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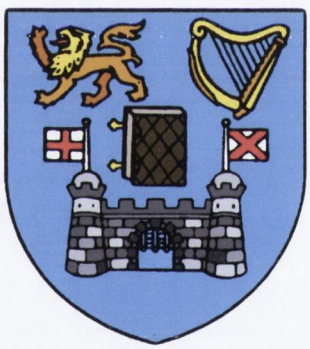
Susceptibility and mortality in honeybees (*Apis mellifera*) infested with the tracheal mite (*Acarapis woodi*)

John B. McMullan



A thesis submitted to the University of Dublin, Trinity College in fulfilment of the requirement for the Degree of Doctor in Philosophy

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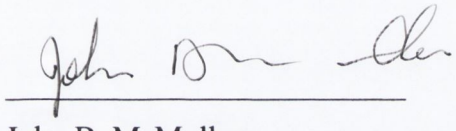

John B. McMullan

Summary

The tracheal mite, *Acarapis woodi* (Rennie), is an obligate parasite of the honeybee. It was first discovered in 1919 by Rennie during his investigation into the widespread deaths of honeybee colonies in Europe in the early 1900s, later known as the 'Isle of Wight' disease. While the mite has been associated with honeybee colony mortality since that time, there is still no consensus that tracheal mite infestation was the cause of the Isle of Wight disease or that tracheal mites can cause colony mortality. In previous research it has been shown that mites transfer from old to young host bees via body hair, that this migration is limited to very young bees (normally less than 4 days old) and that bee strain affects susceptibility. This thesis contains an investigation into infestation of honeybees by tracheal mites and considers the factors that determine susceptibility and mortality in colonies.

The research included a series of inoculation experiments to identify the infestation response of young bees, that had been given different treatments, when exposed to old bees that were heavily infested with tracheal mites. The treatments involved reduced pupation temperature, restricted grooming, high relative humidity and small-cell brood combs. Mite infestations were measured by dissecting the prothoracic tracheae of the bees and counting the mite stages. To complete the laboratory work on susceptibility two-choice experiments were undertaken to determine the attraction of the mites to cuticular compounds of bees across different treatments. In addition, a production colony in the field with a heavy tracheal mite infestation was observed and monitored on a daily basis over a six-month period until its death in early spring. Daily temperatures (hive and ambient) and bee mortality counts were taken as well as regular monitoring of diseases, honey stores consumption, weather conditions and winter cluster. Finally, modelling of mite infestation, colony mortality and mite biology was undertaken.

I identified that bees pupated at a reduced temperature had a large and significant increase in mite susceptibility. I established that autogrooming ability largely accounted for the differences in colony infestation in bees pupated at normal temperature but did not account for the increased infestation in bees pupated at a reduced temperature. In fact, there was only evidence that reduced autogrooming ability made a contribution to increased infestation in the case of colonies with low susceptibility to tracheal mites. There was no evidence that cuticular compounds acted as a mechanism in the increased infestation due to

reduced pupation temperature. The two-choice bioassay used to detect cuticular compounds was that used by two previous researchers, but it did not elicit any response from the mites. A new methodology was devised using a 'hair contact' method and this did give mite responses, indicating that mites use gustatory senses to detect chemical cues and not olfactory senses as required by the original method. An alternative migration strategy is suggested whereby mites 'taste' the bee hair (setae) of the new host to establish bee age prior to transfer contrary to the generally accepted position that mites invariably transfer before establishing the age of the bee. I concluded that the increased susceptibility in bees that have pupated at a low temperature is due to changes in the physiology of the callow bees either through reduced bee mobility, under-development of the sensory system or changes in hair structure thereby facilitating easier mite transfer.

I showed experimentally that there was no evidence that bees raised in small-cell combs had any difference in susceptibility to tracheal mites compared to those raised in normal sized cells. The experiment did demonstrate that native Irish bees of the strain *Apis mellifera mellifera* had no difficulty in making comb and raising brood in small cell sizes of the mid 1800s. Also, the reduction in size of these bees was much less than proportional to the cell-size change, contrary to widespread belief of proportionality. Furthermore, the fill factor (ratio of bee thorax to cell width) was much higher for these bees compared with other strains in published data.

The mite biology model indicated that the mites in Ireland have a more aggressive reproductive rate than those recently encountered in the US, while the infestation model showed that bee strain was the major and significant determinant of susceptibility. Bees that were most strongly of the *A. m. mellifera* strain were most susceptible. The study of the heavily infested tracheal mite colony demonstrated that tracheal mite infestation can cause colonies of bees to die in the late winter/early spring period. There was a significant correlation between the number of bee deaths outside the hive when attempting winter defecation trips and the presence of sunshine. A comprehensive qualitative mortality model was developed based on the results of the experiments and studies carried out and also drawing on published data. This model proposes, for the first time, that colonies heavily infested with tracheal mites will die out in late winter/early spring period due to their inability to thermoregulate the colony. Finally, based on the research, a number of non-chemical techniques were proposed to help the beekeeper manage tracheal-mite-infested colonies in the field.

Acknowledgements

I would like to thank Trinity College for the opportunity to undertake this research. In particular thanks is due to my supervisor, Dr. Mark Brown, for 'taking on' a mature student and providing continuous advice and assistance. Mark, many thanks for everything. Thanks to the Heads of Department for their encouragement; during my first year Dr. Julian Reynolds, and for most of my tenure Professor Celia Holland and for her strategic decision regarding the incubator that made life a lot easier. Dr. Ken Irvine chaired those annual PAC meetings and was generous in his counsel and encouragement. Peter, Alison, Martyn, and Richard were willing to answer my calls for help at short notice and always with a bit of humour thrown in.

Access to colonies of members of Fingal North Dublin Beekeepers' Association was always freely given and, in particular, thanks is due to John and Dorothy Stapleton who effectively gave me 'the run' of their farm in North County Dublin.

I would like to thank the International Bee Research Association in Cardiff for their many searches and copies of old and obscure honeybee papers and in particular Richard Jones and Barbara Chick.

That I was a mature student there can be no doubt, but it was to the credit of my postgraduate colleagues (and Zoology Department) that I kept forgetting that I was actually older than the other students. I would like to thank those in parasitology that I came most in contact with, Mario (and Rejane), Samina, Ranjith, Clare, Paddy and Mike for making my time a pleasant one.

Lastly, thanks to my family for their continuous support. My wife Rosaleen, my greatest supporter, who encouraged me at every stage. My daughters, Sinéad and Niamh, who didn't pass any comment when their father decided to take a sabbatical and get involved in an unorthodox pursuit. The tolerance shown by my family saw no limits. I started out with a love for the honeybee and arrived at the end of my research with a love for its tracheal parasite, *Acarapis woodi*. I am told that this is common in parasitology research, but when my family saw this attachment to my new *Acarapis* 'family' they must have been worried about my sanity but thankfully showed no surprise.

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CHAPTER 1

General introduction

1.1 Introduction

This research investigates a host-parasite relationship. The host is the well-known and much studied honeybee (*Apis mellifera* L.), a social insect of major ecological and economic value. The parasite, a tracheal mite *Acarapis woodi* (Rennie), is not well known and is poorly understood despite having been discovered in the early 1900s.

Honeybees (overview)

The honeybee is one of about 2 million species of insect and the most researched (Gould and Gould, 1995). Like all bees they are considered to have evolved from wasps and shifted from predation to a nectar and pollen diet (Michener, 1974). Plumose hair and the ability to collect and carry pollen was part of this evolution which is likely to have occurred about 100 million years ago during the Cretaceous period. Due to their ability to handle pollen all bees are classified within the super-family, Apoidea (Culliney, 1983). Bees and plants have evolved together with the plants providing pollen, an essential food of the bee, and the bee providing a pollination service (Winston, 1987). There are currently about 11 families of bee with up to 20,000 living species (Michener, 1974). Honeybees are classified in the social bee family, Apidae and are closely related to bumble bees (Bombinae) and stingless bees (Melliponea). Honeybee colonies are perennial and are classified in the sub-family Apini and in the genus *Apis* which includes the common honeybee (*Apis mellifera*), its closely related Indian honeybee (*Apis cerana*), the tropical giant honeybee (*Apis dorsata*) and the tropical dwarf honeybee (*Apis florea*).

It is considered that *Apis mellifera* originated in the African tropics or sub-tropics during the Tertiary period and moved to eastern Asia and later to the colder European regions. Originally *Apis* was only found in the 'Old World' and was moved by European settlers to the Americas, Australia and New Zealand (Winston, 1987). The honeybee species *Apis mellifera* is made up of a number of geographical strains or subspecies that have developed largely as a result of natural selection as the species evolved from southern Africa and throughout Europe to southern Scandinavia. In

Europe the Northern European dark bee, *A. m. mellifera* is larger than its more southerly counterparts - the Italians, *A. m. ligustica*; the Carniolans, *A. m. carnica* and the Caucasians, *A. m. caucasica.* (Ruttner, 1988a).

Honeybee colonies are perennial due to their ability to thermoregulate and store surplus honey to survive the winter in cold or temperate regions and to maintain colony temperature for brood rearing in the late winter - early spring period.

