

CHAPTER 1

INTRODUCTION

1.1 SHORT NOTES ON BEEKEEPING HISTORY IN SOUTH AFRICA

Other than robbing wild nests there is no indication that there were any indigenous beekeeping activities in South Africa before European settlement. This could be because there was no suitable indigenous vegetation from which to produce reliable nectar flows and also because there were no trees that were suitable for making bark hives (Johannsmeier, 2001). Anderson (1985) suggested that at the earlier stages of European settlement, domestication of honeybees was not deemed necessary because there was such an abundance of wild honeybee nests. Only when the fruit industry started to develop in the Western Cape, did the need for domesticated honeybees for use in pollination services arise.

During the Anglo-Boer War (1899-1902) and the depression years of the 1930's, people used honey as a survival food, again mainly by robbing wild nests. Where colonies were kept in hives, prevailing conditions necessitated that use was made of available materials such as the wooden boxes in which paraffin containers were transported. Modern or commercial beekeeping started only when the first Langstroth hives were imported from England and the first beekeepers' association was founded in 1907 in Johannesburg (Johannsmeier, 2001).

In 1923 the Department of Agriculture made the first appointment of a honeybee specialist, namely Dr. A.E. Lundie who encouraged the use of Langstroth hives and the standardisation of beekeeping equipment. He presented many well-attended beekeeping courses. Only in 1950 was a second appointment made, that of Dr. Andy Anderson (Johannsmeier, 2001).

Between 1930 and 1965 Lundie imported Italian queens as he wanted to breed more docile honeybees. But the Italian bees could not get established in southern Africa. When the queen was introduced the brood pattern was good. But over time as the number of African worker bees dropped and the number of Italian worker bees increased, the colony dwindled (Fletcher, 1977).

Books on beekeeping were sought after and the bulletin of F. Taylor, "*Beekeeping for the beginner*" was widely used during the late 1930's. In 1945 a revised edition named "*Beekeeping*" was published. "*Beekeeping in South Africa*" edited by R.H. Anderson, or the well known "Blue Book" was published in 1973, 1983 and a revised edition in 2001 edited by M.F. Johannsmeier is used as a standard reference book by the beekeeping fraternity in South Africa.

Various associations were formed of which the Pollination Services Association (POSA) formed in 1984 is well known. As early as 1907 the first beekeepers Association was formed, namely the South African Beekeepers Association (SABA). In 1909 the beekeepers in Natal followed and in 1927 the Natal Honey Producers co-operative was formed. There are currently at least 15 different associations in South Africa. A list is available on the back page of every issue of the *South African Bee Journal*.

During the 1970's the honey industry boomed. Honey was exported and honey prices climbed. The main source of nectar was from saligna (*Eucalyptus saligna*) gum plantations. But this boom was followed by the arrival of droughts, acid rain, young trees (that do not have so many flowers as the older established trees) and nectar flies (*Drosophila flavohirta* Malloch), that made the nectar unacceptable for the honeybees, all of which had negative impacts on the industry (Johannsmeier, 2001).

Migratory beekeeping - the large scale movement of hives - developed as part of the apicultural industry to keep up with the demand for pollination services and honey production.

1.2 BACKGROUND TO THE CAPENSIS CALAMITY

There are two races of honeybees that occur naturally in South Africa, *Apis mellifera capensis* Escholtz (Cape honeybee) and *Apis mellifera scutellata* Lepeletier (African savanna honeybee). *A. m. capensis* is mainly restricted to the winter rainfall region of the Cape as far as the Bokkeveld (1), Cedarberg (2), Swartberg (3) and Suurberg (4) mountains (Fig. 1.2). *A. m. scutellata* occurs in the summer rainfall region north of the Roggenveldberg (5), Nuweveldberg (6), Sneeuwberg (7), Kikvorsberg (8), Stormberg (9) and Drakensberg (10) mountains. A hybridisation zone occurs between these two groups (Hepburn & Crewe, 1991) (Fig. 1.2).

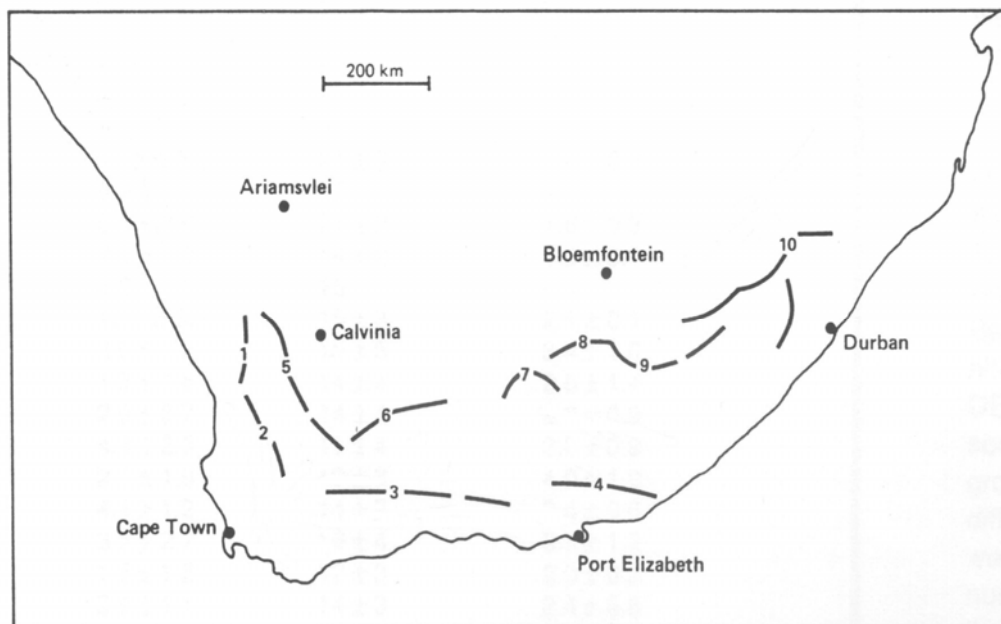


Figure 1.2 Geographical distribution of *A. m. capensis* (South of mountain ranges 1 - 4), *A. m. scutellata* (North of mountain ranges 5 - 10) and the hybrid zone between mountain ranges 1 - 4 and 5 - 10 as drawn from Hepburn & Crewe (1991). The numbers are described in the paragraph above.

In 1909 G.W. Onions (1912) discovered that queenless workers of *A. m. capensis* lay eggs resulting in female offspring rather than male offspring that is the rule in all other honeybee populations. Onions (1912) describe the laying

workers in colonies of *A. m. capensis*, as a pseudo queen, a little dark bee, a menace to the welfare of the apiary and a fatal obstacle to queen-rearing operations. In 1914 Onions ascertained the existence of a well-developed spermatheca in Cape worker bees. He describes the spermatheca as conspicuous, but filled with a clear fluid like those found in unmated queens. This confirmed his theory that the laying workers were not fertilised by drones, but were instead reproducing by parthenogenesis.

This phenomenon of producing diploid offspring without fertilisation is called thelytokous parthenogenesis. *A. m. capensis* workers possess the ability to produce eggs that give rise to female offspring instead of male offspring as is the case with other honeybee races (Tribe & Allsopp, 2001).

Lundie (1954) stumbled by chance onto the phenomenon that laying workers of *A. m. capensis* could invade colonies of *A. m. scutellata*. In his article he described that five colonies of *capensis* honeybees were brought to Pretoria for Italian queen rearing purposes. The bees brought in were black in colour whereas the bees from Pretoria were yellow. After the colonies of Cape bees were kept together with local colonies, some black bees were seen in the yellow-bee colonies. Drifting as the sole explanation for this was ruled out, as the number of black bees in the colonies steadily increased. Some careful notes were taken and he found that these black bees, recognised by their larger abdomens and characteristic attention from the other worker bees, would invade strong as well as weak colonies. Although many would be stung to death, some would succeed in entering the hives and lay eggs. In the beginning they would lay 15 to 25 eggs in each cell, but this would change and become more regular, with fewer eggs per cell. Another important note, was that relatively large areas of brood were found on the outside frames of the brood nest. This never happens with a normal *A. m. scutellata* colony.

Anderson (1963) did some anatomical investigations of *A. m. capensis* worker bees and found that the number of ovarioles in each ovary ranged from 5 to 59 with an average of 19.61. The incidence of asymmetry was high with only 12% of the bees having the same number of ovarioles in the two ovaries. The size of the spermatheca of a laying worker could reach a diameter of about half that

of a queen's. On investigating the spermatheca for the presence of sperm, no sperm could be found. This excludes the drone as a partner in the production of diploid female eggs in this honeybee race. Anderson also describes the fighting in a colony directly after the loss of the queen. This fighting reached a maximum quickly and subsided slowly again.

In his first experiences with Cape bees, Fletcher (1975) recorded the following. The colonies brought from the Cape weakened from December to April, even though the savanna bee colonies in close proximity increased in size. The brood cappings of the Cape colonies were irregular and some prepupa and pupa were uncapped, i.e. bald brood. The colonies from the Cape were less aggressive than the Savanna bees. When comparing the colour of a whole colony, the Cape bees seemed to be darker and he had the impression that they are also larger. He could not use colour differences of individual bees outside a colony as indication of racial origin, as a very yellow bee would probably not be a Cape bee, but a very black bee could be of either race.

Johannsmeier (1983) warned beekeepers not to introduce Savanna bees into the Cape region and *vice versa*. In his experiences with Cape bees in Gauteng, it was clear that the problem of invasion of Savanna bee colonies by Cape worker bees (pseudo-queens) could persist for some years, unless drastic steps were taken to eliminate them. He described the Cape honeybee syndrome affecting Savanna bee colonies as follows:

Bees with black abdominal segments, including those closest to the thorax;
The segments clearly separated by bands of whitish or light grey hairs;
Abdomens longish and dull in contrast to shorter, shiny abdomens of older Savanna bee foragers;
Scattered worker brood that is not diseased;
Increase of the number of black bees;
Disappearance of the African queen and a decrease in colony strength.

The worker laid eggs observed by Johannsmeier (1983) were laid singly, but the position of the eggs was not noted. A queen laid egg would be at the bottom of the cell, while a worker laid egg would be on the side of the cell as the abdomen is too short to reach the bottom of the cells. He also mentioned that

the irregular pattern of colony invasions within an apiary suggested that the Cape bee spread through actively flying workers. Physical removal of these pseudo-queens proved to be futile and gassing of the whole colony (brood included) was the only option to clean an apiary of Cape laying workers.

1.3 HISTORY AND CONSEQUENCES OF THE ‘CAPENSIS CALAMITY’

Migratory beekeeping is one of the best ways to make a good living from honeybees, especially through pollination services and the contracts associated with them. But these migrations of hives across the country have an associated risk of the dissemination of parasites and diseases. According to Allsopp (1993), some 400 *A. m. capensis* colonies were moved from the Lamberts Bay region in the Cape to the Rust-der-Winter region in the Northern Province in 1990. These colonies were incorporated into apiaries with *A. m. scutellata* colonies. The beekeepers took these colonies again to the Rust-der-Winter area during the 1991 winter aloe (*Aloe greatheadii* var. *davyana*) season where further contamination with *capensis* bees occurred. In the same year some colonies originally from the Highflats in Natal that were moved to the Langkloof in the Cape for pollination and returned to Natal. Subsequently, they were moved to the Douglas area in the Northern Cape during August (Allsopp, 1994).

These two major movements of large numbers of colonies into the *A. m. scutellata* region are all that is known to the researcher (Allsopp, 1993 & 1994). One of the movements produced the so-called *capensis* problem bee.

In February 1992 Martin Johannsmeier found some *capensis* laying workers in an apiary of a commercial beekeeper in the Pretoria area. Queen rearing in these colonies would not flourish as in the past. On enquiry it was confirmed that commercial beekeepers had observed the symptoms of *capensis* laying workers a year earlier (M.F. Johannsmeier, pers. comm). He compiled a leaflet (Johannsmeier, 1993) for distribution to beekeepers listing the symptoms as follows:

Outside the hive: reduced foraging; many dead bees, bees involved in fighting. Inside the hive: scattered non-diseased brood with all larval stages next to each other; queenlessness; black *capensis* laying workers together with yellow-banded *scutellata* honeybees; multiple eggs in worker or queen cells; mature queen cells chewed open on the sides. In an advanced stage of take-over these laying worker colonies showed no defensiveness, rapidly weakened and died out or absconded.

The result of this infestation originating from the introduced *capensis* colonies, was that at least 54 000 colonies died or were destroyed in the summer rainfall region. Honey production was drastically reduced and honey prices almost doubled (Allsopp, 1993). Honey production reduced from 32 kg/hive in the seventies to 18 kg/hive in the nineties (Johannsmeier, 2001). Imports of honey were allowed for the first time, with the associated risks of foreign diseases if imports are not adequately monitored. The lack of bees for commercial pollination negatively affected beekeepers, crop producers and consumers (Allsopp, 1993).

For example, pollination of hybrid seed sunflower in the summer rainfall region uses more than 20 000 colonies, while the apple and pear producers in the western Cape need 18 000 colonies. In 2001 there were an estimated 60 000 active colonies in South Africa. According to Du Toit (2001), the honeybee industry contributes R2,5 billion annually to South Africa's GDP, of which the major portion can be ascribed to the value of bee-dependent agricultural crops. Honey, beeswax and other hive products contribute R60 million annually.

With this severe impact on apiculture, beekeepers asked the South African Government for help. Government officials, researchers and beekeepers had various meetings and proposed the so-called Z-line dividing South Africa into *A. m. capensis* and *A. m. scutellata* regions. Transport of colonies across this line was prohibited. But as it had already happened and the damage was already done, various research projects were proposed (see Chapter 1 for details). In addition, it was proposed that all colonies that could be classified as hopelessly queenless should be killed within 72 hours.

1.4 ACTION AGAINST THE 'CAPENSIS CALAMITY'

A general meeting of beekeepers, bee scientists and agricultural officials was held as soon as the problem was detected. Bee scientists of the Plant Protection Research Institute did a survey of the Transvaal (now known as Gauteng, North West, Mpumalanga and Northern Province), Natal and Northern Cape. They found that the problem was widespread and established (Allsopp, 1993). Prominent beekeepers visited the Minister of Agriculture with a petition for relief and support. The Minister appointed a Task Team comprised of beekeepers, members of organised agriculture and the government researchers to investigate the problem. The Task Team recommended legislation dividing South Africa into two bee zones. The Inspectorate of Plant and Quality Control together with the Beekeepers Co-op investigated colonies in infected areas. Only colonies that were moved since September 1990 were included. A financial support system was instituted for beekeepers who had lost colonies to *capensis* invasion, and beekeepers were compensated for some 57 000 colonies.

Legislation was implemented to prevent further spread by *A. m. capensis* laying workers. Regulation R 159 of 5 February 1993 under the Agricultural Pests Act 1983, prohibits the movement of honeybees across a demarcated line. All honeybees north of this line infested with Cape laying workers bees, had to be destroyed. This regulation was amended on 24 December 1998 as R 1674, and it prohibited the keeping of Cape bees north of the borderline. It also stated that any colony that was queenless, or that had *A. m. capensis* laying workers, must be killed within 72 hours.

But all the legislation and control mechanisms failed to eradicate the problem. One of the reasons may have been that only colonies that were moved were inspected and killed. Colonies that were not moved during this period were ignored but could have been infected. Another reason may have been that some beekeepers with infected colonies did not comply with the regulation requiring that infected colonies be killed.

A Working Group on the Capensis problem was established with representatives from the honeybee industry, the Department of Agriculture, the University of Pretoria, Rhodes University and the Agricultural Research Council (ARC). The ARC-Plant Protection Research Institute, University of Pretoria, and Rhodes University conducted the research projects identified by the Working Group in July 1996. A brief description of each project is discussed below.

The author is employed by the ARC-Plant Protection Research Institute and was personally involved in some of the research projects pertaining to this problem and this thesis records these results obtained.

1.5 RESEARCH PROJECTS ON THE CAPE PROBLEM BEE

The following brief descriptions of projects are taken from the research proposals submitted to the Department of Agriculture (Sandmann, E.R.I.C. (Ed.), 1996) who funded this research.

1.5.1 Information transfer

The first and very important project was to get the information on the honeybee problem and possible solutions to this problem, to as many beekeepers and crop producers who used honeybees for pollination, as possible. This information transfer project ended with the publishing of a set of recommendations by Johannsmeier (1997). Copies of this edition of the *South African Bee Journal* were distributed free of charge to all beekeepers and other interested parties. Copies were also distributed from various beekeeping equipment retailers.

