Prevalence of parasites and diseases in the African honeybee (*Apis mellifera scutellata* Lepeletier): with a particular focus on *Varroa destructor*

by

Ursula Strauss

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Department of Zoology and Entomology

University of Pretoria

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DECLARATION

I, **Ursula Strauss**, declare that the thesis, which I hereby submit for the degree **Magister Scientiae** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Ursula Strauss **Date**

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ABSTRACT

The status of honeybee pathogens and parasites in the Gauteng region of South Africa was examined by collecting adult honeybee and worker brood samples from 13 *Apis mellifera scutellata* apiaries. The prevalence of pathogens and parasites were compared per season between sedentary (permanently stationed colonies) and migratory (transportation of colonies for pollination purposes) apiaries. Honeybee pathogens (Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Chronic bee paralysis virus (CBPV), Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Sacbrood virus (SBV), *Varroa destructor* Macula-like virus (VdMLV), *Varroa destructor* virus 1 (VDV-1), American foulbrood (AFB), European foulbrood (EFB), *Nosema apis*, *Nosema ceranae*) were diagnosed with PCR methods. Parasites (Bee lice, *Capensis* social parasites, Small hive beetles, Wax moths, *Varroa destructor*) and Chalkbrood were identified by visual inspection of adult honeybee and worker brood samples. No significant differences were found per season in the prevalence of pathogens and parasites between sedentary and migratory apiaries and consequently all results were pooled. Three (BQCV, VDV-1 and IAPV) of the eight viruses screened were detected in honeybees, while two of these viruses (VDV-1 and IAPV) were also confirmed in *Varroa* mites. This is the first report of IAPV and VDV-1 in South African honeybees as well as in *Varroa* mites infesting *A. m. scutellata* colonies. BQCV was the most common virus and was detected in eight of the 13 screened apiaries. EFB and *N. apis* were also detected in one and five of the apiaries, respectively. ABPV, CBPV, DWV, VdMLV, SBV, AFB and *N. ceranae* were not detected in the 13 apiaries. Honeybee parasites were frequently encountered in the majority of the apiaries

with the most common parasite being the *Varroa* mite. A total of 12 pathogens and parasites were found in 13 apiaries in the Gauteng region of South Africa over a period of 14 months. The impact of *Varroa* mites on the development of honeybee colonies were examined from May to October 2011 in nine chemically treated and nine untreated colonies. The population dynamics of *Varroa* mites were examined by recording the number of mites that fell daily on the bottom boards of the hives (pre-, during and post- treatment), as well as the infestation rates of these mites in adult honeybees and worker brood cells. Honeybee colony development was measured by counting the number of adult honeybees, as well as the surface area of sealed and unsealed brood. *Varroa* mite fall was significantly higher in the treated apiary during treatment and in the three months following treatment. No significant differences were found in the adult honeybee and worker brood infestation rates in both apiaries during May, July and September. Honeybee colony development was similar for both apiaries indicating that colonies in the untreated apiary, that received no chemical treatment, survived just as well as the colonies that were treated. Honeybee (*A. m. scutellata*) colonies were thus able to survive without chemical treatment, in the presence of diverse pathogens and parasites.

LIST OF ABBREVIATIONS

ABPV	Acute bee paralysis virus
AFB	American foulbrood
A. m. c.	*Apis mellifera capensis*
BQCV	Black queen cell virus
CBPV	Chronic bee paralysis virus
cDNA	Complimentary DNA
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DWV	Deformed wing virus
EFB	European foulbrood
Fig.	Figure
IAPV	Israeli acute paralysis virus
M	Molarity
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar
M-MLV RT	Moloney-Murine Leukemia Virus Reverse Transcriptase
N. apis	*Nosema apis*
N. ceranae	*Nosema ceranae*
NaCl	Sodium chloride

No.	Number
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SHB	Small hive beetle
SBV	Sacbrood virus
µg	Microgram
µl	Microliter
UV	Ultraviolet light
VDV-1	*Varroa destructor* virus 1
VdMLV	*Varroa destructor* Macula-like virus

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CHAPTER 1

Introduction to honeybees (*Apis mellifera* L.) and their associated pathogens and parasites

The honeybee (*Apis mellifera* L.) colony

Honeybees (Hymenoptera, Apidae, *Apis mellifera* L.) are social insects that live in colonies consisting of a queen, a number of drones (male) and thousands of workers (female) (Harris 1985; Winston 1987; Fries & Camazine 2001; Martin 2001). Queens are mainly responsible for laying eggs within the colony (Winston 1987). Drones do not perform any tasks in the hive, and their main purpose is to mate with queens on their mating flights (Bailey & Ball 1991; Tribe & Allsopp 2001). Worker honeybees perform the majority of their tasks within a hive and the division of labour within a colony is related to the age of a worker honeybee (Seeley 1982; Nowogrodzki 1984; Winston 1987). Tasks include, cleaning the hive, capping brood cells, feeding the queen and developing larvae, building of comb, ventilating the hive, accepting and storing of pollen and nectar, guarding the hive and foraging (Rothenbuhler 1964; Seeley 1982; Winston 1987; Seeley 1995). This type of social system offers a perfect environment for pathogens to thrive in due to the presence of a high number of likely hosts (Schmid-Hempel 1995; Fries & Camazine 2001). Indeed, the regular and close interaction between individual honeybees provides an easy means of transmission for pathogens within a colony (Naug & Camazine 2002; Chen & Siede 2007).

Pathogens such as viruses, bacteria, fungi and microsporidian parasites can be transmitted either horizontally (between honeybees of the same age group) or vertically (passed on to the progeny by the queen) within honeybee colonies (Schmid-Hempel 1995; Chen *et al*. 2006). Vertically transmitted pathogens are generally less virulent compared to pathogens transmitted horizontally (Lipsitch *et al*. 1996; Fries & Camazine 2001; Chen & Siede 2007). In order for both the host (the queen) and the vertically transmitted pathogen to stay alive and reproduce, the pathogen has to be less virulent to ensure its survival into the next generation (Lipsitch *et al*. 1996). Pathogens

can also be transmitted horizontally within honeybee colonies via food, air, vectors, faecal-oral pathways and through mating (Chen & Siede 2007). Pathogens can enter honeybee colonies by means of contaminated food and water, as well as foraging, robbing and drifting honeybees (Bailey 1968; Koenig *et al*. 1987; Bailey & Ball 1991). Moreover, honeybee parasites such as small hive beetles and parasitic mites can also carry viruses and bacteria into and within honeybee colonies (Dainat *et al*. 2009; Eyer *et al*. 2009; Rosenkranz *et al*. 2010; Schäfer *et al*. 2010a).

The ectoparasitic mite - *Varroa destructor*

The ectoparasitic mite, *Varroa destructor*, (Acari: Varroidae) remains the most important parasite of honeybees (De Jong *et al*. 1982c; De Jong & De Jong 1983; Koeniger *et al.* 1983; Boot *et al*. 1994; Guzman-Novoa *et al*. 1996; Martin 1998; Anderson & Trueman 2000; Rosenkranz *et al*. 2010; Dietemann *et al*. 2012) and has been implicated in the death or collapse of numerous honeybee colonies (Martin 1999; Shen *et al*. 2005a; Dahle 2010; Guzman-Novoa *et al*. 2010; Rosenkranz *et al*. 2010; Schäfer *et al*. 2010b; Dainat *et al*. 2012). *Varroa destructor* was previously described as *Varroa jacobsoni* by Oudemans in 1904 (Oudemans 1904; Rath 1999) but was re-classified as *V. destructor* in 2000 by Anderson and Trueman. *Varroa jacobsoni* is considered to be a species complex and two (Japan/Thailand and Korea) of the 18 genetically distinct mite types (haplotypes) identified from its original host (*Apis cerana*) can reproduce on *A. mellifera* (Anderson 2000; Anderson & Trueman 2000). *Varroa* mites are currently present in most countries around the world (except Australia) (Bradbear 1988; Matheson 1993; Ellis & Munn 2005; Rosenkranz *et al*. 2010; vanEngelsdorp & Meixner 2010).

Varroa mites are visible to the naked eye (Shimanuki & Knox 2000) and can easily be observed on honeybees (Bailey & Ball 1991). Adult *V. destructor* females (± 1.1 x 1.5 mm) (Fig. 1) are reddish brown whereas males (± 0.7 x 0.5 mm) (Fig. 2) are pale in colour and notably smaller than females (Sammataro *et al*. 2000; Boecking & Genersch 2008).

Figure 1. Ventral view of adult female *Varroa destructor*.

Figure 2. Dorsal view of adult male *Varroa destructor*.

Varroa mites can spend their complete life-cycle inside honeybee hives where they feed on the haemolymph of adult and developing honeybees (De Jong *et al*. 1982a; Peng *et al*. 1987; Bailey & Ball 1991; Boot *et al*. 1994; Donzé & Guerin 1994; Shen *et al*. 2005b). Mite reproduction (reproductive phase) takes place inside capped brood cells of honeybees (Donzé & Guerin 1994; Kanbar & Engels 2004) and adult females who are not in their reproductive phase are attached to adult honeybees (phoretic phase) (Kuenen & Calderone 1997; Sumpter & Martin 2004). During this phoretic phase, mites can be transported by honeybees both within and between colonies, and can generally be found attached to the abdomen and thorax of adult honeybees (Boot *et al*. 1994; Shen *et al*. 2005a). In contrast, male mites are only present inside the brood cells, are not phoretic and cannot live on adult honeybees (Donzé *et al*. 1996; Boecking & Genersch 2008).

In *A. mellifera*, *Varroa* mites can reproduce successfully in both worker and drone brood, although they prefer drone brood (De Jong 1988; Fuchs 1990). During the reproductive phase female mites enter drone or worker brood cells just before they are sealed (Martin 2001; Sumpter & Martin 2004). They conceal themselves in the larval food to evade detection and subsequent removal by the nurse honeybees (Calderón *et al*. 2010; Rosenkranz *et al*. 2010). A female will initiate egg laying approximately 60 hours after the brood cell has been sealed (Ifantidis 1983; Boot *et al*. 1995). A male always develops from the first egg and the rest of the eggs which are laid, at 30 hour intervals, develop into females (Ifantidis 1983; Rehm & Ritter 1989; Boot *et al*. 1995; Martin *et al*. 1997; Sumpter & Martin 2004). Mating occurs between offspring (Garrido & Rosenkranz 2003) and after a period of time, which corresponds with the development of the honeybee, only fully developed females (mother and mated female offspring) emerge from the cell with the young honeybee, leaving behind males and immature females that eventually die

(Ball & Allen 1988; Martin *et al*. 1997; Boecking & Genersch 2008). The approximate development time for females is 6.2 days, and 6.9 days for males (Rehm & Ritter 1989).

The damage caused by the feeding activity of *Varroa* mites to honeybees (workers and/or drones) includes a reduction in weight (De Jong *et al*. 1982b; Duay *et al*. 2003), haemolymph protein content (Glinsky & Jarosz 1984), haemolymph volume (Weinberg & Madel 1985), carbohydrate titre (Bowen-Walker & Gunn 2001), hypopharyngeal gland development (Schneider & Drescher 1987) and lifespan (De Jong & De Jong 1983; Schneider & Drescher 1987). *Varroa* mites can supposedly also cause honeybees to have physical defects in the form of shortened abdomens (De Jong *et al*. 1982b) and deformed wings, with these symptoms mostly occurring when Deformed wing virus was transmitted to honeybees by these mites (De Jong *et al*. 1982b; Le Conte *et al*. 1989; Marcangeli *et al*. 1992; Yue & Genersch 2005; Gisder *et al*. 2009). Moreover, *Varroa* mites are capable of lowering the immune response of honeybees thereby increasing the vulnerability of honeybees to viral infections (Yang & Cox-Foster 2005).

Honeybee pathogens

- **Viral pathogens**

Varroa mites and their associated viruses have been linked to many of the recorded honeybee colony losses worldwide (Bailey & Ball 1991; Berthoud *et al*. 2010; Brodschneider *et al*. 2010; Guzman-Novoa *et al*. 2010; Schäfer *et al*. 2010b; vanEngelsdorp & Meixner 2010). It still remains unclear exactly how *Varroa* mites cause colony death or collapse but the role they play in transmitting and activating viruses is thought to contribute (Bailey & Ball 1991; Bowen-Walker *et al*. 1999; Shen *et al*. 2005a). In the absence of *V. destructor*, honeybee viruses

generally persist as covert infections (Allen & Ball 1996; Benjeddou *et al*. 2001; de Miranda *et al*. 2004; Sumpter & Martin 2004; Rosenkranz *et al*. 2010), but since the worldwide introduction of this parasitic mite, virus occurrence and pathogenicity have increased significantly (Ball 2004; Aubert 2008; Ratnieks & Carreck 2010). The majority of viruses affecting honeybees have an almost worldwide distribution (Allen & Ball 1996; Ellis & Munn 2005) and most of these honeybee viruses are single-stranded positive RNA (Chen *et al*. 2004; Tentcheva *et al*. 2004; Ribière *et al*. 2008). The following honeybee viruses are relevant to this study (see Chapter 2).

Acute bee paralysis virus (ABPV) was first detected by Bailey *et al*. (1963) in seemingly healthy honeybees. ABPV has a global distribution (Allen & Ball 1996; Ellis & Munn 2005) and can be activated or transmitted by *V. destructor* to both adult honeybees as well as brood (Ball 1988; Ball & Allen 1988; Ball 1989; Bailey & Ball 1991; Hung *et al*. 1996; Chen & Siede 2007). ABPV infection became far more apparent in honeybee colonies after the arrival of *Varroa* mites (Genersch 2010a) and has since been linked to increased rates of honeybee mortality (Ball & Allen 1988; Nordström *et al*. 1999; Berényi *et al*. 2006; Siede *et al*. 2008; Genersch *et al*. 2010).

Black queen cell virus (BQCV) was first noted in queen cells containing dead prepupae and pupae (Bailey & Woods 1977) and is found in honeybees around the world (Allen & Ball 1996; Ellis & Munn 2005). Co-infection of BQCV and the microsporidian parasite, *Nosema apis* is very common in honeybees (Bailey 1982; Bailey *et al*. 1983). BQCV is more prevalent during spring and summer when associated with *N. apis*, and this virus is known to cause premature mortality in adult honeybees (Bailey 1982; Ribière *et al.* 2008).

Chronic bee paralysis virus (CBPV) was first isolated from seemingly healthy honeybees (Bailey *et al*. 1963) and occurs in most parts of the world (Allen & Ball 1996; Ellis & Munn 2005). CBPV has been detected in all developmental stages of honeybees (Blanchard *et al*. 2007) and symptoms of this virus in adult honeybees can be divided into two types. The first type of symptoms include, paralysed, trembling honeybees with bloated abdomens that are crawling at the entrances of colonies, while symptoms of the second type include honeybees with shiny, hairless, black appearances (Bailey 1968; Bailey 1976; Allen & Ball 1996; Ribière *et al*. 2010). The detection of CBPV in *Varroa* mites suggests that they can possibly act as vectors of this virus within honeybee colonies (Celle *et al*. 2008).

Deformed wing virus (DWV) occurs worldwide (Allen & Ball 1996; Berényi *et al*. 2007; Ribière *et al*. 2008) and can be transmitted to honeybees by *V. destructor* (Bowen-Walker *et al*. 1999; de Miranda & Genersch 2010). DWV affects both adult honeybees and brood (Allen & Ball 1996; Chen & Siede 2007) and the wings of infected honeybees are usually deformed (Fig. 3) or under-developed (Bailey & Ball 1991; Yue & Genersch 2005; Gisder *et al*. 2009). In addition infected adult honeybees can have a reduced body mass, reduced lifespan and show some degree of discolouration (Chen & Siede 2007; Dainat *et al*. 2012).

Figure 3. Honeybee (*Apis mellifera scutellata*) with deformed wings.

Israeli acute paralysis virus (IAPV) was only recently discovered in honeybees from Israel (Maori *et al*. 2007) and has subsequently been found in many countries including Argentina (Reynaldi *et al*. 2011), China (Ai *et al*. 2012), France (Blanchard *et al*. 2008; Gauthier *et al*. 2011), Japan (Kojima *et al*. 2011), Jordan (Al-Abbadi *et al*. 2010), Poland (Pohorecka *et al*. 2011), Spain (Garrido-Bailón *et al*. 2010; Kukielka & Sánchez-Vizcaíno 2010) and the U.S.A. (Chen & Evans 2007; Cox-Foster *et al*. 2007; Palacios *et al*. 2008; Bromenshenk *et al*. 2010; Runckel *et al*. 2011). There have been no reports of IAPV in Africa yet (excluding current study), after a recent survey in Uganda failed to detect any traces of IAPV in honeybees (Kajobe *et al*. 2010). IAPV is closely related to ABPV and Kashmir bee virus (Maori *et al*. 2007; de Miranda *et al*. 2010; vanEngelsdorp & Meixner 2010) and it was recently discovered that *Varroa* mites can act as vectors of this virus (Di Prisco *et al*. 2011).

Sacbrood virus (SBV) occurs worldwide (Matheson 1993; Allen & Ball 1996) and mainly affects honeybee brood, but can also affect adult honeybees without causing any noticeable symptoms (Bailey & Ball 1991; Ribière *et al*. 2008). SBV infected larvae do not pupate, form a characteristic sac and become light yellow in colour as opposed to white (Bailey 1968; Bailey 1976; Bailey & Ball 1991; Shen *et al*. 2005a). It is mostly two day old larvae and young worker honeybees that are prone to SBV (Bailey & Ball 1991). Ball (1989) found that *Varroa* mites can transmit SBV to honeybee pupae and recently SBV was found in *Varroa* mites (Tentcheva *et al*. 1994; Chantawannakul *et al*. 2006) and their saliva (Shen *et al*. 2005a).

***Varroa destructor* virus 1 (VDV-1)** was first detected in *Varroa* mites and is highly related to two other honeybee viruses namely, Kakugo virus (KV) and DWV (Ongus 2006). VDV-1 appears to be quite prevalent in parts of Europe (Ongus 2006) and Israel (Zioni *et al*. 2011). It

was also found to be widespread in queens collected in France (Gauthier *et al.* 2011). The recent discovery that VDV-1 and DWV can form recombinants that might spread more easily within honeybee colonies is concerning (Moore *et al.* 2011; Zioni *et al*. 2011).

- **Bacterial pathogens**

American foulbrood (AFB) occurs worldwide (Ellis & Munn 2005) and is an extremely infectious and harmful disease that normally kills infected honeybee larvae (Gregorc & Bowen 1998; Hansen & Brødsgaard 1999; Neuendorf *et al*. 2004; Genersch 2010b). AFB is caused by the spore producing, gram-positive bacterium *Paenibacillus larvae* (Bakonyi *et al*. 2003; Fries *et al*. 2006; Genersch *et al*. 2006) and larvae usually get infected when they ingest spore contaminated food (Bailey 1968; Genersch *et al*. 2005). Less than one day old larvae are most vulnerable to infection (Bailey 1968). The screening of AFB in this study is of importance (Chapter 2) given the recent outbreak of AFB in South Africa (Baxter 2009; Human *et al.* 2011).

The causative agent of **European foulbrood (EFB)** is *Melissococcus plutonius* (Bailey 1961; McKee *et al*. 2003). EFB presence in honeybee colonies has been recorded across the world (Matheson 1993; Ellis & Munn 2005). Mortality of unsealed EFB infected larvae typically occurs when they are approximately 3-5 days old and severe EFB infections can cause colony losses (Bailey 1968; Waite *et al*. 2003; Budge *et al*. 2010; Forsgren 2010). Although EFB only affects brood, adult honeybees can carry *M. plutonius* spores between colonies and apiaries (Belloy *et al*. 2007).

- **Microsporidian parasites**

The microsporidian parasites, ***Nosema apis*** and ***N. ceranae*** are responsible for causing Nosemosis in adult honeybees (Wang & Moeller 1970; Chen *et al*. 2008; Williams *et al*. 2008) and have a fairly widespread distribution (Bradbear 1988; Ellis & Munn 2005; Klee *et al*. 2007; Fries 2010). Honeybees can become infected with the disease when they consume food or water that contains *Nosema* spores and/or when they come into contact with faeces of infected honeybees (faecal-oral pathway) (Bailey 1968; de Graaf *et al*. 1994; Chen *et al*. 2008). *Nosema* spores germinate inside the mid-gut of honeybees and cause an infection in the epithelial cells (Fries 1988; Bailey & Ball 1991; Shimanuki & Knox 2000; Fries 2010). *Nosema apis* have long been associated with *A. mellifera*, whereas *N. ceranae* only recently shifted from its original host, *Apis cerana* (Fries *et al*. 1996) to *A. mellifera* (Klee *et al.* 2006; Paxton *et al*. 2007). Both these pathogens can have a negative effect within honeybee colonies. *Nosema apis* infection can cause a reduction in worker hypopharyngeal gland development (Wang & Moeller 1969), pollen gathering (Anderson & Giacon 1992) and honey production (Farrar 1947). There have been conflicting reports on the virulence and effect of *N. ceranae* in *A. mellifera* colonies (Genersch 2010a). High colony losses were attributed to *N. ceranae* infections in Spain (Higes *et al*. 2008). In addition, high mortality rates of caged worker honeybees fed *N. ceranae* spores were also reported (Higes 2007; Paxton 2007) as well as weakened immune responses in honeybees infected with *N. ceranae* (Antunez *et al*. 2009). In contrast, Gisder *et al*. (2010) found no direct association between increased honeybee colony mortality and *N. ceranae* infection. Forsgren & Fries (2010) compared the virulence of *N. apis* and *N. ceranae* in adult worker honeybees from Sweden with in vivo infection experiments. They found that in caged honeybees mortality

caused by both species was relatively similar thereby showing that *N. ceranae* is no more virulent than *N. apis*.

- **Fungal pathogens**

The widely distributed heterothallic (+ and -) fungus, *Ascosphaera apis*, causes **Chalkbrood** disease in honeybee brood when the fungal spores are consumed by larvae (Gilliam *et al*. 1988; Bailey & Ball 1991; Shimanuki & Knox 2000; Aronstein & Murray 2010). Even though adult honeybees are not affected they can still spread *A. apis* among and within colonies (Aronstein & Murray 2010). Infected larvae (with only the + or - strain present) are enlarged and covered in a whitish mould and soon after they develop into smaller, white, dried up larvae with mummy-like exteriors (Fig. 4) (Gilliam *et al*. 1978; Heath 1982; Bailey & Ball 1991; Flores *et al*. 2005). When both strains (+ and -) are present the mummies are usually black due to the formation of fruiting bodies (Fig. 5) (Gilliam *et al*. 1978; Shimanuki & Knox 2000). Chalkbrood is lethal to individual larvae, but generally not to the colony as a whole (Swart & Rong 1999; Fries & Camazine 2001).

Figure 4. White Chalkbrood (*Ascosphaera apis*) mummy in an open brood cell.

Figure 5. Black Chalkbrood (*Ascosphaera apis*) mummy with fruiting bodies.

The African situation

In Africa, no serious colony losses have been reported so far (Neumann & Carreck 2010) even though honeybee pathogens and parasites are present in many countries on the continent (Matheson 1993; Allen & Ball 1996; Hussein 2000; Swart *et al*. 2001; Fries *et al*. 2003; Ellis & Munn 2005; Higes *et al*. 2009; Frazier *et al*. 2010; Kajobe *et al*. 2010). African honeybees are either not as susceptible to pathogens or evidence to show otherwise is not available, especially since there is a lack of surveys on honeybee health, pathogens and parasites in Africa (Matheson 1993; Allen & Ball 1996; Hepburn & Radloff 1998; Davison *et al*. 1999; Ellis & Munn 2005; Dietemann *et al*. 2009).