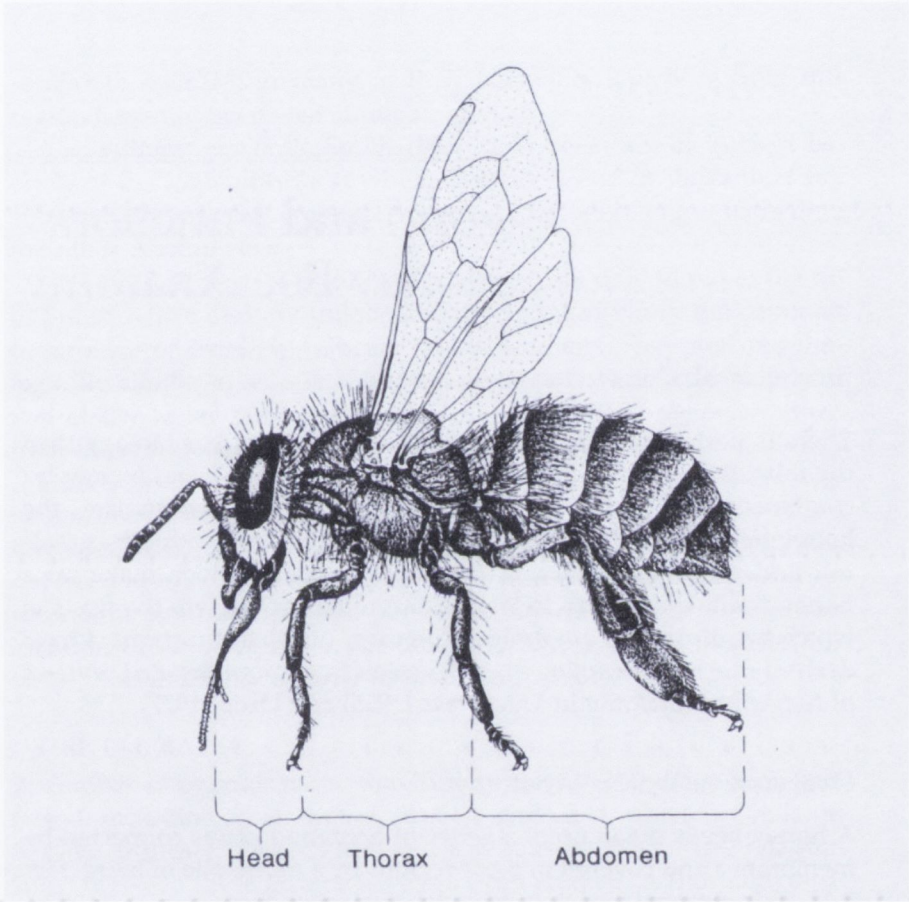


Figure 1.1 The body plan of the worker honeybee showing the three main body parts (*Winston, 1987*).

Individual bees have three main body parts; head, thorax and abdomen (Figure 1.1). The head has highly developed mouthparts and glands for handling, preparing and digesting food and acts as the main sensory region using eyes and antennae (Winston, 1987). The thorax contains the large and powerful wing muscles for flight and heat generation through 'shivering' and has three pairs of legs and two double wings attached. In common with the abdomen, the thorax also contains a large spiracle and tracheal network that channels oxygen to the air sacs of the respiratory system. The abdomen

contains most of the internal organs including the honey sac and rectum. A feature of the rectum is its ability, when the colony is confined to the cluster in winter, to expand to fill a large part of the abdomen in order to hold the accumulated waste matter.

There are three castes in a honeybee colony; queen, worker and drone. There is normally only one queen in a colony and she will mate once and live for several years. The predominant caste is the worker, an unfertile female, and they normally make up over 95% of the bee population in a hive-bee colony that can rise to over 50,000 in summer (Winston, 1987). Drones constitute the male caste and their primary purpose is to fertilise new queens. This caste has a population of a few hundred to a few thousand members and is only typically present in the active spring – summer period. Reproduction is generally by swarming where the old queen departs the hive with about half of the colony leaving behind developing queens in cells. One of these new queens will eventually be mated and head the new colony.

Mites (overview)

Mites are second only to insects in terms of the number of species among arthropods (Eickwort, 1988). However, in contrast to insects they have no wings, eyes or antennae and use their sensory hairs to navigate in their habitat (Figure 1.2). The fused body has two main areas; the gnathosoma (head and mouth parts) and the other part of the body which makes up the idiosoma. The gnathosoma has a pincer-style mouthpart, chelicerae, and surrounding appendages, palps or pedipalps. Four pairs of legs (I–IV) are attached to the idiosoma which contains the respiratory, digestive, reproductive and nervous/sensory functions. *Acarapis woodi* has a tracheal system with a pair of spiracles opening near the gnathosoma (Rennie, 1921). Mites are physically small, normally less than 0.5 mm, but this belies their complexity and their impact on other organisms (Needham *et al.*, 2001).

Mites have recently been classified in the subclass (superorder) Acari (Johnston, 1982). The tracheal mite occurs in the Prostigmata of the order Acariformes (Eickwort, 1988). The genus *Acarapis* occurs within the family Tarsonemidae and has three species, the tracheal mite *A. woodi*, and two closely related and morphometrically almost identical external mites *A. externus* and *A. dorsalis* (Delfinado-Baker and Baker, 1982).

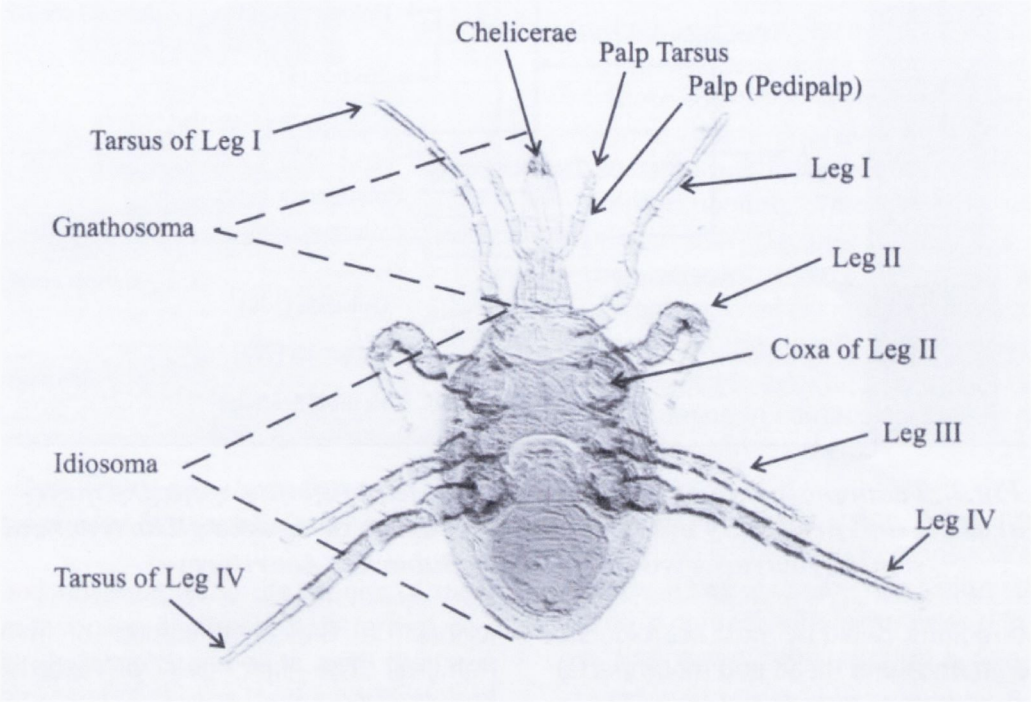


Figure 1.2 Ventral view illustrating the main body parts of a mite, not *Acarapis woodi* (*Photograph Sammataro 2006*).

According to Rennie (1921) the adult female tracheal mite conforms closely with the general character of the Tarsonemidae family.

> *The tarsonemidea are a small family of soft-bodied mites, the females of which are tracheate, and which usually exhibit prominent hairs upon the tarsi of the last pair of legs. The body is more or less clearly segmented dorsally. The mandibles are needle-like, the palps slender and minute. The females possess in most instances, between the first and second pair of legs, a pair of delicate rounded or club-shaped organs which have been designated pseudo-stigmata by OUDEMANS. The legs are short, with six or fewer joints. They are bedecked with a limited number of stout hairs, and terminate in claws. The tarsi of the first pair possess a single claw, the second and third, two.*

Rennie (1921) describes the fourth legs in the tracheal mite, *Acarapis woodi.*

In contrast to what is usual in other species, instead of being long and slender, this limb is moderately stout at its basal segment and slender beyond. But the whole limb consists of only two segments and is much reduced in length. Upon it are four hairs, two of which are terminal and of considerable length. One of the remaining two arises nearer the base, but is usually seen along with the terminal hairs, forming a group of three projecting beyond the body.

1.2 Honeybees as hosts

The honeybee in Europe, *A. mellifera*, was traditionally a cavity dweller living mainly in hollow trees (Gould and Gould, 1995). Today bee colonies generally live in hives. The colony habitat is temperature and humidity controlled with the temperature for a large part of the year, during brood rearing, held at ~34°C. In fact honeybees are the only insects to control their brood-nest temperature (Morse, 1997). Even in winter the cluster temperature is held between 20-30°C (Gates, 1914). The colony maintains quantities of food (honey and pollen) all year round. Large bee populations usually mean that they are living in close proximity to each other. These living conditions make bee colonies attractive habitats for potential parasites. A parasite may be considered as, "an organism living in or on another living organism, obtaining from it part or all of its organic nutriment, commonly exhibiting some degree of adaptive structural modification, and causing some real damage to its host" (Webster's International Dictionary). Accordingly, honeybees play host to a wide range of pathogens or parasites (Bailey and Ball, 1991) that vary in size from the nano-range in the case of viruses to large ectoparasites (> 1 mm) such as *Varroa destructor*.

