Parasite–host interactions between *Varroa destructor* Anderson and Trueman and *Apis mellifera* L.: Influence of parasitism on flight behaviour and on the loss of infested foragers

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich Biologie und Informatik
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
Jasna Kralj
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Parasit-Wirtsbeziehungen zwischen *Varroa destructor* Anderson and Trueman und *Apis mellifera* L.: Einfluss der Parasitierung auf das Flugverhalten and auf den Verlust befallener Arbeiterinnen

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1. Literature review

1.1. Introduction

Varroa destructor, commonly referred to as *Varroa* mite, is one of the most serious pests of the honey bee *Apis mellifera* and has caused numerous losses of honey bee colonies worldwide. *Varroa* is an ectoparasitic mite of honeybees of a large size, feeding on the hemolymph of bees in development stages ranging from larvae until hatching and/or adults (Martin, 2001a). The female is a crab shaped brown reddish mite of 1.1mm in length and 1.6mm in width. The male is pale white and much smaller than the female (length: 0.8mm, width: 0.7mm), and lives only in the sealed honey bee brood cells (Martin, 2001a).

Damage caused by ectoparasitism of the mite to the individual bee that hatched from infested brood cells includes reduced size, weight loss, wing deformities (De Jong et al., 1982a; Schneider and Drescher, 1987), reduction of life span (Schneider and Drescher, 1987; De Jong, 1990), flight frequency of infested drones (De Jong, 1990) and reduction of hypopharyngeal glands of workers (Schneider and Drescher, 1987). The damage caused by *V. destructor* depends on the infestation level. The infestation level of bee brood with mites correlates with the death of bees or damaged wings of hatched bees. Weight loss and life span of workers are more reduced in the case of multi infestation during development in brood cells (De Jong, 1990). In addition, *V. destructor* is a vector for viral (Akey et al., 1995; Ball, 1996; Martin, 1997a) and bacterial infections (Glinski and Jarosz, 1992). Death of infested colonies is occasionally related to viral infections (Akey et al., 1995; Ball, 1996; Martin, 1997a, 2001b).

V. destructor has been a serious threat to the Western honey bee *Apis mellifera* for almost three decades, nevertheless the classification of the virulent mite as a new species of *V. destructor* was recently made. *V. destructor* was know as *V. jacobsoni*, a species described from Java,

which infest the Asian honey bee *A. cerana*. Other mites infesting *A. cerana* were similar to this species and were therefore classified as *V. jacobsoni* as well. Anderson and Trueman (2000) showed that *V. jacobsoni* is a species complex consisted from at least two species; *V. jacobsoni* and *V. destructor* both containing several haplotypes including the Korean haplotype and the less virulent Japan haplotype. *V. destructor* is only one species which has become a serious parasite of the Western bee *A. mellifera*. The original host of *V. destructor* is the Asian honey bee *A. cerana. V. destructor* was initially spread to *A. mellifera* by importation of *A. mellifera* to Asia (Primorsky region, former USSR). It is likely that the *Varroa* mite from *A. cerana* colonies infested *A. mellifera* colonies by mutual robbing and drifting between colonies in close vicinity or/and the efforts of beekeepers to strengthen their *A. mellifera* colonies with brood of *A. cerana* (De Jong et al., 1982b). Further spread to areas outside the original range of *A. cerana* occurred through transportation of infested *A. mellifera* colonies and/or queens to western former USSR from eastern former USSR and further to Europe. By the end of the 1970` the mite had spread over almost all of Europe (De Jong et al., 1982b).

V. destructor has spread at remarkable speed throughout most of the world by now. The particular biology of the mite and modern migratory beekeeping have contributed to its successful spread. Once *Varroa* mites become established in an area, spread is very fast. It was documented that the rate of natural movement of the mite is about 3 km per year in Eastern Europe and Germany, and 6-11 km in 3 months in the area of former USSR (De Jong et al., 1982b). The spread of *V. destructor* from infested to uninfested colonies is accomplished by migration of female mites on foragers to another colony (Greatti et al., 1992) and robbing among colonies (Sakofski, 1990). Swarming, or colony reproduction, is also a possibility for the mite to spread. The mite migrates on bees in a swarm that will establish the new colony (Martin, 2001a; Fries et al., 2003).

V. destructor is, in terms of co-evolution with its host, a relatively new parasite of the western bee *Apis mellifera* which is not adapted to the mite, resulting in high loss of colonies (Oldroyd, 1999). In contrast, the

original host of *V. destructor*, the Asian bee *Apis cerana* is well adapted through long co-existence with the mite. In *A. cerana*, *V. destructor* reproduces exclusively in drone brood cells (Koeninger et al., 1981; Boot et al., 1999) whereas in *A. mellifera* it reproduces on both worker and drone brood. The mite could enter the worker brood of *A. cerana* and eventually start egg laying, however it fails in reproduction as bees rapidly remove infested brood (Boecking and Ritter, 1994). The restriction of mite reproduction to drone cells by effective detection and removal of infested worker brood is crucial for the tolerance of *A. cerana* toward *V. destructor* (Boot et al., 1999). Reproduction of the mite on *A. mellifera* results in high build up of mite populations over time and collapse of the infested colonies. Further, *A. cerana* workers are unable to open sealed drone brood because of the thick cocoon spun by the drone larvae. Consequently, mites are entombed and die with infested drones which die during development or do not succeed in opening the special caps of the cells (Rath. 1992).

A. mellifera is far less successful in defense against *V. destructor*, nevertheless some mechanisms to decrease mite infestation are present. To understand better the mite and bee relationship, defense mechanisms in honey bees have been extensively studied and differences in susceptibility of species and races of honeybees to *V. destructor* have been found. Many efforts have been made to propagate lines of bees with more pronounced defense mechanisms which may permit beekeepers to cease or at least reduce chemical control, thereby lowering operating costs and ensuring pesticide free bee products.

1.2. Population of parasites in relation to *V. destructor*

A population is defined as a group of individuals of the same species which is breeding and occupying the same area (Tarman, 1992). Bush et al. (2001) viewed associations of parasites and hosts as a hierarchy of communities. The most fundamental level of parasite communities is the infra community level consisting of the parasite´s infra populations in the single host individuals. An infra population of parasites

is defined as a sum of all individuals within a single host at particular time. In contrast, with respect to *Varroa* infestation, honey bee colonies consist of numerous bees and the individual bee is usually infested by one or in some occasions by a few mites. Consequently, the classical definitions in parasitology that view the host parasite associations as infra populations of parasites in a single host could not be applied in the same way. In social insects a host population consists of a number of groups such as colonies, each containing a number of host individuals (Schmitd-Hempel, 1998). A colony is considered as a superorganism due its complexity and coordination between individuals. Since the colony behaves as a unit and is an individualized system of activities (Wilson, 1972), for practical purposes, the literature deals mostly with infra populations of *V. destructor* in colonies by referring to the *Varroa* mite population within colonies.

1.3. Population of *V. destructor*

The infestation of colonies by *V. destructor* is commonly estimated by counting dead mites in debris (Liebig et al.,1984; Fries et al., 1991a; Moretto and Mello, 2000), mites in brood and on adult bees (Fuchs, 1985; Martin, 1998; Fries 1991a). The infestation of colonies is also measured by counting dead mites killed after treatment with acaracides (Calatayud and Verdu, 1993). The population growth of *V. destructor* is exponential (Calatayud and Verdu, 1993; Calis et al., 1999) and could increase more than 10 fold in a year in cold climates (Fries et al., 1991b; Korpela et al., 1992; Martin, 1998) and by more than 100 fold in temperate climates where an extended brood period occurs (Branco et al., 1999; Kraus and Page, 1995). In contrast, colonies in tropical climates appear to be less infested, a consequence of a smaller population growth rate of the mite, regardless that bees rear brood virtually all year round (De Jong et al., 1984).

Despite all the research carried out, the precise events leading to colony collapse are still unclear. The typical sign of collapse of an infested colony is rapid loss of bees resulting in a queen with few workers

and patchy brood (Martin, 1997a). The numbers of mites that colonies reach before collapse differ widely. Martin (2001b) reported collapse of colonies having a few thousand mites (2600-1600), whereas some colonies with the same population of mites did not collapse. Delaplane and Hood (1997) reported the threshold of 2500-3500 mites in the colony. There is also a report that colonies containing between 24000 and 25000 mites and did not show any sign of disease (Martin, 1997b). This strongly suggest other factors being involved in the colony collapse. One factor that may explain colony collapse is viral infection transmitted by mites. A relatively small number (2000-3600) of deformed wing virus (DWD) transmitting mites can cause the collapse of colonies with 30000-40000 workers (Martin, 2001b).

Detailed knowledge of the changes in population size of *V. destructor* aids in understanding the biological aspects of the mite and bee relationship and consequently contributes to prediction of population growth of the mite. Some attempts have been made to model population dynamics, which mathematically describe the population changes of the mite in a colony. There are several estimates of population growth based on the life cycle of *V. destructor.* Models including the main factors that influence population growth of the mite (Fries et al., 1994; Martin, 1998; Calis et al., 1999) help to understand basic biology of the mite and predict which factors have a large impact on population dynamics. In addition, the models are useful tools to optimise *Varroa* control in the field. Nevertheless, there is little known about factors influencing mite population dynamics outside the colony. These factors are mostly neglected in the population models, though some do incorporate mortality due to natural death of foragers (Fries et al., 1994; Calis et al., 1999).

1.4. Life history of *V. destructor*

The mite's life history is divided into two distinct phases: 1) the reproductive phase in which the mite reproduces within the sealed honey bee brood 2) and the phoretic (carrying) phase in which the adult female mite is attached to the adult bee (Martin, 2001a).

A mated female mite enters a worker or a drone brood cell prior to capping (workers: 8 day, drones: 8-9 day of bee development) and immerses itself in the brood food under the larva. Once the mite enters brood food it is immobilized (Martin, 2001a). The mite enters drone cells 10-12 times more frequently than workers cells (Fuchs, 1992; Boot et al., 1995b) which could be partly explained by the larger size of drone larvae (Martin, 1998). Oviposition starts after the bee larva eats the food and liberates the mite (60h after capping). The first egg laid by the mother mite is a male egg (haploid), followed by four to five female diploid eggs at intervals of 30 h. (Ifantidis, 1983; Rehm and Ritter, 1989). The female daughter mite develops in 6.2 days and the male in 6.9 days after the fertilized egg is laid (Rehm and Ritter, 1989). Mating of a brother with mature daughters occurs on the faecal accumulation of the mother mite. Mature offspring undertake several matings, each lasting at least 6 min (Donze et al., 1996). The mother mite is estimated to produce on average about 1 mite in workers cell and 1 or 2 mites in drone cell (Ifantidis, 1983; Fuchs and Langebach 1989; Martin 1994; Donze et al., 1996). The number of daughters per mite decreases in multiply infested cells (Fuchs and Langebach, 1989; Eguaras et al., 1994; Donze et al., 1996). When the parasitized bee emerges, the mother mite and adult mated daughters leave the cell on the bee and the male die. The duration of phoretic period, when mites live on the bee and feed on hemolymph, depends on the mites' chance to find a suitable brood cell to enter. In the brood rearing period, the phoretic period is estimated to be between 4.5 to 11 days (Martin, 2001a). Mites that would enter the brood for the subsequent reproductive cycle without passing any time on the bee could still reproduce, but at a reduced rate (Beetsma and Zonneveld, 1992). The mean number of reproductive cycles per single mite is estimated to be between 1.5 and 2 (Fries and Rosenkranz, 1996) with a range of 0 to 7 (Ruijter, 1987).

1.5. Factors inside the colony that influence population dynamics of *V. destructor*

Reproduction and death are the major factors influencing the mite population in colonies. Mechanisms that reduce mite reproduction and facilitate mite mortality are referred as resistant mechanisms of honey bees against the mite. In general, resistance is the ability of organisms to remain unaffected or only slightly affected by pathogens (Dorland, 1990). Resistance mechanisms counteract the population growth and are discussed as possible effective mechanisms of defence toward the mite.

1.5.1. Reproduction

Reproduction of the mite has been recognized as an important factor that influences population dynamics of *V. destructor* (Fries et al., 1994). Several factors cause the mite to reproduce less. The most important is infertility of mites which contributes much to reduce mite populations (Harbo and Harris, 1999). Reproduction of the mite is also reduced by other factors such as shortened post capping period and lower brood attraction which are described in this chapter.

1.5.1.1. *Mite fertility*

The major interest in breeding for *Varroa* resistance are mechanisms to limit reproduction of the mite. The most important case is complete non reproduction of female mites. Infertile mites are females without offspring. The occurrence of non reproduction of mites in our bee, *A. mellifera carnica* in middle Europe ranged from 10%-15% (Rosenkranz and Bartalszky, 1996). In contrast, 70-90% of mites do not reproduce in worker brood of *A. m. carnica* in open mated Carnica colonies in Uruguay (Ruttner and Marx, 1984). Low reproductive success of the female mites after invading brood cells has also been reported from Africa, South and Central America and was a subject of considerable research. The proportion of mites that do not reproduce was reported by Ritter and De

Jong (1984) to be approximately two times greater in tropical Brazil than in Europe. Moretto et al. (1991a) demonstrated that bees of the same origin (Africanized and Italian), when transferred to different climatic regions showed differences in infestation for a single race. Infestation of bees in the cooler regions was higher than those in tropical region. Africanized bees were also less infested compared to Italian colonies. The investigation of the resistant colonies of *A. mellifera* in a temperate zone in Argentina revealed that 40% of female mites are infertile (Eguaras et al., 1995). This rate of infertility is similar to the rate reported for the Africanized honey bees (Camazine, 1986; Ritter, 1988).

Certainly, low fertility of mites is a characteristic with a great impact on population growth according to the model of Fries et al. (1994). Regardless of research and a strong interest in the topic, it is still not clear what influences physiology and triggers mite oviposition. Reproductive success of mites decreased when mites were limited to length of phoretic period on older bees during summer for several weeks (Rosenkranz et al., 1996). Harris and Harbo (1999) found that non-reproductive female mites had few or no spermatozoa in the spermatheca. To reproduce successfully a female mite should obtain enough spermatozoa gathered during several matings (Donze et al., 1996). Mites that fail in mating enter the brood cell but produce only males in the subsequent reproductive cycle (Martin et al., 1997), while normal mites still reproduce. This support Fuchs (1994) finding that non reproduction of the mite is mainly related to the status of the mite. Low mite fertility, especially those reported from South America, could be due to differences in virulence of different haplotypes of *V. destructor* described by Anderson and Trueman (2000).

From the other perspective, Harris and Harbo (2000) found that the genotype of bees has a strong effect on fertility of mites. Replacing a susceptible queen with a queen that had been bred for suppression of mite reproduction (SMR) led to a decrease in reproductive success of mites in the colony. Due to high heritability of SMR, Harbo and Harris (1999) propose that it is one of the most promising traits to select in order

to obtain bees resistant to *Varroa* mite. Nevertheless, it has little effect when it occurs at levels less than 30% (Harbo and Harris, 1999).

1.5.1.2. *Post-capping period*

The practical consequence of *V. destructor* reproduction in the capped brood and death of immature mites upon emergence of its adult bee host is that the duration of the post-capping period influences the average number of fertile daughters produced by a single mother. The average post-capping period for drones in *A. mellifera* is 2 days longer than that of workers (Winston, 1987). Consequently, 3 daughter mites could reach maturity in drone brood and 1.8 in worker brood (Donze et al., 1996). The African cape bee, *A. mellifera capensis* has about 2 day shorter post-capping period compared to *A. mellifera*. This may partly explain the failure of reproduction of 42% of the mites that could not complete a reproductive cycle (Moritz and Hanel, 1984). Büchler and Drescher (1990) demonstrated that the reduction of the post-capping period by 1 hour results in an 8.7% reduction of the colony infestation. Wilkinson and Smith (2002) in the model predict 30% and 60% of reduction in the population growth of *V. destructor* if the post-capping period is reduced about 10% in drone and worker brood respectively.

The post-capping period is a heritable characteristic (Moritz, 1985, Harbo, 1992), therefore could be used in the programs to breed bees resistant to *Varroa*. Harbo (1992) estimated a 5.4h change in duration of worker development by selecting the top 10% bee population for rapid development. Nevertheless, there are some concerns about the post-capping period as a parameter for breeding since the selection for rapid development of bees would simultaneously result in the selection of *Varroa* population for more rapid development in the colony (Bozic, personal communication).

1.5.1.3. *Brood attraction*

Varroa mites prefer drone brood versus worker brood. The high attractiveness of drone brood can be partly explained by a 2-3 times longer attraction period of drone brood to mites and by the larger surface of drone cells compared to workers cells. Combining all these affects, the attraction of *Varroa* to drone brood exceeds the expectation indicating that the mite in about 70% of the encounters rejects the available worker brood (Boot et al., 1995a). Fuchs (1992) suggested that this pronounced preference of the mite for drone brood is the result of a selection for high preference of mites to invade drone brood. With extended search time for drone brood, mites enter workers cells to minimize a cost of delay in reproduction (Fuchs, 1992). Boot (1995a), in his model, predicted that if the phoretic period of mites is less than 7 days, mites enter drone cells, if available. Attraction of mites to bee larvae is explained by chemical signals of the brood. Using an olfactometer, Le Conte et al. (1989) confirmed that *Varroa* mites are attracted preferentially to the odour of drones. Nazzi et al. (2001), in a bioassay, showed that the larval food collected from drones cells or chemicals of larval food, before capping elicited a strong response of *V. destructor*.

Brood from different origins also varied in attraction of *V. destructor* (De Guzman et al., 1995). A low attraction of brood results in low invasion of mites and consequently an increase of the phoretic period of the mite. The model of Fries et al. (1994) predicts that variation in the duration of the phoretic period of the mite does not change the mite population growth much. Their model includes the invasion of worker and drone brood by mites according to the number of available drone and worker cells and the ratio of mites to drone or worker cells. Similarly, the model of Calis et al. (1999) includes different attraction to drones and workers according to the number of available worker/drone cells and the colony size without considering the phoretic period of the mite.

The invasion rates of the mite to brood of different origin are still largely unknown. Knowledge of the invasion rates for different races of bees would be valuable in simulations of population growth of *V.*

destructor and thus could be included in the programs selecting bees for mite resistance.

1.5.2. Behavioural defence facilitating mite mortality

Honeybees have developed defence mechanisms against pathogens. Behavioural defence of bees against the mite including grooming and hygienic behaviour is well researched. Both mechanisms increase the mortality of mites and thus decrease the colony infestation although not sufficiently for the colony to survive.

1.5.2.1. *Grooming behaviour*

Grooming behaviour allows bees to remove mites from their bodies and was well described by Peng et al. (1987). Grooming behaviour includes self- grooming and nest-mate grooming performed by uninfested workers. The infested worker which could not remove the mite by itself performs the grooming dance by vibrating its body laterally. Workers triggered by the dance approach the bee, which stops dancing and stretches the wings and legs and raises up its thorax and abdomen. The nest-mates examine the body with antennae and remove the mite with the mandibles. In the process of removing mites from the bee's body, mites could be damaged (Peng et al., 1987). Many authors have compared grooming behaviour between the Asian bee, *A. cerana* and the Western bee, *A. mellifera*. Regardless of the differences in the proportion of mites removed by bees, all reports are conclusive that *A. cerana* showed more intensive grooming behaviour than *A. mellifera* (Peng et al., 1987; Büchler et al., 1992; Fries et al, 1996). *A. cerana* is capable of removing almost all mites, compared to *A. mellifera* which removes only a small portion of mites (Peng et al., 1987; Büchler et al., 1992). In addition, significantly more mites were injured by *A. cerana* than *A . mellifera* and consequently fewer mites recover (Peng et al., 1987; Büchler et al., 1992; Fries et al., 1996). There is no direct evidence that grooming under

natural conditions in *A. cerana*, plays a significant role in resistance to *Varroa destructor* (Boecking et al., 1993).

Pronounced grooming behaviour after half an hour of inoculation with the *Varroa* mite was reported in Africanized bees, which removed 38% in the contrast to *A. mellifera* which removed only 5.7% (Moretto et al., 1991b). A negative correlation between mite population growth and the number of injured mites found by Arechavaleta-Valasco and Guzman-Novoa (2001) and Moosbeckhofer (1992) suggests that grooming may play an important role in reducing population growth of *V. destructor*.

1.5.2.2. *Hygienic behaviour*

Hygienic behaviour is a mechanism of resistance to American foulbrood (Spivak and Reuter 1997), chalk brood (Gilliam et al., 1983) and *V. destructor* (Boecking and Drescher, 1992). The hygienic behaviour of honey bees is a defensive mechanism including uncapping brood cells and removing diseased or dead brood. Hygienic behaviour is genetically determined (Moritz, 1988), however, Spivak and Gilliam (1993) suggested it is also affected by colony strength and composition of workers within the colony.

Hygienic behaviour is tested by several methods including removing artificially infested brood with *V. destructor*, removing freeze killed brood or pin killed brood after some period. Workers removed double infested brood and freeze killed brood more frequently than single infested brood (Boecking and Drescher, 1992). *A. cerana* is very effective in detecting and removing mites in worker brood (Peng et al., 1987; Rath and Drescher, 1990). *Cerana* workers are not inclined to remove infested drone brood (also see 1.1.) but in four days remove almost all mites (94%) in worker brood (Rath and Drescher, 1990). In contrast, *A. mellifera* in general showed lower removal response toward worker infested brood than *A. cerana*. Boecking and Drescher (1992) reported a removal rate of 24% in single infested cells and 41% removal response in double infested cells. However, there is high variability in removal responses between strains of *A. mellifera*, ranging from 5.5% to 96% in

single infested cells and 5% to 100% in double infested worker cells (Boecking and Drescher, 1992). Janmaat and Winston (2000) reported the influence of pollen storage on removal rate of workers infested with *V. destructor*. Low pollen colonies with a high demand for pollen performed high foraging that leads to a reduction in the number of bees engaged in cell removal behaviour.

Spivak and Reuter (1997) demonstrated that colonies established with open mated queens from hygienic stock had greater hygienic behaviour than unselected stock.

