromonal blend is reached. Within the quantitative pheromonal continuum, virgin queens hold intermediate positions between both reference groups, compositionally however they form a distinct group. Their relatively increased 9-ODA levels may enhance mating and swarming-success by increasing pheromonal attractiveness to drones and workers during this specific life stage, however their dominance status may be impaired due to relatively less queen-like pheromonal spectra. Dominance therefore appears to be determined by a function of compositional and quantitative pheromonal patterns, where individuals, which produce compositionally the most queen-like blends in highest quantities, occupy the highest positions.

Tab. 5.1. Mean masses and standard errors (µg; mean ± SE) of five major mandibular gland compounds in female reproductives of *A. m. capensis*

Compound	laying workers 'pseudoqueens' N=11	queen-derived virgin queens N=93	worker-derived virgin queens N=77	mated queens N=17
HOB	$0.22^{a} \pm 0.04$	$0.47^{a} \pm 0.11$	$0.55^{a} \pm 0.14$	$4.77^{b} \pm 1.44$
9-ODA	$6.97^{a} \pm 2.09$	$35.88^{b} \pm 6.69$	$41.20^{b} \pm 8.51$	$76.27^{b} \pm 25.36$
9-HDA	$1.07^{a} \pm 0.30$	$2.58^{a} \pm 0.38$	$2.75^{a} \pm 0.48$	$15.55^{b} \pm 4.68$
10-HDAA	$0.09^{a} \pm 0.03$	$0.92^{a} \pm 0.37$	$0.69^{a} \pm 0.24$	$2.52^{b} \pm 1.05$
10-HDA	$0.79^{a} \pm 0.27$	$0.61^{a} \pm 0.06$	$0.62^{a} \pm 0.08$	$5.36^{b} \pm 2.86$

*Means within one row followed by the same letter are not significantly different (P>0.05, Scheffé)

Tab. 5.2. Mean percentages and standard errors (mean ± SE) of five major mandibular gland compounds in female reproductives of *A. m. capensis*

Compound	laying workers 'pseudoqueens' N=11	queen-derived virgin queens N=93	worker-derived virgin queens N=77	mated queens N=17
HOB	$3.13^{a} \pm 0.53$	$1.17^{b} \pm 0.12$	$1.27^{b} \pm 0.14$	$5.34^{a} \pm 0.82$
9-ODA	$72.85^{a} \pm 3.31$	$84.80^{b} \pm 0.97$	$84.69^{b} \pm 1.17$	$71.28^{a} \pm 2.21$
9-HDA	$12.10^{ab} \pm 2.00$	$8.16^{b} \pm 0.56$	$8.37^{b} \pm 0.55$	$16.93^{a} \pm 1.45$
10-HDAA	$1.18^{a} \pm 0.38$	$2.07^{a} \pm 0.32$	$1.48^{a} \pm 0.27$	$1.57^{a} \pm 0.33$
10-HDA	$10.74^{a} \pm 3.04$	$3.81^{b} \pm 0.49$	$4.19^{b} \pm 0.65$	$4.88^{ab} \pm 1.38$

*Means within one row followed by the same letter are not significantly different (P>0.05, Scheffé)

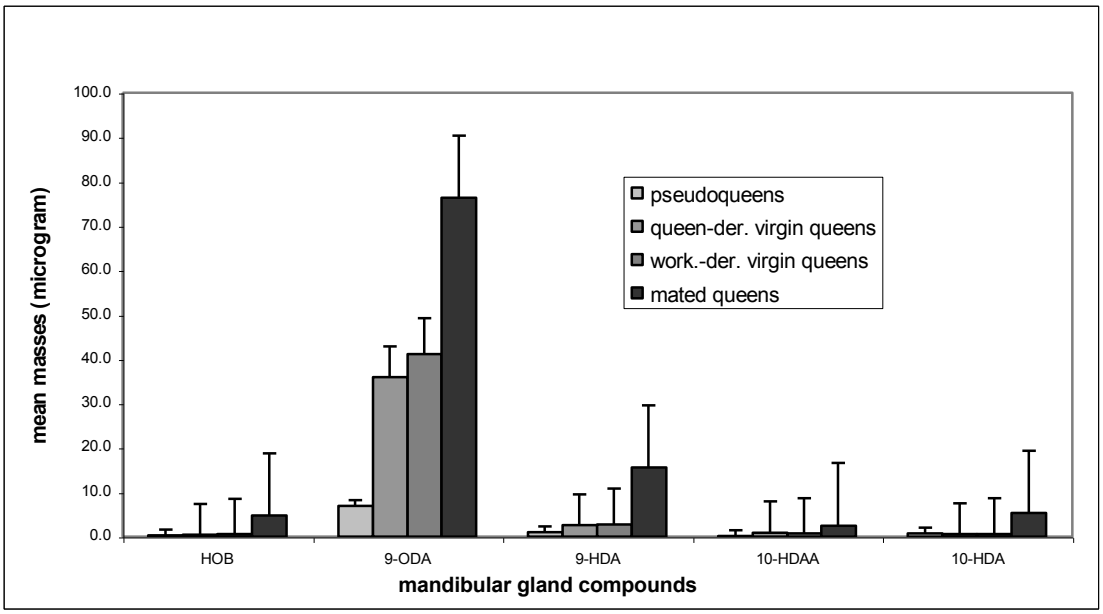

Fig. 5.1. Mean masses of mandibular gland compounds (µg) in *A. m. capensis* female reproductives

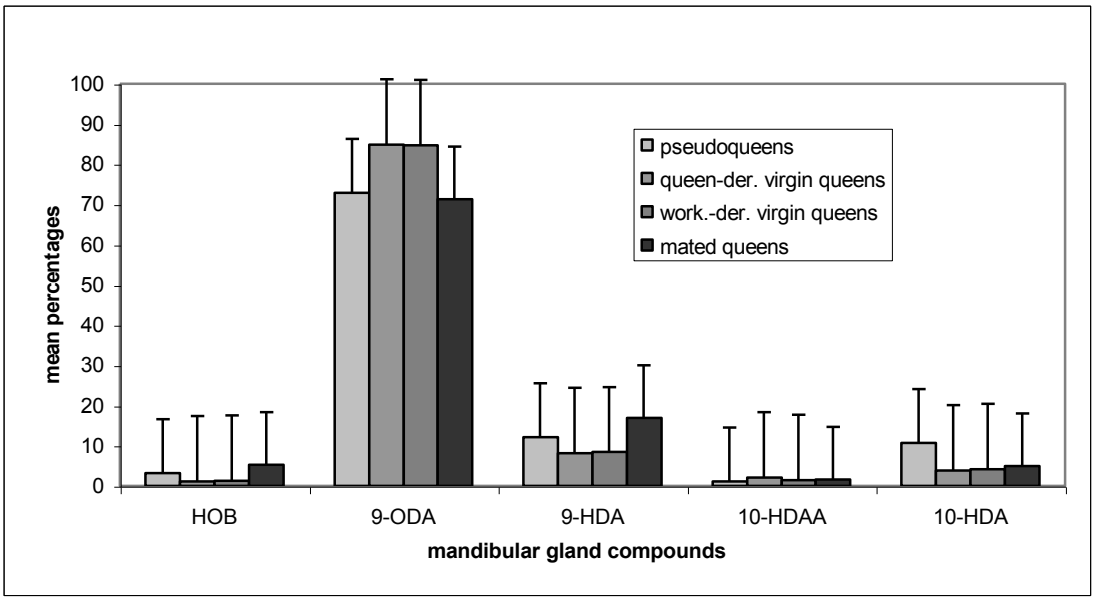

Fig. 5.2. Mean percentages of mandibular gland compounds in *A. m. capensis* female reproductives

CHAPTER 6

Worker clustering induced by queens and laying workers in *Apis mellifera capensis* Eschscholtz

SUMMARY

Behavioural bioassays were carried out to assess differences in attractiveness of *Apis mellifera capensis* virgin queens and laying workers to queenless workers. Samples of caged virgin queens reared from fertilised queen brood, from worker-derived unfertilised clonal offspring and samples of physically ovipositing laying workers were simultaneously offered to queenless workers in preference tests. The strongest cluster formation was observed in response to the virgin queen samples, a relatively reduced cluster formation was observed in response to laying worker samples. The behavioural data were positively correlated to differences in mandibular gland pheromonal spectra (relative 9-ODA

percentages and ratios of absolute masses of 9-ODA / (9-ODA + 10-HDA)). Virgin queens had pheromonal bouquets high in the queen substance 9-ODA with low levels of worker substance 10-HDA. In contrast, laying worker signals were less queen-like with higher percentages of 10-HDA. Differences in attractiveness to workers and differences in the composition of the pheromonal bouquets were insignificant for queen- and laying worker-derived virgin queens, indicating equal potential for reproductive success.

INTRODUCTION

Eusocial insect colonies differ from most other animal societies in that the entire group's reproductive output typically hinges on one reproductive female, the queen (Gilley et al. 2003). The colony's fitness therefore depends largely on her reproductive quality (Hatch et al. 1999). Colonies, with high-quality queens, which are successfully mated and able to produce a large number of viable offspring, will outcompete colonies headed by queens of lower quality (Gilley at al. 2003). Honeybee (*Apis mellifera* L.) colonies produce queens mainly during the reproductive stage of their lifecycle in a process of colony fission known as 'swarming' (Wilson 1971). This process includes three different stages (Gilley et al. 2003): (1) the rearing of 15-25 virgin queens (Winston 1987); (2) the departure of the old queen with about half of the colony's worker force in a primary swarm; and (3) elimination of supernumerary virgin queens or their departure from the nest in a number of secondary swarms until a single successor remains to inherit the natal nest (Gilley and Tarpy 2005). Secondary swarms, which are considered beneficial to the survival, propagation and dispersal of the species (Hepburn 2006), typically depart with fewer workers, eventually settle at a new nesting site and found a new colony.

Swarming in honeybees is generally a pheromonally mediated process. The departure of secondary swarms combines separate pheromones produced by queens and workers (Schmidt et al. 1993). Queen mandibular gland secretions are a blend of more than 50 compounds (Engels et al. 1997), of which the five main components are commonly referred to as the queen's mandibular gland pheromone (QMP) (Winston and Slessor 1998). QMP, with the 'queen substance' (*E*)-9-keto-2-decenoic acid (9-ODA) and (*R,E*)-(-)- and (*S,E*)-(+)-9-hydroxy-2-decenoic acid (9-HDA) as its most prevalent components (Free 1987), besides other activities, acts as a short-range attractant to queenless workers and induces clustering. Other constituents of the pheromonal bouquet of queens may act synergistically with QMP (Velthuis 1970; Free 1987). The worker-produced 'Nasanov pheromone' compliments the pheromonal secretions of the queen as a long-range attractant

especially during airborne and migratory swarm phases (Schmidt et al. 1993). Obviously, very small secondary swarms are less likely to survive than larger swarms (Getz et al. 1982). Virgin queens, which are able to attract more workers to accompany them during swarming and to settle as stronger clusters, will be able to utilise resources more efficiently, expand more quickly and reproduce more successfully (Moritz and Southwick 1992). Consequently it can be assumed that in secondary swarms the reproductive success of a virgin queen is strongly linked to the number of workers, which constitute the swarm. The ability to induce clustering in workers can therefore be used to quantify potential reproductive success.

After accidental queen loss, and in the absence of queen-derived brood, the development of laying workers is common in honeybees (Bourke 1988). Laying workers however, are unable to mate. Due to the hymenopteran haplodiploid sex determination system, they produce exclusively male offspring, until the queenless colony eventually dies (Hepburn and Radloff 1998). In strong contrast, laying workers in the honeybee subspecies *A. m. capensis* produce clonal diploid female offspring from unfertilised eggs via thelytokous parthenogenesis (Onions 1912; Anderson 1963; Moritz and Haberl 1994). Moreover, *A. m. capensis* laying workers quickly develop a queen-like pheromonal bouquet, with relatively high levels of 9-ODA (Crewe and Velthuis 1980), which enables them to act as 'pseudoqueens' by dominating reproduction and taking over queen functions (Onions 1912; Velthuis et al. 1990). Queenless *A. m. capensis* colonies are therefore not necessarily doomed, but can persist as laying worker-headed colonies and re-queen from worker-derived female offspring (Hepburn and Radloff 1998). Two different reproductive castes and queens from two different origins therefore exist in *A. m. capensis* populations: Laying workers, which take over queen-functions as 'pseudoqueens' in queenless colonies; queens from fertilised queen-derived brood, which are mainly produced in queenright swarming colonies; and queens from clonal unfertilised laying worker-derived offspring, which are produced in queenless colonies in an effort to reinstate a queenright condition. Due to their different developmental and genetic histories, differences in reproductive success and their ability to attract queenless workers can be assumed.

Here I present data of bioassays assessing the ability of caged virgin queens and laying workers to induce worker clustering behaviour around their cages. I simultaneously tested combinations of four caged samples: One (empty) control, and one of each of the following groups: Virgin queens reared from queen-derived brood; virgin queens reared from laying worker-derived brood; and laying workers. I assumed differences in the

number of attracted workers between laying worker- and queen-derived queens, indicating differences in reproductive quality. Laying workers, on the other hand, obviously of inferior reproductive quality than queens due to their physical limitations (Hepburn and Allsopp 1994) may, however, induce clustering behaviour in a range similar to real queens due to their queen-like pheromonal bouquet. To test my hypothesis I compared my observations with GC-data of the mandibular gland pheromonal profiles of the assessed queens and laying workers, correlating worker response to differences in pheromonal composition.

MATERIALS AND METHODS

Honeybee colonies

In January 2005, a honeybee colony pool was stocked at three apiaries in Grahamstown (Eastern Cape, South Africa) with unrelated, queenright colonies of: (1) *A. m. capensis* from the Eastern and Western Cape (N = 51) and (2) *A. m. scutellata* from Pretoria (N = 7); (see Hepburn and Radloff (1998) for a detailed review on the distribution and biology of these honeybee populations). The *A. m. scutellata* colonies were kept at a separate apiary to minimise intersubspecific drifting, dispersing, naturally occurring swarm mergers and infestations by socially parasitic workers (Neumann and Hepburn 2002). All colonies were kept in standard Langstroth hives (one breeding chamber and one shallow honey chamber) and had sufficient food (honey and pollen).

Production of laying workers

Freshly emerged *A. m. capensis* workers (N=60) were individually marked and singly introduced into small hoarding cages (10 x 12 x 10 cm^3) containing a piece of comb (6 x 5 cm^2) and 70 young *A. m. scutellata* nurse bees. *A. m. capensis* workers differentiate quickly into pheromonally, trophallactically and reproductively dominant laying workers when kept singly within groups of nurse bees from innately less dominant honeybee subspecies (e.g. *A. m. carnica*, Lattorff et al. 2005; e.g. *A. m. scutellata*, Schäfer et al. 2006). Food was supplied *ad libitum* (sugar candy, pollen and water). The cages were kept in a dark room at 28^o C and a relative humidity of 70-75%. Successful ovipositing was recorded from day 12 until the end of the experiment on day 19. All inspections were carried out under red light conditions to keep the bees undisturbed. Worker samples for bioassays were removed from hoarding cages as required. After termination of the experiments *A. m. capensis* workers were live decapitated and their heads placed in 200 µl dichloromethane in order to extract

mandibular gland secretions. The extractions lasted for at least 24 h after which gas-chromatographic (GC) analyses were performed (Simon et al. 2001).

In this study I used only those differentiated workers where ovipositing was observed (N=21 actively laying workers) for bioassays and comparisons of mandibular gland pheromonal activity.

Production of virgin queens

All donor colonies (N = 6 *A. m. capensis* colonies) intended to provide queen-derived female larvae for grafting were selected from the colony pool 24 h prior to grafting. All donor colonies (N = 3 *A. m. capensis* colonies) intended to provide laying worker-derived female larvae for grafting were selected from the colony pool at least three weeks prior to grafting and de-queened. All emergency queen cells were removed from these colonies and worker ovipositing was monitored at weekly intervals until sufficient larvae of the appropriate age were available.

Queen-rearing from queen-derived and laying worker-derived 12-24 h old female *A. m. capensis* larvae in queenright breeding colonies (N = 20 *A. m. capensis* breeding colonies), was carried out at an experimental apiary in Stones Hill, Eastern Cape, South Africa, in October 2006, according to standard protocols (Zander and Böttcher 1982). On the grafting day, one hour prior to the introduction of the breeding frame with the grafted larvae, the open brood was removed and the old mated queen was confined to a peripheral part of the colony (Anderson 1965). Grafting followed routine procedures (Laidlaw 1979). The queen-cell cups were attached to standard wooden cell bases. Each breeding frame contained alternating groups of individually labelled queen-cell cups with queen-derived and laying worker-derived female larvae (N = 36 larvae on each frame; 18 larvae queen-derived, 18 larvae laying worker-derived). Eight days after grafting (about three days prior to virgin queen emergence), all capped queen cells were secured in a queen cell cage together with five workers. About one day after queen emergence, eclosed virgin queens were individually marked and transferred into standard plastic 'introduction' queen cages (8.0 x 3.4 x 1.0 cm^3; with 30 holes 3.0 x 3.0 mm^2), containing 9 young worker bees and queen candy (castor sugar and honey), as required for the cages. The old mated queens were removed from the breeding colonies for dissections and gas-chromatographically (GC) analyses. All caged virgin queens were stored (banked) within their breeding colonies for at least seven days to reach pheromonal maturity for mating (Wossler et al.

2006). The queen cages allowed optimal nutritional provision by the breeding colonies' nurse bees.

Aged virgin queens (N=21 queen derived and N=21 laying worker-derived) were selected for bioassays from all breeding colonies (queen banks) pair-wise but were later mixed and used in random combinations. After termination of the experiment, all virgin queens were live decapitated for GC analyses and dissections as described above.

Gas-chromatographic analysis

For each extract, the dichloromethane was evaporated to dryness under a stream of nitrogen and the residue was re-dissolved in 20 µl internal standard (0.38 mg octanoic acid and tetradecane in 0.25 ml dichloromethane) and 20 µl bis-trimethylsilyl-trifluoroacet-amid were added (Simon et al. 2001). One µl of this solution was injected into a gas-chromatograph (Hewlett Packard© 5890), using the analytical conditions described by Simon et al. (2001).

Behavioural bioassay

A swarm box (35 x 35 x 35 cm^3) consisting of a wooden frame, closed bottom board, closed lid and wire mesh side-walls (mesh size 3 x 3 mm^2) was used for bioassays testing worker preferences and clustering behaviour around simultaneously offered samples of female honeybees (Fig. 6.1.). Worker honeybees (150g, equal to about 2000 *A. m. capensis* workers) for clustering, were collected from the honey chambers of strong *A. m. capensis* colonies and kept queenless in the swarm box for 1h until the queenless status was clearly perceived by the bees (fanning, nervousness, no clustering). All tests were carried out in a dark room at 20^o C and manipulations or observations were carried out under red light conditions to reduce worker attraction to light stimuli. Before introduction of the test-samples, the bees were slightly water mist sprayed, shaken and the swarm box was quickly knocked on the floor to collect all workers at the bottom of the box. The closed lid was removed and immediately replaced with a test-lid. The test-lids were made from polystyrene (35 x 35 cm^2, strength 2 cm), providing perfect grip for clustering bees. At each corner about 6 cm inwards towards the centre and with a distance of 25 cm from another, 4 holes (∅ 2 cm) held 4 cylindrical queen cages (∅ 2 cm, height 8 cm, with 120 holes 3 x 3 mm^2), which protruded 6 cm into the swarm box (Fig. 6.1.). For each trial a combination of one sample of the following test-groups were assessed: one queen-derived

virgin queen (group 1); one laying worker-derived virgin queen (group 2); and one laying worker (group 3). Three of the cages each contained one of the live test samples. A fourth cage remained empty and served as a control (group 4). After 1 h, worker preferences and clustering behaviour were assessed for all four cages and estimated as follows: 0 = no response (< 10 workers); 1 = aggregation (10 - 100 workers); 2 = small cluster (100 - 500 workers); 3 = cluster (500 - 1000 workers); 4 = large cluster (> 1000 workers). After assessment of the worker responses, the bees were shaken from the cages to the bottom of the box and the test-lid was replaced with the original lid of the box. After 30 min of recovery time, the test procedure was repeated in a new trial as described, using a new lid and new cages containing a different set of live samples. Batches of worker bees to assess clustering behaviour were only used during four consecutive trials and then returned to their natal colony and replaced by a new batch of worker bees from another colony. The experiment was repeated with 21 different sets of live samples, which were prepared for GC analyses and dissected, as described above, immediately after each trial.

Statistical analysis

I compared clustering behaviour of worker bees to samples of (1) queen-derived virgin queens; (2) laying worker-derived virgin queens; (3) laying workers; and (4) controls (empty cages). Pearson χ^2 tests were used to test for significant differences in the clustering behaviour of worker bees within and between the four different groups.

I correlated the clustering behaviour of worker bees to the relative percentage compositions of five essential mandibular gland compounds (HOB, 9-ODA, 9-HDA, 10-HDAA, 10-HDA) of the test groups, to verify biological activity of specific compounds. I also correlated the observed worker behaviour responses to the quantitative ratios of the amounts of 9-ODA / (9-ODA+10-HDA). These ratios indicate a queen-like pheromonal blend when close to 1.0 and a worker-like pheromonal composition when close to 0.0 (Moritz et al. 2000, 2004).

ANOVA procedures were used to test for significant group effect in the relative percentage compositions of 9-ODA, 10-HDA and quantitative ratios (Zar 1996). Heterogeneity of the variances of 9-ODA and 10-HDA was stabilised after an arcsin transformation of the relative percentages. Scheffé post-hoc multiple comparisons tests were used for significant group effects. All tests were performed using Statistica© (StatSoft 2004).

RESULTS

Fifty-seven worker clustering-responses (clustering behaviour 1 - 4) and 27 cases with no response (clustering behaviour 0) were observed to a total number of 63 live samples and 21 empty control cages. Chi-square test analyses revealed significant differences in the clustering behaviour of worker bees both between and within the different groups (Between: $\chi^2 = 76.7$, df=12, P<0.0001; within: group 1: $\chi^2 = 9.2$, df=4, P<0.055, group 2: $\chi^2 = 20.2$, df=4, P<0.0005, group 3: $\chi^2 = 10.2$, df=4, P<0.037, group 4: $\chi^2 = 74.5$, df=4, P<0.0001; Tab. 6.1.). When considering clusters of more than 10 workers, 'clusters' (500-1000 workers) were most frequent (38.6%), followed by 'small clusters' (100-500 workers) (26.3%), 'large clusters' (>1000 workers) (21.1%) and 'aggregations' (10-100 workers) (14.0%). Significantly more 'large clusters' (>1000 workers) formed around queen-derived virgin queens (58.3%) and worker-derived virgin queens (33.3%), than around laying workers (8.3%). Similarly, significantly more 'clusters' (500-1000 workers) formed around queen-derived virgin queens (36.4%) and worker-derived virgin queens (54.5%), than around laying workers (9.1%). In contrast, 'small clusters' (100-500 workers) formed significantly more frequently around laying workers (60.0%) than around queen- or laying worker-derived virgin queens (both 20.0%). Similarly significantly more 'aggregations' (10-100 workers) were observed around laying workers (37.5%), than around queen- or laying worker-derived virgin queens (both 25.0%). A lack of response (clustering behaviour 0 = 0-9 workers) was most frequently observed around empty (control) cages (74.1%) followed by laying workers (22.2%) and finally by queen-derived queens (3.7%) (Tab. 6.1.)

There were no significant differences in the overall ability to induce worker-clustering between queen-derived and laying worker-derived virgin queens. For both groups a high percentage (74.4% and 76.2%, respectively) of the workers formed clusters of more than 500 workers, and a low percentage (both 23.8%) of clusters of 10 to 500 workers. Clustering, on the other hand, was significantly reduced in response to laying workers compared to both virgin queen groups with only 14.3% of the workers forming clusters of more than 500 workers, whilst 57.2% formed clusters of 10 to 500 workers (Tab. 6.1.).

9-ODA was the major constituent of the tested pheromonal blend of all samples and a significant positive correlation between 9-ODA percentages and worker responses was observed (r = 0.42, N=62, P=0.001). A weaker significant positive correlation was found

between worker responses and the quantitative ratios of 9-ODA / (9-ODA+10-HDA) (r = 0.28, N=62, P=0.030). However, a weak but significant negative correlation was found between worker responses and percentages of 9-HDA (r = -0.36, N=62, P=0.003).

There were highly significant differences of 9-ODA and 10-HDA percentages, and ratios among the 3 groups (9-ODA%: $F_{2,59}$=16.3, P<0.0001; 10-HDA%: $F_{2,59}$=7.4, P=0.0014; ratio: $F_{2,59}$=8.5, P=0.0006). Post-hoc multiple comparison tests revealed no significant differences of 9-ODA percentages and ratios between queen- and laying worker-derived virgin queens (9-ODA%: 86.7±0.9% and 78.8±2.9%, respectively; Scheffé: P=0.247; ratio: 0.96±0.01 and 0.90±0.02, respectively), but highly significant differences in 9-ODA percentages between virgin queens (both queen- and laying worker-derived) and laying workers were observed (9-ODA%: 59.1±5.6%, queen-derived: Scheffé: P<0.0001; worker-derived: Scheffé: P=0.0011; ratio: 0.78±0.05, queen-derived: Scheffé: P=0.0007; worker-derived: Scheffé: P=0.029). Virgin queens had low levels of the worker substance 10-HDA (queen-derived: 3.2±0.5%; worker-derived: 7.8±1.9%). In contrast, laying worker signals were less queen-like with significantly higher percentages of 10-HDA (16.7±4.4%; queen-derived: Scheffé: P=0.0011; worker-derived: Scheffé: P=0.054).

DISCUSSION

Behavioural bioassays were used to test for significant quantitative differences in *A. m. capensis* worker responses to *A. m. capensis* virgin queens reared from queen-derived brood versus virgin queens reared from laying worker-derived brood. Queen attractiveness to workers (worker clustering behaviour around cages containing queens or laying workers) was recorded as an indicator for potential reproductive success. Differences in fitness between both queen types have been suggested (Onions 1912; Mowbray 1916; Hartmann and Hartmann 1991). Moreover, queens from unfertilised laying worker brood, derived via the clonal thelytokous pathway, may express a different pheromonal bouquet than queens from fertilised queen brood. The bioassays, however, revealed no significant differences in attractiveness between both queen types, suggesting equally active pheromonal spectra. The bulk of the stronger worker responses with cluster formation >500 workers (clustering behaviour 3 and 4) was observed around samples of the two different virgin queen types. In contrast weaker responses with smaller clusters of 10-500 workers (clustering behaviour 1 and 2) were recorded mainly around the laying workers or around empty control cages. Laying workers therefore appeared to be significantly less attractive to queenless workers

than virgin queens. Ovary development co-varies in *A. m. capensis* workers with the development of a more queen-like pheromonal bouquet, dominated by the 'queen substance' 9-ODA (Hepburn 1992a). Physically ovipositing laying workers can therefore be assumed to express queen-like pheromonal blends and to attract workers in a range similar to real queens. However, in my study I observed that they obviously do so less successfully than virgin queens.

We used five essential pheromonal compounds of the mandibular gland secretions in honeybee queens and workers (the 'queen substances' HOB, 9-ODA, 9-HDA and the 'worker-substances' 10-HDA and 10-HDAA) to support my behavioural observations by comparing quantitative and qualitative data of the different pheromonal spectra. The quantitative ratio 9-ODA / (9-ODA+10-HDA) indicates whether 'worker substance' or 'queen substance' dominates a pheromonal bouquet (Moritz et al. 2000, 2004). I found a positive correlation of those ratios to clustering behaviour and a significant difference in mean ratios between virgin queens and laying workers, confirming my behavioural observations. Obviously the more worker-like pheromonal compositions of laying workers are less attractive and have only reduced activity in cluster formation. Similarly the more queen-like pheromonal ratio in queen-derived virgin queens may explain the high frequency of 'large clusters' (>1000 workers) in contrast to worker-derived virgin queens, which attracted mainly 'clusters' (500-1000 workers), possibly due to their slightly less queen-like pheromonal ratio (Tab. 6.1.).

In addition I observed strong positive correlations of the percentages of 9-ODA in the pheromonal blends to the number of clustering workers. However, Pain (1961) and Simpson (1963) stated that synthetic 9-ODA was unattractive to queenless workers. On the other hand, Velthuis and van Es (1964) demonstrated that worker bees from a queenless swarm were attracted by synthetic 9-ODA, but less so than by the complete extracts from a queen's head. In contrast, Butler and Simpson (1967) found that 9-ODA, especially when combined with 9-HDA, could fully match the attractiveness of a live queen. Moreover, as they would with a live queen, the queenless workers formed compact, stable clusters around a lure containing a mixture of synthetic 9-ODA and 9-HDA. These observations, however, were not confirmed by Morse and Boch (1971), who stated that combinations of synthetic 9-ODA and 9-HDA were less attractive than extracts from queen heads. Winston et al. (1982) suggested that the different activities of the enantiomeres of 9-HDA may well have played a part in the variable results reported for 9-HDA and demonstrated that a

mixture of the two enantiomeres rich in R-(-)-9-HDA was most stimulating and an active compound stabilising swarms of queenless workers.

My behavioural data confirm the significant influence of 9-ODA on attractiveness to workers, notwithstanding synergy effects with other compounds from the complete pheromonal bouquet in live samples. I could not confirm a significant influence of 9-HDA, on the contrary, I observed a weak negative correlation between worker responses and relative percentage composition of this 'queen substance' compound. I was able to demonstrate that virgin queens from queen-derived brood and from laying worker-derived offspring were equally attractive to clustering workers. This would allow them to depart in secondary swarms of equal size, indicating a similar potential for reproductive success in newly founded colonies.

Tab. 6.1. Frequencies of cluster formation around different groups of honeybee queens and workers and their pheromonal ratio indicating 'queen-likeness'

live samples	**large clusters (N=12) >1000 workers**	**clusters (N=22) 500-1000 workers**	**small clusters (N=15) 100-500 workers**	**aggre-gations (N=8) 10-100 workers**	**no response (N=27) 0-9 workers**	**mean pheromonal mass ratio* 9-ODA / (9-ODA+ 10-HDA) (queen-likeness)**
queen-derived virgin queens	7 58.3%	8 36.4%	3 20.0%	2 25.0%	1 3.7%	0.96 ± 0.03
worker-derived virgin queens	4 33.3%	12 54.5%	3 20.0%	2 25.0%	0 0.0%	0.90 ± 0.11
laying workers	1 8.3%	2 9.1%	9 60.0%	3 37.5%	6 22.2%	0.79 ± 0.23
empty cages (controls)	0 0.0%	0 0.0%	0 0.0%	1 12.5%	20 74.1%	not applicable

* 0-0.5 = worker-like; 0.5-0.9 = intermediate; 0.9-1.0 = queen-like (Schäfer et al. 2006)

Fig. 6.1. Swarm box with test-lid for worker preference tests

CHAPTER 7

Do *Apis mellifera capensis* Eschscholtz honeybee drones compete for more attractive virgin queens in a drone congregation area?

SUMMARY

Apis mellifera capensis drones preferentially responded towards apparently more attractive live caged samples of different female *A. m. capensis* test groups, when simultaneously exposed in mid-air within a drone congregation area (DCA). Virgin queens, reared from fertilised queen-derived brood, were equally attractive to drones as virgin queens reared from clonal, unfertilised laying worker-derived offspring, suggesting equal potential reproductive success for both virgin queen types in *A. m. capensis*. In contrast, laying workers were significantly less attractive to drones. A strong positive correlation between

drone preferences and 9-ODA percentage composition in mandibular gland profiles were observed when comparing different test groups. This clear correlation, however, was lacking when comparing individuals within the test groups. Preferential drone response, however, strongly supports the assumption that drones may well compete for pheromonally more attractive individual mating partners within a DCA.

INTRODUCTION

Mating in honeybees (*Apis mellifera* L.) occurs in the air, 10 - 40 m above ground within spatially defined drone congregation areas (DCAs) (Zmarlicki and Morse 1963; Ruttner and Ruttner 1965; Königer et al. 2005). Drones and queens of honeybees are able to locate DCAs independently of each other (Jean-Prost 1957; Zmarlicki and Morse 1963; Ruttner 1966; Tribe 1982), with matching time periods for mating flights (Ruttner and Ruttner 1972). During one or several nuptial flights, queens consecutively mate in a rapid sequence (Königer et al. 1979) with up to 40 drones (Palmer and Oldroyd 2000). This form of extreme polyandry in honeybees, resulting in genotypically highly variable colonies was suggested to be adaptive by enhancing fitness correlates (e.g. more effective division of labour or increased disease resistance) (Palmer and Oldroyd 2000; Crozier and Fjerdingstad 2001) and by reducing the probability of inbreeding (Page 1980). Because thousands of drones can be present in flight during the mating season (Gary 1963; Königer et al. 2005), and virgin queens are relatively rare, the operational sex ratio in honeybees is generally strongly skewed towards males (Paxton 2005). Drones follow a virgin queen, which enters a DCA in a typical 'comet' formation (Gary 1963), comprising about 50 or more drones (median number of drones = 31 per comet, Königer et al. 2005; about 60 drones per comet, Gary 1963). Strong competition between drones for mating success when pursuing a single queen was therefore suggested and is well documented (Königer et al. 2005). Drones entering and leaving the comet compete for the optimal position in close proximity behind the queen to increase their chances for successful mating (Königer et al. 2005).

However, observations concerning drone competition and preferences for more than one queen are scarce (Taylor 1984), but may be of significance when the numbers of drones in a DCA are reduced. In the beginning or at the end of the mating season for example, during off season when supersedure queens may still be produced (Ruttner and Hesse 1981), or in the case of the honeybee subspecies *A. m. capensis* during winter, when absconding and migrating peaks (Hepburn et al. 1993). Those relatively small *A. m. capensis* swarms are often queenless or lose their queens and subsequently may requeen

from worker-derived brood (Hepburn and Radloff 1998) in times when drone production is strongly reduced or absent. Moreover, observations of insufficiently or completely unmated queens, which quickly stop worker production or even only produce drone brood are commonly described in commercial apiculture and may sometimes be ascribed to the limited number of available drones (Zander and Böttcher 1982). Therefore, due to the extreme polyandrous mating behaviour in honeybees, DCAs may quickly become drone depleted when drone densities are low. More attractive queens would then have a better chance for successful mating by attracting more drones more effectively.

Drone attraction in a DCA follows visual and pheromonal cues (Gary 1963; Pain and Ruttner 1963), with artificial lures often providing very strong stimuli, attracting more drones from longer distances than live queens (Boch et al. 1975; Tribe 1982). Visual drone response to wooden queen models was dependent on size and motion of the lures, with larger (upper limit 6.0 cm length), and moving objects being more attractive (Gerig 1985). The 'queen substance' (*E*)-9-keto-2-decenoic acid (9-ODA), the main component of the mandibular gland secretions and the most prevalent constituent of the pheromonal bouquet of honeybee queens (Free 1987), proved to be its most attractive component, with long range activity (Boch et al. 1975). Other constituents of the pheromonal bouquet of queens may act synergistically with 9-ODA (Velthuis 1970; Free 1987), increasing the range of active attractants and possibly widening individual attractiveness differences between individual queens. Synthetic pheromone lures were more effective in attracting drones with increasing 9-ODA quantities (Boch et al. 1975). In high concentrations, 9-ODA lures were able to reduce the *A. m. scutellata* drone response threshold, allowing drone attraction during the peak flight times even at a distance from DCAs (Tribe 1982). Salmon (1938) reported that if several queens were exposed at some distance from one another within a DCA, the drones only responded to one. Boch et al. (1975) similarly observed long distance interference of strong synthetic 9-ODA lures with weaker lures when simultaneously exposed in a DCA, the latter being hardly noticed by the drones. Preferential drone responses were observed when different lures were offered within close proximity, with stronger responses to concentrated synthetic 9-ODA lures over live queens or queen models lacking pheromonal cues (Gerig 1971). Drone response therefore appears to be graded and dependent on differences in the quality of the stimuli. Because a honeybee queen's reproductive success and individual fitness largely depends on her mating success, drone response may therefore be used in preference or choice tests to assess differences in potential reproductive success between queens. Drone preferences would indicate a higher

probability for successful matings and therefore potentially increased individual fitness of a queen.

Here I present data of bioassays assessing differences in drone response to virgin *A. m. capensis* queens and laying workers. *A. m. capensis* laying workers have the unique ability to produce diploid clonal female offspring from unfertilised eggs via thelytokous parthenogenesis (Onions 1912; Anderson 1963; Moritz and Haberl 1994) and in general a more queen-like mandibular gland profile with a relatively high proportion of 9-ODA (Hemmling et al. 1979). I tested combinations of four samples by exposing them simultaneously in some proximity within a DCA. One (empty) control and one of each of the following groups: *A. m. capensis* virgin queens reared from queen-derived brood; *A. m. capensis* virgin queens reared from laying worker-derived brood; and *A. m. capensis* laying workers. I assumed there would be graded drone competition for more attractive individuals. I also assumed differences in attractiveness between both virgin queen groups and the laying worker group, but no differences between the two different queen groups. To test my hypothesis I compared my observations with GC-data of the mandibular gland pheromonal profiles of the assessed queens and laying workers, correlating drone response to differences in pheromonal composition.

MATERIALS AND METHODS

Honeybee colonies

In January 2005, a honeybee colony pool was stocked at three apiaries in Grahamstown (Eastern Cape, South Africa) with unrelated, queenright colonies of: 1) *A. m. capensis* from the Eastern and Western Cape (N = 51) and 2) *A. m. scutellata* from Pretoria (N = 7); (see Hepburn and Radloff (1998) for a detailed review on the distribution and biology of these honeybee populations). The *A. m. scutellata* colonies were kept at a separate apiary to minimise intersubspecific drifting, dispersing, naturally occurring swarm mergers and infestations by socially parasitic workers (Neumann and Hepburn 2002). All colonies were kept in standard Langstroth hives (one breeding chamber and one shallow honey chamber) and had sufficient food (honey and pollen).

Production of laying workers

Freshly emerged *A. m. capensis* workers (N=60) were individually marked and singly introduced into small hoarding cages (10 x 12 x 10 cm^3) containing a piece of comb (6 x 5 cm^2) and 70 young *A. m. scutellata* nurse bees. *A. m. capensis* workers differentiate quickly into pheromonally, trophallactically and reproductively dominant laying workers when kept singly within groups of nurse bees from innately less dominant honeybee subspecies (e.g. *A. m. carnica*, Lattorff et al. 2005; e.g. *A. m. scutellata*, Schäfer et al. 2006). Food was supplied *ad libitum* (sugar candy, pollen and water). The cages were kept in a dark room at 28^o C and a relative humidity of 70-75%. Successful ovipositing was recorded from day 12 until the end of the experiment on day 19. All inspections were carried out under red light conditions to keep the bees undisturbed. Worker samples for bioassays were removed from hoarding cages as required. After termination of the experiments the *A. m. capensis* workers were live decapitated and their heads placed in 200 µl dichloromethane in order to extract mandibular gland secretions. The extractions lasted for at least 24 h after which gas-chromatographic (GC) analyses were performed (Simon et al. 2001).

In this study I used only those differentiated workers where ovipositing was observed (N=19 actively laying workers) for bioassays and comparisons of mandibular gland pheromonal activity.

Production of virgin queens

All donor colonies (N = 6 *A. m. capensis* colonies) intended to provide queen-derived female larvae for grafting, were selected from the colony pool 24 h prior to grafting. All donor colonies (N = 3 *A. m. capensis* colonies) intended to provide laying worker-derived female larvae for grafting, were selected from the colony pool at least three weeks prior to grafting and de-queened. All emergency queen cells were removed from these colonies and worker ovipositing was monitored at weekly intervals until sufficient larvae of an appropriate age were available.

Queen-rearing from queen-derived and laying worker-derived 12-24 h old female *A. m. capensis* larvae in queenright breeding colonies (N = 20 *A. m. capensis* breeding colonies), was carried out in an experimental apiary at Stones Hill, Eastern Cape, South Africa, in October 2006, according to standard protocols (Zander and Böttcher 1982). On the day of grafting, one hour prior to the introduction of the breeding frame with the grafted larvae, the open brood was removed and the old mated queen was confined to a peripheral part of the colony (Anderson 1965). Grafting followed routine procedures (Laidlaw 1979). The queen-cell cups were attached to standard wooden cell bases. Each breeding frame

contained alternating groups of individually labelled queen-cell cups with queen-derived and laying worker-derived female larvae. (N = 36 larvae on each frame; 18 larvae queen-derived, 18 larvae laying worker-derived). Eight days after grafting (about three days prior to virgin queen emergence), all capped queen cells were secured in a queen cell cage together with five workers. About one day after queen emergence, successfully eclosed virgin queens were individually marked and transferred into standard plastic 'introduction' queen cages (8.0 x 3.4 x 1.0 cm^3; with 30 holes 3.0 x 3.0 mm^2), containing 9 young worker bees and queen candy (castor sugar and honey), as required for the cages. The old mated queens were removed from the breeding colonies for dissections and gas-chromatographic (GC) analyses. All caged virgin queens were stored (banked) within their breeding colonies for at least seven days to reach pheromonal maturity for mating (Wossler et al. 2006). The queen cages, allowed optimal nutritional provision by the breeding colonies' nurse bees.