Viruses have been associated with honeybees for well over 100 years but have only been isolated in the second half of the 20th century (Bailey and Ball, 1991). Many viruses of *A. mellifera* have been detected including; chronic, acute and slow paralysis viruses; sac brood virus; deformed wing virus and Kashmir bee virus. Both brood and adult bees can be damaged by viruses but these viruses are generally most harmful when present with other pathogens and mites e.g. varroa (Downey and Winston, 2001).

Bacteria have been known as pathogens of the honeybee since the 19th century. The two well-known brood diseases American foul brood (*Paenibacillas larvae*) and European foul brood (*Mellisococcus plutonius*) are highly contagious (Shimanuki, 1997). The pathogens are globally widespread and are a persistent cause of colony mortality in some countries.

Fungi can cause considerable damage to honeybee brood. The most prominent form is chalkbrood (*Ascosphaera apis*) which has been present in Europe for a large part of the last century but is presently not considered a serious condition (Heath, 1982). In contrast, chalkbrood has spread to the US, Canada and Australia in the recent past and is still causing widespread damage (Bailey and Ball, 1991).

Microsporidia (until recently classified as protozoa) are spore-forming organisms that infect honeybees globally. The best known of these is *Nosema apis* which develops within the mid gut of the honeybee. It has been present in honeybee stocks for over 100 years but rarely causes major damage (Bailey and Ball, 1991; Fries, 1997).

The two most important parasitic mite species affecting honeybees are the tracheal mite (*A. woodi*) and the ectoparasite (*Varroa destructor*). While there are about 100 mite species associated with honeybees most of these with the exception of the tracheal and varroa mites are harmless (Eickwort, 1997; Sammataro *et al.,* 2000). The tracheal mite has been in Britain and Ireland for at least 100 years and has since spread around the world (for a detailed account see paragraph 1.4). The varroa mite arrived in Ireland in 1998, from mainland Europe *via* Britain, and is an example of a parasite that has changed host species having transferred from *Apis cerana* to *Apis mellifera.* The tracheal mite only affects adult bees while varroa directly affects both brood and adult bees and is currently a virulent disease worldwide. Parasitic mites can act as vectors for microparasites, such as varroa mites' association with various viruses.

From the forementioned it is apparent that the honeybee can offer a favourable habitat for parasites. This habitat has shelter, food, and perennial hosts in large numbers and in close proximity. However, the specific parasite's lifecycle must align or have adapted to that of the honeybee if it is to be able to take advantage of this potential host. Furthermore, the honeybee offers an effective transmission system to allow parasites to

invade new colonies. Horizontal transmission by drifting and robbing and vertical transmission through swarming is part of normal honeybee behaviour (Fries and Camazine, 2001).

1.3 Mites as parasites

The life cycle of parasitic mites can be broken down into a number of critical stages. Normally a female mite must be able to mate, have sufficient offspring to maintain population levels and these offspring must be able to remain on the host long enough to mature and transfer to a new host. The issue of mating and the number of offspring are largely determined by the biology of the mites. Migrating to a suitable host is a precarious process and success is directly determined by the interaction between the mite and its host. This migratory stage presents different challenges for parasites on different hosts.

Invertebrates such as bumblebees have colonies that are annual with only the young queens overwintering. Host caste recognition is therefore important for dispersal of mites. It has been shown that mites preferentially attach to queens and host switching is practiced even during copulation and on flowers where bees of all castes forage (Huck *et al.*, 1998). Furthermore, mites of bumblebees actively discriminate among bumblebee species to obtain host features best suited to their needs (Schwarz *et al.*, 1996).

In the case of mites of the honeybee, the migration of young mated mites to their new breeding sites presents many challenges. *Varroa destructor* mites can only survive for a few days away from the host. During its phoretic phase a mated female mite may fall off, be groomed off or damaged by the honeybee. It must subsequently identify and enter open brood within 2 days of being sealed, feed on the brood food and later on the haemolymph of the prepupa (De Jong, 1997). Eggs are laid and the resulting offspring must mate and emerge from the cell with the emerging callow bee.

The tracheal mite, *Acarapis woodi*, faces many challenges. A new mated female tracheal mite leaves the trachea of its honeybee host and seeks a new young bee in

which to raise its family (Wilson *et al.*, 1997). The old or the new host bee may groom it off or if the process of finding a young host takes too long, it will desiccate on the pleural hair of the bee (for a detailed account see paragraph 1.5).

Understanding this migratory process will form a central part of this research work.

Interactions between mites and honeybees are dynamic relationships. The much shorter life cycle of the parasite is likely to give it an evolutionary advantage over its honeybee host enabling it to adapt faster and, for example, evolve a more virulent behaviour (Hafner *et al.*, 1994; discussed in greater detail in Chapter 9). This evolutionary advantage is already apparent in the case of varroa infestation of *Apis mellifera*. The mite transferred from *A. cerana* to *A. mellifera* and the two species were kept together for 50-100 years in Japan and China, before any colony deaths were reported in *A. mellifera* (Martin, 2001).

1.4 History of tracheal mite infestation

Isle of Wight disease

In 1906 on the Isle of Wight there was widespread mortality of honeybee colonies. The condition, later known as the 'Isle of Wight disease' spread to the mainland and eventually throughout Britain and Ireland over a twenty-year period (Butler, 1945; Adam, 1968). There was much speculation as to the cause, with a bacterium or a protozoan being some of the earlier pathogens considered. In fact the protozoan, *Nosema apis,* which had just been identified in 1907 by Zander (1909) was accepted in 1912 by the Board of Agriculture in Britain as the cause (Adam, 1968). It was not until December 1919 that the team under Rennie in Scotland first discovered a mite in the trachea of the honeybee (Rennie *et al.,* 1921). The tracheal mite was initially classified as *Tarsonemus woodi,* after the benefactor Woods, and later given the generic designation by Hirst of *Acarapis* (Hirst, 1921). *Acarapis* mites are part of the order Acari and the mite became formally known as *Acarapis woodi* (Rennie) (Delfinado-Baker and Baker, 1982).

There was uncertainty in 1921 as to whether the mite was the real cause of the epidemic. The Editor of *Bee World* at the time, Miss Betts, after reviewing Rennie's (1921) paper stated: *The verdict of* T. woodi *(whether or not it was the causative organism of Isle of Wight disease) must therefore be 'not proven', at any rate until more cogent evidence of its guilt is forthcoming* (Crane, 1988). To this day there remains no consensus that the 'Isle of Wight disease' was caused by the tracheal mite *Acarapis woodi.*

While the first major outbreak of the 'Isle of Wight disease' on the Isle of Wight was in 1906 it is generally accepted that the condition first occurred on the island about 1904. In an interesting comment on the origin of the outbreak Adam (1968) relates that, "some ascribed it to several colonies imported from France or Switzerland about four years previously". The spread of the 'disease', up until Rennie *et al.* (1921) had identified the mite, was measured in terms of the spread of a fatal condition afflicting honeybees resembling that which occurred on the Isle of Wight. The disease was believed to have spread to the South of England mainland in 1909 and by 1918 to all beekeeping districts of Great Britain (Herrod-Hempsall, 1937). It had already reached Ireland in mid-summer 1912 when the first infested colony was discovered in an apiary in Dún Laoghaire, Dublin (Watson, 1981). This colony contained bees that were reported to have been imported from England. By August of 1912 it was discovered in Lurgan, County Armagh and continued to spread to all adjacent areas. By March 1921 Watson (1981) stated that it was claimed that 'the country was swept clean of bees'. He further stated that the virulence of the disease in Ireland appeared to peak in 1924 and thereafter reports of colony mortalities were less common.

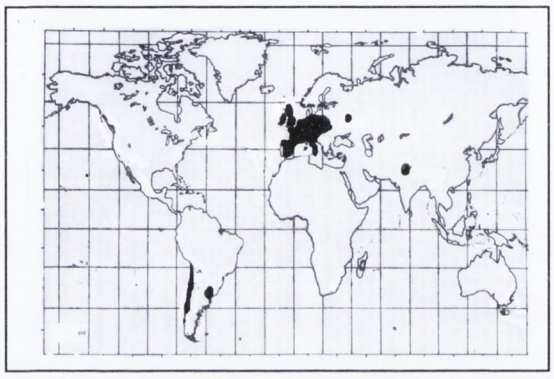
Global spread of mite

The mite was discovered (post Rennie *et al.,* 1921 identification of the mite) in Switzerland and Russia in 1922 and spread rapidly to adjacent countries.