1.5.2 Survey of *capensis* laying workers and diseases

The second project involved the survey of the commercial and hobbyist beekeeping stocks in the summer rainfall region of South Africa. The aim was to determine the extent, nature and range of the Cape bee infestation. Six "snapshot" surveys were planned. A greater understanding of the dynamics of

the problem would be gained with the evaluation of the snapshots over a period of time. This would form the baseline data for subsequent further research and management actions that would be scientifically based. The chapter on Materials and Methods gives more information on this project, which is the topic of the thesis. This is one of the projects in which the author was personally involved.

Simultaneously with the above-mentioned survey, a honeybee disease survey was carried out where samples of the brood, honey and workers were taken from four colonies in each apiary for analysis. During the visual inspections of the colonies, obvious disease symptoms were also recorded. This data does not form part of the thesis.

1.5.3 Penetration of the wild population

The aim of this project was to determine whether *capensis* laying workers could become established and survive in the wild population of *A. m. scutellata*. Trap boxes were to be set out, at White River, outside the western border of the Kruger National Park, on the border itself, and 20km inside the park. These trap hives were to be visited at three monthly intervals. Trapped colonies were to be moved to Pretoria for inspection and sampling.

1.5.4 Laying worker characteristic - inheritance

The aim of this project was to determine whether the trait for thelytokous parthenogenesis and its association with social parasitism could be transferred into the *A. m. scutellata* population. This project was abandoned when it became clear that there was no gene transfer between the two subspecies. The problem bees reproduced without hybridisation and were later identified as closely related.

1.5.5 Controlling element

The aim of this project was to identify and isolate the chemical components of the mandibular gland of the *A. m. capensis* queen that control the *A. m. capensis* workers. If this could be accomplished, such components could theoretically be introduced into colonies affected by *capensis* laying workers in order to control or eradicate the problem bees.

1.5.6 Management practices

Three management strategies were tested for controlling *capensis* laying workers or to diminish this effect. The first was to compare common apiary management with the newly recommended management practices. The second was to demonstrate the effect of the number of supers (honey chambers) per colony in exacerbating the *capensis* problem: The more supers on a brood chamber, the higher the risk that a colony would be taken over by laying workers. Thirdly: Limited transport of colonies could prevent further dispersal of *capensis* laying workers. Reduced stress related to transport and multiple flows, as well as fewer opportunities of invading opened hives, would theoretically decrease take-overs by problem bees.

1.5.7 *A. m. scutellata* reservoir

To obtain and maintain a source of unambiguously pure *A. m. scutellata* colonies that would be required for some of the other projects.

1.5.8 Modes of infestation/invasion of *A. m. scutellata* colonies

To determine whether the infestation of the *A. m. scutellata* colonies is directed (socio-parasitic) or non-directed (chance).

1.5.9 Absconding swarms

To determine whether absconding swarms die or continue to live. If the latter these small remnants can add to the *capensis* problem bee infestation.

1.5.10 Other projects resulting from original Working Group Projects

Two more projects in which the author was involved resulted from the original Working Group Projects.

1.5.10.1 Hybridisation of *Apis mellifera capensis* and *Apis mellifera scutellata*: Does it occur and contribute to the *capensis* problem?

The aim of this project was to investigate the genetic relationship between bees sampled in the Piet Retief area in one apiary of a commercial beekeeper. If *capensis* laying workers invaded neighbouring hives, these workers would be

genetically closely related. If hybridisation occurred, the invading bees would be less related or even unrelated to each other. This project was funded by the South African Bee Industry Executive (SABIE) (Kryger & Van der Schyf, 1998).

1.5.10.2 Dispersal of the pseudoclon in survey apiaries.

After the survey (Project 2) was completed it became clear that there were certain apiaries that showed a below average infection rate and an above average survival and queen retention rate. These colonies would be suitable to use to follow the spread of the pseudoclon in an apiary when they are deliberately exposed to infected colonies. In addition, this project will overcome the problem of the time intervals that were too big during the survey.

1.6 SCOPE OF THIS THESIS

The thesis is mainly concerned with the second research project of the Working Group, namely "Survey of *capensis* laying workers and diseases", but will exclude the data collected on diseases.

Using the survey data, we hoped to determine if the use of low risk beekeeping areas (not used by commercial beekeepers) and low risk beekeeping practices (following the recommendations, Johannsmeier 1997) would reduce the rate of infection by the *capensis* laying workers, and to have a positive effect on the survival and production of the colonies in comparison to high-risk areas (used by commercial beekeepers) and high-risk beekeeping practices (based on migration). A third group was included for high-risk areas but with low risk beekeeping practices.

Secondly to report on the findings after DNA studies to determine the genetic background of the problem bees. The relatedness of the *capensis* laying workers would give an indication to whether or not hybridisation occurred between *A. m. scutellata* and *A. m. capensis*. Gene flow between the two subspecies could contribute to the persistence of the problem.

Thirdly to report on the dispersal of the *capensis* laying workers in an apiary using high risk beekeeping practices. Short time intervals between

observations and detailed records would give indications if the *capensis* laying workers actively disperse or are helped by the beekeepers.

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CHAPTER 2

SURVEY FOR *CAPENSIS* LAYING WORKERS IN APIARIES

2.1 INTRODUCTION

Migratory beekeeping is one of the best ways to make a good living from honeybees, especially through pollination services and the contracts associated with them. But the movement of hives across the country brought with it a potential problem. According to Allsopp (1993), some 400 *Apis mellifera capensis* colonies were moved from the Lamberts Bay region in the Cape to the Rust-der-Winter region in the Northern Province in 1990. These colonies were placed together with *Apis mellifera scutellata* colonies in apiaries. The beekeepers returned these colonies to the Rust-der-Winter area again during the 1991 winter aloe (*Aloe greatheadii* var. *davyana*) season where further interaction with *capensis* bees occurred. In the same year some colonies originally from Highflats in Natal that were moved to the Langkloof (*A. m. capensis* region) in the Cape for pollination, were returned to Natal. These colonies were subsequently moved to the Douglas area in the Northern Cape during August (Allsopp, 1994). The origin of the *capensis* laying worker problem is thought to have originated from one these large scale movements of colonies.

The importance of determining the full impact of the effect of *capensis* laying workers was stressed and a survey of the summer rainfall regions honeybee stock was proposed. The aim was to determine the extent, nature and range of the Cape bee infestation. Six surveys were planned. A greater understanding of the dynamics of the problem would be gained from subsequent evaluation of

the surveys over a period of time. This would form the baseline data for further research and management actions that would be scientifically based.

2.2 MATERIALS & METHODS

2.2.1 Regions and background

For survey purposes, the summer rainfall area was divided into eight regions, namely, Northern Province (NP), Mpumalanga (MP), North Gauteng (NG), South Gauteng (SG), Northern Cape (NC), Free State (FS), Northern KwaZulu-Natal (NKN) and Southern KwaZulu-Natal (SKN). These regions are shown in Figure 2.2.1.

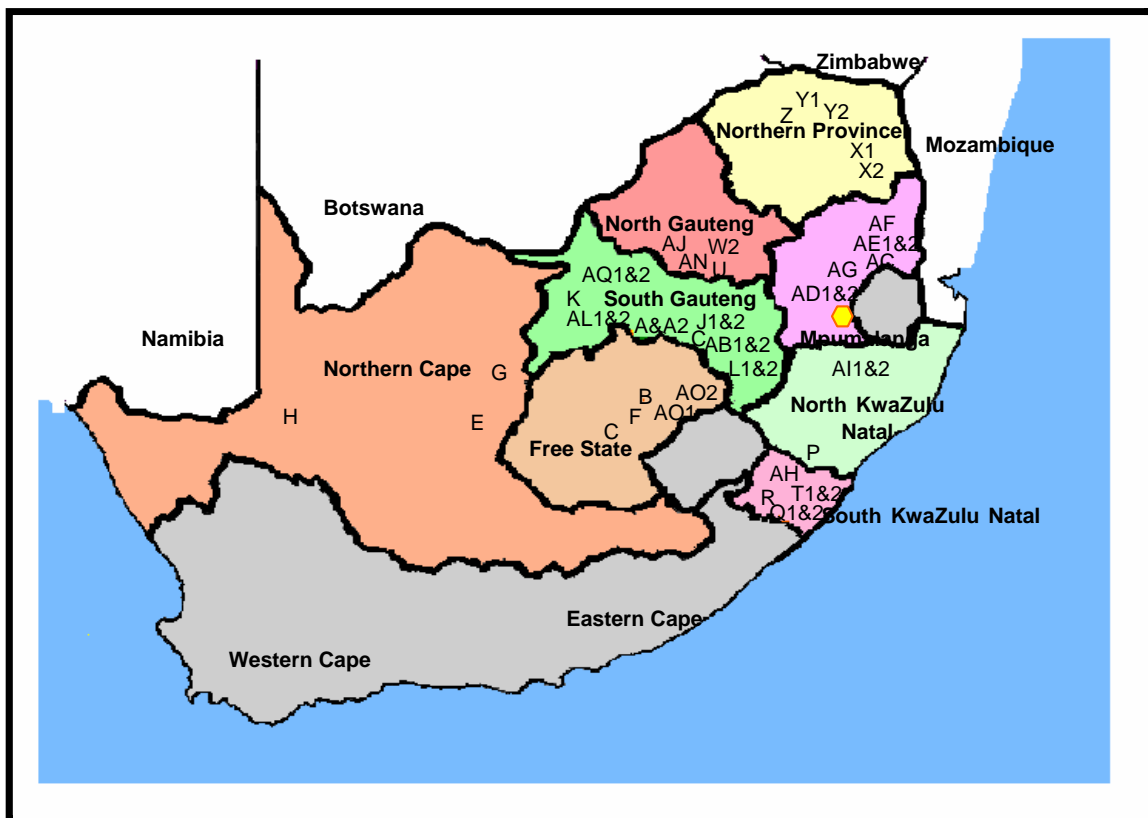


Figure 2.2.1 Map of South Africa showing the regions and sites where the surveys were conducted. The sites are indicated with letters close to where the apiaries were geographically located.

For the eight regions the closest towns to the survey apiaries are given below. If more than one apiary per town was visited, their numbers are given in brackets.

- (i) Free State: Perdeberg, Winburg, Arlington(2), Ventersburg.
- (ii) Mpumalanga: Ermelo (2), Nelspruit (2), Witrivier (2), Hoedspruit, Hazyview.
- (iii) North Gauteng: Pretoria (2), Warmbad, Rayton, Skeerpoort.
- (iv) North KwaZulu-Natal: Vryheid (2), Greytown.
- (v) Northern Cape: Kakamas, Douglas, Warrenton.
- (vi) Northern Province: Louis Trichardt (3), Tzaneen (2).
- (vii) South Gauteng: Vanderbijlpark (2), Parys (3), Klerksdorp, Evander (2), Vredefort (2), Witpoortjie, Randfontein, Derby(2).
- (viii) South KwaZulu-Natal: Richmond, Ixopo, Harding (2), Eston (2).

The beekeeping industry co-operated in the planning of this project and beekeepers were asked by their various associations to participate. Guidelines of what would be expected from each beekeeper were distributed.

Commercial and hobbyist beekeepers operating migratory and sedentary colonies were involved in the survey. Sedentary colonies were not moved at all for the duration of the three surveys, whereas migratory colonies were moved from three to six times.

At least 20 colonies per apiary were needed for inclusion in the survey. Beekeepers selected the colonies and in most cases these colonies were their best stock. Beekeepers were asked to manage the marked colonies in the same way as unmarked ones, i.e. all normal procedures had to be performed, including the killing of infested colonies.

Each beekeeper was expected to give a detailed history of the colonies in each apiary. The period of time that they had the colonies in their apiaries was recorded. In the majority of cases the colonies were all newly caught with presumably young queens. Nectar flows visited by the colonies were also noted. The participating beekeepers were expected to accompany the survey team, which consisted of two technicians and/or researchers from the

Agricultural Research Council's Plant Protection Research Institute. This assured that the beekeepers observed what was being done with their colonies and infestation symptoms were shown to the beekeeper on site.

Beekeepers were called in advance by the author, to make appointments to visit their apiaries. Apiaries in the same area were inspected on consecutive days to cut down on travel and accommodation expenses. Survey 1 started on 4 August 1997 and ended on 10 December 1997. The second survey started on 10 February 1998 and ended on 22 July 1998. The third survey started on 18 August 1998 and ended on 10 December 1998.

All the survey colonies were initially marked, and then inspected for signs of infection and sampled every 5-7 months, irrespective of where they were subsequently located. Each colony received a unique number, comprising one or two letters and two numbers, written on the brood chamber with a Milborrow[®] No-fade ink marker that would not fade in the sun or wash off in the rain. These numbers were used to identify each colony individually. All notes and samples from each colony were marked with this unique number.

All inspections were carried out during the day, mostly during the forenoon. If it rained, an apiary would be inspected on a later date. During the inspection of the colony, Form no. ARC001 (see Appendix A) was completed. The minimum and maximum temperature for the day was recorded. One member of the survey team did the inspections (removing frames etc) while the other member recorded the observations. Internal observations were recorded with each frame that was carefully removed from the hive. Frames were transferred to an empty brood chamber until the queen was found. Supers were covered with a piece of canvas during the inspections to minimise robbing. All frames were transferred back into the original brood chamber in the same order as they were found to minimise disruption of the colony.



Figure 2.2.2 The Inspector, on the left, inspects a brood frame while the recorder, (the author of this thesis) on the right, recorded the observations.

2.2.2 Observations

All observations were made, by the inspection teams, on Form no ARC001 (see Appendix A).

2.2.2.1 External observations

External observations were made before the colony was opened or given smoke. These observations were as follows:

- a. Normal activity at hive entrance? Yes/no.

This observation would give the first indication if the colony under observation might be infested or not.

Normal activity would be when there were workers (foragers) leaving the colony and foragers arriving at the hive entrance carrying pollen or nectar. Those foragers would enter without a fight. If this was the case the colony would be healthy and uninfected.

Abnormal activity would be workers at the hive entrance in groups, without foragers leaving or arriving. Fighting would also be seen as abnormal. This would be indicative of an infected colony.

b. Fighting at the hive entrance? Yes/no.

Bees fighting at the hive entrance or many dead bees outside the colony were an indication of an infected colony (Johannsmeier, 1997). Fighting would be visible between workers in front of the hive or at the hive entrance. Fighting might be between foragers returning and guard bees. Dead workers in front of the hive entrance would be an indication of fighting and recorded as such.

c. Number of supers on hive?

The number and condition of honey chambers on the hive was recorded, as this could be indicative of colony condition. Supers containing honey and filled with workers would indicate a healthy productive colony, while empty supers would indicate a weak colony. Johannsmeier (1997) recommended that hives should be kept as small as possible to ensure that the queen pheromone was present throughout the hive. If the queen's pheromone was absent, as in queenless colonies or in a honey chamber too far removed from the brood chamber, workers started to develop reproductively (Allsopp, 1992).

d. Level of foraging: Ranked from 1-5

Before a hive was opened or given smoke an estimate of the level of foraging was made. Classification of foraging at level 3, would be normal with foragers leaving and returning. Level one (1) foraging would be when only a very few or no foragers left or arrived in the 10 to 20 seconds of observation. This would normally be the case when it was very cold or windy and also in the cases of infestation. At level five (5), foragers would be leaving and arriving in such large numbers that they could not be counted. This would normally happen when there was a good honey flow and fine weather. Allsopp (1992) reported that foraging by the workers decreased after infection.

2.2.2.2 Internal observations

Internal observations were made for each brood frame in the hive. These observations were as follows:

a. Colony size / frames of bees.

When the lid was taken off and smoke was blown from the top into the hive, the number of frames covered with bees was counted. This would be an estimate, since workers could be concentrated at the top of the frames, giving an impression of more workers. When all the frames were removed from the hive, this estimate would be changed if necessary. The lower the numbers of bees in a colony the higher the chance that it might be infected as reported by Allsopp (1992).

b. Estimates of area of brood, pollen and honey on each frame.