Aged virgin queens (N = 19 queen-derived and N = 19 laying worker-derived) were selected for bioassays from all breeding colonies (queen banks) pair-wise but were later mixed and used in random combinations. After termination of the experiment, all virgin queens were live decapitated for GC analyses and dissections as described above.

Gas-chromatographic analysis

For each extract, the dichloromethane was evaporated to dryness under a stream of nitrogen and the residue was re-dissolved in 20 µl internal standard (0.38 mg octanoic acid and tetradecane in 0.25 ml dichloromethane) and 20 µl bis-trimethylsilyl-trifluoroacet-amid were added (Simon et al. 2001). One µl of this solution was injected into a gas-chromatograph (Hewlett Packard© 5890), using the analytical conditions described by Simon et al. (2001).

Behavioural bioassay

I tested *A. m. capensis* drone preferences towards simultaneously offered different caged live female *A. m. capensis* samples, when exposed within a drone congregation area at Donkerbosch Outspan Farm, Stones Hill, Eastern Cape, South Africa. All test trials were carried out on sunny warm (20^o - 28^o C) days between 13.00 and 16.00, during the natural drone flight period (Tribe 1982) and mating season (Hepburn and Radloff 1998) of *A. m. capensis* (October 2005-February 2006). Prior to each trial the presence of sufficient drones at the DCA was confirmed by throwing a dark object (in this instance a hat) several times

into the air to a height of about 10 m. Only when 15 or more drones were immediately attracted to the 'airborne hat' were trials carried out. Four cylindrical queen cages (∅ 2 cm, height 8 cm, with 120 holes 3 x 3 mm^2) were suspended vertically at 5.50 m (=level 1), 6.00 m (=level 2), 6.50 m (=level 3) and 7.0 m (=level 4) from the ground on a cord between two pine trees across the centre of the DCA.

For each trial a combination of one sample of the following test-groups was assessed: one queen-derived virgin queen (group 1); one laying worker-derived virgin queen (group 2); and one laying worker (group 3). Three of the cages contained each one of the live test samples. A fourth cage remained empty and served as a control (group 4). Each trial comprised four successive observation periods of 2 min. Between the observation periods, the cages were lowered and their position was changed in a fixed pattern (pattern a, b, c, d)* which ensured that after termination of the trial, every cage had been suspended at each level (level 1, 2, 3, 4)* for 2 min. Two persons carried out the observations. The counts of drones approaching the lures ('hits') were made by an assistant who was unaware as to the test sequence. The numbers recorded included multiple drone visits because it was not feasible to eliminate drones that were attracted repeatedly during the brief observation period. In cases where drones approached the cages from below, following them one by one consecutively upwards, every approached cage was counted. Small 'comets', which formed for split seconds were counted as five, larger comets as ten approaching drones, and those estimates were photographically confirmed (Figs. 7.1.; 7. 2.).

After every trial the sum of all 'hits' per cage was calculated and the relative attractiveness of the different samples was rated as follows. Most 'hits' (= 4), second most 'hits' (= 3), third most 'hits' (= 2), least 'hits' (= 1). When equal numbers of 'hits' occurred, these samples were rated equally. The experiment was repeated in 19 trials, with 19 different sets of live samples (each in new, unused queen cages). After each trial the specimens were immediately prepared for GC analyses and dissected as described above. Five additional repetitions (trials) were carried out without live samples, using only empty cages as controls.

*(positional patterns (a, b, c, d) of samples at different levels above ground (1, 2, 3, 4):
level 1 (1 = 5.50 m); level 2 (2 = 6.00 m); level 3 (3 = 6.50 m); level 4 (3 = 7.00 m)
a = 4: queen-derived queen; 3: worker-derived queen; 2: laying worker; 1: control
b = 4: control; 3: laying worker; 2: worker-derived queen; 1: queen-derived queen
c = 4: laying worker; 3: queen-derived queen; 2: control; 1: worker-derived queen

d = 4: worker-derived queen; 3: control; 2: queen-derived queen; 1: laying worker)

Statistical analysis

We compared drone preferences (absolute numbers of approaching drones = 'hits') to samples of the four test groups: queen-derived virgin queens (group 1), laying worker-derived virgin queens (group 2), laying workers (group 3) and controls (empty cages) (group 4). ANOVA procedures were used to test for significant group effect and positional patterns effect in the average number of 'hits' (Zar 1996). Prior to analysis, homogeneity of variances of data was examined using Levene's test. Heterogeneity was stabilised after logarithmic transformation of the number of 'hits'. Scheffé post-hoc multiple comparisons tests were used for significant group effects.

For every trial I denoted relative attractiveness of the individual samples (4 = highly attractive; 3 = attractive; 2 = less attractive; 1 = least attractive) from the relative rating of drone counts per cage. Pearson χ^2 tests were used to test for significant differences in the relative attractiveness to drones within and between the four different groups.

We correlated the average number of 'hits' and relative attractiveness to drones to the relative percentage compositions of five essential mandibular gland compounds (HOB, 9-ODA, 9-HDA, 10-HDAA, 10-HDA) of the test groups, to verify biological activity of specific compounds. All tests were performed using Statistica© (StatSoft 2004).

RESULTS

We found significant differences in drone response among the four groups, with no significant difference due to the positional patterns (a, b, c, d) of the samples, which allowed pooling the data, and a significant interaction effect (ANOVA: Groups: $F_{3,284}$ = 25.9, P<0.0001; Position: $F_{3,284}$ = 1.1, P=0.3642; Group*position: $F_{9,284}$ = 4.9, P<0.0001). Scheffé post-hoc tests revealed that differences in average numbers of 'hits' were insignificant between the two virgin queen groups 1 and 2 (Scheffé: P=0.9993) and between laying workers and empty control cages (groups 3 and 4) (Scheffé: P=0.6221). However, differences in average 'hit' numbers between virgin queens (both queen- and laying worker-derived) and laying workers as well as empty (control) cages were highly significant (Scheffé: P<0.0001). No significant differences in average 'hit' numbers were found within the five additional repetitions (trials) carried out without live samples, using only empty cages as controls (ANOVA: $F_{3,16}$ = 0.11, P=0.9539).

A total number of 57 live samples and 19 empty control cages were rated as to their attractiveness to drones. Chi-square test analyses revealed significant differences in the relative attractiveness to drones both between and within the different groups (Between: $\chi^2 = 83.6$, df=9, P<0.0001; within: group 1: $\chi^2 = 19.1$, df=3, P<0.0003, group 2: $\chi^2 = 15.7$, df=3, P<0.0013, group 3: $\chi^2 = 30.5$, df=3, P<0.0001, group 4: $\chi^2 = 19.1$, df=3, P<0.0003; Tab. 7.1.). Queen-derived virgin queens and laying worker-derived virgin queens were significantly more frequently rated 'highly attractive' (45.0% and 50.0%, respectively) than laying workers (5.0%) and empty (control) cages (0.0%). Similarly significantly more queen-derived virgin queens and laying worker-derived virgin queens were rated 'attractive' (52.6% and 42.1%, respectively) than laying workers and empty (control) cages (0.0% and 5.3%, respectively).

In contrast, significantly more laying workers (68.2%) and empty (control) cages (27.3%) were rated 'less attractive' than queen-derived virgin queens and laying worker-derived virgin queens (0.0% and 4.5%, respectively). Similarly significantly more laying workers (20.0%) and empty (control) cages (80.0%) were rated 'least attractive' than queen-derived virgin queens and laying worker-derived virgin queens (both 0.0%; Tab. 7.1.). There were no significant differences in attractiveness to drones between queen-derived virgin queens and laying worker-derived virgin queens. In both groups almost all the ratings (100% and 94.7%, respectively) were 'highly attractive' or 'attractive'. Attractiveness was significantly reduced in laying workers and empty (control) cages when compared to queen-derived virgin queens and laying worker-derived virgin queens. In both laying workers and empty (control) cages 94.7% of the ratings were ‘less’ or ‘least attractive’.

9-ODA was the major, and 9-HDA the second most dominant constituent of the tested pheromonal blend of all samples. I observed a highly significant positive correlation between 9-ODA percentages and drone responses ($r = 0.58$, P<0.0001). The correlation was similar for the mean numbers of absolute counts of approaching drones ('hits') as well as for the relative attractiveness ratings. There were no significant differences of 9-ODA percentages between queen- and laying worker-derived virgin queens (87.2% and 87.0%, respectively; Scheffé: P=0.9899). However, highly significant differences in 9-ODA percentages between virgin queens (both queen- and laying worker-derived) and laying workers were observed (45.2%; Scheffé: P<0.0001).

DISCUSSION

I observed *A. m. capensis* drone responses to different caged *A. m. capensis* virgin queens and laying workers and to empty (control) cages, when simultaneously suspended mid-air within a drone congregation area. Drones obviously did not react without bias towards equal visual cues. Moreover, drones did not respond in an all or none manner but gradually in higher numbers towards apparently more attractive stimuli, suggesting drone competition for more attractive potential mating partners. This is not surprising, as mating with a virgin queen of high reproductive quality may increase a drone's individual fitness. However, differences in drone responses were insignificant between the two different virgin queen test groups (queen-derived and laying worker-derived virgin queens), suggesting equal mating chances and potentially similar reproductive success for both groups of *A. m. capensis* virgin queens. Drone response was significantly reduced towards caged laying workers and empty (control) cages. Moreover, drones did not differentiate between caged laying workers and empty (control) cages and only an insignificant trend indicating a slightly more intense drone response towards caged laying workers was observed. On the other hand, I recorded significant differences in absolute numbers of drone 'hits' and relative attractiveness between different virgin queens within the test groups suggesting differences in potential reproductive success of individual queens. Similarly I observed significant differences in absolute numbers of drone 'hits' and relative attractiveness between different samples within the laying worker test group.

I used five essential pheromonal compounds of the mandibular gland secretions in honeybee queens and workers (HOB, 9-ODA, 9-HDA, 10-HDA, 10-HDAA) to support the behavioural observations by comparing relative compositional data (mean percentage composition) of the different pheromonal spectra. I observed a statistically significant positive correlation of the mean percentages of 9-ODA in the pheromonal blends to my behavioural data, indicating involvement of this component in drone attraction. It is widely accepted that 9-ODA is the most active compound of queen mandibular gland secretions, in attracting drones to potential mating partners within drone congregation areas (Gary 1962; Pain and Ruttner 1963; Strang 1970). Since sometimes relatively large amounts of synthetic amounts of 9-ODA were necessary to match the attractiveness of the whole mandibular gland pheromonal spectrum from head extracts of live queens, the authors assumed synergy effects with other pheromone components. Butler and Fairey (1964) suggested that 9-HDA may have had some activity in drone attraction, however they observed that the attractiveness of 9-ODA was not increased by the addition of 9-HDA. Blum et al. (1971) and Boch et al. (1975) could not confirm the attractiveness of 9-HDA.

Both virgin queen test groups could not be separated using pheromonal data (9-ODA both 87.2%; 9-HDA 6.7%) but were significantly separated from the (less attractive) laying workers, which had relatively lower mean percentages of 9-ODA (45.2%) and higher mean percentages of 9-HDA (33.4%) in their pheromonal blends, confirming my behavioural observations. Similar observations were described for differences between virgin queens and mated queens. Boch et al. (1975) found that mature mated queens were more attractive than virgin queens and ascribed this to the larger absolute amounts of 9-ODA found in their mandibular glands. However, despite significant differences in pheromonal composition and attractiveness to drones between mated queens or laying workers and virgin queens, only the latter are, when adequately matured, mating partners for drones. Both virgin queen groups tested here were equally attractive to drones.

There were, however, significant differences in drone response to individual samples within the virgin queen test groups as well as within the laying workers, suggesting a graded drone response towards different individuals. On the other hand, significant attractiveness differences within the control group were recorded as well. Moreover, the positive correlation of behavioural and pheromonal data between the different test-groups could not be confirmed for individual samples within test-groups. Therefore, the subject of drone preferences and competition towards more attractive individual mating partners remains elusive. The observed significant variation in drone response towards individual samples within all test groups may easily be ascribed to environmental fluctuations, which cannot be completely excluded in honeybee mating bioassays. At any time, the number of flying drones, height of flight, their responses to caged live samples and discriminating ability depend on many variables, including wind speed, wind duration, temperature, cloud cover, thermal turbulence and many more (Free 1987). This may have obscured clear correlations between behavioural observations and pheromonal data. More experiments with a higher number of different individuals of the same test groups are necessary to resolve these issues. My observations, however, which demonstrate preferential drone response to pheromonally more attractive test groups strongly support my contention that drones may well compete for more attractive individual mating partners.

Tab. 7.1. Observed frequencies and column percentages of attractiveness and average

total number of approaching drones (mean ± SE) in response to different samples of caged live female honeybees.

live samples	highly attractive (N = 20)	attractive (N = 19)	less attractive (N = 22)	least attractive (N = 15)	average number of approaching drones ('hits') per trial
queen-derived virgin queens	9 45.0%	10 52.6%	0 0.0%	0 0.0%	29.4 ± 3.7
worker-derived virgin queens	10 50.0%	8 42.1%	1 4.5%	0 0.0%	29.2 ± 3.4
laying workers	1 5.0%	0 0.0%	15 68.2%	3 20.0%	15.7 ± 1.7
empty cages (controls)	0 0.0%	1 5.3%	6 27.3%	12 80.0%	11.4 ± 0.7

Fig. 7.1. Drones approaching queen cages at level 1 (bottom) and 4 (top)

Fig. 7.2. Drone 'comet' formation at level 2 (second from bottom)

CHAPTER 8

Developmental patterns of colonies headed by queens from worker and queen offspring in *Apis mellifera capensis* Esch.

SUMMARY

We surveyed the development of (N = 40) *Apis mellifera capensis* colonies headed by queen- and laying worker-derived queens during the course of one year. Long term observations of honeybee colonies allow the evaluation of a queen's reproductive output/success via worker-force counts using standard evaluation methods or via developmental pathways observed. Following the introduction of artificially reared virgin honeybee queens, seven different developmental pathways were distinguished. Only 10% of the colonies maintained the introduced queen until the end of the observation period. Most prevalent were absconding and/or events of queen exchange via supersedure, swarming and emergency queen-rearing. Colonies headed by queen- and laying worker-derived queens could not be distinguished from comparison of their developmental pathways or worker-force. No statistically significant differences in reproductive success of queen-derived versus worker-derived queens were observed.

INTRODUCTION

Beekeeping in southern Africa is based on the capture of wild swarms and apicultural queen-rearing is virtually non-existent (Johannsmeier 2001b). Furthermore, introductions of European honeybee subspecies have consistently failed (Hepburn and Radloff 1998). Genetic variation of honeybee populations in southern Africa is therefore considered to be natural and virtually undisturbed by man (Hepburn et al. 2004).

The natural adaptive reproductive and behavioural traits of African honeybee populations are actually counter-productive to artificial queen-rearing. Absconding, migrations, prolific swarming, queen supersedure (Hepburn and Whiffler 1988; Hepburn et al. 1993; Hepburn 1994; Allsopp and Hepburn 1997; Hepburn and Radloff 1998) and invasions (Johannsmeier 1983; Allsopp 1992b; Neumann and Hepburn 2002; Wossler 2002) can quickly render sophisticated breeding programs fruitless.

In the honeybee subspecies *A. m. capensis*, laying workers produce clonal diploid female offspring via thelytokous parthenogenesis (Onions 1912; Anderson 1963; Moritz

and Haberl 1994). In contrast to all other honeybee subspecies where laying workers produce haploid male offspring, *A. m. capensis* colonies can rear new queens from laying worker offspring. This provides an additional pathway to restore a queenright condition in the case of queen loss (Hepburn 1994). Queens from two different origins therefore exist in populations of *A. m. capensis*: those from fertilised queen offspring and others from clonal laying worker offspring (Moritz 1986, 1989; Greeff 1996a,b).

The thelytokous trait in the Cape honeybee has been modelled (Moritz 1986, 1989; Greeff 1996a,b; Moritz et al. 1998) and numerous mechanisms for laying worker based reproduction have been described (Hepburn 1994; Hepburn and Radloff 1998), which generally assume that the reproductive quality of both queen- and worker-derived queens is more or less equal. However, for nearly one century it has been an untested and unproven tenet of southern African beekeeping that laying worker-derived queens are reproductively inferior to queen-derived queens (Onions 1912; Mowbray 1916; Hartmann and Hartmann 1991). In the absence of detailed comparative observations indicating fitness differences between both queen types, the reproductive significance of queen and laying worker based pathways remains obscure.

After loss of the queen, female laying worker offspring in *A. m. capensis* colonies represent dominant genotypes, because their mothers were able to successfully dominate reproduction following a phase of intracolonial competition (Moritz and Hillesheim 1985; Moritz et al. 2000, 2004). Genetic differences between queens reared solely from dominant laying worker offspring and queens reared from queen offspring can therefore be assumed. Phenotypes of laying worker-derived queens in wild populations of *A. m. capensis* generally occur after several unsuccessful requeening attempts in dwindling queenless colonies, which exhibit reduced nursing and hygienic behaviour (Hepburn and Radloff 1998). These queens, described as smaller and more worker-like (Onions 1912; Mowbray 1916) were considered reproductively of lower quality than naturally occurring well nursed queen-derived queens. A honeybee queen's reproductive success depends on a function of her mating success, fecundity and offspring viability (Futuyama 1998; Gilley et al. 2003), culminating in successful colony build-up and reproduction (colony fission by swarming) (Wilson 1971; Moritz and Southwick 1992). Performance differences between queens can therefore be quantified, using colony strength (number of adult workers and worker brood) as an indicator for their reproductive quality.

Here I assessed the differences in reproductive success of queen- and laying worker-derived queens which were reared under uniform conditions, by assessing the

observed developmental pathways and by evaluating the worker-force of colonies headed by queens of both origins after a twelve months period.

MATERIALS AND METHODS

Observation period and location

All observations were made in apiaries at Stones Hill and Grahamstown, Eastern Cape, South Africa, within the range of distribution of *A. m. capensis* where thelytoky and other traits characteristic of *A. m. capensis* are extensively expressed (compare Hepburn and Crewe (1991a,b); Hepburn and Radloff (1998, 2002) for detailed reviews on the distribution of this honeybee subspecies). The observation period, including the artificial rearing of honeybee queens, started on the 1st of October 2005, and ended on the 29th of September 2006, at the beginning of the natural mating season of *A. m. capensis* (Hepburn and Radloff 1998).

Queen-rearing

Queen-rearing in queenright breeding colonies (N = 20 *A. m. capensis* breeding colonies), was carried out in an experimental apiary at Stones Hill, in October 2006, according to standard protocols after nine days of restricted ovipositing by the queen (Zander and Böttcher 1982). On the day of grafting, one hour prior to the introduction of the breeding frame with the grafted larvae, the open brood was removed and the queen was confined to a peripheral part of the colony (Anderson 1965). Grafting of young female larvae (12 - 24 h old) followed routine procedures (Laidlaw 1979). The queen-cell cups were attached to standard wooden cell bases. Each breeding frame contained alternating groups of individually labelled queen-cell cups with queen-derived and laying worker-derived female larvae (N = 36 larvae on each frame; 18 queen-derived larvae, 18 laying worker-derived larvae).

Mating colonies

Two days before eclosion, the queen cells were caged together with five workers and returned to their breeding colonies. After successful eclosion, all queens were individually marked and transferred singly into standard introduction cages, sealed with a plug of queen candy (icing sugar and honey). All breeding colonies (each comprising eight standard Langstroth frames occupied by workers) were split into two, generating N = 40 mating colonies of equal strength. All mating colonies were housed in standard 5-frame nucleus

boxes and supplied with food (one frame of honey). Virgin queens (25 queen-derived and 25 laying worker-derived) were introduced into 40 mating colonies as follows. To increase the probability of queen acceptance, virgin queens were introduced into mating colonies generated from their own former breeding colonies. 15 mating colonies were supplied with one queen-derived virgin queen. 15 mating colonies were supplied with one laying worker-derived virgin queen. 10 mating colonies were supplied with two virgin queens (one queen-derived and one laying worker-derived virgin queen). Queens were introduced between two combs in standard introduction cages and eventually released within the mating nucleus after removal of the candy plug by nursing worker bees. All 40 mating colonies were immediately transferred to an experimental mating apiary in Grahamstown, 6 km away, where they remained undisturbed for three weeks.

Colony inspections

Three weeks after installation, all mating colonies were returned to the experimental apiary at Stones Hill. To allow undisturbed development and to limit disturbance-induced absconding, only two major colony inspections were carried out during the observation period.

First inspection: 25 days after installation, the queen stages of all colonies were recorded (acceptance and mating success (ovipositing) of the initially introduced queen or alternatively, occurrence of laying workers, queen cells and/or new queens). New emergency queens from worker-derived brood were individually marked. All colonies were transferred into standard Langstroth brood boxes, and supplied with five additional empty frames containing wax foundation starter strips.

Final inspection: At the end of the observation period (29th September 2006) the queen stages of all colonies were recorded (initially introduced queen or new queen from first inspection present or alternatively occurrence of laying workers, queen cells and/or new un-marked queens) and the colony strength was determined as the sum of adult workers plus the number of cells containing worker brood. The number of adult workers and the amount of worker brood per colony was assessed as follows (the number of adult drones and drone brood was negligible at the time of the observation). All bees were shaken into a calibrated swarm box and weighed. The weight of a sample of 100 bees was then used to estimate the number of adult worker bees per colony. The areas of capped and open worker brood were measured and recorded in dm^2, applying standard procedures (Imdorf et al. 1987), then multiplied by 500 (Hepburn and Radloff 1998), to determine the

number of cells containing worker brood. The sum of adult workers and cells containing worker brood per colony was used to express the reproductive success of a queen.

Additional minor inspections of individual colonies were carried out during the observation period to verify external observations. Swarming or absconding events as well as dwindling or death were recorded after opening the respective colonies and assessing their strength and queen status.

Developmental pathways

All colonies were grouped according to different alternative developmental pathways and their frequency and success were determined using data from the inspections (Tab. 8.1.).

Differences in the reproductive quality of queens

Differences in reproductive quality of queens were assessed by comparing frequencies of acceptance of queen-derived versus laying worker-derived virgin queens and by comparing their reproductive success (colony strength). Colonies initially supplied with two virgin queens (one queen-derived and one worker-derived virgin queen) were used to test for acceptance preferences.

Statistical analysis

Pearson χ^2 tests were used to test for significant differences in the acceptance rates of queen-derived versus laying worker-derived virgin queens, and non-parametric Mann-Whitney tests were used to test for differences in reproductive quality of queen-derived versus laying worker-derived virgin queens. The probability of not detecting significant differences in the reproductive quality of queens (denoted by β) when in actuality differences occurred, were also determined for each test (Zar 1996). All tests were performed using Statistica© (StatSoft 2004).

RESULTS

Developmental pathways

Seven developmental pathways were observed, three of these followed an initially queenright situation including acceptance, successful mating and ovipositing of the introduced queen. Four pathways followed an initially queenless situation with the rejection of the introduced queen.

When following an initially queenright situation, colonies either carried on queenright with the introduced queen present until the end of the observation period (pathway 1; 10%) or absconded to an unknown fate (pathway 2; 22.5%), or carried on queenright but eventually superseded the initial queen or swarmed or otherwise lost/replaced the initial queen with a newly mated queen heading the colony (pathway 3; 15.0%).

When following an initially queenless situation with rejection of the introduced queen, colonies either carried on queenless and remained queenless until the end of the observation period (laying worker regime); or eventually dwindled and died or absconded (pathway 4; 20.0%); or colonies eventually requeened from laying worker offspring with a new (possibly supersedure) queen heading the colony (pathway 5; 12.5%); or already had successfully requeened from laying worker brood at the time of the first inspection but later superseded (or otherwise lost/replaced) the queen with a new mated queen heading the colony (pathway 6; 7.5%); or already had successfully requeened from laying worker brood at the time of the first inspection but later absconded to an unknown fate (pathway 7; 12.5%) (Tab. 8.1.).

47,5 % of the colonies initially accepted the artificially reared and introduced queen, but only 10.0% of the colonies maintained this queen until the end of the observation period (Tab. 8.1.). 52.5% of all colonies were lost, due to absconding (45.0%) or dwindling and death (7.5%). The pathways 1, 3, 5 and 6 resulted in significantly more viable queenright colonies with a mean strength of 16 817.2 ± 9402.8 than pathways 2, 4 and 7 which resulted in absconding or death or in weakened colonies with a mean strength of 242.5 ± 685.9 (Mann-Whitney test: $U=0.0$, $P<0.0001$). The strength of the colonies following pathways 2 and 7 (colonies absconded to an unknown fate) could not be assessed.

Differences in reproductive success of queens

In colonies following a queenright situation (pathways 1, 2 and 3), I found a non-significant trend, with more initially accepted laying worker-derived (57.9%) than queen-derived (42.1%) queens ($\chi^2 = 0.47$, 1 df, $P=0.4913$; $\beta = 0.122$). More colonies, although not significantly more, which initially accepted a worker-derived queen (66.7%) absconded to an unknown fate than colonies which had initially accepted a queen-derived queen (33.3%), (pathway 2; $\chi^2 = 0.54$, 1 df, $P=0.4625$; $\beta = 0.026$). On the other hand, more colonies (not significant) superseded initially accepted queen-derived queens (75.0%) than

initially accepted worker-derived queens (25.0%), (χ^2 = 1.7, 1 df, P=0.1967; β = 0.684) (Tab. 8.2.).

There were no significant differences in colony strength between colonies headed by laying worker-derived queens (mean colony strength: 18618.3±11936.9) when compared to a colony headed by a queen-derived queen (mean colony strength: 10949.0±0.0) (pathway 1; Mann-Whitney: U=1.0, P=0.655; $\beta < 0.10$) or when compared to all other assessed colonies from all pathways (mean colony strength: 15803.0±10680.2; Mann-Whitney: U=7.0, P=0.881; $\beta < 0.10$). Moreover, no significant differences in colony strength were observed in colonies which superseded a queen-derived queen compared to colonies which superseded a worker-derived queen (mean colony strength: queen-derived: 17017.3±11927.3; worker-derived: 10021.0±8311.3; Mann-Whitney: U=3.0, P=0.6434; β = 0.200) (pathway 3, Tab. 8.2.).

From the 10 colonies initially supplied with two virgin queens (one queen- and one laying worker-derived) to test for acceptance preferences, only two colonies (20.0%, χ^2 = 3.6, 1 df, P<0.0578) followed a queenright pathway. One of these colonies initially accepted a worker-derived queen and later absconded (pathway 2); the other one accepted a queen-derived queen, which was later superseded (pathway 3) (Tab. 8.2.). Eight of these colonies followed a queenless pathway and rejected both queens offered. I found a non-significant trend in that initial queen acceptance in colonies supplied with two virgin queens was lower (20.0%) compared to initial queen acceptance in colonies supplied with only one virgin queen (56.7%, χ^2 = 3.8, 1 df, P<0.0511).

DISCUSSION

Only 10% of all observed colonies maintained the initially introduced queen until the end of the observation period and 52.5% of all colonies were lost due to absconding, dwindling and death. More, but not significantly more, worker-derived than queen-derived queens were initially accepted. I also found no difference in reproductive success, when comparing developmental pathways of colonies initially provided with queen-derived versus laying worker-derived queens. It can therefore be assumed, that the observed inferiority of the phenotypes of laying worker-derived queens in wild *A. m. capensis* colonies, is mainly environmentally determined.

After loss of their queen and without queen-derived brood, *A. m. capensis* colonies in general only successfully re-queen from worker-derived brood, when already weakened, after an extended phase of worker ovipositing (Hepburn and Radloff 1998). These small

colonies, comprised only of genetically dominant workers from clonal laying worker offspring, not only lack the necessary worker force for successful requeening, but also express generally reduced nursing and hygienic behaviour (Hepburn and Radloff 1998). In contrast, queenright colonies produce most of their female sexual reproductives (swarming or supersedure queens) during times of high colony strength, with optimal provision of their queen larvae. My data therefore do not necessarily reflect queen fitness in wild populations, but demonstrate the potential for a congruent range of reproductive performance for queen and laying worker-derived queens when reared under uniform environmental conditions.

Developmental pathways

Artificial queen-rearing in *A. m. capensis* colonies is difficult, because the rapid occurrence of female laying worker-derived brood generally results in the rejection or abortion of artificially grafted queen larvae (Swart et al. 2001a). Only queen-rearing techniques using queenright colonies, with a reduced supply of queen pheromones to suppress worker ovipositing, are successful. However, female laying worker-derived brood was observed during queen-rearing, despite the presence of the old queen in a peripheral part of the colony, in all 20 of the breeding colonies. During the course of the rearing process (nine days of restricted ovipositing by the queen, followed by 16 days of queen confinement during the development of the virgin queens), pheromonal suppression of worker reproduction was obviously reduced in a way which allowed both queen-rearing and worker ovipositing without the destruction of queen cells. All mating colonies therefore contained worker-laid brood and active laying workers at the time of introduction of the reared queens, which may have reduced queen acceptance. Colonies which rejected the introduced queens therefore never followed the queenless and broodless pathways described by Hepburn (1994), with worker differentiation after queen loss, but had, at once, differentiated active laying workers and worker-laid eggs or larvae from which to re-queen. The occurrence of un-marked mated queens after three weeks was a result of this rapid requeening process (pathways 6, 7).

In addition, I extended the description of the queenless pathways described by Hepburn (1994), by including supersedure, swarming, or other cases of queen turnover during colony development. It became clear that the presence of mated queens after initial colony decisions is no guarantee for the presence of the same queens after one year, and

processes resulting in exchange of queens appeared to be more frequent than previously assumed.

The presence of only four out of 40 artificially reared and introduced queens after one year (pathway 1), high colony losses due to absconding, dwindling and death (52.5%) (pathways 2, 6, 7), as well as the generally high turn-over of queens in established colonies (pathway 3, 5) confirmed observations by Allsopp and Hepburn (1997), who recorded 66 events of queen and/or colony turnover in a group of 30 established *A. m. capensis* colonies within the period of four years. More than 50.0% of the colonies therefore exchanged or otherwise lost their queens every year. Uncontrolled queen turn-over in high frequencies interferes with classical apicultural queen rearing techniques. These techniques are based on queen selection for breeding and controlled queen replacements in order to improve the quality of the entire colony-stock.

The described reproductive characteristics, however, enhance simple extensive apicultural techniques to increase the colony stock. The capture of absconding or migrating wild colonies in trap boxes is common practice all over southern Africa (Swart 2001d), as well as 'making increase' by generating splits using wild swarm cells during rapid colony development in strong pollen flows (Swart 2001d). Beekeeping practices therefore clearly reflect reproductive patterns of southern Africa's wild honeybee populations.

Differences in the reproductive success of queens

Measuring fitness in a colony of social insects is a difficult task, because selection operates at least at three distinct levels: direct individual selection (classical Darwinian selection), indirect individual selection (through kin-selection, Hamilton 1964) and directly at the colony level (Kraus et al. 2003). It is, however, obvious that the fitness of a colony largely depends on the reproductive success (mating ability, fecundity and offspring viability) of its queen (Gilley et al. 2003). Furthermore, it is generally assumed that colony performance is correlated to its strength (number of adult workers, drones, and brood). Indeed, reproductive success (the number of viable swarms issued and the number of fertile drones produced) (Moritz and Southwick 1992; Kraus et al. 2003), the ability to tolerate adverse conditions (Swart et al. 2001b) and honey yield (Neumann et al. 2000b) or queen-rearing success (Farrar 1968; Zander and Böttcher 1982), increase with increasing colony strength. I therefore used the sum of the number of adult workers together with the number of cells containing worker brood to assess the reproductive success of a queen.

From 19 mating colonies following a queenright pathway more worker-derived (11) than queen-derived (8) queens were initially accepted, suggesting a non-significant trend towards increased attractiveness of worker-derived queens, which genetically represent dominant patrilines from established laying workers (Moritz and Hillesheim 1985). However, when mating colonies were offered both types of queens (queen-derived and worker-derived) simultaneously, the initial acceptance of queens was reduced (20.0%) compared to acceptance when only one queen was offered (56.7%), and acceptance rates for both queen types were equal, suggesting similar attractiveness. In all pathways with initial acceptance of the introduced queen, no significant differences in worker-force between colonies provided with worker-derived queens was observed when compared to colonies provided with queen-derived queens, suggesting similar reproductive success for both groups of queens.

My data do not *per se* contradict anecdotal evidence, and some published accounts (Onions 1912; Mowbray 1916; Hartmann and Hartmann 1991), describing a generally reduced reproductive quality of worker-derived queens in wild populations of *A. m. capensis*. Due to their occurrence mainly in queenless, dwindling laying worker colonies, where nursing quality and hygienic behaviour are reduced, inferiority of laying worker-derived queens may be the rule. Models, however, which assume a similar range of reproductive performance between both queen groups to simulate biogeography and fixation of the thelytokous trait (Tribe 1983; Moritz 1986, 1989; Greeff 1996a,b; Moritz et al. 1998) in wild *A. m. capensis* populations are strongly enhanced and confirmed by my data.

Tab. 8.1. Frequencies of developmental pathways and resulting colony strength

path-way	description of pathway	number of colonies	colony strength * mean ± s.d.
1	initial queen accepted and maintained	4	16701.0 ± 10473.7
2	initial queen accepted, later absconded	9	not applicable
3	initial queen accepted, later superseded	6	14685.2 ± 10593.6
4	initial queen rejected, laying worker colony	8	242.5 ± 685.9
5	initial queen rejected, laying worker colony, later requeened	5	17825.0 ± 7423.6
6	initial queen rejected, requeened and superseded	3	19556.7 ± 12989.3
7	initial queen rejected, requeened and absconded	5	not applicable

*sum of the number of adult workers and cells containing worker brood at final inspection

Tab. 8.2. Acceptance of queen-derived versus laying worker-derived virgin queens and differences in colony strength

pathway	# of colonies	# of introduced queens	accepted queen: queen-derived	accepted queen: worker-derived	strength of colony *
1	1	1	1	-	10949
	1	1	-	1	28752
	1	1	-	1	21643
	1	1	-	1	5460
2	1	1	1	-	not applicable colonies absconded
	1	1	1	-	
	1	1	1	-	
	1	1	-	1	
	1	1	-	1	
	1	1	-	1	
	1	1	-	1	
	1	1	-	1	
	1	2	-	1	
3	1	1	1	-	34677
	1	1	1	-	13675
	1	1	1	-	10648
	1	2	1	-	9069
	1	1	-	1	15898
	1	1	-	1	4144
1, 2, 3	19	21	8	11	-

*sum of the number of adult workers and cells containing worker brood at final inspection

Section 3

GENERAL DISCUSSION, CONCLUSION, REFERENCES, APPENDICES

CHAPTER 9

GENERAL DISCUSSION

Differences in reproductive performance between queen-derived and worker-derived honeybee queens in *A. m. capensis*

Moritz (1986) emphasised, when modelling thelytokous versus arrhenotokous parthenogenetical worker reproduction strategies in populations of the honeybee (*Apis mellifera* L.), that there are as yet no quantitative data concerning the parameters that are related to fitness for laying worker-derived reproductives. Those parameters could be determined by assessing the number and reproductive quality of laying worker-derived queens (and drones) and such data may provide a stronger indication of the actual selective mechanisms operating at the level of laying workers. In the Cape honeybee, *Apis mellifera capensis,* laying workers typically produce mainly diploid female offspring from unfertilised eggs thelytokously (Onions 1912; Anderson 1963).

The occurrence of a small percentage of worker-produced drones within populations of *A. m. capensis* may indicate permanent gene flow from the neighbouring honeybee subspecies *A. m. scutellata* with arrhenotokous worker reproduction (Hepburn and Radloff 1998; Moritz et al. 1998). A honeybee queen's reproductive success depends, as a prerequisite, on egg or larval acceptance in a queen cell by nurse bees, nursing quality (food supply and temperature regulation) and finally survival until eclosion (Weiss 1983b). For an adult queen reproductive success depends on a function of her mating success, fecundity and offspring viability (Futuyama 1998; Gilley at al. 2003).

To address those parameters adequately, I tested different behavioural and physiological aspects during the life history of both queen- and laying worker-derived queens. For the first time, I was able to present data quantifying reproductive success of queen- and laying worker-derived *A. m. capensis* queens comparatively. A major result from my studies is that I was able to accumulate evidence indicating the potential for a congruent range of reproductive performance in queen- and laying worker-derived queens of the Cape honeybee, when reared under uniform conditions. Lack of differences in potential reproductive success between both queen types was clearly established in the

eclosing success of virgin queens, their attractiveness to workers and drones, their pheromonal spectra and, when mated, their ability to head colonies of increasing strength. I observed significant preferential acceptance of laying worker-derived larvae during emergency queen-rearing, which was, however, counterbalanced by the significantly reduced survival of laying worker-derived queen stages post-acceptance until eclosion. I suggested the ability of early brood stages to elicit brood care more efficiently (compare Calis et al. 2002; Allsopp et al. 2003), thereby influencing worker decisions, which may account for preferential acceptance of larvae from laying workers.

Laying worker-derived offspring represent (Moritz and Haberl 1994) genotypes of dominant patrilines, which were able to monopolise reproduction after queen loss (Moritz et al. 2000, 2004). However, I found no evidence for increased pheromonal dominance in adult laying worker-derived virgin queens. Queen- and laying worker-derived virgin queens could not be separated from one another using pheromonal data from the analysis of five essential mandibular gland compounds. The genetic and pheromonal dominance of their mothers therefore seemed not to translate significantly into adult queen life stages. Reduced survival of worker-derived brood is poorly documented in the literature, but may be ascribed to a lack of hygienic behaviour in dwindling queenless colonies (Allsopp 1995). I observed increased mortality of laying worker-derived brood in queenless donor colonies and in strong recipient colonies with an excess of nurse bees. This would support an idea of yet unknown reasons for larval mortality as opposed to mortality from lack of hygienic behaviour.

Caged queen- and laying worker-derived virgin queens (9-13 d old) were equally attractive to queenless workers. Clusters around laying workers or around empty (control) cages were significantly smaller. Both virgin queen types therefore can be assumed to attract workers and leave their natal colonies in secondary swarms of similar strength indicating an equal potential for reproductive success in newly founded colonies. Moreover, caged queen- and laying worker-derived virgin queens (9-13 d old) were equally attractive to drones when simultaneously exposed in a drone congregation area and both queen types were significantly more attractive than laying workers or empty (control) cages, indicating a similar potential for mating success. I observed no significant differences in life histories of queen- and laying worker-derived queens pre- and post-mating when introduced into queenless split colonies. Where the initially introduced queen was still present after one year, reproductive success could be assessed directly via worker-

force for both groups of queens, but no significant differences for colony strength were observed.

The thelytokous capacity of *A. m. capensis* workers is positively selected for if viable (and reproductively fit) queens are ultimately produced from worker-laid eggs (Ruttner 1977b). My observations would be of reduced significance if laying worker-derived queens in wild *A. m. capensis* populations could not reach their reproductive potential due to malnourishment in dwindling colonies. If worker-produced queens only occurred in weak queenless colonies after up to four months or more of laying worker regime (Winston 1987) and were generally of reduced reproductive quality (Onions 1912; Mowbray 1916; Hartmann and Hartmann 1991), the fixation of the thelytokous trait could hardly be explained by their deficient contribution to the gene-pool alone.

Furthermore, only the assumption of increased incidences of accidental queen loss and therefore high frequencies of requeening events from laying worker brood could outweigh reduced individual reproductive quality of worker-derived queens. Indeed, it has been argued that the thelytokous trait may have evolved in *A. m. capensis* as an adaptation to strong winds and extreme predation pressure resulting in high frequencies of queen loss during mating flights (Tribe 1983). Alternatively it has been reported that mating flights were most successful during strong wind conditions (Allsopp and Hepburn 1997) and that requeening from worker-derived brood is generally a rare event in wild *A. m. capensis* populations (Hepburn and Radloff 1998). The latter arguments would probably result in low numbers of reproductively inferior queens in wild populations, which clearly could not account for the fixation of the thelytokous reproductive trait. Obviously orphaning and subsequent queen production from worker-derived brood in queenless colonies are insufficient to explain the fixation and stable distribution of the thelytokous trait in wild populations of *A. m. capensis*, which readily hybridise with the neighbouring subspecies *A. m. scutellata* (Hepburn and Radloff 1998). My observations that laying worker-derived queens and queen-derived queens appear to have a congruent range of reproductive performance was a precondition for my hypothetical assumption of an additional worker-dependent pathway within the life history of *A. m. capensis* which would result in high frequencies of queen production from worker-derived brood in strong colonies with optimal nurse bee densities resulting in high numbers of worker-derived queens within wild *A. m. capensis* populations.