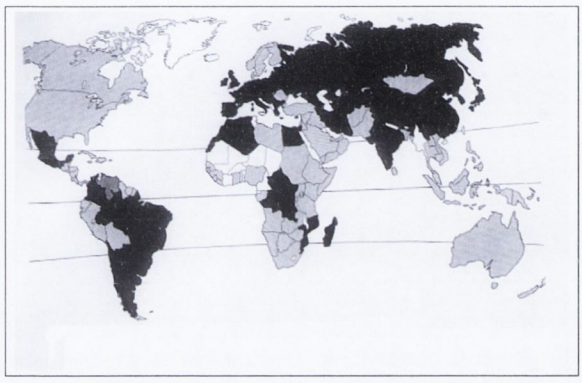
Until 1950 the mite was largely confined to Europe although peripheral countries in Europe were not infested until much later. It reached Argentina in 1951, Uruguay in 1955, and Chile and India in 1956 (Jeffree, 1959). Figure 1.3(a) shows the position in

1959 (Jeffree, 1959). Jeffree suggested that the spread of the mite globally would be limited by climatic conditions, particularly temperature, but this has not been the case. Figures 1.3(b), 1.3(c) and 1.3(d) show the global spread of the mite up to 1982 (Nixon, 1982), 1988 (Bradbear, 1988) and 1993 (Matheson, 1993). The mite has now spread throughout the beekeeping world with a few major exceptions such as Australia, New Zealand, Turkey, Sweden and limited areas of Africa, South America and the Caribbean (Ellis and Munn, 2005).

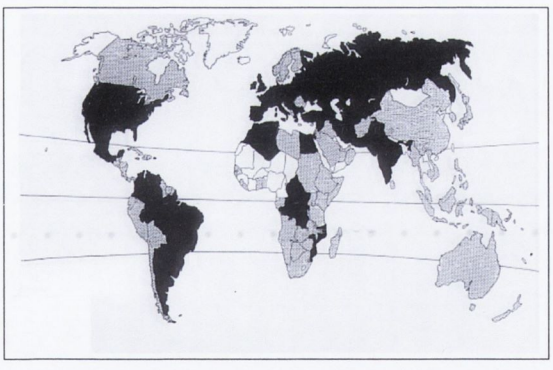
(a) 1959 (Jeffree, 1959)



(b) 1982 (Nixon, 1982)



(c) 1988 (Bradbear, 1988)



(d) 1993 (Matheson, 1993)

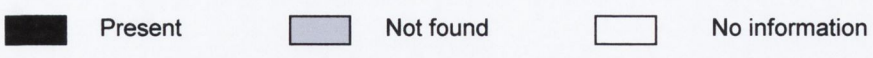


Figure 1.3 Global spread of *Acarapis woodi* infestation 1959 to 1993.

The incidence of *A. woodi* in Britain and Ireland reduced significantly in the period 1925 to 1980 (Bailey, 1985). The usual explanations given for this reduction, according to Bailey (1985), are natural selection of resistant bee strains, various chemical

treatments to control mite infestation and climate change, although he believed that reduced foraging density was the main reason for this reduction. Data from bee diagnostic testing of adult bees undertaken in Ireland by the State Body Teagasc (pers. comm.) suggest that the mite is still widespread. A limited survey that I undertook of 25 colonies during spring 2003 in North County Dublin, an area with a history of tracheal mite infestation, indicated 60% of colonies infested with an average prevalence of 14%. Analysis of bee samples in Britain over the last decade, "from colonies that are in trouble" indicated that almost half of these colonies had tracheal mites with 85% of those infested having prevalence levels greater than 50% (Mike Brown, pers. comm.).

The external *Acarapis* mites were first reported in Switzerland in 1926 several years after the discovery of the tracheal mites. *Acarapis externus,* which resides on the neck, was the first to be discovered. It has also been recorded in Australia, New Zealand, Europe, the former USSR, North and South America, and Africa (Guzman *et al.*, 2001). It has also been recorded on *Apis cerana* in Japan (Bailey and Ball, 1991). *Acarapis dorsalis* resides in the groove on the thorax between the mesoscutum and mesoscutellum. It was found in Britain in 1930 and is more widespread than *Acarapis externus* (Bailey and Ball, 1991). There is no evidence of either of the two external mite ever having been recorded in Ireland. Both forms of the external mites have a wide global distribution and are considered to be present everywhere *Apis mellifera* exists (Morse, 1978). However, they are not considered to be harmful to honeybees.

Response to the infestation

In Britain and Ireland the initial response to the widespread mortality due to the 'Isle of Wight disease' was to restock the colonies with imported bees from the continental mainland. In the case of Ireland, hundreds of skeps of Dutch bees were imported which subsequently demonstrated a dramatic swarming propensity and added little to the solution (Watson, 1981). Following the discovery of the tracheal mites in Europe in 1919, the United States Congress responded in 1922 by prohibiting the importation of all bees. South America allowed bee importation and tracheal mites were first identified in the southern countries in the 1950s and by 1980 the mites were reported in Colombia. In 1984 the mites had reached Texas and seven other States in the same year. Losses of

colonies of up to 90% were recorded only two years after initial infestation (Sammataro *et al.*, 2000).

Another reaction to the infestation was to treat bees with chemicals. The most popular treatments were the 'Frow' mixture (containing nitrobenzene, safrol and petrol), burning sulphur and methyl salicylate (wintergreen). Currently menthol crystals and vegetable patties (made from shortening and vegetable oil) are the most popular treatments in the US (Wilson *et al.,* 2001). The only registered treatment in Britain and Ireland is one based on thymol.

Yet another response was to breed bees with resistance to the mite. There is a natural variation in honeybee resistance to the mite that makes it suitable for selective breeding (Page and Gary, 1990). This has been a large part of the breeding effort of Brother Adam in Devon throughout most of the twentieth century and through his work he has been able to achieve resistance in his 'Buckfast' hybrid (Gary *et al.,* 1990; Danka and Villa, 1998).

1.5 Tracheal mite – honeybee interaction

Mite Biology

The *Acarapis woodi* mite lives within the trachea of its honeybee host and only newly mated females leave to enter a young host bee (Bailey and Ball, 1991). The mites are not visible to the naked eye; the females are120-190 microns long and 77-80 microns wide, while the males are 125-136 microns long and 60-77 microns wide (Sammataro *et al*, 2000).

The mites in the larval and adult stages feed on the haemolymph of the bee by piercing the wall of the trachea. It has not been possible to culture the mites in the laboratory although they have lived for a few days on bee larvae (Giordani, 1967). There are three stages of mite development; egg, larva and adult (Figure 1.4). About one egg per day is laid and these hatch in 3-4 days. Males develop from the egg in 11-12 days while the

females take 14-15 days (Pettis and Wilson, 1996). There are wide variations in published data on the male:female ratio and also on the number of eggs that are laid ranging from 5 up to 21 per female mite (Morgethaler, 1931; Otis *et al.,* 1988; Pettis and Wilson, 1996). All three castes of bees in a colony can be infested.

Impact of mites on the health of honeybees

The spread of tracheal mite infestation appears to have had a similar pattern in each country that it has appeared. Initially there has been widespread mortality of honeybee

Figure 1.4 The *Acarapis woodi* family showing egg, larvae and adult female (bronze).

colonies (Adam, 1987). This eventually peaks and the infestation reduces and stabilises at a reduced level. While the cause of the mortality has not been established, a high correlation has been observed between the health of the bees and the existence of the mites (Rennie, 1921; Adam, 1987). In particular, where bee mortality and evidence of bee crawling on grass were present in a colony of bees it was invariably accompanied by the presence of tracheal mites. The reciprocal of this did not necessarily occur; there were many apparently normal colonies that had high levels of mite infestation (Rennie, 1921; Giordani, 1967; Bailey, 1985 and pers. obser.). It is widely accepted that there are no clinical signs of mite infestation and detection is only by dissection of the trachea

(Bailey, 1964). According to Adam (1987) there are no objective external signs of *Acarapis woodi* until a colony is about to collapse. He believes that a careful observer will, however, be able to predict impending collapse by drowsiness and lack of response in such colonies. Bailey (1985) maintains that the only harmful effect of a tracheal mite infestation was a slight shortening of the lives of individual bees. He also considered that when more than 30% of the bees were infested the chance of the colony dying in late winter was increased, although in reasonable foraging conditions the infestation would typically decrease rapidly in the spring. The death of bees according to Bailey was associated with the chronic paralysis virus that causes rapid death and has the symptoms that were present with the 'Isle of Wight disease'. Giordani (1977) reporting on the Italian experience stated, "From the data it clearly emerges the appreciable losses that uncontrolled acarine disease can inflict, even if other causes have exerted a minor influence. Among the losses one must include the reduced activity and the poor honey yields of the surviving colonies". The mite was first discovered in the US in 1984 and was followed by heavy losses within two years. It has been claimed that heavy tracheal mite loads cause diminished brood area, smaller bee populations, looser winter clusters, increased honey consumption and lower honey yields (Eischen, 1987; Bailey and Ball, 1991; Otis and Scott-Dupree, 1992).

The affect of the tracheal mites on honeybee colonies has been in marked contrast to that of the two external mites. Both external mites also feed on the haemolymph but are generally considered to be harmless parasites of the honeybee. However, *Acarapis externus* has been reported to cause wing loss and abnormality (De Jong *et al.*, 1982).