In South Africa there are two honeybee sub-species, namely the Cape honeybee (*Apis mellifera capensis*) and the African honeybee (*Apis mellifera scutellata*) (Anderson 1963; Ruttner 1977; Eardley *et al*. 2001; Hepburn & Radloff 2002). The Cape honeybee occurs in the fynbos region

of the Cape and the African honeybee occupies the rest of South Africa (Hepburn & Crewe 1991). Both honeybee sub-species are separated by a well defined hybrid zone (Hepburn & Crewe 1991; Crewe *et al*. 1994). A unique situation of social parasitism exists in South Africa where workers of one sub-species (*A. m. capensis*), that lay diploid eggs, can become a social parasite of another sub-species (*A. m. scutellata*) (Onions 1912; Ruttner 1977). The transfer of *A. m. capensis* colonies by a local beekeeper into the distribution area of *A. m. scutellata* approximately 20 years ago resulted in the death of thousands of *A. m. scutellata* colonies (Allsopp 1992; Allsopp & Crewe 1993). The invasion of *A. m. scutellata* colonies by *A. m. capensis* workers was termed the '*capensis* calamity' by Allsopp (1992). *Apis mellifera scutellata* queens are usually lost and parasitic workers have no interest in raising brood or foraging, which ultimately leads to the death of the host colony (Magnuson 1995; Martin *et al*. 2002b; Neumann & Moritz 2002). The movement of colonies for crop pollination remains one of the major reasons for the persistent infestation of *A. m. scutellata* colonies by *A. m. capensis* social parasites (Martin *et al*. 2002a; Neumann & Moritz 2002; Kryger *et al*. 2003).

Varroa destructor was first observed in South Africa in 1997 in Stellenbosch (Western Cape) (Allsopp 1997; Martin & Kryger 2002) and additional surveys have revealed that it is now present throughout South Africa (Allsopp 2006). Extensive colony losses were reported by South African beekeepers during the early spread of *Varroa* (Allsopp 2004), but a country wide loss of honeybee colonies did not occur and this was attributed to a lack of secondary pathogens (viruses) being present (Allsopp 2006).

Information on the distribution and occurrence of honeybee pathogens in South Africa is very limited (Govan 2000; Allsopp 2006) and therefore more data are required to gain a better insight

into the status of honeybee pathogens in South Africa. The following honeybee pathogens have previously been reported in South Africa: Deformed wing virus (Allen & Ball 1996; Martin 2001), Filamentous virus, Chronic bee paralysis virus (Allen & Ball 1996), Sacbrood virus, Acute bee paralysis virus, Black queen cell virus, European foulbrood, Chalkbrood (Davison *et al*. 1999), *Nosema apis* (Fantham 1920; Skaife 1954; Swart 2003) and American foulbrood (Baxter 2009). In addition to the pathogens mentioned above, parasites such as small hive beetles, wax moths, bee lice and tracheal mites are also frequently encountered in South African honeybee colonies (Reviewed in Swart *et al*. 2001).

In South Africa, most of the high colony losses recorded in *A. m. scutellata* colonies specifically has been attributed to the infestation by *A. m. capensis* social parasites. In order to get a better overview on the possible roles of other pathogens and parasites in *A. m. scutellata* colonies, adult honeybee and worker brood samples were collected from 13 apiaries and analysed for their presence (Chapter 2). The seasonal presence and prevalence of pathogens and parasites were compared between sedentary (permanently stationed) and migratory (moved for pollination purposes) apiaries. The long term monitoring of honeybee colonies (vanEngelsdorp *et al*. 2008; Genersch *et al*. 2010; vanEngelsdorp *et al*. 2010) is vital to better understand the effects that pathogens and parasites have on the overall survival and health of honeybees.

The effect of *Varroa destructor* mites on the population growth and overall health of honeybee colonies is receiving much attention. Globally, the majority of honeybee colonies have to be chemically treated against this parasite in order to survive, while in South Africa, honeybee colonies are generally not treated against *Varroa* mites. Chemical treatment is applied to honeybee colonies in order to reduce the *Varroa* mite population sizes to very low, less

damaging levels. In Chapter 3, the population dynamics of *Varroa destructor* mites and their impact on honeybee (*A. m. scutellata*) colony development was compared between chemically treated and untreated colonies. A summary of my results and concluding remarks are given in Chapter 4.

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CHAPTER 2

Seasonal prevalence of pathogens and parasites in the African honeybee (*Apis mellifera scutellata*)

INTRODUCTION

Honeybees (*Apis mellifera* L.) are extremely valuable insects, known for their importance as pollinators and honey producers (Camazine & Morse 1988; Morse & Calderone 2000; Chen & Siede 2007; Aebi *et al*. 2012). The health of honeybees has been one of the most important topics of discussion and research in recent years (Genersch 2010). This is primarily due to the recent recordings of high honeybee colony losses in many parts of the world (Stokstad 2007; vanEngelsdorp *et al*. 2008; Le Conte *et al*. 2010; Neumann & Carreck 2010) and the vulnerability of honeybees to parasitic mites, microsporidian parasites, fungi, viruses and bacteria (Bailey & Ball 1991; Sammataro *et al*. 2000; Martin 2001; Chen *et al*. 2006; Dietemann *et al*. 2009; Genersch 2010; Genersch *et al*. 2010; Ribière *et al*. 2010). These pathogens and parasites can have harmful effects on honeybee health and the services they offer which in turn can lead to severe economic losses (Morse & Calderone 2000; Shen *et al*. 2005; Genersch 2010). The majority of pathogens and parasites affecting honeybees have an almost worldwide distribution (see Chapter 1) with many of these pathogens, especially viruses having a seasonal incidence (Heath 1982; Fries 1993; Allen & Ball 1996; Ribière *et al*. 2008). The single and/or combined role of pathogens and parasites in contributing to honeybee colony losses and reduced honeybee health still needs to be explained (Genersch 2010).

Varroa destructor, an ectoparasitic mite in honeybee colonies, has been linked to many of the observed colony losses and remains the biggest threat to beekeeping in most countries (Ritter 1981; Bailey & Ball 1991; Shimanuki *et al*. 1994; Brodschneider *et al*. 2010; Guzman-Novoa *et al*. 2010; Rosenkranz *et al*. 2010; Schäfer *et al*. 2010; Dietemann *et al*. 2012). *Varroa* mites are known to transmit and activate several of the 18 viruses affecting honeybees (Ball & Allen 1988; Bailey & Ball 1991; Allen & Ball 1996; Bowen-Walker *et al*. 1999; Shen *et al*. 2005; Chen &

Siede 2007; Ribière *et al*. 2008). In addition, it has been shown experimentally that *Varroa* mites can transmit bacteria to honeybees (Strick & Madel 1988; Glinski & Jarosz 1990) and they can act as carriers of the fungal pathogen, Chalkbrood (Liu 1996). Furthermore, the presence of *Varroa* mites can increase the susceptibility of honeybee colonies to stress-related diseases such as European foulbrood (EFB) (Sammataro *et al*. 2000) and Chalkbrood (Finley *et al*. 1996; Medina & Mejia 1999; Hedtke *et al*. 2011). Moreover, Delaplane *et al*. (2010) observed increased levels of tracheal mites (*Acarapis woodi*) in colonies with high *Varroa* mite infestation rates, while Downey & Winston (2001) found that the presence of both these mites in honeybee colonies led to increased levels of winter mortality.

The simultaneous detection of pathogens (viruses, bacteria and *Nosema* spp.) and parasites (parasitic mites and insects) is vital in order to increase our understanding of colony losses and the role each of them play in the weakening of honeybee colonies (Faucon *et al*. 2002; Genersch *et al*. 2010; Runckel *et al*. 2011; Soroker *et al*. 2011). Here I investigate the health status of the African honeybee (*Apis mellifera scutellata*) in relation to pathogen and parasite presence. Adult honeybee and worker brood samples were collected from winter 2010 to autumn 2011 from both migratory and sedentary apiaries. Pathogen and parasite prevalence was compared between the two management types in order to assess whether the movement of colonies for pollination purposes increases the susceptibility of colonies to parasites and pathogens. It has been suggested that the constant movement of migratory colonies cause added stress to honeybees which in turn increases their vulnerability to pathogens and parasites (Swart 2001; Swart *et al*. 2001; Welch *et al*. 2009). Given the role of *Varroa* mites as parasites in honeybee colonies, I also measured the infestation rates in adult honeybees and worker brood from the collected samples.

MATERIALS AND METHODS

1. Sample collection

Apparently healthy honeybee (*Apis mellifera scutellata*) colonies from 13 apiaries belonging to eight beekeepers were randomly selected and sampled each season from July 2010 to August 2011 (Table 1, Fig. 1). Sampled apiaries were situated in and around the Gauteng region of South Africa. Sedentary apiaries (n = 5) remained at these locations permanently, while migratory apiaries (n = 8) were transported to other regions of South Africa (including the North West, Mpumalanga and Limpopo provinces) for pollination services before and between sampling occasions. All colonies were marked to ensure correct identification. Lost, dead or absconded colonies were replaced with new colonies (if available) in order to allow for continued monitoring of the selected apiaries. Two to five honeybee colonies were sampled per apiary (Table 1). On average, 202 ± 103 adult worker honeybees and 100 sealed worker brood cells were collected from each colony for the identification of pathogens and parasites. All samples were kept in a freezer (-20 °C) until analysis.

2. Pathogen and parasite diagnosis

The following honeybee pathogens were screened using PCR methods: Deformed wing virus (DWV), Black queen cell virus (BQCV), *Varroa destructor* virus 1 (VDV-1), Israeli acute paralysis virus (IAPV), Acute bee paralysis virus (ABPV), Chronic bee paralysis virus (CBPV), Sacbrood virus (SBV), *Varroa destructor* Macula-like virus (VdMLV), *Nosema apis*, *Nosema ceranae*, American foulbrood (AFB) and European foulbrood (EFB). Viruses were screened in both adult honeybees and *Varroa* mites whereas *N. apis*, *N. ceranae*, AFB and EFB were screened in adult honeybees only. Even though AFB and EFB are brood diseases, adult

honeybees were screened as they can act as carriers of these pathogens (Wilson 1971; Belloy *et al*. 2007). All eight viruses screened in this study are RNA viruses (Chen *et al*. 2004; Ribière *et al*. 2008) whereas *N. apis, N. ceranae,* AFB and EFB are organisms with DNA as their genetic material. For the purpose of this study in relation to the screening of pathogens, as mentioned above, an apiary refers to the 2-5 colonies that were sampled and subsequently pooled at a specific location. Therefore pathogen analysis was done per apiary and each apiary was represented by the pooled colonies sampled in this study and not by all the other colonies present in the same apiaries that were not sampled in this study.

Parasite and fungal pathogen presence was diagnosed by visual inspection of the adult honeybee and worker brood samples and included: *Varroa destructor*, bee louse (*Braula coeca*), *A. m. capensis* social parasite, small hive beetle (SHB) (*Aethina tumida*), wax moth (*Galleria mellonella*) and Chalkbrood (*Ascosphaera apis*). Parasite and fungal pathogen presence was presented per colony and were therefore not pooled.

Table 1. Sampling locations and number of honeybee colonies screened per season for the presence of pathogens and parasites.

Location code	Locality	Management type	Winter 2010	Spring 2010	Summer 2010-2011	Autumn 2011	Winter 2011
1	Krugersdorp	Sedentary	5	5	-	5	5
2	Magaliesberg Site 1	Migratory	4	4	2	5	5
3	Magaliesberg Site 2	Migratory	5	5	5	5	5
4	Pretoria (Hatfield)	Sedentary	5	5	5	5	5
5	Soshanguve Site 1	Migratory	5	-	-	-	-
6	Soshanguve Site 2	Migratory	5	-	-	-	-
7	Meyerton	Migratory	-	5	5	-	-
8	Rustenburg	Sedentary	-	-	5	-	-
9	Randfontein	Sedentary	-	-	5	-	-
10	Parys	Migratory	-	-	3	-	-
11	Pretoria (Brooklyn)	Sedentary	-	-	5	5	5
12	Pretoria North Site 1	Migratory	-	-	5	-	-
13	Pretoria North Site 2	Migratory	-	-	5	-	-

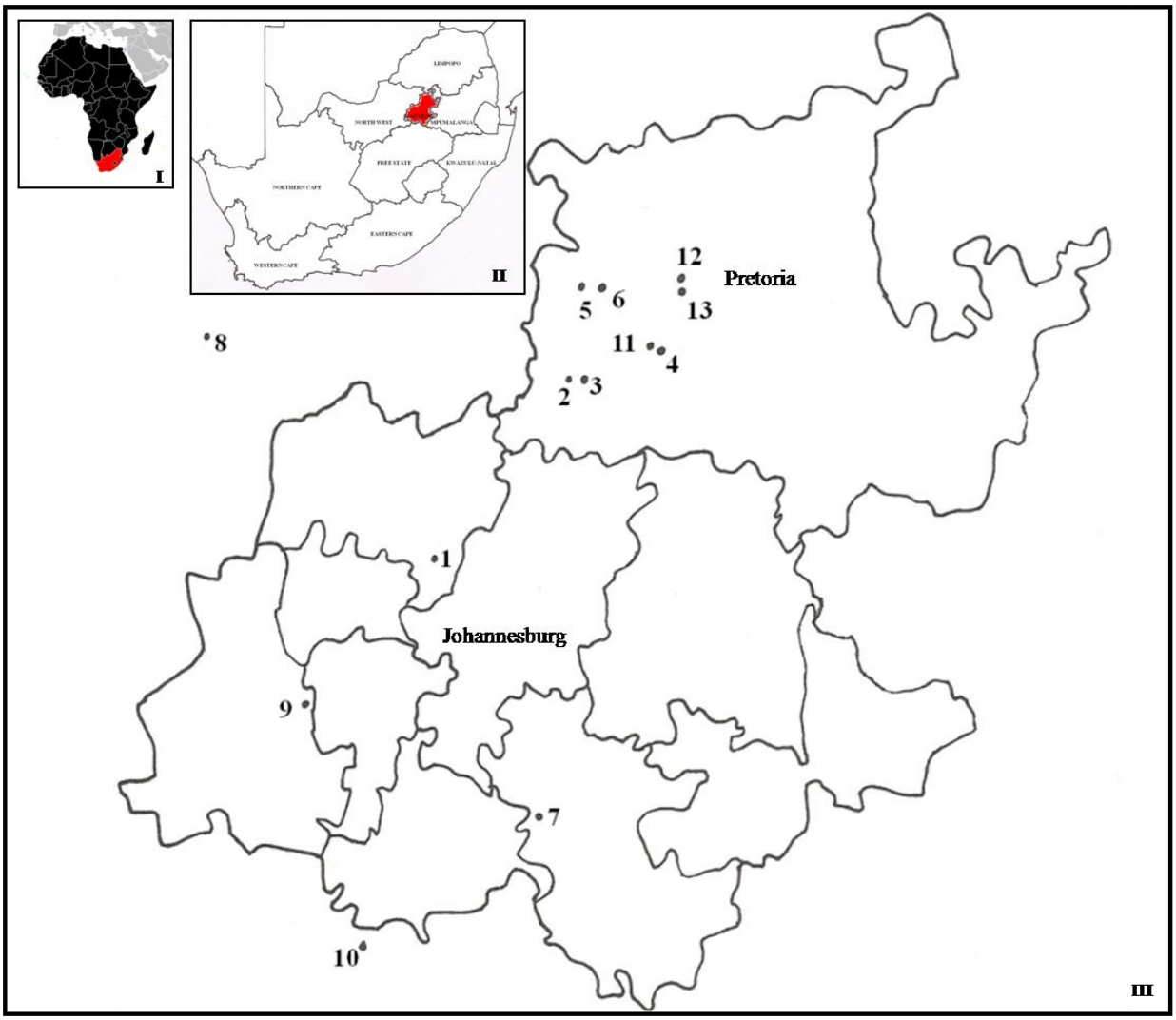

Figure 1. (I) Map of Africa with South Africa highlighted in red. (II) Map showing the nine provinces of South Africa with Gauteng highlighted in red. (III) Map of the Gauteng province of South Africa showing the localities from which samples were collected. Localities 8 and 10 are in the North West and Free State provinces, respectively. The remaining localities are situated in the Gauteng province. (Maps I-III were adapted and modified from Wikipedia.org).

2.1. Pathogen analysis

For PCR diagnosis, six honeybees were pooled per colony from each apiary. When available, up to 45 adult female *Varroa* mites (phoretic on adult honeybees or in the brood) were pooled per apiary. Honeybees and *Varroa* mites were homogenized separately in TN buffer (0.4 M NaCl, Tris 10 mM: pH 7.5) (0.2 ml per honeybee and 200 µl per mite). The samples were placed into Eppendorf tubes and centrifuged at 5000 x g for 10 minutes. Fifty microlitres of the supernatant of each sample were transferred into Eppendorf tubes for total RNA/DNA extraction.

- RNA/DNA extraction

For DWV, BQCV, VDV-1, IAPV, ABPV, CBPV, SBV and VdMLV identification, total RNA was extracted from honeybees and *Varroa* mites using a Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer's protocols. cDNA synthesis was performed with the M-MLV Reverse Transcriptase enzyme from (Invitrogen). For each sample, 9 µl of total extracted RNA, 1 µl of random primers (500µg/ml) and 2 µl dNTP's (2.5mM) were added and incubated at 65 ºC for 5 minutes. This was followed by the addition of 4 µl of 5X cDNA buffer, 1 µl DTT (0.1M), 1 µl RNAse inhibitor, 1 µl water and 1 µl M-MLV RT with a final volume of 20 µl for each sample. Samples were centrifuged and then incubated at 25 °C for 10 minutes, 37 °C for 60 minutes and finally at 70 °C for 15 minutes to inactivate the enzyme. cDNA was diluted with 180 µl of water resulting in a final volume of 200 µl per sample. For *N. apis*, *N. ceranae*, EFB and AFB, total DNA was extracted using a Genomic DNA from Tissue kit (Macherey-Nagel) according to the manufacturer's protocols.

- PCR and product visualization

In order to gain better insight into the general occurrence of viruses in these apiaries, honeybee cDNA from all apiaries (22 µl per apiary) were pooled into one Eppendorf tube and the same was done for *Varroa* mite cDNA. For the rest of the pathogens, extracted honeybee DNA from all apiaries (22 µl per apiary) were pooled into one Eppendorf tube. If a positive result was obtained, additional PCR analysis per apiary was done to determine the specific apiary/apiaries infected. Seven primers (DWV 1-7) were used to screen for DWV in the winter 2010 samples. Primers for DWV (3-5) were designed using Primer Express® software (Applied Biosystems) and primers for DWV (1, 2, 6 and 7) were obtained from the literature (Appendix A - Table I). Since none of these primers gave positive results, only DWV (1), DWV (2) and DWV (7) were used to screen the samples from the remaining seasons to save costs. Also, it was not possible to screen the winter 2010 pooled *Varroa* mite sample with DWV (2), DWV (7) and VDV-1 (2) due to unanticipated circumstances. Primers for BQCV, VDV-1 (1), VDV-1 (2) IAPV, ABPV, CBPV, VdMLV, SBV, *N. apis*, *N. ceranae*, EFB and AFB were obtained from the literature (Appendix A - Table I).

- Reverse Transcriptase-PCR (Qualitative PCR)

For PCR the following master mix was added: 5 µl of cDNA/DNA, 4 µl dNTP's (2.5 mM), 5 µl of 10X reaction buffer, 1 µl each of the forward and reverse primers (10 µM), 1.5 µl $MgCl_2$ (25 mM), 32.3 µl water and 0.2 µl Platinum Taq polymerase (Invitrogen). The final volume of each sample was 50 µl. Positive (previously identified infected samples) and negative (water) controls were also included in all the PCR reactions. The PCR cycling conditions were set at 94 °C for 2 minutes, 35 cycles (94 °C for 30 seconds, 48-56 °C for 20-30 seconds, 72 °C for 1 minute) and finally at 72 °C for 7 minutes (See Appendix A - Table I for primer specific

annealing temperatures of all screened pathogens). Each sample was loaded onto a 1.5% agarose gel stained with Goldview and PCR products were visualized under UV light.

- Real-time Reverse Transcriptase-quantitative PCR (RT-qPCR)

For AFB and EFB, quantitative PCR analysis was done for the winter 2010 samples in a Rotor Gene following the program described in Roetschi *et al*. (2008). Samples were run for 2 minutes at 50 °C, 10 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C and finally for 1 minute at 60 °C.

- Sequencing of amplified PCR products

To confirm the accuracy of the diagnosis, a selected number of amplified PCR products of each pathogen was sequenced, with the exception of EFB which will still be sequenced in due time. PCR products were purified with a high pure PCR template preparation kit (Roche) according to the manufacturer's protocols. Obtained sequences were compared to entries in the NCBI database using the nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Visual inspection

Varroa mite presence and infestation rates were determined in adult honeybee and worker brood samples. *Varroa* mites were separated from adult honeybees using only warm water (De Jong *et al*. 1982; Azizi *et al*. 2008). To allow for a comparison with the previous South African survey on *Varroa* mite infestation rates of adult honeybees, the exact method followed by Allsopp (2006) was used. Warm water was added to honey jars containing approximately 200 honeybees. The honey jars were shaken for 20 seconds to dislodge the *Varroa* mites from the adult honeybees. Two sieves were used to separate the adult honeybees from the *Varroa* mites. The first sieve (King Test laboratory Test Sieve (SABS), Aperture: 2000µm) collected all the

honeybees as well as other material and the second sieve (King Test laboratory Test Sieve (SABS), Aperture: 53µm) collected the *Varroa* mites. The honeybees were placed in the first sieve and washed twice with large amounts of warm water. The number of *Varroa* mites remaining on the second sieve was counted. *Varroa* mite infestation rates in the adult honeybees were summarized as the number of *Varroa* / 100 honeybees.

One hundred sealed worker cells were opened to determine the presence of *Varroa* mites. Cells were opened on both sides of the brood comb when available and generally 50 cells were opened on each side of the comb. In the event that there were not enough sealed cells to open, the maximum number of sealed cells available was opened. Each cell was carefully opened with a toothpick and the content (larvae, pupae or young fully developed honeybee) removed with forceps. *Varroa* mites (mature and immature) present on the larvae, pupae or ready to emerge honeybees as well as in the cells were counted using a dissecting microscope. Even though mites of all developing stages were counted, only adult females were used to determine the *Varroa* infestation rates in the brood cells. *Varroa* mite infestation rates in worker brood were summarized as the number of *Varroa* / 100 worker cells.

Bee lice and *Varroa* mites were separated simultaneously from adult honeybees (see above). The presence of *A. m. capensis* social parasites was diagnosed based on the presence of multiple eggs in open brood cells, noticeable black honeybees and confirmation from beekeepers of *capensis* presence. SHB adults were identified in the adult honeybee samples whereas SHB larvae and eggs were identified in the sealed brood cells. The presence of wax moths was identified based on the occurrence of larvae and their excreta in the sealed brood cells and on developing larvae/pupae/about to emerge honeybees. Chalkbrood was diagnosed on the

presence of either white or black mummies in sealed and unsealed worker brood cells.

3. Statistical analysis

A Chi-square test was performed to compare pathogen and parasite prevalence in migratory and sedentary honeybee colonies. A Mann-Whitney U Test was performed to compare *Varroa* mite infestation rates of adult honeybee and worker brood cells in migratory and sedentary apiaries. All statistical analyses were performed with STATISTICA Version 10.

RESULTS

No significant differences in relation to pathogen and parasite presence were found between migratory and sedentary apiaries (df = 1, $0.01 < \chi^2 < 3.61$, $P > 0.05$). The total number of pathogens and parasites detected in 13 apiaries across all seasons is presented in Table 2. A general overview of the presence and prevalence of pathogens and parasites per season is presented in Tables 3 - 7. The prevalence (%) of pathogens and parasites from both migratory and sedentary apiaries per season were pooled because of the absence of significant differences between the apiaries. *Varroa* mite infestation rates and parasite presence (including Chalkbrood mummies) per colony in the 13 apiaries are presented in Appendix A - Table II and Table III, respectively.