Direct competition between queen and laying workers for reproductive success in *A. m. capensis*

Laying workers, or workers with developed ovaries, are present in low numbers in queenright honeybee (*A. mellifera* L.) colonies with arrhenotokous worker reproduction (Visscher 1989), but especially, and in higher numbers in queenright *A. m. capensis* colonies (Neumann and Moritz 2002). In arrhenotokous honeybee subspecies, workers may maximise their direct fitness by producing fertile male reproductives (drones) (Visscher 1989). The reproductive success of these laying workers, however, is reduced, because worker-laid eggs are removed by policing workers (Ratnieks and Visscher 1989). In contrast, laying *A. m. capensis* workers may maximise their direct fitness more efficiently by producing fertile female reproductives (queens) thelytokously, with increased reproductive success, because removal of worker-laid eggs is reduced in this subspecies (Greeff 1996a,b; Moritz et al. 1999). Visscher (1989) argued that in arrhenotokous subspecies, despite worker policing, it may be advantageous for workers to be able to lay eggs even in queenright colonies, due to competitive advantages in the case of queen loss. This selection for readiness, to be able to lay eggs in the event of queen loss, may result in ovarial development and physiological mechanisms enhancing worker ovipositing even under queenright conditions (Visscher 1998). Similarly in *A. m. capensis*, due to potentially immense gains in direct fitness, selection for readiness to lay eggs may have shaped worker ovipositing behaviour, culminating in active competition with the queen to oviposit in queen cell cups in swarming or superseding colonies.

Thelytokous reproduction appears to be scattered among many taxa, indicating multiple independent phylogenetic origins (White 1984). In social Hymenoptera only a few cases of thelytokous parthenogenesis are known (currently six ant species and *A. m. capensis*; Wenseleers and Billen 2000). Mackensen (1943) and Tucker (1958), working with European honeybee subspecies, found that a low percentage of worker eggs develop into female offspring thelytokously, suggesting that the allele for thelytoky may be present in very low frequencies in other races of honeybees as well. Butler (1954a) noted that arrhenotokously reproducing laying workers were sometimes observed to lay eggs in queen cell cups, but that such unfertilised eggs gave rise to males, except in very rare cases when a female larva is produced, which may develop into a queen. The attraction to old queen cell cups by laying workers at the lower margins of combs was frequently observed in queenless *A. m. capensis* colonies, and regularly multiple eggs in and around these queen cells were found. These eggs, however, either did not hatch or, if so, it was not recorded

whether the larvae were reared until eclosion of adult queens (Onions 1912; Johannsmeier 1983; Swart et al. 2001b; Martin et al. 2002b).

In contrast, I observed, if only at low frequencies, laying worker ovipositing of single eggs in artificial (empty) wax queen cell cups in queenright colonies. Some of these eggs hatched successfully, were readily accepted by nurse bees and a few successfully eclosed as virgin queens. In my study, the old queen was confined behind a wire mesh in a peripheral compartment of the hive, simulating reduced queen pheromone distribution as in overcrowded, swarming or superseding colonies. The presence of some queen pheromone possibly triggered queen-rearing and may have restricted ovipositing mainly to these laying workers, which were already actively ovipositing prior to the queen's confinement. Simulation of swarming or superseding, by separating the queen and offering artificial queen cell cups, however, may not completely imitate a natural situation. Colonies, preparing to swarm or to supersede an old queen, produce numerous queen cell cups (Butler 1957; Lensky and Slabezki 1981). This was also observed in my experiments during queen confinement, however, the number of newly constructed queen cell cups was low. The pheromonal clues which trigger the production of numerous queen cell cups may also not only enhance queen but also worker ovipositing at frequencies much higher than in my simulated situation. Moreover, my artificially produced queen cell cups on wooden bases in breeding frames may have deterred workers from ovipositing when compared to possibly far more attractive natural queen cell cups.

My study, however, gives strong evidence that successful worker ovipositing in queen cell cups most probably occurs in wild *A. m. capensis* colonies, possibly in frequencies much higher than observed in my experiments. It is likely that worker-dependent queen-rearing pathways are therefore not restricted to dwindling queenless *A. m. capensis* colonies, but may occur frequently and regularly in queenright colonies at the height of their development during swarming or superseding. The reproductive significance of worker-derived queen production pathways may therefore not be restricted to requeening events after accidental queen loss, as assumed until recently, but may be more often and more strongly linked to selective advantages from immense gains in direct fitness by individual workers when ovipositing in queen cell cups in swarming or superseding *A. m. capensis* colonies.

CONCLUSION AND PERSPECTIVES

i) Laying worker-derived queens of high reproductive quality in a range comparable to queen-derived queens may occur frequently in wild *A. m. capensis* populations and may be produced in strong queenright colonies preparing to swarm or to supersede more frequently than in dwindling queenless *A. m. capensis* colonies.

ii) Mated worker-derived queens produce workers, which, dependent on their father's allele for worker reproduction, produce females thelytokously or drones arrhenotokously, and queens, which may or may not confer the thelytokous gene to their daughters or sons. Worker-derived queens, however, produce drones, which invariably carry the allele for thelytoky. Potentially high frequencies of drones from reproductively successful worker-derived queens could explain the fixation of the thelytokous trait. Frequent worker-derived queen-rearing pathways therefore could trigger and maintain the self-perpetuation of the thelytokous trait despite gene flow from arrhenotokous subspecies into the *A. m. capensis* territory.

iii) Requeening in dwindling queenless *A. m. capensis* colonies may be only an insignificant by-product of the more significant worker-dependent queen production pathways in queenright colonies.

iv) Only the exact quantification of frequencies of laying worker-derived queens and laying worker dependent queen-production in queenright colonies of wild *A. m. capensis* populations can resolve the issues in question.

CHAPTER 10

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CHAPTER 11 APPENDICES

APPENDIX 1 (General methods and materials)

Appendix 1.1.

Development and management of experimental honeybee colonies during the year 2005 in the vicinity of Grahamstown, Eastern Cape, South Africa

SUMMARY

Low rainfall intensity during spring and early summer 2005, a critical phase for honeybee colony development, resulted in reduced performance, dearth-induced absconding and damage from veldt-fires, in the Grahamstown region, Eastern Cape, South Africa. Continuously restocking with strong colonies to compensate for losses and small-scale transportations to counteract adverse conditions were used to maintain stock number and quality in a range sufficient to meet requirements for queen-rearing and behavioural experiments.

INTRODUCTION

Honeybees (*Apis mellifera* L.) are found naturally in all regions of South Africa, but the honey crops they procure from indigenous vegetation are with few exceptions, variable and unreliable for commercial beekeeping (Johannsmeier and Mostert 2001). South Africa is a marginal beekeeping country generally, but only a few areas are totally unsuitable (Swart 2001c). Over-exploitation of indigenous southern and eastern Cape forest during the 19th century resulted in the establishment of a forest industry based on quick-growing pines and eucalypts. The latter form the basis of the present-day beekeeping industry (Johannsmeier and Mostert 2001).

Colony development in African honeybees closely corresponds with local climate and the available forage. Rainfall drives flowering and its intensity is directly bound to rainfall intensity (Hepburn and Radloff 1995). Flowering, a pollen dispersing mechanism in turn drives brood production and colony growth. The well-defined sequence of colony events form a linear chain of relationships: rainfall-peak, peak-flowering, maximum brood rearing (Hepburn and Radloff 1995). Systematic collection of relevant apicultural data has

been common practice in Europe for more than 100 years (Wyder 1985), but is unknown in South Africa, and only very little information is available documenting honeybee colony development over extended time periods.

Here I provide some data documenting colony development in the vicinity of Grahamstown during the year 2005. Sufficient honeybee colonies of the subspecies *Apis mellifera capensis* and *A. m. scutellata* were required to conduct queen-rearing and behavioural experiments to assess fitness differences of *A. m. capensis* queens, reared from queen-derived and from laying worker-derived female brood. To meet all requirements, and to make reasonable choices among colonies, I observed and recorded the development of a pool of 58 hived honeybee colonies in different apiaries during spring and summer of the year 2005. All management decisions, described here, were based on developmental data and were aimed towards optimal colony performance and prevention of disturbances from vandalism or fire. The data provided here were of exceptional value during the conduct of the experiments and must be mentioned and described in the context of this thesis.

Grahamstown, situated within the band of hybridisation between *A. m. capensis* and *A. m. scutellata* (Hepburn and Crewe 1991a,b, Hepburn and Radloff 1998, 2002), is transitional not only for those two South African honeybee subspecies, but also for its climate and rainfall patterns (Lubke 1998). In the Grahamstown region, the year 2005 was characterised by low rainfall intensity during winter, spring and early summer from May until October, with an accumulation of only ~167 mm (compare long year mean May - October, 1878 - 1998 for Grahamstown = 371 mm) of precipitation for this 6 month period (Fig. 11.1.1.).

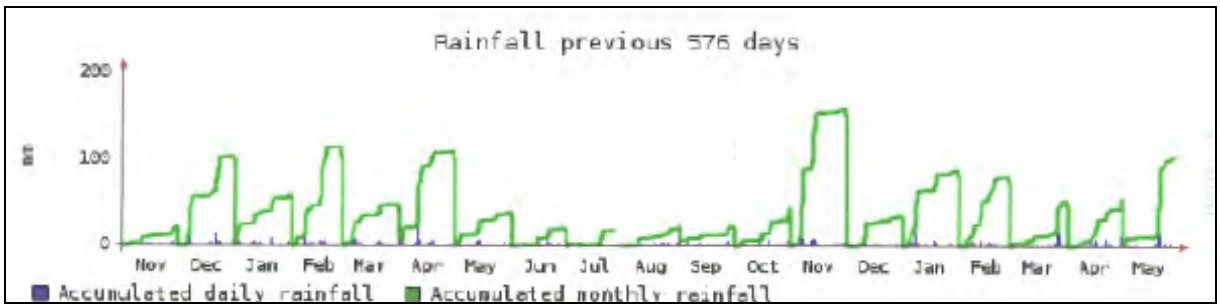


Fig. 11.1.1. Accumulated monthly rainfall November 2004 - May 2006 in Grahamstown (from Rhodes University 2006, Weather Data Collection)

Heavy rains only commenced during November, resulting in late colony peaks without reproductive swarming and relatively good honey yields late in January 2006. Colony management therefore reflects a critical dearth situation during spring and summer development.

MATERIALS AND METHODS

Apiaries

Four different apiary sites were used:

1. Trevor's Farm (=T) (Slaaikraal; S 33^{o} 17' 49.1"; E 26^{o} 26' 32.5"; elevation 669 m), situated 5 km west of Grahamstown within dry grassland used for sheep farming. Minor (pollen/nectar) flows were observed from *Acacia karroo* (summer), *Aloe candelabris* (winter), and from *Eucalyptus cameludensis* (variable flowering periods).
2. Nick's Farm (=N) (Rivendell; S 33^{o} 21' 46.7"; E 26^{o} 30' 50.6"; elevation 507 m), situated 4 km southwest of Grahamstown, within dry grassland used for cattle farming. Main (pollen/nectar) flows were observed from *Eucalyptus grandis* (variable, spring/summer).
3. Fishery Science Department (=F) (S 33^{o} 18' 37.0"; E 26^{o} 31' 06.9"; elevation 549 m), situated at the border of Rhodes University Campus within Grahamstown (beekeeping is restricted in Grahamstown, the apiary can only be used for short periods). Cities are artificial oases and often yield extraordinary honey crops. Pollen and nectar flows of different intensity were observed all year through. The apiary was used for 'emergency' colony build-up.
4. Basil's Farm (= C, G, M, K), (Donkerbosch Outspan; S 33^{o} 19' 50.2"; E 26^{o} 34' 52.7"; elevation 664 m), situated 4 km southeast of Grahamstown, within uncultivated hillside farmland, mainly covered by invasive *Acacia longifolia* and *Acacia mearnsii*. Only unpredictable (pollen/nectar) flows (spring/summer) were observed. The apiary consists of a circular queen-rearing section (C), a 'maintenance' section (M) for splits, an area called garden (G) and the top of a small hill, called kopje (K). The site was used as main experimental apiary because its sections (C, G, M), surrounding the 'bee-lab', a small, inhabited cottage, are fenced and guarded by dogs.

Honeybee colony pool

The apiaries T and N were stocked in January 2005 with (N=42) queenright, unrelated swarms from: 1) the naturally occurring transition zone between *A. m. capensis* and *A. m. scutellata* (= Grahamstown *A. m. capensis*; N=29), 2) *A. m. capensis* from the Heidelberg

area, Western Cape (= Heidelberg *A. m. capensis*; N=9) and 3) *A. m. scutellata* from the Pretoria area (N=4) (compare Hepburn and Crewe (1991a,b), Hepburn and Radloff (1998) and Hepburn and Radloff (2002) for detailed reviews on distribution and biology of the investigated honeybee populations). The queens of all colonies were individually marked. The *A. m. scutellata* colonies were kept separated from all other colonies to minimise intersubspecific drifting, dispersing, naturally occurring swarm mergers and infestations by socially parasitic workers (Neumann et al. 2000b; Neumann et al. 2001a,b; Neumann and Hepburn 2002). From here colonies were moved to different apiary sites according to experimental requirements. The colony pool was continuously restocked during spring/summer 2005 with Grahamstown *A. m. capensis* and *A. m. scutellata*. A total number of N=58 colonies formed the colony pool (Tab. 11.1.2.).

Rating of colonies and general trends in colony development

All colonies were rated for strength at three-week intervals beginning on the 24.08.05 (ex-winter revision), and then on 6 further occasions, ending on the 10.01.06 (late summer revision) (= observation period). Combs in standard Langstroth frames, well occupied on both sides by bees were counted. Following rating system was applied.

1 = occupying ≥ 10 combs; 2 = occupying 8 combs; 3 = occupying 6 combs; 4 = occupying 4 combs; 5 = occupying ≤ 3 combs; (intermediates = 1.5; 2.5; 3.5, 4.5). Events of absconding or other reasons for loss or gain of colonies were recorded as well.

Trends in colony development were roughly estimated according to general observations of climate, flowering periods and colony activity between rating occasions, as well as by analysing the rating results.

Colony movements

Colonies were moved between apiaries according to experimental and developmental requirements, or to avoid disturbances from dearth, fire or vandalism. Transport was carried out after cessation of worker flight early in the morning or in the late evening, to avoid loss of foragers.

Estimate of required colonies

An estimation of colonies required for the experiments:

N=17 *A. m. capensis* (recipient) colonies for queen-rearing (rating 1).

N=3 *A. m. scutellata* (recipient) colonies for queen-rearing (rating 1).

N=7 *A. m. capensis* (laying worker) donor colonies, for worker-derived brood (rating 2-3).
N=3 *A. m. capensis* (queenbank) colonies, for the storage of virgin queens (rating 2).
N=3 *A. m. scutellata* ('meat') colonies as donors of brood, workers and combs (rating 1).
N=8 *A. m. capensis* ('meat') colonies as donors of brood and combs, or as back-ups (rating 2-3).
N=4 *A. m. capensis / A. m. scutellata* colonies for the production of honey (rating N=1).
N total = 45 colonies were required.
The required colonies were chosen from the colony pool according to their developmental progress.

Splits

For long-term developmental experiments, about 40 splits had to be generated, headed by specifically reared queens. To increase the chances of queen acceptance, the starter-finisher (= breeding) colonies from all queen-rearing cycles were divided into splits and provided with queens, which had been nursed within them. To generate the required number of splits, additional colonies had to be utilised, to provide comb and nurse bees.
All splits were generated from 3 combs in standard Langstroth frames occupied by nurse bees and (if any) capped brood, and virgin queens caged temporarily in introduction queen cages. The frames and caged queens were placed in the centre of 5-frame nucleus boxes, flanked by one comb of honey and one frame containing a wax foundation starter strip. After set-up, the splits were transported to a mating apiary, where they stayed undisturbed for 20 days. From there they were reinstalled within the fenced area (M) for maintenance and long-term observation.

RESULTS AND DISCUSSION

Apiaries

1. Trevor's Farm (T): Pollen flow was observed from *Aloe candelabris* during June and July 2005. No other relevant flows were observed until October. The apiary however was never vandalised and no veldt-fires occurred, during the observation period.
2. Nick's Farm (N): Nectar and pollen flow was observed from *Eucalyptus grandis* in early spring (August 2005). The apiary however was vandalised on 05.09.05 and had to be abandoned. Furthermore, a veldt-fire went through the apiary site during October 2005.
3. Fishery Science Department (F): The apiary was successfully used for enhancing colony development during October and November 2005, with uninterrupted pollen and nectar

flows despite lack of precipitation. Chain-locking of all colonies prevented vandalism, which was observed regularly in previous years at this site.

4. Basil's Farm (= C, G, M, K): Only minor pollen and nectar flows were observed from September until October 2005. Relatively strong and persistent nectar flows (presumably from *Eucalyptus* spp. some distance away) resulted in honey yields from November 2005 until the end of January 2006. Several veldt-fires went through the entire farm area during October 2005, fortunately with only minor colony losses at the apiary (K). Vandalism was observed at the apiary (K) from February 2006 onwards. Less than three months after the termination of the short-term experiments, 15 hived colonies were completely destroyed or stolen from apiary K. The fenced area was not vandalised, the relatively large accumulation of honeybee colonies in close proximity to houses and livestock however were a permanent risk, and were removed as soon as the long-term experiments were terminated in the summer of 2006.

Honeybee colony pool

A steady decline of pool colonies from initially 42 to finally 20 was recorded during the observation period due to losses from experimental procedures, absconding, vandalism or fire. This decline was buffered, but not stopped, by restocking colonies or by trapping swarms. A total of 37 colonies were lost during the observation period. Twelve of these colonies absconded, 4 were lost to vandalism or fire, and 21 were used for the generation of splits. A total of 16 colonies were gained during the observation period, 14 colonies from restocking and 2 from trapped swarms. (Tabs. 11.1.1.; 11.1.2.)

Rating of colonies and general trends in colony development

The results from ratings (Tabs. 11.1.1.; 11.1.2.), general observations of climate, flowering periods, colony activity or disturbances between rating occasions, revealed the following rough estimates of trends in colony development during the observation period. Restocking of colonies was used to compensate for losses or to buffer observed trends.

A. Results from ex-winter rating (24.08.05) averaged 2.6 for all 42 pooled colonies of all subspecies or geographical origins, representing the initial stock from January 2005. *A. m. scutellata* colonies were rated best (average rating = 2.1), followed by Heidelberg *A. m. capensis* colonies (average rating = 2.3) and Grahamstown *A. m. capensis* (average rating = 2.7). No colony losses or gains were recorded during winter (May - August 2005) (Tabs. 11.1.1.; 11.1.2.).

Dry and cold weather during winter resulted in partially depleted honey stores, but winter flowering aloe (T) or minor pollen and nectar flows from unidentified plants (N), resulted in the survival of all colonies of the initial stock during winter. On average colonies occupied 7 standard Langstroth frames, in general a good basis for spring development.

B. Results from spring-1 rating (18.09.05) averaged 2.3 for all 37 pooled colonies of all subspecies or geographical origins. *A. m. scutellata* colonies were rated best (average rating = 1.6), followed by Grahamstown *A. m. capensis* (average rating = 2.5) and Heidelberg *A. m. capensis* (average rating = 2.7). Three (2 Heidelberg, and 1 Grahamstown) *A. m. capensis* colonies were lost due to absconding, two (Grahamstown) *A. m. capensis* colonies were lost due to vandalism (Tabs. 11.1.1.; 11.1.2.).

Very dry, cold and windy weather resulted in further depletion of honey stores, inadequate nectar or pollen flow (T), or with some nectar and pollen flow from Eucalyptus (N). On average, colonies occupied 7-8 standard Langstroth frames, a slight upward trend in colony development was observed. Some dearth absconding was recorded (T), and some colonies were lost due to vandalism (N), resulting in the relocation of all colonies from the apiary (N) to the apiary (T).

C. Results from spring-2 rating (08.10.05) averaged 2.7 for all 35 pooled colonies of all subspecies or geographical origins. *A. m. scutellata* colonies were rated best (average rating = 2.0), followed by Heidelberg *A. m. capensis* colonies (average rating = 2.7) and Grahamstown *A. m. capensis* (average rating = 2.8). Seven (5 Grahamstown, 1 Heidelberg *A. m. capensis* and 1 *A. m. scutellata*) colonies were lost due to absconding. Four *A. m. scutellata* colonies were gained from restocking, one (Grahamstown) *A. m. capensis* colony was gained from a trapped swarm (Tabs. 11.1.1.; 11.1.2.).

Persistent dry, cold and windy weather resulted in further depletion of honey stores, with not enough nectar or pollen flow to enhance colony development. The observed slight upward trend from the spring-1 rating was obviously stopped and reversed. Colonies generally declined in strength to an average of 6-7 occupied standard Langstroth frames. Dearth absconding resulted in a loss of 7 colonies. The restocking with relatively strong colonies (3 *A. m. scutellata* rating = 2; 1 Grahamstown *capensis* rating = 2.5) did not cover the losses, nor did it reverse the general trend. Nine Grahamstown *A. m. capensis* colonies and 2 *A. m. scutellata* colonies were moved to the apiary (F) to enhance colony development by using the permanent nectar and honey flows observed under urban

conditions. Apiary (T) was completely destocked to counteract extremely adverse micro-climatic conditions.

D. Results from spring-3 rating (28.10.05) averaged 2.5 for all 34 pooled colonies of all subspecies or geographical origins. *A. m. scutellata* colonies were rated best (average rating = 2.3), followed by Grahamstown *A. m. capensis* (average rating = 2.5) and Heidelberg *A. m. capensis* (average rating = 3.0). Two (Grahamstown) *A. m. capensis* colonies were lost due to absconding, two (Grahamstown) *A. m. capensis* colonies were lost due to fire and three (1 Grahamstown, 1 Heidelberg *A. m. capensis* and 1 *A. m. scutellata*) colonies were used to generate splits. Six (Grahamstown) *A. m. capensis* colonies were gained from restocking (Tabs. 11.1.1.; 11.1.2.).

Ongoing dry, cold and windy weather resulted in further depletion of honey stores, with no relevant nectar or pollen flow to enhance colony development. A further decline in strength was observed for *A. m. scutellata* and Heidelberg *A. m. capensis* colonies. Restocking with 6 strong Grahamstown *A. m. capensis* colonies reversed the downward trend for this group, and a slight increase in the average strength of all pooled colonies, with an average of 7 occupied standard Langstroth frames observed. Restocking however did not cover the losses from absconding (2 colonies), fire (2 colonies) and experimental procedures (3 colonies used for splits).

E. Results from summer-1 rating (22.11.05) averaged 2.6 for all 30 pooled colonies of all subspecies or geographical origins. *A. m. scutellata* colonies were rated best (average rating = 1.8), followed by Grahamstown *A. m. capensis* (average rating = 2.6) and Heidelberg *A. m. capensis* (average rating = 3.4). Six (5 Grahamstown, 1 Heidelberg) *A. m. capensis* colonies were used to generate splits. One (Grahamstown) *A. m. capensis* colony was gained from restocking, one (Grahamstown) *A. m. capensis* colony was gained from a trapped swarm (Tabs. 11.1.1.; 11.1.2.).

Dry, cold and windy weather at the end of October and heavy rainfall together with low temperatures at the beginning of November, resulted in further depletion of honey stores. Initially only minor nectar or pollen flows were available to enhance colony development. Despite increasing temperatures, colony activity and flow intensities, the development of the colonies seemed generally arrested or even to be declining (Grahamstown- and Heidelberg *A. m. capensis*). The colonies at the apiary (F) gained in strength but did not reverse the general trend. The use of 6 strong colonies for the generation of splits contributed to the slight decrease in average strength of all pooled

colonies. Colony gains (2 Grahamstown *A. m. capensis*) from a trapped swarm and from restocking did not cover the losses.

F. Results from summer-2 rating (20.12.05) averaged 1.8 for all 27 pooled colonies of all subspecies or geographical origins. *A. m. scutellata* colonies were rated best (average rating = 1.1), followed by Grahamstown *A. m. capensis* (average rating = 1.7) and Heidelberg *A. m. capensis* (average rating = 3.3). Six (5 Grahamstown *A. m. capensis* and 1 *A. m. scutellata*) colonies were used to generate splits. Three (Grahamstown) *A. m. capensis* colonies were gained from restocking (Tabs. 11.1.1.; 11.1.2.).

November rain, together with increasing temperatures resulted in increased nectar and pollen flows and a sudden increase in strength with an average of 8-9 occupied standard Langstroth frames for all pooled colonies. Restocking 3 (Grahamstown) *A. m. capensis* colonies did not cover the losses from six strong colonies, which were used for the generation of splits, nor did the loss of those colonies impact on the general increasing trend.

G. Results from summer-3 rating (10.01.06) averaged 2.1 for all 20 pooled colonies of all subspecies and geographical origins. *A. m. scutellata* colonies were rated best (average rating = 1.0), followed by Grahamstown *A. m. capensis* (average rating = 2.1) and Heidelberg *A. m. capensis* (average rating = 3.8). One (Heidelberg) *A. m. capensis* colony was lost due to absconding, six (4 Grahamstown, 1 Heidelberg *A. m. capensis* and 1 *A. m. scutellata*) colonies were used to generate splits (Tabs. 11.1.1.; 11.1.2.).

Due to experimental progress the amount of strong colonies was reduced. Six strong colonies were used for the generation of splits. Swarm-cohesion experiments reduced the strength of a number of worker-bee donor colonies. The reduced strength of all pooled colonies, now occupying only 8 standard Langstroth frames on average, therefore does not reflect trends in colony development but the negative impact of extended experimental activity. Persistent pollen and nectar flows and warm temperatures resulted in good honey yields of about 200 kg from six colonies (33.3 kg / colony), which were predominantly used for honey-production, indicating a positive developmental trend for this period. Nectar and pollen flows and positive colony development was further observed until the end of January 2006, the additional honey yield however was not assessed. The number of pooled colonies was reduced to 20. Until July 2006, subsequent to the observation period, the number of colonies declined to 8, as a result of persistent vandalism at the apiary (K).

Colony movements

Besides colony movements according to experimental progress and requirements, three main events were recorded.

1. Due to vandalism (05.09.05) a total of 14 colonies had to be removed from the apiary (N) and were temporarily relocated to apiary (T) (Tab. 11.1.2.).

2. Due to dearth and adverse micro-climatic conditions, a total of 28 colonies had to be removed from apiary (T) between spring 1 rating and spring 2 rating. Eleven of those colonies were temporarily moved to (F) for quick colony-growth, the remaining colonies were stocked as required at different apiary sites (Tab. 11.1.2.).

3. All colonies, after being used in experiments or otherwise weakened, were finally stocked at apiary (K) for recovery. At the end of the observation period all the other apiaries were vacated of pool-colonies (Tab. 11.1.2.).

Required colonies

Restocking and moving colonies to enhance development proved to be essential in obtaining all requirements for this study as the initial stock would have been insufficient for the conduct of all the experiments. The following colonies were chosen, some of which served as multi-purpose colonies (Tab. 11.1.2.).

A. m. capensis (recipient) colonies for queen-rearing:

L01, L04, L06, L25, L26, L30, L32, L33, L34, L37, L41, L48, L49, L50, L51, L52, H21 (N=17).

A. m. scutellata (recipient) colonies for queen-rearing:

S16, S17, S19 (N=3).

A. m. capensis (laying worker) colonies, donors for worker-derived brood:

L01, L10, L26, L47, L53, H13, H22 (N=7).

A. m. capensis (queenbank) colonies, for the storage of virgin queens:

L35, H08, H15 (N=3).

A. m. scutellata ('meat') colonies as donors for workers, brood and combs:

S44, S45, S46 (N=3)

A. m. capensis ('meat') colonies as donors of brood and combs, or as back-ups:

L03, L05, L11, L14, H15, H20, L24, L36, L47, L54, L56, L57, L58 (N=13)

Colonies for the production of honey:

S44, S45, S46, L55, L56, L57 (N=6).

(For developmental details of specific colonies see Tab. 11.1.2.)

A total of N=43 different colonies was utilised for experiments, roughly confirming the estimates.

Splits

Colonies utilised to generate N = 41 splits are listed in Table 11.1.3. Most colonies were former starter-finisher colonies and yielded 2 splits each (L4, L6, L25, L30, L32, L33, L34, L37, L41, L48, L49, L50, L51, L52, H8, S16, S17, S19). A few colonies yielded only one split (L35, H20, H21), and other splits were made up of combs from 2 colonies, which were combined (L14+L24, L56+L57) (Tab. 11.1.3.). The initial strength of the splits was equal (rating = 4; occupying 4 combs). After three weeks at the mating apiary and one week at the maintenance apiary, all splits were introduced into standard Langstroth brood boxes. The remaining space in the boxes was filled with empty frames containing wax foundation starter strips. Queen status and mating success was recorded. From then on, besides observation of absconding events, swarming or death from dwindling, the colonies remained undisturbed and were only assessed for strength and developmental progress in the spring of 2006.

CONCLUSION

Dry and cold weather during winter, spring and early summer 2005, had a negative impact on colony development. Continuously restocking to compensate for losses and reduced performance, in combination with moving colonies to enhance developmental potential, allowed me to meet all colony requirements to conduct the experiments. Heidelberg *A. m. capensis* colonies were rated worst during most of the observation period, suggesting least adaptation to lack of winter and spring rainfall. The best ratings were obtained throughout by *A. m. scutellata* colonies, possibly due to similarities of the observed climatic conditions to their native summer rainfall distribution. Grahamstown *A. m. capensis* exhibited intermediate developmental capacities, possibly in adaptation to variable rainfall patterns in the Eastern Cape, a transitional zone between summer and winter rainfall areas.

Loss of honeybee colonies due to vandalism, theft and fire are calculable risks and occur in high frequencies. They threaten efficient colony management and beekeeping in the region.

Tab. 11.1.1. Rating results of honeybee colonies on 7 successive dates in three-week intervals

	rating	A ex winter 24.08.05 # colonies	B spring 1 18.09.05 # colonies	C spring 2 08.10.05 # colonies	D spring 3 28.10.05 # colonies	E summer 1 22.11.05 # colonies	F summer 2 20.12.05 # colonies	G summer 3 10.01.06 # colonies
Grahamstown *capensis*	1	3	3	1	1	1	8	8
	1.5	2	2	0	4	9	4	0
	2	4	9	9	11	1	1	1
	2.5	3	2	3	0	0	0	0
	3	11	5	1	2	3	2	3
	3.5	1	1	3	1	2	0	0
	4	5	3	4	2	1	2	2
	4.5	0	1	2	3	4	2	1
total	**colonies**	**29**	**26**	**23**	**24**	**21**	**19**	**15**
average	**rating**	**2.7**	**2.5**	**2.8**	**2.5**	**2.6**	**1.7**	**2.1**
Heidelberg *capensis*	1	1	2	0	0	0	0	0
	1.5	0	1	0	0	0	1	0
	2	3	1	1	0	0	0	0
	2.5	0	0	2	0	0	0	0
	3	3	2	3	5	2	1	1
	3.5	0	0	0	0	1	0	0
	4	2	0	0	0	1	1	0
	4.5	0	1	0	0	0	1	1
total	**colonies**	**9**	**7**	**6**	**5**	**4**	**4**	**2**
average	**rating**	**2.3**	**2.7**	**2.7**	**3.0**	**3.4**	**3.3**	**3.8**
Pretoria *scutellata*	1	0	1	0	0	0	3	3
	1.5	1	2	0	0	2	1	0
	2	1	0	6	2	3	0	0
	2.5	2	1	0	3	0	0	0
	3	0	0	0	0	0	0	0
	3.5	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	4.5	0	0	0	0	0	0	0
total	**colonies**	**4**	**4**	**6**	**5**	**5**	**4**	**3**
average	**rating**	**2.1**	**1.6**	**2.0**	**2.3**	**1.8**	**1.1**	**1.0**
absconded	colonies	0	-3	-7	-2	0	0	-1
vandal. / fire	colonies	0	-2	0	-2	0	0	0
used for splits	colonies	0	0	0	-3	-6	-6	-6
swarm in	colonies	0	0	+1	0	+1	0	0
restocked	colonies	0	0	+4	+6	+1	+3	0
total	**colonies**	**42**	**37**	**35**	**34**	**30**	**27**	**20**
average	**rating**	**2.6**	**2.3**	**2.7**	**2.5**	**2.6**	**1.8**	**2.1**

Tab. 11.1.2. Development of individual colonies and their fates during experiments

nr.	specification	24.08.05	18.09.05	08.10.05	28.10.05	22.11.05	20.12.05	10.01.06	
		A ex-winter	B spring 1	C spring 2	D spring 3	E summer 1	F summer 2	G summer 3	notes and comments
L 01	Graham. *capensis*	2 C	1 **B**	2 **B IV**	3 **IV**	3-4 **IV** C	4-5 **IV** C	4-5	queen L 01 conserved, unsatisfactory lay. work. brood
L 02	Graham. *capensis*	3 C	3	4	burned away				
L 03	Graham. *capensis*	4 C	4	4-5	4-5	4-5	4	3	bee donor for exp. 2 (15.11.02)
L 04	Graham. *capensis*	2 T	2	2	2	1-2 **N**	split21split22		queen L 04 conserved
L 05	Graham. *capensis*	3 T	3	3-4	4	4-5	3	3	bee donor for exp. 2 (15.11.02)
L 06	Graham. *capensis*	2 T	2	2	2	1-2 **T**	1-2 **T**	split36split37	queen L 06 conserved
H 07	Heidelb.*capensis*	4 T	absconded						
H 08	Heidelb.*capensis*	3 T	3	2-3	3	3-4	3 bank3 C	3	fertilized larvae used for grafting in breeding cycle 1, queen H8 returned to hive
H 09	Heidelb.*capensis*	4 T	4-5	absconded					queen H 09 conserved
L 10	Graham. *capensis*	1-2 T	2-3 **II**	3-4 **II**	4-5 **II**	4-5 **II**	4-5 **II** G	4	queen L10 preserved, clonal larvae used for grafting in breeding cycle 1
L 11	Graham. *capensis*	4 T	4-5	4-5	4-5	4-5	3	3	bee donor for exp. 2 (15.11.02)
H 12	Heidelb.*capensis*	3 T	absconded						
H 13	Heidelb.*capensis*	3 T	3	3	3	3 **VII**	4-5 **VII** C	4-5	queen H13 preserved, unsatisfactory lay. work. brood
L 14	Graham. *capensis*	1 T	2	2	2	1-2	1	1	bee donor for exp. 2 (24./27.11; 09./25.12.) and queen cages (twice) and split 29
H 15	Heidelb.*capensis*	2 T	2	3	3	3	1-2 bank2 C	split40split 41	emerging brood for pseudoqueen prod. Set-up 1 and 4, queen H 15 conserved
S 16	Pret. *scutellata*	2-3 T	1-2	2	2	1-2 **O**	split23split24		queen S 16 conserved
S 17	Pret. *scutellata*	2 T	1-2	2	2	1-2 **U**	1-2 **U** F	split38split39	queen S 17 preserved
S 18	Pret. *scutellata*	2-3 T	2-3	absconded					queen S 18 preserved
S 19	Pret. *scutellata*	1-2 T	1 **A**	2 **A**	split1 split2				queen S 19 preserved
H 20	Heidelb.*capensis*	2 T	1-2	2-3	3	split16	varroa!!!		queen H 20 preserved, emerging brood for pseudoqueen prod. Set-up 3
H 21	Heidelb.*capensis*	1 T	1 **D**	2 **D**	split3				queen H 21 preservrd
H 22	Heidelb.*capensis*	2 T	1 **I**	3 **I**	3 **I**	4 **I**	4 **I**	absconded	queen H 22 conserved; clonal larvae used for grafting in breeding cycle 2
L 23	Graham. *capensis*	3 T	absconded						
L 24	Graham. *capensis*	2 T	3	3-4	3-4	3	1	1	bee donor for exp. 2 (17./18.11; 09.12.) and split 29
L 25	Graham. *capensis*	1 T	1 **E**	2 **E**	split4 split5				
L 26	Graham. *capensis*	1 T	1 **C**	2 **C**	3 (**C**) **V** C	3-4 **V** C	4 **V** C	4	queen L 26 preserved; clonal larvae used for grafting in breeding cycle 3
L 27	Graham. *capensis*	3 N	vandalized						
L 28	Graham. *capensis*	4 N	vandalized						
L 29	Graham. *capensis*	3 N	2	absconded					
L 30	Graham. *capensis*	2-3 N	2	3	2	1-2 **Q**	1-2 **Q** F	split30split31	queen L30 preserved
L 31	Graham. *capensis*	4 N	4	4	burned away				
L 32	Graham. *capensis*	3 N	2	2	2	1-2 **S**	1-2 **S**	split34split35	queen L 32 preserved
L 33	Graham. *capensis*	1-2 N	1-2	1	2	1-2 **P**	split25split26		queen L 33 preserved
L 34	Graham. *capensis*	3 N	2-3	2-3	2	1-2 **R**	1-2 **R** F	split32split33	queen L 34 preserved
L 35	Graham. *capensis*	3 N	2	2-3	2	2	2bank1split28		queen L 35 conserved
L 36	Graham. *capensis*	3 N	3	4	4	4	1	1	bee donor for exp. 2 (15.11.02)
L 37	Graham. *capensis*	3 N	2	2	2	1-2 **L**	split17split18		queen L 37 preserved
L 38	Graham. *capensis*	3 N	2	absconded					
L 39	Graham. *capensis*	2-3 N	3	absconded					
L 40	Graham. *capensis*	3-4 N	4	absconded					
L 41	Graham. *capensis*	2-3 N	1-2	2	2	1-2 **M**	split19split20		queen L41 conserved
L 42	Graham. *capensis*	4 N	3-4	absconded					
L 43	Graham. *capensis*			4 swarm in G	absconded				
S 44	Pret. *scutellata*			2	2-3	2	1	1	bee donor for pseudoqueen prod. Set-up 2 and 4; honey producer
S 45	Pret. *scutellata*			2	2-3	2	1	1	bee donor for pseudoqueen prod. Set-up 3 and 4; honey producer
S 46	Pret. *scutellata*			2	2-3	2	1	1	bee donor for pseudoqueen prod. Set-up 1 and 4; honey producer
L 47	Graham. *capensis*			1 **III**	absconded				split made from 3 combs of capped brood and young bees from L58
L 48	Graham. *capensis*				1-2 **F**	split6 split7			queen L 48 conserved
L 49	Graham. *capensis*				1-2 **G**	sp;it8 split9			queen L 49 conserved
L 50	Graham. *capensis*				1 **H**	split10 split11			queen L 50 preserved
L 51	Graham. *capensis*				1-2 **I**	split12 split13			queen L 51 preserved
L 52	Graham. *capensis*				1-2 **K**	split14 split15			queen L 52 conserved
L 53	Graham. *capensis*				2 **VI**	3 **VI**	1	1	queen L 53 preserved; clonal larvae used for grafting in breeding cycle 4
L 54	Graham. *capensis*					(1)3(swarm.)K	2	2	bee donor for queen cages
L 55	Graham. *capensis*					1 swarm in	1	1	honey producer
L 56	Graham. *capensis*						1	1	bee donor for exp.2 (05.12.) and split 27: honey producer
L 57	Graham. *capensis*						1	1	bee donor for exp.2 (05.12.) and split 27: honey producer
L58	Graham. *capensis*						1	1	comb/bee donor for L47 (**III**),; honey producer

Legend:

A/B/C/D/…..U :	20 starter-finisher colonies, finally converted into splits
I / II / III / IV / V / VI / VII :	7 laying worker colonies, continually maintained in queenless condition
bank1 / bank2 / bank3 :	3 queenbanks, maintained in queenless condition during storage of unmated queens, finally converted into splits
split 1 - split 41 :	small mating units initially containing about 3-4 combs with bees and 1 or 2 unmated queen(s) reared from clonal or fertilised brood
1; 1-2; 2; 2-3; 3; 3-4; 4; 4-5:	hive strength: 1 = using ?10 combs; 2 = using 8 combs; 3 = using 6 combs; 4 = using 4 combs; (1-2; 2-3; 3-4; 4-5 = intermediates)
T; N; F; C; G; M; K :	apiary sites: T = Travor's farm; N = Nick's farm; F = Fisheries Department; C = Circle apiary; G = Garden; M = Maintanance
queen conserved :	dead imago or pupa conserved in ethanol
queen preserved :	imago life dissected: head and tergites preserved in dichloromethane, thorax conserved in ethanol, photographs of imago and ovaries
	colonies of *Apis mellifera capensis* from the centre of distribution (Heidelberg) without hybridisation with *Apis mellifera scutellata*
	colonies of *Apis mellifera capensis* from the transition zone (Grahamstown), with potential hybridisation with *Apis mellifera scutellata*
	colonies of *Apis mellifera scutellata* from Pretoria

Tab. 11.1.3. Source colonies and queens for 41 generated splits

nr.	source hive	date of installation	queen from fertilised brood	queen from clonal brood	initial strength	mating apiary	main-tenance apiary	moved into standard Langstroth and first inspection
Split 01	A (S19)	24.10.05	queen 2 (5)	X	4	R	M	18.11.05
Split 02	A (S19)	24.10.05	queen 8 (27)	X	4	R	M	18.11.05
Split 03	D (H21)	24.10.05	queen 30 (10)	X	4	R	M	18.11.05
Split 04	E (L25)	24.10.05	queen 4 (38)	X	4	R	M	18.11.05
Split 05	E (L25)	24.10.05	queen 40 (33)	X	4	R	M	18.11.06
Split 06	F (L48)	12.11.05	queen 58 (13)	X	4	R	M	02.12.05
Split 07	F (L48)	12.11.05	X	queen 48a (16)	4	R	M	02.12.05
Split 08	G (L49)	12.11.05	queen 66 (86)	X	4	R	M	02.12.05
Split 09	G (L49)	12.11.05	X	queen 68a (90)	4	R	M	02.12.05
Split 10	H (L50)	12.11.05	queen 92 (97)	X	4	R	M	02.12.05
Split 11	H (L50)	12.11.05	X	queen 98a (12)	4	R	M	02.12.05
Split 12	I (L51)	12.11.05	queen114 (55)	X	4	R	M	02.12.05
Split 13	I (L51)	12.11.05	X	queen 105a (97)	4	R	M	02.12.05
Split 14	K (l52)	12.11.05	queen 130 (44)	X	4	R	M	02.12.05
Split 15	K (l52)	12.11.05	X	queen 126a (13)	4	R	M	02.12.05
Split 16	H20	18.11.05	X	queen 121a (72)	4	C	M	07.12.05
Split 17	L (L37)	03.12.05	queen 141 (70)	queen136a (45)	4	R	M	22.12.05
Split 18	L (L37)	03.12.05	queen 150 (82)	queen 140a (94)	4	R	M	22.12.05
Split 19	M (L41)	03.12.05	queen 154 (56)	queen 155a (20)	4	R	M	22.12.05
Split 20	M (L41)	03.12.05	queen 157 (27)	queen 154a (60)	4	R	M	22.12.05
Split 21	N (L4)	03.12.05	queen 172 (26)	queen 186a (83)	4	R	M	22.12.05
Split 22	N (L4)	03.12.05	queen 188 (52)	queen 183a (55)	4	R	M	22.12.05
Split 23	O (S16)	03.12.05	queen 206 (12)	queen 203a (67)	4	R	M	22.12.05
Split 24	O (S16)	03.12.05	queen 192 (51)	queen 202a (33)	4	R	M	22.12.05
Split 25	P (L33)	03.12.05	queen 210 (39)	queen 221a (73)	4	R	M	22.12.05
Split 26	P (L33)	03.12.05	queen 208 (78)	queen 217a (6)	4	R	M	22.12.05
Split 27	L56/L57	08.12.05	X	queen 208a (93)	4	M	M	23.12.05
Split 28	bank 1 (L35)	10.12.05	X	queen 221a (73)	4	C	M	23.12.05
Split 29	L14/L24	11.12.05	X	queen197a (19)	4	M	M	23.12.05
Split 30	Q (L30)	25.12.05	X	queen 229a (8)	4	R	M	16.01.06
Split 31	Q (L30)	25.12.05	queen 240 (34)	X	4	R	M	16.01.06
Split 32	R (L34)	25.12.05	X	queen 252a (3)	4	R	M	16.01.06
Split 33	R (L34)	25.12.05	queen 249 (2)	X	4	R	M	16.01.06
Split 34	S (L32)	25.12.05	X	queen 274a (19)	4	R	M	16.01.06
Split 35	S (L32)	25.12.05	queen 268 (35)	X	4	R	M	16.01.06
Split 36	T (L6)	25.12.05	X	queen 292a (31)	4	R	M	16.01.06
Split 37	T (L6)	25.12.05	queen 281 (21)	X	4	R	M	16.01.06
Split 38	U (S17)	25.12.05	X	queen 313a (15)	4	R	M	16.01.06
Split 39	U (S17)	25.12.05	queen 305 (49)	X	4	R	M	16.01.06
Split 40	bank 3 (H8)	29.12.05	X	queen 232a (18)	4	R	M	16.01.06
Split 41	bank 3 (H8)	29.12.05	queen 280 (11)	X	4	R	M	16.01.06

Legend:

A/B/C/D/…..U : 20 starter-finisher colonies, finally converted into splits

bank1 / bank2 / bank3 : 3 queenbanks, maintained in queenless condition during storage of unmated queens, finally converted into splits

split 1 - split 41 : small mating units initially containing 3-4 combs with bees and 1 or 2 unmated queen(s) reared from clonal or fertilised brood

1; 1-2; 2; 2-3; 3; 3-4; 4; 4-5: hive strength: 1 = using ≥10 combs; 2 = using 8 combs; 3 = using 6 combs; 4 = using 4 combs; (1-2; 2-3; 3-4; 4-5 = intermediates)

T; N; F; C; G; M; K; R : apiary sites: T = Travor's farm; N = Nick's farm; F = Fisheries Department; C = Circle apiary; G = Garden; M = Maintenance; R = Randall's Place

Appendix 1.2.