Summary of mite – honeybee interaction

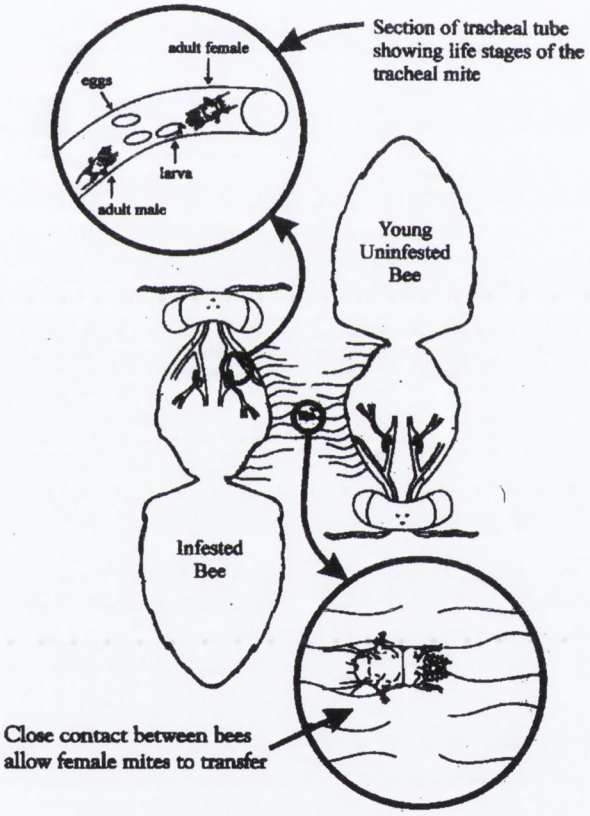
The generally accepted position on the interaction between mites and honeybees is summarised below.

- Mites only disperse to bees under 9 days old and there is a rapid fall off in attractiveness after one or two days old (Morganthaler, 1930, 1931; Lee, 1963; Gary *et al.*, 1989).

- Mites transfer from old host bees to new host bees via the bees' plumose hair Figure 1.5 (Rennie, 1921; Morganthaler, 1930).

- Susceptibility is influenced by bee genetics (Adam, 1968) and this is largely determined by the bee's grooming ability although other unknown factors may also be involved (Danka and Villa 1998).

- Female mites mostly migrate when the host bee is 15-25 days old. (Pettis and Wilson, 1996).



(Diagram, Pettis J., 2001)

Figure 1.5 Dispersal of mated female *Acarapis woodi* mites.

- Mite prevalence in bee colonies increases in autumn and winter but decreases abruptly in the spring (Bailey and Ball, 1991; Otis, *et al.,* 1988).

- High winter prevalence increases the likelihood of mortality (Bailey, 1961; Eischen, 1987; Otis *et al.*, 1992).

- *Acarapis woodi* infestation has no clinical signs (Bailey, 1985; Adam, 1987).

- There is evidence of heavily infested colonies behaving normally (Bailey, 1985; pers. obser.).

1.6 Areas addressed in this research

Aims of research

Despite extensive study of the tracheal mite at different times over the last one hundred years, many areas relating to infestation are still poorly understood. The first period of study was in the early part of the last century following the outbreak of 'Isle of Wight disease' in Britain, Ireland and later in mainland Europe. After the first flush of disease the mortality levels reduced and settled down at a much lower level. The reason for the mortality in the first place still remained a mystery. As stated earlier, Bailey (1985) considered that the tracheal mite was not a serious threat to bees. However, it was not found in North America until the mid 1980s and over the next ten years bee colonies were devastated. A lot of research was undertaken in America in the aftermath of this mortality but much of this centred on finding treatments rather than in trying to understand the underlying mechanisms of infestation and the basic biology involved. The level of mortality again reduced after an initial flush and this reduction was accompanied by a reduction in the research effort leaving many questions unanswered.

While *Acarapis woodi* is no longer considered as serious a disease of honeybees as it once was, improving our understanding of the condition is important:

- while lack of understanding exists the condition is still a potential threat to bees both in presently infested as well as new countries.
- in particular, the mite is likely to spread to other major beekeeping areas such as Australia and New Zealand with the normal pattern of initial devastation.
- colonies infested with the tracheal mite have no external signs and colony deaths due to this 'silent' killer will go undiagnosed.
- the insidious nature of the mite infestation means that it is still doing damage to honeybees, in many cases without mortality, on a widespread basis unknown to beekeepers.
- the use of chemical treatment for tracheal mites is globally widespread.
- overcoming the problem primarily through breeding for resistance has the inherent danger of promoting resistance characteristics at the expense of other desirable traits.

The aims of this research are therefore broadly based;

(i) to improve understanding of the factors that influence the susceptibility of honeybees to tracheal mite infestation.

(ii) to use this improved understanding to develop a comprehensive approach to explain colony infestation and mortality.

(iii) to propose methods to manage infested colonies in the field.

Research work undertaken

A series of inoculation experiments was carried out as well as behavioural experiments to assess tracheal mite response to honeybees with different treatments. Using the data from these experiments and from evaluating heavily infested honeybees in the field, models were developed to explain the dynamics of the interactions between mite and honeybee including why colonies with tracheal mite infestation die. Finally, the implications of these findings for the beekeeper in the field are addressed.

(i) Factors affecting susceptibility:

The major part of this research deals with the factors affecting susceptibility of honeybees to tracheal mites (Figure 1.6). Research over the past one hundred years has essentially established two factors that have a prominent influence on susceptibility – the bee strain and the age of the bee (*Summary of mite – honeybee interaction,* above). The strain of the bee was suspected at an early stage although this was not universally accepted (Previous references). In more recent times the grooming ability of bees has been identified as the major genetic characteristic responsible, although other factors may be involved. The age of the bee is widely accepted as being a critical factor with newly issued (callow) bees being particularly vulnerable.

The research described here considers other possible factors in susceptibility, factors that have not been considered in any previously published work (Chapter 3-7). The pupation temperature and the possible associated mechanisms of autogrooming and chemical cues are considered. Two other possible factors, the influence of relative humidity and the brood comb-cell size of the bee colony, are also evaluated.

(ii) Modelling:

The basic biological data gathered during my investigation of the susceptibility factors are included in biology models (Chapter 8). These contain analysis of the reproductive biology of tracheal mites and also on the effect of pupation temperature on honeybee brood development.

Infestation levels in honeybee colonies can undergo rapid fluctuations yet no modeling of this behaviour, to my knowledge, has ever been carried out. Accordingly, I have undertaken modelling analysis to obtain an understanding of the dynamics of infestation (Figure 1.6). An infestation model has been developed using the data from the experiments undertaken during this research (Chapter 9).

A mortality model has been proposed to explain why colonies infested with tracheal mites die (Chapter 10). There is no consensus in this area; in fact other than viruses no suggested cause has been put forward in published work.

(iii) Beekeeping practice:

Where action is taken by beekeepers in response to tracheal mite infestation it tends to involve the use of chemical treatment. Some bee breeding for mite resistance has taken place particularly in the UK by Adam during a large part of the twentieth century and in the 1990s in the US.

A number of non-chemical steps to colony management that can be undertaken by the beekeeper are proposed in Chapter 11 (Figure 1.6). These include findings from the experiments and work that I have undertaken on colonies over the period.