- **Prevalence of pathogens and parasites**

Pathogen prevalence is presented per apiary across all seasons (Fig. 2). Three apiaries (23%) were virus free with the remaining eight apiaries having one or two viruses present. BQCV was the most prevalent virus detected in honeybees. It was found in eight apiaries (62%) and occurred across all seasons (Fig. 2). It was highly prevalent in spring 2010, autumn 2011 and

winter 2011 and less prevalent in summer 2010-2011. BQCV prevalence was noticeably lower in winter 2010 compared to winter 2011. BQCV was absent from all *Varroa* mite samples. IAPV was found in honeybees from two apiaries (16%) only during spring 2010 (Fig. 2). IAPV was also detected in *Varroa* mites from one of these apiaries. VDV-1 presence was confirmed in honeybees from four apiaries (31%) only during summer 2010 (Fig. 2). *Varroa* mites from one of these apiaries also tested positive for VDV-1. EFB was detected in only one apiary (8%) during winter 2010 and was absent during all the other seasons (Fig. 2). *N. apis* was detected in winter 2010, spring 2010 and winter 2011 honeybee samples (Fig. 2). *N. apis* occurrence was limited to five apiaries (39%). ABPV, CBPV, DWV, VdMLV and SBV were not detected in either honeybees or *Varroa* mites. AFB and *N. ceranae* were not detected in the honeybee samples.

The prevalence of parasites and Chalkbrood is given per colony across all seasons (Fig. 3, Appendix A – Table II and Table III). *Varroa* mites were present in 140 out of 149 colonies (94%). Bee lice were detected in 104 colonies (70%) and were highly prevalent in all seasons. *Capensis* social parasites were found in 21 colonies (14%). SHB were detected in 14 colonies (9%) across all seasons. SHB prevalence was intermediate for spring 2010, summer 2010-2011 and autumn 2011. The prevalence of SHB was considerably higher in winter 2011 compared to winter 2010. Wax moths were very common in all seasons except winter 2011. Their presence was recorded in 48 colonies (32%). Wax moths were highly prevalent in autumn 2011 and winter 2010. The prevalence of wax moths was considerably higher in winter 2010 compared to winter 2011. White and black Chalkbrood mummies were found in 12 colonies (8%) across all seasons except winter 2011.

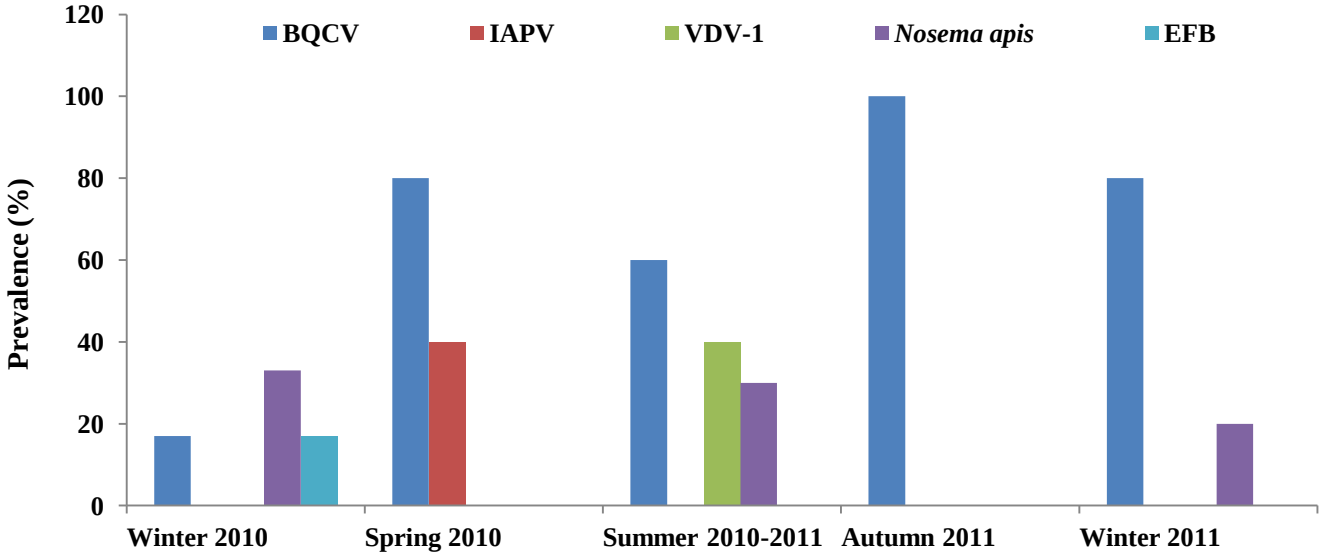

Figure 2. Prevalence (%) of pathogens measured at the apiary level from winter 2010 to winter 2011 in *Apis mellifera scutellata* colonies.

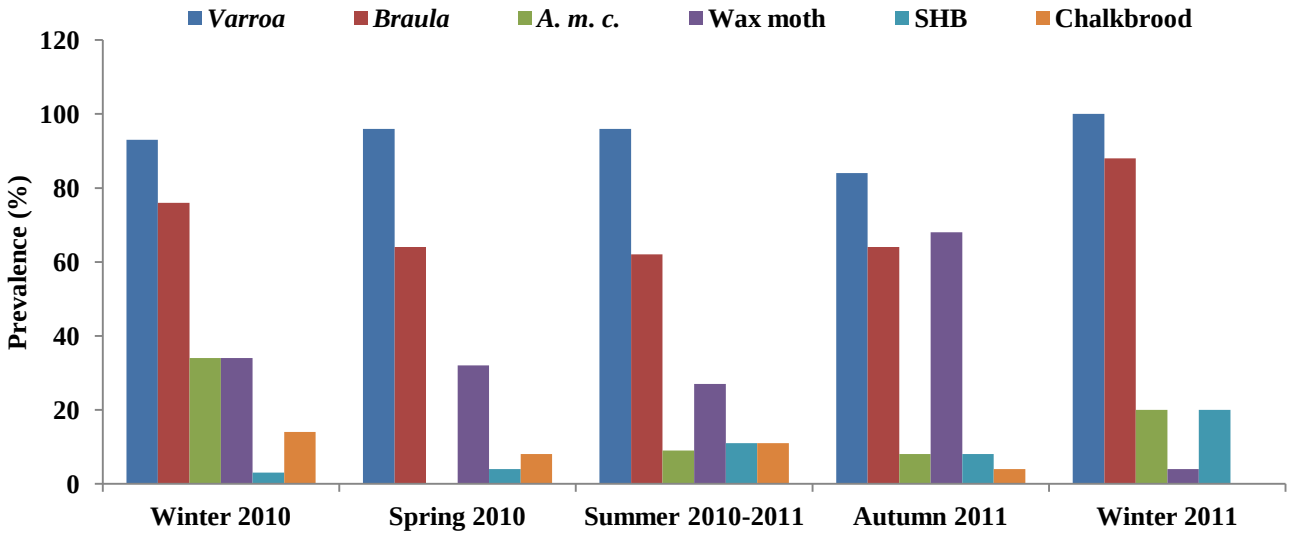

Figure 3. Prevalence (%) of five parasites and Chalkbrood measured at the colony level from winter 2010 to winter 2011 in *Apis mellifera scutellata* colonies.

- **Co-occurrence of multiple pathogens and parasites in apiaries and colony losses**

The co-occurrence of more than one pathogen and parasite per apiary was relatively common across all seasons (Tables 2 - 7). During spring 2010, the same two viruses (BQCV and IAPV) were detected in one sedentary apiary and one migratory apiary. Two more viruses (BQCV and VDV-1) were detected simultaneously in a migratory apiary during summer 2010-2011. The simultaneous detection of BQCV and *N. apis* in honeybees was found in two apiaries. Overall, very few colony losses were reported. Beekeepers attributed most of the colony losses observed in this study to *A. m. capensis* infestation. Damage by honey badgers and overall weakness of colonies due to unknown reasons were also recorded.

Table 2. The total number of different pathogens and parasites detected in 13 *Apis mellifera scutellata* apiaries across all seasons.

Apiary code	Viruses (*n* = 8)	Bacteria (*n* = 2)	Microsporidian parasites (*n* = 2)	Chalkbrood (*n* = 1)	Parasites (*n* = 5)
1	1	0	0	0	5
2	2	0	1	1	5
3	1	0	1	1	4
4	2	1	1	1	5
5	0	0	0	0	3
6	0	0	0	1	4
7	1	0	0	0	5
8	1	0	0	0	3
9	0	0	0	1	4
10	2	0	0	0	1
11	2	0	1	0	4
12	1	0	0	1	2
13	1	0	1	0	5

Table 3. Presence and prevalence of pathogens and parasites screened during Winter 2010 in six *Apis mellifera scutellata* apiaries.

	Viruses								Brood pathogens			Parasites						
Apiary code	**DWV**	**BQCV**	**VDV-1**	**IAPV**	**ABPV**	**CBPV**	**VdMLV**	**SBV**	**EFB**	**AFB**	**Chalk-brood**	***Nosema apis***	***Nosema ceranae***	***Varroa***	***A. m. c.***	***Braula***	**Wax moth**	**SHB**
1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
2	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	+	-
3	-	**(A) +**	-	-	-	-	-	-	-	-	+	+	-	+	-	+	+	+
4	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	+	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
6	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	-
Prevalence (%)	**0**	**17**	**0**	**0**	**0**	**0**	**0**	**0**	**17**	**0**	**50**	**33**	**0**	**100**	**33**	**100**	**83**	**17**

Abbreviations: DWV: Deformed wing virus, BQCV: Black queen cell virus, VDV-1: *Varroa destructor virus* 1, IAPV: Israeli acute paralysis virus, ABPV: Acute bee paralysis virus, CBPV: Chronic bee paralysis virus, VdMLV: *Varroa destructor* Macula-like virus, SBV: Sacbrood virus, EFB: European Foulbrood, AFB: American Foulbrood, *A. m. c.*: *Apis mellifera capensis*, SHB: Small hive beetle. (A) + Indicates presence in honeybees and (V) + indicates presence in *Varroa* mites. – Indicates absence from sample.

Table 4. Presence and prevalence of pathogens and parasites screened during Spring 2010 in five *Apis mellifera scutellata* apiaries.

	Viruses								Brood pathogens			Parasites						
Apiary code	**DWV**	**BQCV**	**VDV-1**	**IAPV**	**ABPV**	**CBPV**	**VdMLV**	**SBV**	**EFB**	**AFB**	**Chalk-brood**	***Nosema apis***	***Nosema ceranae***	***Varroa***	***A. m. c.***	***Braula***	**Wax moth**	**SHB**
1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
2	-	**(A) +**	-	**(A) & (V) +**	-	-	-	-	-	-	+	-	-	+	-	+	-	-
3	-	**(A) +**	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-
4	-	**(A) +**	-	**(A) +**	-	-	-	-	-	-	-	-	-	+	-	+	+	-
7	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
Prevalence (%)	**0**	**80**	**0**	**40/20***	**0**	**0**	**0**	**0**	**0**	**0**	**40**	**0**	**0**	**100**	**0**	**100**	**60**	**20**

Abbreviations: DWV: Deformed wing virus, BQCV: Black queen cell virus, VDV-1: *Varroa destructor virus* 1, IAPV: Israeli acute paralysis virus, ABPV: Acute bee paralysis virus, CBPV: Chronic bee paralysis virus, VdMLV: *Varroa destructor* Macula-like virus, SBV: Sacbrood virus, EFB: European Foulbrood, AFB: American Foulbrood, *A. m. c.*: *Apis mellifera capensis*, SHB: Small hive beetle. (A) + Indicates presence in honeybees and (V) + indicates presence in *Varroa* mites. – Indicates absence from sample.

Table 5. Presence and prevalence of pathogens and parasites screened during Summer 2010-2011 in ten *Apis mellifera scutellata* apiaries.

	Viruses								Brood pathogens			Parasites						
Apiary code	**DWV**	**BQCV**	**VDV-1**	**IAPV**	**ABPV**	**CBPV**	**VdMLV**	**SBV**	**EFB**	**AFB**	**Chalk-brood**	***Nosema apis***	***Nosema ceranae***	***Varroa***	***A. m. c.***	***Braula***	**Wax moth**	**SHB**
2	-	**(A) +**	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-
3	-	**(A) +**	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-
4	-	**(A) +**	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-
7	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
8	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+
9	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+
10	-	**(A) +**	**(A) & (V) +**	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
11	-	-	**(A) +**	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+
12	-	-	**(A) +**	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-
13	-	-	**(A) +**	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+
Prevalence (%)	**0**	**60**	**40/10***	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**40**	**30**	**0**	**100**	**30**	**80**	**60**	**40**

Abbreviations: DWV: Deformed wing virus, BQCV: Black queen cell virus, VDV-1: *Varroa destructor virus* 1, IAPV: Israeli acute paralysis virus, ABPV: Acute bee paralysis virus, CBPV: Chronic bee paralysis virus, VdMLV: *Varroa destructor* Macula-like virus, SBV: Sacbrood virus, EFB: European Foulbrood, AFB: American Foulbrood, *A. m. c.*: *Apis mellifera capensis*, SHB: Small hive beetle. (A) + Indicates presence in honeybees and (V) + indicates presence in *Varroa* mites. – Indicates absence from sample.

Table 6. Presence and prevalence of pathogens and parasites screened during Autumn 2011 in five *Apis mellifera scutellata* apiaries.

	Viruses								Brood pathogens			Parasites						
Apiary code	**DWV**	**BQCV**	**VDV-1**	**IAPV**	**ABPV**	**CBPV**	**VdMLV**	**SBV**	**EFB**	**AFB**	**Chalk-brood**	***Nosema apis***	***Nosema ceranae***	***Varroa***	***A. m. c.***	***Braula***	**Wax moth**	**SHB**
1	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
2	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
3	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
4	-	**(A) +**	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-
11	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
Prevalence (%)	**0**	**100**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**20**	**0**	**0**	**100**	**40**	**100**	**100**	**40**

Abbreviations: DWV: Deformed wing virus, BQCV: Black queen cell virus, VDV-1: *Varroa destructor virus* 1, IAPV: Israeli acute paralysis virus, ABPV: Acute bee paralysis virus, CBPV: Chronic bee paralysis virus, VdMLV: *Varroa destructor* Macula-like virus, SBV: Sacbrood virus, EFB: European Foulbrood, AFB: American Foulbrood, *A. m. c.*: *Apis mellifera capensis*, SHB: Small hive beetle. (A) + Indicates presence in honeybees and (V) + indicates presence in *Varroa* mites. – Indicates absence from sample.

Table 7. Presence and prevalence of pathogens and parasites screened during Winter 2011 in five *Apis mellifera scutellata* apiaries.

	Viruses								Brood pathogens			Parasites						
Apiary code	**DWV**	**BQCV**	**VDV-1**	**IAPV**	**ABPV**	**CBPV**	**VdMLV**	**SBV**	**EFB**	**AFB**	**Chalk-brood**	***Nosema apis***	***Nosema ceranae***	***Varroa***	***A. m. c.***	***Braula***	**Wax moth**	**SHB**
1	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+
3	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+
4	-	**(A) +**	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+
11	-	**(A) +**	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+
Prevalence (%)	**0**	**80**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**20**	**0**	**100**	**40**	**100**	**20**	**80**

Abbreviations: DWV: Deformed wing virus, BQCV: Black queen cell virus, VDV-1: *Varroa destructor virus* 1, IAPV: Israeli acute paralysis virus, ABPV: Acute bee paralysis virus, CBPV: Chronic bee paralysis virus, VdMLV: *Varroa destructor* Macula-like virus, SBV: Sacbrood virus, EFB: European Foulbrood, AFB: American Foulbrood, *A. m. c.*: *Apis mellifera capensis*, SHB: Small hive beetle. (A) + Indicates presence in honeybees and (V) + indicates presence in *Varroa* mites. – Indicates absence from sample.

***Varroa destructor* infestation rates**

In general (excluding season) *Varroa* mite infestation rates of **adult honeybees** were not significantly different between migratory and sedentary apiaries (U = 2805.00; Z = 1.57; P > 0.05). No significant differences were observed when comparing the adult honeybee infestation rates of migratory and sedentary colonies during spring 2010 (U = 174.00; Z = - 0.16; P > 0.05), summer 2010-2011 (U = 229.50; Z = - 0.46; P > 0.05), autumn 2011 (U = 49.50; Z = - 1.39; P > 0.05) and winter 2011 (U = 57.00; Z = - 0.97; P > 0.05). The only significant difference between migratory and sedentary apiaries in relation to *Varroa* mite infestation rates was observed during winter 2010. *Varroa* mite infestation rates were significantly higher in sedentary apiaries compared to migratory apiaries (U = 45.00; Z = - 2.27; P < 0.05) (Fig. 4). Adult honeybee infestation rates were highest in migratory colonies during spring 2010 and lowest during winter 2010 (Fig. 4). In sedentary apiaries the lowest and highest adult honeybee infestation rates were recorded during autumn 2011 and winter 2011, respectively (Fig. 4). *Varroa* mite infestation rates of adult honeybees are given in Appendix A - Table II.

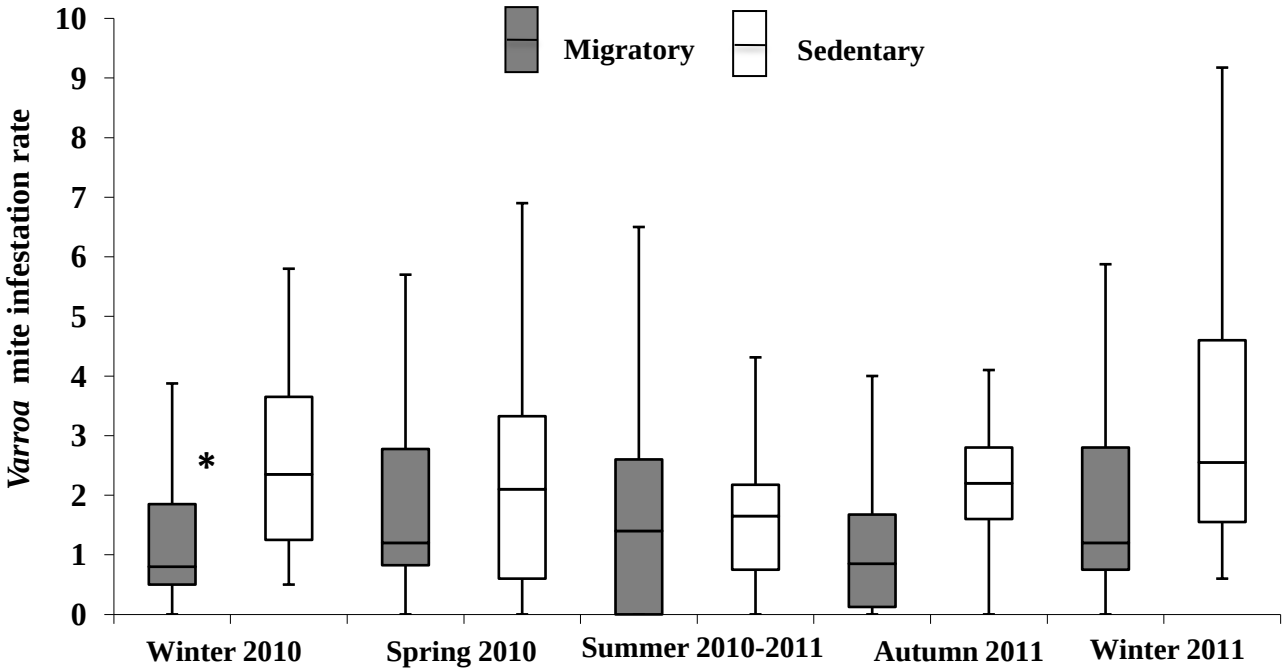

Figure 4. *Varroa destructor* infestation rates (median, minimum, maximum) of adult honeybees (*Varroa* / 100 honeybees) in migratory and sedentary apiaries per season (*Mann-Whitney U Test $P < 0.05$).

A total of 15 683 brood cells were opened to determine the infestation rates of **worker brood** per season of both management types. Generally (excluding season) *Varroa* mite infestation rates of worker brood were not significantly different between migratory and sedentary apiaries (U = 2645.50; Z = 1.87; $P > 0.05$). No significant differences were observed when comparing the worker brood infestation rates of migratory and sedentary colonies during spring 2010 (U = 144.50; Z =- 1.02; $P > 0.05$), summer 2010-2011 (U = 196.50; Z = - 1.21; $P > 0.05$), autumn 2011 (U = 41.00; Z = - 1.67; $P > 0.05$) and winter 2011 (U =55.50; Z = - 0.69; $P > 0.05$). *Varroa*

mite infestation rates of worker brood cells were significantly higher in sedentary apiaries compared to migratory apiaries during winter 2010 (U = 50.00; Z = - 2.04; P < 0.05) (Fig. 5). Brood infestation rates were highest during summer 2010-2011 and lowest during winter 2010 in both migratory and sedentary apiaries (Fig. 5). *Varroa* mite infestation rates of worker brood per apiary are given in Appendix A - Table II.

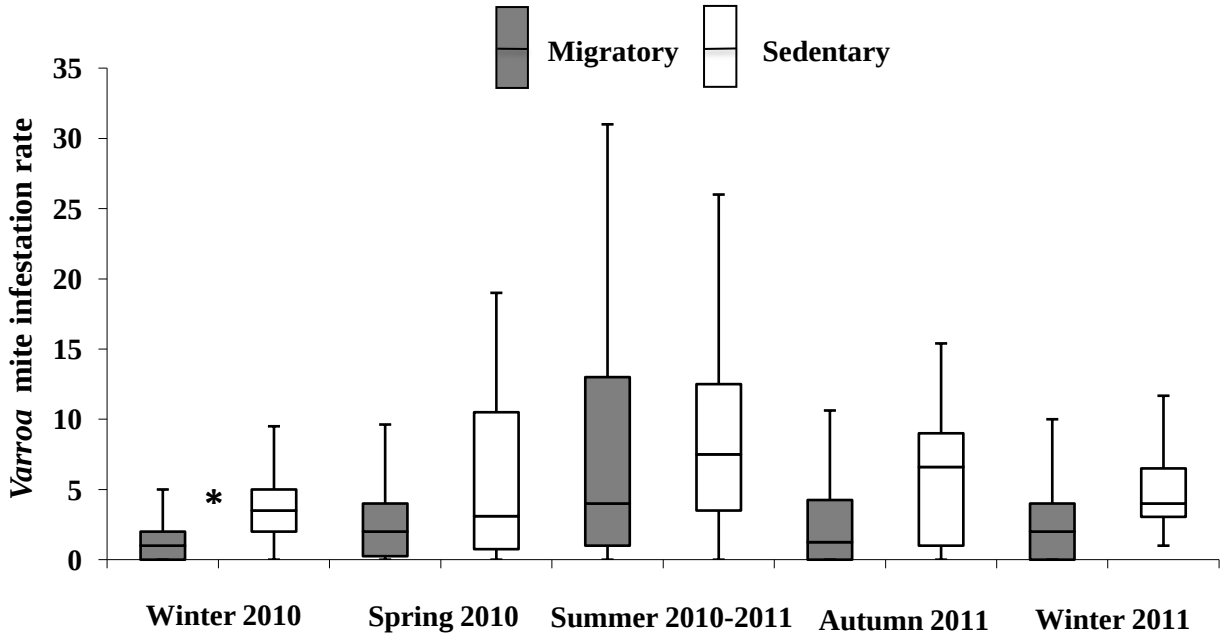

Figure 5. ***Varroa destructor*** **infestation rates (median, minimum, maximum) of worker brood (*Varroa* / 100 worker cells) in migratory and sedentary apiaries per season (*Mann-Whitney U Test P < 0.05).**

DISCUSSION

Honeybee parasites were relatively common across all seasons and pathogens were also present but to a lesser extent (Tables 2 - 7). Bee lice, wax moths, SHB, *Varroa* mites and *capensis* social parasites were found in both sedentary and migratory apiaries. In South Africa, wax moths and SHB are believed to only cause problems in weak honeybee colonies (Lundie 1940; Swart *et al*. 2001) and bee lice are regarded as an irritation to honeybees rather than a threat (Hepburn 1978). However, *capensis* social parasites are much more of a problem in *A. m. scutellata* colonies, since they have the ability to successfully infiltrate *A. m. scutellata* colonies and have been responsible for the death of thousands of *A. m. scutellata* colonies in South Africa (Allsopp & Crewe 1993; Neumann & Moritz 2002). Our results showed no significant difference in the infestation rates of *A. m. capensis* between migratory and sedentary apiaries. This is unexpected given that it was recently demonstrated that migratory apiaries were more vulnerable to *A. m. capensis* infestation (Dietemann *et al*. 2006). Swart *et al*. (2001) also reported that *A. m. capensis* infestation rates were generally higher in migratory *A. m. scutellata* apiaries compared to sedentary apiaries. The possibility that horizontal transmission of this parasite was occurring between migratory (from surrounding beekeepers not part of the study) and sedentary apiaries through close contact could explain why no significant differences were observed (Dietemann *et al*. 2006).