Experimental determination of drone congregation areas for *Apis mellifera capensis*

SUMMARY

An increasingly accurate definition of a drone congregation area (DCA) in the Eastern Cape, South Africa was achieved using drone response limits to exclude areas and locations showing negative results. Putative DCAs were confirmed with a caged, dead mated queen as the lure, and further confirmed several times until the end of the mating season (March 2005) by releasing an empty queen cage. Drone 'comet' formation was observed only within a limited area during two consecutive mating seasons, allowing the empirical definition of the drone response limits of the DCA. Observations on drone responses during two consecutive mating seasons resulted in the confirmation of the DCA and an accurate definition of its centre and approximate area.

INTRODUCTION

On warm summer days honeybee (*Apis mellifera* L.) drones gather high in the air in distinct areas termed drone congregation areas (DCAs) (Zmarlicki and Morse 1963; Ruttner and Ruttner 1965; Drescher 1969; Königer et al. 2005). Drones and queens of honeybees are able to find DCAs independently of each other (Jean-Prost 1957; Zmarlicki and Morse 1963; Ruttner 1966; Ruttner and Ruttner 1972; Tribe 1982). DCAs may be constant over the years and some are known to have existed for more than fifty years (Müller 1950; Jean-Prost 1957; Ruttner and Ruttner 1972; Tribe 1982). DCAs have specific limits and cover areas of between 30-200 m in diameter at heights of 8-40 m, but may vary considerably during any one season (Gary 1962, 1963; Tribe 1982; Loper et al. 1987, 1992). Queens and drones brought into an area from places far away are immediately able to find the local DCAs, indicating general recognisable orientation cues for their detection, independent of any learned behaviour (Ruttner and Ruttner 1966, 1968, 1972). Optical configurations such as depressions in the horizon (Ruttner and Ruttner 1966) as well as wind- and temperature patterns, resulting in air turbulence (Tribe 1982; Cooper 1983) apparently play crucial roles for orientation and detection.

Drones in any DCA are a mixture of a large number of wild and hived colonies in the region (Ruttner and Ruttner 1972; Baudry et al. 1998). However, depending on the location of the colonies, preferences for certain DCAs occur and the energetically most efficient DCAs (the nearer the better) are often favoured, resulting in local 'drone clumping' with considerable genetic diversity within and among different DCAs (Königer et al. 2005). Definite flight routes to and between different DCAs exist, and drones may use those sites to reorientate when switching among DCAs (Loper et al. 1992).

DCAs facilitate mate detection and reduce inbreeding, offering sufficient mating partners within the shortest possible time period and reducing risks of queen losses during mating flights (Woyke 1964; Ruttner and Ruttner 1972; Tribe 1982; Königer et al. 2005). However, the exact factors or local conditions which cause the drones to congregate at the distinct locations, remain elusive (Tribe and Allsopp 2001; Königer et al. 2005). Because mating behaviour cannot be studied under confined artificial conditions (Schmolke 1977), the detection, location and description of accessible, natural, well-defined DCAs are a precondition for selective mating experiments. Here I describe attempts to determine the response limits of DCAs of *A. m. capensis* and consider some constraints and possibilities for successfully defining DCAs. Previous studies often applied the use of synthetic chemical lures with pheromone dosages far higher than those produced by virgin queens. The attractiveness of those artificial lures may have far exceeded natural drone response limits. I report the results of experiments to determine drone response for a DCA by using lures of reduced attractiveness.

MATERIALS AND METHODS

Choice of potential DCA areas

Seven different areas in Grahamstown, Eastern Cape Province, South Africa were chosen for DCA scanning, according to landscape formation and possible turbulence (Ruttner and Ruttner 1966; Tribe 1982; Cooper 1983): Area 1, Rhodes University Athletic Track: situated at the south western border of Grahamstown, this site would allow for the development of thermal as well as for upwind turbulence due to its two-levelled land slope and a row of eucalyptus trees at its western boundary. Sport fields are clear orientation points and have been documented as DCAs (Tribe and Allsopp 2001); Area 2, Rhodes University Great Field and International Library of African Music: in close proximity to an experimental apiary, within the University Campus. The sports field is a clear optical orientation point and thermal conditions could be predicted for this area surrounded by

buildings and vegetation; Area 3, Sports Field Grey Dam: situated at the southern exit of Grahamstown in a valley between the Settlers Monument and Signal Hill Mountain Range. This site could be attractive, due to its proximity to the Botanical Gardens and a small dam where wild colonies can be expected; Area 4, Fairewood: the start of a valley reaching from the eastern boundaries of Grahamstown up to Donkerbosch Outspan farm, forming a clear depression in the landscape and a possible flight pathway for drones; Area 5, Donkerbosch Outspan Farm: the site of an experimental queen-rearing apiary, comprising different landscape formations at the upper end of a long valley 5 km southeast of Grahamstown. The valley is a flight pathway for honeybee swarms that regularly settle in trap boxes. Thickets, clearings, hillocks and mountain ranges could be expected to be good orientation points with distinct possibilities for the development of turbulence or upwind; Area 6, 'Stone Pit': a site forming a distinct white point of orientation within vegetation, with some prospect for the generation of zones of turbulence; Area 7, Road to Beggars Bush: a slight elevation in a landscape of indigenous thicket, where thermal turbulences ('dust-devils') were observed.

Scanning for drone presence

Two different methods for scanning the areas were applied, depending on wind speed and terrain. Under low wind conditions, helium filled balloons with a queen cage containing a dead mated queen suspended 1 m below, were released into the air column up to a height of eight metres. The queens used as lures had been dead for less than 2 months and were kept on ice in sealed Eppendorf-tubes until needed. Whenever the balloons were deflected too strongly by wind, and the height of the lure could not be maintained, the carrying medium was changed. Under wind conditions, a kite was released into the air current to a height of about 200 m. As soon as the kite was in a stable position, a queen cage containing a dead mated queen was fixed to the line and manoeuvred to heights of between 7 and 10 m by releasing or reeling in the kite line as required. Scanning was carried out in January 2005 during the natural mating season (which occurs between October through March) (Hepburn and Radloff 1998), on sunny days with temperatures above 21^{o} C, between 12.30 and 16.00, the natural flight period of drones in summer (Tribe 1982). Depending on the accessibility of the terrain, areas were scanned by walking systematically in big loops and at the same time observing drone response to the exposed lure using a pair of binoculars. Copulation attempts were measured as the number of drones approaching or hitting the

cage ('hits'). A systematic search for DCAs was then carried out in an area with a high numbers of 'hits'.

Systematic search for a DCA

Helium filled balloons or a kite were used as the carrying medium for the lure when systematically searching for a DCA depending on wind conditions (as above). Searching for DCAs was carried out in January 2005 during the natural mating season (Hepburn and Radloff 1998), on sunny days with temperatures above 21^{o} C, between 12.30 and 16.00, the natural flight period of drones in summer (Tribe 1982). A queen cage, containing a dead mated queen, was released into the air column to 8 m above ground and held stationary for 5 min. Copulation attempts ('hits') were recorded. The localities for those stationary assessments were chosen according to accessibility of the terrain and in closer proximity to each other with increasing number of 'hits'. The distances between the localities were extended again as soon as the number of observed 'hits' was decreasing (effectively sampling as a 'Doppler effect').

Confirming and defining a DCA

Localities close to each other with very high numbers of 'hits' were termed 'suspected DCA' and the occurrence of high numbers of drones was confirmed systematically by an assessment as described above. In addition, empirical assessments to confirm the presence of the DCA were carried out during two consecutive mating seasons. Whenever conditions were favourable an empty and unused queen cage was used as a lure and released near the suspected centre of the DCA. Finally, a dark hat, when airborne, proved to be an attractive visual lure for drones only within a DCA. The observation of drone 'comet' formation in response to the airborne hat, thrown to about 6-8 m above ground, could therefore be used to define the DCA's boundaries These quick assessments allowed a frequent confirmation of drone response repeatedly during the entire mating seasons.

RESULTS

Scanning for drone presence

Seven areas were scanned for drone presence during the flight time of drones, 12.30h-16.00h. No drones were observed to approach the lure in the areas 1, 2, 6 and 7 and only a few approaches were observed in scanning areas 3 and 4. However, more than 50 'hits'

were observed in the Donkerbosch Outspan farm area (5) and were concentrated mainly in an area surrounding the experimental apiary (Tab. 11.2.1.).

Systematic search for a DCA:

The Donkerbosch Outspan farm area (5) was systematically assessed at 14 different localities with the following results (Tab. 11.2.2.). No drone responses to the lure were observed at the localities 1, 8, 9, 11, 13 and 14. Very few drone 'hits' were recorded for the localities 10 and 12, indicating probable flight pathways for drones but no DCAs as such. Localities 2 and 7 had slightly increased drone 'hits' and are indicative of DCA edge effects for the more confined micro-localities 3-6. There was an increasing number of 'hits' from northwest to southeast at locations 3 and 4 and declining numbers at 5 and 6; results that further circumscribed a suspected DCA area in the direct vicinity of the experimental apiary.

Confirming and defining the response limits of a DCA

The presence of the suspected DCA was confirmed systematically with a caged dead mated queen as the lure with positive results (Tab. 11.2.3.). It was further assessed empirically several times until the end of the mating season (March 2005) by releasing an empty queen cage up to a height of 8 m into the air column at the suspected centre of the DCA, between the localities 4, 5 and 6, each time with positive results (> 25 'hits' / 5 min). In addition, the presence of this natural DCA was also confirmed several times at the start of the next mating season (September 2005) with positive results (>10 'hits' / 5 min) during the complete absence of hived colonies at the apiary.

'Comet' formation as a response to the 'flying hat' was observed only within a limited area during two consecutive mating seasons (January - March 2005, September 2005 - March 2006). This allowed us to empirically define the boundaries of the DCA, which was determined as having its centre near the west fence of the apiary (S 33º 19’ 50.5"; E 26º 34’ 52.1"; elevation 622 m) and a diameter of about 30 m.

DISCUSSION

Scanning for drone presence

The use of a kite facilitated the scanning and search for DCAs in many cases because even light wind deflects helium filled balloons strongly and it is often impossible to bring the lure to the desired height. In addition, the low carrying capacity of the balloons often

resulted in the line becoming tangled in trees or bushes with no chance of freeing the lure without destroying the balloons. A kite facilitates the scanning of extended areas and even small pathways through thicket can be manoeuvred 'tangle-free' due to the carrying strength of the kite and the stability of the line.

Tribe (1982) observed that a small number of *A. m. scutellata* drones could be attracted to a lure (artificial queen substance (9-ODA) on cotton wool) exposed at a height of 8 m, at any place during the peak mating season in summer. I could not confirm this observation for drones of *A. m. capensis*, using a caged dead mated queen as a lure. Certainly, synthetic queen substance in high quantities can be considered as a strong cue and generally more drones might be attracted to it than to a caged dead queen. On the other hand, the much less attractive lure used in my experiments with only residual pheromonal emission was advantageous for detection and definition of DCAs by reducing the possibility of attracting drones from extended distances as described by Tribe (1982). True drone 'comet' formation was never observed when using a caged dead mated queen but this allowed exact quantification of copulation attempts of single drones ('hits'). The marginal number of 'hits' in the areas 3 and 4 indicated probable drone flight pathways, but not DCAs as such. The high number of 'hits' in the vicinity of the apiary at Donkerbosch Outspan farm (area 5) was highly indicative of a DCA.

Search for DCAs

The increased numbers of 'hits' observed at the localities 2, 3, 7, 10 and 12 in the Donkerbosch Outspan area were indicative of flight pathways for drones to and from the DCA and coincided with observations of honeybee swarm flight pathways that were regularly observed coming from Grahamstown into the valley to Stones Hill and then further southeast towards the Mount Pleasant range. The valley forms a clear indentation of the landscape and the suspected DCA localities 4, 5 and 6 are situated at its highest point before it declines again in elevation. Furthermore it is characterised by clearings in the vegetation and a small white cottage, which could serve as orientation point, clearly visible for several kilometres. The position at the 'saddle' of the valley also results in the formation of upwind for the two main wind directions (west and east), as well as for thermal upwind caused by accumulated warm air generated in the valley. Observations of highly increased numbers of 'hits' at the locations 4, 5 and 6 coincided precisely with observed optical and climatical orientation cues, which made the presence of a DCA highly probable.

Confirming and defining a DCA

Even in the absence of any hived colonies at the apiary in the Donkerbosch Outspan area some drone activity could be observed at the DCA during early spring. In addition the DCA was observed and confirmed during two consecutive mating seasons and its boundaries could be empirically defined a good number of times. Furthermore, choice test experiments (unpublished observations) were carried out within the DCA during the mating period 2005-2006, using live, caged virgin queens of different genetic origin as lures, and drone 'comet' formation was regularly observed and photographically confirmed. The DCA could also be detected by its humming noise (Cooper 1977), especially when it was most attractive to drones: highest attractiveness was regularly recorded on hot summer days shortly after thunderstorms with light showers and a light westerly breeze. Drone activity was then observed to extend late into the evening up to 17.30 or even 18.00.

Defining DCAs first requires some experiential insight as to possible DCA sites, then the gradual scanning of suspect areas using a drone response limit approach and finally confirmation by drone response to accurately define the limits of a DCA.

Tab. 11.2.1. Drone presence in different test areas

No	area	drone 'hits'	max. temp	date
1	RU Athletic Track	0	27°C	10.01.05
2	RU Great Field	0	27°C	10.01.05
3	Sports Field Grey Dam	2	27°C	10.01.05
4	Fairewood	3	27°C	10.01.05
5	Donkerbosch Outspan farm	>50	26°C	13.01.05
6	'Stone Pit'	0	26°C	13.01.05
7	Road to Beggars Bush	0	26°C	13.01.05

Tab. 11.2.2. Drone 'hits' at a stationary lure during 5 min of exposure, at 14 different localities (area 5)

No	locality	drone 'hits'	max. temp	date	time
1	Kopje	0	26° C	16.01.05	13.30-13.35
2	Clearing old	8	26° C	16.01.05	13.50-13.55
3	Clearing new	48	26° C	16.01.05	14.10-14.15
4	Apiary west	140	26° C	16.01.05	14.30-14.35
5	Apiary north	90	26° C	16.01.05	14.45.14.50
6	Apiary south east	40	26° C	16.01.05	15.00-15.05
7	Basil's Place	13	26° C	16.01.05	15.25-15.30
8	Old cottage	0	28° C	17.01.05	13.30-13.35
9	Pathway to Radio tower	0	28° C	17.01.05	13.45-13.50
10	Radio tower	6	28° C	17.01.05	14.05-14.10
11	Pathway to Shooting range	0	28° C	17.01.05	14.25-14.30
12	Shooting range	7	28° C	17.01.05	14.40-14.45
13	Pathway to Braai area	0	28° C	17.01.05	15.00-15.05
14	Braai area	0	28° C	17.01.05	15.25-15.30

Tab. 11.2.3. Drone 'hits' at a stationary lure during 5 min of exposure, at the suspected DCA

No	locality	drone 'hits'	max. temp	date	time
4	Apiary west	435	28° C	19.01.05	12.30-12.35
5	Apiary north	98	28° C	19.01.05	12.40-12.45
6	Apiary south east	166	28° C	19.01.05	12.50-12.55

Appendix 1.3.

Queenright colonies as starter-finishers for Cape honeybee (*Apis mellifera capensis*) queen-rearing experiments

SUMMARY

Queen-rearing in *Apis mellifera capensis* requires modifications in standard rearing procedures. Grafted larvae in artificial queen cell cups are not accepted under queenless conditions. A queenright situation with reduced pheromonal supply is required to suppress laying worker development but on the other hand enhance emergency queen-rearing impulses. The confinement of the old queen in a small cage on the periphery of the colony, was sufficient to comply with both requirements. Acceptance and eclosing rates of grafted larvae in queen-rearing experiments were satisfactory and were comparable with rates observed in European honeybees.

INTRODUCTION

The rearing of honeybee queen bees is sometimes referred to as the "crown of apiculture" in the beekeeping literature (Zander and Böttcher 1982; Bro Adam 1987), implicating both highly advanced apicultural skill-requirements and the potential to develop apiculture to its peak performance. Lundie (1929) outlined that apiculture in South Africa "has not reached that stage in its development at which even one beekeeper can earn a living by specialising in the breeding of queens". Not much has changed in this regard, and queen-rearing is greatly neglected by most beekeepers in South Africa today. The huge genetic pressure from wild honeybee colonies, which may quickly revert all achieved improvements in breeding stocks as well as behavioural characteristics like absconding or prolific swarming (Fletcher 1977; Allsopp and Hepburn 1997; Hepburn and Radloff 1998), may be the reasons for this. Regular colony losses due to fire, flooding (Hepburn and Radloff 1998; Lubke 1998), vandalism or theft (Swart et al. 2001b) add to the fact that extensive beekeeping practises seem to be more efficient in South Africa. In addition, extensive colony management appears to be the best insurance against loss of tolerance against diseases and pests as observed in European derived honeybee populations with two centuries of intensive queen-rearing history.

Literature focussing on queen-rearing with the two South African honeybee subspecies (*A. m. capensis* and *A. m. scutellata*) is scarce. It mainly stems from the first half of the twentieth century when beekeepers experimented with the introduction of European derived honeybee subspecies (Onions 1909, 1912; Terrel 1912; Mowbray 1916; Lundie 1929), or from the 'heydays of beekeeping' (Johannsmeier 2001b) the 1970s and 1980s, where influence from overseas boosted the South African apicultural industry (Anderson 1965; Fletcher and Tribe 1977; Anderson et al. 1983; Taber 1983; Cooke 1986; McGregor 1990). A frequent topic in a number of publications concerns the striking difference between *capensis* and *scutellata* colonies in response to queen-rearing techniques. *Scutellata* colonies can generally be used for queen-rearing by applying standard procedures. These are mainly based on queen- and brood removal together with intensive feeding which stimulates emergency queen-rearing impulses, enhancing the acceptance of selected grafted queen larvae.

In strong contrast, standard procedures for queen-rearing generally failed in *capensis* colonies (Onions 1912, 1914; Anderson 1963; Hepburn and Radloff 1998). Queen removal and intensive feeding resulted in the rapid occurrence of laying workers, intracolonial worker conflicts (Anderson 1963; Crewe 1984; Van Der Blom 1991), the establishment of new dominance hierarchies and eventually pseudoqueens (Onions 1912; Hemmling et al. 1979; Crewe and Velthuis 1980). The ability of *A. m. capensis* workers to produce female offspring parthenogenetically (Onions 1912, 1914; Kerr and Laidlaw 1956; Kerr and Portugal Araújo 1958; Anderson 1963) allowed the continuation of the colony headed by pseudoqueens, and artificially offered queen cells and larvae were either not accepted at all, or aborted shortly after acceptance (Anderson 1963, 1968; Hepburn and Radloff 1998).

Following the experimentally based development of ideas that queens pheromonally inhibit both worker ovarian development and queen cell construction in European honeybees (Butler 1956; Free 1987), Anderson (1965) solved the problem of queen-rearing in *capensis* in a series of classical studies. He sought a method to maintain the delicate balance, according to Butler's (1956) theory, between inhibition of queen cell construction and ovarian development. The controlling pheromones of the queen had to be present in sufficient quantities to allow queen cell construction but to inhibit significant laying worker development (Hepburn and Radloff 1998). Anderson's (1965) method requires a strong queenright colony in a single brood box, which is divided equally by a vertical wire screen of 3.2 mm mesh. The bees cannot access the different compartments except via the landing

board, through two different, separated entrances. One compartment, containing the queen, provides a restricted flow of queen pheromones towards the other compartment, maintained by worker bees feeding each other through the screen or by foragers, entering the compartments interchangeably. The section without the queen is the 'starter-finisher' compartment into which the frame of grafted queen cells is introduced (Swart et al. 2001a). Cooke (1986) described a slightly modified method. Here the queenright compartment and the queenless starter section are horizontally divided, allowing the use of standard equipment, but requiring a very strong colony occupying two standard Langstroth brood chambers. Both *capensis* queen-rearing methods require modifications in the structure of the brood nest (Swart et al. 2001a), very strong colonies and were applied in the centre of the subspecies' distribution. No data concerning queen-rearing in the 'transition zone' are available. The 'transition zone', separates the two South African honeybee subspecies, forming a broad band of hybridisation at their natural interface (Hepburn and Crewe 1991a,b; Hepburn and Radloff 1998; Hepburn and Radloff 2002).

Grahamstown, situated within this band of hybridisation, not only is transitional for the two South African honeybee subspecies (*A. m. capensis* and *A. m. scutellata*) but also for its climate and rainfall pattern (getting a good share of both winter and summer rainfall or none) and for all four major South African floristic regions (Lubke 1998; Lubke and van Wijk 1998). As a consequence, very strong honeybee colonies, generally required for queen-rearing, are scarce due to the minor and highly unpredictable nectar and pollen flows (Muerrle unpublished observations). Here I describe a simple method of queen-rearing in relatively weak (one standard Langstroth brood chamber) *A. m. capensis* colonies from the vicinity of Grahamstown, Eastern Cape, South Africa. This method provides a reduced pheromonal emission required for queen-rearing, simply by keeping the old queen confined to a small cage in the periphery of the hive during the entire rearing process. The lack of a specific queen compartment within the colony enables the entire worker force to take part in queen-rearing tasks and reduces preparation work for the beekeeper.

MATERIALS AND METHODS

Breeding cycles

Four honeybee queen-rearing cycles were carried out at three week intervals, starting on the 10th of October 2005, in Grahamstown, Eastern Cape, South Africa,

Recipient colonies

Sixteen *A. m. capensis* colonies (N=16) where used as starter-finisher colonies (= recipient colonies) for a total of 522 grafted female larvae (N = 522). All recipient colonies were selected from apiaries stocked in January 2005 with colonies from the naturally occurring transitional zone between *A. m. capensis* and *A. m. scutellata* (= Grahamstown *capensis*; N = 42), where workers predominantly express thelytoky and other traits of *A. m. capensis* (Hepburn and Crewe 1991a,b; Hepburn and Radloff 1998; Hepburn and Radloff 2002). All colonies occupied one Langstroth 10-frame breeding chamber. All hives had a minimum of six brood frames, one frame of pollen and two of honey. During the first breeding cycle, three queenless recipient colonies served as a control, for 13 queenright recipient colonies used during the cycles 2, 3, and 4. In the first cycle, 54 larvae were grafted (18 per recipient colony) and in the subsequent cycles 468 larvae were grafted (36 per recipient colony).

Preparation of recipient colonies for queen-rearing

Preparation of the recipient colonies followed the standard protocol for queen-rearing after 9-days of restricted ovipositing by the queen (Zander and Böttcher 1982; Weiss 1983b).
Nine days prior to grafting, the queen was confined on one empty comb in the centre of the colony using a queen excluder cage surrounding the entire frame allowing unhindered food and pheromonal transfer within the hive, but restricting queen ovipositing to one comb. One peripheral (mostly empty) comb in the brood chamber was replaced by a Doolittle type feeder (the size of a Langstroth frame), allowing feeding of sugar solution (250 ml; sugar : water = 1 : 1) as required, at least in weekly intervals.

One day prior to grafting, the brood chamber was checked for the absence of open brood and emergency cells, with the exception of the frame containing the confined queen, where open brood was used to confirm queen presence. On the day of grafting, and 1.5 h prior to the introduction of the breeding frame with grafted larvae, the queen-frame (together with all open brood) was removed, providing space for the introduction of the breeding frame. For the queenless recipient colonies (controls; breeding cycle 1), the queens were removed. For the queenright recipient colonies (breeding cycles 2, 3 and 4.) the queens were confined in a small cage together with 6 workers and were immediately reintroduced into their recipient colonies and positioned on top of a peripheral frame where they were kept until successful eclosion of the virgin queens. The queen cages (8.0 x 3.4 x 1.0 cm; with 30 holes 3.0 x 3.0 mm), allowed restricted food and pheromonal exchange

between the queen and workers, imitating a failing queen and thus enhancing queen cell construction and delaying the development of laying workers.

Grafting

Grafting of 12-24 h old larvae from different donor colonies was carried out according to standard procedures (Laidlaw 1979; Zander and Böttcher 1982; Weiss 1983b). A Chinese grafting tool was used, which allowed optimal uptake of the larvae together with their own provision of royal jelly. Larvae were dry-grafted into water mist sprayed artificial wax cell cups (Ø 8.0 mm, height 6.0 mm), attached to standard wooden bases. The completed breeding frames were introduced into the brood nest of each recipient hive, 1.5 h after removal (queenless controls) or confinement of the queen.

Assessment of acceptance of grafted queen larvae

The breeding frames were assessed for cell acceptance 48 h after grafting. The presence of three-day-old larvae in elongated cells was verified and recorded before they were reintroduced into their colonies.

Assessment of successful eclosion of virgin queens

Eight days after grafting (about three days prior to virgin queen emergence), the breeding frames were checked for capped queen cells. Each capped queen cell was secured in a queen emergence cell cage with five workers. The breeding frames with caged cells were then reintroduced into the hives to allow for further pupal development. About one day after queen emergence the breeding frames were checked for successfully eclosed virgin queens and the individual numbers of all live queens were recorded.

Statistical analysis

Pearson χ^2 tests were used to test for significant differences in acceptance of queen larvae and successful eclosion of virgin queens, between different recipient colonies and breeding cycles.

RESULTS

Assessment of acceptance of queen larvae and of successful eclosion of virgin queens

Differences in acceptance of grafted larvae and successful eclosion of virgin queens between different recipient colonies and breeding cycles were observed as follows (Tab. 11.3.1.).

Group **A**: a) Differences in acceptance of female larvae during the first breeding cycle, using queenless (control) recipient colonies were statistically highly significant (χ^2=16.1, 2df, P=0.0003). The recipient colonies 1 and 2 were reluctant to accept any grafted larvae (0.0%) and recipient colony 3 accepted 7 out of 18 grafted larvae (38.9%) (Tab. 11.3.1.). b) Differences for successful eclosion of virgin queens during the first breeding cycle, using queenless (control) recipient colonies, were statistically significant (χ^2=8.6, 2df, P=0.0133), with no eclosing queens in the recipient colonies 1 and 2 (0.0%), and 4 successfully eclosing queens from 18 grafted larvae, in recipient colony 3 (22.2%) (Tab. 11.3.1.).

Group **B**: a) Differences in acceptance of female larvae during the breeding cycles 2, 3 and 4 using queenright recipient colonies (recipient colonies 4 - 16) were statistically not significant (χ^2 =17.9, 12df, P=0.1201), ranging from 19 accepted larvae out of 36 grafted larvae (52.8%; recipient colony 6) to 31 accepted larvae out of 36 grafted larvae (86.1%; recipient colony 16) (Tab. 11.3.1.). b) Differences in successful eclosion of virgin queens during the breeding cycles 2, 3 and 4, using queenright recipient colonies (recipient colonies 4 - 16) were statistically not significant (χ^2 =19.9, 12df, P=0.0686), ranging from 13 successfully eclosed queens from 36 grafted larvae (52.8%; recipient colony 15) to 27 successfully eclosed queens from 36 grafted larvae (75.0%; recipient colony 16) (Tab. 11.3.1.).

Group **C**: a) Differences in acceptance of female larvae between queenright recipient colonies (recipient colonies 4 - 16) and queenless (control) recipient colonies (recipient colonies 1 - 3, were statistically highly significant (χ^2 =56.0, 1df, P=0.0001). The mean acceptance rate of 65.6% in queenright recipient colonies, with 307 accepted larvae out of 468 grafted larvae, was significantly higher than the mean acceptance rate of 13.0% in queenless (control) recipient colonies, with 7 accepted larvae out of 54 grafted larvae (Tab. 11.3.1.). b) Differences in successful eclosion of virgin queens between queenright recipient colonies (recipient colonies 4 - 16) and queenless (control) recipient colonies (recipient colonies 1 - 3), were statistically highly significant (χ^2 =32.8, 1df, P=0.0001). The mean eclosion rate of 48.3% in queenright recipient colonies, with 226 successfully eclosed queens from 468 grafted larvae, was significantly higher than the mean eclosion

rate of 7.4% in queenless recipient colonies (control), with 4 successfully eclosed queens from 54 grafted larvae (Tab. 11.3.1.).

DISCUSSION

Assessment of acceptance of queen larvae and of successful eclosion of virgin queens

Group **A**: a) and b) Acceptance rates and rates of successful eclosion differed significantly during the first breeding cycle when queenless (control) *A. m. capensis* recipient colonies (recipient colonies 1 - 3) were used. Here the significant differences within the same group can clearly be assigned to queen-rearing methodology (Anderson 1965; Hartmann and Hartmann 1991; Swart et al. 2001a). When using queenless *A. m. capensis* recipient colonies, high variability of acceptance among recipients can be predicted, due to differences in the composition of worker bees. Physiologically developed, potential laying workers will start competing for reproductive dominance with queen loss. The more potential laying workers present, the lower the rate of acceptance and eclosion of introduced larvae (Anderson 1963, 1965; Hepburn 1992b). Two recipient colonies (1 and 2) were reluctant to accept any offered larvae, and intense fighting started one hour after removal of the queen. One recipient colony (3) accepted 7 out of 18 offered larvae, and some fighting was observed following queen removal. On average, this group showed an extremely low larvae acceptance rate of only 13.0%. The mean rate of successful eclosion was also extremely low, with only 7.4% successfully eclosed virgin queens.

Group **B**: a) and b) No statistically significant differences in acceptance of grafted larvae and successful eclosion of virgin queens could be observed during the breeding cycles 2 - 3, when queenright recipient colonies were used. The mean acceptance rate of 65.6% accepted larvae was satisfactory. The mean rate of successful eclosion was lower, but still in a satisfactory range with 48.3% successfully eclosed virgin queens. In European honeybees a mean acceptance rate of 66.7% to 75.0% is considered satisfactory (Pfefferle 1984; Liebig 1998), even though higher acceptance rates have been recorded (up to 100%, Bro Adam 1987). However, complete failure of recipient hives due to inappropriate colony composition or completely unsuccessful breeding cycles due to adverse climatic conditions are regularly recorded from European derived honeybee subspecies as well (Weiss 1983b; Liebig 1998).

Most queen-rearing reports consider acceptance rates most critical, assuming successful eclosion after acceptance, and neglect actual eclosion rates. However, queen larvae and pupae do not always survive following initial acceptance, with mortality rates

being mainly influenced by environmental factors (Weiss 1983b). Capped queen cells for example, are highly vulnerable to cooling during pupation (Zander and Böttcher 1982; Weiss 1983b). The reduced rate of successfully eclosed virgin queens (48.3%) observed here, can clearly be assigned to the harsh climatic conditions in the Eastern Cape during the breeding season. Cold fronts passing along the southern coast at regular intervals (10 - 12 days) during winter until early summer (November) cause drastic fluxes in weather conditions, from hot and dry, to cold and wet (with temperatures dropping towards freezing point) (Stone et al. 1998). Every breeding cycle therefore can be assumed to be 'hit' by one or two of these cold fronts resulting in cooled queen brood in the periphery of the hive, clearly negatively affecting queen eclosion rates, especially in relatively weak nursing colonies as used in this study.

Group **C**: Highly significant differences in acceptance of grafted larvae and successful eclosion of virgin queens were observed when comparing queenright and queenless (control) recipient colonies. The increased mean acceptance and eclosion rates (acceptance 65.6%; eclosion 48.3%) in queenright recipient colonies compared to the very low mean acceptance and eclosion rates (acceptance 13.0%; eclosion 7.4%) in queenless recipient (control) colonies are clearly indicative for the necessity of the confined old queen for reduced pheromone emission, confirming earlier studies (Hepburn and Radloff 1998).

In conclusion, the queen-rearing method described in this study facilitated production of virgin queens in satisfactory numbers in relatively weak colonies of *A. m. capensis* of the 'transitional zone'. The confinement of the old queen to a small cage in the periphery of the hive appeared to have sufficiently reduced pheromonal provision. Delayed laying worker development allowed the successful acceptance of grafted larvae and the eclosion of virgin queens in numbers, satisfactory for small-scale apiaries.

Tab. 11.3.1. Differences in acceptance and eclosion of grafted female larvae

group	hive no	queen status	breed. cycle	grafts	(a)* accept.	(a)* % accept.	(a)* statistical results accepted	(b)* eclosed	(b)* % eclosed	(b)* statistical results eclosed
A	1	queenless	1	18	0	0%	χ2=16.1	0	0%	χ2=8.6
	2	queenless	1	18	0	0%	df=2	0	0%	df=2
	3	queenless	1	18	7	38.89%	P=0.0003	4	22.22%	P=0.0133
B	4	queenright	2	36	24	66.67%		16	44.44%	
	5	queenright	2	36	24	66.67%		15	41.67%	
	6	queenright	2	36	19	52.78%		14	38.89%	
	7	queenright	2	36	22	61.11%		14	38.89%	
	8	queenright	2	36	21	58.33%	χ2=17.9	17	47.22%	χ2= 19.9
	9	queenright	3	36	27	75.00%	df=12	18	50.00%	df=12
	10	queenright	3	36	24	66.67%	P=0.1201	23	63.89%	P=0.0686
	11	queenright	3	36	23	63.89 %		19	52.78%	
	12	queenright	3	36	28	77.78%		27	75.00%	
	13	queenright	4	36	21	58.33%		17	47.22%	
	14	queenright	4	36	24	66.67%		16	44.44%	
	15	queenright	4	36	19	52.78%		13	36.11%	
	16	queenright	4	36	31	86.11%		17	47.22%	
C	1 to 3	queenless	1	54	7	12.96%	χ2=56.0	4	7.41%	χ2=32.8
	4 to 16	queenright	3	468	307	65.60%	df=1	226	48.29%	df=1
							P=0.0001			P=0.0001

* (a) = data accepted larvae; (b) = data eclosed queens

Appendix 1.4.

Storage of virgin queens in *Apis mellifera capensis* Eschscholtz

SUMMARY

The pheromonal bouquet of virgin *Apis mellifera capensis* queens reach peak concentration one week after eclosion and then remains at a high level during the entire mating period. The production and maintenance of mature 'ready to mate' honeybee virgin queens requires efficient post-emergence queen storage conditions. I used two different methods for the storage of *A. m. capensis* virgin queens consecutively. Seven-day on-frame-storage within breeding colonies to produce mature, 'ready to mate' virgin queens, followed by 7-day queen-banking in queenless nursing colonies for virgin queen maintenance. High virgin queen survival rates over a period of 14 days suggest that on-frame-storage and queen-banking are viable long-term storage methods in *A. m. capensis*.

INTRODUCTION

Long-term storage of large numbers of mated honeybee (*Apis mellifera* L.) queen bees, termed queen-banking, is well documented and common routine in commercial queen-rearing of European honeybee subspecies (Laidlaw and Eckert 1962; Reid 1975; Zander and Böttcher 1982; Dietz et al. 1983; Swart et al. 2001a). Queen-banking allows for the production of honeybee queens in sufficient numbers to immediately supply the market according to demand. Strong queenless colonies with an abundant number of young nurse bees are used to provide up to several hundred singly caged queens with nutrients under near natural conditions (Roberts and Stanger 1969; Weaver 1969; Laidlaw and Eckert 1962). Queens are usually not held for more than a month in this manner (Weiss 1983b; Swart et al. 2001a), being superseded by the next batch of queens from a new rearing cycle. Banking appears to have no negative impact on the mated queens' fitness (Swart et al. 2001a) and the amount of the mandibular gland pheromone components were observed to be equal for groups of banked queens and non-banked queens (Slessor et al. 1990).

Very little information is available concerning the storage of newly eclosed virgin queens. Long-term storage of virgin queens usually is of minor importance in commercial queen-rearing, where mating nucs are preferentially and continuously provided with capped queen cells rather than with virgin queens, to facilitate queen acceptance. Long-term

storage of virgin queens, however, is of relevance whenever aged and mature 'ready to mate' virgin queens are required. For example prior to instrumental insemination, for scientific experiments or to supply specific market demands. In general it is recommended that newly eclosed virgin queens be used without delay (Zander and Böttcher 1982), and that storage should not exceed 36 h (Weiss 1983b). Temperature and humidity requirements for virgin queens seem to range within smaller parameters than for mated queens, and slight deviations may result in the rapid death of the queens even if accompanied by nursing bees (Muerrle, unpublished observations). Jones (2000) compared different holding conditions for *A. m. capensis* virgin queens and observed that queen-banking was not successful, with 100% mortality after five days. She reported that small hoarding cages, containing a piece of comb, food, water and about 50 workers, kept under constant temperature conditions (30^{o} C) were most successful for storage. Weiss (1983b) mentioned the possibility of virgin queen storage on top of the frames of a honeybee colony (on-frame-storage) but his recommendations lacked detailed information about colony composition or storage periods.

Newly eclosed European honeybee queens have low levels of all five major mandibular gland components, (*E*)-9-keto-2-decenoic acid (9-ODA), methyl *p*-hydroxybenzoate (HOB), 4-hydroxy-3-methoxyphenylethanol (HVA) and (*R,E*)-(-)- and (*S,E*)-(+)-9-hydroxy-2-decenoic acid (9-HDA). Levels of 9-ODA and 9-HDA however rise and reach about half those found in mated laying queens after one week. HOB appears after one week in virgin queens as well, with HVA levels appearing only after queens are mated (Slessor et al. 1990).