Each frame was removed and scanned for the queen. If she was not seen, the frame would be shaken into the brood chamber to dislodge the workers. Each frame was inspected and divided into 8 squares across of equal size on each side. Each area was very close to 1dm² in size. The number of squares that were filled with worker and/or drone brood were counted and recorded. This number would be between 0 and 16. The procedure was repeated for honey and pollen.

c. Assessment of brood pattern: Ranked from 1-5

During the inspection of each frame the quality of the brood pattern was assessed. A very good brood pattern was scored at level 5. This was only given when there was brood of the same age grouped together forming a solid block of capped brood, or larvae or eggs. A score of 3 was given for brood of a similar age grouped together, with a few larvae or eggs between larvae of a different size or capped brood. A score of 1 was given for scattered brood i.e. brood of different ages with empty cells in-between. Johannsmeier (1997) reported that a scattered worker

brood pattern with unevenly aged brood was indicative of infection. See Figure 2.2.3 for an example of a level 1 brood pattern.

d. Brood examination:

A detailed examination was made of the brood. Eggs in cells were scrutinised and if more than one egg had been laid in a cell, this was recorded. The type of cell (worker, drone or queen) was recorded as well. The position of eggs was recorded, e.g. bottom of cells or walls of cells. The position of such cells on the frames was noted, e.g. on the periphery or centre of the frame; also the position of the frame in the hive. Laying worker brood was recorded, as well as the number of frames with such abnormal brood. Raised or flat cappings were noted. These were symptoms indicating possible infection as described by Allsopp in 1992 and Johannsmeier in 1993 and 1997. When drone brood was found it was recorded separately. The presence of queen cells was recorded. Notes were also made as to whether they were normal, cryptic, chewed open, sealed, open, old, or cups.

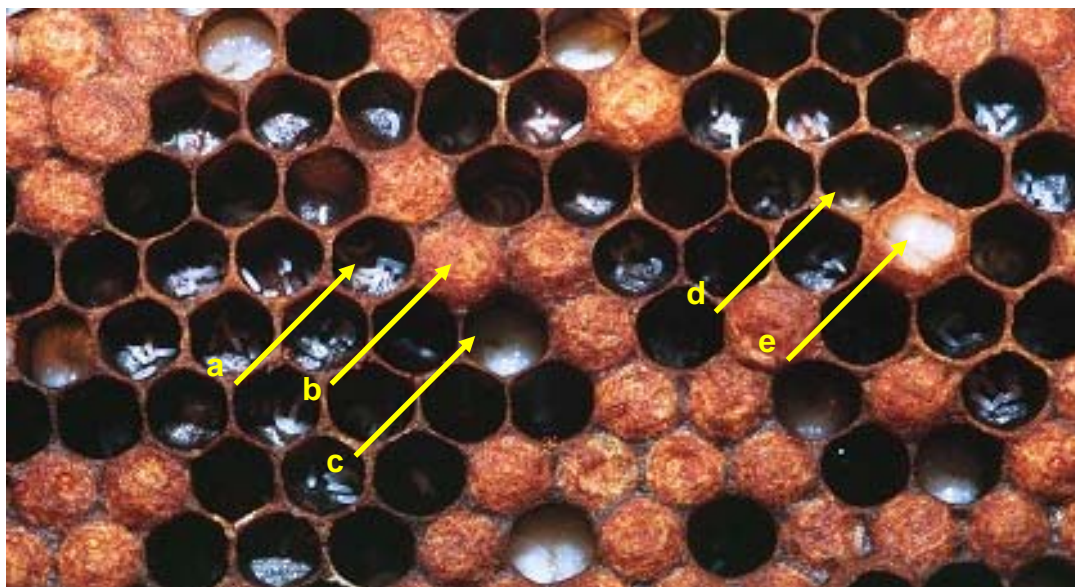


Figure 2.2.3 Laying worker brood showing most of the signs of infection. The arrows point to the following characteristics: a. multiple eggs, b. raised cappings, c. & d. larvae of different ages in a group, e. uncapped prepupa.

e. Fighting on comb: Yes/no

When fighting on the comb was noticed, it was recorded, as this could be indicative of infection according to Allsopp (1992) and Johannsmeier (1997).

f. Colour of the bees: Ranked from 1-5

The colour of the majority of bees on a frame was estimated before the frame was shaken. A score of one (1) was for bees that had very yellow abdomens with no black markings. A score of three (3) was for workers with the more usual yellow abdomen with black markings and sometimes a black tip (see Figure 2.2.4). A score of five (5) was for workers with a black abdomen and no yellow markings (see Figure 2.2.4). Allsopp (1992) and Johannsmeier (1993 & 1997) reported dark or black bees or mixed colours of bees in a colony as indicative of infection. The scutellum colour was not noted, as this would be done during the dissection of workers.



Figure 2.2.4 Yellow (level 3) honeybees, note the yellow scutellum indicated by the arrow marked a. A single black *capensis* bee (level 5) with a black abdomen and black scutellum, indicated by the arrow marked b.

- g. Level of colony defensive behaviour: Ranked from 1-5
Johannsmeier (1993 & 1997) reported that colonies that were infected showed very little or no defensive behaviour. A score of one (1) for colony defensive behaviour was allocated to very docile worker bees, where none came out and flew around the observers. A score of three (3) was the normal behaviour where a fair number of workers were flying around, but not into, the observers. A score of four (4): workers flying around and hitting into the observers with occasional stinging. While a score of five (5) was when workers flew out and attacked the observers and stung the gloves and veil frequently. When the aggression levels reached a level of 5 it was very difficult to work with the colony, as the number of bees gathering on the gloves made it almost impossible to handle frames carefully. Usually a fair amount of smoke was required to calm them down.
- h. Queen present: Yes or no; recording of her colour marking
As the queen was the most important individual in the colony and the one producing the offspring it was necessary to record her presence in the colony. Queenlessness was one of the symptoms of infection according to Johannsmeier (1993 & 1997). When the queen was found, she was caught and marked with Tipp-Ex® on the thorax. During the first survey all queens were marked with a light blue colour. During the second survey unmarked queens were marked pink, and during the third survey unmarked queens were marked light green. Figure 2.2.5 shows the cage in which queens was caught, the light blue Tipp-Ex® and screened lid used for marking. During the second and third surveys queens already marked light blue (in the first survey) or pink (in the second survey) would not be caught again, but the colour on the thorax was noted. On occasion when the queen was not marked, the unmarked queens was noted as unmarked. See the Frontispiece to appreciate the difficulty of finding an unmarked queen between workers. When a queen was not found, this was noted.



Figure 2.2.5 Equipment used to mark queens, TippEx, queen marker lid and queen cage.

2.2.3 Sampling bees for dissection

Sputum bottles (40ml clear plastic) as in Figure 2.2.6 were used to sample the worker bees. Punched holes in the lids prevented the bees from suffocating and becoming moist. Each bottle was labelled with the date of sampling, the region in which it was sampled, the number of the apiary and colony and the beekeeper's code.



Figure 2.2.6 Sample bottles that were used to take samples of worker bees.

Two samples were taken from each colony, of which one was labelled as being the reserve copy. Sample sizes of between 30 and 60 workers were collected per bottle. The second sample was only used if the first sample could not be used for some reason. The original and reserve samples were kept in separate freezers. Samples were taken as randomly as possible, directly from the brood chamber after all the frames were removed. Care was taken not to catch the queen.

The vials of sampled bees were put into a cooler bag with frozen ice bricks to keep them cool until they could be transferred into a freezer. All samples were frozen in a "Minus 40" portable freezer installed in the survey vehicles. The temperature of the freezers ranged from -5 to -10°C. On arrival at the laboratory all samples were transferred to chest freezers. Here they were kept frozen until dissected.

2.2.4 Dissection of bees

Twenty honeybee workers were dissected from each sample and form ARC002 (see Appendix A) was completed for each sample bottle. The colour of the abdomen and scutellum (yellow or black) was recorded before dissection. As the ovarioles disintegrate very quickly, especially in very warm weather, only a few honeybees could be thawed at any one time. These bees were dissected under a binocular stereo microscope, using iridectomy scissors. The ovarioles of each ovary were then transferred to a microscope slide, where they were immersed in distilled water and covered with a cover slip before being counted. By pressing slightly on the cover slip, the ovarioles separated and made counting easier. The development stage of the ovarioles was classified as being, 1 for no development, 2 milky content with no visible oocytes, 3 for milky oocytes just becoming visible and 4 clearly visible mature oocytes (Velthuis, 1970). On dissecting, the size of the spermatheca was estimated as being large (almost queenlike), medium or small (in some cases absent) (See Figure 2.2.7).

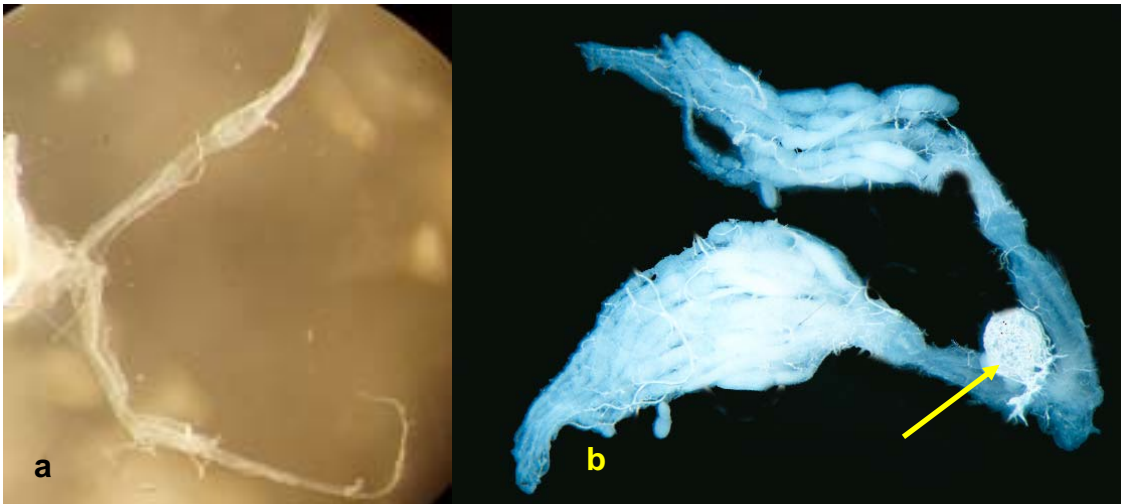


Figure 2.2.7 Stages of ovariole development: a. Stage I ovarioles showing no development and no spermatheca. b. Stage IV ovarioles showing mature oocytes and the large spermatheca is indicated with the arrow.

2.2.5 Classification of apiaries by risk of *capensis* infection

The apiaries were classified into three groupings according to the risk of infection by *capensis* laying workers. The first group of apiaries is the "low risk" group, which includes apiaries of beekeepers in areas that were separated geographically from other beekeepers and their activities. These colonies were not moved and were mainly from hobbyist beekeepers with usually less than 100 colonies.

The "high risk" group consisted of apiaries of commercial beekeepers. These colonies were moved and mixed with colonies from other apiaries and catch swarms. These colonies were also in high risk areas eg. Aloes, sunflower pollination areas, citrus and other fruit pollination areas.

The "medium risk" group were those apiaries that were in high-risk areas, but that were not moved or not exposed to high-risk situations. Alternatively, they were colonies in a low risk area, but were exposed to high-risk situations.

High-risk situations would include the following characteristics:

Migration of colonies.

Mixing of colonies of different origins.

Harvesting honey, or inspecting colonies during the day.

Not killing infected apiaries, but only the infected colonies.

Manipulating colonies by eg. Splitting which leaves one colony queenless for a period of time.

2.2.6 Data Analysis

The data from Forms ARC001 and ARC002 (see Appendix A) were captured in Microsoft Excel spreadsheets. The Agrimetrics Institute of the Agricultural Research Council initially analysed the data with GenStat Release 4.2 for Windows 95. ANOVA tests were done with Statistica Release 6.

2.3 RESULTS

2.3.1 Colonies and Apiary Classification

The survey was done in the summer rainfall area of South Africa from 4 August 1997 until 10 December 1998. The number of apiaries and colonies inspected is given in Table 2.3.1. Samples were taken from all these colonies for dissection.

Table 2.3.1 Total number of apiaries and colonies inspected during the three surveys.

	No of apiaries inspected	No of colonies inspected
Survey 1	61	1142
Survey 2	54	810
Survey 3	39	457
Totals	154	2409

The apiaries were classified into three groupings according to the risk of infection by *capensis* laying workers. The composition of these groups is given in Table 2.3.2.

Table 2.3.2 Number of colonies classified into each of the risk groups with number of apiaries in brackets.

	Low	Medium	High
Survey 1	284 (15)	244 (13)	417 (22)
Survey 2	262 (15)	202 (13)	216 (19)
Survey 3	214 (14)	111 (11)	93 (11)

The number of colonies and apiaries are less than those in Table 2.3.1. This is due to the omission of certain apiaries as discussed in detail in this chapter under 2.4.5 Shortcomings of the survey. This include problems that were unrelated to infection by *capensis* laying workers eg. Honey badgers, baboons, vandalism and the inability to inspect colonies due to problems experienced by the beekeepers.

2.3.2 Observations

2.3.2.1 Colour of worker bees

The colour of the abdomen and scutellum of the worker bees was a good indication of infection by the *capensis* laying workers. With an average colour ranking of between 3 and 4 the non-infected colonies showed a marked difference from the infected colonies with an average between 3,5 and 4,5 (Figure 2.3.1) . The lower values of the non-infected groups indicate that the worker bees were yellow with a few exceptions that raised the averages above 3. The infected group, with the higher values, indicate darker to black worker bees.

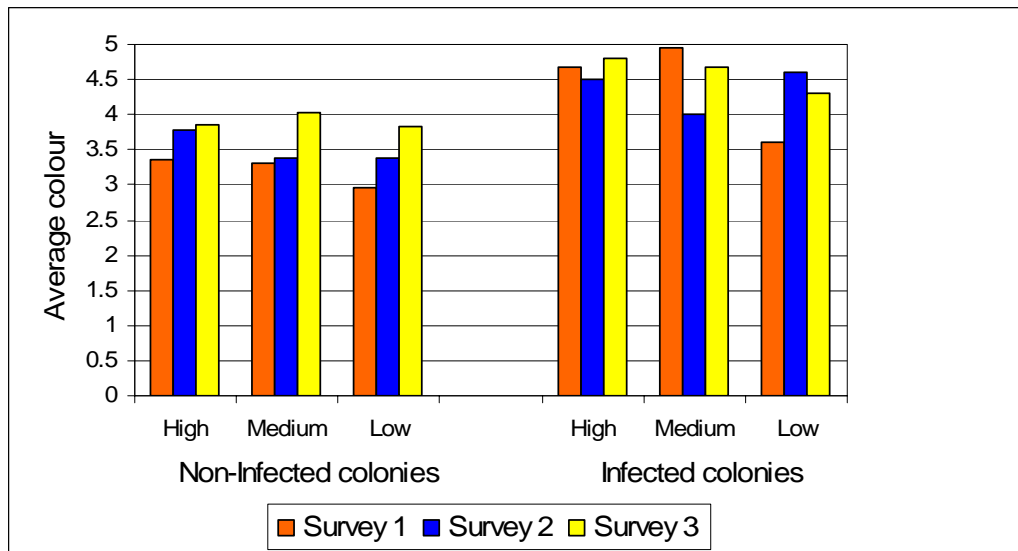


Figure 2.3.1 Colour ranking of worker bees in infected and non-infected colonies in apiaries classified into high, medium and low risk situations.

2.3.2.2 Quality of the Brood pattern

In Figure 2.3.2 the ranking of the quality of the brood pattern in the non-infected colonies is above 2,5 and thus in the range of that of normal colonies. The infected colonies show a decreased quality that varies markedly. This low quality of the brood pattern is regarded as below normal and a definite sign of infection.

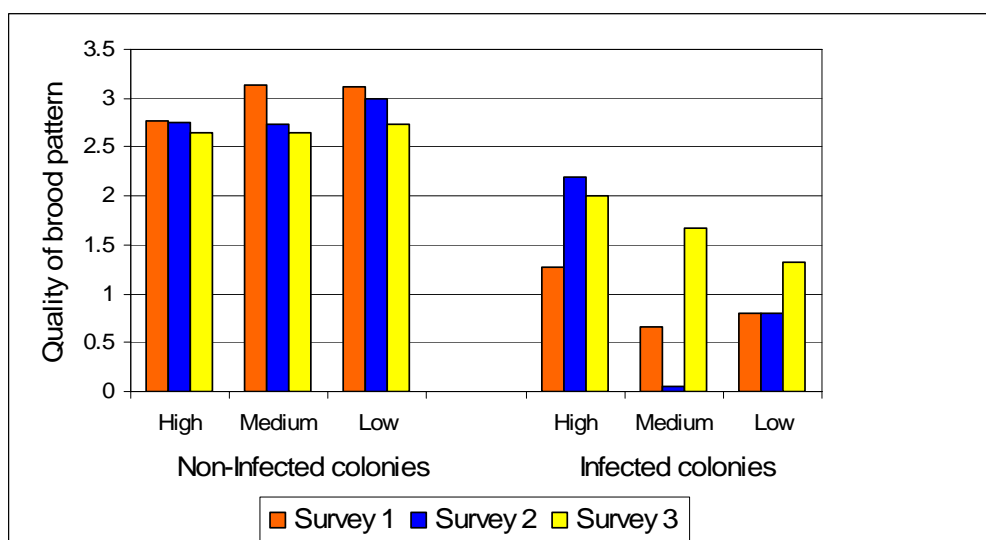


Figure 2.3.2 Ranking of the quality of the brood pattern for infected and non-infected colonies in apiaries classified into high, medium and low risk situations.