In the process of uncapping and removing brood, immature female mites die (Spivak, 1996) and adult female mites could be damaged or killed (Boecking and Drescher, 1992). The main effect of hygienic behaviour is to postpone the phoretic period. Escaped mites may re-enter another available brood or adhere to adult bees (Boecking, 1994). Mangum et al. (1997) in modelling population biology and population genetic dynamics of the honey bee with respect to the mite, estimated that the frequency of hygienic behaviour is insufficient to protect a colony from *Varroa* mite population growth.

1.5.3. Death of mites within colonies

The rate of death obviously has a negative influence on the population growth. Despite protection of the mite inside the brood cell, a small portion of mites (1-3%) fail to escape from bee food in which it was immersed and so become entombed between the cell wall and the cocoon. Further death of mother mites (1-5%) could occur during reproduction (Martin, 2001a). Mite offspring suffer much higher mortality. The greatest juvenile mite mortality occurs at the latest stage of mite development (immobile phase –deutochrysalis) ranging from 16% in the first offspring to 60% in the third offspring in *A. mellifera macedonica* (Ifantidis et al., 1999). Considering the fourth offspring which could not complete development and therefore dies upon bee emergence, the total proportion of offspring that die within the cell, would be even higher. Mechanical factors such as a movement of a moulting pupa or a bee

within the cell could kill developing mites, but only the third or fourth daughter mite in a sequence. The increase in mortality rate of the second offspring compared to the first clearly indicates the presence of other factors influencing death of juvenile mites within the cells (Ifantidis et al., 1999).

The emergence of bees corresponds to the numbers of dead mites registered in debris counts on the bottom of the colony (Lobb and Martin, 1997). The mite fall in hatched worker brood is 2-3 times higher than in drone brood, probably due to shorter development time of workers barely allowing complete development of the last female offspring (Lobb and Martin, 1997). Lobb and Martin (1997) estimated that half of the mites that fall from the nest to the bottom of the colony include those that die within the cells and those which die after emergence due to incomplete development. The other half of fallen mites was still alive and could reproduce when artificially introduced to brood. This portion of mites could represent those that fail to successfully change a bee host. Two days after emergence of infested bees most of the mites change their host (Kovac and Crailsheim, 1986).

The life expectancy of *V. destructor* under natural conditions depends on the biology of a colony. In the period with brood it is estimated to be about 27 days and in broodless winter period it exceeds 5-6 months (Martin, 2001a). Bowen-Walker and Gunn (1998) showed that mites fallen from the winter cluster with dead bees could survive up to 48h by feeding on dead bees. Most mites (75%) effectively left dying or dead bees and found a new host in the winter cluster within 24h. This clearly indicates that mites move between hosts and on the other hand that the survival of mites in the winter cluster is not completely related to death of bees and therefore it is higher than expected (Bowen-Walker and Gunn, 1998). In contrast, Fries and Perez-Escala (2001) could not find that mites become concentrated on the remaining bees in the winter cluster as the number of mites per dead bee was not significantly different from the number of mites per live bee. This difference in results is probably due to different techniques used which result in different

opportunities for mites to re- infest adult bees after falling from the winter cluster.

Mite mortality is monitored by counting fallen mites in debris. This method is recognized as a useful parameter to estimate population levels in honey bee colonies by many authors (Liebig et al., 1984; Calatayud and Verdu 1993; Fries et al., 1991a) except for colonies with well expressed grooming behavior (Arechavaleta-Velasco and Guzman-Novoa, 2001). Nevertheless, the parameter is highly variable and depends on season and the presence of brood and gives rougher estimates of a mite population within the colony. The estimates are most accurate during the broodless period and the period in which colonies have large brood nests (Martin, 1998). The model of Martin (1998) accounts for seasonal differences in brood amount by using different multiplication factors to convert the daily mite mortality into a estimate of the mite population within the colony.

1.6. Factors outside the colony that influence population dynamics of *V. destructor*

Factors such as spread of mites and mite mortality outside the colony influence population dynamics of the mite. Despite the importance of these factors in population dynamics, both the spread of mites and mite mortality have not received much attention. Most research has covered spread of mites which contributes to invasion of large areas by mites, while the mite mortality outside the colony due foraging has been poorly researched and therefore is still not completely understood.

1.6.1. Spread of mites

The spread of the mites has a great impact on population dynamics of *V. destructor* and its virulence. The fitness and virulence of the mite does not depend only on the ability of mites to reproduce and spread within the colony but also on the ability to spread between colonies. In parasitology, spread of parasites is classified as vertical and

horizontal transmission. Vertical transmission occurs with the transfer of parasites from parents to their offspring. Specifically in honey bees, the vertical transmission of mites is achieved by swarming. Horizontal transmission occurs with the transfer from one host to another host and could include intra-colony and inter-colony transmission by contact of infested and uninfested individuals (Fries and Camazine, 2001). The horizontal transmission of a pathogen contributes much more to virulence than the vertical transmission (Schmidt-Hempel, 1998). The vertical transmission of a parasite requires effective reproduction of the host which can be successfully achieved at the low level of virulence. Such a situation can be easily applied to honey bees which divide during swarming (Fries and Camazine, 2001).

1.6.1.1. *Vertical transmission*

Vertical transmission occurs from one host generation to the next (Schmidt Hempel, 1998). In honey bees, the vertical transmission is related to swarming, when the parent colony divides (Fries and Camazine, 2001). The swarm is headed by an old queen (mother) and thousands of workers. The new queen (daughter) stays in the parent colony with the rest of bees. Occasionally a colony produces more swarms which are headed by unmated queens (Winston, 1987). Division of the colony by swarming spreads the mite. The mite infestation of swarms is found to be equal to that of parent colonies in the late fall indicating that swarm survival is similarly affected by *V. destructor* as original colonies (Fries et al., 2003). Martin (2001a) suggested that swarming could be the main source of mite dispersal in its natural host, the Asian bee, *A. cerana*. Vertical transmission undoubtedly serves as a mechanism to spread mites over a large area.

1.6.1.2. *Horizontal transmission*

The horizontal transmission of the pathogen occurs when the pathogen is introduced into a colony by infested bees from another

colony (Fries and Camazine, 2001). Such mistaken entering (drifting) of bees into other colonies is a normal event in honey bee life. Another possibility of transmission is a contact between infested and uninfested bees by robbing in which bees from one colony invade another colony to steal honey. A third possibility is transfer of the pathogen during foraging on flowers. With respect to the mite and honey bees, this transfer is not likely although still not ruled out (Fries and Camazine, 2001).

Bees are known to drift from one to another colony. Drifting between colonies has been extensively researched by Jay (1965), who found that drifting between colonies in a single apiary and between apiaries is large. Infested foragers that drift into other colonies are vectors for mites and spread *V. destructor* via contacts with other bees. On the other hand drifters can pick up mites and transfer them to the original colonies, if they return. Ritter and Leclercq (1987) found that low infested colonies built up mite populations relatively fast when surrounded by infested colonies in the radius of 2 km. Sakofsky and Koeniger (1988) showed that the number of drifting bees corresponds to the number of transferred *V. destructor* and that drifting increases with the infestation level of the colony (Sakofsky, 1990). This might suggest a different approach to drifting which is normally considered as an error of workers entering the foreign colonies. Since the disease can change flight behaviour of bees (Woyciechowski and Kozlowski, 1998), drifting as a means of horizontal transmission, may not be an error made by workers but a behaviour triggered by a parasite to its own advantages (Schmid-Hempel, 1998).

Robbing is another way of spreading mites horizontally by foragers carrying mites. Bees generally rob when little forage is available and they are able to invade weak colonies. Guard bees at the front entrance of the hive protect the colony from intruding bees (Winston, 1987). However, when the colony is weak, the guard bees become ineffective in repelling intruders which leads to robbing. Sakofsky (1990) confirmed that horizontal transmission by robbing activities of foragers is a very effective means of spreading mites to other colonies. In experiments, he provoked robbing of weakened colonies infested by *V. destructor.* Approximately

14% of the mites were transferred in 2 hours from infested bees of the attacked colony to robber bee colonies. At the end of the season, when food is scarce and occurrence of robbing is high, the invasion of mites on foragers increases (Sakofsky, 1990).

There is some evidence that mites can use foraging as means of transfer. In bumble bees, the protozoan, *Crithidia bombi* is transmitted via flowers visited by workers of different colonies and different species. Such horizontal transmission is very efficient and common in bumble bees as sooner or later all colonies around the infection source become infested in their life cycle (Schmid-Hempel, 1998). In honey bees, infection by the mite via a flower would be possible, in principle. *V. destructor* can survive up to several days without a bee; considerable time to change the host on the flower. Even in unsuitable conditions, with low temperatures, the mite survives for at least several hours (De Guzman et al., 1993). Hartwig and Jedruszuk (1987) explored survival of the mite on flowers and found that it can survive on flowers 144 hours and was able to transfer to a bee after 5 days. Kevan et al. (1990) reported *V. destructor* on flowers imported from South America to Florida. In another report from Georgia a live *Varroa* mite was found on cut flowers transported from the Netherlands (Pettis et al., 2003). However, there was no supportive evidence of the presence of mites on flowers near a heavily infested honey bee colony (Pettis et al., 2003) to effect vertical transmission of the mite using flowers as a vectors of transfer.

1.6.2. Death of mites outside colonies

Mites die outside the hive on foragers which fail to return to the hive. Mortality of mites in the field is considered mainly as turnover of bees. Fries et al. (1994) predicted 0.5% daily mite mortality due to turnover of foragers. By subtracting the actual daily increase of mite population from the expected daily birth rate, Fuchs and Kutschker (2000) estimated a daily death rate of 0.032. This estimate results in a daily mite mortality of 32 mites in the colony with a population of 1000 mites. According to many studies of population growth in relation to the number

of dead mites found on the bottom of the colony, it is clear that such a high number of dead mites could not be found in a colony with the population of 1000 mites. Fuchs and Kutschker (2000) concluded that only 1/3 of the expected dead mites could be found in a colony. Loss of mites due to normal turnover of foragers can explain only an additional 12% of missing mites (Fuchs and Kutschker, 2000). Kutscher (1999) found proportionally more infested workers leaving than returning to the colony which indicates that more mites are lost than expected by normal mite mortality of foragers.

Turnover of infested workers could affect the infestation of a colony and consequently, colony survival. The model of a conveyor belt represents a stream of bees from birth until death. The conveyer belt carries the pathogens out from the colony if the pathogens do not change host to younger bees. Potentially dangerous infections are acquired by the forager from outside (Schmid-Hempel, 1998). In a balanced situation, the transmission of infection from outside would be in equilibrium with loss of infection due to turnover of infested foragers. If the conveyer belt runs fast, workers age rapidly which increases turnover of bees. Such a situation will confine the infection because the infection from outside could not keep pace with the belt movement (Schmid-Hempel, 1998).

However, mite mortality on foragers outside the colony might be more pronounced. Kutschker (1999) demonstrated that mites do not return to the colony as would be expected from the normal turnover of bees. She reported that the infestation of outflying workers is 3 times higher than the infestation of returning bees. Such loss of mites exceeding normal mortality of foragers could explain why a proportion of dead mites could not be found in the colony according to mite reproduction (Fuchs and Kutschker, 2000). Pronounced loss of mites also suggests that infested bees do not return to the colony at a higher proportion than uninfested workers. In this respect, failure of infested workers to return could potentially be another strategy of honey bees to eliminate the parasite and so decrease a colony infestation (Fuchs, 2000).

2. Objectives of the research

Vague understanding of fate of mites on foragers and striking differences in infestation between outflying and returning bees demonstrated by Kutschker (1999) raises the question of where mites go during foraging. One possibility would be that mites do not return to the colony as a result of death of foragers. Another would be that mites are removed or they change host during foraging. Both mechanisms could lead to pronounced loss of mites and could be viewed as defence mechanisms, yet unknown, to eliminate a pathogen from the colony.

The main goal of the research was to determine mite loss with foragers and explain the difference in the infestation between outflying and returning bees. Besides the possibility that foragers lost mites outside the colony, I particularly focused on the question whether infested workers do not return to the colony as frequently as uninfested foragers. In this respect I focused on the question whether flight behavior of foragers is altered by *V. destructor* and contributes to higher frequency of non returning infested foragers.

a) The question whether flight behavior is influenced by parasitism of *V. destructor* to explain loss of mites was explored by investigating flight duration, returning time, orientation and the frequency of drifting (entering to other colonies). To determine whether flight duration of infested workers differs from uninfested workers, the flight duration was measured by using a video technique. Similarly, to investigate whether infested bees need more time to return, workers were released from different distances to measure returning time. Whether infested bees return as frequently as uninfested bees was explored by releasing workers from different distances and recording their returning in evening. Further, to investigate whether infested workers show some deficiency in orientation which could explain loss of infested bees, the affect of nest orientation was determined. Since impaired orientation might result in pronounced drifting, the frequency of drifting was investigated for infested and uninfested bees.

b) Mite loss was explored by repeating the experiment of Kutschker (1999) to determine differences in infestation between outflying and returning

bees. Further, the degree to which mites are lost from the colony and the possible ways of mite loss were investigated by using a video technique to record individually marked workers.

c) Effect of infestation of *V. destructor* on a colony level was explored by investigating loss of foragers in high and low infested colonies. Monitoring infestation of outflying bees and the number of bees lost from colonies per day were used to estimate the proportion of mite loss from the mite population in a colony due foraging

d) To explore whether flight pattern differs in bees of different origin, Primorsky and Carnica were compared in infestation of outflying and returning workers, in flight duration and returning time.

The research provides novel information on the influence of *V. destructor* on flight behavior of infested foragers and the importance of foraging as a mean of mite loss.

3. Materials and methods

The evaluation of the influence of *Varroa destructor* on the flight behaviour of parasitised foragers included seven experiments. The research was conducted at the Institut für Bienenkunde in Oberursel in the period from June to September in the years 2001, 2002 and 2003. Honey bee colonies were headed by Carnica queens and Primorsky queens in experiments performed in 2001 and by Carnica queens in experiments performed in 2002 and 2003. I used full-size colonies made up of two boxes, each including 10 frames and small nucleus colonies made up of Kirchhainer box including four frames. Colonies were checked on a weekly basis for the presence of queen, brood and food. The nucleus colonies received sugar candy every week. Full-size colonies were fed at the end of the season with sugar syrup (Apinvert) to supply bees with additional food.

3.1. Infestation of colonies

Full-size colonies were already heavily infested by *V. destructor*. In contrast, the nucleus colonies had lower level of infestation and therefore had to be continuously infested by additional mites. Three different methods to infest nucleus colonies with the mites were used. I introduced mites to colonies a) on adult bees, b) on emerged young workers, and c) by placing mites directly on bees.

a) Colonies were infested by introducing adult infested bees collected from very infested colonies close to colony break down. These bees were collected in other apiaries to avoid that the infested bees return to their home colonies. Young bees were introduced into a nucleus colony in a cage which was placed in the feeder in the nucleus colony to ensure good acceptance of introduced workers (Photo 1). The wired side of the cage was oriented toward the colony to enhance the contact between introduced workers and bees in the colony. The opening of the

cage was covered with sugar candy. Bees ate the candy in one day and freed the introduced bees.

b) The colony was also infested by introducing newly emerged workers infested by *V. destructor*. Brood combs of workers were taken from the very infested colonies and placed in a dark incubator on 34 C^0 and 60% RH. Hatched workers were checked for mites. Infested young workers were collected and introduced in a cage into a nucleus colony to ensure acceptance in a same way as described above (a). I also introduced one day old infested workers directly to nucleus colonies in 2003 because newly hatched workers are readily accepted by bees.

c) I introduced mites in the nucleus colony directly by placing the mite on the workers' body in the years 2002 and 2003. Mites were collected from brood and hatched bees. Larvae or pupae were taken from worker and drone brood cells and examined for mites. Mites were collected with a fine brush from capped brood and hatched bees and placed on bees.

Photo 1. Introduction of one day old marked workers in the cage into the nucleus colony. The opening of the cage was covered with sugar candy. Bees were freed by eating sugar candy.

3.2. Marking bees

To recognize workers in the experiment, I marked one day old workers individually and introduced them to infested colonies. Capped frames of worker brood of healthy *Varroa* free colonies were placed in a dark incubator at 34 C^0 and 60% RH. Hatched workers were collected and individually marked with coloured plates specially made for workers (2r=2mm) and numbered from 1-100. I held the bee at the thorax and abdomen to drop glue on the thorax and to attach the plate. The colour of

the plate represented the day of emergence and the number represented an individual bee.

Young bees were introduced into a nucleus colony in a cage which was placed in the feeder in the nucleus colony to ensure good acceptance of introduced workers as described above (3.1.a).

The method of individually marking bees with the numbered plates was simplified in 2003. Emerged workers in the incubator were brushed from the comb into a container and from there about 100 workers were shaken on a styrofoam plate. I covered the plate with a net with a wooden frame (Photo 2). I pressed the net around an individual bee and glued the plate through the aperture of the net measuring 4mm X 4mm on the thorax of the bee. Marked bees were brushed from the styrofoam plate directly to the nucleus colony.

The same procedure using the styrofoam plate was used to mark workers with colour. Emerged bees were marked through apertures of the net on the thorax with a marker and then introduced directly into colonies. Each colour represented the day of bee emergence.

Occasionally, when many bees emerged, I marked them with the colour and later before the experiment I marked them additionally with the number plates for individual recognition.

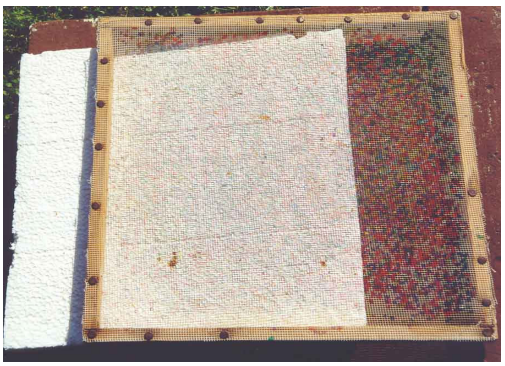


Photo 2. The device for marking workers including the styrofoam plate and the net attached to a wooden frame. Workers were brushed to the syrofoam plate, covered with the net and marked with the numbered plates through the net.

3. 3. **Measurement of infestation of outflying and returning workers**

Out-flying and returning workers were sampled from the beginning of August to the beginning of September in five commercial colonies to compare infestation. Four colonies were headed by Primorski queens and one by a Carnica queen. To sample out-flying and returning foragers separately without sampling the younger bees guarding the entrance, the hive entrance was modified to catch foraging bees in a cage placed in the front of an extended entrance.

The infestation of experimental colonies was monitored regularly by counting dead mites fallen on the bottom inserts of the colony.

3.3.1. Sampling device

Hives were modified in a such a way to enable separate sampling of outflying and returning bees. To avoid collecting the guarding bees, the entire hive entrance was extended into a cage. The cage was made from a wooden frame covered with a net. The cage was attached to the entire hive entrance (modified drone trap measuring 40cm X 30cm X 10cm). The sides of the cage were covered with a cloth to reduce light in the cage. This helped outflying workers to orient toward the light coming through the new hive entrance at the front of the cage and fly out. The new entrance was a short tunnel insert (11cm X 11cm X 4cm), a wooden box, placed in the front of the cage (Photo 3, Photo 4).

During sampling I removed the tunnel insert and replaced it with a sampling tunnel insert of the same size. The sampling insert was partitioned in the middle by a cloth net arranged in such a way to form a tunnel leading to the opening at the top of the sampling insert. This opening led into a plastic jar from which I sampled bees (Photo 4). To collect outflying bees, the sampling tunnel insert was closed at the outer side from where bees were returning back to the colony. This prevented returning bees to enter the sampling insert. To collect returning bees, the

sampling insert was closed at the inner side of the hive entrance to prevent outflying bees to enter the sampling insert.

Photo 3. The sampling device to sample outflying and returning bees separately. The entire hive entrance was extended into a cage (a). The sides of the cage were covered with a cloth to reduce light in the cage. The new entrance was a short tunnel insert made from a wooden box (b) placed in the front of the cage.

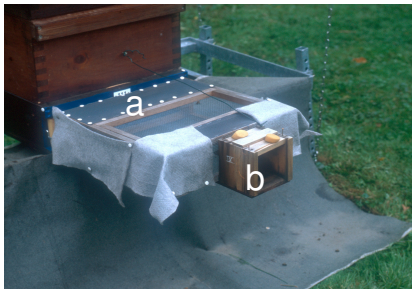
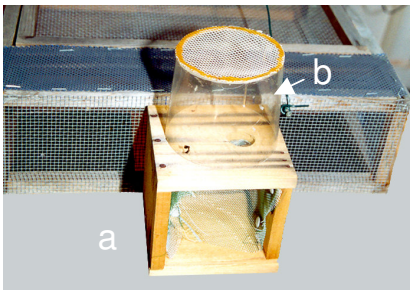


Photo 4. The sampling insert with the jar to collect bees. The sampling insert (a) was partitioned in the middle by a cloth net arranged in such a way to form a tunnel leading to the opening at the top covered with the jar (b).



3.3.2. Conducting the experiment

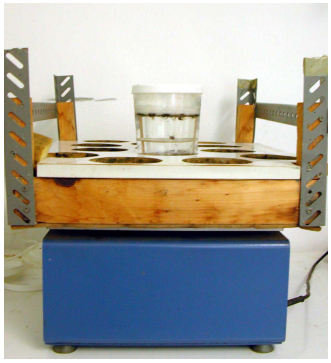
3.3.2.1. *Sampling procedure*

Sampling of outflying and returning bees was conducted approximately three weeks after hive modification. This period was necessary for bees to learn the new entrance. Samples of bees were taken from 11h to 15h to avoid collecting bees having orientation flights which could interfere with the results. Samples of outflying and returning bees per colony were taken only once per day amounting to about 100 bees per sample. In total 54 samples were taken. The sampling jar with bees was deposited into a plastic bag and placed into a freezer. Frozen bees were counted.