A similar pattern was found in queens of the South African honeybee *A. m. capensis* (Wossler et al. 2006). Mandibular gland secretions were also observed to change in relation to age. These changes however were largely quantitative in nature, with the total volume of the secretion and that of most of the individual compounds increasing with age. A progressive increase in the quantities of the major compounds for the first six days, followed by a relatively stable period (peak-plateau) when optimal production occurs between one to two weeks after emergence, characterised the ontogenetic patterns of the mandibular gland secretions of *A. m. capensis* virgin queens (Jones 2000). This corresponded with the period in which virgin queens were most likely to leave the colony for mating. Pheromone levels were observed to decrease in older queens with compositional changes towards higher 9-ODA percentages in mated queens (Wossler et al. 2006).

For behavioural open-air mating experiments, aged and mature, 'ready to mate', virgin *A. m. capensis* queens had to be produced in sufficient numbers. Therefore post-eclosion storage conditions were required, which allowed optimal maturation of virgin queens for a period of one week with increasing pheromone levels. Post-maturation storage conditions then had to support the maintenance of the pheromonal 'peak-plateau', until the queens were used in the experiments.

Here I describe and clearly distinguish two successful methods of *A. m. capensis* virgin queen storage: On-frame-storage and queen-banking. Both methods were applied consecutively. On-frame-storage, for a period of seven days after virgin queen eclosion, provided optimal ageing and maturation conditions. The newly eclosed virgin queens, stacked in queen cages on top of the frames, remained within their own breeding colonies, surrounded by nurse bees which helped rear them. Queen-banking within specific queenless nursing colonies facilitated the maintenance of larger numbers of aged and mature 'ready to mate' virgin queens for an additional period of 7 days. Both methods required only small standard queen cages, with queens remaining in their cages during the two consecutive storage periods of 14 days.

MATERIAL AND METHODS

Breeding cycles

During three successive honeybee queen-rearing cycles started on the 26th October 2005, at an experimental apiary in Stones Hill, Grahamstown, Eastern Cape, South Africa, 15 (five per rearing cycle) starter-finisher colonies (= recipient colonies) were used for the production of 253 virgin queens, intended for use in drone and worker preference tests to assess differences in queen fitness. All queen-rearing cycles (the last one terminated in December 2005) were carried out within the natural breeding and swarming period of *A. m. capensis* (Hepburn and Radloff 1998).

Recipient colonies

All recipient colonies were selected from experimental apiaries, stocked in January 2005 with queenright, unrelated swarms from: 1) the naturally occurring transitional zone between *A. m. capensis* and *A. m. scutellata* (= Grahamstown *A. m. capensis*; N=42), where workers predominantly (Hepburn and Crewe 1991a,b) express thelytoky and other traits of *A. m. capensis*; 2) *A. m. capensis* from the Heidelberg area, Western Cape (= Heidelberg *A. m. capensis*) where thelytoky is extensively expressed (N=9); and 3) *A. m. scutellata*

from the Pretoria area (N=7) (compare Hepburn and Crewe (1991a,b), Hepburn and Radloff (1998) and Hepburn and Radloff (2002) for detailed reviews on distribution and biology of the investigated honeybee populations). All colonies occupied at least one Langstroth 10-frame breeding chamber and had a minimum of six brood frames, one frame of pollen and two of honey prior to the queen-rearing process.

Queen-rearing

Queen-rearing followed standard protocols in starter-finisher (recipient) colonies after 9 days of restricted ovipositing by the queen (Zander and Böttcher 1982; Weiss 1983b). Grafting of 12-24 h old larvae from different donor colonies was carried out according to standard procedures (Laidlaw 1979; Zander and Böttcher 1982; Weiss 1983b). Larvae were dry-grafted into water mist sprayed, handmade wax cell cups (Ø 8.0 mm, height 6.0 mm), attached to standard wooden bases. Eight days after grafting (about three days prior to virgin queen emergence), the breeding frames were checked for capped queen cells. Each capped queen cell was secured in a queen emergence cell cage with five workers. The breeding frames with caged cells were then reintroduced into the recipient colonies to allow further pupal development. About one day after queen emergence the breeding frames were checked for successfully eclosed virgin queens. The virgin queens were individually marked and transferred into standard plastic 'introduction' queen cages, containing 9 young worker bees and queen candy (castor sugar and honey), as required for the cages. The exits of the cages were tape-sealed to prevent the queens from escaping. The queen cages (8.0 x 3.4 x 1.0 cm; with 30 holes 3.0 x 3.0 mm), allowed feeding of the confined queen and workers by nurse bees.

On-frame-storage

After successful eclosion of the virgin queens, all recipient colonies were used for On-Frame-Storage. Only queens, which had been reared within them, were stored in each recipient colony respectively. The queens were reintroduced into their recipient colonies shortly after their transfer into the queen cages, as described above. The queen cages were positioned on top of the frames with 1 cm distance between each other, allowing the colony's nurse bees unhindered access. All recipient colonies were provided with food (500 ml sugar solution; sugar : water = 1 : 1) and closed with a migratory lid, leaving an air space of 5 cm between the top bars of the frames and the upper cover. The frame of the migratory lid had six openings (80 mm x 8 mm), covered with a wire mesh screen (2.5 mm

x 2.5 mm), facilitating the maintenance of optimal climatic conditions by tending worker bees. The lid was protected against heat and rain by polystyrene and metal covers. The stored queens remained undisturbed on top of the frames for a period of seven days. After this period, the mature 'ready to mate' virgin queens, remaining in their cages, were removed from the top of the frames. All queens from one rearing cycle, but from different recipient colonies, were pooled and prepared for Queen-banking as described below.

Queen-banking

One queen bank colony was prepared for all mature 'ready to mate' virgin queens from each rearing cycle. Queen bank colonies were selected from experimental apiaries as described above and were Grahamstown *A. m. capensis* (bank 1; rearing cycle 1), or Heidelberg *A. m. capensis* (bank 2 and bank 3; rearing cycles 2 and 3 respectively). One hour prior to the introduction of the virgin queens for banking, the old queen and three peripheral frames were removed from the colony providing space for one Doolittle-type feeder (the size of a Langstroth frame) and two banking frames.

The pooled queen cages of one rearing cycle were vertically stacked, back to back in empty frames and were centred and held in place by rubber bands cut from an old inner tube of a car tyre and by foam rubber cuts. Feeding of the confined queen by the colony's nurse bees was possible through the holes of the cages along the sides. Two rows of 23 cages allowed the stacking of up to 46 queen cages on one banking frame. The prepared banking frames were introduced between 2 brood combs of the banking colony, 1 h after the removal of the old queen. All banking colonies were provided with food (500 ml sugar solution; sugar : water = 1 : 1). Virgin queens were successively removed from the colony according to experimental requirements on a daily basis during a total banking period of up to seven days.

Statistical analysis

Pearson and Fisher exact χ^2 tests were used to test for significant differences in survival rates of virgin queens, between different recipient hives and rearing cycles, after seven days of On-Frame-Storage. Pearson and Fisher exact χ^2 tests were used to test for significant differences in survival rates of mature 'ready to mate' virgin queens, between different queen banks, within a period of seven days of queen-banking. All tests were performed using Statistica© (StatSoft 2004).

RESULTS

On-frame-storage

Survival rates of virgin queens were observed after seven days of on-frame-storage (Tab. 11.4.1.). No significant differences in survival of stored virgin queens after seven days of on-frame-storage were observed between different recipient colonies of each rearing cycle. The mean survival rate for the colonies of the rearing cycle 1 (colonies F-K) was 89.5% (χ^2 =6.7, 4df, P=0.1533), that for rearing cycle 2 (colonies L-P) 98.1% (χ^2 =3.4, 4df, P=0.4993) and for rearing cycle 3 (colonies R-U) 85.7% (χ^2 =3.6, 4df, P=0.4619) (Tab. 11.4.1.).

Statistically significant differences between survival rates of virgin queens were observed between different rearing cycles when the results from each rearing cycle were pooled (χ^2 =10.0, 2df, P=0.0068): The survival rate of stored virgin queens from rearing cycle 2 (98.1%) was significantly higher than that from rearing cycle 1 (89.5%) (Fisher exact χ^2 =6.5, 1df, P=0.0175) and from rearing cycle 3 (85.7%) (Fisher exact χ^2 =10.3, 1df, P=0.0019). Differences of survival rates between pooled results from cycle 1 and 3 were statistically not significant (Fisher exact χ^2 =0.5, 1df, P=0.6160) (Tab. 11.4.1.). The mean survival rate of all stored virgin queens was high at 92.1% (233 out of 253 queens survived on-frame-storage) (Tab. 11.4.1.).

Queen-banking

Survival rates of banked mature 'ready to mate' virgin queens were observed after a banking period of up to seven days (Tab. 11.4.2.). Statistically significant differences between survival rates of mature ' ready to mate' virgin queens were observed between all queen banks (χ^2 =31.8, 2df, p<0.0001): The survival rate of banked mature 'ready to mate' virgin queens in queen bank 2 (100%) was significantly higher than in queen bank 1 (91.2%) (Fisher exact χ^2 =9.6, 1df, P=0.0032) and queen bank 3 (73.3%) (Fisher exact χ^2 =31.0, 1df, p<0.0001) (Tab.11.4.2.). Survival rates also differed significantly between bank 1 and bank 3 (Fisher exact χ^2 =7.1, 1df, P=0.0096), with bank 3 having the lowest survival rate of only 73.3% (Tab. 11.4.2.). The mean survival rate of all banked mature 'ready to mate' virgin queens was high at 90.6% (211 from 233 queens survived queen-banking) (Tab. 11.4.2.).

There was no significant difference in mean survival rates between stored (on-frame-storage) and banked (queen-banking) virgin queens (Fisher exact χ^2 =0.4, 1df, P=0.6286).

DISCUSSION

On-frame-storage

Mortality during on-frame-storage was probably due to queens suffocating in a too soft and sticky queen patty, which could have been avoided by using a dryer sugar-honey mixture or not providing the caged bees with any food at all. Significant differences in survival between different breeding cycles can therefore probably be assigned to differences in the consistency of the food patty, which was prepared with slight deviations from cycle to cycle. The high overall survival rate of virgin queens with 233 from 253 queens surviving storage (92.1%) indicates, that on-frame-storage is a viable and easy method for virgin queen storage for at least one week. However, the efficiency of this method is reduced due to the requirement of relatively high numbers of queenless recipient colonies. Further experiments could be conducted to assess possibilities for on-frame-storage of a higher number of virgin queens above the queen excluder in strong queenright colonies. These colonies would stay economically intact and virgin queen storage could be an additional valuable feature.

Queen-banking

The high overall survival rate of mature 'ready to mate' virgin queens with 211 from 233 queens surviving queen-banking (90.56%) for an additional period of up to seven days following on-frame-storage indicates that queen-banking is an efficient and viable method for the maintenance of large numbers of mature 'ready to mate' virgin queens for up to one week. The results are clearly contradictory to Jones' (2000) observations, which stated that queen-banking is not a successful storage method for *A. m. capensis* virgin queens. However, statistically significant differences in survival rates between all different queen banks were recorded. Mortality seemed to be mainly due to neglect of specific queens by nurse bees. The holes of the cages of neglected queens were completely propolised, but no data are available as to whether the cages were propolised before or after the death of the queens. Queen banks 2 and 1 had high survival rates of 100% and 91.2% respectively. In queen bank 3 the survival rate was significantly lower (73.3%). Here the highest number of propolised cages was found and the nursing quality appeared to be generally reduced, suggesting a failing queen bank.

Differences in queen bank quality and survival rates of virgin queens may be due to differing worker age-group composition and the presence of laying workers or

pseudoqueens in *A. m. capensis* banking colonies. However, failing queen banks are commonly observed in European honeybees as well, as soon as the workers age (Muerrle unpublished observations). In commercial queen-rearing, queen banks in general are artificial queenless colony constructs, initially comprising mainly capped brood frames collected from different colonies, and very young bees added from brood frames with open brood. Maintenance of functional queen banks requires continuous exchange of eclosed frames with capped brood frames to ensure a steady influx of a high number of very young workers. Success in virgin queen-banking therefore seems to be mainly management dependent and requires the right choice and worker composition of banking colonies.

Tab. 11.4.1. Survival rates of virgin queens after seven days of on-frame-storage

rearing cycle	recipient colony	eclosed queens	surviving queens	mean survival rates per rearing cycle
1	F	16	13	
1	G	15	13	
1	H	14	11	89.5%
1	I	14	14	
1	K	17	17	
2	L	18	17	
2	M	23	22	
2	N	19	19	98.1%
2	O	20	20	
2	P	27	27	
3	Q	17	13	
3	R	16	13	
3	S	13	11	85.7%
3	T	17	16	
3	U	7	7	
1-3	total	253	233	92.1%

Tab. 11.4.2. Survival rates of mature 'ready to mate' virgin queens after queen-banking

rearing cycle	queen bank	banked queens per rearing cycle	surviving banked queens	mean survival rates per rearing cycle
1	bank 1	68	62	91.2%
2	bank 2	105	105	100%
3	bank 3	60	44	73.3%
1-3	banks 1-3	233	211	90.6%

APPENDIX 2 (Production of virgin queens)

Appendix 2.1. Rearing of *A. m. capensis* queens in queenright and queenless breeding colonies during 4 breeding cycles in 2005

queen nr.	cl./ fe.	cycl. nr.	mother hive	start-finish.	cell accept. yes/no	capped cell attractive-ness 1/2/3	hatched queen attractive-ness 1/2/3	matured queen attractive-ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
1		1	H8	A / S19	Y	3	2	no data	1	X	N	p 27.10.05	11	7	X
1	a	1	II / L10	A / S19	Y	aborted									lay.work. present early
2		1	H 8	A / S19	Y	1	1	no data	X	Sp 1	N	lost			rejected by Sp1
2	a	1	II / L10	A / S19	Y	aborted									lay.work. present early
3		1	H 8	A / S19	N										lay.work. present early
3	a	1	II / L10	A / S19	N										lay.work. present early
4		1	H 8	A / S19	Y	3	2	no data		Sp 4	Y				signed blue -38-
4	a	1	II / L10	A / S19	Y	aborted									lay.work. present early
5		1	H8	A / S19	N										lay.work. present early
5	a	1	II / L10	A / S19	N										lay.work. present early
6		1	H8	A / S19	Y	3	1	no data	2	X	N	p 25.10.05	9	1	X
6	a	1	II / L10	A / S19	N										lay.work. present early
7		1	H8	A / S19	Y	3	3	no data	2	X	N	p 25.10.05	9	2	X
7	a	1	II / L10	A / S19	Y	aborted									lay.work. present early
8		1	H8	A / S19	Y	2	3	no data	X	Sp 2	N	lost			rejected by Sp2
8	a	1	II / L10	A / S19	Y	aborted									lay.work. present early
9		1	H8	A / S19	Y	3	dead								pupation incomplete
9	a	1	II / L10	A / S19	N										lay.work. present early
10		1	H8	B / L1	N										lay.work. present early
10	a	1	II / L10	B / L1	N										lay.work. present early
11		1	H8	B / L1	N										lay.work. present early
11	a	1	II / L10	B / L1	N										lay.work. present early
12		1	H 8	B / L1	N										lay.work. present early
12	a	1	II / L10	B / L1	N										lay.work. present early
13		1	H 8	B / L1	N										lay.work. present early
13	a	1	II / L10	B / L1	N										lay.work. present early
14		1	H 8	B / L1	N										lay.work. present early
14	a	1	II / L10	B / L1	N										lay.work. present early
15		1	H8	B / L1	N										lay.work. present early
15	a	1	II / L10	B / L1	N										lay.work. present early
16		1	H8	B / L1	N										lay.work. present early
16	a	1	II / L10	B / L1	N										lay.work. present early
17		1	H8	B / L1	N										lay.work. present early
17	a	1	II / L10	B / L1	N										lay.work. present early
18		1	H8	B / L1	N										lay.work. present early
18	a	1	II / L10	B / L1	N										lay.work. present early
19		1	H8	C / L26	N										lay.work. present early
19	a	1	II / L10	C / L26	N										lay.work. present early
20		1	H8	C / L26	N										lay.work. present early
20	a	1	II / L10	C / L26	N										lay.work. present early
21		1	H8	C / L26	N										lay.work. present early
21	a	1	II / L10	C / L26	N										lay.work. present early
22		1	H 8	C / L26	N										lay.work. present early
22	a	1	II / L10	C / L26	N										lay.work. present early
23		1	H 8	C / L26	N										lay.work. present early
23	a	1	II / L10	C / L26	N										lay.work. present early
24		1	H 8	C / L26	N										lay.work. present early
24	a	1	II / L10	C / L26	N										lay.work. present early
25		1	H8	C / L26	N										lay.work. present early
25	a	1	II / L10	C / L26	N										lay.work. present early
26		1	H8	C / L26	N										lay.work. present early
26	a	1	II / L10	C / L26	N										lay.work. present early
27		1	H8	C / L26	N										lay.work. present early
27	a	1	II / L10	C / L26	N										lay.work. present early
28		1	H8	D / H21	N										lay.work. present early

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
28	a	1	II / L10	D / H21	N										lay.work. present early
29		1	H8	D / H21	Y	2	2	dead				c 25.10.05		3	storage problems
29	a	1	II / L10	D / H21	Y	aborted									lay.work. present early
30		1	H8	D / H21	Y	1	1	no data		Sp 3	Y				signed blue -10-
30	a	1	II / L10	D / H21	N										lay.work. present early
31		1	H8	D / H21	N										lay.work. present early
31	a	1	II / L10	D / H21	Y	aborted									lay.work. present early
32		1	H 8	D / H21	N										lay.work. present early
32	a	1	II / L10	D / H21	N										lay.work. present early
33		1	H 8	D / H21	N										lay.work. present early
33	a	1	II / L10	D / H21	N										lay.work. present early
34		1	H 8	D / H21	N										lay.work. present early
34	a	1	II / L10	D / H21	N										lay.work. present early
35		1	H8	D / H21	N										lay.work. present early
35	a	1	II / L10	D / H21	Y	3	dead								pupation incomplete
36		1	H8	D / H21	N										lay.work. present early
36	a	1	II / L10	D / H21	N										lay.work. present early
37		1	H8	E / L25	N										lay.work. present early
37	a	1	II / L10	E / L25	N										lay.work. present early
38		1	H8	E / L25	N										lay.work. present early
38	a	1	II / L10	E / L25	Y	aborted									lay.work. present early
39		1	H8	E / L25	N										lay.work. present early
39	a	1	II / L10	E / L25	Y	aborted									lay.work. present early
40		1	H8	E / L25	Y	1	1	no data		Sp 5	Y				signed blue -33-
40	a	1	II / L10	E / L25	Y	aborted									lay.work. present early
41		1	H8	E / L25	N										lay.work. present early
41	a	1	II / L10	E / L25	N										lay.work. present early
42		1	H8	E / L25	N										lay.work. present early
42	a	1	II / L10	E / L25	N										lay.work. present early
43		1	H8	E / L25	N										lay.work. present early
43	a	1	II / L10	E / L25	Y	2	1	dead				c 25.10.05		4	storage problems
44		1	H8	E / L25	Y	3	1	dead				c 25.10.05		5	storage problems
44	a	1	II / L10	E / L25	N										lay.work. present early
45		1	H8	E / L25	N										lay.work. present early
45	a	1	II / L10	E / L25	Y	2	1	no data	1	X	N	p 27.10.05	11	6	X
46		2	L52	F / L48	Y	1	1	1	1	X	N	p 15.11.05	9	20	X
46	a	2	I / H22	F / L48	N										X
47		2	L52	F / L48	Y	1	dead								cooled (weather)
47	a	2	I / H22	F / L48	Y	1	1	2	dead			c 13.11.05			suffocated in patty
48		2	L52	F / L48	N										X
48	a	2	I / H22	F / L48	Y	1	1	2	X	Sp 7	X	absconded			queen lost
49		2	L52	F / L48	Y	1	1	1	1	X	N	p 18.11.05	12	42	X
49	a	2	I / H22	F / L48	Y	1	1	2	1	X	N	p 19.11.05	13	58	X
50		2	L52	F / L48	N										X
50	a	2	I / H22	F / L48	Y	1	1	2	1	X	N	p 15.11.05	9	16	X
51		2	L52	F / L48	Y	1	1	2	1	X	N	p 18.11.05	12	37	X
51	a	2	I / H22	F / L48	Y	1	1	2	1	X	N	p 17.11.05	11	25	X
52		2	L52	F / L48	Y	1	2	2	1	X	N	p 17.11.05	11	30	X
52	a	2	I / H22	F / L48	Y	1	1	2	1	X	N	p 18.11.05	12	41	X
53		2	L52	F / L48	Y	1	2	2	1	X	N	p 17.11.05	11	27	X
53	a	2	I / H22	F / L48	Y	1	1	2	dead						suffocated in patty
54		2	L52	F / L48	N										X
54	a	2	I / H22	F / L48	Y	aborted									X
55		2	L52	F / L48	Y	3	dead					c 08.11.05			cooled (weather)
55	a	2	I / H22	F / L48	Y	1	dead					c 08.11.05			cooled (weather)
56		2	L52	F / L48	Y	3	dead								cooled (weather)
56	a	2	I / H22	F / L48	Y	3	dead					c 08.11.05			cooled (weather)
57		2	L52	F / L48	N										X
57	a	2	I / H22	F / L48	N										X
58		2	L52	F / L48	Y	2	2	3	X	Sp 6	X	lost			invaded by 68a
58	a	2	I / H22	F / L48	Y	1	2	2	dead						suffocated in patty
59		2	L52	F / L48	N										X
59	a	2	I / H22	F / L48	Y	aborted									X
60		2	L52	F / L48	N										X
60	a	2	I / H22	F / L48	Y	1	2	2	1	X	N	p 19.11.05	13	49	X
61		2	L52	F / L48	N										X
61	a	2	I / H22	F / L48	Y	1	2	2	2	X	N	p 18.11.05	12	46	X
62		2	L52	F / L48	N										X
62	a	2	I / H22	F / L48	N										X

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
63		2	L52	F / L48	N										X
63	a	2	I / H22	F / L48	Y	2	dead					c 08.11.05			cooled (weather)
64		2	L52	G / L49	N										X
64	a	2	I / H22	G / L49	Y	1	dead								cooled (weather)
65		2	L52	G / L49	Y	aborted									X
65	a	2	I / H22	G / L49	N										X
66		2	L52	G / L49	Y	1	1	1		Sp 8	Y				signed blue 86
66	a	2	I / H22	G / L49	N										X
67		2	L52	G / L49	N										X
67	a	2	I / H22	G / L49	Y	1	1	1	1	X	N	p 19.11.05	13	54	X
68		2	L52	G / L49	Y	1	1	1	dead			c 13.11.05			suffocated in patty
68	a	2	I / H22	G / L49	Y	1	1	2		Sp 9	Y	p 26.12.05	50	142	invaded spl 6 + abscon
69		2	L52	G / L49	Y	1	1	1	1	X	N	p 19.11.05	13	51	X
69	a	2	I / H22	G / L49	Y	1	1	1	dead						storage problems
70		2	L52	G / L49	Y	1	1	1	1	X	N	p 18.11.05	12	44	X
70	a	2	I / H22	G / L49	Y	1	1	1	1	X	N	p 18.11.05	12	45	X
71		2	L52	G / L49	Y	1	1	1	1	X	N	p 19.11.05	13	50	X
71	a	2	I / H22	G / L49	Y	1	1	1	1	X	N	p 18.11.05	12	33	X
72		2	L52	G / L49	N										X
72	a	2	I / H22	G / L49	Y	1	1	2	dead			c 14.11.05			suffocated in patty
73		2	L52	G / L49	N										X
73	a	2	I / H22	G / L49	Y	1	dead					c 08.11.05			cooled (weather)
74		2	L52	G / L49	Y	3	dead					c 08.11.05			cooled (weather)
74	a	2	I / H22	G / L49	Y	1	dead					c 08.11.05			cooled (weather)
75		2	L52	G / L49	N										X
75	a	2	I / H22	G / L49	N										X
76		2	L52	G / L49	Y	2	dead								cooled (weather)
76	a	2	I / H22	G / L49	Y	2	1	1	1	X	N	p 14.11.05	8	11	X
77		2	L52	G / L49	Y	2	2	1	1	X	N	p 18.11.05	12	38	X
77	a	2	I / H22	G / L49	N										X
78		2	L52	G / L49	Y	2	1	1	1	X	N	p 14.11.05	8	9	X
78	a	2	I / H22	G / L49	N										X
79		2	L52	G / L49	N										X
79	a	2	I / H22	G / L49	Y	3	1	1	dead			c 17.11.05			storage problems
80		2	L52	G / L49	Y	2	dead					c 08.11.05			cooled (weather)
80	a	2	I / H22	G / L49	Y	3	dead								cooled (weather)
81		2	L52	G / L49	Y	3	dead								cooled (weather)
81	a	2	I / H22	G / L49	N										X
82		2	L52	H / L50	Y	aborted									X
82	a	2	I / H22	H / L50	Y	1	1	1	dead						suffocated in patty
83		2	L52	H / L50	Y	1	1	2	1	X	N	p 19.11.05	13	52	X
83	a	2	I / H22	H / L50	Y	1	1	2	dead			c 13.11.05			injured from hatch
84		2	L52	H / L50	N										X
84	a	2	I / H22	H / L50	N										X
85		2	L52	H / L50	Y	1	2	2	1	X	N	p 18.11.05	12	40	X
85	a	2	I / H22	H / L50	Y	aborted									X
86		2	L52	H / L50	Y	1	1	2	1	X	N	p 14.11.05	8	12	X
86	a	2	I / H22	H / L50	Y	1	1	2	2	X	N	p 18.11.05	12	39	X
87		2	L52	H / L50	N										X
87	a	2	I / H22	H / L50	N										X
88		2	L52	H / L50	Y	1	1	2	1	X	N	p 17.11.05	11	31	X
88	a	2	I / H22	H / L50	N										X
89		2	L52	H / L50	N										X
89	a	2	I / H22	H / L50	N										X
90		2	L52	H / L50	N										X
90	a	2	I / H22	H / L50	Y	1	1	2	dead						suffocated in patty
91		2	L52	H / L50	N										X
91	a	2	I / H22	H / L50	Y	2	1	2	dead						storage problems
92		2	L52	H / L50	Y	2	1	3		Sp 10	Y				signed blue 97
92	a	2	I / H22	H / L50	Y	2	dead								cooled (weather)
93		2	L52	H / L50	Y	1	1	2	2	X	N	p 19.11.05	13	53	X
93	a	2	I / H22	H / L50	Y	aborted									X
94		2	L52	H / L50	N										X
94	a	2	I / H22	H / L50	Y	1	dead								cooled (weather)
95		2	L52	H / L50	N										X
95	a	2	I / H22	H / L50	N										X
96		2	L52	H / L50	N										X
96	a	2	I / H22	H / L50	N										X
97		2	L52	H / L50	Y	1	1	2	1	X	N	p 14.11.05	8	10	X
97	a	2	I / H22	H / L50	Y	1	2	2	1	X	N	p 18.11.05	12	43	X

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
98		2	L52	H / L50	N										X
98	a	2	I / H22	H / L50	Y	2	2	2		Sp 11	Y				signed red 12
99		2	L52	H / L50	N										X
99	a	2	I / H22	H / L50	N										X
100		2	L52	I / L51	N										X
100	a	2	I / H22	I / L51	N										X
101		2	L52	I / L51	Y	2	dead					c 08.11.05			cooled (weather)
101	a	2	I / H22	I / L51	N										X
102		2	L52	I / L51	Y	2	1	2	1	X	N	p 15.11.05	9	14	X
102	a	2	I / H22	I / L51	N										X
103		2	L52	I / L51	N										X
103	a	2	I / H22	I / L51	N										X
104		2	L52	I / L51	Y	1	1	3	1	X	N	p 17.11.05	11	22	injured
104	a	2	I / H22	I / L51	Y	1	1	2	1	X	N	p 14.11.05	8	8	X
105		2	L52	I / L51	Y	1	1	2	1	X	N	p 17.11.05	11	23	X
105	a	2	I / H22	I / L51	Y	1	1	3	X	Sp 13	X	lost			queen lost
106		2	L52	I / L51	N										X
106	a	2	I / H22	I / L51	N										X
107		2	L52	I / L51	Y	1	dead								cooled (weather)
107	a	2	I / H22	I / L51	Y	1	1	1	2	X	N	p 19.11.05	13	55	X
108		2	L52	I / L51	Y	1	dead					c 08.11.05			cooled (weather)
108	a	2	I / H22	I / L51	Y	1	1	3	dead			c 15.11.05			storage problems
109		2	L52	I / L51	N										X
109	a	2	I / H22	I / L51	Y	aborted									X
110		2	L52	I / L51	Y	1	1	2	1	X	N	p 15.11.05	9	17	X
110	a	2	I / H22	I / L51	N										X
111		2	L52	I / L51	N										X
111	a	2	I / H22	I / L51	Y	3	dead					c 08.11.05			cooled (weather)
112		2	L52	I / L51	Y	1	1	3	2	X	N	p 19.11.05	13	57	X
112	a	2	I / H22	I / L51	Y	1	1	1	1	X	N	p15.11.05	9	13	X
113		2	L52	I / L51	N										X
113	a	2	I / H22	I / L51	Y	aborted									X
114		2	L52	I / L51	Y	2	1	3		Sp 12	Y				signed blue 55
114	a	2	I / H22	I / L51	Y	1	1	1	1	X	N	p 18.11.05	12	35	X
115		2	L52	I / L51	N										X
115	a	2	I / H22	I / L51	Y	2	1	3	1	X	N	p 19.11.05	13	48	X
116		2	L52	I / L51	N										X
116	a	2	I / H22	I / L51	Y	1	1	3	1	X	N	p 17.11.05	11	29	X
117		2	L52	I / L51	Y	3	dead					c 08.11.05			cooled (weather)
117	a	2	I / H22	I / L51	Y	aborted									X
118		2	L52	K / L52	Y	1	dead					c 08.11.05			cooled (weather)
118	a	2	I / H22	K / L52	N										X
119		2	L52	K / L52	Y	1	2	1	dead			c 17.11.05			storage problems
119	a	2	I / H22	K / L52	Y	1	1	2	1	X	N	p 18.11.05	12	47	X
120		2	L52	K / L52	N										X
120	a	2	I / H22	K / L52	Y	1	1	1	1	X	N	p 18.11.05	12	36	X
121		2	L52	K / L52	Y	1	1	1	dead			c 17.11.05			storage problems
121	a	2	I / H22	K / L52	Y	1	1	2		Sp 16	Y				signed red 72
122		2	L52	K / L52	Y	aborted									X
122	a	2	I / H22	K / L52	N										X
123		2	L52	K / L52	N										X
123	a	2	I / H22	K / L52	N										X
124		2	L52	K / L52	N										X
124	a	2	I / H22	K / L52	N										X
125		2	L52	K / L52	N										X
125	a	2	I / H22	K / L52	Y	1	1	1	1	X	N	p 17.11.05	11	32	X
126		2	L52	K / L52	N										X
126	a	2	I / H22	K / L52	Y	1	1	3		Sp15	Y				signed red 13
127		2	L52	K / L52	N										X
127	a	2	I / H22	K / L52	Y	2	1	1	1	X	N	p 15.11.05	9	15	X
128		2	L52	K / L52	N										X
128	a	2	I / H22	K / L52	Y	2	2	2	1	X	N	p 17.11.05	11	28	X
129		2	L52	K / L52	Y	3	2	1	1	X	N	p 17.11.05	11	26	X
129	a	2	I / H22	K / L52	Y	2	2	1	1	X	N	p 15.11.05	9	19	X
130		2	L52	K / L52	Y	2	1	2		Sp14	Y				signed blue 44
130	a	2	I / H22	K / L52	N										X
131		2	L52	K / L52	Y	2	2	1	1	X	N	p 18.11.05	12	34	X
131	a	2	I / H22	K / L52	Y	2	2	1	1	X	N	p 17.11.05	11	21	X
132		2	L52	K / L52	N										X
132	a	2	I / H22	K / L52	Y	2	dead								cooled (weather)

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
133		2	L52	K / L52	Y	2	2	1	1	X	N	p 15.11.05	9	18	X
133	a	2	I / H22	K / L52	N										X
134		2	L52	K / L52	N										X
134	a	2	I / H22	K / L52	Y	3	1	2	1	X	N	p 19.11.05	13	56	X
135		2	L52	K / L52	Y	3	dead					c 08.11.05			cooled (weather)
135	a	2	I / H22	K / L52	Y	3	2	1	1	X	N	p 17.11.05	11	24	X
136		3	L4	L / L37	Y	1	dead								dead in cell
136	a	3	V / L26	L / L37	Y	1	1	2		Sp17					signed red 45
137		3	L4	L / L37	Y	1	1	1	2	X	N	p 08.12.05	11	82	X
137	a	3	V / L26	L / L37	Y	1	1	2	3	X	N	p 09.12.05	12	115	X
138		3	L4	L / L37	N										X
138	a	3	V / L26	L / L37	Y	1	1	2	2	X	N	p 07.12.05	10	75	X
139		3	L4	L / L37	Y	1	1	1	3	X	N	p 06.12.05	9	67	X
139	a	3	V / L26	L / L37	Y	1	dead								dead in cell
140		3	L4	L / L37	Y	1	1	1	1	X	N	p 08.12.05	11	84	X
140	a	3	V / L26	L / L37	Y	1	1	2		Sp18	X	lost		X	signed red 94
141		3	L4	L / L37	Y	1	1	2		Sp17	X	lost		X	signed blue 70
141	a	3	V / L26	L / L37	Y	aborted									X
142		3	L4	L / L37	Y	1	1	1	3	X	N	p 09.12.05	12	100	X
142	a	3	V / L26	L / L37	Y	1	1	2	3	X	N	p 09.12.05	12	104	X
143		3	L4	L / L37	N										X
143	a	3	V / L26	L / L37	Y	1	1	2	3	X	N	p 09.12.05	12	111	X
144		3	L4	L / L37	N										X
144	a	3	V / L26	L / L37	Y	1	dead								dead in cell
145		3	L4	L / L37	N										X
145	a	3	V / L26	L / L37	Y	aborted									X
146		3	L4	L / L37	N										X
146	a	3	V / L26	L / L37	Y	2	dead								dead in cell
147		3	L4	L / L37	Y	1	1	2	3	X	N	p 09.12.05	12	99	X
147	a	3	V / L26	L / L37	Y	1	1	1	1	X	N	p 05.12.05	8	62	X
148		3	L4	L / L37	Y	2	1	2	2	X	N	p 09.12.05	12	114	X
148	a	3	V / L26	L / L37	Y	aborted									X
149		3	L4	L / L37	N										X
149	a	3	V / L26	L / L37	N										X
150		3	L4	L / L37	Y	1	1	3		Sp18					signed blue 82
150	a	3	V / L26	L / L37	Y	1	dead								dead in cell
151		3	L4	L / L37	N										X
151	a	3	V / L26	L / L37	Y	1	1	1	3	X	N	p 08.12.05	11	86	X
152		3	L4	L / L37	Y	2	1	1	2	X	N	p 06.12.05	9	73	X
152	a	3	V / L26	L / L37	Y	2	1	1	2	X	N	p 08.12.05	11	95	X
153		3	L4	L / L37	N										X
153	a	3	V / L26	L / L37	Y	1	dead								dead in cell
154		3	L4	M / L41	Y	1	1	3		Sp19	X	lost		X	signed blue 56
154	a	3	V / L26	M / L41	Y	1	1	3		Sp20	X	lost		X	signed red 60
155		3	L4	M / L41	Y	1	1	1	2	X	N	p 08.12.05	11	93	X
155	a	3	V / L26	M / L41	Y	1	1	2		Sp19	X	lost		X	signed red 20
156		3	L4	M / L41	Y	1	1	1	2	X	N	p 09.12.05	12	110	X
156	a	3	V / L26	M / L41	Y	1	1	1	2	X	N	p 08.12.05	11	81	X
157		3	L4	M / L41	Y	1	1	3		Sp20	X	lost		X	signed blue 27
157	a	3	V / L26	M / L41	Y	1	1	1	2	X	N	p 07.12.05	10	78	X
158		3	L4	M / L41	Y	1	1	1	3	X	N	p 09.12.05	12	106	X
158	a	3	V / L26	M / L41	Y	1	1	1	2	X	N	p 09.12.05	12	109	X
159		3	L4	M / L41	Y	1	1	2	1	X	N	p 05.12.05	8	65	X
159	a	3	V / L26	M / L41	Y	1	1	1	3	X	N	p 09.12.05	12	118	X
160		3	L4	M / L41	N										X
160	a	3	V / L26	M / L41	Y	1	1	1	2	X	N	p 06.12.05	9	69	X
161		3	L4	M / L41	N										X
161	a	3	V / L26	M / L41	N										X
162		3	L4	M / L41	Y	2	2	dead				c 02.12.05			storage problems
162	a	3	V / L26	M / L41	Y	1	1	1	3	X	N	p 09.12.05	12	102	X
163		3	L4	M / L41	N										X
163	a	3	V / L26	M / L41	N										X
164		3	L4	M / L41	Y	1	2	1	1	X	N	p 05.12.05	8	61	X
164	a	3	V / L26	M / L41	N										X
165		3	L4	M / L41	N										X
165	a	3	V / L26	M / L41	N										X
166		3	L4	M / L41	Y	1	1	2	1	X	N	p 06.12.05	9	71	X
166	a	3	V / L26	M / L41	N										X
167		3	L4	M / L41	Y	1	1	2	2	X	N	p 07.12.05	10	76	X
167	a	3	V / L26	M / L41	Y	2	1	2	1	X	N	p 05.12.05	8	60	X

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
168		3	L4	M / L41	Y	1	1	1	1	X	N	p 05.12.05	8	59	X
168	a	3	V / L26	M / L41	Y	2	1	2	1	X	N	p 05.12.05	8	66	X
169		3	L4	M / L41	Y	1	1	1	1	X	N	p 05.12.05	8	64	X
169	a	3	V / L26	M / L41	N										X
170		3	L4	M / L41	N										X
170	a	3	V / L26	M / L41	Y	aborted									X
171		3	L4	M / L41	N										X
171	a	3	V / L26	M / L41	Y	2	2	1	1	X	N	p 05.12.05	8	63	X
172		3	L4	N/ L4	Y	1	1	2		Sp21	X	lost		X	signed blue 26
172	a	3	V / L26	N/ L4	Y	aborted									X
173		3	L4	N/ L4	Y	1	1	1	2	X	N	p 10.12.05	13	133	X
173	a	3	V / L26	N/ L4	N										X
174		3	L4	N/ L4	N										X
174	a	3	V / L26	N/ L4	N										X
175		3	L4	N/ L4	Y	1	1	1	3	X	N	p 08.12.05	11	85	X
175	a	3	V / L26	N/ L4	N										X
176		3	L4	N/ L4	Y	1	1	1	2	X	N	p 08.12.05	11	97	X
176	a	3	V / L26	N/ L4	Y	1	dead								dead in cell
177		3	L4	N/ L4	Y	1	1	1	1	X	N	p 10.12.05	13	127	X
177	a	3	V / L26	N/ L4	Y	1	1	2	2	X	N	p 08.12.05	11	83	X
178		3	L4	N/ L4	N										X
178	a	3	V / L26	N/ L4	N										X
179		3	L4	N/ L4	N										X
179	a	3	V / L26	N/ L4	Y	1	1	1	2	X	N	p 08.12.05	11	91	X
180		3	L4	N/ L4	Y	2	1	1	1	X	N	p 07.12.05	10	77	X
180	a	3	V / L26	N/ L4	Y	1	1	1	2	X	N	p 09.12.05	12	108	X
181		3	L4	N/ L4	Y	1	2	1	2	X	N	p 08.12.05	11	80	X
181	a	3	V / L26	N/ L4	N										X
182		3	L4	N/ L4	Y	1	1	1	2	X	N	p 10.12.05	13	128	X
182	a	3	V / L26	N/ L4	N										X
183		3	L4	N/ L4	Y	1	1	1	3	X	N	p 09.12.05	12	101	X
183	a	3	V / L26	N/ L4	Y	1	2	3		Sp22	X	lost		X	signed red 55
184		3	L4	N/ L4	N										X
184	a	3	V / L26	N/ L4	N										X
185		3	L4	N/ L4	Y	1	1	1	3	X	N	p 09.12.05	12	105	X
185	a	3	V / L26	N/ L4	Y	1	2	1	3	X	N	p 08.12.05	11	90	X
186		3	L4	N/ L4	Y	1	1	1	2	X	N	p 09.12.05	12	116	X
186	a	3	V / L26	N/ L4	Y	1	1	2		Sp21	X	lost		X	signed red 83
187		3	L4	N/ L4	N										X
187	a	3	V / L26	N/ L4	Y	1	dead								dead in cell
188		3	L4	N/ L4	Y	2	1	2		Sp22	X	lost		X	signed blue 52
188	a	3	V / L26	N/ L4	N										X
189		3	L4	N/ L4	Y	1	1	1	2	X	N	p 08.12.05	11	88	X
189	a	3	V / L26	N/ L4	Y	aborted									X
190		3	L4	O / S16	Y	1	1	2	3	X	N	p 10.12.05	13	135	X
190	a	3	V / L26	O / S16	N										X
191		3	L4	O / S16	N										X
191	a	3	V / L26	O / S16	N										X
192		3	L4	O / S16	Y	1	1	2		Sp24	X	lost		X	signed blue 51
192	a	3	V / L26	O / S16	Y	aborted									X
193		3	L4	O / S16	Y	1	1	2	2	X	N	p 08.12.05	11	96	X
193	a	3	V / L26	O / S16	Y	1	dead								dead in cell
194		3	L4	O / S16	Y	1	1	2	2	X	N	p 10.12.05	13	126	X
194	a	3	V / L26	O / S16	Y	1	1	2	3	X	N	p 10.12.05	13	138	X
195		3	L4	O / S16	N										X
195	a	3	V / L26	O / S16	Y	1	1	1	3	X	N	p 10.12.05	13	134	X
196		3	L4	O / S16	N										X
196	a	3	V / L26	O / S16	N										X
197		3	L4	O / S16	Y	1	1	1	3	X	N	p 09.12.05	12	112	X
197	a	3	V / L26	O / S16	Y	1	1	1		Sp29	Y				signed red 19
198		3	L4	O / S16	Y	1	1	1	2	X	N	p 10.12.05	13	137	X
198	a	3	V / L26	O / S16	Y	1	dead								dead in cell
199		3	L4	O / S16	N										X
199	a	3	V / L26	O / S16	Y	1	1	1	3	X	N	p 10.12.05	13	131	X
200		3	L4	O / S16	N										X
200	a	3	V / L26	O / S16	N										X
201		3	L4	O / S16	N										X
201	a	3	V / L26	O / S16	Y	1	1	1	3	X	N	p 09.12.05	12	98	X
202		3	L4	O / S16	Y	1	1	2	2	X	N	p 08.12.05	11	89	X
202	a	3	V / L26	O / S16	Y	2	1	3		Sp24	X	lost		X	signed red 33