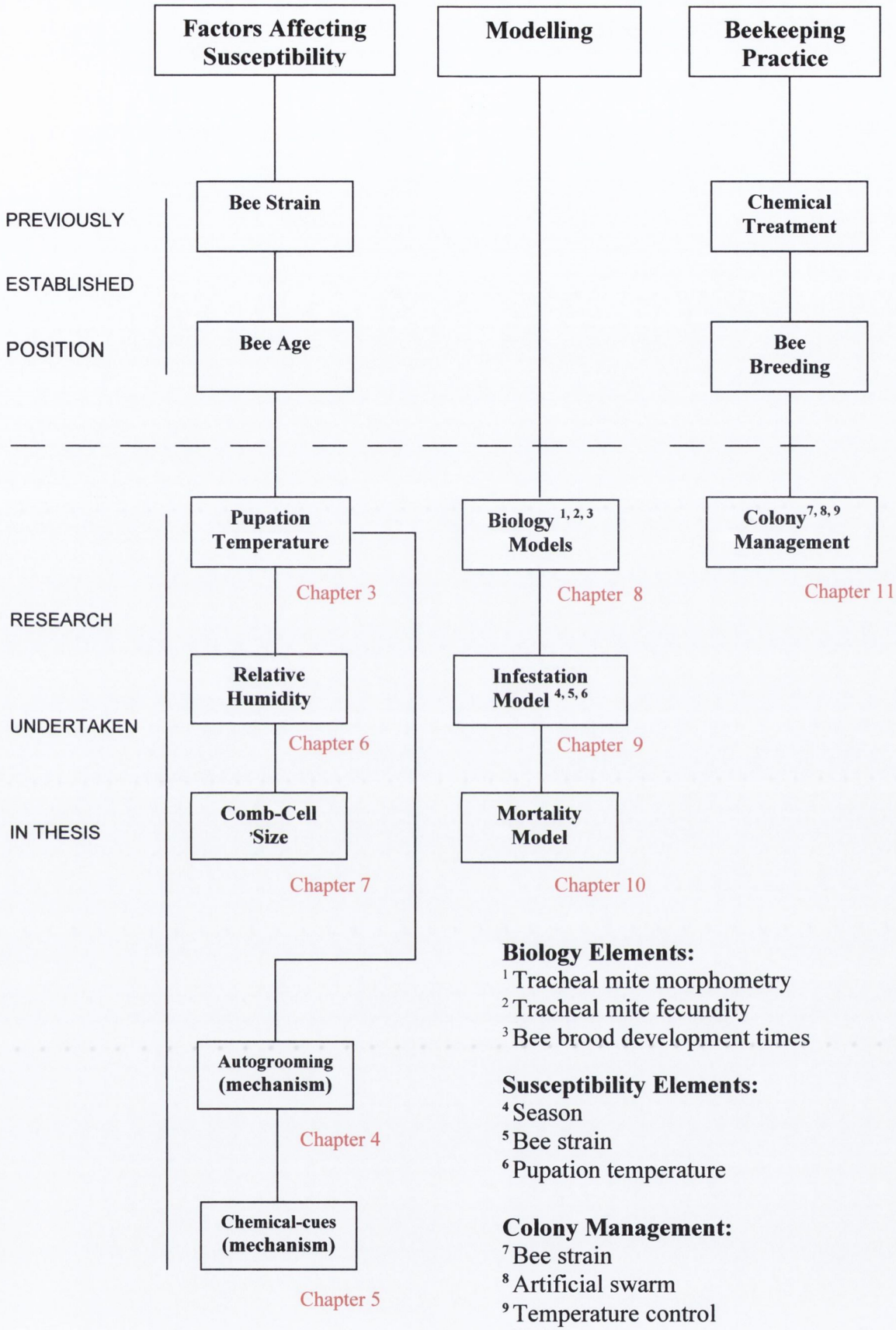


Figure 1.6 Structure of the research work undertaken

CHAPTER 2

General materials and methods

2.1 Introduction

Many of the materials and methods used in the experiments are similar and to avoid repetition common areas are detailed in this chapter.

2.2 Laboratory bioassay

The inoculation process used a cage of mite-infested bees into which were introduced callow (newly-emerged) worker bees to be tested. A similar approach had originally been used by Gary and Page (1987) to measure migration and reproduction of mites. Throughout this research the infested bees will be referred to as 'host' bees and the callow bees to be tested as 'target' bees (terminology adapted from Gary and Page). Previous experiments on honeybee susceptibility have shown a good correlation between the results from short-term bioassays and from field colonies (Page and Gary, 1990; Danka and Villa, 1996; Nasr *et al.*, 2001).

The inoculation cages were 16 cm in height and 6.5 cm in diameter (giving a total volume of 531 cm^2) and were made from mesh screen wire of 3.1 mesh to the cm. A piece of old brood comb (sterilized in 80% acetic acid fumes and well ventilated) measuring 5 cm x 4 cm x 2 cm was suspended from the top of the cage (Figure 2.1).

2.3 Host bees

The host bees came from a colony of infested bees from the same or adjacent apiary. Hence the strain of mite is assumed to be the same in all cases. The mite prevalence can be increased in a lightly infested colony by removing a large proportion of its emerging brood frames a month prior to an experiment. This has the effect of increasing the concentration of mites in the callow bees that emerge from the remaining frames. In the first experiment (Chapter 3) bees were taken from the crown board (inner cover) and the

landing board, shaken into a holding box and given a light misting of water. Host bees, measured by volume, were introduced into the inoculation cage using a funnel. This proved to be cumbersome and crude in terms of handling the bees and estimating host bee numbers. To overcome this a cordless car vacuum cleaner (Black and Decker, Dustmaster V9650) was modified, based on the design of Gary and Lorenzen (1987) and this facilitated the collection of the host bees directly into the inoculation cages (Figure 2.2).

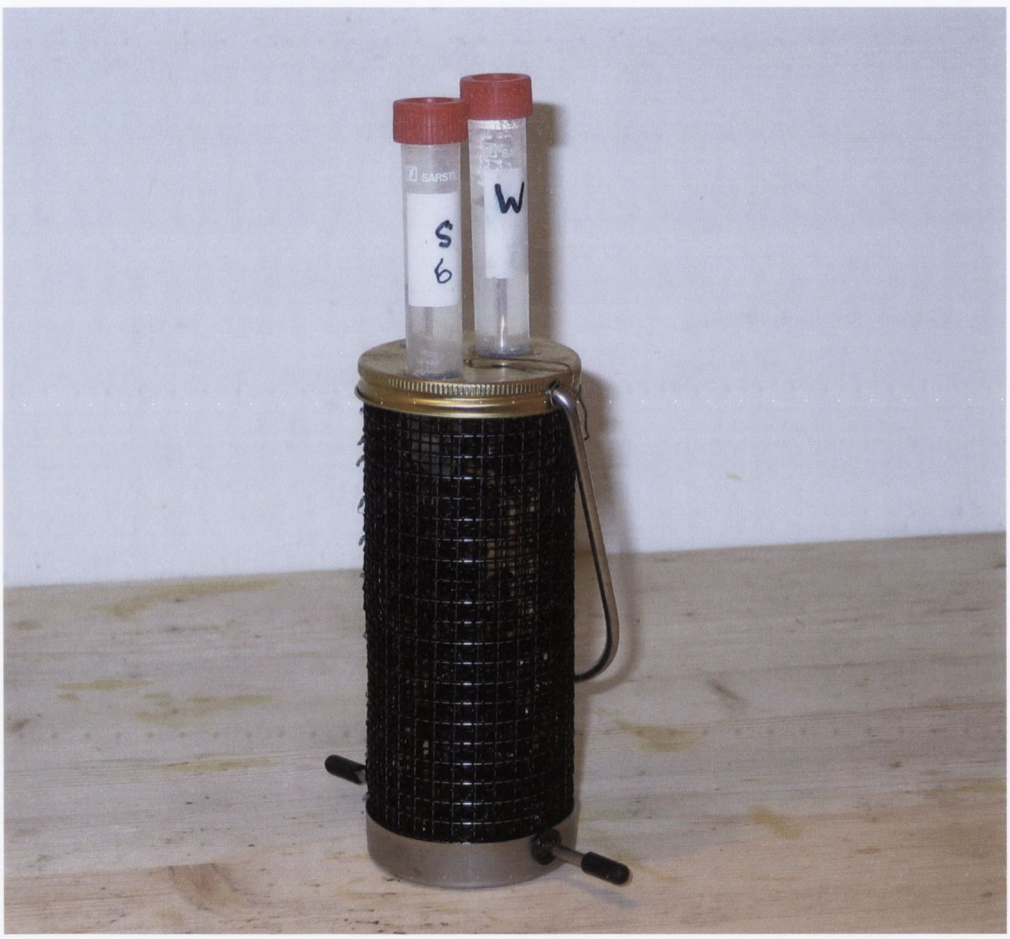


Figure 2.1 Photograph of inoculation cage showing feeding vials for sugar-syrup and water on top.

Figure 2.2 Photograph of a modified cordless car vacuum cleaner (Black and Decker, Dustmaster V9650) showing an inoculation cage inside the perspex tube to enable direct filling of the cages with bees.

2.4 Target bees

The colony from which a sample of test (callow) bees was to be taken had a frame of brood with the adhering bees brushed off, removed from the hive and inserted into a separate perforated stainless-steel frame cage (Figures 2.3 and 2.4). The frame cage was put into insulated nucleus boxes, transported to the laboratory and transferred to a dark incubator within half an hour of leaving the hive.

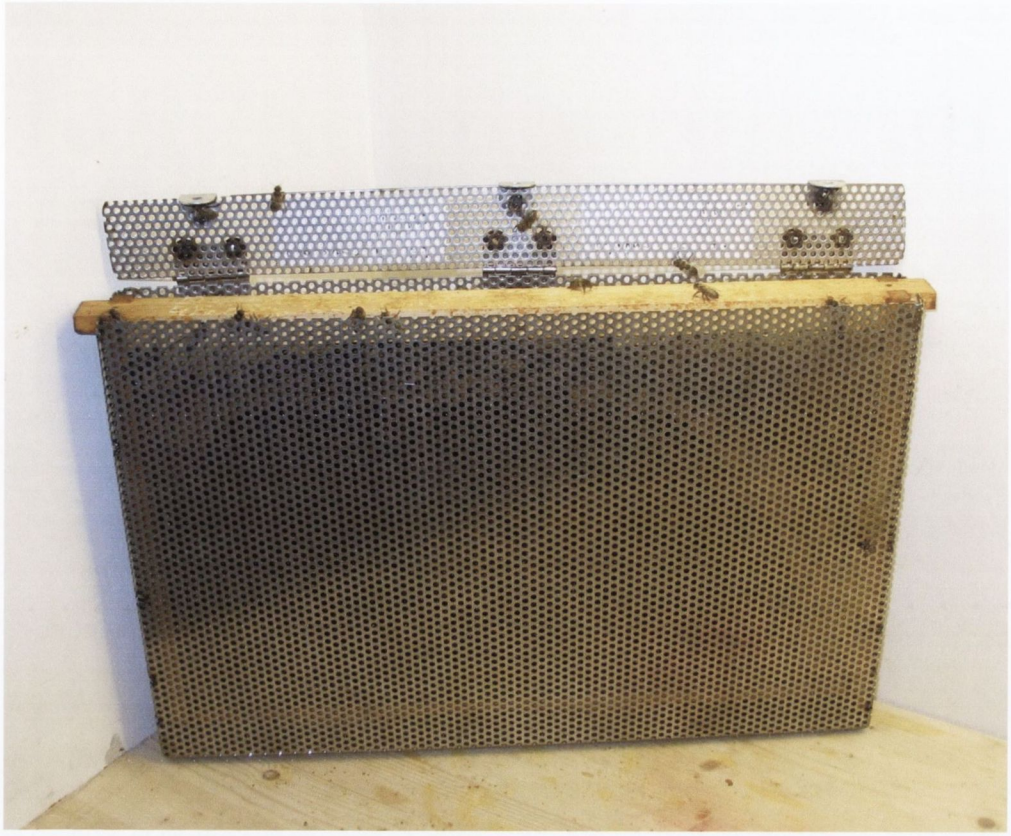


Figure 2.3 Photograph of a frame cage made from perforated stainless-steel sheeting, showing the top bar of the hive brood frame and the hinged top.