Varroa mites were found to be highly prevalent in *A. m. scutellata* colonies and this corresponds to results obtained in a previous survey of *A. m. scutellata* colonies (Allsopp 2006). At the colony level, observed *Varroa* mite infestation rates of adult honeybees were extremely high in some instances with one sedentary and one migratory colony having as many as 17 and 23 mites per 100 honeybees respectively (Appendix A - Table II). Similar and even higher infestation

rates were also previously reported in Cape honeybees and *A. m. scutellata* sampled during 1998 - 2000 (Allsopp 2006). However, on average for all apiaries per season, *Varroa* infestation rates were relatively low and never exceeded 4.0 mites per 100 adult honeybees (Fig. 4). The average infestation rates of untreated *A. m. scutellata* colonies measured during autumn and winter of 1999 were 7.7 and 1.1 mites per 100 adult honeybees, respectively (Allsopp 2006). In Cape honeybees, Allsopp (2006) recorded average infestation rates of 3.7 and 2.1 mites per 100 adult honeybees during the summer and winter months, respectively. In Africanized honeybee colonies adult honeybee infestation rates of 3.5 mites per 100 adult honeybees are also considered to be relatively low (Medina *et al*. 2002). Indeed, susceptible honeybee colonies in Germany with *Varroa* infestation rates of 3.4 mites per 100 adult honeybees were able to survive, but colonies with 15.1 mites per 100 adult honeybees collapsed (Genersch *et al*. 2010). The average worker brood infestation rates for all apiaries per season were never above ten mites per 100 worker brood cells (Fig. 5). In the Cape honeybee an average of 6.2 mites per 100 worker brood cells was recorded (Allsopp 2006). In South Africa brood is produced throughout the year, although to a lesser extent during winter, therefore there is no interruption in *Varroa* mite reproduction at any point during the year. The infestation rates of *Varroa* mites are therefore expected to be higher in adult honeybees and lower in brood during the colder months when less brood is available (Figs. 4 - 5).

During the initial spread of *Varroa* mites in South African honeybee colonies, the prevalence of Chalkbrood was found to be unusually higher than normal (Swart *et al.* 2001; Allsopp 2004; Allsopp 2006). Liu (1996) detected an increased occurrence of Chalkbrood in *Varroa* mite infested colonies compared to colonies that were free of the parasite and Medina & Mejia (1999) found that colonies in Mexico collapsed with a much lower number of *Varroa* mites when

Chalkbrood was also present. Chalkbrood was detected in almost half of the apiaries screened during this study. At the colony level, ten colonies from six apiaries infested with Chalkbrood had on average 2.0 ± 1.7 mites per 100 adult honeybees and 6.3 ± 6.4 per 100 worker brood cells (data not shown). It is difficult to suggest a possible association between increased Chalkbrood presence and *Varroa* mites in this case because all apiaries were infested with *Varroa* mites, only a small piece of worker brood was used to identify Chalkbrood and a whole colony assessment for Chalkbrood was not done. However, of the 94% of honeybee colonies infested with *Varroa* mites in this study, only 8% of these colonies were infested with Chalkbrood as well, thereby suggesting a very weak interaction, if any, between the two species.

In South Africa, *N. apis* is considered to be a widespread pathogen of adult honeybees (Buys 1976). Recently, three surveys were done over a period of 18 months covering eight regions of South Africa to determine the *N. apis* infestation levels in migratory and sedentary honeybee colonies (Swart 2003). *Nosema apis* occurred in all eight regions, with migratory colonies having slightly higher *N. apis* infestation rates than sedentary colonies (Swart 2003). We found no significant differences between sedentary and migratory apiaries and *N. apis* was not as common as expected. *Nosema ceranae* was not detected in any of the adult honeybee samples collected during this study and therefore Algeria remains the only country in Africa thus far that has recorded this microsporidian pathogen (Higes *et al*. 2009).

The most recent assessment on EFB prevalence in eight regions of South Africa showed that 87% of the screened apiaries tested positive for EFB (Davison *et al*. 1999). EFB detection in only one apiary and season was unexpected considering that it is a common infection in South African honeybees (Buys 1976; Davison *et al*. 1999). This positive result came from a colony

that showed no clinical symptoms of EFB presence. This is similar to Belloy *et al.* (2007) who found that EFB can be detected in adult honeybee workers of colonies showing no clinical symptoms. AFB, which was only recently found to be present in the Western Cape region of South Africa (Baxter 2009, Human *et al*. 2011) was not detected in the apiaries sampled in this study. The non detection of AFB in the samples does confirm, at least for the apiaries screened in this study, that this bacterial disease is still absent from some areas in the Gauteng region.

In total eight viruses were screened in honeybees and *Varroa* mites. Three viruses were detected across different seasons of which BQCV, IAPV and VDV-1 were found in honeybees. Only IAPV and VDV-1 were found in *Varroa* mites. IAPV was first diagnosed in Israel where it reportedly caused large scale honeybee mortality and symptoms included trembling wings and paralysis (Maori *et al*. 2007; Palacios *et al*. 2008). The detection of IAPV in the *Varroa* mites sampled during this study is of concern given that it was recently confirmed that *Varroa* mites are capable of transmitting IAPV to honeybees (Di Prisco *et al*. 2011). Moreover, it was shown that IAPV is an important indicator for colony collapse disorder (CCD) (Cox-Foster *et al*. 2007). IAPV was only recorded during spring 2010 in two apiaries. Even though this virus is present in the Gauteng region of South Africa, its prevalence is relatively low (Tables 2 - 7).

In contrast to IAPV and VDV-1, BQCV was present throughout the year and was the most prevalent virus detected. BQCV has previously been reported South Africa (Allen & Ball 1996; Davison *et al*. 1999) and more recently in Uganda (Kajobe *et al*. 2010). BQCV was also detected throughout the year in French apiaries (Tentcheva *et al*. 2004). This virus is closely associated with the microsporidian parasite, *N. apis* (Bailey *et al*. 1983). In this study, a total of eight apiaries tested positive for BQCV of which two of these apiaries also tested positive for *N.*

apis (25% co-occurrence). BQCV presence in honeybees only and not in *Varroa* mites is similar to results obtained in France (Tentcheva *et al*. 2004) and Hungary (Forgach *et al*. 2008), where BQCV was also screened in both honeybees and *Varroa* mites, but only detected in honeybees. Ball & Allen (1988) found that it is unlikely that the feeding action of *V. destructor* plays a role in BQCV incidence and transmission since this virus only infects honeybees when consumed. The observation by Ball & Allen (1988), together with results from the current and previous (Tentcheva *et al*. 2004; Forgach *et al*. 2008) studies suggest that *Varroa* mites might not play a major role in transmitting this virus (Carreck *et al*. 2002; Tentcheva *et al*. 2004; Ribière *et al*. 2008). BQCV was, however, detected in *Varroa* mites from an apiary in Thailand (Chantawannakul *et al*. 2006) and Ball (1989) found that *Varroa* mites can be vectors of BQCV. Laboratory experiments also showed that *V. destructor* was able to transmit BQCV from BQCV infected pupae to uninfected target pupae (Johns 2003). Consequently, more studies are required to clarify the relationship, if any, between *V. destructor* and BQCV (Chen & Siede 2007).

VDV-1 and DWV are very closely linked viruses that share 84% sequence identity (Ongus 2006). Information on the pathogenicity of VDV-1 is relatively scarce, but it appears to be as prevalent as DWV in Europe (Ongus 2006; Zioni *et al*. 2011). Both DWV and VDV-1 can co-occur in the same apiaries and even in the same individual mites and honeybees (Ongus 2006). In our study DWV and VDV-1 were not observed in the same apiaries. VDV-1 prevalence was relatively low and restricted to the warmer months of the year (Table 5).

ABPV, CBPV, DWV, VdMLV and SBV were not detected in the honeybee or *Varroa* mite samples. With the exception of VdMLV, a recently discovered virus (Gauthier *et al*. 2011), all these viruses have previously been detected in South Africa (Allen & Ball 1996; Davison *et al*.

1999). The non detection of five of the eight viruses may indicate, that these viruses are either present at very low undetectable levels in the form of inapparent infections (Sumpter & Martin 2004), or that they are simply not present in these apiaries. The absence of DWV from the honeybee and *Varroa* mite samples was unexpected given the high prevalence of DWV in apiaries around the world (Allen & Ball 1996; Martin *et al*. 1998; Tentcheva *et al*. 2004; Ellis & Munn 2005; Berényi *et al*. 2006; Chen & Siede 2007; Baker & Shroeder 2008) and the close association of DWV with *Varroa* mites (Bailey & Ball 1991; Bowen-Walker *et al*. 1999; Yang & Cox-Foster 2005; Yue & Genersch 2005; Gisder *et al*. 2009; Dainat *et al*. 2012). Recently, DWV was also found to be absent from drones collected in South Africa (Yañez *et al*. 2012) and honeybee samples of Uganda (Kajobe *et al*. 2010). The only record of DWV presence in South African honeybees was in 1993, prior to the arrival of *Varroa* mites (Allen & Ball 1996). The absence of DWV in the samples from this study could explain why the negative effects of *Varroa* mites were apparently absent in these apiaries.

It has been suggested that the constant transportation of migratory colonies can cause stress to honeybees (Swart 2001; Kryger *et al*. 2003; Ostiguy 2010). Migratory colonies are also more likely to come into contact with other colonies, which increases the risk of acquiring pathogens (Welch *et al.* 2009). Recently, migratory apiaries in the USA were found to have a higher number of viruses compared to sedentary apiaries (Welch *et al.* 2009) and in South Africa, *A. m. capensis* parasites were also more frequently encountered in migratory apiaries (Swart *et al*. 2001; Dietemann *et al*. 2006). In this study, however, honeybee pathogens and parasites were equally prevalent in both management types, with no significant differences found. This suggests that parasites and pathogens were present in both management types irrespective of whether the colonies were moved for pollination purposes or remained stationary on a permanent

basis. Recent surveys done in the USA in relation to winter colony losses found that losses experienced by beekeepers were also similar for both management types thereby indicating that the movement of colonies did not necessarily lead to higher colony losses (vanEngelsdorp *et al*. 2008; vanEngelsdorp *et al*. 2010). The presence of more than one pathogen and parasite per apiary was fairly common in this study (Tables 2 - 7). Multiple infections of viruses specifically are frequently diagnosed in apiaries (Tentcheva *et al*. 2004; Berényi *et al*. 2006; Chen *et al*. 2006; Baker & Schroeder 2008; Forgach *et al*. 2008; Nielsen *et al*. 2008; Teixeira *et al*. 2008; Welch *et al*. 2009; Kojima *et al*. 2011; Soroker *et al*. 2011). Multiple viruses have even been detected in 93% and 83% of queens from apiaries in the USA (Chen *et al*. 2005) and France (Gauthier *et al*. 2011), respectively. Moreover, Chantawannakul *et al*. (2006) found as many as five viruses in an individual *Varroa* mite.

In conclusion, a total of 12 parasites and pathogens were found in 13 honeybee apiaries in the Gauteng region of South Africa over a period of 14 months. This is the first report of IAPV and VDV-1 in South African honeybees as well as in *Varroa* mites infesting *A. m. scutellata* colonies. The transmission routes of these viruses in *A. m. scutellata* colonies are not known yet, but the detection of both these viruses in *Varroa* mites does suggest that *Varroa* mites can possibly act as vectors of these viruses. In South Africa, there have been reports of increased honeybee mortality in *Varroa* mite infested colonies that tested positive for BQCV, ABPV and two unidentified viruses (Davison *et al*. 1999; Swart *et al*. 2001). However, the extent and severity of these colony losses were not specified. Colony losses were also recorded just after the initial invasion of *Varroa* mites into South Africa (Allsopp 2006), while *A. m. capensis* social parasites have also caused significant colony losses (Allsopp 1992; Allsopp & Crewe 1993). *A. m. capensis* social parasites were also responsible for most of the observed colony losses in this

study. No colony losses in this study were directly attributed to *Varroa* mite presence or their associated pathogens, with relatively low *Varroa* mite infestation rates being recorded across all seasons (Figs. 2 - 3). These results are in contrast to other studies around the world where *Varroa* mites played a significant or central role in colony losses (see introduction). Colony losses can be reduced by ensuring good beekeeping practices, better control measures to avoid the spread of pathogens and parasites to uninfected colonies and by keeping stress levels in honeybee colonies to a minimum (Fries & Camazine 2001; Swart *et al*. 2001; Dietemann *et al*. 2006). Even though some apiaries in this study were infested with multiple pathogens and parasites, no obvious signs of disease were observed thereby confirming the health of the African honeybee population studied.

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CHAPTER 3

Population dynamics of *Varroa destructor* and the impact on honeybee (*Apis mellifera scutellata*) colony development

INTRODUCTION

The honeybee parasite, *Varroa destructor*, has an almost global distribution, with the exception except of Australia, (see Chapter 1) after shifting host from *Apis cerana* Fabr. to *Apis mellifera* L. (Rath & Drescher 1990; Donzé & Guerin 1994; Solignac *et al*. 2005). Globally, *Varroa* mites are believed to be responsible for the mortality of a large number of honeybee colonies (deGrandi-Hoffman *et al*. 2002; Medina *et al*. 2002; Dahle 2010; Guzman-Novoa *et al*. 2010; Schäfer *et al*. 2010). In most countries honeybee colonies cannot survive without chemical treatment against *Varroa* mites and they usually die within a few years if left untreated (Harbo & Hoopingarner 1997; Martin 1998; Wilkinson *et al*. 2001; Gregorc & Poklukar 2003; Le Conte *et al*. 2010; Rosenkranz *et al*. 2010; vanEngelsdorp & Meixner 2010). Although this is generally the rule, there are several examples of honeybee colonies that have survived in the presence of *Varroa* mites without chemical treatment for a considerable number of years (De Jong & Soares 1997; Fries *et al*. 2006; Le Conte *et al*. 2007; Seeley 2007; Locke & Fries 2011).

Displays of tolerance to *Varroa* mites are most commonly known in the original host of *V. destructor*, the Asian honeybee (*A. cerana*) and Africanized honeybees (see below). *Varroa* mite tolerance in *A. cerana* can be explained by the long-term parasite-host association that exists between these two species (Peng *et al*. 1987; Rosenkranz & Engels 1994; Fries *et al*. 1996; Boot *et al*. 1999; Rosenkranz *et al*. 2010). The ability of *A. cerana* to maintain *Varroa* mite infestations at relatively low levels is mainly due to *Varroa* mite reproduction being limited to drone brood as well as the grooming and hygienic behaviour exhibited by these honeybees (Koeniger *et al*. 1983; Peng *et al*. 1987; De Jong 1988; Büchler *et al*. 1992; Rath 1999). Adult honeybees are able to sense the presence of mites in brood cells and they are very efficient at killing mites and eliminating them from colonies (Peng *et al*. 1987).

In 1956, *A. m scutellata* queens were introduced into Brazil and mixed with previously introduced European honeybee sub-species which established large feral populations (Kerr 1967; Francoy *et al*. 2009). The so called Africanized honeybees spread to most parts of South, Central and North America (Sheppard *et al*. 1991; Winston 1992; Visscher *et al*. 1997; Page 1998; Sheppard & Smith 2000). A significant amount of research has been done on the genetic and behavioural composition of the invasive Africanized honeybee, with most of the research indicating that a high percentage of African characters are conserved within these populations (Schneider *et al*. 2004; Moritz *et al*. 2005; Kraus *et al*. 2007). In general, tolerance to *Varroa* mites in Africanized honeybees has been attributed to the presence of a high number of infertile female mites (Rosenkranz & Engels 1994), the uncapping of worker brood cells by honeybees that contain *Varroa* mites (Corrêa-Marques & De Jong 1998), the removal of *Varroa* mite infested brood (Guerra *et al*. 2000; Vandame *et al*. 2002) and the mortality of both male and female *Varroa* mite offspring which influences the reproductive cycle of the mites (Medina & Martin 1999; Mondragon *et al*. 2005; Mondragon *et al*. 2006). A well known example of long-term *Varroa* mite tolerance is found in Africanized honeybees from Brazil (Rosenkranz 1999). *Varroa* mite infestation rates have remained very low over the years, from when the mite was first observed in the early seventies up until recent times, with no serious reports of honeybee mortality (De Jong *et al*. 1984; Camazine & Morse 1988; Moretto & Leonidas 2003; Carneiro *et al*. 2007; Calderón *et al*. 2010). Although tolerance has not been studied as much in Africa, possible reasons for *Varroa* mite tolerance in honeybees on the African continent have been attributed to a short post-capping stage, good hygienic and grooming behaviour as well as a lack of acaricide use (Moritz & Hänel 1984; Moritz 1985; Boecking & Ritter 1993; Allsopp 2006; Frazier *et al*. 2010).

The introduction of *Varroa* mites into South Africa was a fairly recent occurrence. *Varroa* mites invaded the Western Cape region of South Africa approximately 14 years ago (Allsopp 1997; Martin & Kryger 2002). They spread quite rapidly to most parts of the country where they infested both wild and commercially kept honeybee colonies of both sub-species (Martin & Kryger 2002; Allsopp 2006). During the first few years of invasion by *Varroa* mites, typical symptoms of *Varroa* mite presence in honeybee colonies were observed and included: pink-eyed pupae either dead in their cells or outside the hive, a spotty brood pattern, honeybees with deformed wings and shortened abdomens as well as a large number of Chalkbrood mummies (Allsopp 2006). Also, during this initial stage, *Varroa* mite population sizes were estimated to be between 10 000 - 50 000 per colony and in a comparative study between chemically treated and untreated colonies Allsopp (2006) found colony losses of up to 35% in untreated colonies. However, after extensive examination of both wild and commercial honeybee colonies Allsopp (2006) found that both honeybee sub-species of South Africa were able to survive without treatment in the presence of *Varroa* mites.

My aim is to assess the population dynamics of *Varroa* mites in African honeybee (*A. m. scutellata*) colonies as well as their impact on the development of these colonies some years after the first reports of possible tolerance was exhibited by South African honeybees towards *Varroa* mites (Allsopp 2006). Colony losses were reported during the initial phase of *Varroa* mite introduction into South Africa (Allsopp 2006), so here I determine what the current situation regarding *Varroa* mite tolerance is in *A. m. scutellata* colonies during the winter and spring of 2011. The daily *Varroa* mite fall, *Varroa* mite infestation rates in adult honeybees and worker cells as well as overall honeybee colony development was compared between acaricide treated and untreated apiaries.

MATERIALS AND METHODS

The study was conducted at the Experimental Farm of the University of Pretoria (25°45'11"S, 28°15'29"E) in South Africa from May to October 2011. The population growth of *Varroa* mites was monitored in 18 sedentary honeybee (*Apis mellifera scutellata*) colonies by measuring the daily mite fall as well as adult honeybee and worker brood infestation rates. Two apiaries were established, roughly one kilometer apart to prevent drifting of honeybees between apiaries as well as contamination of the untreated colonies by the acaricide (Allsopp 2006). Both apiaries had nine colonies each that were of similar state and size. Colonies were housed in standard 10 frame Langstroth hives and placed on stands with oil filled cans at the bottom to prevent ants from entering the colonies and removing mites. The queens were marked in July to ensure that the same colonies were monitored for the duration of the experiment. A feeding station was set up close to the colonies in both apiaries during the winter months, due to low food availability. A pollen supplement as well as 1:1 water: sugar solution was provided on a regular basis. The colonies were only opened when honeybee colony sizes were estimated (see below). Adult honeybee and worker brood samples were collected on the same day in order to keep the disturbance of the colonies to a minimum.

Daily *Varroa* mite fall

All 18 honeybee colonies were equipped with screened *Varroa* bottom boards (460 x 360 x 5 mm). Sheets of white paper were inserted into each of the 18 *Varroa* bottom boards. Fallen *Varroa* mites, other hive parasites and debris were collected on the sheets of paper. On each sampling occasion (daily or weekly depending on the intensity of mite fall) the paper in all colonies were carefully removed with forceps, placed into plastic zip loc bags to allow for mite counting in the laboratory and immediately replaced with new sheets of paper. Average daily

mite fall was obtained by dividing the number of mites fallen on the white paper by the number of days since the previous mite count.

Varroa mite fall was recorded for 25 days in both apiaries to obtain the baseline infestation rates in all the colonies before the chemical treatment was applied (pre-treatment period). In order to observe the effect of high *Varroa* mite numbers on *A. m. scutellata* colonies, it was decided that the apiary with the lowest number of mites would be chemically treated. After 25 days, the remaining eight colonies (one colony absconded) in the first apiary (from here on referred to as the treated apiary) were treated with an acaricide (Bayvarol® from Bayer Healthcare) according to the manufacturer's recommendations. The strength of the colonies determined the number of strips given to each colony. A strong colony received four strips and a weaker colony two or three strips. The second apiary (from here on referred to as the untreated apiary) did not receive treatment. Again both dead and live adult female mites were counted daily or weekly for 16 days in both apiaries (during treatment period). Following this, the daily mite fall was recorded for an additional 96 days in the treated and untreated apiaries (post-treatment period). The Bayvarol strips were removed from the treated apiary on day 55 when *Varroa* mite levels were at their lowest. This is in line with the manufacturer's recommendation that the Bayvarol strips should not be left in the colonies for longer than six weeks.

Varroa mite infestation rates

Adult honeybee and worker brood samples were collected from all 18 honeybee colonies once every second month to determine the *Varroa* infestation rates in both apiaries. The same methods as given in Chapter 2 were used. No samples were collected if colonies were too weak

or had insufficient brood available. *Varroa* mite infestation rates were summarized as: number of *Varroa* / 100 adult honeybees and number of *Varroa* / 100 worker cells.

Honeybee colony development

Honeybee colony development (estimate of the number of adult honeybees, sealed and unsealed brood in a colony) was assessed every month for the entire duration of the experiment using the Liebefeld method (Gerig 1983). To determine the number of adult honeybees, sealed and unsealed brood present in the colonies, brood frames were divided into eight squares of 1 dm^2 each. To get a precise estimate of the number of adult *A. m. scutellata* that completely fill 1 dm^2 square, photographs of 21 frames were taken and the number of honeybees in each of the squares was counted. Only fully occupied squares were used for the final estimate. Results showed that one fully occupied square contained on average 170 ± 19.9 honeybees. The number of fully occupied squares on both sides of the brood frames as well as on the lids and walls of the hives was counted and multiplied by 170 to obtain the number of honeybees present. The surface area containing sealed and unsealed brood on both sides of the frames was counted and expressed in dm^2. The estimation of honeybee colony development using the Liebefeld method was performed by the same individuals on every occasion. The Liebefeld method was conducted at the start of the experiment (21st of May 2011), when the treatment was applied (14th of June 2011), when the treatment was removed (14th of July 2011) and finally one (August), two (September) and three (October) months after the treatment was removed. The presence of the queen, queen cells and other parasites were also recorded to get a better idea of the general status of the colonies.

Statistical analysis

A Mann-Whitney U Test was performed to compare average daily *Varroa* mite fall from May to October as well as *Varroa* mite infestation rates between the treated and untreated apiaries. A Mann-Whitney U Test was performed to examine if the treatment had an influence on the incidence of parasites and Chalkbrood mummies on the *Varroa* bottom boards in both apiaries (during and post-treatment periods). A Repeated measures ANOVA was done to compare honeybee colony development (number of adult honeybees, surface area of sealed and unsealed brood) between both apiaries. All statistical analyses were performed with STATISTICA Version 10.

RESULTS

In total, six honeybee colonies absconded over the monitoring period (May to October 2011). In the treated apiary, three colonies absconded on day 24, 43 and 82, respectively. In the untreated apiary, one colony absconded on day 24 and the other two colonies absconded on day 82. Consequently these colonies could not be monitored for the entire duration of the study. There was no effect of treatment on absconding with both apiaries experiencing a loss of the same number of colonies (Gehan's Wilcoxon test, Survival T statistic^2 = - 0.29; $P > 0.05$).