3.3.2.2. *Determination of infestation of bee samples*

To determine mite infestation of samples of outflying and returning bees I used a method of washing described by Fuchs (1985). Frozen bees were placed into plastic jars with inserted smaller cups with a sieve bottom (3mm X 3 mm) that separated jars in the middle. The plastic jars with sampled bees were filled with hot detergent water. The jars were covered with plastic lids and placed into a laboratory device for shaking samples of bees (Photo 5) set on 150 Cycles/min (Hz) for 45 minutes. Shaking caused mites to fall down from bees on the bottom of jars. Bee samples in the inserted cups were washed with water for additional mites to fall in a sieve (1mm X 1mm). The total number of fallen mites per sample was recorded.

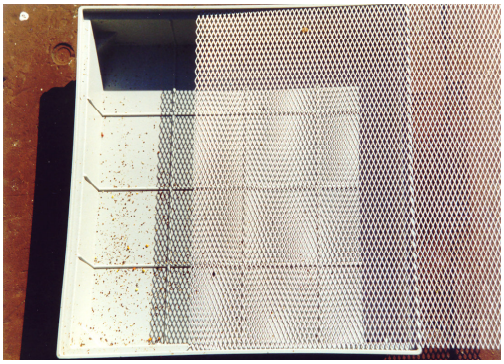
Photo 5. The shuttling device with the plastic jar containing bees in hot detergent water.



3.3.2.3. *Monitoring mite mortality*

Infestation of colonies was monitored two times per week by counting dead mites fallen on bottom boards of screened inserts. The bottom screened inserts consisted of a board with a screen on the top to prevent re-infestation of bees that would enter to the bottom board (Photo 6). The bottom board screened inserts were placed on the bottom of the hive. Dead mites fallen on the bottom board screened inserts were recorded twice per week and the average number of mites per day was calculated.

Photo 6. The bottom board screened insert to record dead mites fallen from the colony.



3.3.3. Statistical procedure

Wilcoxon matched pairs rank test (WMPR) was performed to test differences in the infestation of outflying and returning workers. The test analysed differences in the infestation for pairs of outflying and returning workers per colony sampled once per day to limit differences in the infestation between colonies.

Kruskal Wallis test was performed to test differences in the infestation between colonies for outflying workers, returning workers and to test differences in the daily mite mortality.

3.4. Video recordings of outflying and returning workers

Flight time of infested and uninfested workers was determined by using a video equipment in the summer of 2002 and 2003. Recordings of outflying and returning individual workers were made in an entrance tunnel. About 500 mites were introduced into the nucleus colony during the entire period of observation each year as described in the chapter 3.1.

One day old *Varroa* free workers were marked individually with coloured plates numbered from 1-100 and introduced into the nucleus colony in a cage (see 3.2.). In 2001 I marked both Carnica workers (1-50) and Primorsky workers (51-100), while in 2002 I marked Carnica workers (1-100) only. In total 600 and 800 marked bees were introduced in the years 2001 and 2002, respectively.

3.4.1. Video camera system to record outflying and returning workers

The entrance of the nucleus colony was extended and narrowed into a tunnel made from perspex glass (width: 2cm, depth: 6mm, length: 110mm) to record bees by using 2 video cameras. The tunnel was narrowed in the middle (width: 7mm, depth: 6mm, length: 2cm) to allow only one bee at time to pass through and to prevent the bee from walking on the left and/or right side of the tunnel walls. The passing bee was visible through the tunnel made of glass from both ventral and dorsal sides (Photo 7, Photo 8). This enable recording of the entire bee and determine presence or absence of a mite. The glass on the top of the narrow tunnel part was removable to enable regular cleaning before recording. Regarding that narrowed entrance decrease colony ventilation, the nucleus colony had a large opening at the bottom (2r=6 cm) covered by a net to improve ventilation.

To record every bee leaving and returning to the nucleus colony, one camera was placed under and one above the narrow part of the tunnel to record the ventral and dorsal part of each bee (Photo 7). Four light diodes were used to supply light for the cameras. The focal length of the camera was 7.5 mm in 2001 and 15 mm in 2002 to optimise video recordings. Signals of both cameras were transmitted to a video recorder (Panasonic AG 7355) at the same time by a video splitter to combine both recordings of the ventral and dorsal side of the bee. The bee tag number and mite infestation were determined from video records (Photo 8).

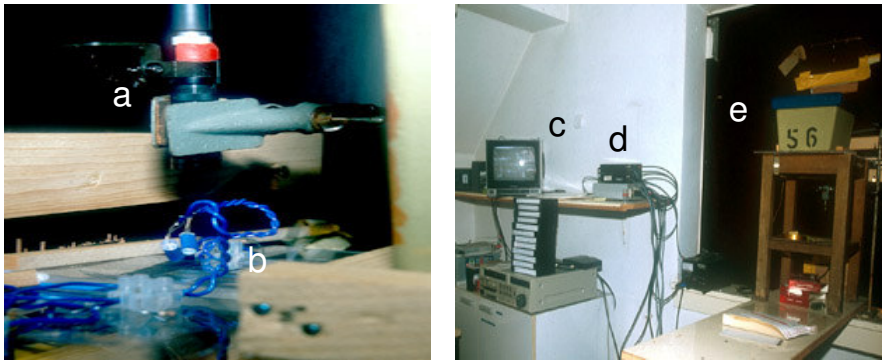


Photo 7. Left: the camera above the flight tunnel (a) with two upper light diodes (b). Right: a setting of the experiment with the monitor (c), video splitter (d) and nucleus colony (e).

Photo 8. The video recording of marked workers infested with *Varroa* mite on the ventral side of the abdomen.

3.4.2. Video data collection

Recordings were made only when weather conditions allowed bees to conduct foraging flights. Temperature and weather conditions were documented for each day I recorded bee flights. Ten video tapes were analysed, including one whole day video recording (8:30-18:40) in 2001 and one whole day video recording in 2002 (8:30-17:30) to determine flight duration, infestation of outflying and returning workers and mite loss and gain.

I observed video recordings in normal speed (25 pictures per s) and slowed down for every marked worker to inspect it frame by frame (Photo 7). The number of workers, the presence or absence of the mites, flight time and age of bees were recorded directly into the computer for every marked worker that left and returned to the colony. When workers returned uninfested and were previously recorded as infested outflying workers, the video recordings were checked once more and vice versa. When outflying workers were recorded as uninfested and returned infested, the video recordings were checked once more frame by frame.

The accuracy of the method was tested in both years 2001 and 2002 before recordings. Infested and uninfested workers were collected and placed in a cage. I removed the colony, took a bee from a cage and checked it for presence of a mite and listed the time number of recording from a videotape and the worker's infestation status. I placed the worker into the tunnel, blocked the tunnel in both ends and started recording. A bee, confined in the tunnel, was searching for the exit. The bee therefore passed the narrow part of the tunnel in both directions which was recorded on the tape. Recordings for each investigated worker were observed afterwards on a monitor frame by frame. I identified workers in the video tape by the time of passing the tunnel and listed the presence of a mite for both directions. The results of this observation were compared with data on infestation of examined bees to determine the accuracy of the method.

3.4.3. Statistical procedure

The flight duration of infested and uninfested workers of the same age that were flying out closest time was compared using Wilcoxon match pairs rank test (WMPR) to ensure similar conditions for bees compared.

Differences in the flight duration between Carnica and Primorsky strains for infested and uninfested workers were analysed using Mann Whitney U test for two independent samples.

Spearman rank correlations were determined for flight duration and age for both infested and uninfested workers. Ranking was performed for the lowest to the highest flight duration and age.

A chi square test was performed to test differences in the infestation between outflying and returning workers, proportion of non returning foragers, mite loss and gain. The test was used to compare Carnica and Primorsky workers in the proportion of mite loss.

3.5. Individual release of workers

The ability of infested and uninfested workers to find home was investigated. Infested and uninfested individually marked workers were released at different distances from the hive. The time that workers needed to return to the colony from different locations was recorded. The distances of release were as follows: 5m, 10m and 50m in 2002 and 20m, 50m and 400m in 2003.The closest locations of 5m, 10m and 20m were measured directly by a meter scale. More distant locations in which the direct line between the colony and a location of release could not be measured, the distance was calculated by triangulation.

The experiment was conducted in three highly infested nucleus colonies between 19.7. and 1.9. in 2002 and in two highly infested colonies between 20.6. and 15.8. in 2003. A colony was infested with *V.*

destructor by introducing mites on emerged bees and by placing mites on bees in the colony (see 3.1.). Marked workers were also artificially infested with mites using a fine brush one day before the experiment. Infested workers were caged overnight and released the next day during the experiment. In total, each nucleus colony received approximately 500 mites in the period from 11.7. until 12.8. in 2002 and from 10.6. until 8.7. in 2003.

To identify released workers, 1900 one day old Carnica and Primorsky workers were marked in 2002 and 1500 one day old Carnica workers were individually marked in 2003. Workers were introduced in the colony in a cage or directly in the colony (see 3.2.). Carnica workers were marked with coloured plates numbered from 1 to 50 and Primorsky workers with plates numbered from 51 to 100. The bees were introduced together in the cage.

3.5.1. Registration of returning bees

The entrance of a nucleus colony was extended into a prolonged landing board to enable identification of returning marked workers. A simple method of observation of released workers to record returning time was used in both years 2002 and 2003. A nucleus colony was placed on a bee stand 1 m high. A long wooden board was placed under the nucleus colony. The board extended in the front of the hive to enabled bees to land before entering the colony. The hive entrance was narrowed with transparent plastic to a 1.5 cm long and 1 cm wide entrance. A piece of transparent plastic was attached with a drawing pin to the front side of the nucleus hive in a such a way to allow closing the nest entrance during the experiment (Photo 9). This enabled recognition of the tag numbers of returned workers when standing beside the nucleus colony.

I improved colony ventilation that was decreased by the narrowed entrance. The plate under the nucleus colony had a large opening covered with a net. The bottom of the nucleus colony had also an opening covered with a net in the same position as the opening in the

plate. This part of the plate bearing the nucleus colony was leaning over the stand which enhanced ventilation of the colony.

The experiment was modified in 2002 to exclude any conceivable influence of an observer standing beside the hive. The hive entrance was extended by a tunnel made from perspex glass which led through a wooden wall (Photo 10). The entrance of the nucleus colony was extended into the tunnel that ended as a new entrance in the white wall (235cm X 178cm). The new entrance of the colony in the wall was marked with a blue square (10cm X 12cm). The tunnel had a movable wooden insert to close the tunnel of the hive during the experiment to ensure recognition of returning marked bees before they enter the hive. Returning bees were observed behind the wall to minimise disturbance of observation.

Photo 9. The nucleus colony to record returning workers and measure their returning time. The narrowed entrance of the nucleus colony was blocked with a piece of a transparent plastic (a) during the experiment.

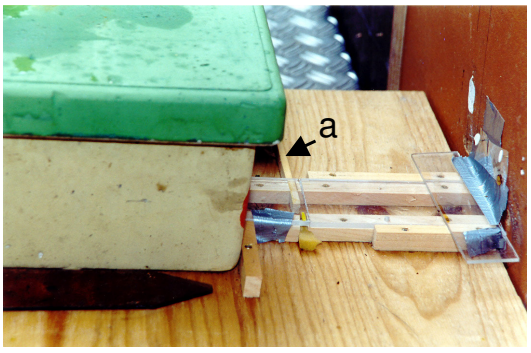


Photo 10. The modified nucleus colony with the new entrance in the wall to observe returning workers and record their returning time beside the wall. The movable wooden stick (a) was inserted to close the tunnel during experiments.

3.5.2. Artificial infestation of marked workers prior to the experiment

Marked workers were artificially infested with *V. destructor* in 2003 to compare returning time of workers infested over a definite time of 20-24h. The same number of infested and uninfested workers of the same age was collected separately into 2 wooden cages. Workers from one cage were infested with the mites which had been collected previously from infested brood. A worker was taken with a forceps from the cage and the mite was placed on the thorax or abdomen of the worker with a fine brush. I observed the mite on the bee for a few seconds to ensure that it stayed firm on the bee. The infested worker was then transferred to another cage. Infested and uninfested workers were stored overnight separately in two different cages with sugar supply (Photo 11). The next day, before the experiment, infested workers were checked again for mites. Workers that lost mites during time of confinement in the cage were excluded from the experiment. Infested and uninfested workers were then released from three different locations to record returning time.

Photo 11. Marked workers of the same age (uninfested) collected from the nucleus colony to be caged overnight.

3.5.3. Conducting the experiment

Infested and uninfested workers were released from different distances from the hive in order to compare their returning time to the colony. The average age of released workers was 22 ± 6 days for both years. Later in the season, in August, during a lack of older bees, I occasionally used bees younger than 14 days with the minimum age of 10 days in 2002 and 11days in 2003.

In 2002, a total 107 infested and 299 uninfested Carnica and Primorski workers were released from nucleus colonies from the following distances: 5m, 10m, and 50m according to their age. Younger bees which had started to forage were released only from the shortest distance of 5m to ensure that they return home in the observation period of 15 min. Bees aged about one month and more were released from the longest distance of 50m. Prior to the experiment, marked workers of foraging age were checked for mites (Photo 12). For each infested worker I collected two or three uninfested workers of the same age and of the same colony as a control. Each marked bee was placed in a small vial with sugar candy attached inside the plastic lid that had a small opening for respiration (Photo 13). For each experiment I took about 6 infested and 6 uninfested marked workers which were placed in the vials separately. The vials containing workers were kept in a box, covered with a cloth to keep bees warm on cool days. Bees were kept in the shadow on hot summer days. Individual bees were kept in the vials for a maximum of half an hour before release. Workers were released individually. I used about 25 sec to reach the colony from the longest distance of 50 m. Returning time of workers was recorded.

During the experiment the entrance of the nucleus colony was covered with a transparent plastic (Photo 9). Such an obstruction caused sufficient delay of the returning bees at the entrance to enable recording the numbers on workers, but did not cause a jam and so disturb foraging. Before I released an individually marked worker from some distance I completely blocked the entrance. I released the bee, ran to the colony and partly opened the entrance to enable bees to return to the colony. I recorded the time of landing of an individual bee by standing beside the colony. The maximum observation time per bee was 15 min in 2002.

The experiment was modified in 2002. The nucleus colony had an extended entrance in the wall (see 3.5.1., Photo 10) to observe bees in a tunnel behind the wall. I closed the tunnel in the middle, released the bee, ran to the colony and partly opened the block of the tunnel to enable bees to return to the colony.

In 2003, a total of 123 infested and 160 uninfested marked workers were released from the distances of 20m, 50m, and 400m. Marked workers were released from all three locations in one experiment. To allow more time for bees to return from the longest distances of release, the maximum observation period per bee was extended to half an hour. During the experiment, the entrance of the nucleus colony was also partly blocked with the transparent plastic to recognize landing marked workers. The experiment included two persons, one was sitting beside the nucleus colony and observed landing bees, the other released bees from all three locations. Both had a stop watch which was triggered at the same time. The time of release of individual bees and the landing time of workers in front of the nucleus entrance was recorded. From the difference between releasing and arrival time, the actual returning time was calculated.

The same number of infested and uninfested workers of the same age was collected from the nucleus colonies for all three locations. I recorded the position of the mite on a bee, the number of a bee, and distance of release. Workers were placed in the vials with sugar candies attached inside the plastic lid. The time workers spent kept in the vials depended on the walking distance to the location of release. Correspondingly, workers released at the longest distance (400m) spent the longest time in the glass containers (approximately 10 min walking).

Photo 12. The marked infested bee on the comb

Photo 13: Marked workers in vials to be released from different locations. The plastic lids have the openings for respiration and attached sugar candies.

3.5.4. Statistical procedure

Returning time of workers released from different distances of different strains was analysed using Wilcoxon matched pairs rank test (WMPR).

Mann Whitney U test for two independent samples was used to determine whether returning time differed between two groups.

To determine whether returning time differ between three locations, I preformed a test for several independent samples (Kruskal Wallis test).

An univarate analysis of variance was performed to analyse the influence of age on returning time of workers as a dependent variable according to locations. The location was used as a fixed factor and the age as a covariance. The test was performed for infested and uninfested workers separately.

A chi square test was used to analyse differences in the position of the mite on workers.

3.6. Returning of workers in a whole day

The success of infested and uninfested workers to return to the original nucleus colony was investigated in the year 2003. The experiment included marked workers from two colonies, specifically 283 workers that were released and measured for returning time from three locations (20m, 50m, 400m, see 3.5.) and additional 35 marked workers

released from the longest distance of 400m. I checked colonies in the evening for the presence of 5-10 marked workers that had not returned within the observation period of half an hour and/or for returning of additionally released workers. Colonies were checked for the presence of workers twice in 20 min. The number of infested and uninfested workers that returned during the whole day was recorded.

The accuracy of finding marked individual workers in the evening was tested by choosing workers from numerous young marked workers (few days old). I marked about 20-30 chosen workers once more with a coloured pencil. I listed chosen workers and checked twice whether they could be re-sampled after half an hour. The numbers of recognized workers and the number of workers, which I could not find, was recorded.

2.6.1. Statistical procedure

A chi square test was performed to test differences in the proportion of infested workers that returned and did not return in the observation period of 15 min and in a whole day. The differences in returning between infested and uninfested workers were analysed for following categories: locations, year of the experiment, source of infestation (natural and artificial) and time of observation (observation period of 30 min and whole day). The proportion of outflying infested workers was compared to the proportion of returning infested workers.

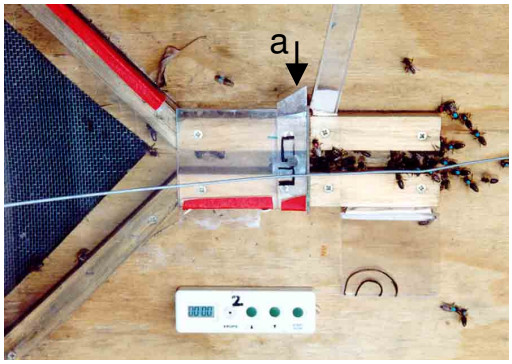
3.7. Group release of bees

The experiment was conducted in two highly infested colonies from 29.8.-8.9. in the year 2002. In total 5429 one day old coloured marked workers were introduced in two highly infested colonies. I tested whether infested workers returned faster and therefore infestation of returning workers changes over time. I released marked workers of the same age from three different locations. The number of infested and uninfested workers that returned in the time interval of 1 min over the entire observation period of 15 min was recorded.

3.7.1. Modification of hive entrance to record workers

The hive entrance was modified in a way to lead bees to a tunnel (Photo 14). The whole construction was situated on a wooden plate (55cm long) and installed at the hive entrance. The entire entrance was narrowed into a shape of a funnel leading to a tunnel. The construction included two wooden sides for the funnel and two wooden sides for the tunnel attached to the plate. The funnel construction was covered with a net for ventilation and the tunnel construction with perspex glass. To prevent bees to enter or leave the colony during the experiment, the tunnel had a removable plastic block inserted in the middle. The perspex glass of the tunnel after the block was removable to enable collecting bees that had entered the tunnel.

Photo 14. The modifications of the hive entrance to collect returned workers released in a group of 30. The perspex glass of the tunnel in front of the block (a) was removed to collect workers during the experiment.



3.7.2. Conducting the experiment

Thirty infested and uninfested marked workers of the same age (marked with the same colour) were collected from the colony into a cage and released from the distances of 4m, 16.5m and 28 m from the hive. On average 8 of 30 workers were infested. The number of collected infested workers was recorded before release. Workers were released in the morning (8:00- 10:30h) to avoid marked workers of the same age, bearing the same colour plate, mixing on their return from foraging and therefore affect the experiment. In total 660 bees were released in 22 groups from different distances.

The tunnel had been blocked before I released workers from the cage at some distance. Workers were landing on the wooden plate and in the tunnel in front of the block. Returned bees were checked for mites. To catch returning marked bees in the tunnel, I removed the perspex glass of the tunnel in front of the block. Every inspected worker was returned to the cage to avoid double counting. The number of returning infested and uninfested workers was recorded over the time period of 1 minute. The number of returned workers was summed in the observational intervals of 5 min over a total period of 15 minutes.

3.7.3. Statistical procedure

The proportions of returned infested and uninfested workers in the intervals of 5 min over a 15 min observational period was compared using a chi square test. Returning of infested and uninfested workers was compared between locations for each observational interval of 5 min. A chi square test was performed also to analyze the proportion of infested workers that did and not return in the total observation period of 15 min.

3.8. Orientation toward the nest entrance

The orientation of infested and uninfested workers toward the nest entrance was tested from 3.9. to 12.9. 2002 and from 8.8.-16.8. 2003 in a modified nucleus colony. The colony was infested by introducing mites (see 3.1.). I collected the same number of marked bees of the same age in two cages. I infested workers in one cage and left the workers in another cage uninfested. Workers were caged overnight and used in the experiment the next day.

To identify workers, one day old *Varroa* free workers were individually marked and introduced into a highly infested nucleus colony in a cage in 2002 or directly to the colony in 2003 (see 3.1. and 3.2.).

3.8.1. Design of the experiment

The infested nucleus colony had an extended hive entrance into a tunnel that opened to a white wall (Photo 15 left). The new entrance in the wall was encircled (2r=16.5cm) and marked with a blue square (10cm X 12cm, Photo 15 right). On the left and right side of the nest entrance in the wall were 2 circles drawn in the same dimension as the circle of the nest entrance. The distance from the circle of the nest entrance and circles on both sides was 4 cm. I presented a dummy to released workers during the experiment. The dummy was a blue square of the same size as the blue square of the nest entrance and attached in the circles on the left or the right side (Photo 15, right).

Photo 15. Left: the nucleus colony with the tunnel opened to the wall as an entrance. Right: the nest entrance (a), the dummy entrance (b) and the empty circle (c) marking on the wall.

3.8.1. Conducting the experiment

Infested and uninfested workers aged at least two weeks and more were individually collected and placed in vials with sugar candies attached to plastic covers (see 2.5.3., Photo 13). Four to ten workers were collected at the same time. Half of the collected workers was

infested and half uninfested. Workers were released individually from the distance of 4 m. Each single bee was kept in the vial for approximately 15 minutes. The number of each bee was recorded before release and also the position of the mite on workers was additionally recorded in the year 2003. In total 118 workers were released in the year 2002 and 335 in the year 2003. A release of a marked infested worker was followed by a release of a *Varroa* free worker of the same age or vice versa. I was sitting in the fixed position of 1.3m in the front of the centre of the blue square of the nest entrance. I observed flights of returning workers (Photo 16). Workers that entered the nest entrance directly got a score for direct return. Workers that searched for the nest entrance and crossed the dummy or the empty circle got a score for the dummy or empty circle respectively. When a bee crossed the dummy or the empty circle again, it received an additional score. The maximum observation time per bee was 15 minutes.