2.3.2.3 Colony defensive behaviour

Although there were no statistically significant differences between colony defensiveness in the risk groupings, there was a slight decrease in the level of colony defensiveness in the infected colonies as can be seen in Figure 2.3.3.

Infected colonies had defensiveness scores of 1 and 2 and rarely 3, while non-infected colonies had values of 2, 3 and higher.

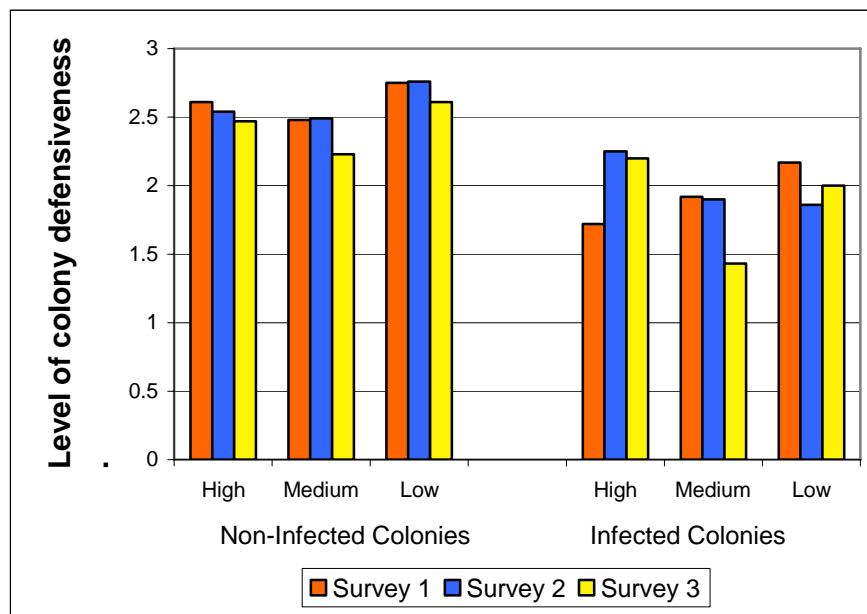


Figure 2.3.3 Level of colony defensiveness in the three risk groupings divided into infected and non infected colonies.

2.3.2.4 Numbers of ovarioles/ovary

There were no statistically significant differences between the mean ovariole counts/ovary obtained during the three surveys and in the three groupings as can be seen in Table 2.3.3. This can be attributed to the fact that there were infected colonies in all three groupings that would give these high counts. Another source of error was that the sampling of workers for dissection was biased to mainly *A. m. scutellata* bees rather than the much less numerous *capensis* laying workers.

Table 2.3.3 Mean number of ovarioles/ovary found in workers sampled from the colonies in the three apiary risk groupings with the maximum count found in each case in brackets.

	Low	Medium	High
Survey 1	4.26 (25)	4.22 (25)	4.31 (35)
Survey 2	4.28 (28)	4.22 (30)	4.42 (36)
Survey 3	4.31 (24)	4.09 (27)	3.99 (34)

2.3.2.5 Ovariole development

In Figure 2.3.4 the ovariole development of workers in the infected colonies is markedly higher than those of the non-infected colonies. The degree of development noticed in the non-infected colonies can be attributed to the fact that in some cases pure *A. m. scutellata* worker bees will start developing their ovaries in the absence of the queen or in early stages of infection these colonies could have been misclassified as uninfected.

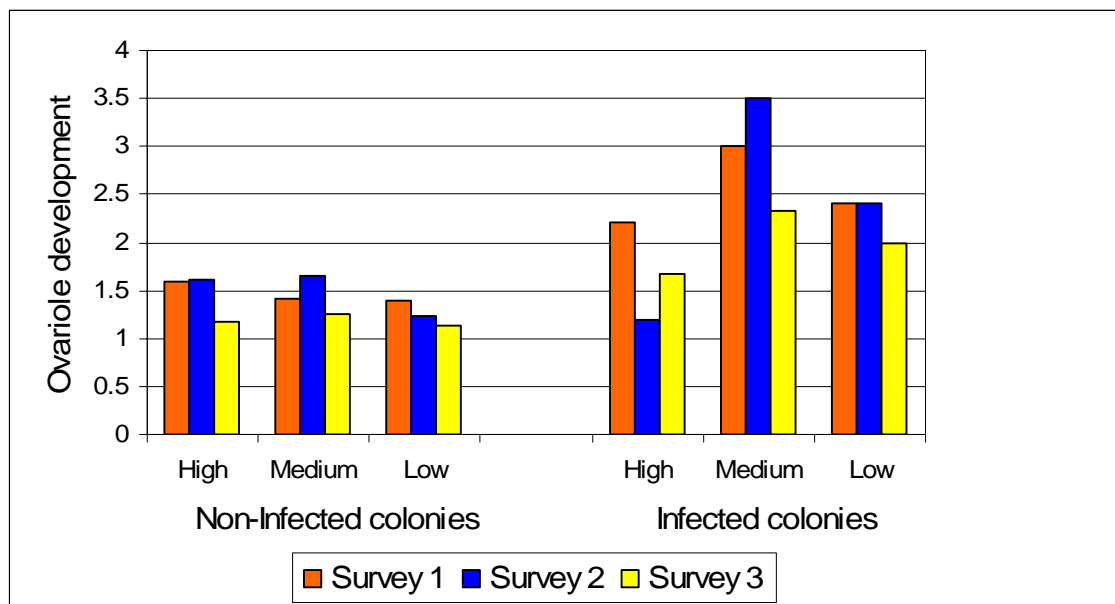


Figure 2.3.4 Level of ovariole development in the workers of infected and non-infected colonies in apiaries classified into high, medium and low risk situations.

2.3.2.6 Queen survival

Table 2.3.4 shows the percentage of queens found and marked in the first survey that were presented in subsequent surveys. The queens found and marked subsequently were not indicated. It shows that the survival of the queens found in the first survey was greater in the low risk group than in the higher risk groups.

Table 2.3.4 Percentage of colonies in Survey 1 in which queens were found and marked with light blue Tippex. The percentage of colonies in surveys 2 and 3 in which queens with a blue mark were identified. Colonies were classified into the three risk groups.

	Low	Medium	High
Survey 1	81.35	88.19	71.68
Survey 2	55.67	49.56	49.52
Survey 3	39.79	33.66	26.54

2.3.2.7 Colony size

The number of worker bees in the colonies decreased with time, but it is clear from Table 2.3.5 that the colonies deteriorated more quickly in the higher risk groups than was the case with the low risk group. The three groups should have been more or less equal for the first survey, but the higher risk groups were already shown to have fewer worker bees.

Table 2.3.5 Mean number of frames filled with worker bees in the three categories of risk for apiaries during the three surveys.

	Low	Medium	High
Survey 1	7.4	6.52	6.79
Survey 2	7.5	6.34	4.17
Survey 3	5.42	2.88	1.79

2.3.3 Infection rates of colonies in three risk situations

As there was no single criterion that could be used to identify an infected colony, the following rules were applied to classify colonies as infected.

The conservative criteria used: If a colony had 5 or more of the following properties it was classified as being infected. The less-conservative criteria were when only 3 of these characteristics were present:

- a. Presence of dark coloured or black bees,
- b. Irregular brood pattern, or absence of brood
- c. No queen,
- d. Very docile workers with little defensiveness,
- e. Multiple eggs in cells,
- f. High numbers of ovarioles/ovary (10 or higher),
- g. A black bee with the highest ovariole count,
- h. A black scutellum on any of the dissected bees,
- i. Ovariole development,
- j. A medium or large spermatheca.

In Figure 2.3.5 it is clear that with the less conservative classification, the low risk group had an infection rate of below 10%. The medium group started with an infection rate of 12% but it increased to 20% within six months and stayed at 20%. The high group started with an infection rate of 18% that increased to 22% within six months and it stayed at 22%.

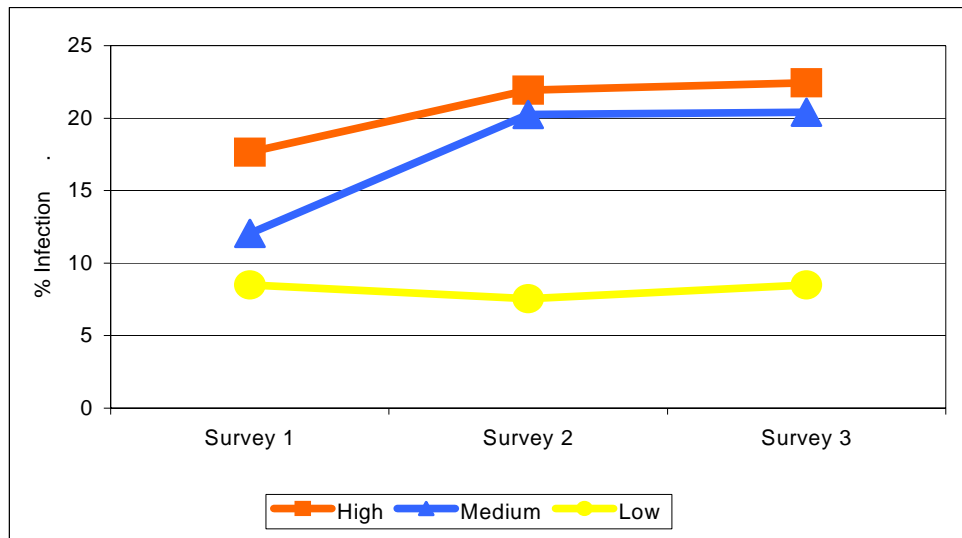


Figure 2.3.5 Percentage infected colonies in three risk situations over time using the less conservative method of classifying colonies as infected.

As the data were the percentage of infected hives in an apiary, they were arcsine transformed to be analysed with a repeated measures ANOVA. The repetitions are the three surveys.

To be able to perform the ANOVA, the data were tested for normal distribution (Shapiro-Wilks W test) and the distribution of all groups was not significantly different from normality at the level 0.01 ($W > 0.84$ and $p > 0.01$ in all cases). Mauchly sphericity test was used to test for sphericity and the assumption holds (Chi-square=1.74, $df=2$, $p=0.418$). The data were also tested for homogeneity of variance with Levene's test and were found to have homogenous variance (df for all F 's: 2,28: Survey 1 $F=0.90$, $p=0.416$; Survey 2 $F=1.33$, $p=0.281$; Survey 3 $F=0.55$, $p=0.585$).

The data showed that there were significant differences in the infection rates in survey 3 (ANOVA: $F_{0.05(2,28)}=5.401$; $p=0.010$), based on the less conservative estimate. The low risk group's infection rate differs significantly from the medium risk group (ANOVA: $F_{0.05(1,28)}=6.391$; $p=0.017$). The medium and high risk group have similar infection rates (ANOVA: $F_{0.05(1,28)}=0.422$; $p=0.521$). The low and high risk groups infection rates differ significantly from each other (ANOVA: $F_{0.05(1,28)}=8.014$; $p=0.008$).

2.3.4 Productivity of colonies in three risk situations

a. Brood production

To be able to perform the ANOVA, the brood production data were tested for normal distribution and the data were normally distributed in all groups ($W > 0.84$, $p > 0.02$), but one (high risk – survey 3, $W = 0.79$, $p < 0.0001$). The Mauchly sphericity test was used and the assumption holds (Chi-square=2.26, $df=2$, $p=0.119$). The data were tested for homogeneity of variance with Levene's test and were found to have homogenous variance (df for all F's: 2,48: Survey 1 $F=0.56$, $p=0.58$; Survey 2 $F=1.03$, $p=0.37$; Survey 3 $p=0.67$).

There were significant differences in brood production among the three risk groups for survey 3 (ANOVA: $F_{0.05(2,48)}=4.682$; $p=0.014$). To determine where the differences were, pairs of groups were compared. Brood production between the low and medium risk groups were similar (ANOVA: $F_{0.05(1,48)}=4.118$; $p=0.048$). Brood production between the medium and high risk groups were similar (ANOVA: $F_{0.05(1,48)}=0.549$; $p=0.462$). But the brood production between the low and high risk groups were significantly different (ANOVA: $F_{0.05(1,48)}=9.049$; $p=0.004$). This data are represented in Figure 2.3.6.

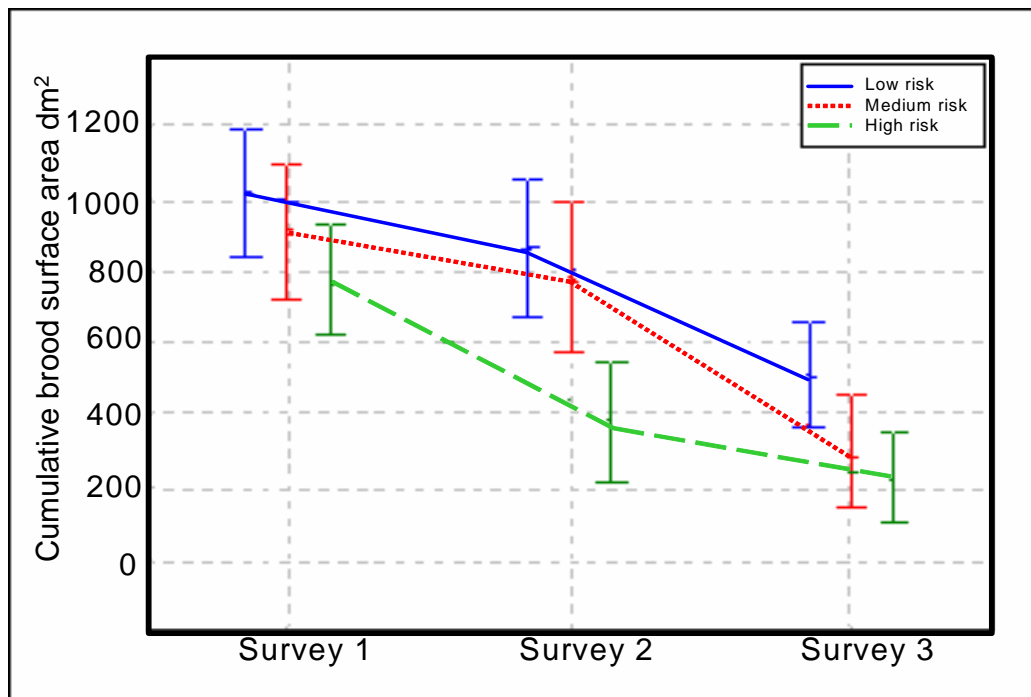


Figure 2.3.6 Brood production in colonies for three risk groups over time. Vertical lines denote 95% confidence intervals.

b. Honey production

To be able to perform the ANOVA, the honey production data were tested for normal distribution. The data of all groups was normally distributed ($w > 0.90$, $p > 0.03$), but two (high risk – survey 1, $W = 0.87$, $p = 0.01$ and high risk – survey 3, $W = 0.79$, $p = 0.001$). Mauchly sphericity test was done to test for sphericity but the assumption fails $p = 0.014$. Nevertheless the ANOVA was performed, but its validity was verified with a multivariate test which is not dependant on the sphericity assumption. The data were tested for homogeneity of variance and were found to have homogenous variance at the level 0.01 (df for all F's: 2,48: Survey 1 $F = 4.74$, $p > 0.01$; Survey 2 $F = 0.73$, $p = 0.49$; Survey 3 $F = 4.22$, $p = 0.02$).