The following parasites were recorded in both apiaries on the *Varroa* bottom boards after treatment was applied: ants, pseudoscorpions, cockroaches, small hive beetles (SHB), *Braula* and wax moths. The fungal pathogen, Chalkbrood was only found on the *Varroa* bottom boards of the untreated apiary in the form of mummies (Chapter 1 & 2). SHB presence was significantly higher in the untreated apiary (U = 9.50; Z = - 2.31; $P < 0.05$). No significant

differences were found in the presence of ants (U = 16.50; Z = - 1.58; P > 0.05), pseudoscorpions (U = 31.50; Z = 0.00; P > 0.05), cockroaches (U = 29.00; Z = - 0.26; P > 0.05), *Braula* (U = 14.50; Z = - 1.79; P > 0.05), wax moths (U = 21.00; Z = 1.10; P > 0.05) and Chalkbrood (U = 28.00; Z = - 0.37; P > 0.05) between both apiaries. All the honeybee parasites as well as Chalkbrood mummies that were collected on the bottom boards (pre-, during and post- treatment periods) are presented in Appendix B (Table III and IV).

Most of the colonies in both apiaries had queens present during the study period. Only one colony in the treated apiary became queenless two months after the study commenced. This colony remained without a queen for the rest of the monitoring period. The only honeybee parasite that was obvious on the frames while estimating the number of honeybees and surface area of brood was SHB. Results on the presence of the queen, queen cells and parasites of both apiaries that were recorded during the assessment of honeybee colony development are presented in Appendix B (Table V and VI). No obvious damage by *Varroa* mites as described by (Allsopp 2006) during the initial infestation period were noticed in the colonies during honeybee colony development assessments.

Daily *Varroa* mite fall

Both live and dead female adult *Varroa* mites were collected on the bottom boards. On some occasions immature females and male mites were observed. Even though immature and male mites were found, only adult females were counted in order to determine average daily mite fall (Appendix B - Table I and II).

- **Pre-treatment period (21st May - 14th June 2011)**

A significant difference was observed in *Varroa* mite fall between the treated and untreated apiaries. Daily mite fall was significantly higher in the untreated apiary compared to the treated apiary (U = 18760.00; Z = - 4.62; P < 0.01) (Table 1, Fig. 1).

Table 1. Daily *Varroa* mite fall in the treated and untreated apiaries 25 days before treatment was applied.

Treated			Untreated		
Colony no.	**Average daily mite fall ± SD**	**Total no. of mites**	**Colony no.**	**Average daily mite fall ± SD**	**Total no. of mites**
1	7.8 ± 5.0	196.0	1	6.9 ± 3.8	172.0
2	0.7 ± 1.0	17.0	2	15.6 ± 7.3	391.0
3	12.3 ± 2.9	307.0	3	27.8 ± 20.4	695.0
4	3.9 ± 3.1	90.0	4	11.7 ± 3.7	292.0
5	7.2 ± 2.6	181.0	5	1.7 ± 1.1	43.0
6	0.9 ± 0.9	22.0	6	1.4 ± 1.4	35.0
7	0.4 ± 0.7	11.0	7	0.8 ± 1.2	20.0
8	2.2 ± 1.6	55.0	8	0.0 ± 0.0	0.0
9	1.4 ± 2.0	42.0	9	19.2 ± 8.1	480.0
Average ± SD	4.1 ± 2.2 P < 0.01	102.3 ± 103.1		9.5 ± 5.2	236.4 ± 245.3

- **During treatment period (15th June - 30th June 2011)**

A significant difference was observed in *Varroa* mite fall between the treated and untreated apiaries. Daily mite fall was significantly higher in the treated apiary compared to the untreated apiary (U = 14298.00; Z = 3.63; P < 0.01) (Table 2, Fig. 1).

Table 2. Daily *Varroa* mite fall in the treated and untreated apiaries over 16 days during the treatment period.

Treated			Untreated		
Colony no.	**Average daily mite fall ± SD**	**Total no. of mites**	**Colony no.**	**Average daily mite fall ± SD**	**Total no. of mites**
1	17.9 ± 15.5	286.0	1	8.1 ± 2.9	129.4
2	3.7 ± 1.5	60.0	2	17.1 ± 3.9	273.9
3	45.1 ± 55.5	721.0	3	5.7 ± 2.9	91.0
4	N/A	N/A	4	11.2 ± 1.7	178.6
5	51.7 ± 54.6	828.0	5	3.1 ± 1.8	49.9
6	8.7 ± 5.0	140.0	6	2.5 ± 1.2	39.9
7	4.0 ± 7.0	65.0	7	1.8 ± 0.8	28.6
8	14.4 ± 7.8	230.0	8	N/A	N/A
9	13.9 ± 11.6	223.0	9	13.2 ± 2.5	210.6
Average ± SD	19.9 ± 19.8 $P < 0.01$	319.1 ± 293.4		7.8 ± 2.2	125.2 ± 89.2

Abbreviation: N/A – Colonies absconded.

- **Post-treatment period (1st July – 4th October 2011)**

Daily *Varroa* mite fall was significantly higher in the untreated apiary compared to the treated apiary during July ($U = 79853.50$; $Z = -12.82$; $P < 0.05$), August ($U = 48485.00$; $Z = -10.24$; $P < 0.05$) and September ($U = 39640.00$; $Z = -7.24$; $P < 0.05$). No significant differences in *Varroa* mite fall was found between the two apiaries during October ($U = 256.00$; $Z = -0.65$; $P > 0.05$) (Table 3, Fig. 1).

Table 3. Daily *Varroa* mite fall in the treated and untreated apiaries during July, August, September and October 2011 (post-treatment period).

Month	Treated			Untreated		
	Colony no.	Average daily mite fall ± SD	Total no. of mites	Colony no.	Average daily mite fall ± SD	Total no. of mites
July	1	1.5	2.0	1	7.1 ± 1.3	219.0
	2	2.3 ± 0.9	71.0	2	14.2 ± 5.9	440.0
	3	1.6 ± 1.2	51.0	3	2.1 ± 1.0	64.0
	4	N/A	N/A	4	5.2 ± 2.3	160.0
	5	0.8 ± 0.4	26.0	5	7.2 ± 2.4	223.0
	6	1.7 ± 0.6	54.0	6	5.7 ± 2.0	177.0
	7	0.1 ± 0.1	2.0	7	1.8 ± 0.7	57.0
	8	3.2 ± 2.4	99.0	8	N/A	N/A
	9	5.1 ± 2.9	159.0	9	10.7 ± 4.2	333.0
	Average ± SD	2.0 ± 1.1 $P < 0.05$	58.0 ± 52.7		6.8 ± 2.5	209.1 ± 129.1
August	1	N/A	N/A	1	3.2 ± 1.1	99.0
	2	1.0	4.0	2	2.1 ± 2.3	65.0
	3	0.9 ± 0.4	29.0	3	0.7 ± 0.4	22.0
	4	N/A	N/A	4	1.5 ± 0.0	6.0
	5	0.9 ± 0.5	29.0	5	7.6 ± 1.9	235.0
	6	0.6 ± 0.1	18.0	6	7.2 ± 2.4	224.0
	7	0.1 ± 0.1	3.0	7	0.7 ± 0.4	21.0
	8	0.5 ± 0.5	14.0	8	N/A	N/A
	9	1.8 ± 1.4	57.0	9	4.7 ± 0.0	19.0
	Average ± SD	0.8 ± 0.4 $P < 0.05$	22.0 ± 18.7		3.5 ± 1.1	86.3 ± 93.4
September	1	N/A	N/A	1	1.0 ± 0.5	31.0
	2	N/A	N/A	2	0.9 ± 0.3	26.0
	3	0.7 ± 0.5	21.0	3	0.4 ± 0.3	11.0
	4	N/A	N/A	4	N/A	N/A
	5	0.3	9.0	5	4.0 ± 2.3	120.0
	6	0.5 ± 0.1	15.0	6	4.0 ± 2.0	120.0
	7	0.1 ± 0.0	1.0	7	0.1 ± 4.2	3.0
	8	0.3 ± 0.1	9.0	8	N/A	N/A
	9	0.6 ± 0.3	17.0	9	N/A	N/A
	Average ± SD	0.4 ± 0.2	12.0 ± 7.1		1.7 ± 1.6	51.8 ± 53.8

		$P < 0.05$				
October	1	N/A	N/A	1	0.0 ± 0.0	0.0
	2	N/A	N/A	2	1.6 ± 0.0	6.0
	3	2.0 ± 0.0	8.0	3	0.4 ± 0.0	2.0
	4	N/A	N/A	4	N/A	N/A
	5	0.4 ± 0.0	2.0	5	3.9 ± 0.0	16.0
	6	0.5 ± 0.0	2.0	6	4.3 ± 0.0	17.0
	7	0.0 ± 0.0	0.0	7	0.1 ± 0.0	0.0
	8	0.6 ± 0.0	2.0	8	N/A	N/A
	9	1.4 ± 0.0	6.0	9	N/A	N/A
	Average ± SD	0.8 ± 0.0	3.3 ± 3.0		1.7 ± 0.0	6.8 ± 7.8
		$P > 0.05$				

Abbreviation: N/A – Colonies absconded.

Varroa mite fall was significantly higher in the untreated apiary before and three months after treatment. As expected, *Varroa* mite fall was significantly higher during the treatment period in the treated apiary. Similar *Varroa* mite fall rates were observed during the month of October in both apiaries. Overall, in the untreated apiary daily mite fall decreased from May until October. At the start of the experiment approximately ten mites fell per day and this number decreased to almost two per day in October. Mite fall in the treated apiary was at its highest (approximately 20 mites per day) during the two weeks of treatment, but after this period mite fall decreased drastically until it reached a mite fall of only one mite per day in October. In addition, mite fall numbers might be a fraction lower than expected given that ants were occasionally found on the bottom boards in most of the colonies of both apiaries despite efforts to keep them away (Appendix B - Table III and IV).

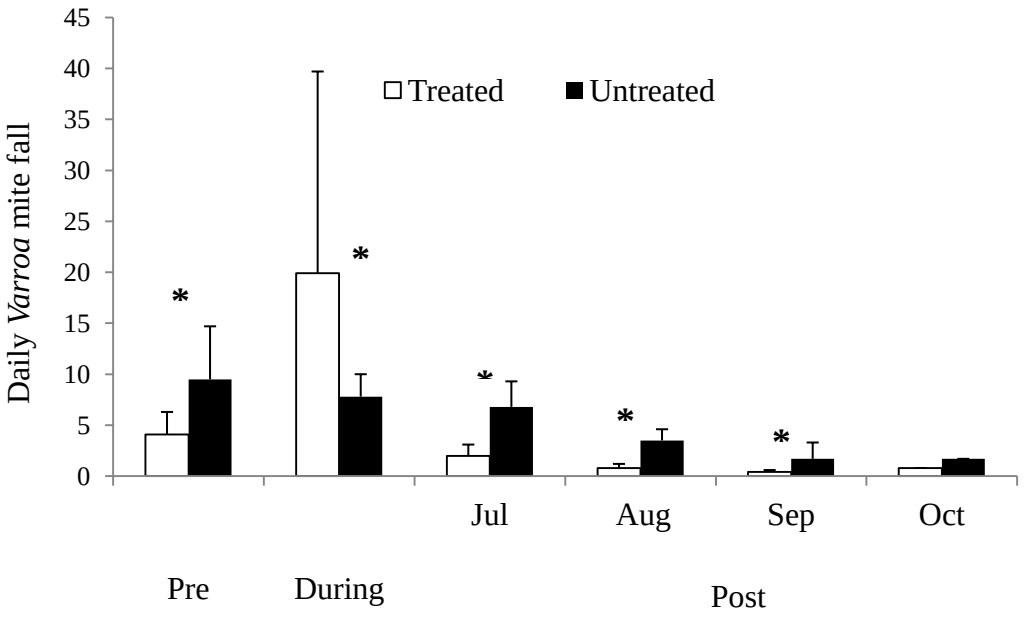

Figure 1. Daily *Varroa* mite fall (mean ± SD) in treated and untreated apiaries before (21st May - 14th June 2011), during (15th June - 30th June 2011) and four months (1st July - 4th October 2011) after treatment. * Indicates significant differences between treated and untreated apiaries (Mann-Whitney U test, *P* < 0.05).

Varroa mite infestation rates

Adult honeybee and worker brood infestation rates per colony per month for the treated and untreated apiaries are presented in Appendix B in Table IX and X, respectively.

- **Adult honeybee infestation rates**

All of the mites were not eliminated from the treated colonies, but *Varroa* infestation rates were generally higher in the untreated apiary compared to the treated apiary. Infestation rates in the treated apiary decreased, as expected, from May to July as a result of the chemical treatment. *Varroa* mite infestation rates were however not significantly different between the treated and untreated apiaries in May ($U = 30.00$; $Z = -0.53$; $P > 0.05$), July ($U = 3.50$; $Z = -1.78$; $P > 0.05$) and September ($U = 3.50$; $Z = -1.47$; $P > 0.05$) (Fig. 2).

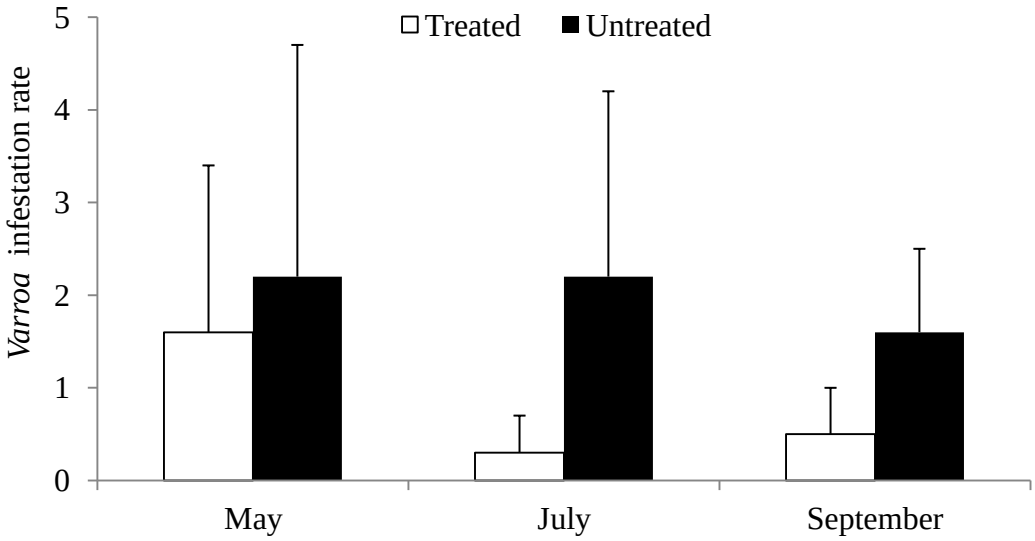

Figure 2. *Varroa* mite infestation rates (mean ± SD) per 100 adult honeybees in the treated and untreated apiaries.

- **Worker brood infestation rates**

Similar to what was observed in the adult honeybee infestation rates (see above), *Varroa* mite numbers were higher in the untreated apiary compared to the treated apiary. *Varroa* infestation rates in the treated apiary decreased after treatment and were lowest during September. Interestingly, there was also a great reduction in the infestation rates of *Varroa* mites from July to September in the untreated apiary. *Varroa* mite infestation rates were not significantly different between the treated and untreated apiaries in May (U = 18.00; Z = - 0.77; $P > 0.05$), July (U = 0.50; Z = - 1.94; $P > 0.05$) and September (U = 4.00; Z = - 0.53; $P > 0.05$) (Fig. 3).

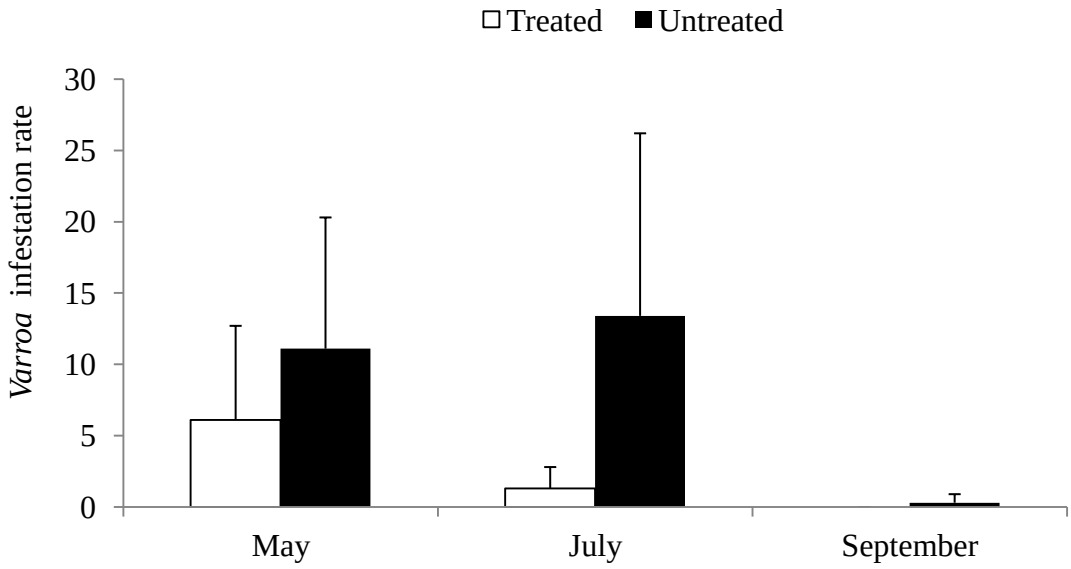

Figure 3. ***Varroa*** **mite infestation rates (mean ± SD) per 100 worker brood cells in the treated and untreated apiaries.**

Honeybee colony development

- **Adult honeybees**

The percentage change in adult honeybee population was only measured from July to October to quantify the honeybee population growth in both apiaries after the treated apiary was treated with Bayvarol. The number of adult honeybees measured from July to October was not significantly different between the treated and untreated apiaries ($F_{3,30} = 1.42$, $P > 0.05$) (Fig. 4, Table 4). The estimated number of honeybees recorded in all colonies of both apiaries from May to October is presented in Appendix B (Table VII and VIII).

The average adult honeybee population in the treated apiary was generally lower from July to September compared to the untreated apiary (Appendix B - Table VII). During October the average adult honeybee population in the treated apiary increased from 5248.8 honeybees

(measured in September) to 8797.5 honeybees. October was the only month where average adult honeybee population sizes were relatively similar in both apiaries with 9080.8 honeybees being recorded in the treated apiary compared to 8797.5 honeybees in the untreated apiary. In the treated apiary, the average adult honeybee population remained relatively similar across the monitoring period (Table 4).

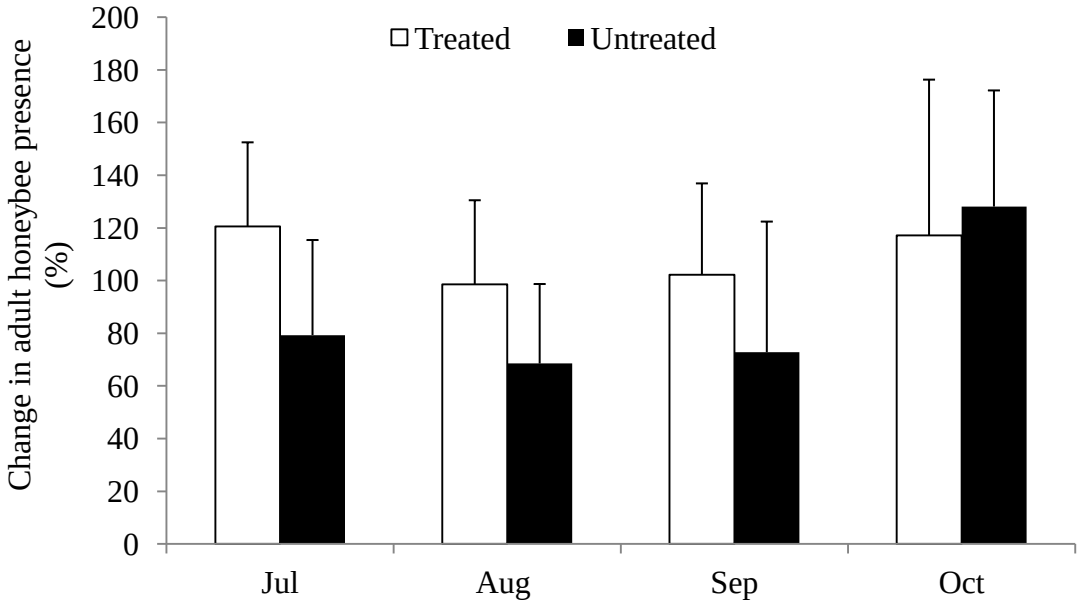

Figure 4. Number of adult honeybees (mean ± SD) measured in the treated and untreated apiaries from July to October 2011.

- **Sealed brood**

The percentage change in the surface area of sealed brood was measured from July to October to determine the presence of sealed brood in both apiaries after the treated apiary was treated with Bayvarol. The estimated surface area of comb containing sealed brood recorded in all colonies

of both apiaries from May to October is presented in Appendix B (Table VII and VIII). The surface area of sealed worker brood measured from July to October was not significantly different between the treated and untreated apiaries ($F_{3,30} = 0.91$, $P > 0.05$) (Fig. 5, Table 4).

The surface area of sealed brood was quite similar in both apiaries during July and August (Appendix B - Table VII and VIII). In the untreated apiary the presence of sealed brood started to increase from August to October. The surface area of sealed brood was much higher in the untreated apiary compared to the treated apiary during September and October.

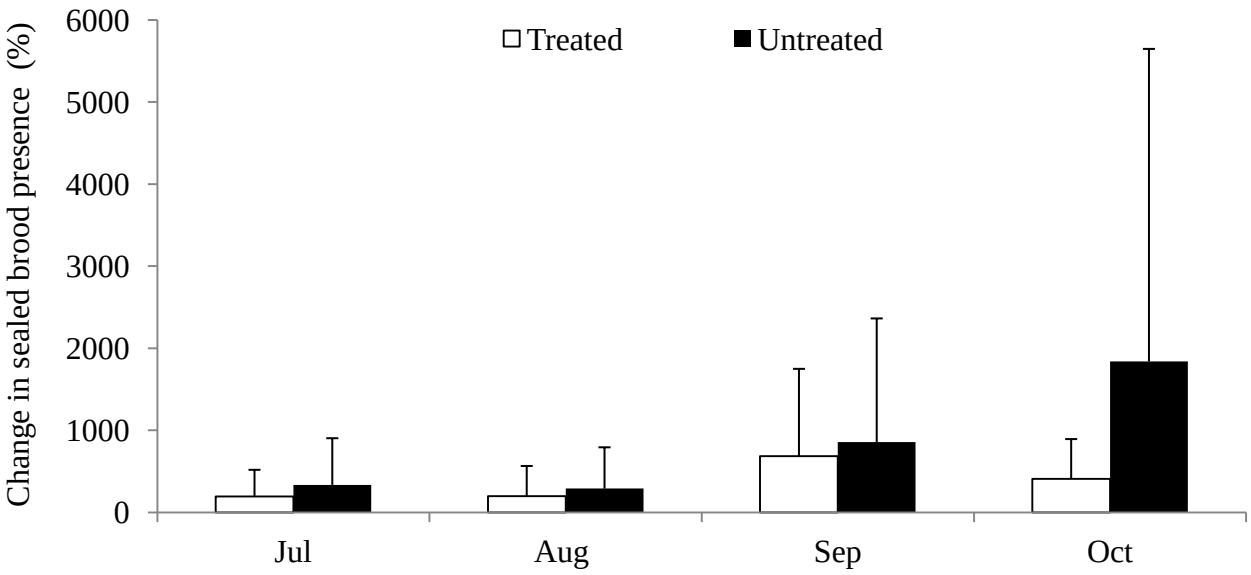

Figure 5. Surface area of sealed worker brood (dm^2) (mean ± SD) in the treated and untreated apiaries from July to October 2011.

- **Unsealed brood**

The percentage change in the surface area of unsealed brood was measured from July to October to determine the presence of sealed brood in both apiaries after the treated apiary was treated with Bayvarol. The estimated surface area of comb containing unsealed brood recorded in all colonies of both apiaries from May to October is presented in Table VII and VIII of Appendix B. No significant differences were found in the surface area of unsealed worker brood measured from July to October between the treated and untreated apiaries ($F_{3,30} = 1.09$, $P > 0.05$) (Table 4, Fig. 6).