Photo 16. Observation of flight of workers toward the nest entrance, dummy and empty circle

3.8.3. Statistical procedure

Orientation toward the nest entrance of infested and uninfested workers was analysed using a chi square test. The test was performed to analyse differences in the proportion of infested workers for those that returned: a) directly to the nest entrance b) crossed the dummy or c) empty circle before finding the nest entrance. Differences in the proportion of infested workers that returned directly or not were compared for both years and for both dummy positions on the left and right side. The proportion of workers that did not return in the observation period was compared for infested and uninfested workers.

3.9. Daily loss of foragers and forager infestation in colonies infested by *V. destructor*

The number of outflying and returning foragers was monitored using an electronic bee counter in the summer 2002 and 2003. Colony infestation was monitored by sampling outflying bees and recording mite mortality. The infestation of outflying workers was recorded over time by sampling outflying workers that were checked for mites. Mite mortality was measured as the number of dead mites fallen on bottom screened inserts per day (see 3.3.2.3., Photo 6). The bottom board inserts were changed two times per week in 2002 and once per week in 2003.

3.9.1. Electronic bee counter

The bee counter (Beescan, Lowland Electronics bvba, Photo 17) is a scanner counting outflying and returning bees separately. Energy supply was provided by a 12V battery. The bee counter consisted of a counter unit attached to the hive entrance which has 32 direction sensitive channels to record the number of leaving and returning bees. Only one bee could pass the sensitive channel at any time. Data were recorded in 15 minute intervals and summarised every day. Data from the bee counter were collected directly by connecting a portable computer to the counter in the field. From the difference in the number of outflying and returning bees, the daily loss of bees was calculated.

One bee counter was installed at the hive entrance to an infested colony from 10.8.-20.10. in 2002 . Two bee counters were in use from 15.7.2003 until lightening destroyed both (4.8. 2003). One bee counter was installed to one highly infested colony and one to a low infested colony (Photo 18) at the same time to compare losses of bees between both colonies. The colonies differed in colour of the entrance to decrease drifting between them. The bee counter was covered with the same coloured wooden plate as the original entrance to help bees to recognize their own colony.

The bee counters were regularly cleaned with alcohol and sensitive channels were checked with a plastic bee dummy in both directions for detection. One channel in one bee counter did not record in both directions. I blocked this channel with a wooden stick to prevent bees from passing through. Data was taken only on days without manipulations of the colony that would affect counting of the electronic device. Days on which I inspected the colony, cleaned the bee counter, introduced infested brood, took samples of outflying bees and changed the bottom boards were excluded from the experiment.

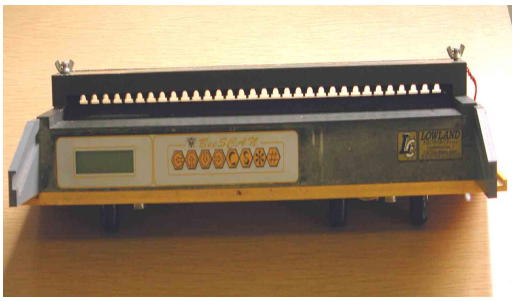


Photo 17. The bee counter with direction sensitive channels

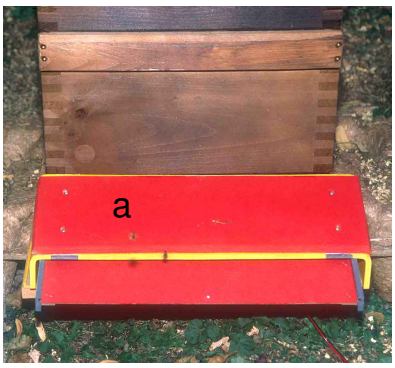


Photo 18. Bee counter installed at the bee colony. The bee counter was covered with the wooden plate (a) of the same colour as the hive entrance before.

3.9.2. Sampling outflying and returning bees

Samples of outflying bees from colonies with installed bee counters were taken to determine bee infestation. Samples were taken every third day if weather conditions were suitable for bees to fly out. The size of the sample varied from 100 to 200 bees. If samples of bees were small, I sampled twice per day to obtain a sufficient number of bees. To collect outflying bees, a special device was built and attached to the hive. The bee collector consisted of a wooden box with a wide opening at the top and a plastic container with an attached funnel that extended its

narrow part inside the container (Photo 19, left). I covered the box opening with the transparent plastic container (Photo 19, right) in a reverse position during sampling. Bees were flying from the box to the funnel into the plastic container. Samples of bees were frozen and washed for fallen mites as described in the chapter 3.3.2.2.

Photo 19. Left: the bee collector to sample outflying workers. The bee collector consisted of a wooden box with a wide opening at the top (a) and a plastic container (b) upside down to catch bees. Right: the plastic container to sample outflying workers. The plastic container had attached a funnel that extended its narrow part inside the container.

3.9.3. Statistical procedure

Pearson correlations were determined for a loss of bees, the infestation of outflying workers and mite mortality (fallen dead mites) over time. The relationship between the proportion of bee loss and the infestation of outflying workers sampled a day before or after the counts on foragers loss was examined using Spearman's rank correlation. The correlations between the bee loss and daily mite mortality and between the infestation of outflying workers and daily mite mortality were also determined.

Mann Whitney U test was performed to compare daily mite mortality, the infestation of outflying workers, and loss of workers between a low infested and high infested colony.

3.10. Drifting

The proportion of infested and uninfested workers that entered the same and different coloured hives as original ones was compared.

3.10.1. Observation of drifting in individual workers

The experiment included marked infested and uninfested workers that were released from the nucleus colony during day to record returning time and did not return in the observation period of 30 min and also additionally 35 workers released during day (see 3.5.). Workers were checked for drifting in a neighbouring nucleus colony in the evening. The number of infested and uninfested workers that drifted in the colony neighbour was recorded.

3.10.2. A choice test for nest recognition

An indirect experiment was set up to investigate whether infested bees drift more to another colony in the last week of August 2003. The experiment was conducted in two highly infested colonies consisting of 4 frames.

Colonies were replaced with an empty hive of same or different colour than of the original hive during the experiment The original colony was replaced for 10 minutes in 6 experiments and for 1.5 minutes in one experiment. Bees were searching to find their nest colony therefore some entered the empty hive. Three and 7 samples of workers that entered empty hives were taken in the experiments when colonies were replaced for 10 minutes and when colonies were replaced for 1.5 min respectively. Before sampling, the colony was closed and lifted in a vertical position. A plastic bag was attached to the whole hive entrance which was opened to enable bees to fly out from the empty hive. To reinforce bees to fly out, the hive was shaken.

Samples of bees were frozen. Samples per experiments were then joined and washed for fallen mites as described in the chapter 3.3.2.2. The infestation per sample was calculated. Infestation in samples of bees entering the hive of same colour was compared to infestation of workers which had entered the hive of different colour as original hive.

3.10.3. Statistical procedure

Occurrence of drifting and a choice test for nest recognition was analysed using a chi square test. The proportion of infested and uninfested workers that entered the same and different coloured hives as original ones was compared.

4. Results

4.1. Infestation of outflying and returning workers

Infestations of bees flying out and back to the colony were determined in 5 colonies by analysing paired samples of outflying and returning bees (N=54). Samples were taken in the period from 8.8. to 31.8. 2001. The samples contained 103±41.6 outflying workers and 99±56.7 returning workers with a range from 31 to 204 and a range from 19 to 266 outflying and returning workers respectively.

Varroa counts in samples varied from no mite to 10 mites. The samples of outflying workers had on an average 1.9±1.96 mites and the samples of returning workers had 0.8±1.29 mites. The infestation of outflying workers was higher than the infestation of returning workers for all colonies except one (Figure 2). In total, pooled samples of outflying workers (N=5553) contained 102 mites and pooled samples of returning workers (N=5326) contained 44 mites. The mean infestation of outflying workers (0.019±0.018) was about twice as high as the mean infestation of returning workers (0.009±0.018). The differences in the infestation between outflying and returning workers for paired samples was highly significant (Wilcoxon test, $P<0.001$, Figure 1). A correlation between the infestation of outflying workers and the infestation of returning workers was positive and close to significance (Pearson correlation, $r=0.256$, $P<0.061$).

I tested differences in the infestation of outflying and returning workers among five colonies. The infestation of outflying workers differed significantly (Kruskal Wallis test, $P<0.003$), but no significant differences were found in the infestation of returning workers among colonies (Figure 2). Accordingly, differences in the infestation of outflying and returning workers between colonies were also significant (Kruskal Wallis test, $P<0.05$, Figure 3).

The number of dead mites fallen on the bottom board inserts (mite mortality) was monitored for each colony during the entire time of the experiment and differed significantly among colonies (Kruskal Wallis test, $P<0.001$). The number of dead mites varied from few to about 65 mites per day (Figure 4). Mite mortality monitored every third day was correlated with the infestation of outflying or returning workers sampled on the same day. Neither the infestation of outflying bees nor the infestation of returning bees was not influenced by colony infestation as measured by a daily mite mortality.

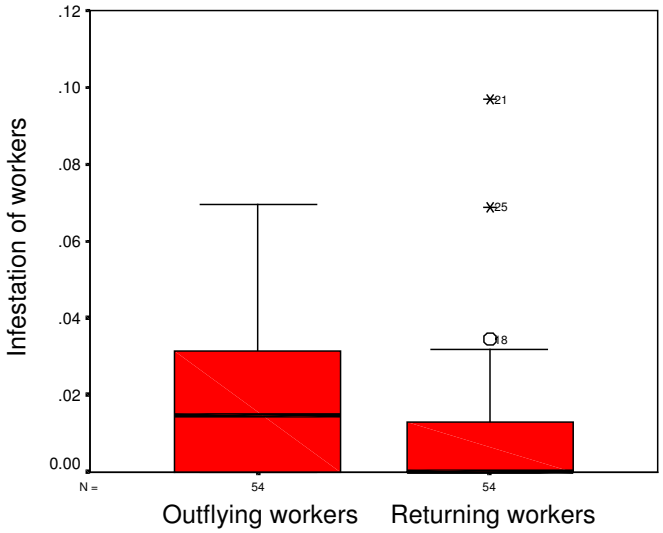


Figure 1. The infestation of outflying and returning workers sampled from 5 colonies. The differences in the infestation between outflying and returning workers for paired samples was highly significant (Wilcoxon test, $P<0.001$). The number of workers in 54 samples of outflying workers: 5553. The number of workers in 54 samples of returning workers: 5326. The chart indicates medians inter quartile ranges, outliers and extreme values (see Appendix 1).

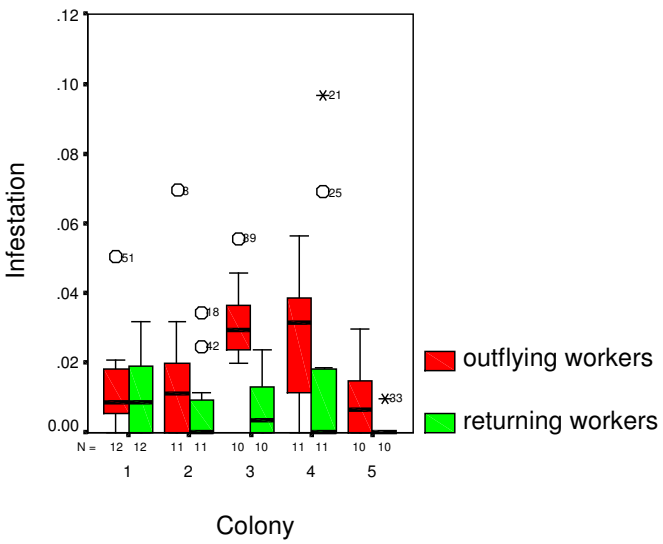


Figure 2. The infestation of outflying and returning workers sampled in five colonies. The infestation of outflying workers differed significantly among colonies (Kruskal Wallis test, P<0.003). The number of workers in 54 samples of outflying workers: 5553. The number of workers in 54 samples of returning workers: 5326. The chart indicates medians, inter quartile ranges, outliers and extreme values.

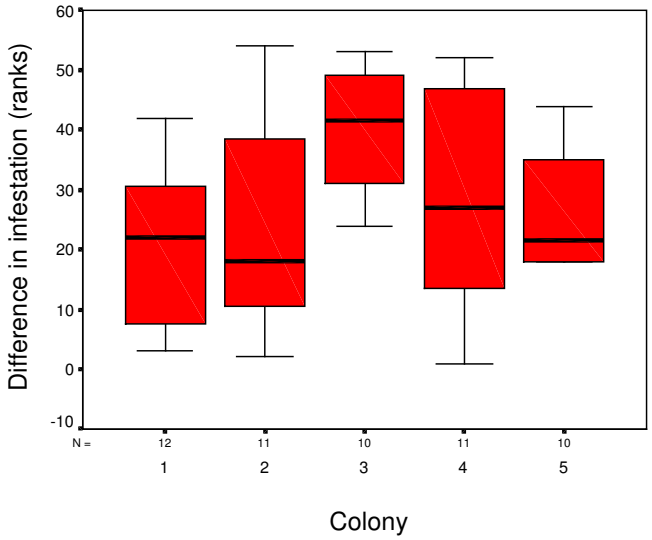


Figure 3. The ranked difference of infestation between outflying and returning workers in five colonies. Colony 3. differed significantly in the differences of the infestation of outflying and returning workers from all other colonies (Kruskal Wallis test, P<0.05). The number of paired samples for comparison: 54. The chart indicates medians and inter quartile ranges.

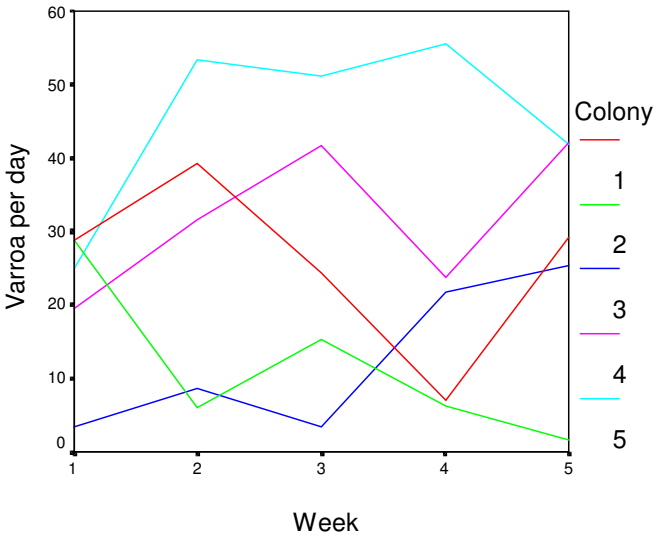


Figure 4. The number of dead mites per day (daily mite mortality) in week intervals during one month period for five colonies (13.8.-13.9. 2001). The number of dead mites differed significantly among colonies (Kruskal Wallis test, $P<0.001$).

4.2. Video recordings of outflying and returning workers

4.2.1. Testing the accuracy of a method

A test to establish the accuracy of the video method to detect the mites on workers was made prior to recordings of outflying and returning workers for both settings of the experiments in the years 2001 and 2002. In total, 200 bees were placed in a tunnel to walk through in both directions. The accuracy of detection of mites on bees was tested for both directions (outflying and returning). In 2001, I examined 37 uninfested and 63 infested workers. For both directions detecting the mite failed in two occasions (3% error). In 2002, 50 infested and 50 uninfested workers were tested for detection of the mite in both directions of the tunnel. The mite detection failed in two occasions (4% error). In one case I could not detect the mite on the bee when it walked in the direction of returning workers and in another case I could not detect the mite on the bee for both directions. There were no erroneously detections of mites on uninfested workers in both years.

4.2.2. Flight duration of workers

The flight duration was recorded for each of the 748 marked outflying workers which returned to the colony. The flight times of infested workers were compared to the flight times of uninfested workers of the same age that were flying out closest in time. In total, 127 pairs of infested and uninfested workers were compared (72 pairs in 2001 and 55 pairs in 2002). The median duration of infested workers (214s) was 1.7 times higher than the median duration of uninfested workers (128 s). The difference between the duration of infested and uninfested workers for the compared pairs was highly significant (WMPR test, $P<0.0005$, Figure 5).

In 2002, half of the marked workers introduced into the nucleus colony were of Primorsky origin, and a half of marked workers were of Carnica origin. The flight duration of Carnica and Primorsky workers was compared. No significant differences in the median flight duration were found in infested and uninfested groups of workers from the two origins (Figure 6).

The average age of the examined workers was 21 ± 10. The age of recorded workers differed from the minimum of 5 days in 2001 and the maximum of 44 days in 2002. Duration of flights showed age dependency for both years. Older workers had longer flights than younger ones. The difference in the flight duration was significant for both infested workers (Spearman´s rank correlation, $r=0.301$, $P<0.01$, $N=117$) and uninfested workers (Spearman´s rank correlation, $r=0.335$, $P<0.01$, $N=631$).

The flight duration of infested and uninfested workers was consistent for both years 2001 and 2002; there were no significant differences in flight duration between the years (Table 1).

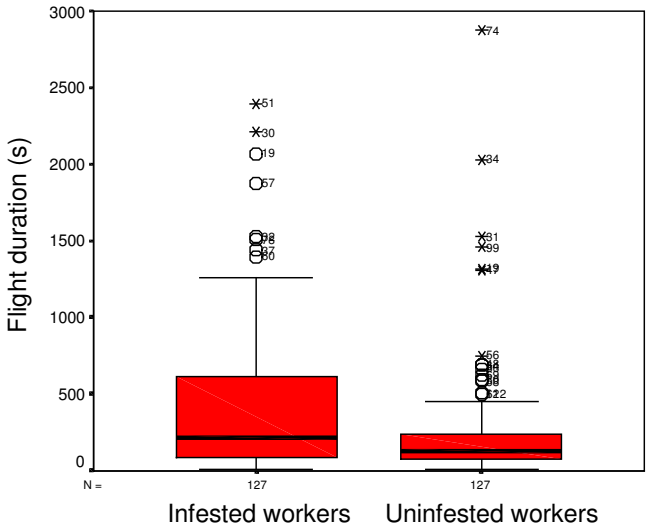


Figure 5. The flight duration of outflying and returning workers for the compared 127 pairs of infested and uninfested workers of the same age that flew closest in time, recorded in both years 2001 and 2002. The difference in duration of infested and uninfested workers was highly significant (P<0.0005, WMPR test). The chart indicates medians, inter quartile ranges, outliers and extreme values.

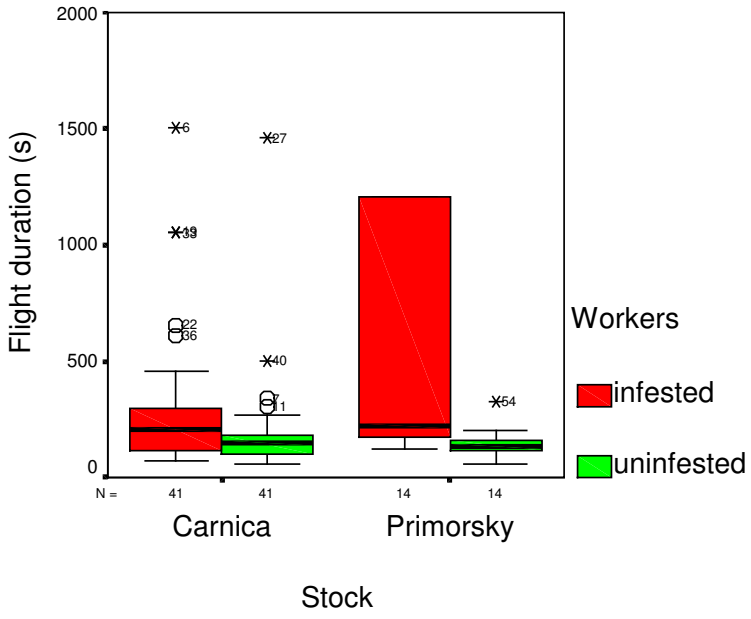


Figure 6. The flight duration for the compared pairs of Primorski (n=14) and Carnica (n=41) infested and uninfested workers of the same age that flew closest in time. The chart indicates medians, inter quartile ranges, outliers and extreme values.

Year	Workers	N	Median (s)	Min. (s)	Max. (s)	Significant differ. between infest. and uninfest. bees
2001	Infested C	72	205.5	6	7979	P<0.0005, WMPR test
	Uninfested C	72	100.5	7	2032	
2002	Infested C	41	205	72	3653	P<0.012, WMPR test
	Uninfested C	41	148	56	5397	
	Infested P	14	219	126	3673	P<0.002, WMPR test
	Uninfested P	14	131	56	330	

Table 1. The number of workers, median, minimum and maximum flight duration of Carnica (C) and Primorsky workers (P) in the years 2001 and 2002. Data included selected pairs of infested and uninfested workers of the same age that flew closest at time. The number of worker pairs in 2001: 72. The number of worker pairs in 2002: 55.

4.2.3. Infestation of outflying and returning workers

In total, 914 video recordings of marked outflying workers were analysed. From these, 179 workers were infested and 735 were uninfested. The infestation of outflying workers (19.6%) was significantly higher than the infestation of returning workers (15.7 %, 117 infested from 748 workers, Chi^2 test, $P<0.038$).

The comparison of the infestation of Carnica and Primorsky workers in the video recordings made in 2002 showed that Primorsky workers leaving the colony were significantly less infested than Carnica workers leaving the colony (Chi^2 test, $P<0.011$, Figure 7). Accordingly, Primorsky workers returning to the colony were also significantly less infested than Carnica returning workers (Chi^2 test, $P<0.001$, Figure 7).

No significant differences in the infestation of outflying and returning workers were found between years.

Fewer mites returned to the colony in comparison with outflying bees. Mites were lost through two processes: a) non returning of infested foragers and b) mite loss from foragers. Small portion of mites was also gained by bees that left the colony uninfested.