Wilks multivariate test showed significant differences in the honey production ($F = 10.55$, $p < 0.0001$) and that the risk group had a significant influence on honey production ($F = 2.82$, $p = 0.000$). The repeated measures ANOVA results were comparable to those of the multivariate test as it showed that there were significant differences between the three risk groups for survey 3 (ANOVA: $F_{0.05(2,48)} = 12.672$; $p = 0.000$). To determine where the differences were, pairs were compared. Between the low and medium groups there was a significant difference of honey production (ANOVA: $F_{0.05(1,48)} = 12.470$; $p = 0.001$), as well as between the low and high risk groups (ANOVA: $F_{0.05(1,48)} = 23.949$; $p = 0.000$). On the other hand, there were no significant differences in honey production between the high and medium risk groups (ANOVA: $F_{0.05(1,48)} = 0.924$; $p = 0.341$). Honey production data is presented in Figure 2.3.7.

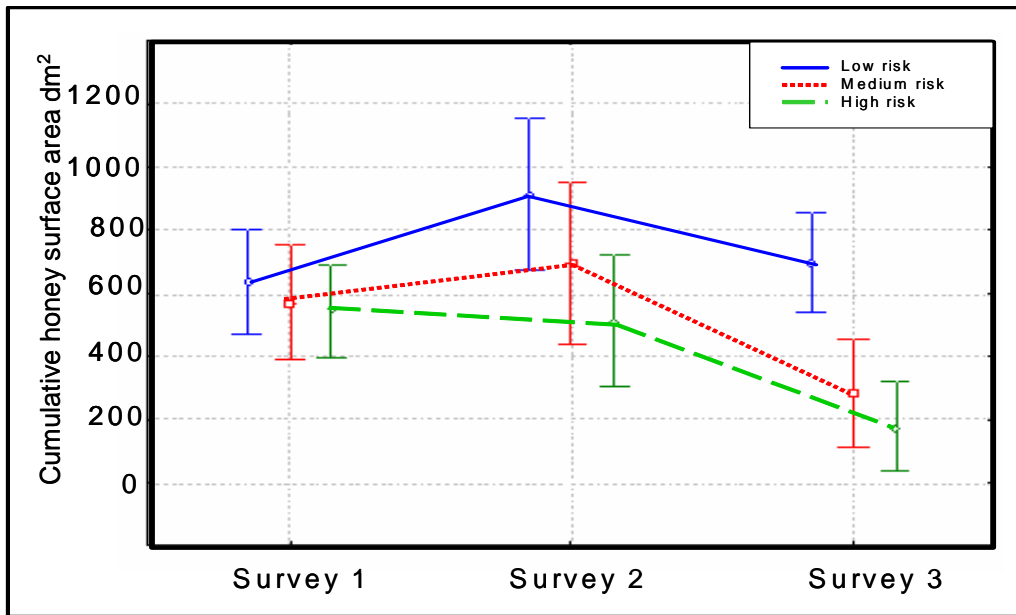


Figure 2.3.7 Honey production in colonies of the three risk groups over time. Vertical lines denote 95% confidence intervals.

2.3.5 Survival of colonies in three risk situations

Survival rate of colonies is a good indicator of the sustainability of an apiary. Figure 2.3.8 shows that the high risk group lost more colonies in the six months between the first and second survey than the other two groups. The medium risk group had losses comparable to that of the low risk group. The losses after 12 months showed that the high risk and medium group lost significantly more colonies than the low risk group.

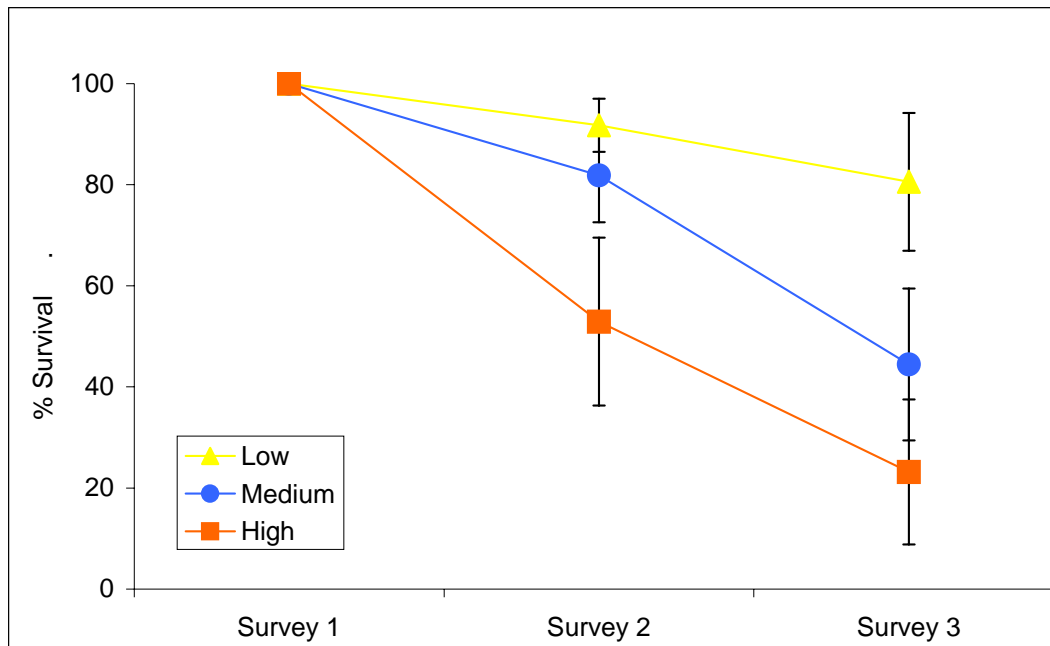


Figure 2.3.8. Percentage survival of colonies in apiaries of the three risk groups during the three surveys.

In the high risk group, only 44% of colonies survived to six months (Figure 2.3.8) of which 22% were infected. This suggests that for every 1000 colonies only 440 will survive for six months, but of those 440 at least 97 would be infected and be destined to die. In addition, the infected colonies also show a decline in brood and honey production (Figure 2.3.6 & 2.3.7).

Survival analysis shows that the survival time of the 3 risk groups is significantly different: $\text{Chi}^2 = 170.86$; $\text{df} = 2$; $p < 0.0001$. A two groups comparison with Gehan's Wilcoxon Test shows that all groups have significantly different survival times.

A regression model was used to determine the variables correlated with survival time. A general Chi square test for the regression model determines that at least some of the variables measured were significantly correlated with survival time: $\text{Chi}^2 = 87.148$; $\text{df} = 8$; $p = 0.000$. The details of the variables are given in Table 2.3.6.

Table 2.3.6 Correlation of various variables with survival time (variables with $p < 0.05$ are significantly correlated).

Variable	p value
Multiple eggs	0.001*
Colour	<0.0001*
Brood pattern	0.030*
Colony defensiveness	0.129 NS
Ovariole count	0.052 NS
Mean number of ovarioles	<0.0001*
Ovariole development	0.032*
Queen loss	<0.0001*

The correlations indicate that the presence of multiple eggs, the colour of the worker bees, the brood pattern, mean number of ovarioles, ovariole development and presence/absence of queen are correlated with the survival of the colony. Their use in the diagnosis of infected colonies is thus strongly supported by this analysis.

2.4 DISCUSSION

The aim of this project was to determine the extent, nature and range of *capensis* infestation. From the data it is clear that the *capensis* laying workers can be found everywhere in the summer rainfall region of South Africa and that they are a serious pest causing the loss of 56% of the sampled colonies in six months.

The main characteristics used for diagnosis of the presence of *capensis* laying workers, as well as the effect of beekeeping practices on the effect and spread of the *capensis* laying workers will be discussed.

2.4.1 Characteristics used for diagnosis

2.4.1.1 Colour of worker bees

The colour of the worker bee in infected colonies corresponded with the reports from Allsopp (1992) and Johannsmeier (1993 & 1997), that darker bees and mixed colours were indicative of infection. Colour of worker bees could not be used as a single criterion to classify a colony as infected or not, but the darker the worker bees the higher the chance that the colonies were infected.

2.4.1.2 Brood pattern

The less uniform brood pattern of infected colonies showed that the queen was either absent or that she could not lay eggs in a normal manner. *Capensis* laying workers can lay single or multiple eggs in the cells prepared for the queen, and thus contribute to the poor brood pattern. This was consistent with the reports from Allsopp (1992) and Johannsmeier (1993 & 1997). A poor brood pattern can be indicative of infection, but should be used in conjunction with the other criteria.

2.4.1.3 Colony defensive behaviour

In general the colonies that were infected by the *capensis* laying workers behaved less aggressively than those that were not infected. This was also described as a symptom of *capensis* dwindling syndrome by Johannsmeier (1993, 1997). Ruttner (1977) described the classical *A. m. capensis* honeybee as less aggressive than *A. m. scutellata* and this trait has been expressed in the *capensis* laying workers.

2.4.1.4 Numbers of ovarioles/ovary

Numbers of ovarioles/ovary were used as one of the criteria for identification of the *capensis* laying workers. Hepburn and Crewe (1991) described the ovariole

number of *A. m. capensis* as 10 and for *A. m. scutellata* as 5, with intermediates in the hybrid zone. With the survey the mean numbers of ovarioles/ovary for the groupings are all in this range of a mean of less than 5 ovarioles, as would be expected in the *A. m. scutellata* region. This corresponds with the literature of various other authors like Anderson (1963) on *A. m. capensis* and Hepburn *et al* (1998) on both races.

But what is notable, is that extremely high numbers of ovarioles/ovary in individual bees were observed. These high numbers can be attributed to the *capensis* laying workers, which act as social parasites. The preferential feeding that the larvae in host colonies get (Beekman *et al*, 2000) favours the development of ovaries with larger numbers of ovarioles.

The use of numbers of ovarioles/ovary for diagnosis of infection is limiting. By calculating the means of the numbers of ovarioles/ovary for a colony cannot be used as a criterion for identifying the presence or absence of the *capensis* laying workers in a colony. Sampling effects in the collection of the worker samples from the colonies show that the majority of individuals in the sample will be host workers with a small and variable number of parasite workers being present. Maximum counts can be misleading as pure *A. m. scutellata* honeybees can have numbers of ovarioles/ovary higher than 5, and in addition to that can *capensis* laying workers on occasion have numbers of ovarioles/ovary lower than 5.

2.4.1.5 Ovariole development

Some of the non-infected colonies showed development of the ovarioles, which can be an indication of early infection. But in non-infected *A. m. scutellata* queenless colonies, the workers' ovarioles can also start developing. The high rate of development in infected colonies shows clearly that ovariole development is a very good indicator to confirm infection.

2.4.1.6 Queen survival

Previous studies have shown that the queen disappears soon after the host colony is infested (Johannsmeier, 1997; Martin *et al*, 2002). From the data it was clear that more queens were retained and over a longer period, if low risk beekeeping practices are performed. This should motivate beekeepers to alter to the low risk beekeeping practices as described by Johannsmeier (1993 & 1997) and Kryger *et al* (2003).

2.4.1.7 Colony size

The mean size of the colonies in the high and medium risk beekeeping practices are in all cases smaller than those of the low risk group. This would have a negative impact on the productivity of the colonies in the higher risk groups.

2.4.2 Infection rates

The method developed to identify infected colonies according to specific criteria as described in 2.3.4, worked well for the analysis of the data. The low risk group showed a significantly lower infection rate than that of the high and medium groups. In the high and medium groups the infection rate increased to 22% and 20% respectively in the first six months, and stayed at the same percentages for the following six months. Thus with low risk beekeeping practices the infection rate was significantly lower than in any other case.

The less conservative estimate is more representative of the natural situation in apiaries. Even though the inspections were punctual in time, characteristics were monitored that might not occur simultaneously in a colony. For example a queen might be still present, but multiple eggs laid by the *capensis* laying workers would be observed. Or there might be just a few *capensis* laying workers with black abdomens in a colony and the observer might overlook them in a colony with 50 000 workers.

Therefore the precautionary principle should be applied: rather decide to kill or isolate an apiary if only a few (not necessarily five or more) signs of infection are present.

2.4.3 Productivity

Brood production showed a gradual decline with increasing beekeeping risk practices, as there were no significant differences between the Low and Medium or Medium and High risk groups. But there were significant differences between the Low and High risk groups that implied that the colonies would be more productive with Low risk beekeeping practices.

Honey production shows a significant difference between the Low and Medium and also between the Low and High risk groups. With more honey being produced in Low risk beekeeping practices this would make adoption of Low risk practices more acceptable.

2.4.4 Survival

The data showed a slight drop in colony survival for the low and medium risk beekeeping practises groups between the first and second surveys. At this stage, survival in high risk groups was much lower and this trend was exacerbated 6 months later. Overall, the survival of colonies was significantly better in the low risk group. This data should act as motivation for beekeepers to adopt low risk beekeeping practises as described by Johannsmeier (1993 & 1997) and Kryger *et al* (2003).

2.4.5 Shortcomings of the survey

Only three of the six planned surveys were done. The Working Group decided after the third survey that the surveys should be stopped as the number of colonies had already decreased by 60%.

The time interval between the consecutive surveys was too long. This makes it difficult to estimate if a colony was indeed infected or not. It also makes it impossible to get a clear picture of the deterioration of a colony from first infection until death or absconding.

Various problems necessitated that apiaries were excluded from data analysis. Two beekeepers experienced problems with honey badgers breaking the hives and the colonies absconded. One beekeeper had problems with baboons that caused the colonies to abscond. Two beekeepers had vandalism problems to such an extent that the colonies could not be inspected while various others had minor problems with vandalism, but the colonies remained. Two beekeepers sold all their colonies. One apiary was killed after the orchard that was being pollinated, was sprayed with an insecticide. One beekeeper fell ill and the apiaries could not be inspected subsequently. One commercial beekeeper could not find the marked colonies in his apiaries. A few of the dissection samples were damaged and could subsequently not be dissected. When it rained the colonies could not be inspected. If possible those colonies were inspected at a later date, but that was not in all cases possible.

A few criteria were left out of the data analysis. Normal activity at the hive entrance, fighting at the hive entrance and the level of foraging were left out as there are too many unrelated factors that could influence these criteria, e.g. weather conditions, temperature, wind speed, birds like the drongo, nectar flows and robbers from nearby colonies. Number of supers on hive was also excluded as in most of the cases there were 1 or no supers on the hive. Fighting on comb was observed on very rare occasions.

2.5 CONCLUSIONS

The relationship between the colour of the worker bees that was positively correlated with the quality of the brood pattern, level of colony defensiveness and negatively correlated with numbers of ovarioles/ovary and ovariole

development suggest the following: on finding black worker bees the incidence of finding a below average brood pattern and docile bees increases. Simultaneously the number of ovarioles increases as well as the incidence of finding developed ovarioles.

As the quality of the brood pattern was also correlated with the incidence of finding marked queens, the colony defensiveness, numbers of ovarioles/ovary and ovariole development, this suggests the following: with a below average brood pattern the incidence of finding a marked queen decreases as well as the level of aggression. The number of ovarioles in workers is likely to increase as well as the incidence of finding developed ovarioles.

If all these findings are taken into account, a simple example of a beekeeper with 1000 healthy productive colonies can expect the following when using high risk beekeeping practices. Of the only 440 (44%) of the colonies surviving the first six months, 22% would probably be infected and be destined to die, thus leaving the beekeeper with 343 productive colonies after only six months.

If the recommendations of Johannsmeier (1993 & 1997) and Kryger *et al* (2003) are followed, the same beekeeper with the 1000 healthy productive colonies would have 920 (92%) of which 7,5% might be infected, leaving the beekeeper with 857 productive colonies after six months. Low risk beekeeping practices are in the long run more economical than medium or high risk practices.

As commercial beekeepers with thousands of colonies, would not be able to stop migrations altogether, they should consider introducing management practices that are less prone to favour infections (Kryger *et al*, 2003).

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CHAPTER 3

GENETIC BACKGROUND OF *CAPENSIS* LAYING WORKERS

3.1 INTRODUCTION

The honeybee, *Apis mellifera* is characterized by a haplodiploid system of reproduction. Workers of the subspecies *A. m. scutellata* are able to lay only haploid male eggs by arrhenotokous parthenogenesis. In contrast, workers of the subspecies *A. m. capensis* native to the Western Cape, are able to produce diploid female eggs by thelytokous parthenogenesis (Onions, 1912). Both these subspecies occur in South Africa with a more or less stable hybrid zone between them (Hepburn & Crewe, 1991).

In 1990 a large number of colonies were moved from the *A. m. capensis* region into the *A. m. scutellata* region leading to the so-called capensis calamity (Allsopp, 1993). Various other migrations took place after this, with the movement of the two subspecies to and from the two regions over the hybrid zone (Allsopp, 1994). This according to Allsopp (1993) led to the loss of 54 000 colonies in the *A. m. scutellata* region.