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
203		3	L4	O / S16	N										X
203	a	3	V / L26	O / S16	Y	1	1	3		Sp23	X	lost		X	signed red 67
204		3	L4	O / S16	N										X
204	a	3	V / L26	O / S16	Y	1	1	1	2	X	N	p 07.12.05	10	79	X
205		3	L4	O / S16	Y	1	1	1	3	X	N	p 10.12.05	13	132	X
205	a	3	V / L26	O / S16	Y	1	1	2	1	X	N	p 10.12.05	13	130	X
206		3	L4	O / S16	Y	1	1	2		Sp23	X	lost		X	signed blue 12
206	a	3	V / L26	O / S16	N										X
207		3	L4	O / S16	Y	1	1	2	1	X	N	p 10.12.05	13	141	X
207	a	3	V / L26	O / S16	Y	1	1	1	2	X	N	p 10.12.05	13	124	X
208		3	L4	P / L33	Y	1	1	2		Sp26	X	lost		X	signed blue 78
208	a	3	V / L26	P / L33	Y	1	1	1		Sp27	Y/N				signed red 93
209		3	L4	P / L33	Y	1	1	1	2	X	N	p 09.12.05	12	119	X
209	a	3	V / L26	P / L33	N										X
210		3	L4	P / L33	Y	1	1	2		Sp25	X	lost		X	signed blue 39
210	a	3	V / L26	P / L33	Y	1	1	2	3	X	N	p 06.12.05	9	72	X
211		3	L4	P / L33	Y	1	1	1	3	X	N	p 09.12.05	12	117	X
211	a	3	V / L26	P / L33	Y	1	1	2	2	X	N	p 10.12.05	13	122	X
212		3	L4	P / L33	Y	1	1	1	2	X	N	p 09.12.05	12	120	X
212	a	3	V / L26	P / L33	N										X
213		3	L4	P / L33	Y	1	1	1	2	X	N	p 10.12.05	13	125	X
213	a	3	V / L26	P / L33	N										X
214		3	L4	P / L33	Y	1	1	1	3	X	N	p 08.12.05	11	94	X
214	a	3	V / L26	P / L33	Y	1	1	2		Sp25	X	lost		X	signed red 43
215		3	L4	P / L33	Y	1	1	1	1	X	N	p 06.12.05	9	70	X
215	a	3	V / L26	P / L33	Y	1	1	1	3	X	N	p 06.12.05	9	68	X
216		3	L4	P / L33	Y	1	1	1	3	X	N	p 09.12.05	12	107	X
216	a	3	V / L26	P / L33	N										X
217		3	L4	P / L33	N										X
217	a	3	V / L26	P / L33	Y	1	1	2		Sp26	X	lost		X	signed red 96
218		3	L4	P / L33	N										X
218	a	3	V / L26	P / L33	Y	1	1	1	2	X	N	p 08.12.05	11	92	X
219		3	L4	P / L33	Y	1	1	1	2	X	N	p 10.12.05	13	139	X
219	a	3	V / L26	P / L33	Y	1	1	1	2	X	N	p 08.12.05	11	87	X
220		3	L4	P / L33	Y	1	1	2	3	X	N	p 10.12.05	13	129	X
220	a	3	V / L26	P / L33	Y	1	1	1	3	X	N	p 09.12.05	12	113	X
221		3	L4	P / L33	N										X
221	a	3	V / L26	P / L33	Y	1	1	1		Sp28	X	lost		X	signed red 73
222		3	L4	P / L33	Y	1	1	1	2	X	N	p 10.12.05	13	123	X
222	a	3	V / L26	P / L33	Y	1	1	1	2	X	N	p 10.12.05	13	136	X
223		3	L4	P / L33	N										X
223	a	3	V / L26	P / L33	Y	1	1	1	2	X	N	p 10.12.05	13	140	X
224		3	L4	P / L33	Y	3	1	1	2	X	N	p 09.12.05	12	103	X
224	a	3	V / L26	P / L33	Y	1	2	1	1	X	N	p 06.12.05	9	74	X
225		3	L4	P / L33	Y	2	dead								dead in cell
225	a	3	V / L26	P / L33	Y	1	1	1	2	X	N	p 10.12.05	13	121	X
226		4	L30	Q / L30	Y	2	1	1	1	X	N	p 26.12.05	8	153	X
226	a	4	VI / L53	Q / L30	Y	2	1	1	dead			c 28.12.05			stor. probl. (bank4)
227		4	L30	Q / L30	Y	aborted									X
227	a	4	VI / L53	Q / L30	Y	2	1	1	1	X	N	p 27.12.05	9	167	X
228		4	L30	Q / L30	N										X
228	a	4	VI / L53	Q / L30	Y	1	1	2	1	X	N	p 27.12.05	9	160	X
229		4	L30	Q / L30	Y	1	1	1	dead			c 28.12.05			stor. probl. (bank4)
229	a	4	VI / L53	Q / L30	Y	1	1	2		Sp30					signed red 8
230		4	L30	Q / L30	N										X
230	a	4	VI / L53	Q / L30	N										X
231		4	L30	Q / L30	N										X
231	a	4	VI / L53	Q / L30	N										X
232		4	L30	Q / L30	N										X
232	a	4	VI / L53	Q / L30	Y	2	1	1		Sp40					signed red 18
233		4	L30	Q / L30	N										X
233	a	4	VI / L53	Q / L30	Y	2	2	1	dead			c 28.12.05			stor. probl. (bank4)
234		4	L30	Q / L30	N										X
234	a	4	VI / L53	Q / L30	N										X
235		4	L30	Q / L30	Y	3	1	1	1	X	N	p 26.12.05	8	150	X
235	a	4	VI / L53	Q / L30	Y	2	1	1	dead			c 26.12.05			injured from hatch
236		4	L30	Q / L30	Y	2	1	1	1	X	N	p 27.12.05	9	159	X
236	a	4	VI / L53	Q / L30	Y	3	1	1	2	X	N	p 27.12.05	9	158	X
237		4	L30	Q / L30	Y	1	1	1	1	X	N	p 26.12.05	8	155	X
237	a	4	VI / L53	Q / L30	N										X

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
238		4	L30	Q / L30	Y	3	1	1	1	X	N	p 26.12.05	8	152	X
238	a	4	VI / L53	Q / L30	Y	2	1	1	1	X	N	p 26.12.05	8	147	X
239		4	L30	Q / L30	N										X
239	a	4	VI / L53	Q / L30	Y	3	dead								empty cell
240		4	L30	Q / L30	Y	3	2	2		Sp31					signed blue 34
240	a	4	VI / L53	Q / L30	Y	3	1	1	1	X	N	p 27.12.05	9	175	X
241		4	L30	Q / L30	Y	aborted									X
241	a	4	VI / L53	Q / L30	N										X
242		4	L30	Q / L30	N										X
242	a	4	VI / L53	Q / L30	Y	3	dead								empty cell
243		4	L30	Q / L30	N										X
243	a	4	VI / L53	Q / L30	N										X
244		4	L34	R / L34	N										X
244	a	4	VI / L53	R / L34	Y	1	1	1	1	X	N	p 27.12.05	9	168	X
245		4	L34	R / L34	Y	1	1	1	dead			c 26.12.05			storage problems
245	a	4	VI / L53	R / L34	Y	1	1	1	dead						storage problems
246		4	L34	R / L34	Y	aborted									X
246	a	4	VI / L53	R / L34	Y	1	1	1	1	X	N	p 27.12.05	9	157	X
247		4	L34	R / L34	N										X
247	a	4	VI / L53	R / L34	Y	1	1	1		Sp34					signed red 19
248		4	L34	R / L34	N										X
248	a	4	VI / L53	R / L34	Y	1	1	1	dead			c 28.12.05			stor. probl. (bank4)
249		4	L34	R / L34	Y	1	1	2		Sp33					signed blue 2
249	a	4	VI / L53	R / L34	N										X
250		4	L34	R / L34	Y	1	1	1	1	X	N	p 27.12.05	9	156	X
250	a	4	VI / L53	R / L34	Y	aborted									X
251		4	L34	R / L34	N										X
251	a	4	VI / L53	R / L34	Y	1	dead					c 20.12.05			hatched but dead
252		4	L34	R / L34	Y	1	dead								dead pupa
252	a	4	VI / L53	R / L34	Y	1	1	2		Sp32					signed red 3
253		4	L34	R / L34	Y	1	1	1	1	X	N	p 27.12.05	9	169	X
253	a	4	VI / L53	R / L34	N										X
254		4	L34	R / L34	Y	1	1	1	dead			c 26.12.05			storage problems
254	a	4	VI / L53	R / L34	N										X
255		4	L34	R / L34	N										X
255	a	4	VI / L53	R / L34	Y	aborted									X
256		4	L34	R / L34	Y	1	1	1	1	X	N	p 26.12.05	8	154	X
256	a	4	VI / L53	R / L34	Y	aborted									X
257		4	L34	R / L34	Y	1	1	1	1	X	N	p 27.12.05	9	171	X
257	a	4	VI / L53	R / L34	Y	1	dead					c 20.12.05			hatched but dead
258		4	L34	R / L34	Y	1	1	1	1	X	N	p 27.12.05	9	172	X
258	a	4	VI / L53	R / L34	Y	1	1	1	dead			c 18.12.05			stor. probl. (bank4)
259		4	L34	R / L34	N										X
259	a	4	VI / L53	R / L34	N										X
260		4	L34	R / L34	N										X
260	a	4	VI / L53	R / L34	Y	1	dead								empty cell
261		4	L34	R / L34	N										X
261	a	4	VI / L53	R / L34	Y	1	2	1	1	X	N	p 26.12.05	8	148	X
262		4	L32	S / L32	Y	1	1	1	dead			c 28.12.05			stor. probl. (bank4)
262	a	4	VI / L53	S / L32	Y	1	1	1	1	X	N	p 27.12.05	9	165	X
263		4	L32	S / L32	Y	1	1	2	1	X	N	p 27.12.05	9	170	X
263	a	4	VI / L53	S / L32	Y	1	dead					c 20.12.05			hatched but dead
264		4	L32	S / L32	Y	1	1	2	dead			c 28.12.05			stor. probl. (bank4)
264	a	4	VI / L53	S / L32	Y	1	dead					c 20.12.05			hatched but dead
265		4	L32	S / L32	N										X
265	a	4	VI / L53	S / L32	Y	1	dead					c 20.12.05			hatched but dead
266		4	L32	S / L32	Y	1	1	1	1	X	N	p 27.12.05	9	174	X
266	a	4	VI / L53	S / L32	N										X
267		4	L32	S / L32	Y	1	1	1	1	X	N	p 27.12.05	9	173	X
267	a	4	VI / L53	S / L32	N										X
268		4	L32	S / L32	Y	1	1	2		Sp35					signed blue 35
268	a	4	VI / L53	S / L32	Y	1	1	1	dead			c 28.12.05			storage problems
269		4	L32	S / L32	N										X
269	a	4	VI / L53	S / L32	Y	1	dead					c 20.12.05			hatched but dead
270		4	L32	S / L32	N										X
270	a	4	VI / L53	S / L32	N										X
271		4	L32	S / L32	N										X
271	a	4	VI / L53	S / L32	Y	1	1	1	dead			c 26.12.05			storage problems
272		4	L32	S / L32	N										X
272	a	4	VI / L53	S / L32	N										X

queen nr.	cl./ fe.	cycl. nr.	mother hive	start- finish.	cell accept. yes/no	capped cell attractive- ness 1/2/3	hatched queen attractive- ness 1/2/3	matured queen attractive- ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
273		4	L32	S / L32	N										X
273	a	4	VI / L53	S / L32	N										X
274		4	L32	S / L32	N										X
274	a	4	VI / L53	S / L32	Y	1	1	1	dead			c 28.12.05			stor. probl. (bank4)
275		4	L32	S / L32	Y	2	1	1	dead			c 28.12.05			stor. probl. (bank4)
275	a	4	VI / L53	S / L32	Y	2	1	2	1	X	N	p 26.12.05	8	151	lame / injured
276		4	L32	S / L32	N										X
276	a	4	VI / L53	S / L32	Y	1	dead					c 20.12.05			hatched but dead
277		4	L32	S / L32	Y	1	1	1	1	X	N	p 27.12.05	9	164	X
277	a	4	VI / L53	S / L32	N										X
278		4	L32	S / L32	N										X
278	a	4	VI / L53	S / L32	Y	1	dead					c 20.12.05			hatched but dead
279		4	L32	S / L32	N										X
279	a	4	VI / L53	S / L32	N										X
280		4	L6	T / L6	Y	1	1	1		Sp41					signed blue 11
280	a	4	VI / L53	T / L6	Y	1	1	1	dead			c 28.12.05			storage problems
281		4	L6	T / L6	Y	1	1	2		Sp37					signed blue 21
281	a	4	VI / L53	T / L6	Y	1	1	1	1	X	N	p 27.12.05	9	163	X
282		4	L6	T / L6	N										X
282	a	4	VI / L53	T / L6	Y	1	dead					c 20.12.05			dead in cell
283		4	L6	T / L6	Y	1	1	2	dead			c 28.12.05			stor. probl. (bank4)
283	a	4	VI / L53	T / L6	Y	aborted									X
284		4	L6	T / L6	Y	1	1	1	1	X	N	p 27.12.05	9	162	X
284	a	4	VI / L53	T / L6	Y	1	dead					c 20.12.05			hatched but dead
285		4	L6	T / L6	N										X
285	a	4	VI / L53	T / L6	Y	1	dead								dead in cell (slime)
286		4	L6	T / L6	Y	1	dead					c 20.12.05			hatched but dead
286	a	4	VI / L53	T / L6	Y	aborted									X
287		4	L6	T / L6	Y	1	1	1	1	X	N	p 27.12.05	9	166	X
287	a	4	VI / L53	T / L6	Y	1	1	1	dead			c 28.12.05			stor. probl. (bank4)
288		4	L6	T / L6	Y	2	1	1	dead			c 28.12.05			stor. probl. (bank4)
288	a	4	VI / L53	T / L6	Y	1	1	1	dead			c 28.12.05			stor. probl. (bank4)
289		4	L6	T / L6	Y	2	1	1	dead			c 28.12.05			stor. probl. (bank4)
289	a	4	VI / L53	T / L6	N										X
290		4	L6	T / L6	Y	aborted									X
290	a	4	VI / L53	T / L6	Y	1	dead								empty cell
291		4	L6	T / L6	Y	2	dead					c 20.12.05			hatched but dead
291	a	4	VI / L53	T / L6	Y	2	dead								dead in cell (slime)
292		4	L6	T / L6	Y	1	1	1	1	X	N	p 26.12.05	8	146	X
292	a	4	VI / L53	T / L6	Y	1	1	2		Sp36					signed red 31
293		4	L6	T / L6	Y	1	1	1	1	X	N	p 26.12.05	8	144	X
293	a	4	VI / L53	T / L6	Y	aborted									X
294		4	L6	T / L6	Y	1	1	1	1	X	N	p 27.12.05	9	161	X
294	a	4	VI / L53	T / L6	N										X
295		4	L6	T / L6	Y	1	dead					c 20.12.05			hatched but dead
295	a	4	VI / L53	T / L6	Y	1	2	2	2	X	N	p 26.12.05	8	145	X
296		4	L6	T / L6	Y	2	1	1	1	X	N	p 26.12.05	8	149	X
296	a	4	VI / L53	T / L6	Y	2	dead					c 20.12.05			hatched but dead
297		4	L6	T / L6	Y	2	dead								dead in cell
297	a	4	VI / L53	T / L6	N										X
298		4	S17	U / S17	Y	1	dead								dead in cell
298	a	4	VI / L53	U / S17	N										X
299		4	S17	U / S17	N										X
299	a	4	VI / L53	U / S17	N										X
300		4	S17	U / S17	N										X
300	a	4	VI / L53	U / S17	Y	1	dead								empty cell
301		4	S17	U / S17	N										X
301	a	4	VI / L53	U / S17	Y	1	dead					c 20.12.05			hatched but dead
302		4	S17	U / S17	N										X
302	a	4	VI / L53	U / S17	Y	1	dead					c 20.12.05			hatched but dead
303		4	S17	U / S17	N										X
303	a	4	VI / L53	U / S17	N										X
304		4	S17	U / S17	Y	1	1	1	dead			c 28.12.05			stor. probl. (bank4)
304	a	4	VI / L53	U / S17	Y	1	dead					c 20.12.05			hatched but dead
305		4	S17	U / S17	Y	2	1	2		Sp39					signed blue 49
305	a	4	VI / L53	U / S17	N										X
306		4	S17	U / S17	Y	2	dead					c 20.12.05			dead in cell
306	a	4	VI / L53	U / S17	Y	2	dead								empty cell
307		4	S17	U / S17	N										X
307	a	4	VI / L53	U / S17	Y	aborted									X

queen nr.	cl./ fe.	cycl. nr.	mother hive	start-finish.	cell accept. yes/no	capped cell attractive-ness 1/2/3	hatched queen attractive-ness 1/2/3	matured queen attractive-ness 1/2/3	ovary size/ developm. 1/2/3	split nr.	mated yes/no	date p= preserved or c = conserved	age days	photo nr.	comments notes
308		4	S17	U / S17	N										X
308	a	4	VI / L53	U / S17	N										X
309		4	S17	U / S17	Y	2	1	2	dead			c 28.12.05			stor. probl. (bank4)
309	a	4	VI / L53	U / S17	Y	aborted									X
310		4	S17	U / S17	N										X
310	a	4	VI / L53	U / S17	N										X
311		4	S17	U / S17	Y	3	dead					c 20.12.05			hatched but dead
311	a	4	VI / L53	U / S17	N										X
312		4	S17	U / S17	N										X
312	a	4	VI / L53	U / S17	Y	3	1	2	dead			c 28.12..05			stor. probl. (bank4)
313		4	S17	U / S17	Y	3	1	1	dead			c 28.12..05			stor. probl. (bank4)
313	a	4	VI / L53	U / S17	Y	3	1	2		Sp38					signed red 15
314		4	S17	U / S17	Y	aborted									X
314	a	4	VI / L53	U / S17	Y	2	1	lost							escaped
315		4	S17	U / S17	N	3	dead								own lay.work product
315	a	4	VI / L53	U / S17	Y	3	2	1	dead			c 28.12.05			stor. probl. (bank4)

legend:

column 1: (queen number) grafted larvae were consecutively numbered

column 2: (cl / fe) a = clonal worker-derived larvae; blank = fertilised queen-derived larvae

column 3: (cycl. nr.) 1 = rearing cycle 1(start 05.10.05); 2 = rearing cycle 2 (start 26.10.05); 3 = rearing cycle 3 (start 16.11.05); 4 = rearing cycle 4 (start 07.12.05)

column 4: (mother colony) compare appendix 1, 2

column 5: (start.-finish.) A-U = 20 breeding colonies; compare appendix 1, 2

column 6: (cell accept. yes/no) Y = larva accepted; N = larva rejected

column 7: (capped cell attractiveness) 1 = 0-10 workers; 2 = 11-20 workers; 3 = 21 and more workers tending

column 8: (hatched queen attractiveness) 1 = 0-10 workers; 2 = 11-20 workers; 3 = 21 and more workers tending

column 9: (mature queen attractiveness) 1 = 0-10 workers; 2 = 11-20 workers; 3 = 21 and more workers tending

column 10: (ovary size/ development 1/2/3) 1 = undeveloped; 2 = slightly developed compartmentalised; 3 = fully developed with ripe eggs

column 11: (split nr.) queen used for production of small mating colony (split); compare appendix 3

column 12: (mated yes/no) yes = mated; no = virgin

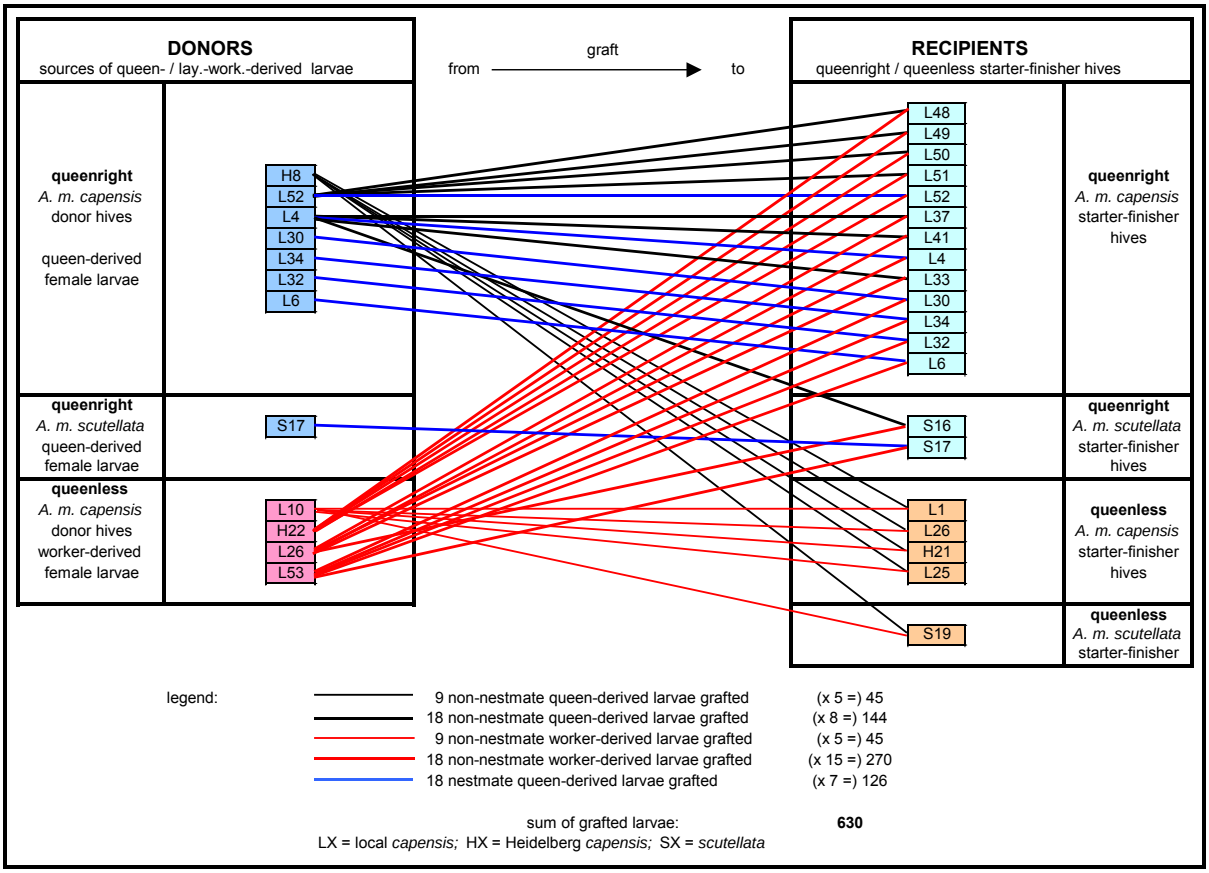
column 13: (date p=preserved or c=conserved) preserved = p: life adult insect dissected, head + tergites preserved in dichloromethane, thorax conserved in ethanol, photographs of imago and ovaries; c = conserved = dead adult, pupa or larvae conserved in ethanol

column 14: (age days) age in days after eclosing

column 15: (photo nr.) key number for photograph file detection

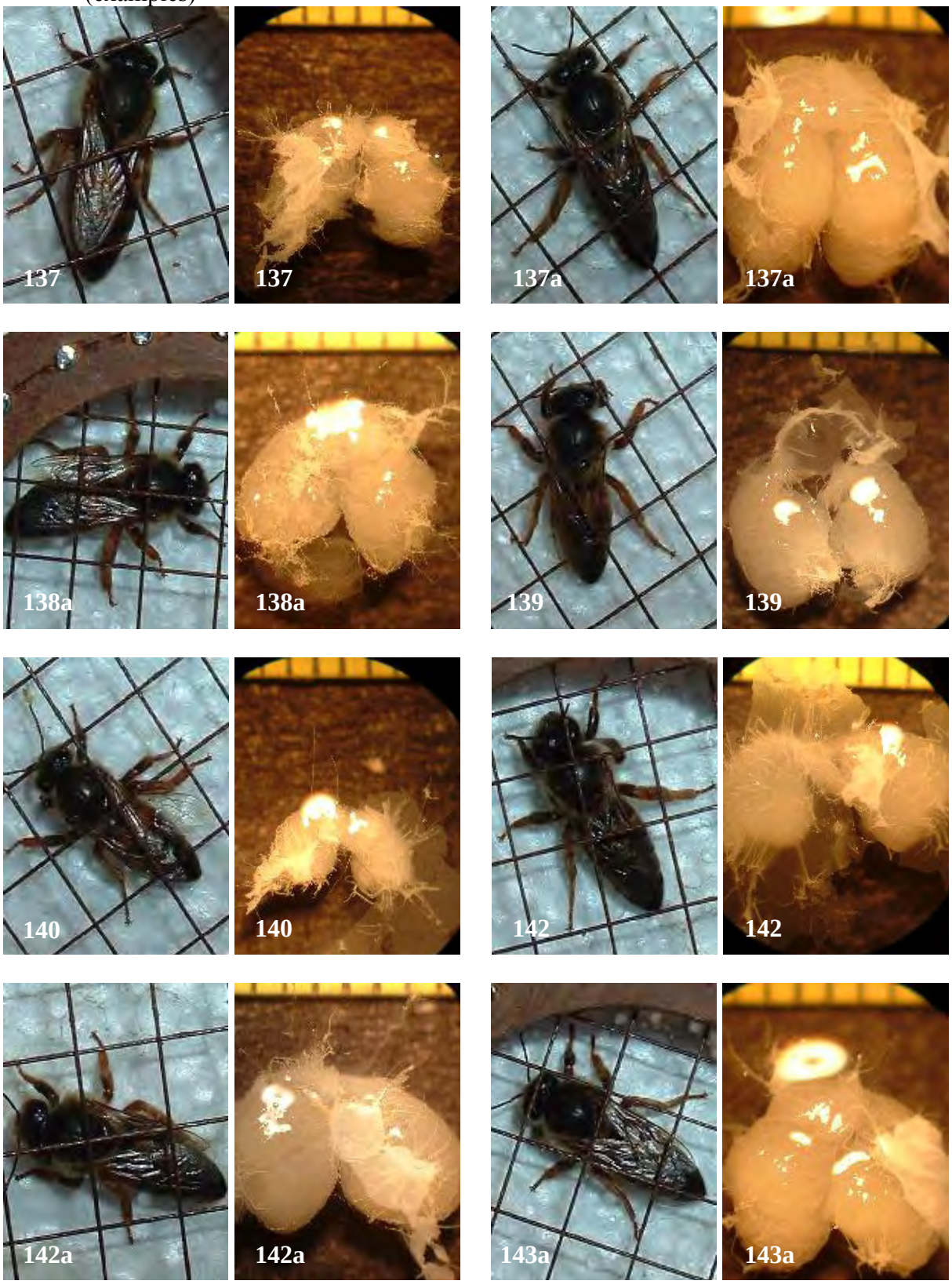
column 16: (comments notes) comments and notes

Appendix 2.2. Overview grafting scheme: donor colonies (for larvae) and recipient (breeding colonies) for queen-rearing*

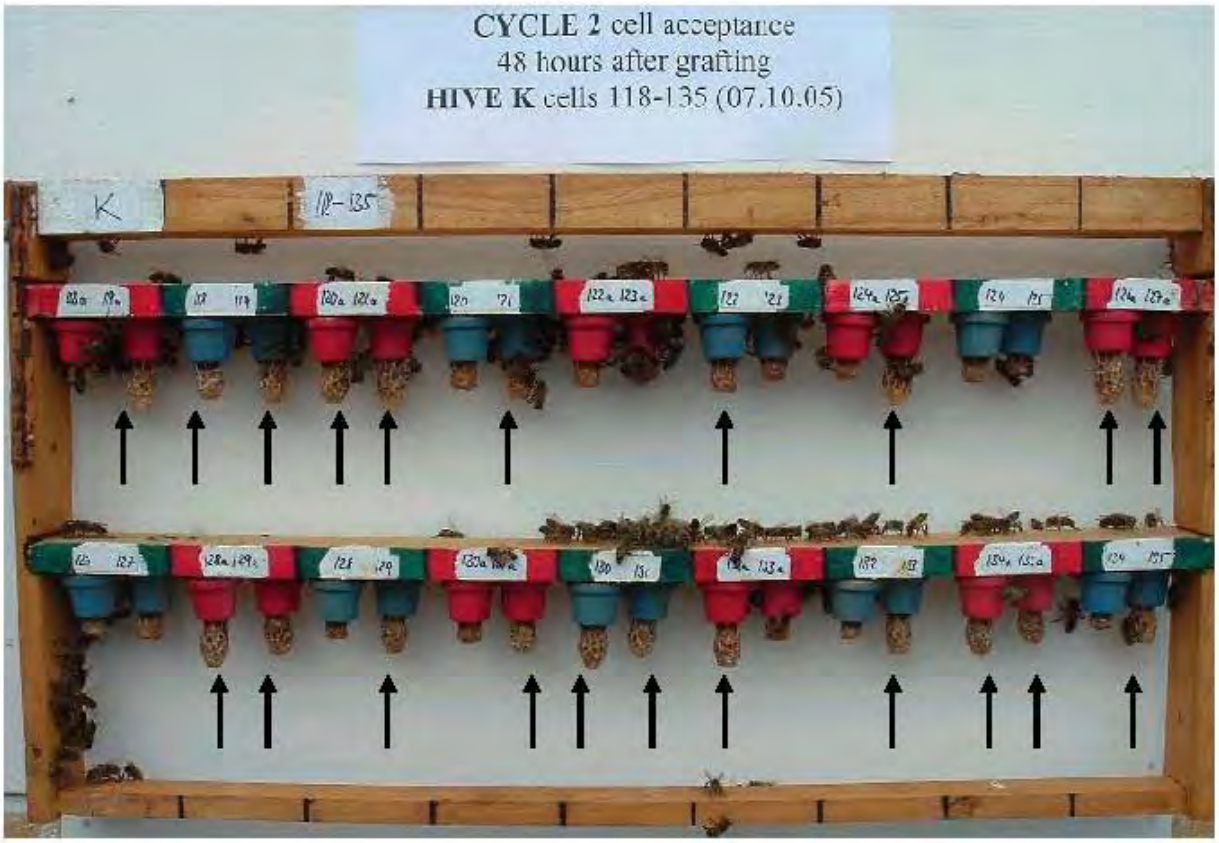


* Compare Appendix 2, for origin and development of donor and recipient colonies

Appendix 2.3. Reared *A. m. capensis* virgin queens, adults and ovaries (photos of adults not to scale, measuring square ~ 5 x 5 mm^2; photos of ovaries not to scale, scale unit = 1mm) (examples)



Appendix 2.4. *A. m. capensis* queen-rearing: acceptance 48 h after grafting (arrow = accepted) (examples)



Appendix 2.5. *A. m. capensis* queen-rearing: attractiveness 8/9 d after grafting (examples)

Appendix 2.6. *A. m. capensis* queen-rearing: attractiveness 1/2 d after eclosion (examples)

Appendix 2.7. *A. m. capensis* queen-rearing: attractiveness 7/8 d after eclosion (examples)

APPENDIX 3 (Development of experimental colonies)

Appendix 3.1. Development of individual colonies and their fates during experiments

nr.	specification	24.08.05	18.09.05	08.10.05	28.10.05	22.11.05	20.12.05	10.01.06	
		A ex-winter	B spring 1	C spring 2	D spring 3	E summer 1	F summer 2	G summer 3	notes and comments
L 01	Graham. *capensis*	2 C	1 **B**	2 **B IV**	3 **IV**	3-4 **IV** C	4-5 **IV** C	4-5	queen L 01 conserved, unsatisfactory lay. work. brood
L 02	Graham. *capensis*	3 C	3	4	burned away				
L 03	Graham. *capensis*	4 C	4	4-5	4-5	4-5	4	3	bee donor for exp. 2 (15.11.02)
L 04	Graham. *capensis*	2 T	2	2	2	1-2 **N**	split21split22		queen L 04 conserved
L 05	Graham. *capensis*	3 T	3	3-4	4	4-5	3	3	bee donor for exp. 2 (15.11.02)
L 06	Graham. *capensis*	2 T	2	2	2	1-2 **T**	1-2 **T**	split36split37	queen L 06 conserved
H 07	Heidelb.*capensis*	4 T	absconded						
H 08	Heidelb.*capensis*	3 T	3	2-3	3	3-4	3 bank3 C	3	fertilized larvae used for grafting in breeding cycle 1, queen H8 returned to hive
H 09	Heidelb.*capensis*	4 T	4-5	absconded					queen H 09 conserved
L 10	Graham. *capensis*	1-2 T	2-3 **II**	3-4 **II**	4-5 **II**	4-5 **II**	4-5 **II** G	4	queen L10 preserved, clonal larvae used for grafting in breeding cycle 1
L 11	Graham. *capensis*	4 T	4-5	4-5	4-5	4-5	3	3	bee donor for exp. 2 (15.11.02)
H 12	Heidelb.*capensis*	3 T	absconded						
H 13	Heidelb.*capensis*	3 T	3	3	3	3 **VII**	4-5 **VII** C	4-5	queen H13 preserved, unsatisfactory lay. work. brood
L 14	Graham. *capensis*	1 T	2	2	2	1-2	1	1	bee donor for exp. 2 (24./27.11; 09./25.12.) and queen cages (twice) and split 29
H 15	Heidelb.*capensis*	2 T	2	3	3	3	1-2 bank2 C	split40split 41	emerging brood for pseudoqueen prod. Set-up 1 and 4, queen H 15 conserved
S 16	Pret. *scutellata*	2-3 T	1-2	2	2	1-2 **O**	split23split24		queen S 16 conserved
S 17	Pret. *scutellata*	2 T	1-2	2	2	1-2 **U**	1-2 **U** F	split38split39	queen S 17 preserved
S 18	Pret. *scutellata*	2-3 T	2-3	absconded					queen S 18 preserved
S 19	Pret. *scutellata*	1-2 T	1 **A**	2 **A**	split1 split2				queen S 19 preserved
H 20	Heidelb.*capensis*	2 T	1-2	2-3	3	split16	varroa!!!		queen H 20 preserved, emerging brood for pseudoqueen prod. Set-up 3
H 21	Heidelb.*capensis*	1 T	1 **D**	2 **D**	split3				queen H 21 preservrd
H 22	Heidelb.*capensis*	2 T	1 **I**	3 **I**	3 **I**	4 **I**	4 **I**	absconded	queen H 22 conserved; clonal larvae used for grafting in breeding cycle 2
L 23	Graham. *capensis*	3 T	absconded						
L 24	Graham. *capensis*	2 T	3	3-4	3-4	3	1	1	bee donor for exp. 2 (17./18.11; 09.12.) and split 29
L 25	Graham. *capensis*	1 T	1 **E**	2 **E**	split4 split5				
L 26	Graham. *capensis*	1 T	1 **C**	2 **C**	3 **(C) V** C	3-4 **V** C	4 **V** C	4	queen L 26 preserved; clonal larvae used for grafting in breeding cycle 3
L 27	Graham. *capensis*	3 N	vandalized						
L 28	Graham. *capensis*	4 N	vandalized						
L 29	Graham. *capensis*	3 N	2	absconded					
L 30	Graham. *capensis*	2-3 N	2	3	2	1-2 **Q**	1-2 **Q** F	split30split31	queen L30 preserved
L 31	Graham. *capensis*	4 N	4	4	burned away				
L 32	Graham. *capensis*	3 N	2	2	2	1-2 **S**	1-2 **S**	split34split35	queen L 32 preserved
L 33	Graham. *capensis*	1-2 N	1-2	1	2	1-2 **P**	split25split26		queen L 33 preserved
L 34	Graham. *capensis*	3 N	2-3	2-3	2	1-2 **R**	1-2 **R** F	split32split33	queen L 34 preserved
L 35	Graham. *capensis*	3 N	2	2-3	2	2	2bank1split28		queen L 35 conserved
L 36	Graham. *capensis*	3 N	3	4	4	4	1	1	bee donor for exp. 2 (15.11.02)
L 37	Graham. *capensis*	3 N	2	2	2	1-2 **L**	split17split18		queen L 37 preserved
L 38	Graham. *capensis*	3 N	2	absconded					
L 39	Graham. *capensis*	2-3 N	3	absconded					
L 40	Graham. *capensis*	3-4 N	4	absconded					
L 41	Graham. *capensis*	2-3 N	1-2	2	2	1-2 **M**	split19split20		queen L41 conserved
L 42	Graham. *capensis*	4 N	3-4	absconded					
L 43	Graham. *capensis*			4 swarm in G	absconded				
S 44	Pret. *scutellata*			2	2-3	2	1	1	bee donor for pseudoqueen prod. Set-up 2 and 4; honey producer
S 45	Pret. *scutellata*			2	2-3	2	1	1	bee donor for pseudoqueen prod. Set-up 3 and 4; honey producer
S 46	Pret. *scutellata*			2	2-3	2	1	1	bee donor for pseudoqueen prod. Set-up 1 and 4; honey producer
L 47	Graham. *capensis*			1 **III**	absconded				split made from 3 combs of capped brood and young bees from L58
L 48	Graham. *capensis*				1-2 **F**	split6 split7			queen L 48 conserved
L 49	Graham. *capensis*				1-2 **G**	sp;it8 split9			queen L 49 conserved
L 50	Graham. *capensis*				1 **H**	split10 split11			queen L 50 preserved
L 51	Graham. *capensis*				1-2 **I**	split12 split13			queen L 51 preserved
L 52	Graham. *capensis*				1-2 **K**	split14 split15			queen L 52 conserved
L 53	Graham. *capensis*				2 **VI**	3 **VI**	1	1	queen L 53 preserved; clonal larvae used for grafting in breeding cycle 4
L 54	Graham. *capensis*					(1)3(swarm.)K	2	2	bee donor for queen cages
L 55	Graham. *capensis*					1 swarm in	1	1	honey producer
L 56	Graham. *capensis*						1	1	bee donor for exp.2 (05.12.) and split 27: honey producer
L 57	Graham. *capensis*						1	1	bee donor for exp.2 (05.12.) and split 27: honey producer
L58	Graham. *capensis*						1	1	comb/bee donor for L47 (III).; honey producer

Legend:

A/B/C/D/…..U :	20 starter-finisher colonies, finally converted into splits
I / II / III / IV / V / VI / VII :	7 laying worker colonies, continually maintained in queenless condition
bank1 / bank2 / bank3 :	3 queenbanks, maintained in queenless condition during storage of unmated queens, finally converted into splits
split 1 - split 41 :	small mating units initially containing about 3-4 combs with bees and 1 or 2 unmated queen(s) reared from clonal or fertilised brood
1; 1-2; 2; 2-3; 3; 3-4; 4; 4-5:	hive strength: 1 = using 10 combs; 2 = using 8 combs; 3 = using 6 combs; 4 = using 4 combs; (1-2; 2-3; 3-4; 4-5 = intermediates)
T; N; F; C; G; M; K :	apiary sites: T = Travor's farm; N = Nick's farm; F = Fisheries Department; C = Circle apiary; G = Garden; M = Maintanance
queen conserved :	dead imago or pupa conserved in ethanol
queen preserved :	imago life dissected: head and tergites preserved in dichloromethane, thorax conserved in ethanol, photographs of imago and ovaries
	colonies of *Apis mellifera capensis* from the centre of distribution (Heidelberg) without hybridisation with *Apis mellifera scutellata*
	colonies of *Apis mellifera capensis* from the transition zone (Grahamstown), with potential hybridisation with *Apis mellifera scutellata*
	colonies of *Apis mellifera scutellata* from Pretoria