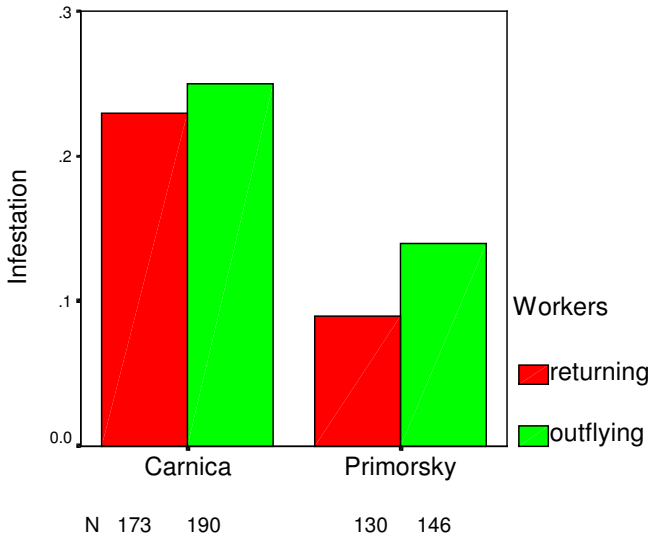


Figure 7. The infestation of outflying and returning Carnica and Primorski workers. The infestation of outflying and returning Pimorsky workers was significantly lower in the comparison with the infestation of Carnica workers (outflying: Chi^2 test, $P<0.011$, returning: Chi^2 test, $P<0.001$). The number of outflying and returning workers (N) is indicated below the figure.

4.2.4. Mite loss by non returning of infested foragers

Infested workers did not return in 21.7% (39 of 179) occasions. Specifically, significantly more infested (30.2%, 33 from 109) than uninfested workers (21.4%, 100 from 467) did not return in 2001 (Chi^2 test, $P<0.048$). Contrary, no significant differences in returning between infested and uninfested workers were observed in the year 2002. The loss of bees in this year was significantly lower than in 2001 for both infested (Chi^2 test, $P <0.001$) and uninfested workers (Chi^2 test, $P <0.001$, Table 2).

When Primorsky and Carnica were compared, more, but not significantly more, infested Primorsky workers did not return in whole day than Carnica workers (Table 2).

Year	Infested (N)				Uninfested (N)			
	Outflying	Return		Not return	Outflying	Return		Not return
		with mite	without mite			without mite	with mite	
2001	109	63	13	33	469	367	2	100
%		*57.8*	*11.9*	*30.3*		*78.3*	*0.43*	*21.3*
2002 C	49	33	14	2	141	120	7	14
%		*67.3*	*28.6*	*4.1*		*85*	*4.9*	*9.9*
2002 P	21	8	9	4	125	110	4	11
%		*38.1*	42.9	19.0		*88.0*	3.2	*8.8*

Table 2. The number of outflying, returning and not returning infested and uninfested workers. The percentage for returning and not returning workers was calculated according to the number of outflying workers. Data for Carnica (C) and Primorski workers (P) used in 2002 are presented separately.

4.2.5. Loss of mites from infested foragers

The additional factor for mite loss was contributed by infested workers which lost mites outside the colony and returned to the colony uninfested (20.1%, 36 of 179). Significantly more mites were lost due to mite disposal in the year 2002 compared to the year 2001 (Chi^2 test, $P<0.001$). More, but not significantly more, infested Primorsky workers lost mites than Carnica workers (Table 2).

4.2.6. Mite gain by uninfested foragers

Only a small portion of mites were gained (1.8%, 13 of 735). Compared to a total mite loss of 42% in the outflying infested workers, mites were lost significantly more often than gained (Chi^2 test, $P<0.0005$). In the contrast to loss, significantly fewer mites (0.4%) were gained in 2001 compared to 2002 (4.2%, Chi^2 test, $P<0.0005$).

4.2.7. Total mite gain and loss

Proportionally more bees that had been infested when they left the colony did not return, or returned without a mite, (62 from 179, 34.6%)

than bees that had no mites or gain mites (104 from 735, 14.1 %, Chi^2 test, P<0.0005, Figure 8, Appendix 2).

Loss of mites on leaving infested workers was more pronounced in Primorsky bees. Workers originated from Primorsky colonies lost almost two times as many mites (61.9%, 13 from 21) as Carnica workers (32.6%, 16 from 49) by non returning and mite loss outside the hive (Chi^2 test, P<0.023, Figure 9). When the mite gain was subtracted from the mite loss there were still significant differences between Carnica and Primorsky workers in mite loss (Chi^2 test, P<0.032).

According to that more mites were lost by mite disposal (see 4.2.5.) and fewer by non returning workers (see 4.2.4., Table 2) in the year 2002 compared to the year 2001, the total mite loss was 42.2% in 2001 and 41.4% in 2002 and did not differ significantly between years.

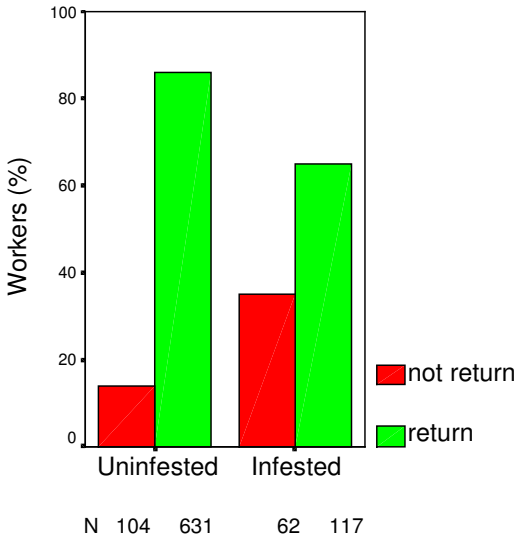


Figure 8. The percentage of returned and not returned workers that had left the colony either infested either uninfested. Proportionally more bees which had been infested when they left the colony did not return or lost mites than bees that had no mites or gained mites (Chi^2 test, P<0.0005). The number of workers (N) is indicated below the figure.

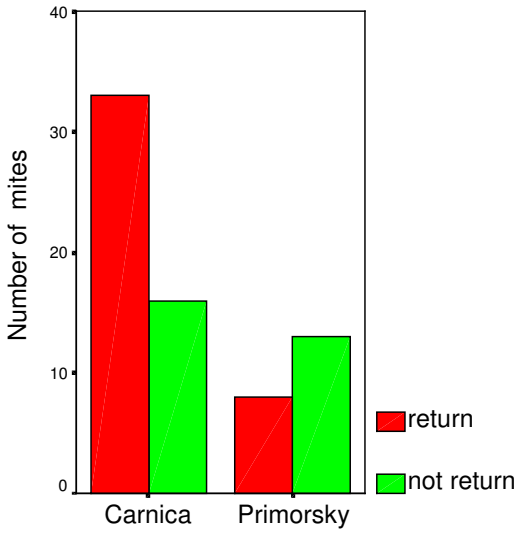


Figure 9. Returning of mites in Carnica and Primorsky workers. Primorsky lost almost two times more mites (61.9 %,) than Carnica (32.6 %) by non returning infested workers and mite loss outside the hive (Chi^2 test, P <0.023).

4.3. Individual release of workers

4.3.1. Returning time of workers

Infested (244) and uninfested (480) individually marked workers were released from different distances from the hive (5m, 10m, 50m in 2002 and 20m, 50m and 400m in 2002). Differences in the median returning time between the infested and uninfested workers were compared for pairs of workers of the same age and the same genetic background that were released from the same distance closest at time. In total 130 pairs of workers that returned in the observation period were analysed. Infested workers returned to the colony 2.3 times later (122s) than uninfested workers (52s, WMPR test, P<0.0005, Figure 10). The difference in returning time between infested and uninfested workers was consistent for both years in 2002 (Mann Whitney U test, P<0.0005) and 2003 (Mann Whitney U test, P<0.016).

The distance also influenced the returning time. A comparison of the returning time of workers in the observation frame observed for 15 min between all locations showed that bees released from more distant

locations need more time to return (Figure 11). The difference was highly significant for the both infested (Kruskal Wallis test, $P<0.0005$) and unifested group of workers (Kruskal Wallis test, $P<0.0005$). The median returning time of infested (387s) and uninfested workers (253 s) released from the larger distances (20m-400m) in 2003 was significantly higher than the median returning time of infested (91.5s) and uninfested (32s) workers released from the shorter distances (5m-50m) in the 2002 (uninfested workers: Kruskal Wallis test, $P<0.0005$, infested workers: Kruskal Wallis test, $P<0.0005$). There was no significant difference in the returning time between locations of closer distances (5m-50m) in 2001 whereas there was a significant difference in the returning time between locations of larger distances (20m-400m) in 2003 (infested workers: Kruskal Wallis test, $P<0.0005$, uninfested workers: Kruskal Wallis test, $P<0.0005$). Workers released from the largest distance of 400m took more time to return to the colony than workers released from the distance of 20m and 50m.

The influence of age on returning time according to locations was found only in the infested group of workers (ANOVA, $P<0.0005$) indicating that older infested workers returned faster than younger.

When infested Primorsky and Carnica workers were compared, the median returning time did not differ. Similarly, returning time did not differ between uninfested Carnica and Primorsky workers.

Two experimental designs to measure returning time of Carnica and Primorsky workers were used in 2002. In one I identified and measured the returning time of single bees by standing besides the nucleus colony. In the second one, I used the modified nucleus colony and observed bees behind the wall. No difference in the returning time for both infested and uninfested group was observed between the experimental sets.

In 2003, some of the infested workers were artificially infested one day prior to the experiment. From 317 workers released, 98 were artificially infested and 39 were naturally infested in the colony. Returning

time of artificially infested workers and returning time of naturally infested workers in the colonies was significantly higher compared to the controls (artificially infested workers: Mann Whitney U test, P<0.032, naturally infested workers: Mann Whitney U test, P<0.049). The median returning time of artificially infested workers was higher than the median returning time of naturally infested workers, but the difference was not significant. Similarly, one day caged uninfested workers used as a control group to the artificially infested workers, had higher returning time than the uninfested workers taken directly from the colony prior to release. The difference was not significant for each location (Table 3).

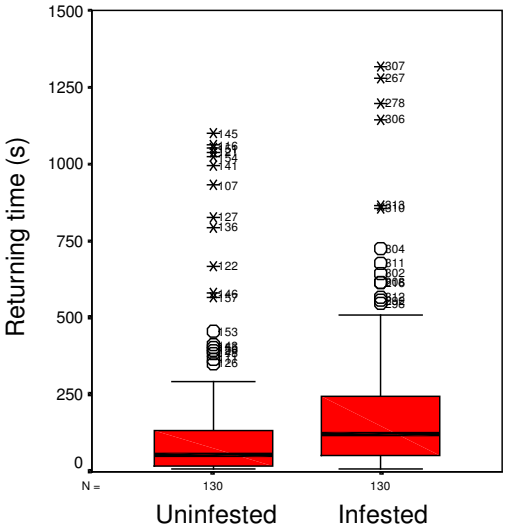


Figure 10. The returning time of workers released from different distances in 2002 and 2003 for the compared 130 pairs. The infested workers returned to the colony significantly later than the uninifested workers (WMPR test, P<0.0005). The chart indicates medians, inter quartile ranges, outliers and extreme values.

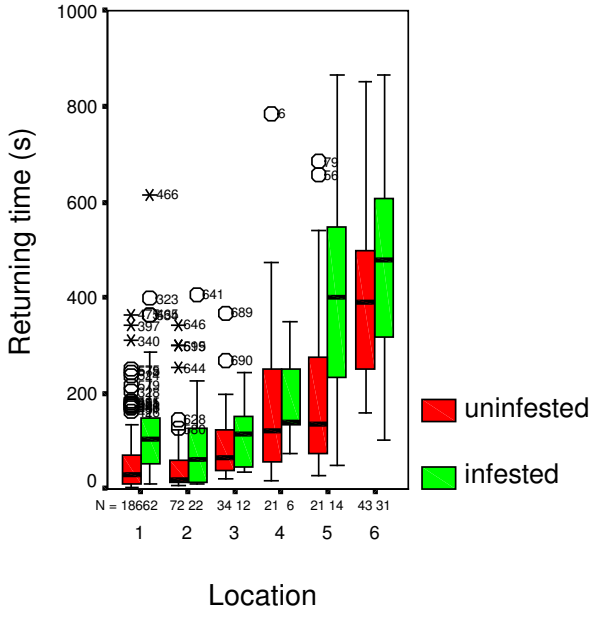


Figure 11. The returning time according to locations (distance) in the years 2001 and 2002 in the observation period of 15 min. Locations in 2001: 1 (5m), location 2 (10m), location 3 (50m). Locations in 2002: location 4 (20m), location 5 (50m) and location 6 (400m). The difference was highly significant for both infested (Kruskal Wallis test, P<0.0005) and uninfested group of workers (Kruskal Wallis test, P<0.0005). The chart indicates medians, inter quartile ranges, outliers and extreme values. The number of workers (N) is indicated below the figure.

	Locations	1 (20m)		2 (50m)		3 (400m)	
Workers		Time (s)	N	Time (s)	N	Time (s)	N
Infested	artificial	134	1	505.5	8	512	33
Uninfested	control	310.5	10	224	8	408	36
Infested	natural	143	5	234	4	300	4
Uninfested	control	69	15	87	13	463	13

Table 3. The median returning time of infested (natural and artificial) and uninfested workers used as a control to the artificially and naturally infested workers. Uninfested workers used as a control to the artificially infested workers were caged one day before the release.

4.3.2. Returning of workers in the observation period of 15 min

I compared the numbers of infested and uninfested workers released from different distances that returned in the observation period of 15 min in the both years 2002 and 2003. From a total of 689 released

workers 166 did not return in the observation period (17.9% uninfested and 36.4% infested). Infested workers did not return to the colony two times more frequently than uninfested workers (Chi^2 test, $P<0.0005$). The difference in returning in the observation period was consistent for the both years 2002 (Fischer test, $P<0.002$) and 2003 (Chi^2 test, $P<0.045$, Figure 12).

The non-return rate of workers within the observation period was significantly greater in 2003 when bees were released from more distant locations than in 2002 (infested: Chi^2 test, $P<0.0005$, uninfested: Chi^2 test, $P<0.0005$).

No difference in the returning was found between artificially infested and naturally infested workers (Fischer test, $P<0.535$).

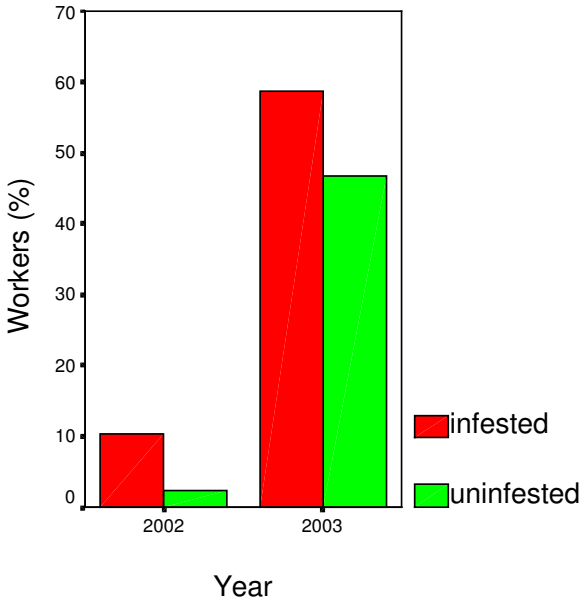


Figure 12. The percentage of workers that did not return in the observation period of 15 min in the year 2002 (release from 5m-50m) and 2003 (release from 50m-400m). The infested workers returned significantly less often to the colony in the observation period of 15 min compared to the uninfested workers. (2002: Fischer test, $P< 0.002$, and 2003: Chi^2 test, $P<0.045$). The total number of released workers in 2002: 406 (107 infested and 299 uninfested). The total number of workers released in 2003: 283 (123 infested and 160 uninfested).

4.3.3. Returning of workers in a whole day

Whether infested or uninfested workers returned during the day after being released from 3 different locations (20m, 50m and 400m) was recorded in the evening. The accuracy of the method to recover marked workers in evening was tested using 101 marked workers. From these, 6 workers were not found in the examination of a nucleus colony after a half an hour indicating an error of non detection about 6%.

In total 318 workers were released from different locations from the hive. From 181 uninfested workers, 52 (28.7%) and from 137 infested workers 58 (42.3%) did not return to the colony until evening. Infested workers, thus did not return to the colony about 1.5 more frequently than uninfested workers and this difference in returning was significant (Chi^2 test, $P<0.012$, Figure 13).

The difference in the frequency of returning between infested and uninfested workers was consistent for the locations 1 (20m) and 2 (50m) showing that significantly more infested workers did not return until evening compared to the uninfested workers (Chi^2 test, $p<0.0005$, Figure 14). This difference was not significant for the most distant location 3 (400m) in which the lower returning rate of infested workers was less pronounced than in the locations 1 and 2.

A comparison of returning frequency in the first 30 min and later until evening revealed that significantly more workers returned during the observation period than later (Chi^2 test, $p<0.0005$). Only 17.3% (32 from 185 returned workers) returned after half an hour until evening. This difference in the returning is more, but not significantly more pronounced in uninfested workers. A higher proportion of uninfested workers returned within 30 min of observation and a lower proportion later until evening compared to infested workers (Table 4).

A comparison of infestation of released and returning workers showed that the infestation of returning workers (37.5%, 78 from 208)

was by 13% lower than the infestation of released workers (43.1%, 137 from 318) but the difference was not significant.

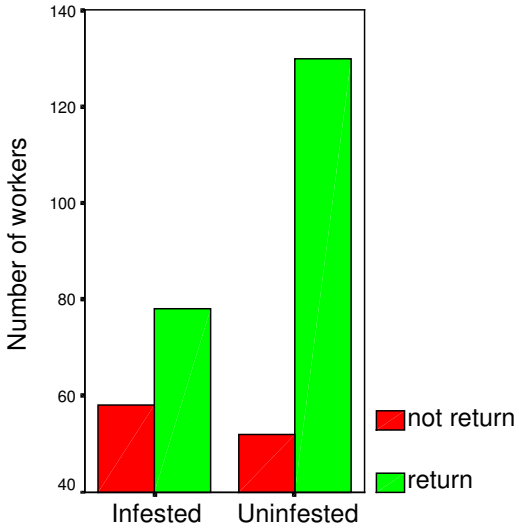


Figure 13. The total number of infested and uninfested workers that did or not return to the colony until evening. Significantly more infested workers did not return to the colony (42.3%) than uninfested workers (28.7%, Chi^2 test, $P<0.012$).

Workers	Release	Return 30 min		Return evening		Total	
		N	%	N	%	N	%
Uninfested	160	95	59.4	16	10	111	69.4
Infested	123	58	47.2	16	13	74	60.2

Table 4. The number and the percentage of returning workers observed during half an hour and later in evening. Significantly more uninfested workers returned within the observation period of 30 min than infested workers (Chi^2 test, $P<0.027$). Significantly more workers returned in the first 30 min than later until the evening (Chi^2 test, $P<0.0005$).

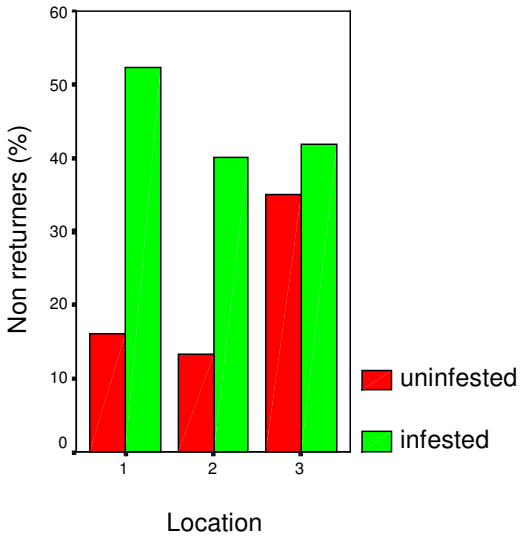


Figure 14. The percentage of workers that did not return to the colony for each location (location 1: 20m, location 2: 50m and location 3: 400m). Significantly more infested workers did not return from the locations 1and 2 until evening than uninfested workers (Chi^2 test, $P<0.0005$). The number of released workers in the locations 1, 2 and 3 was 52, 55 and 211 respectively.

4.4. Group release of workers

A total of 660 marked bees (140 infested and 520 uninfested) were released in groups of 30 workers of the same age from distances of 4m to 28m to test the proportion of infested workers that returned to the colony in 5 min intervals in the period of 15 min. I recorded 452 uninfested and 103 infested returned workers.

More than half of 660 released workers (63.6%) returned in 5 min, additional 20.5% of workers returned in the following l0 min of observation. This difference in returning over three time intervals was highly significant (Chi ^{2} test, $P<0.0005$, Table 5). When the numbers of returned infested workers were compared to the numbers of returned uninfested workers during the observational intervals, proportionally more of the workers which returned the last 10 min were infested compared to the workers returned in the first 5 min (Chi ^{2} test, $P< 0.043$). Correspondingly, the infestation of workers returning back to the colony increased from 0.16 in the first 5 min to 0.244 in the last 10 min of sampling (Figure 15, Table 5). The infestation of workers that did not

return was significantly higher (0.19, 103 from 555) than the infestation of workers did return in the total observation period of 15 min (0.26, 37 from 142, Chi ^{2} test, p< 0.047). Two times more infested workers (26.4%, 37 from 140) did not return to the colony in the observation period of 15 min than uninfested workers (13.1%, 68 from 520). This difference in returning was highly significant (P<0.0005, Chi ^{2} test, Figure 16).

The proportion of returned infested workers did not differ significantly among the three locations of release (4m, 17m, 28m) in each time interval of 5 min (Table 5).

Interval	0-5min			6-10 min			11-15 min		
Location	Bee, N	Mite, N	Inf.	Bee, N	Mite, N	Inf.	Bee, N	Mite, N	Inf.
1 (4 m)	77	9	0.12	18	6	0.33	5	2	0.4
2 (17m)	185	36	0.19	45	10	0.22	5	2	0.4
3 (28m)	158	25	0.16	40	9	0.22	22	4	0.18
Total	420	70	0.16	103	25	0.24	32	8	0.35

Table 5. The total number of returning workers, the number of mites (infested workers) and the infestation in the time intervals of 5 min according to the location. The infestation of workers in the first 5 min was significantly higher than the infestation of workers sampled in the last 10 min (Chi ^{2} test, P< 0.043).