In 1998 various research projects were done on the capensis calamity, and it was decided to investigate the genetic background of the workers that acted as parasites in the *A. m. scutellata* host colonies. As there was such a large number of colonies moved, it was speculated that there would be various strains of *A. m. capensis* workers acting as parasites.

Differentiating the two subspecies on the basis of a few morphological traits has been done (Chapter 2 of this dissertation). The *capensis* laying workers tend to have more as well as fully developed ovarioles in ovaries, a large spermatheca and a darker colour. Yet, hybridisation between the two subspecies is likely to produce intermediate types, more difficult to differentiate.

Moritz and Haberl (1994) have shown that the genetic outcome of thelytokous parthenogenesis in *A. m. capensis* is mostly like a cloning process. They found that the genetic variation among laying workers of *A. m. capensis* is extremely small due to central fusion of the meiotic products and a lack of recombination on the chromosomes. All offspring of an individual *capensis* laying worker are nearly genetically identical to the mother. This allows for easy recognition of multiple *capensis* laying workers' offspring with the use of DNA fingerprinting.

3.2 MATERIALS & METHODS

Eighteen colonies in an apiary in the Piet Retief area were inspected and samples of the workers bees taken in 500ml plastic honeyjars (± 200 worker bees). The genotypes of the bees were analysed with the help of DNA fingerprinting via microsatellite markers (Estoup *et al.* 1993 & 1994, Kryger & Estoup 1994, Kryger & Moritz 1997). This technique makes it possible to determine whether the different bees invading the different hives were closely related i.e. originating from one or a few related *capensis* laying workers in the apiary or surroundings, or whether those bees with very high ovariole numbers were daughters of the hive's queen, i.e. half-sisters of the other workers, but with a *capensis* father.

The worker bees that were selected had black abdomens and scutella. They also had high ovariole counts. For comparison yellow bees from the same colonies were also analysed. Thirty workers per colony were analysed.

3.2.1 DNA extraction procedure

DNA was extracted from the left front leg of the honeybee by boiling it for 15 minutes at 100°C in a 5% Chelex® solution (Walsh *et al*, 1991), in a 2,0ml micro centrifuge tube (Figure 3.2.1). The tube was then vortexed for 5 seconds and centrifuged for 3 minutes at 140 000rpm. The DNA in the top layer of the solution was removed for subsequent analysis.



Figure 3.2.1 Two ml Eppendorf tubes that were used for DNA extraction.

3.2.2 Polymerase chain reaction (PCR) procedures

The extracted DNA was amplified using the polymerase chain reaction (PCR) with primers specific for the honeybee microsatellite loci (Table 3.2.1). DNA was used in PCR reactions under the conditions as in Table 3.2.2. The loci selected were of different sizes (lengths) to cover the range of lengths to be expected for honeybees. For economic reasons they were selected on being compatible at annealing temperatures to minimise the use of chemicals and reduce time spent in the PCR machine.

Table 3.2.1 The micorsattelite loci with their associated primer sequences and PCR conditions for the amplification of the DNA extracted from the sampled worker bees.

Locus	Size	Primer sequence	MgCl ₂ (mM)	Annealing temp.	Number of cycles
A7	131	5'-CCCTTCCTCTTTTCATCTTCC-3' 5'-GTTAGTGCCCTCCTCTTGC-3'	1.2	58°C	35
A8	160	5'-CGAAGGTAGGTAAATGGAAC-3' 5'-GGCGGTAAAGTTCTGG-3'	1.2	55°C	30
A24	100	5'-CACAAAGTTCCAACAATGC-3' 5'-CACATTGAGGATGAGCG-3'	1.2	55°C	30
A28	140	5'-GAAGAGCGTTGGTTGCAGG-3' 5'-GCCGTTTCATGGTTACCACG-3'	1.7	54°C	30
A35	115	5'-GTACACGGTTGCACGGTTG-3' 5'-CCTCGATGGTCGTTGTACCC-3'	1.2	58°C	30
A76	250	5'-GCCAATACTCTCGAACAATCG-3' 5'-GTCCAATTCACATGTCGACATC-3'	1.2	60°C	30
A79	120	5'-CGAAGGTTGCGGAGTCCTC-3' 5'-GTCGTCGGACCGATGCG-3'	1	62°C	30
A88	150	5'-CGAATTAACCGATTTGTGCG-3' 5'-GATCGCAATTATTGAAGGAG-3'	1.2	55°C	30
A113	220	5'CTCGAATCGTGGCGTCC-3' 5'-CCTGTATTTTGCAACCTCGC-3'	1	60°C	30

One µl of the DNA extract was pipetted into a well of a cooled 96-well microplate's well (Figure 3.2.2). Nine µl of the reaction mixture A, B or C was added depending on the loci being analysed (Table 3.2.2). The process was repeated for the 96 honeybee samples and the wells were covered with lids. As the different primers need different annealing temperatures, (Table 3.2.1), their PCR programmes differ. Thus a sample of the DNA of each bee was run under three different cycling conditions.

Table 3.2.2 Composition of the PCR reaction mixtures used for analysing the DNA of the workers sampled (All values are μl).

Chemical	Reaction mixture A	Reaction mixture B	Reaction mixture C
PCR buffer (10X) with MgCl_2	110	110	110
A7 I	22		
A7 II	22		
A35 I	33		
A35 II	33		
A8 I		33	
A8 II		33	
A24 I		22	
A24 II		22	
A88 I		22	
A88 II		22	
A76 I			22
A76 II			22
A113 I			22
A113 II			22
DNTP	22	22	22
TAQ (enzyme)	7	7	7
DNA for calculation	110	110	110
H_2O	741	697	763

For the primers A7 and A35 the programme was set for denaturing at 94°C for 3 minutes, 30 amplification cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and ending with a elongation step of 5 minutes at 72°C . For the primers A8, A24, and A88 the programme was set for denaturing at 94°C for 3 minutes, 30 amplification cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and ending with a elongation step of 5 minutes at 72°C . For the primers A76 and A113 the programme was set for denaturing at 94°C for 3 minutes, 30 amplification cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and ending with a elongation step of 5 minutes at 72°C .

Primers A28 and A113 were not initially used in this analysis as their annealing temperatures were too low and too high respectively for combining into the reaction mixtures. A113 would be used if it became clear that a very long

sequence i.e.220bp would be needed for analysis. A28 would be used if it became clear that the differentiation between 131bp and 150bp needed to be finer as A28 has 140bp.



Figure 3.2.2 Ninety six well Eliza plates used for the PCR procedure.

3.2.3. DNA sequencing

A reaction mixture was prepared by mixing 7 μ l loading buffer, 35 μ l formamid and 14 μ l TAMRA (100bp) (Perkin Elmer) a standard for comparison. The PCR products were then combined from the wells in the three plates eg, the product in well A1 of plate 2 and 3 was added to well A1 of plate 1. 0.5 μ l of the PCR products was put in a marked micro centrifuge tube (Figure 3.2.1) and 1.5 μ l of the reaction mixture added. The amplified sample was then analysed with the DNA automated sequencer (ABI 310). After the sequencing procedure was finished the results were downloaded from the computer system and interpreted.

3.3 RESULTS

The honeybees listed in Table 3.3.1, with the black abdomens, black scutella, high ovariole counts (ranging from 8/5 to 15/24) and big spermathecae were identified as *capensis* laying workers. When their DNA fingerprints were compared, they were found to be identical. These honeybees originated from different colonies.

In the cases where the morphological characteristics were not consistent with the individuals being *capensis* laying workers (14-1, 14-4 and 3-3 in Table 3.3.1) i.e. black abdomen and black scutellum bees but with a small spermatheca and low ovariole counts, the DNA fingerprints were also different.

The DNA fingerprints of the yellow bees from the same colony showed what would be expected from a normal sexually reproducing outbred population - a normal distribution of alleles with homozygosity and heterozygosity for the different alleles. It could be established that they were sisters of one mother (queen) but with various fathers, as would be expected with polyandry.

Table 3.3.1 Physical characteristics of a sample of bees from three colonies (N3, N14 & N18) with an assessment of whether they were *capensis* laying workers.

Bee #	Colour of scutellum	Colour of abdomen	Spermatheca size	Ovariole stage development	Ovariole count - Left	Ovariole count - Right	<i>Capensis</i> laying workers
14-1	Black	Black	Small	I	3	3	No
14-2	Black	Black	Big	I	25	23	Yes
14-3	Black	Black	Big	I	10	9	Yes
14-4	Black	Yellow	Small	I	5	7	No
14-15	Black	Black	Big	I	14	17	Yes
3-2	Black	Black	Big	IV	11	13	Yes
3-3	Black	Yellow	Small	I	2	5	No
18-17	Black	Black	Small	I	8	10	Yes
18-22	Black	Yellow	Small	I	17	19	Yes

Table 3.3.2 shows the data from a colony at an early stage of infection, in which there were only a few workers actively laying eggs. The presence of the clonal *capensis* laying worker was confirmed by the microsatellite studies (bee number 3-2 collected on 11/03/1998 in Table 3.3.1). The queen was still present, but the brood pattern was already irregular and the level of aggression was low. As can be seen from Table 3.3.2, the colony seemed to be healthy with enough food, workers and brood, but was nevertheless infected.

Table 3.3.2 Field data of one colony (N03) at an early stage of infection by the clonal *capensis* laying workers.

N03	01/10/1997	11/03/1998
Total area of brood (dm ²)	40	108.5
Total area of honey (dm ²)	33	26
Colony size (frames of bees)	5	9
Level of foraging (1=poor; 5=good)	1	3
Colour of bees (1=yellow; 5=black)	3	3
Quality of brood pattern (1=poor; 5=good)	4	3
Queen present?	Yes	Yes
Queen colour mark	Blue	Blue
Level of aggression (1=docile; 5=very aggressive)	3	2
Multiple eggs in cells?	No	No

From Table 3.3.3 it is clear that the colony deteriorated from a queen-right active colony to a colony in which clonal laying workers were active. The queen could not be found, and there were no signs that a queen was present. This is an example of a middle stage of infection. The level of foraging, number of worker bees, brood pattern and level of aggression decreased. A greater proportion of the workers were black on 11/03/1998 and multiple eggs were seen. DNA fingerprints of bees collected from this colony showed 3 *capensis* laying workers with identical DNA fingerprints i.e. bees no 14-2; 14-3 and 14-15 in table 3.3.1.

Table 3.3.3 Field data of one colony (N14) where *capensis* laying workers were active.

N14	01/10/1997	11/03/1998
Total area of brood (dm ²)	80	46.75
Total area of honey (dm ²)	23	52
Colony size (frames of bees)	8.5	6
Level of foraging (1=poor; 5=good)	3	2
Colour of bees (1=yellow; 5=black)	2	5
Quality of brood pattern (1=poor; 5=good)	4	2
Queen present?	Yes	Not sure
Queen marked colour	Blue	None
Level of aggression (1=docile; 5=very aggressive)	3	2
Multiple eggs in cells?	No	Yes

Both these colonies were from the same apiary. On the 01/10/1997 there were 20 colonies in this apiary and the number of colonies decreased to 18 on the 11/03/1998 of which 8 had multiple eggs and 9 were queenless. This beekeeper decided to kill all the colonies in this apiary, after the inspection of 11/03/1998, thus the spread of the clonal *capensis* laying workers could not be monitored.

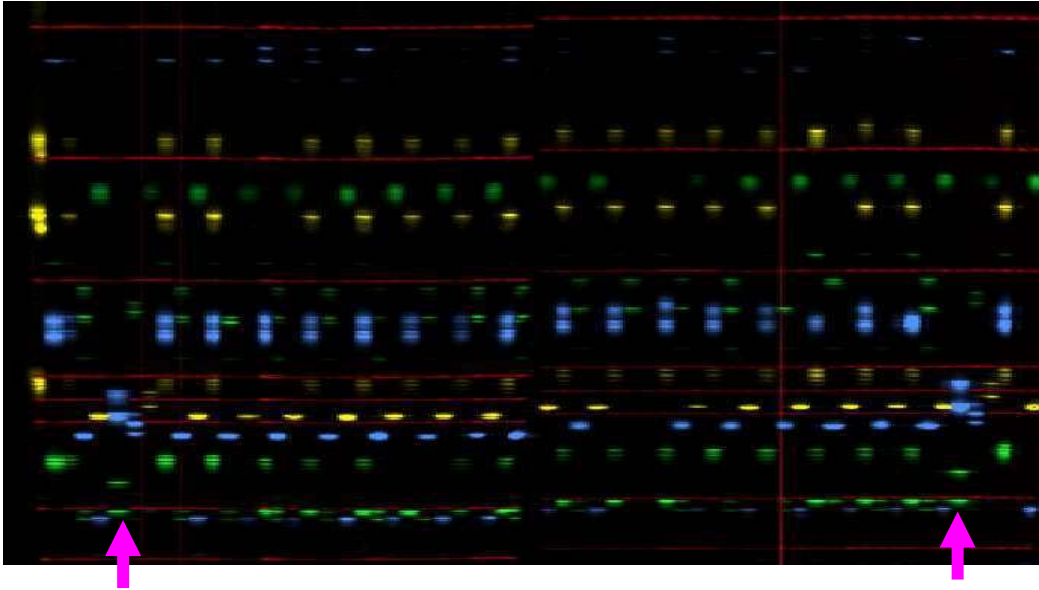


Figure 3.3.1 Electrophoresis gel showing the genetic identity of the clonal *capensis* laying workers. Each bee is represented in three adjacent vertical lanes with different loci (showing as green, blue and yellow bands) in each lane. The red bands are an internal standard. The purple arrows point to the control lanes.

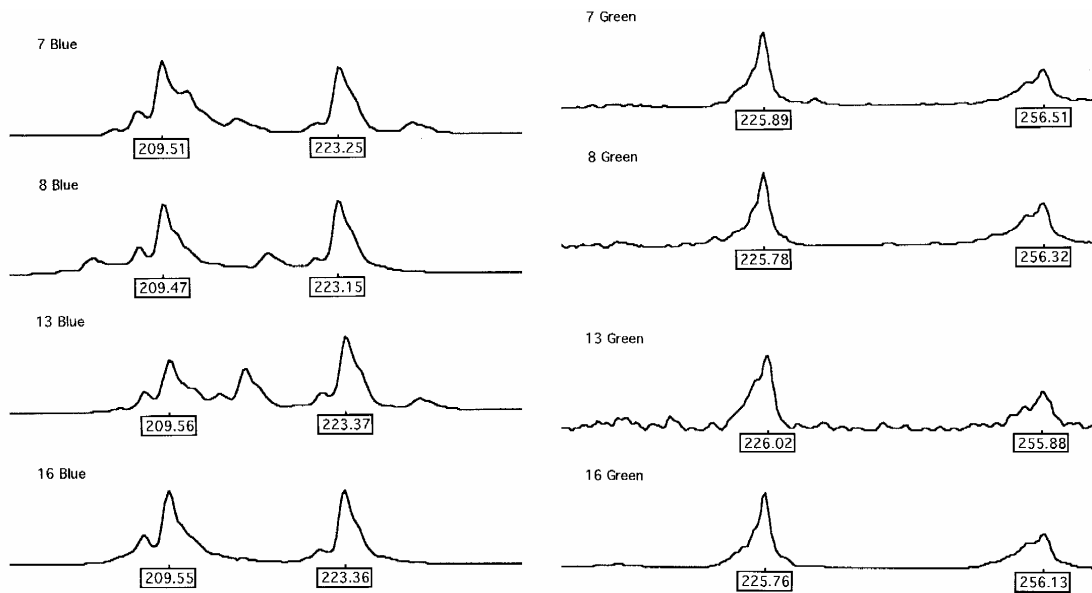


Figure 3.3.2 The alleles for the A113 and A14 loci as found in four *capensis* laying workers, showing the clonal nature of the individuals from colony N14.