The surface area of unsealed brood was similar during July and August (Appendix B - Table VII and VIII). A slight decrease in the surface area of unsealed brood was observed during October in the treated and untreated apiaries.

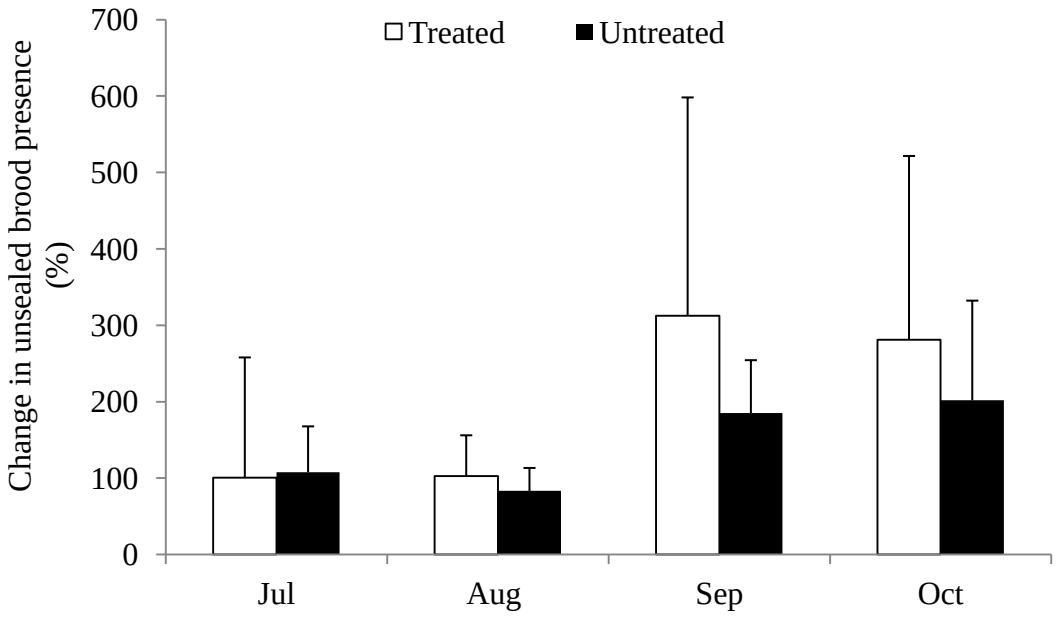

Figure 6. Surface area of unsealed worker brood (dm^2) (mean ± SD) in the treated and untreated apiaries from July to October 2011.

Table 4. The number of adult honeybees (mean ± SD), sealed and unsealed brood (dm^2) (mean ± SD), present in the treated and untreated apiaries sampled from May to October 2011. No significant differences were observed between the treated and untreated apiaries in relation to the number of adult honeybees and surface area of brood (sealed and unsealed).

	Treated				Untreated			
Month	**Sample size**	**Adult honeybees**	**Sealed brood**	**Unsealed brood**	**Sample size**	**Adult honeybees**	**Sealed brood**	**Unsealed brood**
May	9	7357 ± 2711	8.7 ± 6.8	7.6 ± 4.1	9	6309 ± 2402	8.4 ± 4.4	6.2 ± 5.0
June	8	7873 ± 2703	13.8 ± 10.0	7.4 ± 7.0	8	5111 ± 1775	7.1 ± 6.8	5.0 ± 3.6
July	7	8949 ± 3562	8.3 ± 9.8	4.3 ± 4.7	8	5589 ± 3155	10.3 ± 8.4	6.7 ± 5.3
August	6	8217 ± 3474	8.0 ± 7.7	8.0 ± 5.7	6	4817 ± 2709	7.3 ± 6.2	6.5 ± 4.0
September	6	8762 ± 4027	30.8 ± 22.8	20.3 ± 13.7	6	5249 ± 4014	21.2 ± 14.4	13.5 ± 7.0
October	6	9081 ± 5156	37.2 ± 35.8	17.7 ± 10.8	6	8798 ± 3911	29.4 ± 12.8	11.6 ± 6.3

DISCUSSION

One of the main problems facing beekeepers worldwide in terms of acaricides is that *Varroa* mites tend to develop resistance over time, which makes the treatments completely ineffective (De Guzman *et al*. 1996; Rinderer *et al*. 2001; Wilkinson *et al*. 2001; Gregorc & Poklukar 2003; Büchler *et al*. 2010; Rinderer *et al*. 2010). In South Africa, honeybee colonies are generally not treated and therefore no mite resistance to chemical products is to be expected (Allsopp 2006). The immediate effect of treatment on *Varroa* mites in the treated apiary was clearly observed (during treatment period). In the treated apiary mite fall increased from four mites per day before treatment to almost 20 mites per day during treatment and ended with one mite per day by the end of the monitoring period (October). Our results therefore show that Bayvarol was very effective at killing the *Varroa* mites and keeping the number of mites present in the treated colonies at a very low level.

The majority of the parasites and Chalkbrood mummies detected in both apiaries are relatively common in honeybee colonies (Appendix B - Table III and IV) (Swart *et al*. 2001). Of these parasites, only SHB prevalence was significantly higher in the untreated colonies. A possible explanation would be that the Bayvarol treatment had a negative effect on SHB, as was found by Elzen *et al*. (1999) after treating honeybee colonies with another chemical control agent of *Varroa* mites, namely Coumaphos. But at no stage in this study were there large numbers of dead SHB on the *Varroa* bottom boards of the treated apiary. SHB are generally not a problem in strong colonies of South African honeybees and only weaker colonies are vulnerable to infestation (Lundie 1940). In South Africa, litte is known about the interactions of *Varroa* mites and the other parasites found on the bottom boards in this study. Chalkbrood was more pronounced in honeybee colonies during the initial phase of *Varroa* mite introduction in South

Africa, but appears to now be present at very low levels (Allsopp 2006). In this study, Chalkbrood was only detected in three of the colonies (Appendix B - Table III and IV) which correspond to the findings of Allsopp (2006) that Chalkbrood prevalence is low.

Unfortunately, only limited adult honeybees and worker brood were available to determine *Varroa* mite infestation rates in all colonies of both apiaries. Some of the colonies were too weak to collect samples from and this was especially true during the months of July and September. The average number of *Varroa* mites per 100 adult honeybees three months after treatment was one and two for the treated and untreated apiaries, respectively, which represents relatively low adult honeybee infestation rates. Allsopp (2006) measured the *Varroa* infestation rates of adult honeybees (*A. m. scutellata*) in two apiaries (treated and untreated) during 1999 before treatment with Bayvarol and then three and a half months after treatment. *Varroa* infestation rates were 7.7 mites per 100 adult honeybees in both apiaries before treatment. After treatment, *Varroa* infestation rates decreased in both apiaries with the treated and untreated apiaries having 0.02 and 1.0 mites per 100 adult honeybees, respectively. On average *Varroa* mites infested 2% of worker brood in the treated apiary and 8% in the untreated apiary. As expected, although not significantly so, the *Varroa* infestation rates were lower in both adult honeybees and worker brood in the treated apiary.

In both apiaries mite fall decreased over time, with the exception of the increased mite fall rates witnessed during the treatment period in the treated apiary. This overall decrease in mite fall can be attributed to the reduced availability of honeybee brood during the colder months (May to July) of the year in both apiaries and then in particular to the administration of the treatment which caused a significant reduction in mite numbers of the treated apiary. In Germany,

honeybee colonies are generally treated once natural *Varroa* mite fall goes beyond ten mites per day in July or August and mite fall as high as this does suggest that colonies are about to die (Le Conte *et al*. 2010; Genersch *et al.* 2010). In this study, the natural mite fall before the treatment was applied exceeded ten mites per day in one and four colonies of the treated and untreated apiaries, respectively. None of these colonies showed any signs that they were close to collapsing, but two of these colonies (untreated apiary) eventually absconded during September. The absconding of colonies during the monitoring period was to be expected given that absconding is a relatively common occurrence in African honeybees (Fletcher 1978; McNally & Schneider 1992; Hepburn & Radloff 1998). In South Africa between 10-30% of colony losses experienced by beekeepers during a year is due to absconding (Fletcher 1975; Swart 2001). African honeybees generally abscond due to predation pressure (wax moths, ants, SHB and humans), unfavourable environmental conditions as well as constant interference (Michener 1973; Camazine & Morse 1988; Hepburn & Radloff 1998). Although the absconding of only two colonies with initial high *Varroa* numbers is not significant, these two colonies specifically might have absconded to lower the parasite load. When honeybees abscond many of the parasites such as SHB and wax moths remain in the comb and by doing this they decrease the amount of parasites that can affect them at their new nest site (Fletcher 1978).

The number of adult honeybees, sealed and unsealed brood was similar for both apiaries, with no significant differences observed. The average number of adult honeybees and the surface area of sealed brood for both apiaries were highest during October, which is the start of spring in South Africa. Thus, during this study period, no negative effect of *Varroa* mites in terms of decreased honeybee colony development could be seen. Results suggest that colonies in the untreated apiary, that received no chemical treatment, can survive just as well as the colonies that were

treated. No colony losses relating to dead colonies or high parasite loads were observed in either apiary, but six colonies did abscond (33%). This corresponds with the absconding rate that is usually found in South African colonies (Fletcher 1975; Swart 2001), even though the absconding rate was slightly higher in this study. Two and four of the colonies absconded during the colder (May to July) and warmer months (August to October), respectively. The Liebefeld method is considered to be invasive to some degree (Spiewok *et al*. 2006), but because the colonies were opened only once a month it is most likely that the six colonies that did abscond, did so for other reasons. Possible reasons, although unlikely, for the absconding of these colonies under these circumstances could be bad weather conditions (during winter), lack of food (even though honeybees from both apiaries were fed during the winter period) or the presence of parasites (Michener 1973; Camazine & Morse 1988; Hepburn & Radloff 1998).

During his studies on tolerance development in the Cape honeybee, Allsopp (2006) observed that *Varroa* infestation rates of adult honeybees decreased over time in the monitored colonies and that they were much lower than when the mite first arrived in South Africa. Cape honeybees, however, showed no direct aggression towards *Varroa* mites, nor did they exhibit any grooming behaviour (Allsopp 2006). He concluded that a short post-capping stage in Cape honeybees (on average 11.0 days) and the ability of these honeybees to eliminate reproductive *Varroa* mites from brood (hygienic behaviour) contributed to the development of tolerance. No *Varroa* mite tolerance studies were done on *A. m. scutellata* honeybees in South Africa, but it was suggested that the good hygienic behaviour of these honeybees would enable them to tolerate *Varroa* mites (Allsopp 2006). The removal of pin and freeze-killed brood (hygienic behaviour) by honeybees in Africa from Zimbabwe (Fries & Raina 2003) and Kenya (Frazier *et al*. 2010) respectively, have been documented. In Zimbabwe, honeybees (*A. m. scutellata*) removed over 95% of the

pin-killed brood and in Kenya honeybees (believed to be *A. m. scutellata*) also removed more than 95% of the freeze-killed brood at one apiary site but at a second site honeybees failed to do the same. These results suggest that good hygienic behaviour exists in African honeybees but more research is needed especially since colonies from two different apiaries in Kenya gave contrasting results (Frazier *et al*. 2010).

In comparison to honeybees of European subspecies, (hereafter referred to as European honeybees) that have been devastated by *Varroa* mites, African and Africanized honeybees are smaller, develop faster and abscond more often (Michener 1973; Fletcher 1978; Camazine 1986; Hepburn & Radloff 1998). These characteristics as well as their good hygienic behaviour and short post-capping stages all contribute to keeping *Varroa* mites at very low levels (see Introduction). However, even though most of the evidence obtained so far points towards *Varroa* mite tolerance in both sub-species of South Africa, Martin & Kryger (2002) found that *Varroa* mite reproductive rates were similar in *A. m. scutellata* and European honeybees, thereby indicating that *Varroa* mites should have more or less the same negative effect in *A. m. scutellata* as in European honeybees. Also, Martin & Kryger (2002) showed that *Varroa* mites were reproductively more successful in *A. m. scutellata* drone and worker cells compared to Africanized workers cells and that a higher percentage of fertile female mites were produced in *A. m. scutellata* compared to Africanized honeybees. Consequently more studies are needed to give us a better idea on the reproductive potential of *Varroa* mites in both honeybee subspecies of South Africa. We need to determine the percentage infertile mites and the rates of offspring mortality.

In conclusion, the aim of this study was to examine the impact of *Varroa* mites on honeybee colony development and survival. Honeybee colony development was similar in both apiaries, irrespective of whether *Varroa* mites were present in low numbers or not. It was very apparent that honeybees from both apiaries at a colony level did not show any signs of disease or collapse and were developing normally in the presence of *Varroa* mites. It is widely known that *Varroa* mites have a preference to reproduce in drone brood and that the infestation rates are generally higher compared to worker brood (De Jong 1988; Fuchs 1990; Beetsma *et al*. 1999; Calderone & Kuenen 2001). This is due to drones having a longer developmental period that allows for more female mites to reach maturity (Ifantidis 1983; Moritz & Hänel 1984). Martin & Kryger (2002) found that *Varroa* mite reproduction was more successful in *A. m. scutellata* drones compared to workers, due to less mite offspring dying which increased reproductive success. The damage caused by *Varroa* mites when feeding on drones specifically can lead to a reduction in haemolymph protein content (Glinsky & Jarosz 1984), sperm production (Duay *et al.* 2002) and weight (Duay *et al*. 2003). Therefore it would be interesting to examine the effects of *Varroa* mites at the individual level in developing *A. m. scutellata* workers and drones in order to see if there are differences with European and Africanized honeybees.

Although this study was conducted for only a short period of time, some insights into the population dynamics of both *Varroa* mites and honeybees were gained. *Varroa* mite fall and infestation rates decreased from May to October in both apiaries and honeybee colony development was similar in both apiaries. The continuous monitoring of these apiaries is important in order to see the effect of treatment and the lack thereof on the survival of *Varroa* mites and both apiaries over a longer period of time. The mechanism behind the decrease in *Varroa* mite numbers in both apiaries over time with and without treatment is still unclear and

requires further research. A more detailed look into *Varroa* mite reproduction and *A. m. scutellata* hygienic behaviour is necessary to allow for a better understanding of the factors involved in *Varroa* mite tolerance and the long-term survival of these honeybees.

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CHAPTER 4

General conclusion

In this study my first aim was to assess the current pathogen and parasite status of the African honeybee (*A. m. scutellata*) per season between migratory and sedentary apiaries (Chapter 2) situated in the Gauteng region of South Africa. The infestation rates of *Varroa* mites in adult honeybee and worker brood samples collected from beekeepers in the Gauteng region of South Africa were also examined (Chapter 2). My second aim involved looking at the effect of *Varroa* mites on the development of honeybee (*A. m. scutellata*) colonies with and without chemical treatment (Chapter 3). This was in order to understand how tolerance can develop in a host (*A. m. scutellata*) that did not co-evolve with the parasite (*Varroa destructor*).

Varroa mites remain the biggest threat to beekeeping in most countries (Chapter 1 & 2). Through their feeding they are able to cause physical deformities in honeybees as well as transmit viruses and bacteria (Reviewed in Bailey & Ball 1991; Rosenkranz *et al*. 2010). Most of the devastating effects of *Varroa* mites have been observed in European honeybees, while in *A. cerana*, Africanized and African honeybees various degrees of tolerance have been recorded (Chapter 3). The current and previous studies suggest that the situation we find here is similar to what is found in *A. cerana* and Africanized honeybees (Chapter 3). The host-parasite relationship of *Varroa* mites and *A. cerana* is stable due to their long-term association (Chapter 3). Therefore, the tolerance observed in African honeybees is more comparable with Africanized honeybees, since *Varroa* mites were introduced into both populations and due to the shared genetic and behavioural characters of these honeybee populations (Chapter 3). Many factors have been suggested for the observed *Varroa* mite tolerance by Africanized honeybees and include behavioural defence mechanisms such as grooming and hygienic behaviour as well as a short post-capping stage and brood cell size (Chapter 3; Calderón *et al*. 2010). In African honeybees hygienic behaviour and a short post-capping stage have been suggested as possible

reasons for *Varroa* mite tolerance. In addition, grooming behaviour was very apparent in Tunisian honeybees that showed tolerance towards *Varroa* mites (Boecking & Ritter 1993), but Allsopp (2006) found no evidence of grooming behaviour in Cape honeybees. However, a recent study that examined the hygienic behaviour of *A. m. carnica* found that these honeybees were actively removing brood parasitised by *Varroa* mites that could possibly cause lethal DWV infections by transmitting it to the affected brood (Schöning *et al*. 2012). They showed that brood parasitised by *Varroa* mites that were capable of transmitting DWV at lower, less damaging levels were removed less often compared to brood parasitised by *Varroa* mites capable of transmitting lethal DWV infections. This indicated that these honeybees were not only removing brood parasitised by *Varroa*, but that they were actively removing brood that could possibly become infected with a lethal *Varroa* mite transmitted DWV infection. This study by Schöning *et al*. (2012) suggests that there might be more to hygienic behaviour than just removing all the developing honeybees parasitised by *Varroa* mites; especially in colonies where viruses that can replicate in *Varroa* mites are also present.

Honeybees have behavioural defence mechanisms such as hygienic behaviour that enable them to deal sufficiently with pathogens and parasites including *Varroa* mites (Boecking & Spivak 1999). The removal of *Varroa* mite infested brood has been documented in Africanized honeybees, *A. cerana* and to a lesser extent in African honeybees (Chapter 3). The removal of pin and freeze-killed brood by honeybees in Africa from Zimbabwe (Fries & Raina 2003) and Kenya (Frazier *et al*. 2010) respectively, have also been recorded. Although honeybees of only one of the two apiary sites in Kenya showed hygienic behaviour, honeybees in Zimbabwe was considered to be very hygienic (Chapter 3). These results suggest that good hygienic behaviour exists in African honeybees (even though there are exceptions) and may contribute to the

absence of pathogens such as AFB (Fries & Raina 2003). Hygienic behaviour is very important for the control of pathogens and parasites within honeybee colonies (de Guzman *et al*. 2001). The most important aspect of hygienic behaviour and ultimately tolerance is the elimination of pathogens from the honeybee colony before they start to multiply and become infective (Rothenbuhler 1964). The detection, uncapping and removal of larvae infested with Chalkbrood (Gilliam *et al.* 1983; Invernizzi *et al*. 2011) and American foulbrood (Woodrow & Holst 1942; Rothenbuhler 1964) have been documented previously. It would be interesting to have a closer look into the hygienic behaviour of *A. m. scutellata* from South Africa specifically in order to get a better idea as to why the effects of *Varroa* mites are not so pronounced in these colonies. In this study average *Varroa* mite infestation rates remained relatively low and never reached more than 4.0 mites per 100 adult honeybees (Chapter 2 & 3). The hygienic behaviour observed in Zimbabwe and Kenya might also explain the low prevalence of pathogens (with the exception of BQCV) in the 13 screened apiaries (Chapter 2).

There is considerable variation within and between the post-capping stages (in days) of *A. m. scutellata* (10 - 12), Cape honeybees (9.6 - 12), Africanized (11.5 - 11.6) and European honeybees (11.6 - 12.04) (Moritz & Hänel 1984; Moritz 1985; Vandame *et al*. 1999; Tribe & Allsopp 2001; Martin & Kryger 2002; Allsopp 2006; Calderón *et al*. 2010). The somewhat shorter post-capping stage in both African and Africanized honeybees is thought to contribute to *Varroa* mite tolerance (Moritz & Hänel 1984; Moritz 1985; Camazine 1986; Rosenkranz 1999; Allsopp 2006). If the developmental time of honeybees is shorter, *Varroa* mites have less time to produce fertile (mated) female offspring that can infest new brood cells or adult honeybees which ultimately results in lower reproductive rates (Ramirez & Otis 1986; Calderón *et al*. 2010). In order to maximize reproductive success the highest number of mated females should

be produced since all unmated females do not contribute to reproduction (Ball & Allen 1988; Martin *et al*. 1997; Boecking & Genersch 2008). Moritz & Hänel (1984) found that fewer than half of the newly produced female *Varroa* mites were able to reach maturity due to the shorter post-capping stage in Cape honeybees. In a single reproductive cycle, Martin & Kryger (2002) found that 0.9 and 2.2 fertile female offspring were produced on *A. m. scutellata* worker and drone brood, respectively. In worker brood of Africanized honeybees the number of fertile female offspring produced ranged between 0.6 - 0.73 (Medina & Martin 1999; Corrêa-Marques *et al*. 2003) but an even lower value of 0.3 has also been recorded (Calderón *et al*. 2010). In European honeybees 1.0 – 1.01 fertile female offspring were produced in worker brood (Medina & Martin 1999; Corrêa-Marques *et al*. 2003). These results show that *Varroa* mites are more reproductively successful in *A. m. scutellata* and European honeybees compared to Africanized honeybees. However, even with a shorter post-capping stage *Varroa* mite reproduction still takes place and mite development within the honeybee colony is just delayed (Martin 1998), suggesting that more than one tolerance factor is necessary to maintain low *Varroa* mite levels. In this study both adult honeybee and worker brood infestation rates remained relatively low across all seasons (Chapter 2 & 3). The exact mechanism behind these low *Varroa* mite infestation rates requires additional research.

Pesticides, environmental factors, bad beekeeping practices, pathogens and parasites have been linked to honeybee colony losses and a reduction in the overall health status of honeybees (Le Conte *et al*. 2010; vanEngelsdorp & Meixner 2010). The lack of large colony losses observed in this study can possibly be explained by the relative absence of *Varroa* mite associated pathogens (for example DWV and ABPV) that have in recent times caused colony losses on a worldwide scale (Chapter 1 & 2). The absence of DWV is especially interesting given its close association

with *Varroa* mites and because it is such a common virus in honeybees around the world (Chapter 2). The non detection of the newly emergent pathogen, *N. ceranae*, and the fatal bacterial disease, AFB might also have contributed to the absence of colony losses. It is difficult to predict how *N. ceranae* would affect honeybees in South Africa if it was ever to become established here. *Nosema ceranae* presence in three depopulated honeybee colonies in Africa (Algeria) which share a similar climate to Spain where colony losses due to *N. ceranae* have previously been recorded is of concern (Higes *et al*. 2009). However, the virulence of *N. ceranae* is still under debate (Chapter 1) and more research is needed to fully understand the effect of this parasite on honeybees from different regions of the world. In contrast, we know that AFB can have a devastating effect on most honeybee colonies (Chapter 1) and therefore its absence from the collected samples in this study is extremely good news to beekeepers in these sampled regions. Especially considering that AFB was confirmed to be present in the Western Cape province of South Africa in 2009 and that data on whether AFB has spread to the rest of the country is lacking (Chapter 1 & 2).

The most prevalent virus detected in *A. m. scutellata* colonies was BQCV, which is transmitted within honeybee colonies, most probably without the aid of *Varroa* mites (Chapter 2). This, and the fact that a co-occurrence of only 25% between BQCV and *N. apis* was found, might explain why no apiaries were negatively affected. It has been suggested that the presence of *N. apis* in BQCV infested colonies can cause an increase in the infectivity of this virus which can be more harmful to honeybees (Bailey *et al*. 1983; Bailey & Ball 1991). It would be interesting to have a closer look into the transmission pathways of pathogens in *A. m. scutellata* colonies, especially since IAPV and VDV-1 have now been confirmed in honeybees and *Varroa* mites. We might be able to better explain the lack of colony losses as soon as we understand how these viruses are

transmitted or spread within *A. m. scutellata* colonies. The devastating effects of viruses are mostly observed in honeybee colonies infested with *Varroa* mites that are capable of transmitting these viruses to developing and adult honeybees (Chapter 1 & 2). This vector-borne transmission route adds to the vertical and other horizontal transmission pathways that already exist in honeybee colonies and can also increase the virulence of the viruses (Chapter 1).