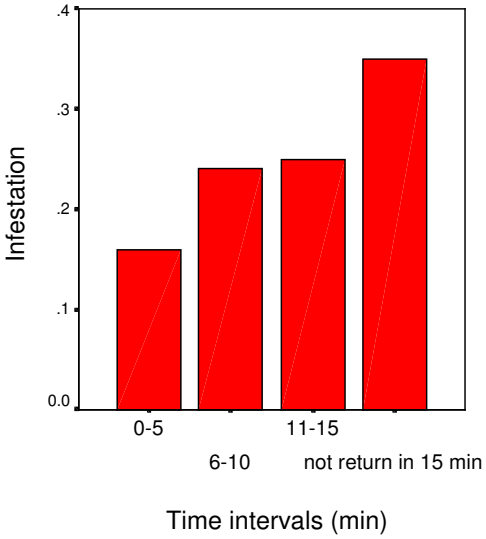


Figure 15. The infestation of returning workers in 5 min intervals and the infestation of workers that did not return back to the colony in the period of 15 min. The infestation of non-returning workers was significantly higher compared to the infestation of returning workers (Chi ^{2} test, P < 0.047).

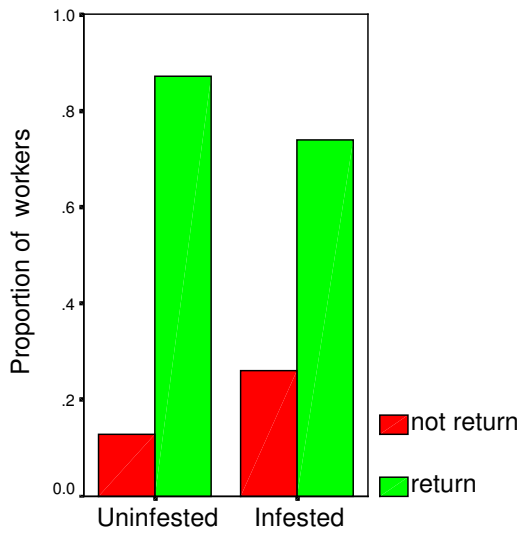


Figure 16. The proportion of workers which did and did not return to the colony in the observation period of 15 min. Significantly more infested workers did not return to the colony compared to uninfested workers (Chi ^{2} test, P <0.0005). The total number of infested workers:140. The total number of uninfested workers: 520.

4.5. Orientation toward the nest entrance

I released 118 workers (57 infested and 61 uninfested) in the year 2001 and 123 (58 infested and 65 uninfested) in the year 2002. From these significantly more infested (19) did not return to the colony than uninfested (7) in the observation period of 15 min (Chi ^{2} test, P <0.0005). Workers that returned flew to the nest entrance directly or approached and crossed the dummy entrance and empty circle on the right or left side before entering the nest entrance.

The most infested workers (76%, 73 from 96) crossed the dummy entrance whereas only more than one third of returned uninfested workers (36%, 43 from 119) searched for the nest entrance and so crossed the dummy entrance once or more times (Chi ^{2} test, p< 0.0005 Figure 17). Correspondingly, the dummy entrance was significantly more often crossed by the infested than by the uninfested workers (Mann Whitney Rank test, P< 0.0005, Figure 18). When only workers that

crossed the dummy were considered, the dummy was crossed more often, but not significantly so by infested than uninfested bees (Figure 19). The median crosses of infested workers was 2 with the maximum of 5 and the median of uninfested workers was 1 with the maximum 3.

Only 11 workers crossed the empty circle. Similarly, more infested workers crossed the empty circle (8.3%, 8 from 96) than the uninfested workers (2.5%, 3 from 119). The difference was close to significance (Fisher test, $p < 0.065$). Only one worker (infested) crossed the empty circle more than once (2 times).

The differences between infested and uninfested workers in the number of bees that returned to the colony directly or crossed the dummy entrance or empty circle were consistent for both years 2002 and 2003 (Table 6).

Half of 240 workers were released when the dummy position was on the left side and the empty circle on the right side and vice versa and the other half of 242 workers were released when the dummy was on the right side and the empty circle on the left side. No significant differences were observed in a relation to the position of the dummy and the empty circle (Table 6).

The age of released workers varied from 12 day to 38 days. No effect of age on orientation toward the nest entrance was found.

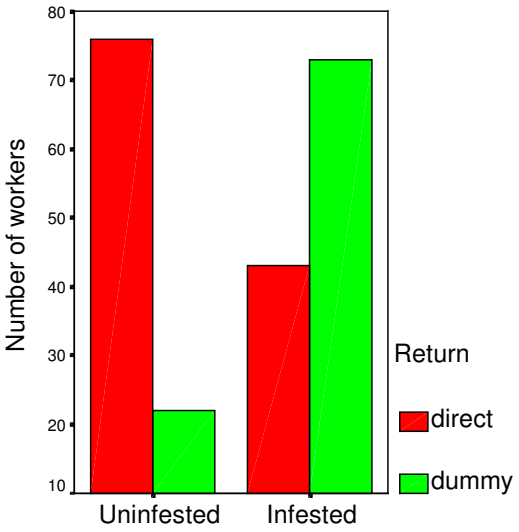


Figure 17. The number of infested and uninfested workers returning to the colony directly or crossing the dummy first before entering the colony. Significantly more uninfested workers (Chi ^{2} test, P<0.0005) flew home directly and significantly fewer crossed the dummy before finding the nest entrance compared to the infested workers (Chi ^{2} test, P<0.0005).

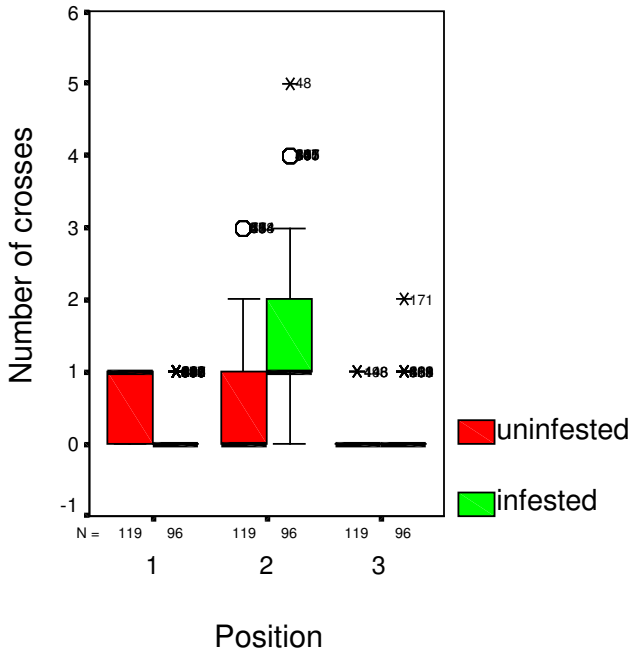


Figure 18. The number of crosses toward the nest entrance (1), dummy (2), and empty circle (3). The dummy entrance was significantly more often crossed by the infested than by the uninfested workers (Mann Whitney Rank test, P<0.0005). The chart indicates medians, inter quartile ranges, outliers and extreme values. The number of workers (N) is indicated below the chart.

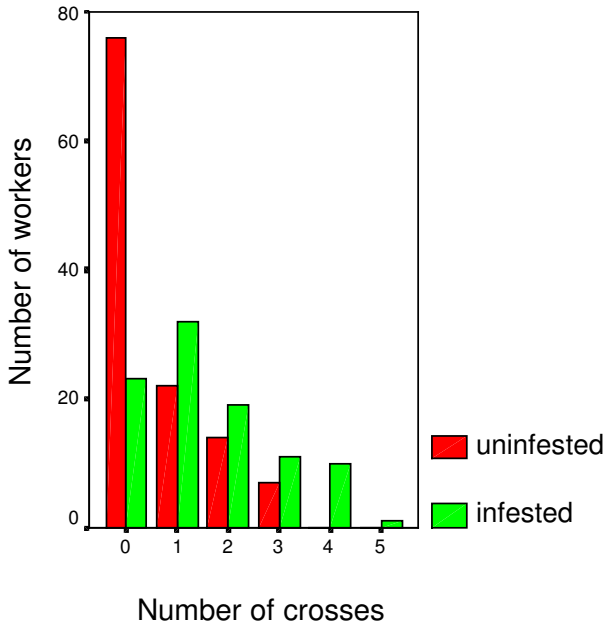


Figure 19. The number of approaches toward the dummy by infested and uninfested workers in both years 2002 and 2003. The number of crosses of infested workers varied from 1-5, while the number of crosses of uninfested workers varied from 1-3.

Year	Workers N	Dummy position	Nest entrance	Dummy	Empty circle
2002	Uninfested	Left	15	14	1
		Right	16	12	0
	Infested	Left	5	19	1
		Right	4	21	4
2003	Uninfested	Left	21	10	0
		Right	24	7	2
	Infested	Left	6	15	1
		Right	7	18	2

Table 6. The number of infested and uninfested workers which returned directly (nest entrance) or crossed the dummy or empty circle before entering the nest entrance for both years 2001 and 2002 and both position of the dummy (right and left).

4.6. Position of *V. destructor* on workers

Records on position of the mite on bees were taken only for bees infested by natural means in 2003. In total 49 infested workers were checked for mite position. The mite was mostly situated on the ventral side (89.8%), piercing between segments. Only 10.2% of workers had

the mite exposed on the surface of the thorax and abdomen. The difference in the position of the mite on a bee was highly significant (Chi ^{2} test, P< 0.0005, Figure 20).

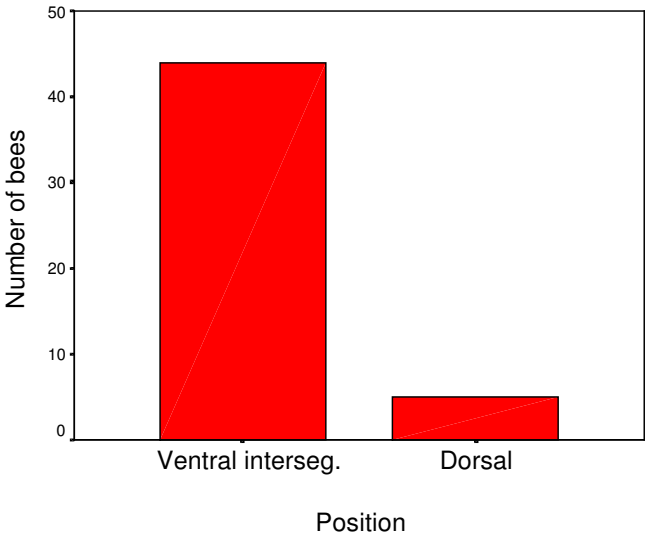


Figure 20. Position of the mites on bees. The mite was significantly more situated between abdominal segments in ventral side of the bee body than on the dorsal side of the abdomen and thorax (Chi ^{2} test, P< 0.0005).

4. 7. Daily loss of foragers in colonies infested by *V. destructor*

4.7.1. Daily loss of foragers in an infested colony over time

Loss of workers, the infestation of outflying bees and a daily mortality of the mites were monitored in a period between 10.8. 2002 and 19.10. 2002 in one highly infested colony.

I took sixteen samples of 2403 outflying workers. The median infestation of outflying workers was 0.02 with the minimum of 0 and the maximum of 0.41. The infestation of outflying workers significantly increased during time (Pearson correlation, r= 0.829, P<0.01, Figure 22). The average infestation of the last three samples was about 17 times higher than the infestation of the first three samples.

Data on worker loss recorded every 15 min included 32 days when the number of outflying workers exceeded 1000 and without handling the

colony (Figure 21). The median loss of workers was about 2.5% per flight per day with the range of 0.04 % and 12.7 % per flight per day. The proportion of bee loss increased significantly during the period of 70 days (Pearson correlation, r=0.534, P<0.002, Figure, 23). When the proportion of bee loss was correlated to the infestation of outflying workers sampled a day before or after the counts on foragers loss, the correlation was close to significance (Spearman rank correlation, r=0.53, P<0.053, n=12). The similar correlation, performed on the number of dead mites counted on bottom board inserts per day was nonsignificant.

The mite counts on the bottom board inserts (mite mortality) included 20 records. The median number of dead mites amounted to 30 mites per day in a range of 7 and 93 mites per day. The daily mite mortality decreased significantly over time (Spearman rank correlation, r=- 0.495, P<0.026. Figure 24). The correlation between the number of dead mites per day and the infestation of outflying bees was negative and nonsignificant.

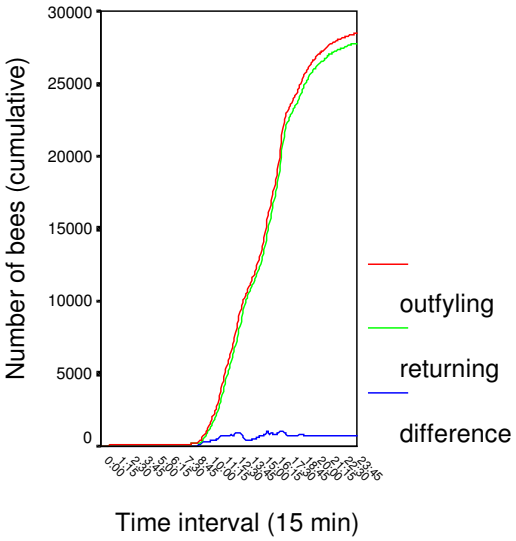


Figure 21. Example of flight recording over a whole day by using the bee counter. The cumulative curves of outflying, returning foragers and the difference between the cumulative number of outflying and returning foragers. Data were recorded every 15 min in the whole day on 10.8. 2002.

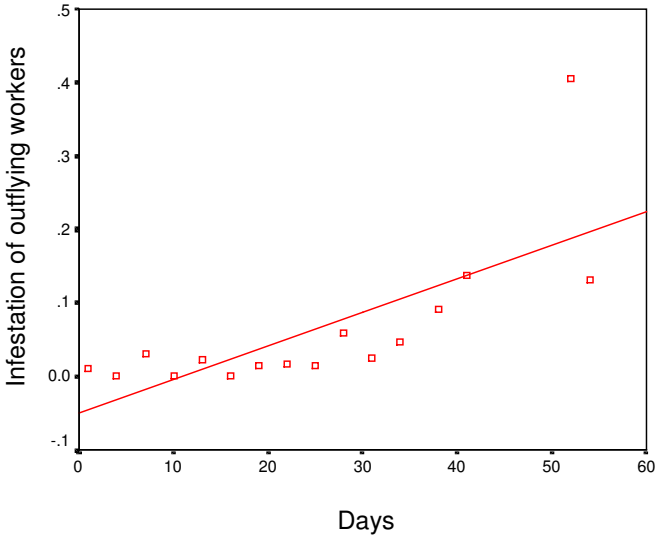


Figure 22. The infestation of outflying workers in 54 days (10.8 - 3.10. 2002). The infestation of outflying workers significantly increased during time (Pearson correlation, r= 0.829, P<0.01, N=16).

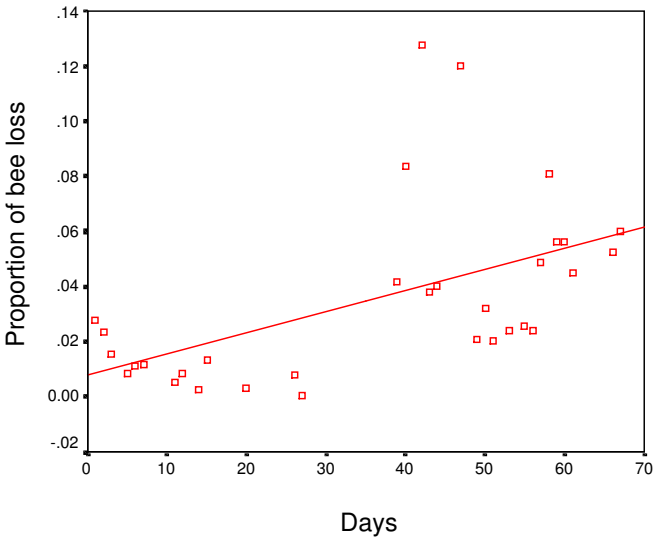


Figure 23. The proportion of bee loss per flight per bee in 70 days (10.8.-19.10. 2002). The proportion of bee loss increased significantly over time (Pearson correlation, r=0.534, P<0.002, N=32).

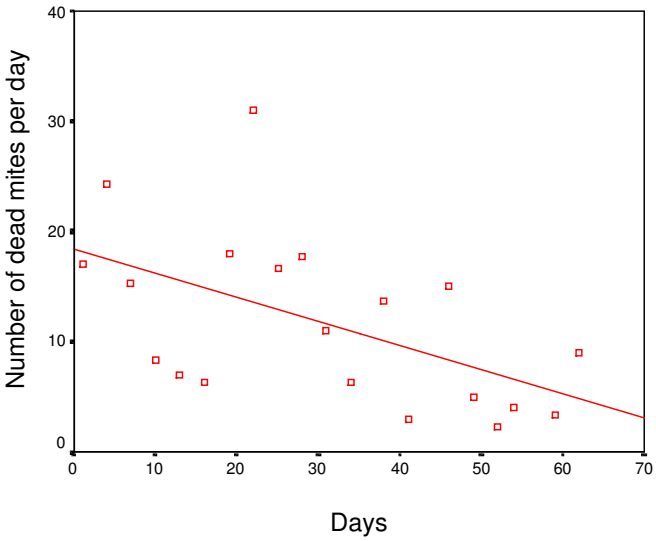


Figure 24. The number of dead mites per day in the period of 62 days (10.8. - 10.10. 2002). The number of dead mites per day significantly decreased over time (Pearson correlation, r=-0.495, P<0.026, N=20).

4.7.2. Simultaneous recording of bee foragers in a colony of high infestation and in a colony of low infestation

The number of outflying and returning foragers was monitored in one highly infested and one lowly infested colony from 11.7. to 3.8. 2003. Fourteen samples of 1883 outflying workers were analysed for mite infestation. The infestation of outflying workers was 7.7 higher in the highly infested colony than in the lowly infested colony (Mann Whitney U test, P<0.002). Specifically, the infestation of outflying bees was 0.015 mites per bee in the highly infested colony and 0.002 mites per bee in the lowly infested colony.

Loss of foragers as determined by the bee counters, included 9 records taken one day before or after sampling of outflying bees. Loss of workers was 0.022 per flight per day in the highly infested colony and 0.01 per flight per day in the lowly infested colony. Thus, bee loss was 2.2 times higher in the highly infested colony compared to the lowly infested colony (Mann Whitney U test, P<0.004, Figure 25). Correspondingly, a significant correlation was found between the proportion of bee loss and the infestation of outflying workers (Spearman

rank correlation, r=0.771, P<0.002, Figure 26) and between the proportion of bee loss and the number of dead mites per day (Spearman rank correlation, r=0.641, P<0.018).

The infestation, measured by the number of dead mites, differed significantly between both colonies (Mann Whitney U test, P<0.002). The median number of dead mites recovered on bottom board inserts was 47.5 and 1.43 mites per day in the highly and the lowly infested colony respectively. The number of dead mites decreased in both colonies over time (Figure 27). The number of dead mites decreased significantly over time in the infested colony (Pearson correlation, r= -0.955, P<0.0005, Figure 27). The mite counts of the lowly infested colony decreased in the period from 3.7. to 24.7. 2003 (in the first 3 weeks) and increased after neighbouring infested colony was robbed (31.7. 2003).

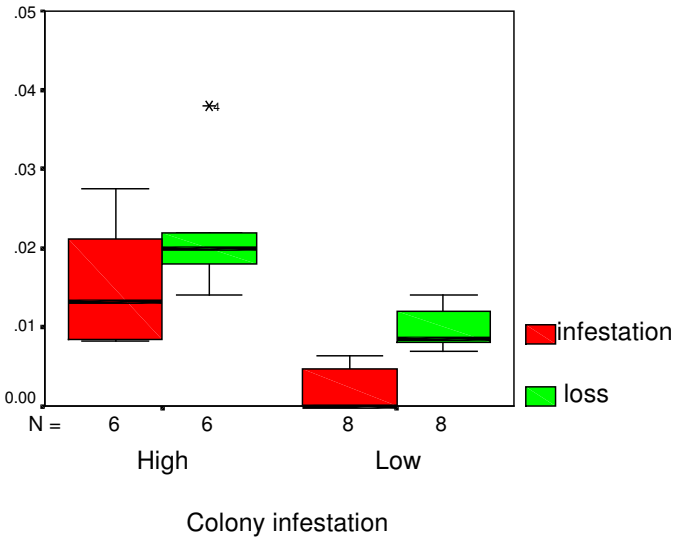


Figure 25. The infestation of outflying bees and loss of foragers in the colony of high infestation and in the colony of low infestation. Samples of outflying bees from the highly infested colony were significantly more infested (Mann Whitney U test, P<0.002) and the colony lost significantly more foragers compared to the lowly infested colony (Mann Whitney U test, P<0.004). The number of measurements is indicated below the figure. The chart indicates medians, inter quartile ranges and extreme values.

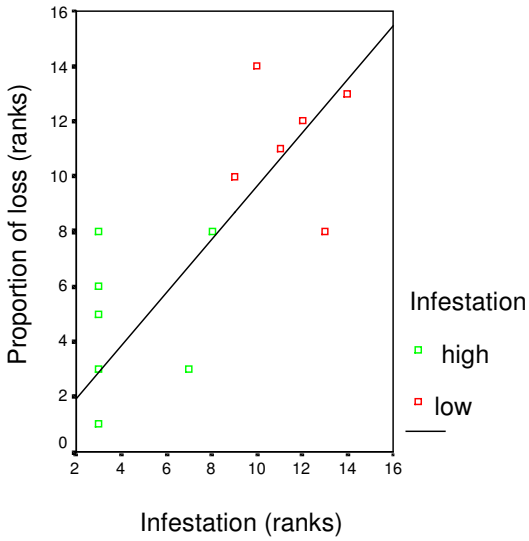


Figure 26. The correlation between the infestation of outflying workers and workers' loss for the highly infested colony and lowly infested colony (Spearman rank correlation, r=0.771, P <0.01).