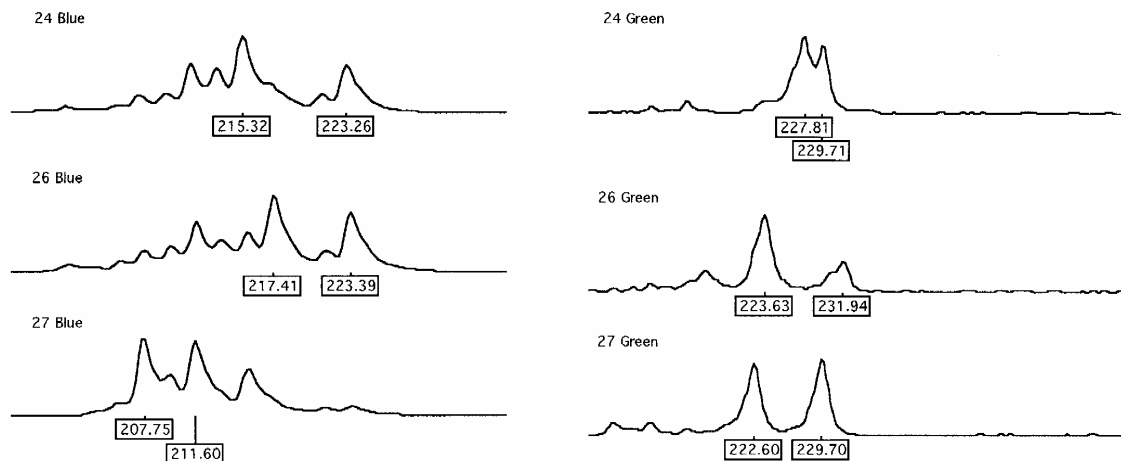


Figure 3.3.3 The alleles for the A113 and A14 loci as found in three *A. m. scutellata* bees from the same colony (N14) as the workers shown in Fig. 3.3.2.

Pseudoclone bees no 7, 8, 13 and 16 from colony N14 are represented in Figure 3.3.2, with the corresponding ovariole counts and morphological characteristics shown in Table 3.3.4. The alleles for the A113 locus, on the left-hand side of the figure, are 209/223. The A14 locus on the right shows the alleles are 226/256. In all cases the *capensis* laying workers share these alleles.

A. m. scutellata bees no 24, 26 and 27 are represented in Figure 3.3.3, with corresponding ovariole counts and morphological characteristics shown in Table 3.3.4. These bees were tested for the same loci as that of the clonal *capensis* laying workers i.e. A113 and A14 but show entirely different alleles from those at the same loci of the *capensis* laying workers. There is some similarity between the alleles of the *A. m. scutellata* bees, but this is expected, as they are daughters of the queen.

Table 3.3.4 The ovariole counts and morphological characteristics of the bees used in the genetic analysis presented in Figures 3.3.2 and 3.3.3. Pseudoclone = clonal *capensis* laying workers

Bee no	Ovariole count	Scutellum colour	Abdomen colour	Spermatheca size	Classification
7	12/12	Black	Black	Big	Pseudoclone
8	14/17	Black	Black	Big	Pseudoclone
13	16/13	Black	Black	Big	Pseudoclone
16	6/8	Black	Black	Big	Pseudoclone
24	2/2	Yellow	Yellow	Small	<i>A. m. scutellata</i>
26	4/1	Yellow	Yellow	Small	<i>A. m. scutellata</i>
27	Nd/nd	Yellow	Yellow	N/d	<i>A. m. scutellata</i>

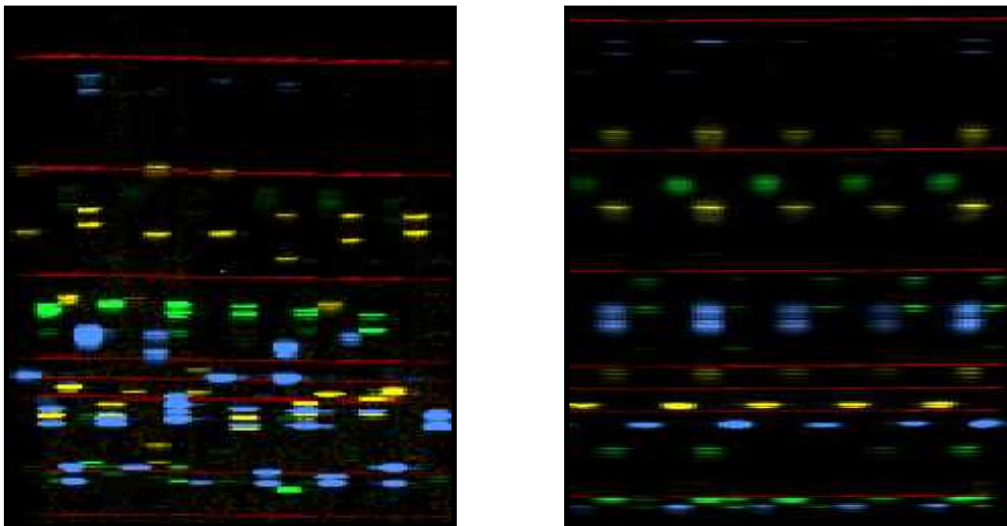


Figure 3.3.4 Electrophoresis gel showing on the left the genetic diversity in *A. m. scutellata* individuals and the lack of genetic diversity of the clonal *capensis* laying workers on the right. Each bee is represented in three adjacent vertical lanes with different loci (showing as green, blue and yellow bands) in each lane. The red bands are an internal standard.

Subsequently workers from all colonies in this apiary (N) showed similar DNA fingerprints. In all cases high ovariole counts per ovary, black scutella, black abdomens and large spermathecae were phenotypically observed, DNA fingerprinting showed that all these individuals were genetically uniform.

In addition samples from other apiaries in the *A. m. scutellata* region with known *capensis* laying worker activity were screened subsequently. The same genetic results were obtained with identical phenotypes. In cases where one or more of the morphological (phenotype) characteristics were absent, no genetic relatedness to the typical clonal *capensis* laying worker line could be found.

3.4 DISCUSSION

The genotypes of the yellow *scutellata* workers showed variability as would be expected of a normal out-breeding population. This showed the contrast between the two different groups of workers honeybees. It was also possible to detect daughters from one mother (queen) originally from a single colony. This demonstrates that the workers from a single colony were genetically related, but not genetically identical.

Worker bees with black abdomens, black scutella, high ovariole counts and big spermathecae were classified as *capensis* laying workers (Chapter 2 of this dissertation). The genotypes of these bees were compared with those of workers with yellow abdomens, yellow scutella, low ovariole counts and small or absent spermathecae. With DNA microsatellites it was possible to identify the individual *capensis* laying workers as members of a single clone.

The finding that the *capensis* laying workers had identical genotypes was unexpected but an interesting and puzzling phenomenon. The DNA microsatellite analysis was repeated and different workers were tested. Samples were also sent to Dr. M. Solignac (Génétique et Evolution, Centre National de la Recherche Scientifique, France) for confirmation by an independent laboratory. The results (Baudry *et al.* 2004) confirmed what had

been found here, and indicated that the laying workers constituted a pseudoclonal population.

Moritz and Haberl (1994) have shown that the genetic outcome of thelytokous parthenogenesis in *A. m. capensis* is mostly like a cloning process. All offspring of an individual *capensis* laying worker are nearly genetically identical to the mother. On careful inspection of the genotypes of the parasitic lineage some variability as a result of a few mutations was detected (Baudry *et al*, 2004). This is the genetic signature of a clone derived from a single individual by uninterrupted generations of thelytokous parthenogenesis (Baudry *et al*, 2004).

Ruttner (1977) found that after a three or four generations of parthenogenetically reproducing *A. m. capensis* worker bees, the spermatheca increased in size and the pigmentation levels of the abdomen and scutellum increased. He speculated that in each thelytokous generation an autoselection occurs towards a homozygous *A. m. capensis* genome.

But this does not explain why a single clonal line was found as hundreds of unrelated colonies were migrated into the *A. m. scutellata* area. The chance that a single clonal lineage would become established was not anticipated. In finding this certain very specific processes had to co-exist which would favour this specific patriline to be able to out compete all the other patrilines.

Which selection processes would be advantageous to result in a single clonal line? Neumann *et al*, (2001) found that *A. m. capensis* workers disperse significantly more often than other races of *Apis mellifera*. This suggests that dispersing represents a host finding mechanism. Individuals from colonies with the best host finding ability would be favoured in relation to individuals that do not disperse and find suitable hosts.

Extreme intracolony selection was reported by Moritz *et al* (1996) showing that only a few of the potential laying workers (pseudoclones) eventually develop to the stage of laying eggs. The pseudoclones that develop a queenlike

pheromone the fastest would suppress the development of the pseudoclone phenotype in the other potential laying workers (Moritz *et al*, 2000).

These potential laying workers have to evade the resident queen, as the queen's pheromones would probably be able to suppress development of the ovaries and pheromonal bouquet in the worker. This evasion of the queen was reported by Moritz *et al* (2002). At the same time the pseudoclone has to evade worker policing for itself as well as for the eggs being laid by it. This was reported by Neumann and Hepburn (2002).

Significant competition was evidenced between the workers of different patriline in an *A. m. capensis* colony (Moritz, *et al*, 1996). Beekman *et al* (2000) found that *A. m. capensis* workers, being raised by non-*capensis* workers, get royal treatment. These workers showed increased queen-like characteristics i.e. large spermatheca and many ovarioles per ovary. Hepburn (1992) found that *capensis* laying workers develop a queenlike pheromonal bouquet. With such competition and queenlike characteristics the result of a single founding pseudoqueen, can be explained.

The positive aspect of these findings is that, in spite of the cohabitation of *A. m. scutellata* and *A. m. capensis* within the same hives, they have nevertheless completely separated genepools. The parasitic clonal *A. m. capensis* laying workers rarely lay drone (haploid) eggs (Hepburn & Crewe, 1991), and they do not mate with *A. m. scutellata* drones as they reproduce via thelytokous parthenogenesis. This in itself is an indication that the process can be stopped, even though it has persisted for more than a decade already.

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CHAPTER 4

EFFECT OF THE PSEUDOCLONE ON COLONIES IN AN APIARY

4.1 INTRODUCTION

The results of the survey of the apiaries (see Chapter 2) showed that there were certain apiaries with below average infection rates and an above average survival and queen retention rate, suggesting resistance to invasion by *capensis* pseudoclones. Colonies with these characteristics would be suitable for a study of the spread of the pseudoclon in an apiary when deliberately exposed to infected colonies. If these colonies proved to be resistant to invasion, they could provide stock for breeding resistant colonies. Colonies that were apparently resistant to invasion, were obtained from beekeepers who had participated in the survey. The experiment described in this chapter was designed to test whether there was any evidence that resistance to the social parasite was evolving.

4.2 MATERIALS & METHODS

Four colonies were bought from each of five beekeepers and placed in the Rietondale-South apiary in Pretoria. The twenty colonies were selected on the basis of the results of the survey. Fifteen of the colonies came from the low risk group while the remaining five were from the medium risk group. All had survived for at least 18 months without any signs of inflection. They came from

Greytown, (North-KwaZulu Natal), Vredefort (Free State), Parys (Free State), Warrenton (Northern Cape) and Kakamas (Northern Cape). On the 22nd of July 1999 the colonies were thoroughly inspected and approximately 50 worker bees sampled for ovariole counts from each colony.

On 25 and 26 July 1999, two heavily infected colonies were placed amongst these colonies. These two colonies had *capensis* laying workers present in them as described by Johannsmeier (1997) with black worker bees, multiple eggs and they were queenless. Both these colonies came from beekeepers in the Pretoria area. One of the infected colonies was replaced with another infected colony at the end of August 1999, because the original colony absconded or died.

All 22 colonies were inspected (completing Form ARC001 - Appendix A) and workers were sampled every three weeks. The sequence and direction in which the colonies were opened and inspected was changed with every inspection. The opening and disruption of the colonies simulated management practices in commercial apiaries, which were classified as high risk beekeeping.

During this same period of time (June 1999 to April 2000) two apiaries were used in another experiment at the Rietondale North apiary and the Rietondale North-East apiary. These two apiaries consisted of 13 and 9 colonies respectively. The Rietondale South apiary is 900m from the Rietondale North apiary and 1200m from the Rietondale North-East apiary. These control colonies were not inspected as frequently as the ones in the Rietondale South apiary. Samples for dissections to monitor the number of ovarioles per ovary were also taken.

4.2.1 Observations

The inspection team recorded all observations, on Form no ARC001 (see Appendix A).

4.2.1.1 External observations

External observations were made before the colony was opened or given smoke. The external observations that were performed were as described in Chapter 2.

4.2.1.2 Internal observations

Internal observations made for each brood frame in the hive, were the same as those described in Chapter 2.

4.2.2 Sampling bees for dissection

Sputum bottles (40ml clear plastic) were used to sample the worker bees. Punched holes in the lids prevented the bees from suffocating and becoming moist. Each bottle was labelled with the date of sampling and the number of the colony. Samples of between 30 and 60 workers were collected per bottle. Samples were taken as randomly as possible, directly from the brood chamber after all the frames were removed. Care was taken not to catch the queen. The bottles of sampled bees were put into a cooler bag with frozen ice bricks to keep them cool until they could be transferred into a freezer in the laboratory. Here they were kept frozen until dissected.

4.2.3 Dissection of bees

Twenty honeybee workers were dissected from each sample and form ARC002 (see Appendix A) was completed for each sample bottle. The colour of the abdomen and scutellum (yellow or black) was recorded before dissecting. These bees were dissected under a binocular stereo microscope, using iriodectomy scissors. The ovarioles of each ovary were then transferred to a microscope slide, where they were immersed in distilled water and covered with a cover slip before being counted. By pressing slightly on the cover slip, the ovarioles separated and made counting easier. The development stage of the ovarioles was classified as being, 1 for no development, 2 milky content with no

visible oocytes, 3 for milky oocytes just becoming visible and 4 clearly visible mature oocytes (adapted from Velthuis, 1970). On dissection, the size of the spermatheca was estimated as being large, medium or small (as described in Chapter 2).

4.3 RESULTS

In table 4.1 the fate of colony 3 during the course of the experiment is shown. Evidence of infection was found on 18/11/99, but it was only on the next sampling date that workers were sampled with very high ovariole counts per ovary and big spermathecae. This can be attributed to the method of sampling worker bees, i.e. at random. If only a very small proportion of the workers were black they were likely to be missed during the sampling process.

Table 4.1 Data collected from colony no 3 showing the criteria used to classify it as infected on 18/11/99. Shaded blocks indicate that infection has taken place.

Date	Brood area (dm ²)	Colony size	Colour of workers	Queen present	Multiple eggs	Multiple eggs where?*	Ovariole counts	Spermatheca size
22/7/99	44	7	2-3	Yes	No	None	12	Small
16/8/99	37	6	3	Yes	No	None	10	Small
8/9/99	83	9	2-3	Yes	No	None	7	Small
4/10/99	88	8	3	Yes	No	None	6	Small
26/10/99	79	11	3	Yes	No	None	9	Small
18/11/99	74	12	2-5	Yes	Yes	W, Q	5	Small
7/12/99	116	7	4-5	No	Yes	W, Q	23	Big
5/1/2000	43	4	3-5	No	Yes	Q	24	Big
2/2/2000	5	2	3-5	No	Yes	W, D, Q	20	Big
29/2/2000	Colony dead or absconded due to pseudoclone laying worker activity.							

* W= worker cells, Q= queens cells, D= drone cells.

As the loss of the queen and the appearance of multiple eggs in worker, queen and drone cells coincided with the appearance of black worker bees in the colony, these were used as the main criteria for classifying a colony as infected. These criteria were also the ones that were found to be the significant ones in Chapter 2 of this dissertation (Shaded columns in Table 4.1).

Table 4.2 Data showing the cumulative area of brood (dm²) present in different colonies, the highest mean number of ovarioles per ovary in dissected workers and the size of the spermatheca before a colony was infected, during the early stage of infection and when it was irreversibly infected.

Colony 2

Date	Classification	Brood	Highest ovariole count	Spermatheca size
4/10/99	Healthy	60	8	Small
26/10/99	Infected	74	25	Big
18/11/99	Infected	9	19	Big

Colony 9

Date	Classification	Brood	Highest ovariole count	Spermatheca size
26/10/99	Healthy	98	8	Small
18/11/99	Infected	60	23	Big
5/01/2000	Infected	0	24	Big

Colony 10

Date	Classification	Brood	Highest ovariole count	Spermatheca size
04/10/99	Healthy	59	9	Small
26/10/99	Infected	69	19	Big
18/11/99	Infected	17	17	Big

Colony 11

Date	Classification	Brood	Highest ovariole count	Spermatheca size
2/2/2000	Healthy	49	5	Small
29/2/2000	Infected	20	20	Big
31/3/2000	Infected	5	29	Big

Colony 13

Date	Classification	Brood	Highest ovariole count	Spermatheca size
18/11/99	Healthy	102	7	Small
7/12/99	Infected	74	9	Small
5/01/2000	Infected	0	23	Big

From table 4.2 it can be seen that the area of brood (dm²) decreased considerably as the colony went from being uninfected to being infected. Furthermore workers sampled from all the colonies showed significant shifts in the mean number of ovarioles per ovary and an increase in the size of the spermathecae.