The lack of significant differences between migratory and sedentary apiaries in terms of pathogen and parasite prevalence was unexpected. It has been suggested that the increased and constant movement of colonies cause added stress to honeybees (Chapter 2) which can increase their susceptibility to pathogens and parasites (Swart *et al.* 2001). In South Africa, it is mostly the *A. m. capensis* parasite that is especially problematic for migratory beekeepers (Chapter 1 & 2), but in this study migratory apiaries were no more affected by this parasite than sedentary apiaries. The management practices employed by beekeepers might explain why differences are absent or present. In South Africa, the trapping of wild swarms to replace lost, unproductive and absconded colonies plays a very important role in migratory beekeeping (Dietemann *et al*. 2009). South African beekeepers can therefore easily compensate for their losses by simply catching new swarms.

The results obtained in this study showed that *Varroa* mites are omnipresent in *A. m. scutellata* colonies. Even though the more virulent Korea haplotype (Anderson & Trueman 2000) that has caused significant damage to honeybee populations worldwide is present in South Africa, *A. m. scutellata* colonies were able to survive with pathogens and parasites as well as without chemical treatment (Chapter 2 & 3). The overall low *Varroa* mite infestation rates observed in Chapter 2 and 3 suggest that *A. m. scutellata* honeybees are dealing rather well with these mites. In this

study, no colony losses were recorded as a result of *Varroa* mites. Therefore, it is most likely that South African honeybees are able to survive without treatment because *Varroa* mite infestation rates and their direct impact within honeybee colonies remain below the damage threshold.

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Appendix A

Table I. Primers used for the screening of DWV, BQCV, VDV-1, IAPV, ABPV, CBPV, SBV, VdMLV, *Nosema apis*, *Nosema ceranae*, EFB and AFB.

Primer	Primer sequence (5' - 3')	Base pair length	Annealing temp (°C)	Reference
DWV (1)	TTT GCA AGA TGC TGT ATG TGG GTC GTG CAG CTC GAT AGG AT	395	56	Tentcheva *et al*. 2004a
DWV (2)	ATT AAA AAT GGC CTT TAG TTG CTT TTC TAA TTC AAC TTC ACC	694	55	de Miranda & Fries 2008; Forsgren *et al*. 2009
DWV (3)	CC TAG AAT CCA TAG ATT GCC TTC TCG AAT GCC AAT AAC TGA GT	583	55	Gauthier unpublished
DWV (4)	TGA GGT TAT ACT TCA AGG AG TCC GTG AAT ATA GTG TGA GG	806	55	Gauthier unpublished
DWV (5)	AAT CCT GCA GCG CGT TTG TGC TTA CAT CCT CGC TTC TTC TCT	736	55	Gauthier unpublished
DWV (6)	CGG CCT ATC AAA GAG TAC CTT TTC TAA TTC AAC TTC ACC	430	55	de Miranda & Fries 2008
DWV (7)	CCT GCT AAT CAA CAA GGA CCT GG CAG AAC CAA TGT CTA ACG CTA ACC C	355	54	Genersch 2005

BQCV	GTC CAG TGT GAT ATT GCC AA TCA TTA GAA AGC GCC AGA CT	550	50	Gauthier *et al.* 2007
VDV-1 (1)	GCC CTG TTC AAG AAC ATG CTT TTC TAA TTC AAC TTC ACC	430	50	de Miranda & Fries 2008; Gauthier *et al.* 2011
VDV-1 (2)	CGA AAC GAA GAG AGC ATG TAT CGA CTC TTC CCC AGC TAA G	1130	55	Ongus *et al*. 2004
IAPV	CCA TGC CTG GCG ATT CAC CTG AAT AAT ACT GTG CGT ATC	203	55	de Miranda *et al*. In preparation
ABPV	CTC AAG TTA TAC GTA AAA TAG CTG GAA TT AAC CAA CCT TGC TTC CCT TTA	646	55	Gauthier *et al.* 2007
CBPV	TCA GAC ACC GAA TCT GAT TAT TG TCT AAT CTT AGC ACG AAA GCC GAG	1113	56	Ribière *et al*. 2002 & Blanchard *et al*. 2009
SBV	GGA TGA AAG GAA ATT ACC AG CCA CTA GGT GAT CCA CAC T	426	48	Tentcheva *et al*. 2004b
VdMLV	ATC CCT TTT CAG TTC GCT AGA AGA GAC TTC AAG GAC	438	50	Gauthier *et al.* 2011
N. apis	GAA CCA GGC GAT TTT GTT CCT A CAC GCA TTG CTG CAT CAT TGAC	250	56	Chen *et al*. 2008; de Miranda *et al*. In preparation
N. ceranae	CGG ATA AAA GAG TCC GTT ACC TGA GCA GGG TTC TAG GGAT	250	50	Chen *et al*. 2008

EFB	CAG CTA GTC GGT TTG GTT CC TTG GCTG TAG ATA GAA TTG ACA AT	79	48	Roetschi *et al*. 2008
AFB	GCA AGT CGA GCG GAC CTT GT GCA TCG TCG CCT TGG TAA GC	237	50	Bakonyi *et al*. 2003

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Table II. ***Varroa destructor*** **infestation rates in adult honeybee and worker brood samples collected from beekeepers during July 2010 to August 2011.**

				Adult honeybees			Worker brood				
Apiary code	**Colony no.**	**Month**	**Management type**	**No. of adult honeybees**	**No. of mites on adult bees**	***Varroa* / 100 bees**	**No. of cells opened**	**No. of *Varroa* infested cells**	**Infested cells (%)**	**No. of *Varroa* in cells**	***Varroa* / 100 cells**
1	1			116	1	0.9	100	11	11	11	11
	2			103	6	5.8	57	0	0	0	0
	3	Jul-10	Sedentary	151	3	2.0	100	4	4	4	4
	4			187	7	3.7	100	7	7	10	10
	5			267	7	2.6	100	5	5	5	5
	1			83	0	0	86	0	0	0	0
	2			332	2	0.6	40	0	0	0	0
	3	Sep-10	Sedentary	304	0	0	93	3	3.2	3	3.2
	4			169	1	0.6	68	0	0	0	0
	5			295	2	0.7	54	0	0	0	0
	1			313	8	2.6	100	3	3	3	3
	2			249	1	0.4	100	0	0	0	0
	3	Nov-10	Sedentary	173	3	1.7	100	4	4	5	5
	4			206	0	0	100	1	1	1	1
	5			310	9	2.9	100	12	12	14	14
	1	Apr-11	Sedentary	345	10	2.9	100	7	7	7	7

	2			183	0	0	100	0	0	0	0
	3			181	3	1.7	100	1	1	1	1
	4			177	5	2.8	97	6	6.2	6	6.2
	5			268	4	1.5	NS	NS	NS	NS	NS
	1			232	5	2.2	100	2	2	2	2
	2			127	1	0.8	100	0	0	0	0
	3	Aug-11	Sedentary	100	1	1.0	100	0	0	0	0
	4			91	2	2.2	100	6	6	6	6
	5			158	3	1.9	100	1	1	1	1
2	1			73	1	1.4	100	2	2	2	2
	2	Jul-10	Migratory	279	2	0.7	100	0	0	0	0
	3			189	8	4.2	100	9	9	9	9
	5			147	5	3.4	100	14	14	15	15
	1			143	3	2.1	100	0	0	0	0
	2	Sep/Oct-10	Migratory	110	1	0.9	100	5	5	5	5
	3			73	17	23.3	100	15	15	18	18
	5			236	8	3.4	100	2	2	2	2
	1			154	1	0.6	100	0	0	0	0
	2	Nov-10	Migratory	235	5	2.1	100	4	4	4	4
	3			143	9	6.3	100	8	8	8	8
	5			297	9	3.0	100	4	4	4	4
	3	Jan-11	Migratory	102	1	1.0	NS	NS	NS	NS	NS
	5			227	12	5.3	100	26	26	32	32

	1			317	3	1.0	65	1	1.5	1	1.5
	2			307	9	2.9	100	2	2	2	2
	3	Apr-11	Migratory	138	0	0	100	0	0	0	0
	4			226	0	0	87	0	0	0	0
	5			373	2	0.5	100	0	0	0	0
	1			140	4	2.9	NS	NS	NS	NS	NS
	2			165	6	3.6	100	20	20	25	25
	3	Aug-11	Migratory	156	2	1.3	100	1	1	1	1
	4			220	2	0.9	100	0	0	0	0
	5			148	1	0.7	100	4	4	4	4
3	1			705	9	1.3	100	0	0	0	0
	2			454	14	3.1	100	1	1	1	1
	3	Jul-10	Migratory	373	3	0.8	100	2	2	2	2
	4			70	0	0	100	2	2	2	2
	5			732	5	0.7	100	0	0	0	0
	1			168	2	1.2	100	0	0	0	0
	2			195	2	1.0	100	4	4	4	4
	3	Nov-10	Migratory	159	9	5.7	100	7	7	7	7
	4			335	1	0.3	100	2	2	2	2
	5			245	3	1.2	100	1	1	1	1
	1			152	0	0	100	2	2	2	2
	2			171	0	0	100	0	0	0	0
	3	Jan-11	Migratory	186	3	1.6	100	1	1	1	1
	4			193	4	2.1	100	6	6	6	6
	5			269	0	0	100	3	3	3	3

	1			186	3	1.6	100	1	1	1	1
	2			282	0	0	100	0	0	0	0
	3	Apr-11	Migratory	130	11	8.5	100	35	35	44	44
	4			353	6	1.7	100	5	5	5	5
	5			282	2	0.7	100	6	6	7	7
	1			160	4	2.5	100	4	4	5	5
	2			134	1	0.7	100	0	0	0	0
	3	Aug-11	Migratory	98	10	10.2	100	9	9	9	9
	4			88	1	1.1	NS	NS	NS	NS	NS
	5			149	0	0	100	2	2	3	3
4	1			294	3	1.0	100	2	2	2	2
	2			342	12	3.5	100	3	3	3	3
	3	Aug-10	Sedentary	254	11	4.3	100	5	5	5	5
	4			188	4	2.1	100	1	1	1	1
	5			209	1	0.5	100	2	2	2	2
	1			105	3	2.9	100	13	13	15	15
	2			239	10	4.2	77	13	16.9	16	20.8
	3	Oct-10	Sedentary	87	6	6.9	100	9	9	9	9
	4			152	7	4.6	100	2	2	2	2
	5			197	4	2.0	100	2	2	2	2
	1			274	11	4.0	100	13	13	13	13
	2	Nov-10	Sedentary	393	21	5.3	100	19	19	20	20
	3			228	7	3.1	100	10	10	11	11
	4			224	5	2.2	100	5	5	6	6

	5			394	5	1.3	100	2	2	2	2
	1			176	2	1.1	100	16	16	17	17
	2			240	5	2.0	100	5	5	5	5
	3	Jan-11	Sedentary	243	4	1.6	100	9	9	10	10
	4			132	1	0.8	100	2	2	2	2
	5			308	1	0.3	100	16	16	18	18
	1			144	3	2.1	100	8	8	8	8
	2			228	0	0	100	1	1	1	1
	3	Mar-11	Sedentary	323	7	2.2	100	9	9	9	9
	4			105	0	0	100	1	1	1	1
	5			133	3	2.3	100	9	9	11	11
	1			177	30	17.0	100	22	22	24	24
	2			164	4	2.4	94	3	3.2	3	3.2
	3	Jul-11	Sedentary	227	7	3.1	100	3	3	3	3
	4			147	4	2.7	100	17	17	22	22
	5			195	10	5.1	100	7	7	12	12
5	1			160	1	0.6	100	0	0	0	0
	2			206	0	0	100	0	0	0	0
	3	Aug-10	Migratory	126	0	0	100	0	0	0	0
	4			139	1	0.7	100	1	1	1	1
	5			276	1	0.4	100	1	1	1	1
6	1	Aug-10	Migratory	212	4	1.9	100	0	0	0	0
	2			110	2	1.8	100	0	0	0	0

	3			118	3	2.5	100	14	14	14	14
	4			300	1	0.3	100	0	0	0	0
	5			191	3	1.6	100	2	2	2	2
7	1			129	1	0.8	100	0	0	0	0
	2			118	2	1.7	100	3	3	3	3
	3	Nov-10	Migratory	133	1	0.8	100	1	1	1	1
	4			114	1	0.9	100	1	1	1	1
	5			111	0	0	100	0	0	0	0
	1			109	0	0	100	0	0	0	0
	2			172	3	1.7	100	4	4	5	5
	3	Jan-11	Migratory	111	0	0	100	6	6	6	6
	4			141	0	0	100	2	2	2	2
	5			162	5	3.1	100	1	1	1	1
8	1			456	8	1.8	100	8	8	9	9
	2			290	6	2.1	100	14	14	15	15
	3	Dec-10	Sedentary	63	1	1.6	100	12	12	13	13
	4			86	0	0	100	1	1	1	1
	5			168	4	2.4	100	7	7	8	8
9	1			137	0	0	100	4	4	4	4
	2	Dec-10	Sedentary	127	3	2.4	100	6	6	6	6
	3			115	11	9.6	100	8	8	8	8
	4			154	1	0.6	100	1	1	1	1

	5			217	3	1.4	100	0	0	0	0
10	1			41	1	2.4	100	4	4	4	4
	2	Feb-11	Migratory	96	0	0	100	5	5	5	5
	3			35	0	0	100	14	14	15	15
11	1			177	3	1.7	100	1	1	1	1
	2			280	8	2.9	100	18	18	25	25
	3	Feb-11	Sedentary	147	12	8.2	100	27	27	37	37
	4			212	4	1.9	100	7	7	9	9
	5			222	0	0	100	12	12	13	13
	1			156	6	3.8	100	3	3	3	3
	2			282	8	2.8	100	13	13	16	16
	3	Apr-11	Sedentary	208	5	2.4	100	11	11	12	12
	4			169	3	1.8	100	1	1	1	1
	5			148	6	4.1	65	10	15.4	13	20
	1			152	3	2.0	100	4	4	5	5
	2			177	1	0.6	100	1	1	1	1
	3	Aug-11	Sedentary	114	7	6.1	100	4	4	4	4
	4			147	2	1.4	100	5	5	5	5
	5			73	1	1.4	100	3	3	4	4
12	1	Feb-11	Migratory	224	12	5.4	100	13	13	14	14
	2			223	9	4.0	100	18	18	22	22

	3			329	14	4.3	100	26	26	29	29
	4			381	10	2.6	100	15	15	15	15
	5			395	3	0.8	100	3	3	3	3
13	1	Feb-11	Migratory	111	2	1.8	100	4	4	4	4
	2			214	3	1.4	100	1	1	2	2
	3			201	18	9.0	100	32	32	45	45
	4			148	1	0.7	100	2	2	2	2
	5			196	2	1.0	100	1	1	1	1

Abreviation: NS: No sample was collected.

Table III. Number of Chalkbrood mummies and parasites found in adult honeybee and worker brood samples collected from July 2010 to August 2011 in 13 *Apis mellifera scutellata* apiaries.

Apiary code	Colony no.	Month	Sedentary/Migratory	Chalkbrood	Parasites			
					A. m. c.	*Braula*	Wax moth	SHB
1	1			0	0	1	0	0
	2			0	0	0	0	0
	3	Jul-10	Sedentary	0	0	1	0	0
	4			0	0	7	Excreta	0
	5			0	0	2	Excreta	0
	1			0	0	0	0	0
	2			0	0	0	0	0
	3	Sep-10	Sedentary	0	0	5	0	0
	4			0	0	2	0	0
	5			0	0	4	0	0
	1			0	0	1	0	0
	2			0	0	0	0	0
	3	Nov-10	Sedentary	0	0	2	Excreta	0
	4			0	0	1	Excreta	0
	5			0	0	4	0	0
	1			0	0	2	0	0
	2			0	0	4	0	0
	3	Apr-11	Sedentary	0	0	2	Excreta	0
	4			0	0	6	Excreta	0
	5			0	8	1	0	1 Adult

	1			0	0	3	0	0
	2			0	0	9	0	0
	3	Aug-11	Sedentary	0	0	1	0	0
	4			0	0	4	0	0
	5			0	0	2	0	0
2	1			0	0	1	Excreta	0
	2	Jul-10	Migratory	0	0	2	Excreta	0
	3			0	0	3	Excreta	0
	5			Y	0	0	0	0
	1			0	0	0	0	0
	2	Sep-Oct 2010	Migratory	0	0	1	0	0
	3			0	0	1	0	0
	5			0	0	0	0	0
	1			0	0	0	0	0
	2	Nov-10	Migratory	0	0	0	0	0
	3			4	0	1	0	0
	5			0	0	1	0	0
	3	Jan-11	Migratory	0	Multiple eggs	2	0	0
	5			0	0	0	0	0
	1			0	0	2	Excreta	0
	2	Apr-11	Migratory	0	11	5	Excreta	0
	3			0	0	1	Excreta	0

	4			0	0	0	Excreta	0
	5			0	0	0	Excreta	0
	1			0	1	0	0	0
	2			0	3	1	0	0
	3	Aug-11	Migratory	0	7	1	0	0
	4			0	0	0	0	1 Adult
	5			0	1	0	0	0
3	1			0	0	6	Excreta	0
	2			0	0	6	Excreta	0
	3	Jul-10	Migratory	0	0	4	0	0
	4			0	0	0	0	0
	5			17	0	10	0	1 Adult
	1			57	0	0	0	0
	2			0	0	0	0	0
	3	Nov-10	Migratory	0	0	8	0	0
	4			0	0	0	0	0
	5			0	0	0	0	0
	1			0	0	0	0	0
	2			0	0	0	0	0
	3	Jan-11	Migratory	0	0	2	Excreta	0
	4			11	0	1	0	0
	5			0	0	2	0	0
	1	Apr-11	Migratory	0	0	2	0	0
	2			0	0	0	Excreta	1 Adult

	3			0	0	0	Excreta	0
	4			0	0	0	Excreta	0
	5			0	0	0	0	0
	1			0	0	3	0	0
	2			0	0	1	0	0
	3	Aug-11	Migratory	0	0	1	0	0
	4			0	0	1	0	1 Adult
	5			0	0	5	0	0
4	1			0	0	1	Excreta	0
	2			0	0	6	0	0
	3	Aug-10	Sedentary	0	0	3	0	0
	4			0	0	2	Excreta	0
	5			0	0	0	0	0
	1			0	0	1	0	0
	2			0	0	0	Excreta	0
	3	Oct-10	Sedentary	0	0	0	0	0
	4			0	0	12	Excreta	0
	5			0	0	1	Excreta	0
	1			0	0	8	Excreta	0
	2			0	0	7	Excreta	0
	3	Nov-10	Sedentary	0	0	1	0	0
	4			0	0	9	0	0
	5			0	0	0	0	0
	1	Jan-11	Sedentary	0	0	4	Excreta	0

	2			0	0	5	Excreta	0
	3			0	0	0	Excreta	0
	4			0	0	0	Excreta	0
	5			5	0	1	Excreta	0
	1			0	0	8	Excreta	0
	2			0	0	3	Excreta	0
	3	Mar-11	Sedentary	0	0	6	Excreta	0
	4			0	0	1	Excreta	0
	5			12	0	0	Excreta	0
	1			0	0	9	0	0
	2			0	0	2	0	0
	3	Jul-11	Sedentary	0	2	11	0	1 Adult
	4			0	0	14	0	0
	5			0	0	6	Excreta	1 Adult
5	1			0	Y	1	0	0
	2			0	Y	2	0	0
	3	Aug-10	Migratory	0	Y	2	0	0
	4			0	Y	0	0	0
	5			0	Y	0	0	0
6	1			0	Y	5	0	0
	2			3	Y	0	0	0
	3	Aug-10	Migratory	0	Y	1	Excreta	0
	4			2	Y	4	0	0
	5			0	Y	2	0	0

7	1			0	0	1	Excreta	0
	2			0	0	0	0	0
	3	Nov-10	Migratory	0	0	1	Excreta	2 Adults
	4			0	0	0	0	0
	5			0	0	0	0	0
	1			0	0	0	0	0
	2			0	0	3	Excreta	0
	3	Jan-11	Migratory	0	Y	0	0	0
	4			0	0	2	Excreta	0
	5			0	Y	2	0	0
8	1			0	0	3	0	0
	2			0	0	6	0	0
	3	Dec-10	Sedentary	0	0	0	0	0
	4			0	0	3	0	0
	5			0	0	5	0	1 Adult
9	1			0	0	0	0	0
	2			0	0	0	0	0
	3	Dec-10	Sedentary	0	0	1	Excreta	0
	4			1	0	0	0	7 Larvae & 194 Eggs
	5			0	0	0	Excreta	0

10	1			0	0	0	0	0
	2	Feb-11	Migratory	0	0	0	0	0
	3			0	0	0	0	0
11	1			0	0	2	0	0
	2			0	0	0	Excreta	0
	3	Feb-11	Sedentary	0	0	0	0	0
	4			0	0	1	0	0
	5			0	0	1	0	1 Adult
	1			0	0	0	0	0
	2			0	0	3	Excreta	0
	3	Apr-11	Sedentary	0	0	1	0	0
	4			0	0	0	Excreta	0
	5			0	0	1	0	0
	1			0	0	1	0	0
	2			0	0	1	0	0
	3	Aug-11	Sedentary	0	0	3	0	1 Adult
	4			0	0	4	0	0
	5			0	0	2	0	0
12	1			0	0	3	0	0
	2			2	0	1	0	0
	3	Feb-11	Migratory	0	0	3	0	0
	4			0	0	5	0	0
	5			1	0	1	0	0

13	1			0	0	1	0	0
	2			0	Multiple eggs	7	0	1 Adult
	3	Feb-11	Migratory	0	0	3	0	0
	4			0	0	2	Excreta	6 Adults
	5			0	0	6	0	0

Abreviation: Y indicates the presence of a particular pathogen or parasite; otherwise exact numbers are given if known.

Appendix B

Table I. Daily *Varroa* mite fall in nine colonies of the treated apiary from 21st of May to 4th of October 2011.