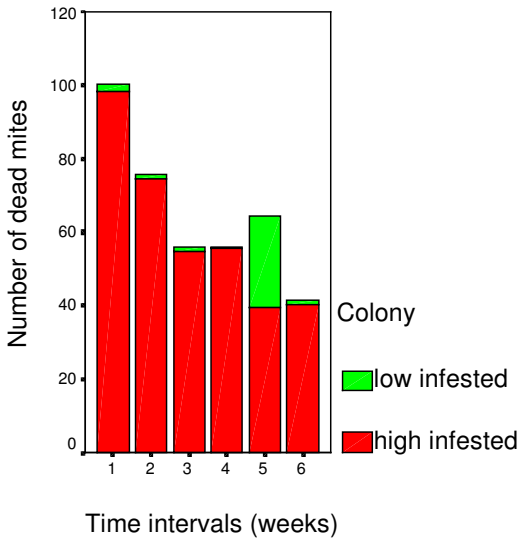


Figure 27. The total number of dead mites combined for both the lowly infested and highly infested colony in the time intervals of one week from 3.7.- 28.8. 2003. The number of dead mites significantly decreased during time (Pearson correlation, r= - 0.916, P<0.004).

4.8. Drifting

4.8.1. Observation of drifting in individual workers

A total of 318 infested and uninfested workers were released from different distances (20m, 50m, 400m) from the hive to record bee returning in the original colony and the neighbouring colony in the evening. Drifting to the neighbouring nucleus colony was observed only in 4 occasions in the whole experimental period from 20.6. until 16.8. 2003. From 4 drifting workers, one was naturally infested, one artificially infested and two were uninfested. The occurrence of drifting was about 1% (1.1%: 2 from 181 uninfested, and 1.4%: 2 from 137 infested workers).

4.8.2. A choice test for nest recognition

Differences in drifting between infested and uninfested workers to similar hives were tested in 8 experiments using two highly infested colonies. The original colony was replaced with the hive of the same and different colour. In total 472 workers were sampled from the empty hive of the same colour and 359 workers were sampled from the hive of different colour. More infested workers (2.6%) entered the different coloured hive than the same coloured hive (1.9 %, Table 7). The differences in the proportion of infested and uninfested workers that entered empty hives was, however, not significant.

Colony replacement (Hive colour)	Infested workers (N)	Uninfested workers (N)	Total
Same colour	9	463	472
Different colour	10	395	405

Table 7. The number of infested and uninfested workers drifting to the same coloured and different coloured hive.

5. Discussion

The fate of *Varroa destructor* mites on foragers is scarcely known. The main focus of detailed studies has been mite reproduction while equally important factors of population dynamic such as transportation of mites on foragers, immigration into colonies, death of infested foragers and removal of mites in the field have been largely neglected. Attention has been paid only to mechanisms of bees such as grooming and hygienic behavior, causing the removal of mites from adults in a colony and from brood respectively. Considering that workers spend about half of their lives engaged in tasks within the colonies and a half of their lives engaged outside the colony in foraging for nectar and pollen (Winston, 1987) it would seem as likely that foragers may have evolved some mechanisms to remove pathogens outside the colony.

As a main support of such a view, Kutschker (1999) showed that the infestation of outflying workers is substantially higher than the infestation of returning workers, which indicates a loss of mites during foraging. Several possibilities exist to explain this phenomenon. Mites might be actively removed during foraging or might accidentally fall from bees due to their rapid movements. Mites also might leave bees intentionally in order to change host, either on foraging sources as flowers, or when workers temporarily enter other colonies. Lower infestation of returning workers would, however, also result if infested workers do not return to the colony. Experiment of Kutschker (1999) did not allow separation of these possible causes.

Substantial loss of foragers during flights is a normal element of worker force turnover, mostly due to death of foragers and occasionally to “drifting”, that is entering other colonies in the vicinity. However, to decrease infestation, the non returning of infested foragers must exceed that of uninfested workers. Extensive loss of infested workers could be of adaptive value to bees, as these might not return in order to remove pathogens from the colony.

The main goal of this research was to provide more information about the fate of mites on foragers during their stay outside the colony. An emphasis was given to investigation whether flight behavior of workers infested by *V. destructor* as foragers is changed and could contribute to loss of mites from the colony. To determine influence of mites on returning success of foragers, I tested whether infested foragers return to the colony as frequently as healthy foragers. In investigating loss of mites with a two camera system I explored possible ways of mite loss from foragers outside the colony. Further, I determined loss of foragers in highly and lowly infested full size colonies to test whether infestation by *V. destructor* causes considerable loss of workers. To determine if changes in flight behavior might have impact on returning rate of workers I focused on the question whether the mites influences flight duration and orientation of infested workers.

Mite loss with foragers was investigated in 3 experiments. The experiment of Kutschker (1999) was repeated to confirm consistency of her results showing lower infestation of returning bees compared to outflying bees. Because this experiment could not be differentiated between loss of infested foragers and loss of mites in the field, I conducted the experiment using a two camera video system. Individual outflying and returning marked workers were recorded in an entrance tunnel to determine mite loss and gain from individual bees and the infestation of outflying and returning workers. To determine whether infested workers are more prone to fail to return to the colony, I released individually marked workers of the same age and checked for their return in evening. It was also investigated, whether infested bees erroneously enter a nearby colony equally often as uninfested bees.

The flight behavior of workers as a factor influencing returning success was investigated by using two approaches. The flight duration was determined by recording outflying and returning workers using the video method mentioned above. To determine whether infested returning workers need more time to return, I released workers of the same age individually or in groups from close vicinity of the colony. Further, the orientation of infested workers toward the nest entrance was tested

because orientation plays important role in returning of bees to the colony.

Loss of foragers in lowly and highly infested full size colonies was measured using a bee scanner that counted outflying and returning workers. Recording bee loss in relation to infestation may provide substantial support for the investigation of mite influence on flight behavior of foragers. The effect of mite loss with foragers on a decrease of colony infestation was evaluated and discussed.

I investigated whether changes in flight behavior differ in bees of different origin; in particular in their expression of resistance against *V. destructor*. Primorsky and Carnica workers were compared in infestation of outflying and returning workers, flight duration and returning time. Differences in expression of altered flight behavior might provide the evidence that the response of infested bees is a trait that may be selected and used in selection programs to breed bees resistant to *V. destructor.*

5.1. Loss of *V. destructor* on flight bees: loss of mites

Higher infestation of outflying workers compared to the infestation of returning workers in Kutschker (1999) and my investigations indicates that mites are lost from the colony at a considerable rate. Two possibilities exist to explain this phenomenon. One possibility would be that infested workers do not return to the colony at as high a rate as uninfested workers. Normal death of foragers due to turn over of workers would lead to lower infestation of returning bees if non-returning of infested workers exceeds turn over of uninfested individuals. This would mean that infested workers experience death earlier and/or do not return to the colony. Another possibility would be that mites are removed or leave workers during foraging.

Mite loss due to non-returning of infested workers was investigated in 2 whole days of video recordings. Non-returning in infested workers occurred in nearly a quarter of occasions (22%), while non returning of uninfested bees occurred only at the lower rate (17%). The net loss of infested foragers for both years of investigation, 2001 and 2002,

explained half of mite loss from foragers. Significantly more infested workers did not return to the colony compared to uninfested workers in 2001 indicating that infested workers were more rapidly lost from the colony than uninfested individuals. However, the next year, no difference in returning between infested and uninfested workers was found. Loss of bees in general that year was also significantly lower compared to loss in 2001. These differences might be explained by different colony conditions. The colony broke down at the end of season in 2001 very likely due to high infestation. The same reason might have contributed to higher loss of bees and accounted for higher rate of non-returning between infested workers in 2001.

5.1.1. Loss of infested foragers

That infested workers did not return to the colony at as high a rate as uninfested workers could be explained either by death rate of the infested foragers which exceeds the death of healthy individuals, or by the drifting of infested foragers to another colony. It is largely known that workers infested in pupae stage have shorter life spans (Sakofsky, 1990), so it would be possible that the life span of workers infested as adults is also shortened. However, from analyzing video recordings it was not possible to determine whether the foragers die in the field, get lost and therefore die, or drift into other colonies.

Loss of infested workers was also supported by the experiment in which workers were released from different locations. Released infested workers returned to the colony significantly less frequently compared to uninfested workers. The exception was the most distant location of 400m, where the colony was not perceived from the release location. However, even here more infested bees did not return compared to uninfested bees. Long distance orientation requires learning the signals and the retrieval of context-specific memories (Menzel,1993). In long distance orientation the sun orientation dominates over landmarks in sunny days (Menzel et al., 1990; Chitka and Geiger, 1995). Discrepancy in returning frequency between short and large distances may be interpreted as

different cues used by bees to return home. Bees released in the close vicinity from where they perceive the colony and its surrounding might use more land marks then sun compass orientation to return home.

Monitoring bee loss by using the Bee scan in the full size colonies supported the pervious observations indicating higher loss of infested bees. Number of non returning of foragers was investigated by monitoring the number of outflying and returning workers and the infestation of a colony was monitored by sampling outflying bees. Monitoring outflying and returning bees indicated a relation between loss of bees and the infestation of outflying workers. Loss of bees increased during a more than 2 month period of monitoring in 2002. Similarly, the colony encountered significant increase of infestation of outflying workers during this period. Accordingly, results suggest the influence of mite infestation on loss of bees. However, from this experiment, I could not conclude whether the increase in bee loss was a result of season or infestation by *V. destructor*. The comparison of loss of workers in highly and lowly infested colonies monitored at the same time suggested indeed that it was the influence of infestation rather than that of the influence of season on bee loss. Significant correlations between loss of workers and colony infestation as measured by the infestation of outflying workers or by the number of fallen mites, indicate that the colony with higher mite infestation lost more bees.

5.1.2. Loss of mites from foragers

Mites might be lost from foragers by unloading mites during foraging. Analyses of video recordings showed that 20% of infested workers did nor return to the colony with mites, a factor which explained about half of mite loss. In relation to mite loss, mites might be removed actively by workers or by accidental fall from bees during foraging. On the other hand, mites might leave bees when infested workers temporally enter other colonies (drift) or on foraging sources as flowers and change a host. In bumble bees, a protozoa *Crithidia bombi* efficiently uses flowers as vectors to spread (Schmid-Hempel, 1998). Theoretically it would be

possible that *Varroa* mite change host on flowers though there is no direct evidence. A few reports noted *Varroa* mites on flowers. Kevan et al. (1990) reported mites from honey bees found on flowers transported from South America to U.S.A. and Pettis et al. (2003) reported an individual mite found on flowers transported from Netherlands to USA. Hartwig and Jedruszuk (1987) and De Guzman et al. (1993) showed that *Varroa destructor* survives on flowers up to several days and can change hosts after 5 days (Hartwig and Jedruszuk, 1987). This is sufficient time to change hosts on flowers or even to change host after being shipped into new areas. This would however have tremendous impact on spread of the mite between colonies. Nevertheless the lack of evidences of mites on flowers around infested colonies (Pettis et al., 2003) might indicate that host change on flowers is not common for *V. destructor*. The benefit of mites using flowers as a vectors of transfer is dispersal to other colonies. Two colonies in the same foraging environment with abundant flowers rarely share foraging patches on the same day (Waddington et al., 1994). From this perspective the host change if it occurred would be more likely between the members of the same colony and therefore would not result in the removal of mites from the colony. However, such a situation might specifically refer to a colony in an environment with reach foraging source. Controversy, bees in poor foraging environment might share foraging source which opens the possibility of inter colony transfer of mites using flowers as vectors.

From the video analysis, it could not be determined whether workers lost mites outside the colony or in the tunnel and at the entrance of the colony. Consequently, it could not be excluded that some mites change host in the tunnel or at the entrance and were therefore not removed from the colony. Despite this the video observation supported the experiment in which bees were released from close proximity of the colony showing higher non returning in infested workers. However, the lower difference in returning between infested and uninfested workers for the most distant location of 400m could not be explained by this experiment.

5.1.3. Gain of mites

The analyses of video recordings showed that some uninfested workers gained mites. Several possibilities exist to explain mite gain in returning foragers. One possible explanation would be that mites are gained as a result of host change on flowers (discussed above) or host change when workers temporary drift (enter) into other infested colonies. Again it could not be excluded that the mite gain was a result of mites that changed host in the recording tunnel or at the entrance. Further, the mite gain possible could have been at least impaired, a result of misdetection of mites on outflying workers. The mite gain was recorded to be less than 2%, while the expected error in detection of mite is found to be in the range of 3-4%.

Gain of mites was 21 times lower than loss of mites indicating that considerably more mites are lost than gained. The loss of mites amounting to 20% exceeded possible misdetection of mites. The net loss of mites resulted in significantly lower infestation of returning workers. This is in an agreement with my and Kutschker (1999) results showing that the infestation of outflying workers is significantly higher than the infestation of returning workers. However, video recordings of individual outflying and returning workers showed a less marked difference in the infestation between outflying and returning workers compared to the infestation obtained by sampling outfyling and returning workers. The difference in infestation between experiments might be related to the colony conditions. In the experiment using the video technique, the nucleus colony was used. The colony received food supply every week so the foraging pressure for finding profitable food source far from the institute is rather low, while samples of outflying and returning workers were taken from full size colonies which did not receive food supply and had to be self sufficient. It is very likely that foraging distances traveled by bees are much longer in the full size colonies compared to foraging distances in the small nucleus colonies. Foraging in more distant locations is encountered by higher risk and requirements for more

complex orientation. This might explain higher loss of mites in full size colonies.

Observation of returning of infested and uninfested workers in a whole day by using the video equipment and the returning of released workers supported Kutschker (1999) and my experiment showing loss of mites due foraging. The video technique provided supporting evidence that mites are lost with foragers by non return of infested foragers or by mite loss from foragers. However, this technique might have involved more close contact of forager bees in the entrance tunnel and thus possible increased host change of the mites. Therefore this part of results should be taken with caution. Nevertheless results were consistent with the possibility that mites might be lost during flights.

5.1.4. Comparison between Primorsky and Carnica workers

Primorsky colonies show lower colony infestation than Carnica colonies (Berg et al., 2003). In my experiment, Primorsky workers were also less infested than Carnica workers. Primorsky workers lost almost two times more mites than Carnica workers due to non returning or loss of mites and this resulted in a considerably lower infestation of returners. One possibility would be that mites are indeed more rapidly removed from the colony by Primorsky than Carnica workers. However, another explanation due to the experimental setup cannot be excluded. It would also be possible that mites change from Primorsky hosts to the more attractive Carnica workers in the entrance tunnel. The low infestation of Primorsky workers could thus have been resulted from a low attractiveness of Primosky workers compared to Carnica workers.

5.2. Parasite host interaction - changes in forager behaviour

Loss of infested foragers might have a base in changes of flight pattern of infested foragers. Woyciechowski and Kozlowski (1998) showed that loss of bees might be enhanced by changed flight behavior of diseased workers. For this reason I compared flight duration and returning time of infested and uninfested workers after release from close proximity of the colony. Another factor that may effect returning is orientation. In this respect I tested nest orientation of infested bees. Bees which do not return to the colony may enormously enter the other colony (Free, 1958) and if infested they disperse the mite. In such a case non-returning of workers would be of great benefit of the mite. In contrast non-returning of infested bees is of great benefit of bees to reduce infestation of the colony. It is of interest to know whether infested workers drift more than uninfested ones and in what rate in regards to loss of bees.

5.2.1. Flight times

Marked loss of mites due to non-returning of infested workers and mite disposal during foraging trips (discussed above) may indicate the possibility that the parasitation of the mite changes flight behavior of foragers. Sakofsky (1999) showed that workers from highly infested colonies drift more compared to the lowly infested colonies. This supports the hypothesis that the mite could effect flight behaviour. However, from this experiments it is not clear whether such changes would be a general response of workers from infested colony or an individual response of infested workers. The evidence that disease influence flight pattern was reported by Woyicechowski and Kozlowski (1998) showing that workers infested by nosema more frequently take flights unsuitable weather conditions. That disease changes the flight behaviour was supported also in my examinations. The duration as a parameter of flight behaviour was prolonged by the mite parasitism. Both, recording the flight duration of workers and measuring the time that workers need to return to the colony after release showed that parasited workers have prolonged flights.

5.2.1.1. *Flight duration*

The duration of the absence of marked workers from a colony measured by recording the leaving and returning time of workers was referred to as flight duration. The indirect method of measuring flight duration of workers is based on assumption that the most workers perform foraging during the recording period. In support, the average age of recorded workers was 21 days, which corresponds to intermediate foraging age assuming that workers undertake their first foraging trips at the age of 12-13 days until their death at the age of 30 days (Winston, 1987). Nevertheless it could not be excluded that some recorded workers were engaged in other tasks such as ventilation, guarding the entrance or orientation flights which take about 3-7 min. (Frisch, 1967).

The flight duration of infested workers was 1.7 higher than the duration of uninfested workers. Specifically, the flight duration of infested workers was 3.6 min and the flight duration of uninfested workers was 2.1 min. Prolonged flights of infested workers were consistent for both years indicating influence of *V. destructor* on flight behaviour of workers.

No significant differences were found in flight duration of Primorsky and Carnica workers. Both strains showed prolonged flights of infested bees. The investigation of Kovac and Crailsheim (1988) showed that flight activity measured by the frequency and duration of flights of workers that were infested in pupal period was not affected by *V. destructor.* However, Sakofsky (1990) found an impact of mite infestation in developmental stages on the age of foraging. Infested workers have orientation flights earlier and so start foraging earlier than workers which were not infested in the larval stage.

In regards to my experiment, results showed that the infestation by *V. destructor* has an effect on flight duration when workers are parasited as foragers. Prolonged flight might partly explain higher loss of infested bees due to higher risk involved in foraging.

5.2.1.2. Returning time

The influence of *V. destructor* on workers causing prolonged flights of infested foragers was supported also by the experiment in which workers were released from different locations. Infested workers took twice as long to return to the colony as uninfested workers when released from the same distance. Significantly more infested workers did not return in the observation period of 15 min indicating late returning or even non returning to the colony. Similarly, when groups of infested and uninfested workers were released, proportionally more uninfested workers returned in the first 5 min. The proportion of infested workers increased significantly during the last 10 min assuming that no mites were lost and gained after release. Significantly more infested workers did not return during the observation period of 15 min suggesting prolonged flights of infested workers or higher non-returning rate of infested workers.

Workers released from the largest distance of 400m took more time to return. This agrees with flight duration directly corresponding to distances traveled by bees as the speed is independent from the distance (Frisch, 1967). However, the returning time of uninfested bees was higher compared to infested workers for all locations.

Returning time of infested workers was influenced by age indicating that older bees return faster. No influence of age on returning time was found in uninfested group of workers. This might be interpreted that older, more experienced workers are not influeneced by mite parasitism in to such an extend as younger workers.

No significant differences were found in returning time of Primorsky and Carnica workers. Both strains showed prolonged returning time in infested bees.

5.2.2. Orientation toward the nest entrance

During first orientation flights bees gradually acquire knowledge about the appearance and location of the colony based on optical and olfactory cues (Frisch, 1967). Recognizing nest entrance by a position of

hive, color, smell, enable bees to successfully orient toward the nest entrance when returning from foraging trips (Frisch, 1967). In the test of nest orientation, infested workers compared to uninfested workers twice as often approached a dummy entrance before finding the nest entrance. On contrast, more than half of uninfested workers flew back directly to the nest entrance. This result indicates that *V. destructor* causes impaired orientation of infested workers toward the nest entrance. Impaired orientation might be a result of decreased ability of infested workers to recognize the position of the entrance and detect smell of the nest colony. Only few workers crossed the empty circle. These results are in an accordance with largely known fact that bees use visual cues to find the colony (Frisch, 1967).

Impaired orientation skills could explain prolonged flights of infested workers and loss of bees. Nevertheless some caution should be noted regarding orientation. The orientation from distance uses different mechanisms than close orientation where the goal is perceived directly (Frisch, 1967). Impaired orientation ability of infested workers toward the nest entrance therefore could not be directly applied to orientation from a longer distance from the colony. In this perspective, impaired nest orientation of infested workers only suggest a possibility of the impact of *V. destructor* on orientation in a narrow sense.

Flight behaviour is influenced by parasitism of *V. destructor*. Prolonged flights of infested workers might be caused by impaired orientation ability. Both extended flight duration and impaired orientation of infested bees suggest that infested bees are less likely to return to the colony. Prolonged stay outside the colony involves higher risk which could explain low returning success of infested workers observed in my experiments.

5.2.3. Drifting

Infested bees might erroneously enter into other colonies (drift) due to impaired orientation. Drifting of workers from unhealthy to healthy colonies is a potential means of transmitting disease (Free, 1958) and consequently of great benefit for the mite. My experiment was not conclusive whether infested workers drift more than uninfested workers. Drifting into the neighboring colony of infested bees was slightly higher but of any means did not differ significantly from drifting of uninfested bees. The occurrence of drifting between colonies half a meter apart was relatively low (1%). On contrast Jay (1966) reported much higher rate of drifting between healthy colonies one meter apart. Low drifting in my experiment could be explained by foraging age of released workers regarding that drifting occurs mostly in the first two weeks of bee life (Free, 1958). Further, drifting is influenced by colony position which is much lower at the end of a row compared to intermediate positions of colonies (Jay, 1965). Because experiment included only two colonies in a row it is likely that such position of colonies also contributed to low drifting. Recording of the occurrence of drifting in the evening could however miss workers that temporarily enter the neighboring colony during day. In this respect it would be possible that drifting was underestimated. Observing the occurrence of drifting of infested workers is rather difficult. It would require the observer to check drifting bees for mite infestation for a whole day.

Similar results were obtained with a choice test to same and different colored hives. Infested workers did not differ significantly in a choice test to the same and differently colored hive from uninfested workers, although there were more infested workers that entered the differently colored hive.