Figure 2.4 Photograph of a hive brood frame showing brood in the centre and sealed honey (stores) on top and side of the frame

Callow bees were collected at eight-hour intervals from the frame cages and kept in separate polystyrene cups that had a smear of petroleum jelly along the top to prevent them from climbing out (Figure 2.5). Bees were chosen randomly from the polystyrene cups and (using surgical gloves to handle them) a 1-2 mm spot of Testor's™ gloss enamel paint was applied to the V–VI tergites on the abdomen of the callow bees; i.e. six different colours were used to mark the target bees when six brood frames were involved. Previous work has shown that there is no effect on mite migration of marking in this way (Smith *et al.*, 1991). The marked callow bees were introduced into the inoculation cages using a funnel.

Figure 2.5 Photograph of inoculation cage and six coloured polystyrene retaining cups.

2.5 Inoculation process

The inoculation cages in normal mode were maintained in a dark incubator at 32°C and 50% RH. The bees were provided with sugar syrup (50%) *ad libitum* and water via

gravity feeding vials on the top of the cages (Figure 2.1). A supply of freshly gathered pollen was available on the floor of each cage. This pollen was delivered through the vial-feed openings on top thus enabling access even when dead bees gathered on the floor. The experiment was normally concluded after 7 days, based on the evidence that the first adult mites to mature are the males after 8 days (Pettis and Wilson, 1989). Hence after 7 days the only adult mites present are the founding females. The dead bees were removed from the bottom of the cage, segregated on the basis of colour and counted. The live bees were subdued with CO_2, held at 0°C (only held on ice for 4–8 hours in Chapter 3 experiment), and then taking each cage in turn were segregated into host and the different categories of target bees, and counted. A sample of sixty host bees was randomly chosen from each cage, placed in numbered boxes (Figure 2.6) and stored at -30°C. The target bees from each cage were also placed in numbered boxes and stored at -30°C to await dissection.

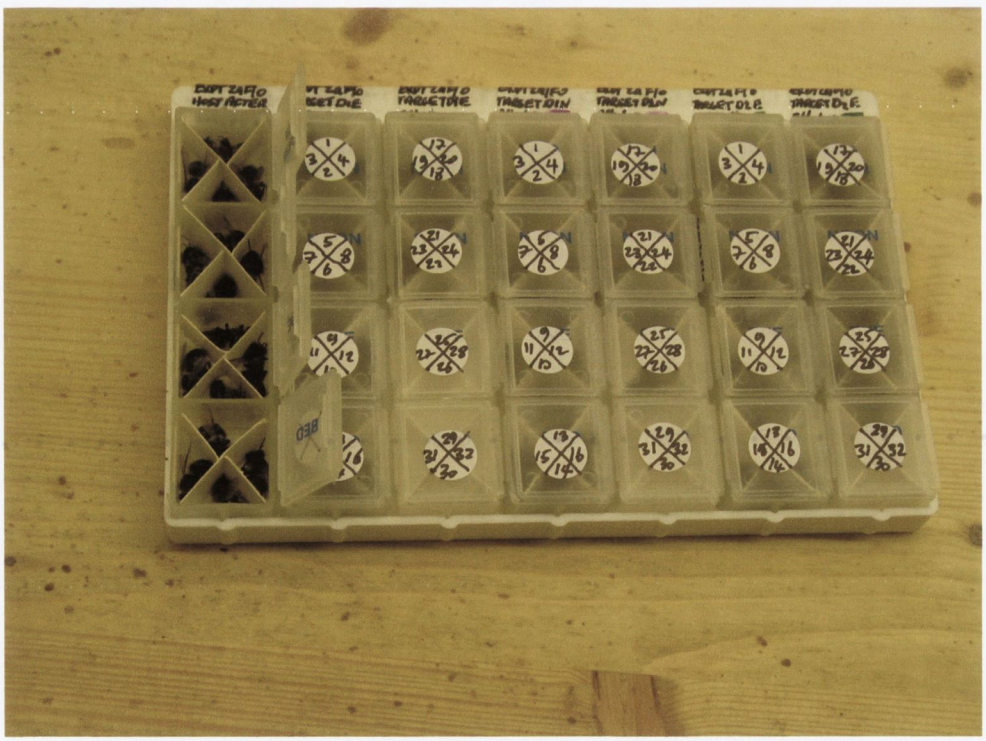


Figure 2.6 Photograph of numbered storage box for host and target bees. The boxes are modified pill boxes with each pill compartment split into four.

2.6 Dissection technique

The thoracic disk method is the most commonly used technique of dissecting bees for tracheal mite examination of large numbers of bees (Shimanuki and Knox, 2000). This method requires the use of 5-10% potassium hydroxide solution, and incubation of the disks for 16–24 hours at 37°C to clear the bees' tissues. A preliminary test of this technique was conducted but was abandoned because of its inability to provide accurate estimates of mite intensity. Difficulty in distinguishing the different stages of mite development was also a major problem.

The bees were dissected using a modification to the classic technique as described by Shimanuki and Knox (2000) of removing the head and thoracic collar (Figure 2.7).

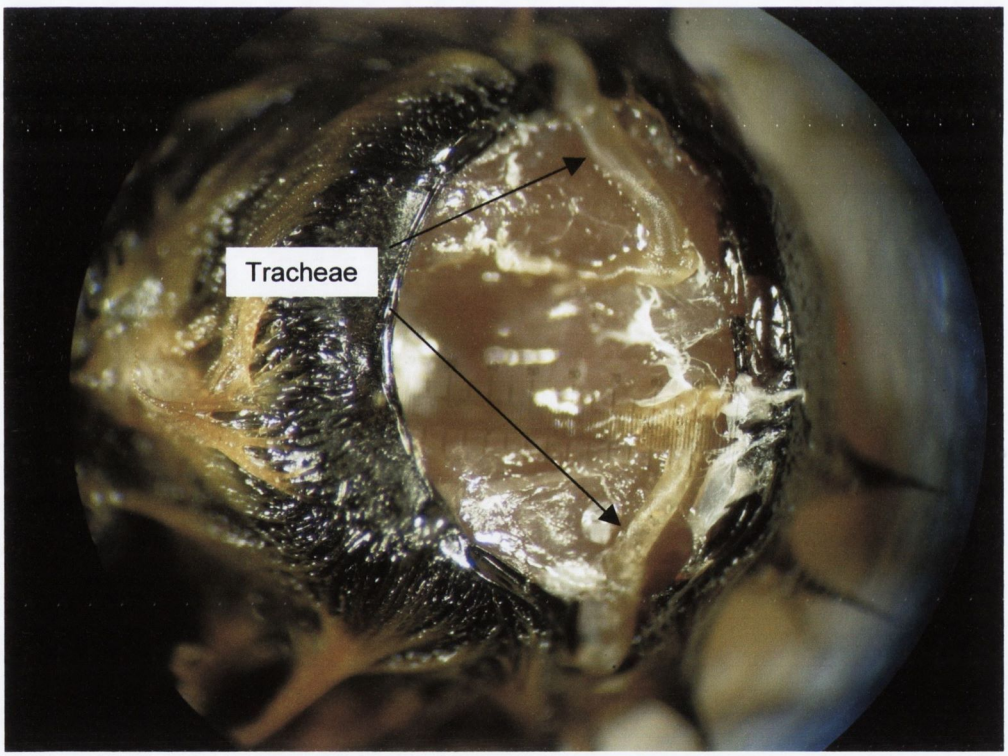


Figure 2.7 Photograph of a bee after removal of the head and pronotum (thoracic collar) to expose the prothoracic tracheae.

The prothoracic tracheae were removed and placed on double-sided tape attached to a glass slide. In order to protect the trachea at the spiracle end (to avoid bursting eggs or damaging larvae clustered behind the operculum) a modification was introduced whereby part of the adjacent cuticle (including the spiracle) was cut with scissors and left attached to the trachea. In all cases an effort was made to retain the maximum length of trachea beyond the first branch. Under a stereomicroscope at 60-100X magnification (Figure 2.8), each trachea was opened with a dissecting needle and the number of adult mites, larvae and eggs were counted and recorded.

Figure 2.8 Photograph of a stereomicroscope magnification (20-100X) being used during dissection of bee tracheae on double-sided tape on a glass slide.

2.7 Wing morphometry

The strains of the honeybees were determined using wing venation parameters; (i) cubital index and (ii) discoidal shift as indicated in Figure 2.9.