APPENDIX 4 (Development of mating colonies)

Appendix 4.1. Generation of mating colonies (splits + virgin queens)

nr.	source hive	date of installation	queen from fertilised brood	queen from clonal brood	initial strength	mating apiary	main-tenance apiary	first revision
Split 01	A (S19)	24.10.05	queen 2 (5)	X	4	R	M	18.11.05
Split 02	A (S19)	24.10.05	queen 8 (27)	X	4	R	M	18.11.05
Split 03	D (H21)	24.10.05	queen 30 (10)	X	4	R	M	18.11.05
Split 04	E (L25)	24.10.05	queen 4 (38)	X	4	R	M	18.11.05
Split 05	E (L25)	24.10.05	queen 40 (33)	X	4	R	M	18.11.06
Split 06	F (L48)	12.11.05	queen 58 (13)	X	4	R	M	02.12.05
Split 07	F (L48)	12.11.05	X	queen 48a (16)	4	R	M	02.12.05
Split 08	G (L49)	12.11.05	queen 66 (86)	X	4	R	M	02.12.05
Split 09	G (L49)	12.11.05	X	queen 68a (90)	4	R	M	02.12.05
Split 10	H (L50)	12.11.05	queen 92 (97)	X	4	R	M	02.12.05
Split 11	H (L50)	12.11.05	X	queen 98a (12)	4	R	M	02.12.05
Split 12	I (L51)	12.11.05	queen114 (55)	X	4	R	M	02.12.05
Split 13	I (L51)	12.11.05	X	queen 105a (97)	4	R	M	02.12.05
Split 14	K (l52)	12.11.05	queen 130 (44)	X	4	R	M	02.12.05
Split 15	K (l52)	12.11.05	X	queen 126a (13)	4	R	M	02.12.05
Split 16	H20	18.11.05	X	queen 121a (72)	4	C	M	07.12.05
Split 17	L (L37)	03.12.05	queen 141 (70)	queen136a (45)	4	R	M	22.12.05
Split 18	L (L37)	03.12.05	queen 150 (82)	queen 140a (94)	4	R	M	22.12.05
Split 19	M (L41)	03.12.05	queen 154 (56)	queen 155a (20)	4	R	M	22.12.05
Split 20	M (L41)	03.12.05	queen 157 (27)	queen 154a (60)	4	R	M	22.12.05
Split 21	N (L4)	03.12.05	queen 172 (26)	queen 186a (83)	4	R	M	22.12.05
Split 22	N (L4)	03.12.05	queen 188 (52)	queen 183a (55)	4	R	M	22.12.05
Split 23	O (S16)	03.12.05	queen 206 (12)	queen 203a (67)	4	R	M	22.12.05
Split 24	O (S16)	03.12.05	queen 192 (51)	queen 202a (33)	4	R	M	22.12.05
Split 25	P (L33)	03.12.05	queen 210 (39)	queen 221a (73)	4	R	M	22.12.05
Split 26	P (L33)	03.12.05	queen 208 (78)	queen 217a (6)	4	R	M	22.12.05
Split 27	L56/L57	08.12.05	X	queen 208a (93)	4	M	M	23.12.05
Split 28	bank 1 (L35)	10.12.05	X	queen 221a (73)	4	C	M	23.12.05
Split 29	L14/L24	11.12.05	X	queen197a (19)	4	M	M	23.12.05
Split 30	Q (L30)	25.12.05	X	queen 229a (8)	4	R	M	16.01.06
Split 31	Q (L30)	25.12.05	queen 240 (34)	X	4	R	M	16.01.06
Split 32	R (L34)	25.12.05	X	queen 252a (3)	4	R	M	16.01.06
Split 33	R (L34)	25.12.05	queen 249 (2)	X	4	R	M	16.01.06
Split 34	S (L32)	25.12.05	X	queen 274a (19)	4	R	M	16.01.06
Split 35	S (L32)	25.12.05	queen 268 (35)	X	4	R	M	16.01.06
Split 36	T (L6)	25.12.05	X	queen 292a (31)	4	R	M	16.01.06
Split 37	T (L6)	25.12.05	queen 281 (21)	X	4	R	M	16.01.06
Split 38	U (S17)	25.12.05	X	queen 313a (15)	4	R	M	16.01.06
Split 39	U (S17)	25.12.05	queen 305 (49)	X	4	R	M	16.01.06
Split 40	bank 3 (H8)	29.12.05	X	queen 232a (18)	4	R	M	16.01.06
Split 41	bank 3 (H8)	29.12.05	queen 280 (11)	X	4	R	M	16.01.06

Legend:

column 1: (split nr.) 1-41 = splits were consecutively numbered
column 2: (source hive) compare appendix 2
column 3: (date of installation) date of generation of the mating colony
columns 4+5: (queen from fertilised/clonal brood) compare appendix 1
column 6: (initial strength) 4 = four combs occupied by workers + one comb of food
column 7: (mating apiary) apiary sites: T = Trevor's farm; N = Nick's farm; F = Fisheries Department; C = Circle apiary; G = Garden; M = Maintenance; R = Mating apiary
column 8: (date of first revision) date of first revision

Appendix 4.2. Mating colonies (splits + virgin queens) long term development

pathway	split nr.	initial queens	initial queen accepted and mated first revision	fate of colony revisions as required	situation second revision (after 360 d)	number of adult workers	worker brood capped (cells)	worker brood open (cells)	total worker offspring *
1	5	queen-derived	yes	no changes	initial queen	7789	3160	0	**10949**
	11	work.-derived	yes	no changes	initial queen	19632	8960	160	**28752**
	29	work.-derived	yes	no changes	initial queen	14778	6280	585	**21643**
	30	work.-derived	yes	no changes	initial queen	1120	3300	1040	**5460**
2	3	queen-derived	yes	absconded February 2006	X	X	X	X	
	8	queen-derived	yes	absconded February 2006	X	X	X	X	
	9	work.-derived	yes	absconded January 2006	X	X	X	X	
	10	queen-derived	yes	absconded August 2006	X	X	X	X	
	15	work.-derived	yes	absconded June 2006	X	X	X	X	
	16	work.-derived	yes	absconded April 2006	X	X	X	X	
	17	w.-and q.-der.	w.-der. yes	absconded January 2006	X	X	X	X	
	27	work.-derived	yes	absconded January 2006	X	X	X	X	
	40	work.-derived	yes	absconded August 2006	X	X	X	X	
3	4	queen-derived	yes	initial queen superseded	new queen unsigned	19566	10613	4498	**34677**
	12	queen-derived	yes	initial queen superseded	new queen unsigned	4573	5400	675	**10648**
	14	queen-derived	yes	initial queen superseded	new queen unsigned	6410	4678	2587	**13675**
	18	w.-and q.-der.	q.-der. yes	swarmed September 2006	new queen unsigned	8264	805	0	**9069**
	34	work.-derived	yes	initial queen superseded	new queen unsigned	8608	6380	910	**15898**
	36	work.-derived	yes	queen lost (September 2006)	new queen unsigned	3949	195	0	**4144**
4	23	w.-and q.-der.	no	no changes	queenless	740	975	225	**1940**
	6	queen-derived	no	absconded	X	X	X	X	
	7	work.-derived	no	absconded January 2006	X	X	X	X	
	25	w.-and q.-der.	no	absconded May 2006	X	X	X	X	
	31	queen-derived	no	absconded August 2006	X	X	X	X	
	32	work.-derived	no	absconded August 2006	X	X	X	X	
	22	w.-and q.-der.	no	dwindled dead	X	X	X	X	
	24	w.-and q.-der.	no	dwindled dead	X	X	X	X	
5	2	queen-derived	no	eventually self-requeened	new queen unsigned	8181	5095	500	**13776**
	13	work.-derived	no	eventually self-requeened	new queen unsigned	20126	6990	1770	**28886**
	20	w.-and q.-der.	no	eventually self-requeened	new queen unsigned	9091	3945	1400	**14436**
	21	w.-and q.-der.	no	eventually self-requeened	new queen unsigned	12250	5360	4045	**21655**
	26	w.-and q.-der.	no	eventually self-requeened	new queen unsigned	4987	4770	615	**10372**
6	1	queen-derived	no	new queen white superseded	new queen unsigned	26233	3910	100	**30243**
	35	queen-derived	no	new queen white superseded	new queen unsigned	14878	7880	570	**23328**
	33	queen-derived	no	queen lost (September 2006)	new queen unsigned	2554	2495	50	**5099**
7	19	w.-and q.-der.	no	absconded September 2006	X	X	X	X	
	37	queen-derived	no	absconded September 2006	X	X	X	X	
	38	work.-derived	no	absconded January 2006	X	X	X	X	
	39	queen-derived	no	absconded February 2006	X	X	X	X	
	28	work.-derived	no	dwindled dead	X	X	X	X	

*sum of the number of adult workers and cells containing worker brood

Legend	**to appendix 3.2.:**
path-way 1-7	short description of pathway
1	initial queen accepted and maintained
2	initial queen accepted later absconded
3	initial queen accepted, later superseded
4	initial queen rejected, laying worker colony
5	initial queen rejected, laying worker colony, later requeened
6	initial queen rejected, requeened and superseded
7	initial queen rejected, requeened and absconded
column 2:	(split nr.) 1-40 = splits were consecutively numbered
column 3:	(initial queen) work.- / queen derived = reared from clonal / fertilised brood
column 4:	(initial queen accepted / mated yes / no) yes = accepted and mated; no = rejected
columns 5-10:	self explanatory

APPENDIX 5 (Production of differentiated (laying) workers)

Appendix 5.1. Production of *A. m. capensis* laying workers / pseudoqueens

lay. work. name	batch nr.	set up date	mother hive	tending workers from hive	rating a)	rating b)	rating c)	rating halo only	total rating	ovary develop. 1/2/3	ovariole nr.	pollen stage 2/1/0	date of preservation / conservation	age (d)	comments / notes
a	1	11.10.05	L26	S46	X	X	X	0	11	1	5 / ?	0	p 27.10.05	17	X
b	1	11.10.05	L26	S46	X	X	X	2	12	2	8 / 7	1	p 28.10.05	18	X
c	1	11.10.05	L26	S46	X	X	X	0	7	1	no data	2	p 25.10.05	14	X
d	1	11.10.05	L26	S46	X	X	X	0	3	2	4 / ?	2	p 25.10.05	14	X
e	1	11.10.05	L26	S46	X	X	X	0	5	1	no data	1	p 25.10.05	14	X
f	1	11.10.05	L26	S46	X	X	X	X	X	lost					X
g	1	11.10.05	L26	S46	X	X	X	0	5	1	3 / 3	2	p 27.10.05	17	X
h	1	11.10.05	L26	S46	X	X	X	0	X	lost					X
I	1	11.10.05	L26	S46	X	X	X	0	8	1	3 / 3	2	p 27.10.05	17	X
k	1	11.10.05	L26	S46	X	X	X	0	8	1	3 / 3	0	p 27.10.05	17	X
l	2	01.11.05	H15	S44	4	3	5	1	12	3	12/12	0	p 15.11.05	15	eggs detected
m	2	01.11.05	H15	S44	2	5	4	2	11	2	8/7	1	p 17.11.05	17	X
n	2	01.11.05	H15	S44	X	X	X	X	X	lost					X
o	2	01.11.05	H15	S44	4	4	4	2	12	3	11/9	0	p 15.11.05	15	eggs detected
p	2	01.11.05	H15	S44	0	1	4	1	5	3	8/9	1	p 17.11.05	17	eggs detected
q	2	01.11.05	H15	S44	2	3	4	1	9	3	16/16	0	p 18.11.05	18	eggs detected
r	2	01.11.05	H15	S44	0	0	4	1	4	3	8/8	2	p 18.11.05	18	eggs detected
s	2	01.11.05	H15	S44	2	4	4	2	10	3	11/10	0	p 15.11.05	15	eggs detected
t	2	01.11.05	H15	S44	1	3	2	0	6	3	10/10	0	p 18.11.05	18	eggs detected
u	2	01.11.05	H15	S44	0	2	1	0	3	lost					X
v	2	01.11.05	H15	S44	3	1	4	1	8	3	12/11	0	p 18.11.05	18	eggs detected
w	2	01.11.05	H15	S44	2	5	4	2	11	3	12/12	0	p 15.11.05	15	eggs detected
x	2	01.11.05	H15	S44	0	4	3	2	7	3	11/8	1	p 18.11.05	15	eggs detected
y	2	01.11.05	H15	S44	1	4	3	2	8	lost					X
z	2	01.11.05	H15	S44	1	3	4	1	8	2	9/7	2	p 17.11.05	17	X
a'	2	01.11.05	H15	S44	1	3	2	0	6	3	11/10	0	p 19.11.05	19	eggs detected
b'	2	01.11.05	H15	S44	3	3	4	1	10	1	11/11	0	p 17.11.05	17	X
c'	2	01.11.05	H15	S44	3	3	3	0	9	lost					X
d'	2	01.11.05	H15	S44	1	2	2	0	5	3	4/?	1	p 19.11.05	19	eggs detected
e'	2	01.11.05	H15	S44	0	3	3	0	6	1	9/10	2	p 17.11.05	17	X
f'	2	01.11.05	H15	S44	5	5	4	3	14	lost					X
g'	2	01.11.05	H15	S44	0	2	1	0	3	lost					X
h'	2	01.11.05	H15	S44	0	2	1	0	3	2	17/17	1	p 17.11.05	17	X
l'pink a)	3	22.11.05	H8	S45	5	5	5	3	15	3	15/15	0	p 08.12.05	17	eggs detected
l'pink b)	3	22.11.05	H8	S45	5	5	5	3	15	3	8/4	0	p 08.12.05	17	eggs detected
k'white	3	22.11.05	H20	S45	4	4	2	0	10	3	13/13	0	p 08.12.05	17	eggs detected
k'pink	3	22.11.05	H8	S45	4	4	2	0	10	lost					X
l'white	3	22.11.05	H20	S45	5	5	1	2	11	3	10/10	1	p 08.12.05	17	eggs detected
l'pink	3	22.11.05	H8	S45	5	5	1	2	11	lost					X
m'white	3	22.11.05	H20	S45	5	4	5	3	14	lost					X
m'pink	3	22.11.05	H8	S45	5	4	5	3	14	3	5/7	0	p 10.12.05	19	eggs detected
n'white	3	22.11.05	H20	S45	5	2	5	2	12	3	3/3	1	p 08.12.05	17	X
n'pink	3	22.11.05	H8	S45	5	2	5	2	12	2	4/?	2	p 08.12.05	17	X
o'white	3	22.11.05	H20	S45	5	5	5	3	15	3	11/10	0	p 10.12.05	19	eggs detected
o'pink	3	22.11.05	H8	S45	5	5	5	3	15	3	9/7	0	p 10.12.05	19	eggs detected
p'white	3	22.11.05	H20	S45	5	3	5	2	13	removed					X
p'pink	3	22.11.05	H8	S45	5	3	5	2	13	removed					X
q'white	3	22.11.05	H20	S45	1	1	4	0	6	lost					X
q'pink	3	22.11.05	H8	S45	1	1	4	0	6	3	7/7	0	p 08.12.05	17	eggs detected
r'white	3	22.11.05	H20	S45	5	5	5	3	15	2	3/3	0	p 07.12.05	16	X
r'pink	3	22.11.05	H8	S45	5	5	5	3	15	3	13/13	0	p 07.12.05	16	eggs detected
s'white	3	22.11.05	H20	S45	5	5	5	3	15	2	9/9	0	p 07.12.05	16	X
s'pink	3	22.11.05	H8	S45	5	5	5	3	15	1	9/8	2	p 07.12.05	16	X
t'white	3	22.11.05	H20	S45	5	4	5	2	14	3	11/11	0	p 10.12.05	19	eggs detected
t'pink	3	22.11.05	H8	S45	5	4	5	2	14	lost					X
u'white	3	22.11.05	H20	S45	5	1	0	1	6	removed					X

lay. work. name	batch nr.	set up date	mother hive	tending workers from hive	rating a)	rating b)	rating c)	rating halo only	total rating	ovary develop. 1/2/3	ovariole nr.	pollen stage 2/1/0	date of preservation / conservation	age (d)	comments / notes
u'pink	3	22.11.05	H8	S45	5	1	0	1	6	removed					X
v'white	3	22.11.05	H20	S45	5	1	0	1	6	lost					X
v'pink	3	22.11.05	H8	S45	2	4	3	0	9	3	6/3	1	p 09.12.05	18	X
w'white	3	22.11.05	H20	S45	5	5	5	3	15	2	12/10	1	p 05.12.05	14	X
w'pink	3	22.11.05	H8	S45	5	5	5	3	15	3	14/13	1	p 05.12.05	14	eggs detected
x'white	3	22.11.05	H20	S45	5	5	5	3	15	3	11/11	0	p 06.12.05	15	eggs detected
x'pink	3	22.11.05	H8	S45	5	5	5	3	15	1	6/6	2	p 06.12.05	15	X
y'white	3	22.11.05	H20	S45	1	4	5	1	10	2	4/4	1	p 09.12.05	18	X
y'pink	3	22.11.05	H8	S45	1	4	5	1	10	lost					X
z'white	3	22.11.05	H20	S45	4	1	0	1	5	removed					X
z'pink	3	22.11.05	H8	S45	4	1	0	1	5	removed					X
a''white	3	22.11.05	H20	S45	5	5	5	3	15	escaped					X
a''pink	3	22.11.05	H8	S45	5	5	5	3	15	3	16/16	0	p 06.12.05	15	eggs detected
b''white	3	22.11.05	H20	S45	4	5	5	3	14	2	5/5	2	p 10.12.05	19	X
b''pink	3	22.11.05	H8	S45	4	5	5	3	14	3	9/11	0	p 10.12.05	19	eggs detected
c''white	3	22.11.05	H20	S45	5	4	5	3	14	lost					X
c''pink	3	22.11.05	H8	S45	5	4	5	3	14	3	18/18	0	p 09.12.05	18	eggs detected
d''white	3	22.11.05	H20	S45	4	2	0	1	6	3	5/5	0	p 10.12.05	19	eggs detected
d''pink	3	22.11.05	H8	S45	4	2	0	1	6	lost					X
e''white	3	22.11.05	H20	S45	2	1	0	0	3	removed					X
e''pink	3	22.11.05	H8	S45	2	1	0	0	3	removed					X
f''white	3	22.11.05	H20	S45	5	5	3	2	13	3	10/?	1	p 05.12.05	14	eggs detected
f''pink	3	22.11.05	H8	S45	5	5	3	2	13	1	9/?	2	p 05.12.05	14	X
g''	4	15.12.05	H15	S44	4	5	2	1	11	1	13/13	2	p 26.12.05	12	eggs detected
h''	4	15.12.05	H15	S44	5	5	5	3	15	1	7/7	0	p 26.12.05	12	eggs detected
l''	4	15.12.05	H15	S44	2	1	2	0	5	removed					X
k''	4	15.12.05	H15	S44	2	2	1	0	5	lost					presence >1 *cap*
l''	4	15.12.05	H15	S44	5	5	5	3	15	removed					X
m''	4	15.12.05	H15	S44	4	1	4	1	9	1	13/12	2	p 26.12.05	12	eggs detected
n''	4	15.12.05	H15	S44	2	5	5	2	12	lost					presence >1 *cap*
o''	4	15.12.05	H15	S44	1	2	1	0	4	lost					presence >1 *cape*
p''	4	15.12.05	H15	S44	2	4	3	0	9	1	14/14	2	p 26.12.05	12	eggs detected
q''	4	15.12.05	H15	S44	3	1	3	0	7	1	11/11	2	p 27.12.05	13	eggs detected
r''	4	15.12.05	H15	S44	4	5	3	2	12	3	10/9	0	p 27.12.05	13	eggs detected
s''	4	15.12.05	H15	S44	4	2	3	1	9	1	12/12	1	p 27.12.05	13	eggs detected
t''	4	15.12.05	H15	S44	X	X	X	X	X	lost					presence >1 *cap*
u''	4	15.12.05	H15	S44	4	5	2	2	11	1	12/11	2	p 27.12.05	13	eggs detected
v''	4	15.12.05	H15	S44	X	X	X	X	X	lost					presence >1 *cap*
w''	4	15.12.05	H15	S44	1	0	0	0	1	lost					presence >1 *cap*
x''	4	15.12.05	H15	S44	3	4	0	0	7	removed					X
y''	4	15.12.05	H15	S44	X	X	X	X	X	lost					presence >1 *cap*
z''	4	15.12.05	H15	S44	0	0	4	0	4	1	11/11	2	p 27.12.05	13	eggs detected
a'''	4	15.12.05	H15	S44	1	1	0	0	2	lost					presence >1 *cap*
b'''	4	15.12.05	H15	S44	0	0	0	0	0	lost					presence >1 *cap*
c'''	4	15.12.05	H15	S44	3	0	5	1	8	lost					presence >1 cap
d'''	4	15.12.05	H15	S44	1	5	4	2	10	3	15/15	1	p 27.12.05	13	eggs detected
e'''	5	12.01.06	L55	L57	1	2	2	0	5	lost					control, eggs+
f'''	5	12.01.06	L55	L57	1	1	2	0	4	lost					control, eggs+
g'''	5	12.01.06	L55	L57	2	2	2	0	6	lost					control, eggs+
h'''	5	12.01.06	L55	L57	1	2	2	0	5	lost					control, eggs+
l'''	5	12.01.06	L55	L57	1	2	2	0	5	lost					control, eggs+
k'''	5	12.01.06	L55	L57	1	1	2	0	4	lost					control, eggs+
l'''	5	12.01.06	L55	L57	2	2	2	0	6	lost					control, eggs+
m'''	5	12.01.06	L55	L57	1	2	2	0	5	lost					control, eggs+
n'''	5	12.01.06	L55	L57	2	2	2	0	6	lost					control, eggs+
o'''	5	12.01.06	L55	L57	4	2	4	0	10	1	3 / 3	2	p 26.01.06	14	control, eggs +
p'''	5	12.01.06	L55	L57	1	2	2	0	5	lost					control, eggs+
q'''	6	12.01.06	S45	S44	0	0	0	0	0	lost					control

lay. work. name	batch nr.	set up date	mother hive	tending workers from hive	rating a)	rating b)	rating c)	rating halo only	total rating	ovary develop. 1/2/3	ovariole nr.	pollen stage 2/1/0	date of preservation / conservation	age (d)	comments / notes
r'''	6	12.01.06	S45	S44	1	2	2	0	5	1	2 / 2	1	p 26.01.06	14	control
s'''	6	12.01.06	S45	S44	2	1	4	0	7	1	3 / 3	2	p 26.01.06	14	control
t'''	6	12.01.06	S45	S44	0	0	2	0	2	1	2 / 2	1	p 26.01.06	14	control
u'''	6	12.01.06	S45	S44	2	2	2	0	6	lost					control,
v'''	6	12.01.06	S45	S44	2	1	1	0	4	1	3 / 3	2	p 26.01.06	14	control
w'''	6	12.01.06	S45	S44	3	4	2	0	9	1	2 / 2	1	p 26.01.06	14	control
x'''	6	12.01.06	S45	S44	2	1	2	0	5	1	2 / 2	1	p 26.01.06	14	control
y'''	6	12.01.06	S45	S44	1	3	2	0	6	1	2 / 2	2	p 26.01.06	14	control
z'''	6	12.01.06	S45	S44	0	0	1	0	1	lost					control
a''''	6	12.01.06	S45	S44	0	2	4	0	6	1	2 / 2	1	p 26.01.06	14	control
b''''	6	12.01.06	S45	S44	0	0	2	0	2	lost					control

Legend:

column 1: (laying worker 'name') alphabetical order, with ', " , '" denoting new start of alphabet

column 2: (batch nr.) 1-6 = number of experimental set-ups

column 3: (set-up date) = date of experimental set-up

column 4: (mother hive) compare appendix 1

column 5: (tending workers from hive) compare appendix 1

columns 6-8: 0-5 = sum of rating: signed worker detectable on comb / unit calm, most workers on comb / comb clean, worked on and some food in cells / signed worker "queen-like cruising" on comb / signed worker with distinct court / for each: 1 pt

column 9: (rating halo only) 0-3 = sum of 'halo' rating column 6-8: (signed worker with clear 'halo')

column 10: (total rating) 1-15 = sum of columns 6-7

column 11: (ovary size/ development 1/2/3) 1 = undeveloped; 2 = slightly developed compartmentalised; 3 = fully developed with ripe eggs

column 12: (ovariole nr) # / # = number of ovarioles of left and right ovaries

column 13: (pollen stage) 0-2 = 0 = no pollen; 1 = some pollen; 2 = massive pollen (undigested pollen residues) in rectum

column 14: (date of preservation / conservation) p/c date : p = life adult insect dissected, head + tergites preserved in dichloromethane, thorax conserved in ethanol photographs of imago and ovaries; c = conserved = dead adult, pupa or larvae conserved in ethanol

column 15: (date (d)) # = age in days after eclosion

column 16: (comments notes) = comments and notes

Appendix 5.2. Produced *A. m. capensis* differentiated workers, adults and ovaries (photos of adults not to scale; photos of ovaries not to scale: scale unit = 1mm) (examples)

Appendix 6.1. Produced *A. m. capensis* queens: gas-chromatographical analysis of mandibular gland components

queen nr.	cl./ fe.	mother hive	cell accept. yes/no	matured queen attractive-ness 1/2/3	ovary size/ developm. 1/2/3	mated yes/no	age days	HOB micro gram	9-ODA micro gram	9 H D A micro gram	10 HDAA micro gram	10-H D A micro gram	9-ODA / (9-ODA + 10-H DA) ratio
1		H8	Y	no data	1	N	11	0.45	19.98	0.83	0.07	0.41	0.980
6		H8	Y	no data	2	N	9	3.10	194.40	10.41	1.72	0.33	0.998
7		H8	Y	no data	2	N	9	0.35	16.98	2.55	0.08	0.77	0.957
29		H8	Y	no data	?	N	?	4.58	242.22	1.18	12.55	0.42	0.998
43	a	II / L10	Y	no data	?	N	?	0.15	16.48	2.02	0.48	0.43	0.975
44		H8	Y	no data	?	N	?	0.06	16.00	1.59	0.04	0.21	0.987
45	a	II / L10	Y	no data	1	N	11	0.81	32.83	5.62	0.38	3.47	0.904
46		L52	Y	1	1	N	9	0.08	4.77	0.62	0.07	0.35	0.932
49		L52	Y	1	1	N	12	0.20	22.15	2.39	0.31	0.12	0.995
49	a	I / H22	Y	2	1	N	13	0.04	6.96	0.84	1.52	0.77	0.900
50	a	I / H22	Y	2	1	N	9	0.20	16.86	1.60	0.06	0.33	0.981
51		L52	Y	2	1	N	12	0.06	6.34	0.84	0.08	0.25	0.962
51	a	I / H22	Y	2	1	N	11	0.17	39.34	4.59	0.07	1.09	0.973
52		L52	Y	2	1	N	11	0.02	3.11	0.29	0.06	0.05	0.984
52	a	I / H22	Y	2	1	N	12	0.06	1.18	0.36	0.06	0.34	0.776
53		L52	Y	2	1	N	11	0.05	17.97	2.73	0.08	0.28	0.985
60	a	I / H22	Y	2	1	N	13	0.38	15.76	7.04	0.04	0.77	0.953
61	a	I / H22	Y	2	2	N	12	0.45	35.24	2.54	0.08	0.79	0.978
67	a	I / H22	Y	1	1	N	13	0.08	10.53	0.99	0.03	0.84	0.926
68	a	I / H22	Y	2	3	Y	50	0.11	4.92	0.53	0.09	1.07	0.821
69		L52	Y	1	1	N	13	0.07	2.89	0.67	0.13	0.72	0.801
70		L52	Y	1	1	N	12	0.32	38.43	5.67	0.04	0.43	0.989
70	a	I / H22	Y	1	1	N	12	0.27	38.11	4.80	0.07	0.08	0.998
71		L52	Y	1	1	N	13	1.25	69.41	4.69	0.28	2.31	0.968
71	a	I / H22	Y	1	1	N	12	0.15	15.99	1.91	0.03	0.32	0.980
76	a	I / H22	Y	1	1	N	8	4.65	223.25	1.52	12.01	0.47	0.998
77		L52	Y	1	1	N	12	0.39	22.30	3.74	0.02	0.30	0.987
78		L52	Y	1	1	N	8	8.58	270.53	1.69	17.26	1.81	0.993
83		L52	Y	2	1	N	13	0.12	15.15	1.58	0.07	0.61	0.961
85		L52	Y	2	1	N	12	0.50	47.49	2.29	0.13	0.85	0.982
86		L52	Y	2	1	N	8	0.25	26.76	2.95	0.05	0.29	0.989
86	a	I / H22	Y	2	2	N	12	0.68	20.37	4.85	0.24	2.59	0.887
88		L52	Y	2	1	N	11	0.07	12.08	1.18	0.04	0.93	0.929
93		L52	Y	2	2	N	13	2.67	153.43	11.65	0.14	0.21	0.999
97		L52	Y	2	1	N	8	0.03	6.70	1.56	0.34	0.46	0.936
97	a	I / H22	Y	2	1	N	12	0.17	25.06	2.30	0.03	0.39	0.985
102		L52	Y	2	1	N	9	0.08	15.98	1.56	0.08	0.26	0.984
104		L52	Y	3	1	N	11	7.02	259.73	20.88	1.50	1.72	0.993
104	a	I / H22	Y	2	1	N	8	0.34	53.26	4.65	0.09	0.44	0.992
105		L52	Y	2	1	N	11	0.07	6.39	0.13	0.04	0.26	0.961
107	a	I / H22	Y	1	2	N	13	0.05	2.36	0.30	0.16	0.31	0.884
110		L52	Y	2	1	N	9	0.20	10.14	2.56	0.26	2.20	0.822
112		L52	Y	3	2	N	13	0.03	4.70	0.58	0.03	0.10	0.979
112	a	I / H22	Y	1	1	N	9	0.09	0.86	0.33	0.10	0.69	0.555
114	a	I / H22	Y	1	1	N	12	0.00	19.35	1.41	0.13	0.27	0.986
115	a	I / H22	Y	3	1	N	13	0.09	4.61	0.81	0.07	0.51	0.900
116	a	I / H22	Y	3	1	N	11	0.51	99.35	8.58	0.21	0.17	0.998
119	a	I / H22	Y	2	1	N	12	0.06	13.29	0.62	0.19	1.04	0.927
120	a	I / H22	Y	1	1	N	12	0.33	45.45	4.13	0.20	0.90	0.981
125	a	I / H22	Y	1	1	N	11	0.13	48.00	5.82	0.14	1.25	0.975
127	a	I / H22	Y	1	1	N	9	0.19	75.53	2.62	0.12	1.17	0.985
128	a	I / H22	Y	2	1	N	11	1.89	234.98	19.33	4.21	0.32	0.999
129		L52	Y	1	1	N	11	0.18	61.34	4.25	0.08	1.29	0.979
129	a	I / H22	Y	1	1	N	9	0.36	88.11	10.37	0.12	0.28	0.997
131		L52	Y	1	1	N	12	0.09	1.41	0.51	0.02	0.18	0.887
131	a	I / H22	Y	1	1	N	11	0.13	4.78	0.65	0.05	0.47	0.910
133		L52	Y	1	1	N	9	0.07	22.79	1.88	0.06	0.79	0.966
134	a	I / H22	Y	2	1	N	13	0.04	11.40	2.27	0.06	0.31	0.974
135	a	I / H22	Y	1	1	N	11	5.15	208.33	16.74	6.89	0.62	0.997
137		L4	Y	1	2	N	11	0.07	17.73	1.02	0.29	0.75	0.959
137	a	V / L26	Y	2	3	N	12	0.11	9.39	0.45	0.09	0.38	0.961
138	a	V / L26	Y	2	2	N	10	0.24	6.82	0.16	0.63	0.68	0.909
139		L4	Y	1	3	N	9	0.21	1.99	0.09	0.36	0.78	0.718

queen nr.	cl./ fe.	mother hive	cell accept. yes/no	matured queen attractive-ness 1/2/3	ovary size/ developm. 1/2/3	mated yes/no	age days	HOB micro gram	9-ODA micro gram	9 H D A micro gram	10 HDAA micro gram	10-H D A micro gram	9-ODA / (9-ODA + 10-H DA) ratio
142		L4	Y	1	3	N	12	0.02	9.39	0.02	0.19	0.02	0.998
142	a	V / L26	Y	2	3	N	12	0.05	10.21	0.04	0.35	0.36	0.966
143	a	V / L26	Y	2	3	N	12	0.75	26.48	3.55	0.52	2.10	0.927
147		L4	Y	2	3	N	12	0.07	2.92	0.33	0.03	0.24	0.924
147	a	V / L26	Y	1	1	N	8	0.47	21.43	1.62	0.03	0.31	0.986
148		L4	Y	2	2	N	12	0.08	22.62	1.90	0.08	0.42	0.982
151	a	V / L26	Y	1	3	N	11	0.00	13.41	0.11	0.39	0.35	0.975
152		L4	Y	1	2	N	9	0.06	21.53	3.69	0.21	1.45	0.937
152	a	V / L26	Y	1	2	N	11	0.04	4.44	0.82	0.03	0.20	0.957
155		L4	Y	1	2	N	11	0.85	30.74	3.01	0.04	0.56	0.982
156		L4	Y	1	2	N	12	0.09	12.43	1.16	0.07	0.39	0.970
156	a	V / L26	Y	1	2	N	11	0.11	59.29	7.00	0.13	1.59	0.974
157	a	V / L26	Y	1	2	N	10	0.09	18.11	0.32	0.13	0.18	0.990
158		L4	Y	1	3	N	12	0.00	1.69	0.02	0.25	0.22	0.885
158	a	V / L26	Y	1	2	N	12	0.66	14.82	3.27	0.25	1.07	0.933
159		L4	Y	2	1	N	8	4.22	189.48	18.17	0.20	0.64	0.997
159	a	V / L26	Y	1	3	N	12	0.09	11.35	0.11	0.26	1.45	0.887
160	a	V / L26	Y	1	2	N	9	0.18	1.90	0.06	0.26	0.27	0.876
162	a	V / L26	Y	1	3	N	12	0.08	7.90	1.42	0.27	0.18	0.978
164		L4	Y	1	1	N	8	0.66	34.35	4.81	0.14	0.62	0.982
166		L4	Y	2	1	N	9	0.01	4.98	0.08	0.20	0.05	0.990
167		L4	Y	2	2	N	10	0.94	182.75	22.29	5.79	2.01	0.989
167	a	V / L26	Y	2	1	N	8	0.02	6.83	0.12	0.24	0.18	0.974
168		L4	Y	1	1	N	8	0.01	3.07	0.07	0.23	0.13	0.959
168	a	V / L26	Y	2	1	N	8	3.54	237.46	20.15	4.98	0.63	0.997
169		L4	Y	1	1	N	8	0.21	12.58	1.17	0.16	0.35	0.973
171	a	V / L26	Y	1	1	N	8	0.61	20.82	2.49	0.06	0.56	0.974
173		L4	Y	1	2	N	13	0.06	3.90	0.06	0.34	0.25	0.940
175		L4	Y	1	3	N	11	0.10	30.51	1.93	0.04	0.39	0.987
176		L4	Y	1	2	N	11	0.14	38.53	2.25	0.11	0.60	0.985
177		L4	Y	1	1	N	13	0.12	27.01	2.63	0.39	0.30	0.989
177	a	V / L26	Y	2	2	N	11	0.03	3.55	0.53	0.05	0.51	0.874
179	a	V / L26	Y	1	2	N	11	0.13	38.45	6.35	0.55	0.64	0.984
180		L4	Y	1	1	N	10	0.17	15.13	1.71	0.03	0.67	0.958
180	a	V / L26	Y	1	2	N	12	0.12	30.71	5.09	0.09	1.56	0.952
181		L4	Y	1	2	N	11	0.17	9.07	0.92	0.76	0.64	0.934
182		L4	Y	1	2	N	13	0.19	29.54	4.72	1.35	2.57	0.920
183		L4	Y	1	3	N	12	0.04	31.67	2.93	0.07	0.58	0.982
185		L4	Y	1	3	N	12	0.15	8.82	0.30	0.22	0.48	0.948
185	a	V / L26	Y	1	3	N	11	0.13	32.49	2.03	0.08	0.70	0.979
186		L4	Y	1	2	N	12	1.87	43.42	3.89	0.14	1.72	0.962
189		L4	Y	1	2	N	11	0.11	17.47	1.37	1.08	1.87	0.903
190		L4	Y	2	3	N	13	0.26	13.02	2.23	0.04	0.31	0.977
193		L4	Y	2	2	N	11	0.16	17.70	2.05	0.05	0.39	0.978
194		L4	Y	2	2	N	13	0.37	14.93	0.96	0.01	0.13	0.991
194	a	V / L26	Y	2	3	N	13	0.04	1.65	0.60	0.04	0.06	0.965
195	a	V / L26	Y	1	3	N	13	0.42	24.73	2.77	0.19	1.46	0.944
197		L4	Y	1	3	N	12	1.34	37.65	3.38	0.08	0.29	0.992
198		L4	Y	1	2	N	13	0.07	1.56	0.28	0.24	0.48	0.765
199	a	V / L26	Y	1	3	N	13	0.05	7.41	0.27	0.04	0.16	0.979
201	a	V / L26	Y	1	3	N	12	0.11	4.50	0.66	0.06	0.28	0.941
202		L4	Y	2	2	N	11	0.88	38.26	4.63	0.09	0.87	0.978
204	a	V / L26	Y	1	2	N	10	1.10	25.04	2.25	0.10	0.53	0.979
205		L4	Y	1	3	N	13	0.52	23.43	2.93	0.10	0.89	0.963
205	a	V / L26	Y	2	1	N	13	0.82	24.36	2.75	0.03	0.48	0.981
207		L4	Y	2	1	N	13	0.05	0.97	0.38	0.04	0.19	0.836
207	a	V / L26	Y	1	2	N	13	0.13	8.23	1.79	0.05	0.18	0.979
209		L4	Y	1	2	N	12	0.07	2.54	0.53	0.13	0.19	0.930
210	a	V / L26	Y	2	3	N	9	0.20	13.86	1.99	0.02	0.18	0.987
211		L4	Y	1	3	N	12	0.04	0.80	0.41	0.11	0.60	0.571
211	a	V / L26	Y	2	2	N	13	1.28	37.88	6.12	0.12	0.75	0.981
212		L4	Y	1	2	N	12	0.00	0.90	0.00	0.04	0.03	0.968
213		L4	Y	1	2	N	13	0.13	7.03	0.78	0.38	0.29	0.960
214		L4	Y	1	3	N	11	0.09	11.87	1.76	0.09	0.41	0.967
215		L4	Y	1	1	N	9	0.41	2.97	0.28	1.48	1.78	0.625
215	a	V / L26	Y	1	3	N	9	0.05	11.72	0.09	0.37	0.46	0.962
216		L4	Y	1	3	N	12	0.52	19.99	1.49	0.07	0.69	0.967
218	a	V / L26	Y	1	2	N	11	0.52	20.08	1.27	0.05	0.23	0.989
219		L4	Y	1	2	N	13	1.14	40.33	3.71	0.19	1.40	0.966
219	a	V / L26	Y	1	2	N	11	1.02	32.09	6.71	0.29	2.24	0.935
220		L4	Y	2	3	N	13	0.24	15.06	2.58	0.18	0.96	0.940
220	a	V / L26	Y	1	3	N	12	0.68	16.02	2.62	0.10	2.77	0.853

queen nr.	cl./ fe.	mother hive	cell accept. yes/no	matured queen attractive-ness 1/2/3	ovary size/ developm. 1/2/3	mated yes/no	age days	HOB micro gram	9-ODA micro gram	9 H D A micro gram	10 HDAA micro gram	10-H D A micro gram	9-ODA / (9-ODA + 10-H DA) ratio
222		L4	Y	1	2	N	13	0.75	60.53	4.90	0.13	0.46	0.992
222	a	V / L26	Y	1	2	N	13	0.18	13.89	0.07	0.42	0.83	0.944
223	a	V / L26	Y	1	2	N	13	0.29	34.92	4.07	0.13	0.42	0.988
224		L4	Y	1	2	N	12	0.05	4.77	0.28	0.03	0.18	0.964
224	a	V / L26	Y	1	1	N	9	0.08	11.96	0.02	0.48	0.31	0.975
225	a	V / L26	Y	1	2	N	13	0.04	10.85	0.85	0.02	0.11	0.990
226		L30	Y	1	1	N	8	0.06	15.17	0.72	0.14	0.04	0.997
227	a	VI / L53	Y	1	1	N	9	0.27	10.94	1.38	0.02	0.12	0.989
228	a	VI / L53	Y	2	1	N	9	0.13	24.23	2.32	0.13	0.23	0.991
235		L30	Y	1	1	N	8	0.17	21.39	0.55	0.33	0.17	0.992
236		L30	Y	1	1	N	9	0.07	12.80	0.53	0.18	0.13	0.990
236	a	VI / L53	Y	1	2	N	9	0.01	1.48	0.01	0.12	0.19	0.886
237		L30	Y	1	1	N	8	0.16	9.23	1.49	0.04	0.28	0.971
238		L30	Y	1	1	N	8	0.61	378.28	1.90	10.95	0.29	0.999
238	a	VI / L53	Y	1	1	N	8	0.07	17.40	1.00	0.02	0.35	0.980
240	a	VI / L53	Y	1	1	N	9	0.05	13.98	0.99	0.08	0.12	0.991
244	a	VI / L53	Y	1	1	N	9	0.07	14.71	0.32	0.10	0.21	0.986
246	a	VI / L53	Y	1	1	N	9	0.27	22.68	1.39	0.01	0.04	0.998
250		L34	Y	1	1	N	9	0.05	11.83	0.01	0.18	0.10	0.992
253		L34	Y	1	1	N	9	0.00	4.83	0.25	0.02	0.04	0.992
256		L34	Y	1	1	N	8	0.00	4.21	0.57	0.02	0.12	0.972
257		L34	Y	1	1	N	9	0.02	4.82	0.46	0.04	0.21	0.958
258		L34	Y	1	1	N	9	0.06	16.22	0.01	0.17	0.26	0.984
261	a	VI / L53	Y	1	1	N	8	0.00	2.74	0.44	0.07	0.07	0.975
262	a	VI / L53	Y	1	1	N	9	0.79	408.98	2.44	26.97	0.17	1.000
263		L32	Y	2	1	N	9	0.04	8.29	0.71	0.00	0.04	0.995
266		L32	Y	1	1	N	9	0.05	6.77	0.68	0.05	0.32	0.955
267		L32	Y	1	1	N	9	0.04	6.82	0.03	0.27	0.14	0.980
275	a	VI / L53	Y	2	1	N	8	0.99	318.93	2.69	5.45	2.26	0.993
277		L32	Y	1	1	N	9	0.97	108.52	9.07	1.04	0.45	0.996
281	a	VI / L53	Y	1	1	N	9	0.06	7.43	0.02	0.35	0.37	0.953
284		L6	Y	1	1	N	9	0.07	28.97	4.64	0.07	0.49	0.983
287		L6	Y	1	1	N	9	0.17	15.96	1.86	0.04	0.61	0.963
292		L6	Y	1	1	N	8	0.03	5.94	0.08	0.19	0.08	0.987
293		L6	Y	1	1	N	8	0.07	12.95	1.02	0.08	0.76	0.945
294		L6	Y	1	1	N	9	0.05	11.45	2.64	0.03	0.19	0.984
295	a	VI / L53	Y	2	2	N	8	3.53	67.87	6.71	3.15	0.67	0.990
296		L6	Y	1	1	N	8	6.26	67.37	24.24	2.41	1.16	0.983