Table 4.3 The fate of the 20 colonies in the experimental apiary following the introduction of colonies infected with the pseudoclone workers. The experiment was initiated on 22 July 1999.

Colony No.	Date	Survival (No. weeks)	Fate of colony
1	16/8/99	4	Absconded
2	7/12/99	20	Infected by pseudoclone workers and died or absconded
3	29/2/2000	32	Infected by pseudoclone workers and died or absconded
4	16/8/99	4	Absconded
5	5/7/2000	50	Alive with no signs of infection
6	29/2/2000	32	Diseases and or infected by pseudoclone
7	18/11/99	17	Diseases and or infected by pseudoclone
8	2/2/2000	28	Absconded without any signs of infection
9	2/2/2000	28	Infected by pseudoclone workers and died or absconded
10	2/2/2000	28	Infected by pseudoclone workers and died or absconded
11	5/5/2000	41	Infected by pseudoclone workers and died or absconded
12	31/3/2000	36	Absconded without any signs of infection
13	2/2/2000	28	Infected by pseudoclone workers and died or absconded
14	22/7/99	48	Absconded
15	7/12/99	20	Infected by pseudoclone workers and died or absconded
16	7/12/99	20	Infected by pseudoclone workers and died or absconded
17	5/1/2000	24	Infected by pseudoclone workers and died or absconded
18	13/6/2000	47	Infected by pseudoclone workers and died or absconded
19	8/9/99	7	Infected by pseudoclone workers and died or absconded
20	13/6/2000	47	Infected by pseudoclone workers but remains in hive

In Table 4.3 it can be seen that only one of the original 20 challenged colonies survived at the end of the experiment. This corresponded to a 95% loss of active colonies due mainly to laying workers of the pseudoclone as a result of

using high risk beekeeping activities. The colonies survived on average for 28 weeks i.e. almost 7 months. All colonies died within 3 to 4 weeks of being infected.

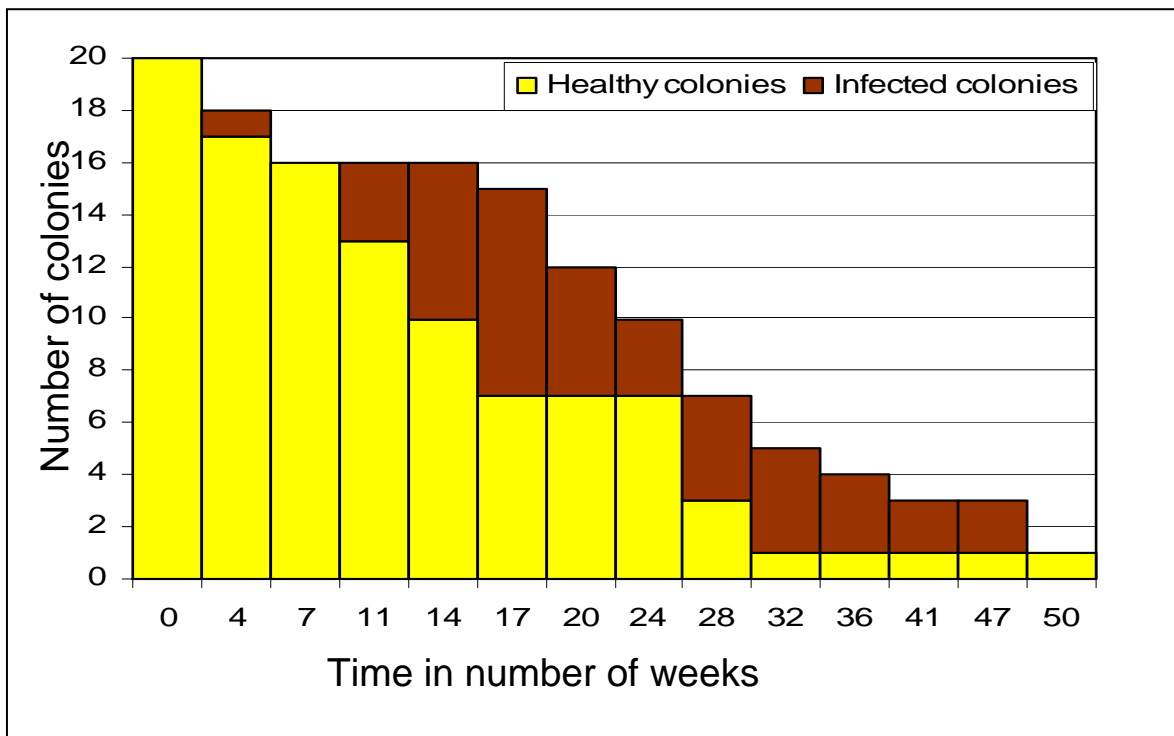


Figure 4.1 Decrease in the number of surviving colonies over time in weeks.

Figure 4.1 shows the decline in the number of colonies over time. The number decreased to 50% after 24 weeks and further decreased to only 5% after 50 weeks. After 17 weeks 50% of the surviving colonies showed signs of infection. At 32 weeks only one healthy colony was left with four infected colonies still present. This showed that the infection spread through the apiary and the infected colonies were being killed.

Brood production by healthy colonies and by colonies that had been infected was tested for normal distribution (Shapiro-Wilks W test) and the data were not significantly different from normality at the level 0.01 ($W > 0.90$, $p > 0.01$). The Wilcoxon matched pairs test showed significant differences between the healthy

colonies and the infected colonies at $p < 0.05$ ($T=252.5$; $Z=2.11$) indicating that there was a significant decline in brood production after infection.

The colour of the abdomen and scutellum of the worker bees was a good indication of infection by the *capensis* laying workers as was seen in Chapter 2 of this dissertation. The average colour ranking of 3.11 in the non-infected colonies showed that the workers were mainly yellow with a few exceptions. The average colour of the workers in the colony after infection rose to 3.96, which reflects the fact that the bees were mainly darker with many black workers amongst them.

Dissection of the worker bees from the infected colonies revealed the highest mean number of ovarioles per ovary of 19.65 vs. 9.41 for the colonies before infection. In the infected colonies the ovarioles were developed in all cases up to stage 4, with mature eggs being visible. A large spermatheca was visible in all cases.

Only colony no 5 survived during the period 22/7/1999 to 5/7/2000 (50 weeks). It had a mean brood production of 46,57 dm², a mean colony size of 6,36 and the colour of the worker bees were mainly a 3 i.e. yellow. The same queen was present and no multiple eggs were observed. Dissection of sampled workers showed that the highest ovariole count per ovary was 9 and no large spermathecae were observed.

The 22 control colonies in the Rietondale North and North-East apiaries survived for the entire duration of the experimental period with none of them being infected, absconding or dying. Some of these colonies were queenless for a few weeks, due to splits as part of another experiment, and even in this case they remained uninfected. A few of the sampled yellow workers in the queenless colonies showed ovariole development on dissection, but there were no eggs observed in those colonies. Mean numbers of ovarioles per ovary in the dissected workers stayed below 7. This showed that the pseudoclone workers had not spread over a distance of 900m and 1200m, even though most

of the area between the apiaries is open grassland with only a few rows of Eucalypts and some indigenous trees.

4.4 DISCUSSION

The aim of this project was to challenge healthy productive colonies with pseudoclones to be able to follow the spread within the apiary. This enabled us to observe the progression of the infestation by having more frequent inspections of the colonies than was possible during the survey described in Chapter 2.

All of the experimental colonies had survived for at least 18 months before being used for this experiment. All the colonies were healthy and productive at the start of the experiment. But with the high risk beekeeping practices that were used in this experiment, 95% of the colonies become infected, died or absconded within twelve months.

It was shown that when black worker bees are observed in the colony the queen disappeared soon afterwards, multiple eggs were observed and high ovariole counts per ovary were found. Brood production decreased dramatically and the colony only survived for a limited period.

The colonies that were used in this experiment were colonies that had come from apiaries that had shown some resistance to infection by the pseudoclon. Only one of the original 20 'resistant' colonies showed effective resistance to infestation by the pseudoclon. Since this experiment was undertaken, additional resistant colonies have been found (Allsopp, 2004).

With the regular disruption of the colonies using high risk beekeeping practices, the likelihood of infection was enhanced. The effectiveness of the pseudoclon as a parasite was demonstrated in that it killed 95% of the test colonies in the Rietondale South apiary in 50 weeks.

The spread of the pseudoclone between colonies appears to be poor (Kryger *et al*, 2003), but within an apiary setting such as the one used here, the risk of infection is high. The dispersal of the pseudoclone between apiaries appears to be poor (Sandmann, 2000), as all the colonies in the control apiaries stayed uninfected and productive.

Johannsmeier's (1997) practical recommendations for limiting infection are still valid. He recommended that apiaries should not be mixed, and not to add recently trapped or acquired colonies to existing apiaries. Keep apiaries small to limit the likelihood of infection and to reduce losses should the apiary be infected. No exchange of any hive parts as this helps with the spread of the pseudoclone. Inspection of colonies should be done late in the afternoon to minimize the risk of robbing and the spread of the pseudoclone. Infected colonies should be killed as soon as possible and at night to ensure all foragers are killed with the colony. Colonies should be placed as far as possible from each other to minimise drifting. He gave additional recommendations, but these were the relevant ones to this study.

Recommendations from this study are that apiaries should be kept as discreet units with no introduction of colonies from different origins. Inspection of colonies during daytime assists the pseudoclone in spreading, thus inspection should be done in the late afternoon. Legislation dictates that infected colonies should be killed within 72 hours and this should be adhered to. An infected colony in an apiary can lead to the loss of all the colonies in the apiary.

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CHAPTER 5

SUMMARY

5.1 SUMMARY OF RESULTS OBTAINED

This study of the *capensis* laying workers acting as social parasites in *Apis mellifera scutellata* colonies, found that infection rates of *A. m. scutellata* colonies correlates positively with the pigmentation (colour) of the abdomen and scutellum of the workers in the colony. Colonies in which workers were found to have larger number of ovarioles per ovary, more developed ovaries, and with a large spermatheca increased the probability of being infected. In the absence of a queen, a poor brood pattern was observed and defensive behaviour of the colony decreased. This has been described as the “colony dwindling syndrome”, leading to the death of thousands of *A. m. scutellata* colonies.

It was further established that high risk beekeeping practices favour the spread and maintenance of the social parasite in *A. m. scutellata* colonies. These practices include the migrating of colonies, mixing of apiaries, exchange of hive parts between colonies, manipulating of colonies during daytime to favour drifting and robbing. Practical control measures as suggested by Johannsmeier (1997) would limit the spread and continuation of the invasions by the *capensis* laying workers.

On studying the genotype of the *capensis* laying workers, acting as social parasites, it was found that they were all genetically related to such an extent that they are pseudoclones, i.e. more or less identical to each other. A single clonal line was found and this finding was confirmed by Baudry *et al* (2004).

By following the effect of the pseudoclone in an *A. m. scutellata* apiary that was deliberately infected with pseudoclone workers, it was found that 95% of the

colonies were killed within 50 weeks. On average the colonies survived 27,71 weeks, with many being infected.

5.2 POSSIBLE CONSEQUENCES

Possible consequences of the invasion of *A. m. scutellata* colonies by the pseudoclone would include the loss of productive colonies used for honey production and pollination. Importation of honey is expensive and brings with it the possibility of importing other honeybee diseases and or pests. When looking at pollination of fruits, seeds and other vegetation the loss of this service to farmers and natural flora would constitute both an economic loss from beekeeping activities and a loss of jobs of those involved in the industry.

The pseudoclone on its own will most probably not be a threat to biodiversity as few wild colonies were found that showed signs of infestation (Dr. Per Kryger, pers. comm.). This situation will continue as long as the number of wild colonies exceeds the number of managed colonies (Moritz, 2002). However, wild bee populations are under threat of being over-exploited by beekeepers trying to replace colonies killed by *capensis* laying workers (Kryger *et al*, 2004).

This lethal host – parasite relationship suggests that the invasion will be self limiting (Oldroyd, 2002). If all host colonies are killed the social parasite (pseudoclone) will perish as it will have no host to infect. To kill all colonies in an infected apiary would accomplish this. But as the problem is more widespread than just a few apiaries it might not be economical. Kryger *et al* (2003) suggested that infected colonies be killed and the apiary be left for few months to limit the spread of the pseudoclone.

But as this is a human assisted parasite-host relationship with the beekeeper as the vector (Kryger *et al*, 2003) the invasions will continue as long as there are non-resistant *A. m. scutellata* colonies being exposed to the pseudoclone. According to Allsopp (2004) recent experiments have found colonies that show resistance to being usurped by the pseudoclone.

Schmid-Hempel (1998) describes social parasitism in social insects as a relationship between two species in which the parasite benefits in many ways from brood care or other socially managed resources at the expense of the host society. The activity of the pseudoclone which has arisen from an *A. m. capensis* colony, fits this description.

Neumann and Moritz (2002) stated that scientists might be in the fortunate position to study the sympatric evolution of a social parasite in real time. The inability to produce sexual reproductives (queens and or drones) in infected colonies, suggests that the pseudoclone is reproductively isolated from its host, resulting in a separation of the two gene pools.

5.3 RECOMMENDATIONS TO BEEKEEPERS

Identifying and killing of infected colonies is of paramount importance. This would restrict the spread of the pseudoclone to neighbouring colonies. Introduction of the low risk management techniques could reduce or eliminate the parasite within a few years. These techniques would include the following: limiting migration of colonies, keeping apiaries as discreet units, no exchange of hive parts between colonies, manipulating of colonies during late afternoon or at night to limit drifting and robbing. Manipulations should be limited as this disturbs the colony and makes it more vulnerable to infection (Kryger *et al*, 2003).

Identification of infected colonies would include the following criteria: workers with different pigmentation levels in one colony, i.e. workers with black scutella and black abdomens between yellow workers. These workers would also have high ovarioles numbers per ovary and would also show development of the ovaries. A large spermatheca would be observed. But as these last mentioned criteria needs specialised equipment, other visual criteria would include the finding of multiple eggs in worker cells. An uneven brood pattern, a lack of colony defensiveness and the absence of a queen would be regarded as

possible signs of infection. Under South African law a colony that is hopelessly queenless should be killed within 72 hours. By killing an infected colony at night when all the foragers are in the hive, spread of the infection will be drastically curtailed.

As this problem is a human assisted condition, where the beekeepers, are acting as the vector in this parasite – host relationship, they are also the solution to the problem. If the vector no longer assists in the spread of the parasite, the parasite will perish (Kryger *et al*, 2003).

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Appendix A

Form ARC001

This form was used for all field observations. A new form was used for each colony.

Form ARC002

This form was used for dissections in the laboratory. A new form was used for each colony.

As all the data obtained is confidential, the names of the beekeepers can not be disclosed with regard to the regions or towns where their apiaries were located.

CAPENSIS SURVEY

Apiary Site:
 Region:
 Colony no.:

Date:
 Time of day:
 Name of recorder:

Frame No	Brood (dm ²)	Pollen (dm ²)	Honey (dm ²)	Remarks
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				

Normal activity at hive entrance (yes/no)	Y / N	Multiple eggs in cells: (yes/no)	Y / N
Fighting at hive entrance (yes/no)	Y / N	- In drone/worker/queen cells	
Fighting on comb (yes/no)	Y / N	- Side of walls/bottom of cells	
No. of supers on hive		Laying worker brood: (yes/no)	Y / N
Colony size (frames of bees)		- Cappings raised / flat	
Level of foraging (0 = poor; 5 = good)		- Number of frames	
Colour of bees (1 = yellow; 5 = black)		- Peripheral or central frames	
Quality of brood pattern (1= poor; 5=good)		- Periphery or centre of frames	
Queen present? (yes / no / not sure)		Queen cells: (yes/no)	Y / N
Queen no./colour		- Sealed/open //old/cup	
Level of Aggression (1=docile; 5=very aggressive)		- Normal, chewed open, cryptic	

General Comments:

CAPENSIS DISSECTIONS

CONFIDENTIAL



INFORMATION AS ON LABEL: _____

No	Date of dissection	Colour* of scutellum	Colour* of abdomen	Ovarioles touch at ends (Y/N)	Spermatheca (Description)	Ovariole development and count		
						Stage	Left	Right
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								

Dissector: _____
 Signature _____ Print name _____ Date _____

*Colour designation: Yellow (Y); Yellowish-Brown (YB); Brown (BR); Black (BL)