Date	Colony no.								
	1	2	3	4	5	6	7	8	9
21-May-11	6	0	10	5	1	0	0	1	1
22-May-11	5	1	10	5	5	0	0	2	0
23-May-11	4	1	11	12	9	0	0	0	0
24-May-11	10	0	18	6	9	0	2	2	2
25-May-11	4	0	10	4	10	1	0	0	0
26-May-11	4	0	12	5	6	0	0	5	0
27-May-11	9	0	14	6	8	1	0	4	3
28-May-11	14	2.3	10	5	11.3	0.3	0.3	2.3	2
29-May-11	14	2.3	10	5	11.3	0.3	0.3	2.3	2
30-May-11	14	2.3	10	5	11.3	0.3	0.3	2.3	2
31-May-11	19	4	15	6	11	0	0	2	6
01-Jun-11	12	0	18	5	8	1	1	2	1
02-Jun-11	16	0	11	8	7	2	0	4	1
03-Jun-11	13	0	18	7	4	2	0	0	1
04-Jun-11	8	0.3	11.3	0.7	7	1	1	1.7	3.3
05-Jun-11	8	0.3	11.3	0.7	7	1	1	1.7	3.3
06-Jun-11	8	0.3	11.3	0.7	7	1	1	1.7	3.3
07-Jun-11	0	0	5	2	6	1	0	7	8

08-Jun-11	5.3	1	13.3	0.3	7.3	1.7	0.3	1	0.3
09-Jun-11	5.3	1	13.3	0.3	7.3	1.7	0.3	1	0.3
10-Jun-11	5.3	1	13.3	0.3	7.3	1.7	0.3	1	0.3
11-Jun-11	4	0	13.3	0.3	5	1.7	0.7	3.3	0.7
12-Jun-11	4	0	13.3	0.3	5	1.7	0.7	3.3.	0.7
13-Jun-11	4	0	13.3	**0.3 (A)**	5	1.7	0.7	3.3	0.7
14-Jun-11	0	0	11	N/A	5	0	1	1	0
15-Jun-11	12	2	237	N/A	244	15	30	41	49
16-Jun-11	13.5	1.5	61.5	N/A	55	2.5	3.5	9	16.5
17-Jun-11	13.5	1.5	61.5	N/A	55	2.5	3.5	9	16.5
18-Jun-11	43.3	4.5	54.8	N/A	51.5	12.5	3.5	10.3	19.5
19-Jun-11	43.3	4.5	54.8	N/A	51.5	12.5	3.5	10.3	19.5
20-Jun-11	43.3	4.5	54.8	N/A	51.5	12.5	3.5	10.3	19.5
21-Jun-11	43.3	4.5	54.8	N/A	51.5	12.5	3.5	10.3	19.5
22-Jun-11	13.0	5.0	18.0	N/A	53.0	17	3.0	21	3.0
23-Jun-11	7.8	2.8	22.4	N/A	41.0	9.2	2.0	14.2	5.0
24-Jun-11	7.8	2.8	22.4	N/A	41.0	9.2	2.0	14.2	5.0
25-Jun-11	7.8	2.8	22.4	N/A	41.0	9.2	2.0	14.2	5.0
26-Jun-11	7.8	2.8	22.4	N/A	41.0	9.2	2.0	14.2	5.0
27-Jun-11	7.8	2.8	22.4	N/A	41.0	9.2	2.0	14.2	5.0
28-Jun-11	10	5.5	2.5	N/A	3.5	2.5	0	14	16.0
29-Jun-11	10	5.5	2.5	N/A	3.5	2.5	0	14	16.0
30-Jun-11	1.5	6.5	7.0	N/A	2.5	1.5	0.5	9.5	2.5
01-Jul-11	1.5	6.5	7.0	N/A	2.5	1.5	0.5	9.5	2.5
02-Jul-11	**0 (A)**	2.4	1.7	N/A	0.6	1.7	0	5.7	6.9
03-Jul-11	N/A	2.4	1.7	N/A	0.6	1.7	0	5.7	6.9
04-Jul-11	N/A	2.4	1.7	N/A	0.6	1.7	0	5.7	6.9
05-Jul-11	N/A	2.4	1.7	N/A	0.6	1.7	0	5.7	6.9

06-Jul-11	N/A	2.4	1.7	N/A	0.6	1.7	0	5.7	6.9
07-Jul-11	N/A	2.4	1.7	N/A	0.6	1.7	0	5.7	6.9
08-Jul-11	N/A	2.4	1.7	N/A	0.6	1.7	0	5.7	6.9
09-Jul-11	N/A	2.3	2.7	N/A	0.5	2.2	0.2	5.0	9.5
10-Jul-11	N/A	2.3	2.7	N/A	0.5	2.2	0.2	5.0	9.5
11-Jul-11	N/A	2.3	2.7	N/A	0.5	2.2	0.2	5.0	9.5
12-Jul-11	N/A	2.3	2.7	N/A	0.5	2.2	0.2	5.0	9.5
13-Jul-11	N/A	2.3	2.7	N/A	0.5	2.2	0.2	5.0	9.5
14-Jul-11	N/A	2.3	2.7	N/A	0.5	2.2	0.2	5.0	9.5
15-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
16-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
17-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
18-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
19-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
20-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
21-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
22-Jul-11	N/A	2.3	1.3	N/A	1.1	2.4	0	1.5	3.9
23-Jul-11	N/A	1.9	0.7	N/A	0.9	1	0	0.6	1.6
24-Jul-11	N/A	1.9	0.7	N/A	0.9	1	0	0.6	1.6
25-Jul-11	N/A	1.9	0.7	N/A	0.9	1	0	0.6	1.6
26-Jul-11	N/A	1.9	0.7	N/A	0.9	1	0	0.6	1.6
27-Jul-11	N/A	1.9	0.7	N/A	0.9	1	0	0.6	1.6
28-Jul-11	N/A	1.9	0.7	N/A	0.9	1	0	0.6	1.6
29-Jul-11	N/A	1.9	0.7	N/A	0.9	1	0	0.6	1.6
30-Jul-11	N/A	1	0.3	N/A	0.7	0.5	0	1.7	4.3
31-Jul-11	N/A	1	0.3	N/A	0.7	0.5	0	1.7	4.3
01-Aug-11	N/A	1	0.3	N/A	0.7	0.5	0	1.7	4.3
02-Aug-11	N/A	1	0.3	N/A	0.7	0.5	0	1.7	4.3

03-Aug-11	N/A	1	0.3	N/A	0.7	0.5	0	1.7	4.3
04-Aug-11	N/A	1	0.3	N/A	0.7	0.5	0	1.7	4.3
05-Aug-11	N/A	0	0.8	N/A	1.8	0.7	0.3	0.3	3.2
06-Aug-11	N/A	0	0.8	N/A	1.8	0.7	0.3	0.3	3.2
07-Aug-11	N/A	0	0.8	N/A	1.8	0.7	0.3	0.3	3.2
08-Aug-11	N/A	0	0.8	N/A	1.8	0.7	0.3	0.3	3.2
09-Aug-11	N/A	0	0.8	N/A	1.8	0.7	0.3	0.3	3.2
10-Aug-11	N/A	**0 (A)**	0.8	N/A	1.8	0.7	0.3	0.3	3.2
11-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
12-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
13-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
14-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
15-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
16-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
17-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
18-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
19-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
20-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
21-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
22-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
23-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0	0.3	1.4
24-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0.1	0.3	0.3
25-Aug-11	N/A	N/A	1.3	N/A	0.9	0.5	0.1	0.3	0.3
26-Aug-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
27-Aug-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
28-Aug-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
29-Aug-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
30-Aug-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3

31-Aug-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
01-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
02-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
03-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
04-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
05-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
06-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
07-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
08-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
09-Sep-11	N/A	N/A	0.5	N/A	0.3	0.7	0.1	0.2	0.3
10-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
11-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
12-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
13-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
14-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
15-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
16-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
17-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
18-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
19-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
20-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
21-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
22-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
23-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
24-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
25-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
26-Sep-11	N/A	N/A	0.5	N/A	0.3	0.4	0	0.3	0.5
27-Sep-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4

28-Sep-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4
29-Sep-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4
30-Sep-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4
01-Oct-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4
02-Oct-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4
03-Oct-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4
04-Oct-11	N/A	N/A	2	N/A	0.4	0.5	0	0.6	1.4

Abreviation: (A) Indicates that the colony absconded and as a result no data could be collected from these colonies (N/A).

Table II. Daily *Varroa* mite fall in nine colonies of the untreated apiary from 21st of May to 4th of October 2011.

Date	Colony no.								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
21-May-11	6	18	54	12	3	1	1	0	21
22-May-11	7	24	59	12	5	0	0	0	34
23-May-11	3	5	51	19	1	0	0	0	31
24-May-11	14	13	53	16	2	0	0	0	34
25-May-11	14	8	47	15	1	1	0	0	39
26-May-11	6	10	35	9	3	0	1	0	17
27-May-11	12	21	48	6	0	1	0	0	24
28-May-11	8.7	28.7	53.7	7.3	1.3	1.7	1.7	0	17.3
29-May-11	8.7	28.7	53.7	7.3	1.3	1.7	1.7	0	17.3
30-May-11	8.7	28.7	53.7	7.3	1.3	1.7	1.7	0	17.3
31-May-11	3	15	23	13	2	3	2	0	10
01-Jun-11	7	8	16	12	1	4	3	0	25
02-Jun-11	3	23	15	16	1	5	0	0	17
03-Jun-11	3	25	35	10	2	0	0	0	26
04-Jun-11	2.7	8.3	9	12.3	0.7	1.3	0.7	0	13.7
05-Jun-11	2.7	8.3	9	12.3	0.7	1.3	0.7	0	13.7
06-Jun-11	2.7	8.3	9	12.3	0.7	1.3	0.7	0	13.7
07-Jun-11	3	16	14	12	1	1	1	0	8
08-Jun-11	6.3	13.3	9.7	11.7	1.7	1.7	1	0	13.7
09-Jun-11	6.3	13.3	9.7	11.7	1.7	1.7	1	0	13.7
10-Jun-11	6.3	13.3	9.7	11.7	1.7	1.7	1	0	13.7
11-Jun-11	7.3	11.7	6.3	8.3	2	1	0.3	0	14.7
12-Jun-11	7.3	11.7	6.3	8.3	2	1	0.3	0	14.7
13-Jun-11	7.3	11.7	6.3	8.3	2	1	0.3	**0 (A)**	14.7

14-Jun-11	16	19	9	21	4	2	1	N/A	16
15-Jun-11	11	16	7	13	0	1	1	N/A	9
16-Jun-11	1	6	5	14	2	1	0	N/A	9
17-Jun-11	9	20	14	15	6	1	1	N/A	10
18-Jun-11	7.3	16.3	8	9.7	4.3	3.3	2.7	N/A	11.7
19-Jun-11	7.3	16.3	8	9.7	4.3	3.3	2.7	N/A	11.7
20-Jun-11	7.3	16.3	8	9.7	4.3	3.3	2.7	N/A	11.7
21-Jun-11	7	15	6	9.5	1.5	1.5	2.5	N/A	12.5
22-Jun-11	7	15	6	9.5	1.5	1.5	2.5	N/A	12.5
23-Jun-11	7.4	17.8	3.4	10.8	2.2	2.2	1.6	N/A	14.8
24-Jun-11	7.4	17.8	3.4	10.8	2.2	2.2	1.6	N/A	14.8
25-Jun-11	7.4	17.8	3.4	10.8	2.2	2.2	1.6	N/A	14.8
26-Jun-11	7.4	17.8	3.4	10.8	2.2	2.2	1.6	N/A	14.8
27-Jun-11	7.4	17.8	3.4	10.8	2.2	2.2	1.6	N/A	14.8
28-Jun-11	13.5	23.5	4.5	12.5	6	4	2.5	N/A	16
29-Jun-11	13.5	23.5	4.5	12.5	6	4	2.5	N/A	16
30-Jun-11	8.5	17	3	9.5	3	5	0.5	N/A	16.5
01-Jul-11	8.5	17	3	9.5	3	5	0.5	N/A	16.5
02-Jul-11	7.9	16	3.7	6.9	4.4	3.6	1.7	N/A	16.9
03-Jul-11	7.9	16	3.7	6.9	4.4	3.6	1.7	N/A	16.9
04-Jul-11	7.9	16	3.7	6.9	4.4	3.6	1.7	N/A	16.9
05-Jul-11	7.9	16	3.7	6.9	4.4	3.6	1.7	N/A	16.9
06-Jul-11	7.9	16	3.7	6.9	4.4	3.6	1.7	N/A	16.9
07-Jul-11	7.9	16	3.7	6.9	4.4	3.6	1.7	N/A	16.9
08-Jul-11	7.9	16	3.7	6.9	4.4	3.6	1.7	N/A	16.9
09-Jul-11	5	12.6	1.8	7.4	5.8	5.4	3.4	N/A	9.6
10-Jul-11	5	12.6	1.8	7.4	5.8	5.4	3.4	N/A	9.6
11-Jul-11	5	12.6	1.8	7.4	5.8	5.4	3.4	N/A	9.6

12-Jul-11	5	12.6	1.8	7.4	5.8	5.4	3.4	N/A	9.6
13-Jul-11	5	12.6	1.8	7.4	5.8	5.4	3.4	N/A	9.6
14-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
15-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
16-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
17-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
18-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
19-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
20-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
21-Jul-11	7.1	22.1	1.9	5.6	7.4	5	1.6	N/A	11.3
22-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
23-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
24-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
25-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
26-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
27-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
28-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
29-Jul-11	8.1	6.9	1	2.4	9.9	7	1.5	N/A	6.3
30-Jul-11	4.2	7.8	1.5	1.5	11	11.8	1.5	N/A	4.7
31-Jul-11	4.2	7.8	1.5	1.5	11	11.8	1.5	N/A	4.7
01-Aug-11	4.2	7.8	1.5	1.5	11	11.8	1.5	N/A	4.7
02-Aug-11	4.2	7.8	1.5	1.5	11	11.8	1.5	N/A	4.7
03-Aug-11	4.2	7.8	1.5	1.5	11	11.8	1.5	N/A	4.7
04-Aug-11	4.2	7.8	1.5	1.5	11	11.8	1.5	N/A	4.7
05-Aug-11	3.2	2.5	0.3	0	9.5	9.5	0.7	N/A	0
06-Aug-11	3.2	2.5	0.3	0	9.5	9.5	0.7	N/A	0
07-Aug-11	3.2	2.5	0.3	0	9.5	9.5	0.7	N/A	0
08-Aug-11	3.2	2.5	0.3	0	9.5	9.5	0.7	N/A	0

09-Aug-11	3.2	2.5	0.3	0	9.5	9.5	0.7	N/A	0
10-Aug-11	3.2	2.5	0.3	**0 (A)**	9.5	9.5	0.7	N/A	**0 (A)**
11-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
12-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
13-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
14-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
15-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
16-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
17-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
18-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
19-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
20-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
21-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
22-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
23-Aug-11	3.9	1.1	0.6	N/A	5.8	5.0	0.8	N/A	N/A
24-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
25-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
26-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
27-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
28-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
29-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
30-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
31-Aug-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
01-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
02-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
03-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
04-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
05-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A

06-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
07-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
08-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
09-Sep-11	1.5	0.5	0.8	N/A	7.3	6.9	0.1	N/A	N/A
10-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
11-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
12-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
13-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
14-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
15-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
16-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
17-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
18-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
19-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
20-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
21-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
22-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
23-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
24-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
25-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
26-Sep-11	1	0.9	0.1	N/A	2.3	2.4	0.1	N/A	N/A
27-Sep-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A
28-Sep-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A
29-Sep-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A
30-Sep-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A
01-Oct-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A
02-Oct-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A
03-Oct-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A

04-Oct-11	0	1.6	0.4	N/A	3.9	4.3	0.1	N/A	N/A

Abreviation: (A) Indicates that the colony absconded and as a result no data could be collected from these colonies (N/A).

Table III. Number of parasites and Chalkbrood mummies found in nine colonies of the treated apiary on the *Varroa* bottom boards from 21st of May to 4th of October 2011.

Colony no.	SHB	Wax moths	*Braula*	Ants	Pseudoscorpions	Cockroaches	Chalkbrood
1	2	0	3	5	0	0	0
2	2	3	1	38	1	1	0
3	1	4	23	1	0	0	0
4	0	1	1	0	0	0	0
5	6	17	21	8	0	6	0
6	4	0	4	2	0	1	0
7	0	2	4	27	0	0	0
8	33	13	1	24	0	0	0
9	13	29	7	112	1	0	0

Abreviation: SHB: Small hive beetles.

Table IV. Number of parasites and Chalkbrood mummies found in nine colonies of the untreated apiary on the *Varroa* bottom boards from 21st of May to 4th of October 2011.

Colony no.	SHB	Wax moths	*Braula*	Ants	Pseudoscorpions	Cockroaches	Chalkbrood
1	8	0	7	0	0	1	0
2	9	3	4	0	0	0	0
3	4	0	5	5	0	0	1
4	17	0	7	0	0	0	0
5	47	9	0	2	2	0	2
6	24	5	1	3	0	0	29
7	18	5	1	26	1	1	0
8	2	0	0	1	0	1	0
9	31	0	12	0	0	1	0

Abreviation: SHB: Small hive beetles.

Table V. The presence of queens, queen cells and parasites in nine colonies of the treated apiary from May to October 2011.

	Colony no.								
Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
May	Queen	Queen SHB	SHB Queen cell	-	Queen SHB Queen cell	Queen	-	Queen	SHB Queen cell
June	-	Queen SHB	Queen Queen cell	N/A	SHB	Queen Queen cell	Queen	Queen SHB	SHB Queen cell
July	N/A	Queen SHB	Queen Queen cell	N/A	Queen	Queen cell Multiple eggs	Queen	Queen SHB	SHB Queen cell
August	N/A	N/A	Queen Queen cell	N/A	Queen	Queen cell	Queen	Queen SHB	SHB Queen cell
September	N/A	N/A	Queen Queen cell	N/A	Queen SHB	Queen cell Multiple eggs	Queen	Queen SHB	SHB Queen cell
October	N/A	N/A	Queen cell	N/A	Queen	SHB Queen cell	Queen	-	Queen SHB Queen cell

Abreviation: N/A indicates that the colonies absconded and – indicates that no queen, queen cells or parasites were observed. SHB: Small hive beetles.

Table VI. The presence of queens, queen cells and parasites in nine colonies of the untreated apiary from May to October 2011.

	Colony no.								
Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
May	Queen SHB	Queen SHB	SHB	Queen SHB	Pseudo-scorpion	Queen SHB	Queen SHB	Queen SHB	Queen SHB
June	Queen	Queen SHB	Queen	Queen SHB	Queen cell	Queen SHB	SHB	N/A	Queen SHB
July	Queen SHB	Queen	Queen SHB Multiple eggs	Queen SHB Multiple eggs	Queen SHB	Queen	Queen SHB Multiple eggs	N/A	Queen SHB
August	Queen SHB	Queen	Queen SHB Multiple eggs	N/A	SHB Queen cell	Queen	Queen SHB	N/A	N/A
September	Queen SHB	Queen SHB	Queen SHB	N/A	Queen	Queen SHB Multiple eggs	Queen SHB Queen cell	N/A	N/A
October	Queen SHB	Queen	Queen Multiple eggs	N/A	Queen cell	-	SHB	N/A	N/A

Abreviation: N/A indicates that the colonies absconded and – indicates that no queen, queen cells or parasites were observed. SHB: Small hive beetles.

Table VII. Number of adult honeybees, sealed and unsealed worker brood measured in nine colonies of the treated apiary from May to October 2011.

		Colony no.								
	Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
No. of adult honeybees	May	6885	4037.5	7905	6077.5	8245	7140	4165	8670	13090
	June	4760	4250	10370	-	9605	8670	5100	10710	9520
	July	-	5355	10030	-	8075	10625	3867.5	14535	10157.5
	August	-	-	6970	-	7905	9095	2337.5	12452.5	10540
	September	-	-	10285	-	7310	9435	1657.5	10285	13600
	October	-	-	9435	-	17340	6205	4420	12665	4420
Sealed brood (dm^2)	May	13.00	0.00	12.00	10.50	5.00	5.50	8.00	22.5	2.00
	June	0.00	10.25	10.00	-	9.50	19.00	13.50	34.75	13.50
	July	-	4.75	11.25	-	0.00	5.75	0.25	27.50	17.00
	August	-	-	9.00	-	3.50	1.25	0.25	14.75	19.00
	September	-	-	45.25	-	33.75	0.00	6.00	44.00	56.00
	October	-	-	87.00	-	62.00	0.00	20.50	53.50	0.00
Unsealed brood (dm^2)	May	10.50	2.25	11.50	8.50	10.5	6.00	3.75	13.00	2.50
	June	0.00	3.00	5.75	-	7.00	14.00	3.00	21.50	5.00
	July	-	3.00	5.25	-	0.00	0.50	0.00	10.00	11.00
	August	-	-	12.00	-	10.50	2.00	2.50	16.00	4.75
	September	-	-	36.00	-	30.75	0.00	8.25	25.75	21.25
	October	-	-	24.75	-	30.50	0.00	11.00	21.75	18.00

Table VIII. Number of adult honeybees, sealed and unsealed worker brood measured in nine colonies of the untreated apiary from May to October 2011.

		Colony no.								
	Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
No. of adult honeybees	May	4080	8160	6545	6885	6290	9775	6460	1360	7225.00
	June	2720	7565	3400	3995	6417.5	6885	4165	-	5737.50
	July	2805	5865	3910	3187	9265	11305	5440	-	2932.50
	August	2337.5	4420	1955	-	7310	8755	4122.5	-	-
	September	1530	3485	1530	-	9562.5	10795	4590	-	-
	October	6035	10030	2805	-	9095	14025	10795	-	-
Sealed brood (dm^2)	May	14.00	10.00	10.5	8.50	13.50	5.50	0.50	4.00	9.50
	June	6.50	5.00	1.00	4.0	20.00	16.50	8.00	0.00	3.00
	July	6.00	12.00	5.00	4.0	21.50	26.00	8.50	0.00	10.00
	August	3.25	0.00	4.50	-	15.00	14.75	6.50	-	-
	September	6.50	21.00	5.00	-	39.75	35.50	19.50	-	-
	October	27.50	34.25	11.75	-	19.50	35.50	48.00	-	-
Unsealed brood (dm^2)	May	4.00	6.00	4.00	1.50	13.00	16.00	6.00	1.75	3.50
	June	4.75	8.00	0.00	3.50	9.50	9.00	3.75	-	1.00
	July	1.75	4.50	5.50	3.50	16.00	13.50	6.50	-	2.00
	August	3.75	2.00	3.75	-	10.25	12.00	7.50	-	-
	September	7.00	17.50	7.00	-	25.25	12.00	12.00	-	-
	October	13.50	17.50	9.50	-	0.00	13.75	15.50	-	-

Table IX. ***Varroa destructor*** **infestation rates in adult honeybees and worker brood in the treated apiary measured during May, July and September 2011.**

Colony no.	Month	No. of adult bees sampled	No. of mites on adult bees	*Varroa* / 100 bees	No. of cells opened	No. of *Varroa* infested cells	Percentage infested cells	No. of *Varroa* in cells	*Varroa* / 100 cells
1		139	3	2.2	100	7	7	7	7
2		76	4	5.3	NS	NS	NS	NS	NS
3		171	4	2.3	100	11	11	11	11
4		147	4	2.7	100	8	8	9	9
5	May	180	4	2.2	100	17	17	24	24
6		143	0	0	78	0	0	0	0
7		106	0	0	100	0	0	0	0
8		226	0	0	100	0	0	0	0
9		133	0	0	NS	NS	NS	NS	NS
1		NS	NS	NS	NS	NS	NS	NS	NS
2		NS	NS	NS	NS	NS	NS	NS	NS
3		81	0	0	100	0	0	0	0
4		NS	NS	NS	NS	NS	NS	NS	NS
5	July	143	0	0	NS	NS	NS	NS	NS
6		132	0	0	NS	NS	NS	NS	NS
7		NS	NS	NS	NS	NS	NS	NS	NS
8		145	1	0.7	100	1	1	1	1
9		118	1	0.8	100	3	3	3	3

1	September	NS	NS	NS	NS	NS	NS	NS	NS
2		NS	NS	NS	NS	NS	NS	NS	NS
3		145	1	0.7	100	0	0	0	0
4		NS	NS	NS	NS	NS	NS	NS	NS
5		121	0	0	100	0	0	0	0
6		100	0	0	NS	NS	NS	NS	NS
7		NS	NS	NS	NS	NS	NS	NS	NS
8		173	2	1.2	100	0	0	0	0
9		128	1	0.8	100	0	0	0	0

Abreviation: NS – No sample was collected.

Table X. ***Varroa destructor*** **infestation rates in adult honeybees and worker brood in the untreated apiary measured during May, July and September 2011.**

Colony no.	Month	No. of adult bees sampled	No. of mites on adult bees	*Varroa* / 100 bees	No. of cells opened	No. of *Varroa* infested cells	Percentage infested cells	No. of *Varroa* in cells	*Varroa* / 100 cells
1		65	2	3.1	100	5	5	5	5
2		85	2	2.4	100	15	15	17	17
3		220	15	6.8	100	18	18	20	20
4		160	7	4.4	100	18	18	21	21
5	May	130	0	0	100	0	0	0	0
6		127	0	0	67	0	0	0	0
7		74	0	0	NS	NS	NS	NS	NS
8		NS	NS	NS	NS	NS	NS	NS	NS
9		87	1	1.1	100	22	22	25	25
1		NS	NS	NS	NS	NS	NS	NS	NS
2		88	1	1.1	100	34	34	48	48
3		NS	NS	NS	NS	NS	NS	NS	NS
4		NS	NS	NS	NS	NS	NS	NS	NS
5	July	120	4	3.3	100	9	9	9	9
6		155	2	1.3	100	4	4	4	4
7		85	0	0	100	3	3	3	3
8		NS	NS	NS	NS	NS	NS	NS	NS
9		99	5	5.1	100	17	17	20	20

1	September	NS	NS	NS	NS	NS	NS	NS	NS
2		74	2	2.7	NS	NS	NS	NS	NS
3		NS	NS	NS	NS	NS	NS	NS	NS
4		NS	NS	NS	NS	NS	NS	NS	NS
5		171	2	1.2	100	1	1	1	1
6		160	3	1.9	100	0	0	0	0
7		155	1	0.6	100	0	0	0	0
8		NS	NS	NS	NS	NS	NS	NS	NS
9		NS	NS	NS	NS	NS	NS	NS	NS

Abreviation: NS – No sample was collected.