Legend:

column 1: queen number, compare Appendix 1

column 2: a = worker-derived queen, blank = queen-derived queen

column 3: (mother colony) compare Appendix 1, 2

column 4: (cell accept. yes/no) Y = larva accepted; N = larva rejected

column 5: (mature queen attractiveness) 1 = 0 -10 workers; 2 = 11-20 workers; 3 = 21 and more workers tending

column 6: (ovary size/ development 1/2/3) 1 = undeveloped; 2 = slightly developed compartementalised; 3 = fully developed with ripe eggs

column 7: (mated yes/no) yes = mated; no = virgin

column 8: (age days) age in days after eclosing

columns 9-13: mandibular gland compounds in micro grams

column 14: 9-ODA/10-HDA ratio

Appendix 6.2. Produced *A. m. capensis* differentiated workers: gas-chromatographical analysis of mandibular gland compounds

lay. work. name	rating halo only	total rating	ovary develop. 1/2/3	ovariole nr.	pollen stage 2/1/0	age (days)	HOB micro gram	9-ODA micro gram	9-H DA micro gram	10 HDAA micro gram	10-H DA micro gram	9-ODA / (9-ODA+ 10-H DA ratio	comments / notes
a	0	11	1	5 / ?	0	17	0.77	2.80	28.01	0.23	0.20	0.933	X
b	2	12	2	8 / 7	1	18	0.47	2.34	0.49	0.29	0.93	0.716	X
c	0	7	1	no data	2	14	0.03	0.81	7.11	0.84	0.37	0.686	X
d	0	3	2	4 / ?	2	14	0.16	1.31	1.55	0.06	0.88	0.598	X
e	0	5	1	no data	1	14	0.11	3.78	0.69	1.10	0.34	0.917	X
g	0	5	1	3 / 3	2	17	0.77	2.80	28.01	0.23	0.11	0.962	X
l	0	8	1	3 / 3	2	17	0.63	8.45	22.14	0.14	0.38	0.957	X
k	0	8	1	3 / 3	0	17	0.17	2.20	1.26	0.91	18.48	0.106	X
l	1	12	3	12/12	0	15	0.41	1.90	0.28	0.12	1.97	0.491	eggs detected
m	2	11	2	8/7	1	17	0.22	1.43	0.24	0.29	2.43	0.370	X
o	2	12	3	11/9	0	15	0.13	2.05	0.34	0.21	1.66	0.553	eggs detected
p	1	5	3	8/9	1	17	0.25	2.86	0.37	0.13	1.48	0.659	eggs detected
q	1	9	3	16/16	0	18	0.13	3.48	27.78	0.31	0.54	0.866	eggs detected
r	1	4	3	8/8	2	18	0.07	0.19	2.94	0.15	0.13	0.594	eggs detected
s	2	10	3	11/10	0	15	0.16	7.02	3.27	0.21	0.61	0.920	eggs detected
t	0	6	3	10/10	0	18	0.08	8.36	0.96	0.13	1.74	0.828	eggs detected
v	1	8	3	12/11	0	18	0.20	2.37	0.45	0.03	0.60	0.798	eggs detected
w	2	11	3	12/12	0	15	0.23	13.78	2.10	0.26	2.34	0.855	eggs detected
x	2	7	3	11/8	1	15	0.18	1.08	0.12	0.06	0.28	0.794	eggs detected
z	1	8	2	9/7	2	17	0.23	1.07	3.70	0.68	2.53	0.297	X
a'	0	6	3	11/10	0	19	0.30	9.39	1.79	0.02	0.21	0.978	eggs detected
b'	1	10	1	11/11	0	17	0.41	3.90	0.55	1.65	27.46	0.124	X
d'	0	5	3	4/?	1	19	0.18	0.22	0.15	0.03	0.47	0.319	eggs detected
e'	0	6	1	9/10	2	17	0.22	4.52	1.24	0.11	0.65	0.874	X
h'	0	3	2	17/17	1	17	0.49	7.97	2.00	0.23	4.01	0.665	X
l'pink a)	3	15	3	15/15	0	17	0.06	1.42	0.13	0.01	0.22	0.866	eggs detected
l'pink b)	3	15	3	8/4	0	17	0.15	1.31	0.35	0.05	0.32	0.804	eggs detected
k'white	0	10	3	13/13	0	17	0.09	3.60	0.36	0.06	0.80	0.818	eggs detected
l'white	2	11	3	10/10	1	17	0.21	3.09	1.23	0.06	0.57	0.844	eggs detected
m'pink	3	14	3	5/7	0	19	0.22	4.49	1.85	0.01	0.06	0.987	eggs detected
n'white	2	12	3	3/3	1	17	0.27	16.88	2.38	0.05	0.45	0.974	X
n'pink	2	12	2	4/?	2	17	0.49	3.21	1.98	0.04	0.30	0.915	X
o'white	3	15	3	11/10	0	19	0.15	2.64	0.46	0.00	0.32	0.892	eggs detected
o'pink	3	15	3	9/7	0	19	0.37	6.59	0.70	0.01	0.20	0.971	eggs detected
q'pink	0	6	3	7/7	0	17	0.15	5.89	0.74	0.10	0.70	0.894	eggs detected
r'white	3	15	2	3/3	0	16	0.38	5.79	0.61	0.02	0.36	0.941	X
r'pink	3	15	3	13/13	0	16	0.16	4.89	1.24	1.05	0.37	0.930	eggs detected
s'white	3	15	2	9/9	0	16	0.07	4.41	0.39	0.06	0.07	0.984	X
s'pink	3	15	1	9/8	2	16	0.51	1.56	0.64	0.10	0.41	0.792	X
t'white	2	14	3	11/11	0	19	0.51	16.33	2.01	0.11	1.31	0.926	eggs detected
v'pink	0	9	3	6/3	1	18	0.13	3.43	0.40	0.02	0.07	0.980	X
w'white	3	15	2	12/10	1	14	0.33	11.46	1.26	0.09	1.36	0.894	X
w'pink	3	15	3	14/13	1	14	0.43	2.41	3.54	0.12	1.14	0.679	eggs detected
x'white	3	15	3	11/11	0	15	0.11	2.29	0.28	0.03	0.19	0.923	eggs detected
x'pink	3	15	1	6/6	2	15	0.07	1.29	15.09	0.06	0.20	0.866	X
y'white	1	10	2	4/4	1	18	0.30	11.60	1.64	0.02	0.14	0.988	X
a"pink	3	15	3	16/16	0	15	0.30	6.27	0.73	0.30	2.91	0.683	eggs detected
b''white	3	14	2	5/5	2	19	0.05	11.69	1.50	0.03	0.30	0.975	X
b"pink	3	14	3	9/11	0	19	0.21	5.70	1.25	0.00	0.06	0.990	eggs detected
c"pink	3	14	3	18/18	0	18	0.34	23.55	2.09	0.06	0.58	0.976	eggs detected
d''white	1	6	3	5/5	0	19	0.00	4.63	0.51	0.02	0.20	0.959	eggs detected
f"white	2	13	3	10/?	1	14	0.25	5.19	0.94	0.07	0.90	0.852	eggs detected
f'pink	2	13	1	9/?	2	14	1.15	6.85	43.01	0.29	0.83	0.892	X
g"	1	11	1	13/13	2	12	0.05	1.94	0.29	0.03	0.07	0.965	eggs detected
h"	3	15	1	7/7	0	12	0.07	1.05	0.18	0.04	0.17	0.861	eggs detected
m"	1	9	1	13/12	2	12	1.13	1.30	2.55	1.05	1.53	0.459	eggs detected

lay. work. name	rating halo only	total rating	ovary develop. 1/2/3	ovariole nr.	pollen stage 2/1/0	age (days)	HOB micro gram	9-ODA micro gram	9-H DA micro gram	10 HDAA micro gram	10-H DA micro gram	9-ODA / (9-ODA+ 10-H DA ratio	comments / notes
p''	0	9	1	14/14	2	12	0.09	4.88	4.28	0.12	0.70	0.875	eggs detected
q''	0	7	1	11/11	2	13	0.06	0.44	0.92	0.03	0.23	0.657	eggs detected
r''	2	12	3	10/9	0	13	0.04	3.58	0.47	0.22	1.92	0.651	eggs detected
s''	1	9	1	12/12	1	13	0.06	4.62	0.43	0.08	0.62	0.882	eggs detected
u''	2	11	1	12/11	2	13	0.20	1.61	11.99	2.50	2.37	0.405	eggs detected
z''	0	4	1	11/11	2	13	0.00	0.10	0.80	0.06	0.19	0.345	eggs detected
d'''	2	10	1	15/15	1	13	0.06	0.15	0.06	0.05	0.29	0.341	eggs detected
o'''	0	10	1	2/2	2	14	0.04	2.98	0.78	0.10	1.37	0.685	eggs detected
r'''	0	5	1	3/3	1	14	0.04	0.13	0.50	0.06	0.78	0.143	X
s'''	0	7	1	2/2	2	14	0.27	0.24	0.76	1.25	0.52	0.316	X
t'''	0	2	1	3/3	1	14	0.01	0.07	0.18	0.01	0.08	0.467	X
v'''	0	4	1	2/2	2	14	0.05	0.17	0.14	0.03	0.31	0.354	X
w'''	0	9	1	2/2	1	14	0.04	0.04	0.10	0.04	0.29	0.121	X
x'''	0	5	1	2/2	1	14	0.07	0.17	0.31	0.23	1.22	0.122	X
y'''	0	6	1	2/2	2	14	0.06	0.13	0.06	0.04	0.40	0.245	X
a''''	0	6	1	2/2	1	14	0.10	0.16	0.43	0.47	1.13	0.124	X

Legend:

column 1: (laying worker 'name') alphabetical order, with ', '', ''' denoting new start of alphabet, compare appendix 4

column 2: (rating halo only) 0-3 = sum of 'halo' rating column 6-8: (signed worker with clear 'halo'); compare appendix 4

column 3: (total rating) 1-15 = : 0-5 x 3 = sum of 3 ratings, each: signed worker detectable on comb / unit calm, most workers on comb / comb clean, worked on and some food in cells / signed worker "queen-like cruising" on comb / signed worker with distinct court / for each: 1 pt

column 4: (ovary size/ development 1/2/3) 1 = undeveloped; 2 = slightly developed compartmentalised; 3 = fully developed with ripe eggs

column 5: (ovariole nr) # / # = number of ovarioles of left and right ovaries

column 6: (pollen stage) 0-2 = 0 = no pollen; 1 = some pollen; 2 = massive pollen (undigested pollen residues) in rectum

column 7: (date (d)) # = age in days after eclosion

columns 8-12: mandibular gland compounds in micro grams

column 13: 9-ODA / 10-HDA ratio

column 14: (comments/notes) comments and notes; compare Appendix 4

Appendix 6.3. Mated queens: gas-chromatographical analysis of mandibular gland compounds

queen/hive name	ovary size/ developm. 1/2/3	date p= preserved or c= conserved	HOB micro gram	9-ODA micro gram	9-H DA micro gram	10-HDAA micro gram	10-H DA micro gram	9-ODA / (9-ODA + 10-H DA) ratio	age (days)	other names / functions of hive	comments / notes
L10	3	p 02.10.05	3.53	67.87	6.71	3.15	0.67	0.909	>100	II	laying worker hive II, clonal larvae used for grafting in cycle 1
Lx	3	p 02.10.05	6.26	67.37	24.24	2.41	1.16	0.954	>100	X	wild swarm, queen test-preservation
H9	3	c 08.10.05	1.44	23.84	4.38	0.07	0.69	0.864	>100	(1)	hive H9 (weak) united with H8 after removal of queen H9
S19	3	p 08.10.05	5.02	57.09	14.39	1.66	3.57	0.801	>100	A	*scutellata* queen from KZN
S18	3	p 08.10.05	5.31	60.65	15.04	0.44	1.38	0.916	>100	(B) / (IV)	hive L18 (weak) united with hive L, but queen S18 rejected
L26	3	p 08.10.05	2.97	52.84	14.03	0.60	1.35	0.912	>100	C / V	start.-finish. C not successful, converted into lay.work. V
H21	3	p 08.10.05	1.48	26.74	8.05	1.90	11.63	0.409	>100	D	start.-finish.
L25	3	p 08.10.05	4.17	31.85	11.82	1.56	2.31	0.837	>100	E	start.-finish
H13	3	p 28.10.05	22.45	314.40	66.48	11.24	12.14	0.846	>100	4 / VII	queen H13 removed to produce additional lay. worker colony
L51	3	p 14.11.05	1.55	16.13	3.31	0.09	0.94	0.779	>100	I	start.-finish.
L50	3	p 14.11.05	0.14	10.80	1.97	0.12	1.46	0.574	>100	H	start.-finish.
H20	3	p 14.11.05	15.45	300.62	60.55	15.41	48.86	0.553	>100	3 / queenbank 1	weak, bad brood pattern, converted into queenbank cycle 2
L53	3	p 23.11.05	1.77	40.78	14.74	1.20	0.66	0.957	>100	VI	queen L53 removed to produce additional lay. worker colony
L37	3	p 30.11.05	9.48	258.34	22.22	4.55	5.02	0.816	>100	L	start.-finish.
L33	3	p 30.11.05	1.10	15.98	4.01	0.03	0.31	0.928	>100	P	start.-finish
S17	3	p 26.12.05	0.29	2.73	0.91	0.03	0.14	0.867	>100	U	start.-finish.
L30	3	p 26.12.05	1.16	14.47	5.01	0.08	1.49	0.771	>100	Q	start.-finish.
L32	3	p 26.12.05	0.33	13.26	1.38	0.02	0.35	0.798	>100	S	start.-finish.
L34	3	p 26.12.05	3.61	14.97	3.41	0.06	1.33	0.719	>100	R	start.-finish.
Lxa	3	p 26.12.05	4.14	26.31	12.10	0.33	0.79	0.939	>100	X	small wild swarm, probably absconded split with new queen

Legend:

column 1: (queen / hive name) compare Appendix 2 for origin and fate of individual colonies

column 2: (ovary size/ development 1/2/3) 1 = undeveloped; 2 = slightly developed compartementalised; 3 = fully developed with ripe eggs

column 3: (date preserved or conserved) preserved = p: life adult insect dissected, head + tergites preserved in dichloromethane, thorax conserved in ethanol, photographs of imago and ovaries; conserved = c: dead adult, pupa or larvae conserved in ethanol

columns 4-8: mandibular gland compounds in micro grams

column 9: 9-ODA / 10-HDA ratio, denoting queen-likeness

column 10: (age days) age in days after eclosing

column 11: (other names / functions of colony) compare Appendix 2 for origin an fate of individual colonies

column 12: (comments / notes) comments and notes; compare Appendix 2 for origin an fate of individual colonies

APPENDIX 7 (Swarm cohesion bioassay)

Appendix 7.1. Worker responses (clustering) to caged queens and workers in swarm cohesion bioassays

	quality	number/ name	age (d)	rating attract	9-ODA/ (9-ODA+ 10-H DA) ratio	HOB	9-ODA	9-H DA	10-HDAA	10-HDA	ovary develop	date of experim.	time of experim.	worker bees from hive	notes / comments
TRIAL 1	queen (fertilised)	105	11	**4**	0.98	0.08	15.98	1.56	0.08	0.26	1	17.11.05	0930	L24	confined in swarm box
	queen (clonal)	135a	11	**3**	0.91	0.13	4.78	0.65	0.05	0.47	1				
	pseudoqueen	h'	17	**0**	0.67	0.49	7.97	2.00	0.23	4.01	2				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 2	queen (fertilised)	129	11	**4**	0.97	0.13	48.00	5.82	0.14	1.25	1	17.11.05	11.30	L24	confined in swarm box
	queen (clonal)	51a	11	**3**	0.90	0.04	6.96	0.84	1.52	0.77	1				
	pseudoqueen	b'	17	**2**	0.12	0.41	3.90	0.55	1.65	27.46	1				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 3	queen (fertilised)	53	11	**4**	0.97	0.17	39.34	4.59	0.07	1.09	1	17.11.05	13.15	L24	confined in swarm box
	queen (clonal)	128a	11	**1**	0.98	0.33	45.45	4.13	0.20	0.90	1				
	pseudoqueen	m	17	**1**	0.37	0.22	1.43	0.24	0.29	2.43	2				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 4	queen (fertilised)	52	11	**4**	0.98	0.20	16.86	1.60	0.06	0.33	1	17.11.05	15.00	L24	confined in swarm box
	queen (clonal)	116a	11	**3**	0.55	0.09	0.86	0.33	0.10	0.69	1				
	pseudoqueen	e'	17	**0**	0.87	0.22	4.52	1.24	0.11	0.65	1				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 5	queen (fertilised)	88	11	**3**	0.98	0.50	47.49	2.29	0.13	0.85	1	17.11.05	16.45	L24	confined in swarm box
	queen (clonal)	125a	11	**2**	1.00	0.51	99.35	8.58	0.21	0.17	1				
	pseudoqueen	p	17	**4**	0.66	0.25	2.86	0.37	0.13	1.48	3				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 6	queen (fertilised)	131	12	**0**	1.00	1.89	234.98	19.33	4.21	0.32	1	18.11.05	13.15	L24	confined in swarm box
	queen (clonal)	114a	12	**4**	0.82	0.20	10.14	2.56	0.26	2.20	1				
	pseudoqueen	t	18	**0**	0.83	0.08	8.36	0.96	0.13	1.74	3				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 7	queen (fertilised)	49	12	**1**	0.99	0.06	16.00	1.59	0.04	0.21	1	18.11.05	14.45	L24	confined in swarm box
	queen (clonal)	97a	12	**4**	0.93	0.07	12.08	1.18	0.04	0.93	1				
	pseudoqueen	q	18	**0**	0.87	0.13	3.48	27.78	0.31	0.54	3				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 8	queen (fertilised)	70	12	**4**	0.93	0.08	10.53	0.99	0.03	0.84	1	18.11.05	16.45	L24	confined in swarm box
	queen (clonal)	70a	12	**3**	0.82	0.11	4.92	0.53	0.09	1.07	1				
	pseudoqueen	v	18	**2**	0.80	0.20	2.37	0.45	0.03	0.60	3				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 9	queen (fertilised)	152	9	**3**	0.98	0.08	22.62	1.90	0.08	0.42	2	06.12.05	12.40	L56 L57	confined in swarm box
	queen (clonal)	224a	9	**3**	0.99	0.29	34.92	4.07	0.13	0.42	1				
	pseudoqueen	b''w	15	**2**	0.97		11.69			0.30	X				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 10	queen (fertilised)	215	9	**3**	0.96	0.13	7.03	0.78	0.38	0.29	1	06.12.05	14.30	L56 L57	confined in swarm box
	queen (clonal)	160a	9	**3**	1.00	4.22	189.48	18.17	0.20	0.64	2				
	pseudoqueen	a''p	15	**1**	0.68	0.30	6.27	0.73	0.30	2.91	3				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 11	queen (fertilised)	139	9	**3**	0.96	0.07	17.73	1.02	0.29	0.75	3	06.12.05	16.00	L56 L57	confined in swarm box
	queen (clonal)	215a	9	**4**	0.97	0.09	11.87	1.76	0.09	0.41	3				
	pseudoqueen	x'w	15	**2**	0.92	0.11	2.29	0.28	0.03	0.19	3				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 12	queen (fertilised)	137	10	**3**	0.97	0.07	22.79	1.88	0.06	0.79	2	07.12.05	13.30	L56 L57	confined in swarm box
	queen (clonal)	156a	10	**3**	0.98	0.85	30.74	3.01	0.04	0.56	2				
	pseudoqueen	s'w	16	**2**	0.98	0.07	4.41	0.39	0.06	0.07	2				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 13	queen (fertilised)	181	10	**3**	0.96	0.17	15.13	1.71	0.03	0.67	2	07.12.05	15.15	L56 L57	confined in swarm box
	queen (clonal)	204a	10	**4**	0.94	0.11	4.50	0.66	0.06	0.28	2				
	pseudoqueen	s'p	16	**2**	0.79	0.51	1.56	0.64	0.10	0.41	1				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 14	queen (fertilised)	180	10	**2**	0.87	0.03	3.55	0.53	0.05	0.51	1	07.12.05	16.45	L56 L57	confined in swarm box
	queen (clonal)	157a	10	**3**	0.97	0.09	12.43	1.16	0.07	0.39	2				
	pseudoqueen	r'w	16	**3**	0.94	0.38	5.79	0.61	0.02	0.36	2				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 15	queen (fertilised)	167	10	**4**	0.98	0.66	34.35	4.81	0.14	0.62	2	07.12.05	18.15	L56 L57	confined in swarm box
	queen (clonal)	138a	10	**3**	1.00	5.15	208.33	16.74	6.89	0.62	2				
	pseudoqueen	r'p	16	**0**	0.93	0.16	4.89	1.24	1.05	0.37	3				r'p died during experiment
	control	X	X	**1**	X	X	X	X		X	X				
TRIAL 16	queen (fertilised)	147	12	**3**	0.97	0.05	10.21	0.04	0.35	0.36	3	09.12.05	11.15	L14 L24	confined in swarm box
	queen (clonal)	201a	12	**2**	0.76	0.07	1.56	0.28	0.24	0.48	3				
	pseudoqueen	c''p	18	**2**	0.98	0.34	23.55	2.09	0.06	0.58	3				
	control	X	X	**0**	X	X	X	X		X	X				

	quality	number/ name	age (d)	rating attract	9-ODA/ (9-ODA+ 10-H DA) ratio	HOB	9-ODA	9-H DA	10-HDAA	10-HDA	ovary develop	date of experim.	time of experim.	worker bees from hive	notes / comments
TRIAL 17	queen (fertilised)	224	12	**2**	0.94	0.18	13.89	0.07	0.42	0.83	2	09.12.05	13.15	L14 L24	confined in swarm box
	queen (clonal)	162a	12	**3**	0.89	0.09	11.35	0.11	0.26	1.45	3				
	pseudoqueen	v'p	18	**3**	0.98	0.13	3.43	0.40	0.02	0.07	3				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 18	queen (fertilised)	185	12	**2**	0.92	0.19	29.54	4.72	1.35	2.57	3	09.12.05	15.30	L14 L24	confined in swarm box
	queen (clonal)	142a	12	**3**	0.72	0.21	1.99	0.09	0.36	0.78	3				
	pseudoqueen	y'w	18	**2**	0.99	0.30	11.60	1.64	0.02	0.14	2				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 19	queen (fertilised)	235	8	**4**	0.99	0.27	10.94	1.38	0.02	0.12	1	26.12.05	13.30	L14	confined in swarm box
	queen (clonal)	275a	8	**1**	0.95	0.05	6.77	0.68	0.05	0.32	1				
	pseudoqueen	p''	12	**1**	0.87	0.09	4.88	4.28	0.12	0.70	1				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 20	queen (fertilised)	292	8	**3**	0.98	0.07	28.97	4.64	0.07	0.49	1	26.12.05	15.30	L14	confined in swarm box
	queen (clonal)	238a	8	**2**	0.97	0.16	9.23	1.49	0.04	0.28	1				
	pseudoqueen	m''	12	**0**	0.46	1.13	1.30	2.55	1.05	1.53	1				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 21	queen (fertilised)	293	8	**1**	0.96	0.17	15.96	1.86	0.04	0.61	1	26.12.05	17.15	L14	confined in swarm box
	queen (clonal)	295a	8	**3**	0.94	0.07	12.95	1.02	0.08	0.76	2				
	pseudoqueen	h''	12	**2**	0.86	0.07	1.05	0.18	0.04	0.17	1				
	control	X	X	**0**	X	X	X	X		X	X				
TRIAL 22	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x	28.12.05		L36	
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
TRIAL 23	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x	28.12.05		L36	
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
TRIAL 24	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x	28.12.05		L36	
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
TRIAL 25	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x	28.12.05		L36	
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
TRIAL 26	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x	28.12.05		L36	
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				
	empty cage contr.	X	X	**1**	x	x	x	x	x	x	x				

Legend:

column 1: (TRIAL #) 1-25 = number of experimental repeats

column 2: (quality) queen-derived queen / worker-derived queen / pseudoqueen (= differentiated worker) compare Appendices 1, 4

column 3: (number / name) = name (letter) or number of queens and workers used in the experiment, compare Appendices 1, 4

column 4: (age days) age in days after eclosing

column 5: (rating attract.) 0 = <10 bees gathering, 1 = 10-30 bees gathering, 2 = small cluster, 3 = cluster <50% of bees, 4 = large cluster >50% of bees

column 6: (9-ODA / 10-HDA ratio) denotes queen-likeness of pheromonal composition

columns 7-11: mandibular gland compounds in micro grams

column 12: (ovary size/ development 1/2/3) 1 = undeveloped; 2 = slightly developed compartementalised; 3 = fully developed with ripe eggs

columns 13-14: (date and time of experiment) = date and time of experiment

column 15: (worker bees from hive) = compare Appendix 2, for source colonies for workers for bioassays

column 16: (notes / comments) = notes and comments

APPENDIX 8 (Drone competition bioassay)

Appendix 8.1. Drone responses (preferences) to caged queens and workers in mating bioassays

	quality	number/ name	age (d)	hits at a)	hits at b)	hits at c)	hits at d)	total hits	rating attract.	HOB	9-ODA	9-HDA	10-HDAA	10-H DA	9-ODA/ (9-ODA+ 10-HDA) ratio	ovary develop	date of experim.
TRIAL 1	queen (fertilised)	1	8	2	9	4	6	21	**4**	0.45	19.98	0.83	0.07	0.41	0.98	1	24.10.05
	queen (clonal)	45a	8	3	3	3	1	10	**3**	4.58	242.22	1.18	12.55	0.42	1.00	1	
	pseudoqueen	b	18	0	2	0	4	6	**2**	0.47	2.34	0.49	0.29	0.93	0.72	2	
	control	X	X	2	1	0	1	4	**1**	X	X	X	X	X	X	X	
TRIAL 2	queen (fertilised)	133	9	9	10	4	11	34	**4**	0.36	88.11	10.37	0.12	0.28	1.00	1	15.11.05
	queen (clonal)	129a	9	3	7	12	8	30	**3**	0.19	75.53	2.62	0.12	1.17	0.98	1	
	pseudoqueen	s	15	1	0	3	3	7	**2**	0.16	7.02	3.27	0.21	0.61	0.92	3	
	control	X	X	2	0	2	1	5.	**1**	X	X	X	X	X	X	X	
TRIAL 3	queen (fertilised)	52	11	4	10	4	6	24	**4**	0.20	16.86	1.60	0.06	0.33	0.98	1	17.11.05
	queen (clonal)	116a	11	3	2	13	1	19	**3**	0.09	0.86	0.33	0.10	0.69	0.55	1	
	pseudoqueen	e'	17	6	3	1	6	16	**2**	0.22	4.52	1.24	0.11	0.65	0.87	1	
	control	X	X	3	1	7	3	14	**1**	X	X	X	X	X	X	X	
TRIAL 4	queen (fertilised)	88	11	0	7	5	3	15	**4**	0.50	47.49	2.29	0.13	0.85	0.98	1	17.11.05
	queen (clonal)	125a	11	0	4	6	0	10	**3**	0.51	99.35	8.58	0.21	0.17	1.00	1	
	pseudoqueen	p	17	1	1	1	2	5	**1**	0.25	2.86	0.37	0.13	1.48	0.66	3	
	control	X	X	1	0	3	2	6	**2**	X	X	X	X	X	X	X	
TRIAL 5	queen (fertilised)	51	12	1	5	9	6	21	**3**	0.20	22.15	2.39	0.31	0.12	0.99	1	18.11.05
	queen (clonal)	120a	12	11	8	8	5	32	**4**	0.09	4.61	0.81	0.07	0.51	0.90	1	
	pseudoqueen	u	18	7	5	0	8	20	**2**	0.20	2.37	0.45	0.03	0.60	0.80	3	
	control	X	X	5	1	3	8	17	**1**	X	X	X	X	X	X	X	
TRIAL 6	queen (fertilised)	77	12	8	8	7	9	32	**3**	1.25	69.41	4.69	0.28	2.31	0.97	1	18.11.05
	queen (clonal)	86a	12	7	10	6	11	34	**4**	0.12	15.15	1.58	0.07	0.61	0.96	2	
	pseudoqueen	r	18	4	6	4	4	18	**2**	0.07	0.19	2.94	0.15	0.13	0.59	3	
	control	X	X	2	3	7	6	18	**2**	X	X	X	X	X	X	X	
TRIAL 7	queen (fertilised)	85	12	2	6	2	5	15	**4**	0.39	22.30	3.74	0.02	0.30	0.99	1	18.11.05
	queen (clonal)	52a	12	1	2	6	1	10	**3**	0.06	6.34	0.84	0.08	0.25	0.96	1	
	pseudoqueen	x	15	2	0	0	2	4	**1**	0.18	1.08	0.12	0.06	0.28	0.79	3	
	control	X	X	4	0	1	4	9	**2**	X	X	X	X	X	X	X	
TRIAL 8	queen (fertilised)	159	8	1	7	4	3	15	**4**	0.00	1.69	0.02	0.25	0.22	0.88	1	05.12.05
	queen (clonal)	168a	8	2	5	5	3	15	**4**	0.02	6.83	0.12	0.24	0.18	0.97	1	
	pseudoqueen	f'w	14	3	2	1	3	9	**2**	0.25	5.19	0.94	0.07	0.90	0.85	3	
	control	X	X	3	1	4	4	12	**3**	X	X	X	X	X	X	X	
TRIAL 9	queen (fertilised)	169	8	2	9	7	10	28	**3**	0.01	3.07	0.07	0.23	0.13	0.96	1	05.12.05
	queen (clonal)	171a	8	5	7	10	11	33	**4**	3.54	237.46	20.15	4.98	0.63	1.00	1	
	pseudoqueen	f'p	14	6	2	1	12	21	**2**	1.15	6.85	43.01	0.29	0.83	0.89	1	
	control	X	X	4	3	7	7	21	**2**	X	X	X	X	X	X	X	
TRIAL 10	queen (fertilised)	164	8	8	13	14	8	43	**3**	0.18	1.90	0.06	0.26	0.27	0.88	1	05.12.05
	queen (clonal)	147a	8	16	16	15	4	51	**4**	0.75	26.48	3.55	0.52	2.10	0.93	1	
	pseudoqueen	w'w	14	12	8	4	6	30	**2**	0.33	11.46	1.26	0.09	1.36	0.89	2	
	control	X	X	10	1	8	4	23	**1**	X	X	X	X	X	X	X	
TRIAL 11	queen (fertilised)	168	8	5	2	7	2	16	**3**	0.94	182.75	22.29	5.79	2.01	0.99	1	05.12.05
	queen (clonal)	167a	8	4	2	8	1	15	**2**	0.01	4.98	0.08	0.20	0.05	0.99	1	
	pseudoqueen	w'p	14	7	1	2	7	17	**4**	0.43	2.41	3.54	0.12	1.14	0.68	3	
	control	X	X	4	0	2	2	8	**1**	X	X	X	X	X	X	X	
TRIAL 12	queen (fertilised)	166	9	1	8	3	6	18	**3**	0.08	7.90	1.42	0.27	0.18	0.98	1	06.12.05
	queen (clonal)	210a	9	3	8	9	4	24	**4**	0.13	8.23	1.79	0.05	0.18	0.98	3	
	pseudoqueen	x'p	15	1	4	3	6	14	**2**	0.07	1.29	15.09	0.06	0.20	0.87	1	
	control	X	X	0	0	3	4	7	**1**	X	X	X	X	X	X	X	
TRIAL 13	queen (fertilised)	296	8	4	10	3	4	21	**3**	0.05	11.45	2.64	0.03	0.19	0.98	1	26.12.05
	queen (clonal)	261a	8	7	10	10	2	29	**4**	0.02	4.82	0.46	0.04	0.21	0.96	1	
	pseudoqueen	g''	12	8	1	2	3	14	**2**	0.05	1.94	0.29	0.03	0.07	0.97	1	
	control	X	X	5	0	1	0	6	**1**	X	X	X	X	X	X	X	
TRIAL 14	queen (fertilised)	287	9	10	8	19	6	43	**4**	0.06	7.43	0.02	0.35	0.37	0.95	1	27.12.05
	queen (clonal)	227a	9	7	9	11	4	31	**3**	0.04	10.85	0.85	0.02	0.11	0.99	1	
	pseudoqueen	z''	13	3	4	4	1	12	**2**	0.00	0.10	0.80	0.06	0.19	0.34	1	
	control	X	X	4	1	4	3	12	**2**	X	X	X	X	X	X	X	
TRIAL 15	queen (fertilised)	277	9	8	7	17	22	54	**3**	0.04	6.82	0.03	0.27	0.14	0.98	1	27.12.05
	queen (clonal)	262a	9	12	23	7	23	65	**4**	0.06	16.22	0.01	0.17	0.26	0.98	1	
	pseudoqueen	r''	13	5	7	7	4	23	**2**	0.04	3.58	0.47	0.22	1.92	0.65	3	
	control	X	X	5	3	5	5	18	**1**	X	X	X	X	X	X	X	

	quality	number/ name	age (d)	hits at a)	hits at b)	hits at c)	hits at d)	total hits	rating attract.	HOB	9-ODA	9-HDA	10-HDAA	10-H DA	9-ODA/ (9-ODA+ 10-HDA) ratio	ovary develop	date of experim.
TRIAL 16	queen (fertilised)	284	9	4	10	6	X	20	**3**	0.97	108.52	9.07	1.04	0.45	1.00	1	27.12.05
	queen (clonal)	281a	9	10	15	10	X	35	**4**	0.99	318.93	2.69	5.45	2.26	0.99	1	
	pseudoqueen	d'''	13	6	13	8	X	27	**2**	0.06	0.15	0.06	0.05	0.29	0.34	3	
	control	X	X	2	3	7	X	12	**1**	X	X	X	X	X	X	X	
TRIAL 17	queen (fertilised)	294	9	13	25	23	16	77	**4**	0.03	5.94	0.08	0.19	0.08	0.99	1	27.12.05
	queen (clonal)	228a	9	9	14	14	16	53	**3**	0.06	15.17	0.72	0.14	0.04	1.00	1	
	pseudoqueen	s''	13	6	10	4	3	23	**2**	0.06	4.62	0.43	0.08	0.62	0.88	1	
	control	X	X	1	1	7	4	13	**1**	X	X	X	X	X	X	X	
TRIAL 18	queen (fertilised)	236	9	5	1	14	7	27	**3**	0.13	24.23	2.32	0.13	0.23	0.99	1	27.12.05
	queen (clonal)	236a	9	5	9	8	6	28	**4**	0.17	21.39	0.55	0.33	0.17	0.99	2	
	pseudoqueen	u''	13	4	9	5	2	20	**2**	0.20	1.61	11.99	2.50	2.37	0.40	1	
	control	X	X	2	1	6	1	10	**1**	X	X	X	X	X	X	X	
TRIAL 19	queen (fertilised)	250	9	12	13	3	6	34	**4**	0.07	14.71	0.32	0.10	0.21	0.99	1	27.12.05
	queen (clonal)	246a	9	10	11	4	6	31	**3**	0.05	13.98	0.99	0.08	0.12	0.99	1	
	pseudoqueen	q''	13	3	4	3	3	13	**1**	0.06	0.44	0.92	0.03	0.23	0.66	1	
	control	X	X	3	3	4	5	15	**2**	x	x	X	X	X	x	X	
TRIAL 20	empty (control)	X	X	2	2	2	2	8	**2**	x	x	X	X	X	x	X	14.02.06
	empty (control)	X	X	2	3	2	2	9	**3**	x	x	X	X	X	x	X	
	empty (control)	X	X	1	4	1	4	10	**4**	x	x	X	X	X	x	X	
	empty (control)	X	X	2	0	4	4	10	**4**	x	x	X	X	X	x	X	
TRIAL 21	empty (control)	X	X	0	9	4	4	17	**4**	x	x	X	X	X	x	X	14.02.06
	empty (control)	X	X	4	5	2	2	13	**3**	x	x	X	X	X	x	X	
	empty (control)	X	X	3	2	2	2	9	**1**	x	x	X	X	X	x	X	
	empty (control)	X	X	0	3	4	3	10	**2**	x	x	X	X	X	x	X	
TRIAL 22	empty (control)	X	X	1	3	4	3	11	**2**	x	x	X	X	X	x	X	14.02.06
	empty (control)	X	X	3	6	2	1	12	**3**	x	x	X	X	X	x	X	
	empty (control)	X	X	4	5	2	3	14	**4**	x	x	X	X	X	x	X	
	empty (control)	X	X	2	2	4	3	11	**2**	x	x	X	X	X	x	X	
TRIAL 23	empty (control)	X	X	1	3	4	3	11	**3**	x	x	X	X	X	x	X	16.02.06
	empty (control)	X	X	4	3	2	0	9	**2**	x	x	X	X	X	x	X	
	empty (control)	X	X	5	3	2	2	12	**4**	x	x	X	X	X	x	X	
	empty (control)	X	X	4	1	5	2	12	**4**	x	x	X	X	X	x	X	
TRIAL 24	empty (control)	X	X	0	3	3	3	9	**3**	x	x	X	X	X	x	X	16.02.06
	empty (control)	X	X	4	1	3	1	9	**3**	x	x	X	X	X	x	X	
	empty (control)	X	X	5	1	2	1	9	**3**	x	x	X	X	X	x	X	
	empty (control)	X	X	1	1	5	3	10	**4**	x	x	X	X	X	x	X	

Legend:

column 1: (TRIAL #) 1-24 = number of experimental repeats

column 2: (quality) queen-derived queen; worker-derived queen; pseudoqueen (= differentiated worker) compare Appendices 1, 4

column 3: (number / name) = name (letter) or number of queens and workers used in the experiment, compare Appendices 1, 4

column 4: (age days) age in days after eclosing

columns 5-8: ('hits' at a/b/c/d) = compare * = positional combination of lures at different levels and observed drone response ('hits')

column 9: (total 'hits') = sum of 'hits' on one lure during one experimental trial (repeat)

column 10: (rating attract.) 4 = most attractive; 3 = second most attractive; 2 = attractive; 1 = least attractive

columns 11-15: mandibular gland compounds in micro grams

column 16: (9-ODA / 10-HDA ratio) denotes queen-likeness of pheromonal composition

column 17: (ovary size/ development 1/2/3) 1 = undeveloped; 2 = slightly developed compartementalised; 3 = fully developed with ripe eggs

column 18: (date of experiment) = date of experiment

*

position of lures	a)	b)	c)	d)
level 4 = 7.00m	fertil.	contr.	work.	clonal
level 3 = 6.50m	clonal	work.	fertil.	contr.
level 2 = 6.00m	work	clonal	contr.	fertil.
level 1 = 5.50m	contr.	fertil.	clonal	clonal