Sakofsky showed (1990) that the rate of drifting directly corresponds to colony infestation. From this experiment it is not evident if drifting is a general response of workers in highly infested colonies or a disorientation response of infested workers. Free's (1958) finding that week colonies drift more supports the hypothesis that the loss of bees in

the colonies weaken by mite infestation might be a general response of workers. Nevertheless it is not excluded that colonies in the Free (1958) investigation were weak due to presence of some diseases. Consequently it would be equally possible that the pronounced drifting investigated by Sakofsky (1990) was a result of disorientation of diseased workers.

Although my experiments are not conclusive about drifting of infested bees, they suggest that drifting of infested bees is not elevated in relation to uninfested bees from the same colony. In this respect detectable loss of infested bees rather fail as an explanation of loss of infested bees.

5.2.4. Possible mechanisms by which *V. destructor* influences flight behaviour

Several possibilities exist to explain influence of *V destructor* on flight behaviour. *Varroa* weight less than 1mg (personal observation). Weight of the mite as an explanation of prolonged flights is rather unlikely. Bees loaded with 40 mg do not show any changes in flight speed (Frisch, 1967). In addition, weight also could not explain impaired orientation of infested workers to the nest entrance.

A more likely explanation would be that the mites influences flight behavior of workers due to feeding on haemolymph of workers. Phoretic *V. destructor* is mainly situated on the ventral side of abdomen of foragers, between segments. Such a position prevents the mite from removal or falling due to rapid movements and consequently enable it to feed on haemolymph without interuption.

Haemolymph is composed of blood cells (haemocytes) and blood plasma (haemolymph, Glinski and Jarosz, 1995). Mites can take 0.1 mg haemolymph in 2 h, which represents 0.5% of haemolymph (Ritter, 1988). Weinberg and Madel (1985) estimated 23.6% reduction of haemolymph of worker pupae infested by 1-3 mites. Deprivation of haemolymph might affect flight behaviour for 2 reasons. Conzten et al. (2003) showed a decrease in energy content in bees ready to hatch which were infested by

4-6 mites. Weakened infested forager might need rest during flights or fly at slower speed which result in prolonged flights during foraging or returning after release.

Another explanation is related to changes in haemolymph content. The main components of blood plasma are sugars, lipids and proteins (Glinski and Jarosz, 1995). Several studies showed that infestation by *V.destructor* in developmental stages results in considerable decrease of proteins. The content of proteins decrease rapidly with the number of parasited mites (Weinberg and Madel, 1985; Schatton-Gadelmayer and Engels, 1988). Infestation by one to three mites causes a reduction of the protein content by 27% (Weinberg and Madel, 1985). Regarding the importance of protein synthesis in memory (Menzel, 1995) it would be possible that mite infestations have an impact on learning which in turn weakens foragers orientation and consequently causes prolonged flights. However, Menzel (1993) showed that long term olfactory memory in bees does not require protein synthesis. The conclusion does not deny the possibility of protein synthesis on very low, not detectable level.

Another possibility would be that the mite induces immune response by itself or by infecting bees with other pathogens. The immune response inhibits associative learning (Mallon et al., 2003). In this respect increased immune response of infested bees might contribute to impaired orientation of foragers causing prolonged flights and enhance loss of bees.

Another factor influencing flight behavior might be stress caused by the mite. Titer of juvenile hormone changes under stress (Lin et al., 2004). Treatment with juvenile hormone increase short term memory in young bees (Maleszka and Helliwell, 2001), and a possible decrease in juvenile hormone would affect returning of infested bees due to impaired maturation of short term memory.

The influence of *V. destructor* on flight behavior is apparently affected over short time of exposure to the parasite. Returning time and the rate of returning of artificially infested workers overnight did not differ from naturally infested workers. This indicates that only one day infestation with *Varroa* mite or even less is necessary to cause delay in

returning and increase the loss of infested workers. This is very striking as it implies that infestation by the mites may have rapid impact on foragers´ flight behavior. However, from my experiment it is not evident if the effect of infestation lasts only for the time of infestation or more.

5.3. Does loss of mites have an effect on colony infestation ?

To assess whether loss of mites from forager bees is of significant impact on mite population dynamics within the colonies, it is required to estimate the proportion of mite loss to the total mite population. As loss of mites in our experiments is linked to the foraging flights of infested bees, the total number of foraging flights and mean forager infestation was used as a starting point to estimate the mite population in the colony. The number of foragers and the number of nest bees was calculated assuming that foragers make on average 3.5 foraging trips per day (Thom et.al., 2000) and that 34% of workers are involved in foraging (Thom et.al., 2000). The number of mites on foragers was calculated according to the number of foragers infestation. Further, assuming, the mite preference of a 4-fold for nest bees (Kutschker, 1999) and that 60% of mites are in brood (Schulz, 1984), the number of mites in brood and bees was calculated. As a result, mite loss from foragers is calculated to be 3.1% of the mite population per day, if half of the mites on outflying foragers are lost (see Appendix 3).

This figure covers the estimated mite mortality of 3.2% of the mite population per day (Fuchs and Kutschker, 2000), as calculated by the difference between actual growth of mite population and the population growth expected from the daily birth rate. Of this death rate, less than half has been explained so far. Only about 1% of the expected 3.2% daily mortality relates to mites found dead in debris (Fuchs and Kutschker, 2000). Another portion could be explained by loss of mites on foragers due to natural death of foragers, assumed to be 0.5% per day by Fries et al. (1994) and experimentally determined to be 0.3 % per day by Fuchs and Kutschker (2000). The remaining 1.9% of unexplained mortality per day can easily be covered by the higher rate of non returning of infested

bees and or by loss of mites from foragers found in my experiments. The estimated mite loss on forager bees is 1.6 times higher than the still unexplained mite mortality. There are several possibilities for this difference. The model is sensitive for a) the number of foraging trips, and b) and the proportion between infestation of returning and outflying bees. Any deviation from the expected mean foraging frequency of 3.5 and the proportion between infestation of returning and outflying workers of 0.5 would have a great impact on mite loss.

Foraging performed only once per day would result in considerable lower loss of mites (1.7%). Assuming the ratio between infestation of returning and outflying workers of 0.8 obtained by analyzing video recordings instead of 0.5 obtained from sampling flight bees in full size colonies, the estimated daily loss would also be considerably lower (1.9%). As a last possibility, it would be that the daily reproduction rate of the mites might be higher as assumed in models (Fries et al., 1994; Martin, 1998; Calis et al., 1999) with a corresponding higher daily mite mortality.

5.4. Loss of mites as a defensive strategy

The difference in infestation of outflying and returning workers indicating pronounced loss of mites supports the idea that that bees respond with behavioral changes to the presence of mites. Such behavior might be reflected in a typical break down of colonies due to high infestation characterized by rapid loss of workers. Typically the majority of workers disappear and only a queen with few workers on patchy brood remains in the colony (Martin, 1997a). Though some portion of dead workers might have been carried out by the workers, such a striking loss of workers strongly suggests that the infestation by *V. destructor* might change flight behavior. That disease can influence flight pattern was demonstrated by Woyciechowski and Kozlowski (1998) who showed that workers vary their foraging activities according to age and health status. Workers infested by nosema (*Nosema apis*) foraged in unsuitable weather condition more frequently as healthy individuals. This was interpreted as diseased workers with low life expectancy more readily

engage in risky tasks in order to optimize the collection of nectar in relation to life expectancy.

However, changes in foraging behavior may be also interpreted differently. It appears conceivable, that diseased workers sacrifice themselves for the benefit of the colony to reduce infestation of the colony. To sacrifice for benefit of a colony is not uncommon in honeybees. Workers defend the colony by stinging intruders regardless of fatality of such action to themselves. In this perspective it would be possible that workers do not return to the colony as a result of a suicidal mechanism of infested workers to decrease infestation and increase survival of the colony. Both explanations are not mutually exclusive but might have worked in the same direction. It must be noted, that such a change in bee behavior facilitating non returning of infested foragers might also benefit the parasite as it increases its chance to spread.

It is of considerable interest whether the response on infestation with the mite is a general response of diseased workers that has and may have a genetic basis. This would mean that colonies of different genetic origin might differ in response of diseased workers contributing to a decrease of colony infestation. In the two tested bee lines, Primorsky and Carnica, a difference in mite loss was not conclusive though there is a slight indication that loss of mites is more pronounced in Primorsky workers, which lost almost two times more mites than Carnica workers due to non returning and/or loss of mites resulting in considerable low infestation rate of returners. Though a genetic background was not conclusively demonstrated, a response by not returning to the colony can be considered as highly adaptive as a defense mechanism of bees against pathogens and could possibly be used in the programs selecting bees for resistance to *V. destructor* and other diseases.

6. Summary

Life of *Varroa destructor*, Anderson and Trueman, an ectoparasitic mite of honeybees, is divided into a reproductive phase in the bee brood and a phoretic phase during which the mite is attached to the adult bee. Phoretic mites leave the colony with workers involved in foraging tasks. Little information is available on the mortality of mites outside the colony. Mites may or not return to the colony as a result of death of the infested foragers, host change by drifting of foragers, or removal of mites outside the colony. That mites do not return to the colony was indicated by substantially higher infestation of outflying workers compared to the infestation of returning workers (Kutschker, 1999).

The main objective of the study was to provide information whether *V. destructor* influences flight behaviour of foragers and consequently returning frequency of foragers to the colony. I first repeated the experiment of Kutschker (1999) examining the infestation of outflying and returning workers. Further, I registered flight duration of foragers using a video method. In this experiment I compared also the infestation and flight duration of bees of different genetic origin, Carnica from Oberursel and bees from Primorsky region. I investigated returning time of workers, returning frequency until evening, drifting to other colonies and orientation toward the nest entrance in the experiments in which workers were released in close vicinity of the colony. At last, I measured the loss of foragers in relation to colony infestation using a Bee Scan. Results from this study, listed below, showed considerable influence of *V. destructor* on flight behavior of foragers translating into loss of mites. Loss of mites with foragers add substantial component to mite mortality and was underestimated in previous studies. Such loss might be viewed as a mechanism of resistance against *V. destructor*.

a) The mean infestation of outflying workers (0.019±0.018) was twice as the mean infestation of returning workers (0.009±0.018). The difference in

the infestation between outflying and returning workers was more marked in highly infested colonies.

b) Investigation of individually tagged workers by use of a two camera video recording device showed significantly higher infestation of outflying workers compared to returning workers. Mites were lost by the non returning of infested foragers (22%) and by loss of mites from foragers that returned to the colony without the mite (20%). A small portion of mites (1.8%) was gained. Loss of mites significantly exceeded mite gain.

c) The flight duration of infested workers determined by using the same two camera video system was significantly higher in infested compared to uninfested workers of the same age that flew closest at time. The median flight duration of infested workers was 1.7 higher (214s) than the median duration of unifested workers (128s).

d) Infested workers took 2.3 times longer to return to the colony than uninfested workers of the same age when released from the same locations, closest at time. The returning time increased with the distance of release. In a group of bees released simultaneously the infestation was higher in bees returning later and in those that did not return in the observation period of 15 min.

e) Released workers did not return to the colony 1.5 more frequently than uninfested workers in evening. The difference in returning was significant for locations of 20 and 50m from the colony. No difference in returning between infested and uninfested workers were observed for the most distant location of 400m.

f) No significant difference was found in returning time and/or in the returning frequency until evening between workers artificially infested overnight and naturally infested workers. Artificially infested workers

returned later and less frequently than a control group indicating rapid influence of *V. destructor* on flight behavior of foragers.

g) The orientation ability of infested workers toward the nest entrance was impaired. Infested workers compared to uninfested workers twice as often approached a dummy entrance before finding the nest entrance.

h) No significant differences were found in drifting between infested and uninfested workers. Drifting in the neighboring nucleus colony occurred in about 1% occasions after release of marked workers. Similarly, more infested, but not significantly more infested workers (2.6%) entered a different colored hive than the same colored hive (1.9%). However, the number of drifting bees were to low to make results conclusive.

i) The comparison between Carnica and Primorsky workers revealed higher infestation in Carnica compared to Primorsky. Further, Primorsky workers lost more mites during foraging due to mite loss from foragers and non returning of infested workers. No significant differences in flight duration were observed between the two bee stocks.

j) Loss of foragers, as determined by the Bee Scan counts of outflying and returning foragers, and the infestation of outflying bees increased significantly over a period of 70 days. A colony with 7.7. higher infestation of outflying foragers lost 2.2. time more bees per flight per day compared to a low infested colony.

k) The estimates of mite loss with foragers from mite population per day up to 3.1% exceeds approximately mite mortality of 1% within the colony as represented by counting dead mites on bottom board inserts.

6. Zusammenfassung

Der Lebenszyklus von *Varroa destructor* Anderson und Trueman, einer ektoparasitischen Milbe der Honigbienen, unterteilt sich in eine reproduktive Phase innerhalb der Bienenbrutzellen und in eine phoretische Phase, während der die Milben an den adulten Bienen sitzen. Während der Sammelaktivitäten halten sich die phoretischen Milben mit den Sammlerinnen außerhalb der Bienenvölker auf. Über die Mortalität der Milben während dieser Zeiten ist sehr wenig bekannt. Die Milben könnten außerhalb der Bienenvölker die Arbeiterinnen verlassen oder von diesen entfernt werden. Sie könnten durch Verflug der Bienen einen Wirtswechsel vornehmen oder durch den Tod der Sammlerinnen umkommen. Die Untersuchungen von Kutschker (1999) hatten bereits gezeigt, dass der Befall heimkehrender Arbeiterinnen deutlich geringer ist als der ausfliegenden Bienen und damit belegt, dass in der Tat Milben außerhalb der Völker verloren gehen.

Das hauptsächliche Ziel der Arbeit war zu untersuchen, ob *V. destructor* das Flugverhalten der Sammlerinnen und die Häufigkeit ihrer Rückkehr in die Völker beeinflusst. Zuerst wiederholte ich die Untersuchung des Befalls der ausfliegenden und heimkehrenden Arbeiterinnen. Danach erstellte ich eine Videoregistrierung und untersuchte die Dauer der Sammelflüge der Arbeiterinnen. Hierbei wurden genetisch unterschiedliche Herkünfte, Carnica aus Oberursel und Bienen aus der Primorski-Region, miteinander verglichen. In Rückkehr-experimenten untersuchte ich die Dauer von Rückflügen, die Rückkehrhäufigkeit, die Orientierung zum Nesteingang und den Verflug in andere Völker. Zuletzt bestimmte ich den täglichen Verlust an Sammlerinnen mit einem elektronischen Bienenzähler (Bee Scan) in Bezug auf den Befall der Arbeiterinnen. Die unten aufgelisteten Ergebnisse belegen einen deutlichen Einfluss des Parasiten auf das Flugverhalten der Sammlerinnen, der zu einem beträchtlichen Milbenaustrag führt. Dieser Einfluss stellt eine wesentliche und in früheren Untersuchungen unterschätzte Komponente der gesamten

Milbenmortalität dar, und kann als ein Resistenzmechanismus der Bienen gegen *V. destructor* gedeutet werden.

a) Der mittlere Befall der ausfliegenden Arbeiterinnen (0.019 ± 0.018) war doppelt so hoch wie der der zurückkehrenden Arbeiterinnen (0.009 ± 0.018), der Unterschied war in hochbefallenen Völkern deutlicher.

b) Die Untersuchung individuell markierter Arbeiterinnen mit einer mit zwei Kameras ausgestatteten Videoeinrichtung zeigte ebenfalls einen signifikant höheren Befall der ausfliegenden Arbeiterinnen. 22% der Milben gingen verloren, indem befallene Arbeiterinnen nicht in die Völker zurückkehrten, weitere 20% befanden sich nicht mehr auf den rückkehrenden Arbeiterinnen. Geringe Milbenanzahlen wurden zugewonnen (1.8%), der Verlust überstieg bei Weitem den Zugewinn.

c) Mit dem gleichen Videosystem wurde die Dauer der Flüge bestimmt. Diese war bei befallenen Arbeiterinnen signifikant länger als bei den unbefallenen, wobei möglichst zeitnah ausfliegende Arbeiterinnen gleichen Alters verglichen wurden. Der Median der Flugdauer war bei den befallenen Sammlerinnen 1.7 fach höher als der der unbefallenen (214s bzw 128s).

d) Am gleichen Ort aufgelassene befallene Arbeiterinnen benötigten 2.3 mal länger um in die Völker zurückzukehren als unbefallene. Die Rückkehrzeit nahm mit der Entfernung zu. Bei einer gleichzeitig aufgelassenen Bienengruppe war der Befall der spät zurückkehrenden und der innerhalb von 15 min nicht zurückkehrenden Bienen höher.

e) Befallene aufgelassene Arbeiterinnen kehrten bis zum Abend 1.5 mal häufiger nicht in ihre Völker zurück. Der Unterschied war bei den 20m und 50m entfernten Auflassstellen signifikant, nicht aber bei der weiter entfernten Auflassstelle (400m).

f) Künstlich infizierte und über Nacht außerhalb der Völker gehaltene Arbeiterinnen unterschieden sich nicht von den natürlich infizierten Arbeiterinnen und zeigten ebenfalls signifikant längere Rückkehrzeiten im Vergleich zur Kontrollgruppe. Dies deutet auf eine rasche Wirksamkeit des Befalls.

g) Die Orientierungsfähigkeit der befallenen Arbeiterinnen zum Nesteingang war negativ beeinflusst. Befallene Arbeiterinnen flogen doppelt so häufig eine simultan angebotene Eingangsattrappe an bevor sie den Nesteingang fanden.

h) Es konnten keine Unterschiede im Verflugverhalten festgestellt werden. In etwa 1% der Fälle flogen aufgelassene Arbeiterinnen ein benachbartes Volk an. Stärker befallene Arbeiterinnen flogen mit 2.6% bzw. 1.9% einen Bienenkasten mit vom ursprünglichen Volk unterschiedlicher Farbe an. Die erreichbaren Verflugzahlen waren zu gering, um sichere Aussagen machen zu können.

i) Ein Vergleich von Carnica und Primorski zeigte einen höheren Befall der Carnica-Bienen. Weiterhin gingen bei den Primorski-Bienen während des Sammelns mehr Milben verloren, teils durch Verlust der Milben, teils indem die Sammlerinnen nicht zurückkehrten. In der Flugdauer unterschieden sich die Carnica-Bienen nicht von den Primorski-Bienen.

j) Der mit einem elektronischen Bienenzähler (Bee Scan) ermittelte tägliche Verlust von Sammlerinnen sowie der Befall der Flugbienen stieg parallel über die Versuchsdauer von 70 Tagen an. Die 7 mal höher befallenen Sammlerinnen eines hochbefallenen Volkes hatten eine 2.2 mal höhere tägliche Verlustrate als die Sammlerinnen eines niedrig befallenen Volkes.

k) Eine Abschätzung der auf den Milbenverlust über Sammlerinnen zurückzuführenden Mortalität ergab, dass hierdurch pro Tag bis zu 3.1%

der Population abgehen. Die bisher über Bodeneinlagen erfasste Milbenmortalität innerhalb der Bienenvölker beträgt etwa 1%.

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Appendix 1. Description of a box plot

Charts were made using SPSS statistical program package (SPSS 10.1). The box plot chart indicates medians, inter quartile ranges, outliers and extreme values. Specifically, horizontal lines on bars (box plots) represent medians and vertical lines on bars represent interquartile ranges (1 and 4).

Outliers are single values that are 1.5-3 box length away from the rim of the box. The outliers are indicated as circles.

Extreme values are described as a values that are more than 3 box plot length away from the box rim. The extreme values are indicated as asterixis.

Appendix 2. The number of outflying infested and uninfested workers and the number of returning infested and uninfested workers in the years 2001 and 2002.

Video recordings in 2002 included Carnica (C) and Primorsky workers (P). Proportionally more bees that had been infested when they left the colony did not return, or returned without a mite than bees that had no mites or gained mites (14.1%, Chi^2 test, $p<0.0005$). Primorsky workers lost significantly more mites than Carnica workers (Chi^2 test, $p<0.032$).

Year	Direction of bee fly	Infested N	Uninfested N	Total N
2001	Outflying	109	469	578
	Returning	65	380	445
2002	Outflying C	49	141	190
	Outflying P	21	125	146
	Outflying total	70	266	336
	Returning C	40	133	173
	Returning P	12	118	130
	Returning total	52	251	303

Appendix 3. Calculation of mite loss from population due to foraging

The number of 50000 foraging trips per day were assumed to calculate the number of bees in the colony. The calculation considered that foragers on average made 3.5 trips per day (Thom et al., 2000), and that 34% workers were involved in foraging (Thom et al., 2000).

Number of foragers=N. flights/ N. trips per forager= 50000/3.5=14286

Total number of bees in the colony=
N. foragers X 100/34=14286 X 100/34= 42017

Number of nest bees = Total N. of bees- N. of foragers= 42017-14286= 27731

The number of lost mites on foragers was calculated according to the number of foragers and their infestation. An infestation of outflying workers of 0.01 that made 3.5 trips per day under the assumption that half of the mites are lost on outflying foragers would result in a loss of 130 mites.

Number of mites leaving the colony on foragers=
N. foragers X infestation =14286 X 0.01=143

Mite loss in 3.5 forager flights=
N. mites on foragers X (infest. of returning workers/infest. of outflying workers)

1 foraging flight =143X 0.5=	72
2 foraging flight=72X 0.5=	36
3.5 foraging flight=36X0.5+ 9X0.5=	22.5
Total loss of mites =	130

The number of mites on nest bees was calculated from the number of mites on foragers and the number of foragers under the assumption that mites are 4 times likely in the nest bees (Kutschker, 1999) and that 34% of bees are foragers (Thom et al., 2000). Mites on brood and adults are calculated assuming that 30 % of mites are on adult bees during summer and additional 60 % of mites are in brood (Schulz, 1984).

Number of mites on nest bees = 27731X4X0.01=1109

Total number of mites on bees=1109+143=1252

Total number of mites = 1252 X 100/30=4173

Mite loss from the colony was calculated by dividing the number of mite loss from foragers by the total number of mites.

Mite loss= N. mite loss from foragers/total N. of mites= 130/4173=0.031

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Oberursel, 12.8. 2004 Jasna Kralj