(i) The cubital index is the ratio of BC to CD on the cubital cell of the forewing (Figure 2.9). It was measured using the Beemorph© program and gives an assessment of the genetic strain of the bee colony (Ruttner, 1988a, b). A mean sample value less than 1.9 and individual bees under 2.2 are taken as indicative of *A. m. mellifera*. in the Breeding Regulations of the German B.K.A. (D.I.B.) 1986.

(ii) The discoidal shift is the angle (in degrees, negative angles are measured clockwise) between the perpendicular on the radial cell length through node A and the line through node E (Figure 2.9). It was also measured using the Beemorph© program. A negative value (as illustrated) is indicative of *A. m. mellifera* while in the case of *A. m. ligustica* it is positive, and positive or zero for *A. m. carnica* (Ruttner, 1988a, b).

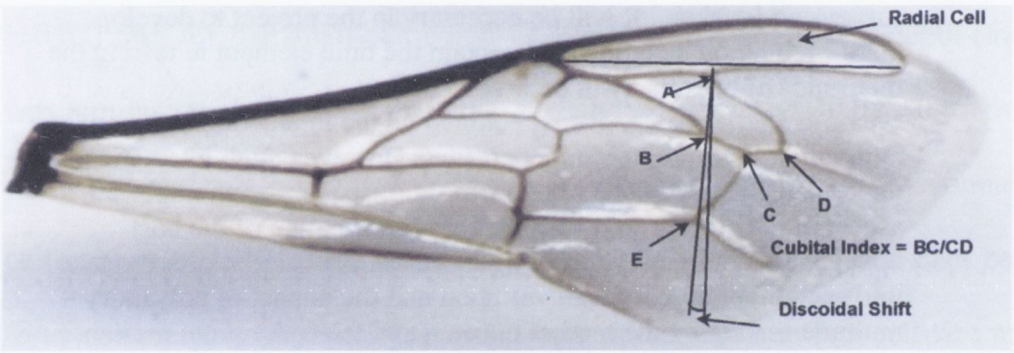


Figure 2.9 The right forewing of a test honeybee showing the measurement of the cubital index and discoidal shift.

2.8 Definitions

The infestation terms used in this thesis are defined below, following Bush *et al.*, (1997).

: Prevalence; number of infested bees in a sample (expressed here as a %).

: Abundance; total number of mites per bee in a sample.

: Intensity; total number of mites per infested bee in a sample

: Mean fecundity; mite offspring per female mite over a given time period.

CHAPTER 3

Influence of brood pupation temperature on honeybee susceptibility to tracheal mites

An extract from this chapter has been published:

McMullan J.B. and Brown M.J.F. (2005) Brood pupation temperature affects the susceptibility of honeybees (*Apis mellifera*) to infestation by tracheal mites (*Acarapis woodi*). *Apidologie* **36:** 97-105.

3.1 Introduction

Recent research on honeybee infestation by tracheal mites has concentrated on the use of chemicals to control infestation in colonies or on the influence of bee strain in providing resistance to the mite (Wilson *et al.,* 1997). Little recent work has been undertaken to try to improve understanding of the dynamics of the interaction between mite and honeybee.

It has long been established that foundress female mites prefer young, newly emerged bees and that resistance in all bees increases with age (Morgenthaler, 1930; Hirschfelder and Sachs, 1952; Bailey, 1958; Lee, 1963; Giordani, 1977; Gary *et al.*, 1989). What additional factors, other than honeybee strain, might affect variations in susceptibility to to infestation? One potential factor is temperature stress during pupation. In other biological systems stress has been shown to play an important role in susceptibility (Maurizio, 1934; Saarinen and Taskinen, 2005). There are certain indications that brood temperature may have a similar effect in the honeybee-mite interaction. First, deviations from normal brood temperature delay or speed up the rate of emergence of callow bees. The susceptibility of a honeybee to tracheal mite infestation is at its peak at emergence and reduces rapidly with age (Morgenthaler, 1930; Gary *et al.*, 1989). This critical initial condition of the bee may be influenced by an abnormal rate of development. Second, mortality in tracheal mite infested colonies is highest in cold climates (Eischen, 1987, Otis and Scott-Dupree, 1992) and this mortality typically occurs in the temperature-stressed late winter/early spring period. While healthy colonies of *Apis mellifera* have good regulation of brood temperature, it is also known that in adverse conditions the brood temperature held by the bees can be well below typical levels. Kronenberg and Heller (1982) established that in adverse conditions the bees maintained the capped brood and uncapped brood cell mean temperatures (± s.d.) at 31.3°C (± 1.9°C) and 27.5°C (± 4.1°C) respectively. Owens (1971) demonstrated that at an ambient temperature of -14°C the temperature within the brood area ranged from 29.5-34.5°C. Conversely, in normal conditions bees that are not healthy would have difficulty regulating brood temperature through reduced bee numbers and restricted ability of wing muscles to generate heat. The rapid collapse of colonies that die with

A*carapis woodi* infestation may be partly explained by a relationship between reduced brood temperature and an increased susceptibility to infestation.

Here the hypothesis is tested that callow bees from brood pupated at a low temperature are more susceptible to tracheal mite infestation than brood pupated at a more typical temperature.

3.2 Materials and methods

The experiment was undertaken in the March/April period 2003 using bees from colonies in North County Dublin. The experimental design is shown in Figure 3.1.

Host Bees

The infested bees were taken from the crown board (inner cover) and the landing board of the host colony, shaken into a holding box and given a light misting of water.

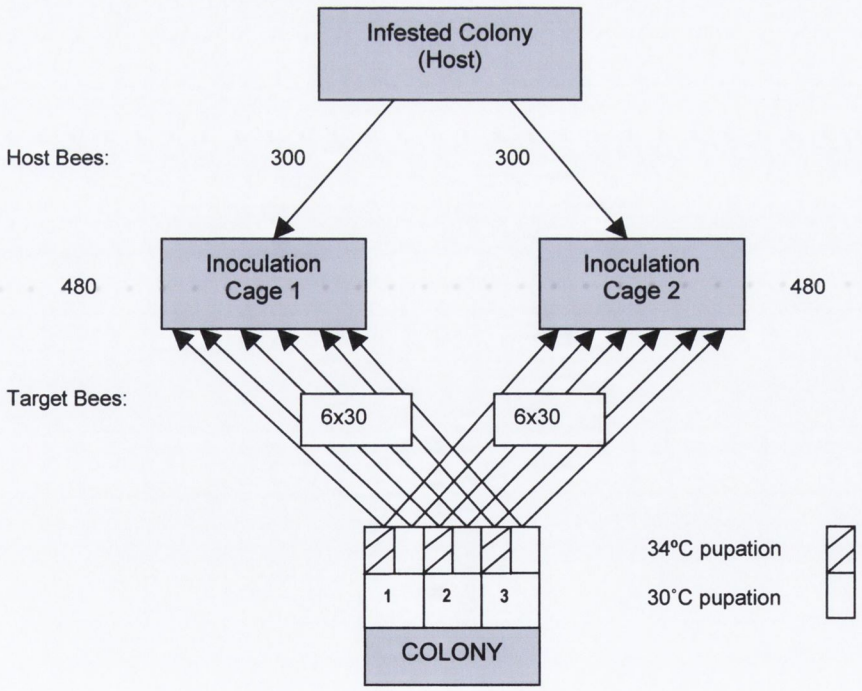


Figure 3.1 Schematic of the experimental design.

Each inoculation cage was filled in turn with an estimated 300 infested worker bees (Figure 3.1). The bees, measured by volume, were introduced into inoculation cage 1 using a funnel. A sample of sixty bees was taken from the holding box, placed in numbered boxes and stored at -30˚C. The bees were later dissected to give a measure of the host bee infestation in cage 1. The procedure was repeated for inoculation cage 2.

Target Bees

The target bees were obtained from three medium/strong colonies (bees covering 7-10 frames of a 12 frame brood box) from three different apiaries in North County Dublin. One apiary was located in an urban coastal area, another in a mixed urban/rural area 3 kilometres from the coast and the third in a rural area 7 kilometres from the coast (Appendix 1). A frame of brood with the adhering bees brushed off was taken from each of the three colonies on 27th March 2003 and transferred to a dark incubator at 30˚C and 50% RH. For the following 9 days callow bees were removed morning and evening from each of the three frame cages. These bees were not included in the experiment. On 5th April (9 days later) a second frame of brood, adjacent in the hive to the frame already removed, was taken from each of the three target colonies and incubated at 34˚C. The brood temperature of the three target colonies had been monitored while the frames were in the hive to ensure that the brood was being pupated at the normal hive temperature (34˚C) for the preceding 9 days. Temperature readings, median (range) of 34.4ºC (± 0.5ºC), 34.6ºC (± 0.7ºC) and 34.6ºC (± 0.5ºC) for colonies 1, 2 and 3 respectively were recorded during the period. From sample readings, the relative humidity in the hives was in the range 54% (± 10%). Consequently, because pupating bees are isolated from all other aspects of the hive environment, the only difference between the control and treatment bees was the temperature at which they were pupated, with the control bees pupating at 34ºC for 9 days and the treatment bees pupating at 30ºC for 9 days.

At eight-hour intervals over the next 24 hours callow bees emerging from the six frames were collected in separate polystyrene cups, marked with six different colours. Over the 24-hour period, thirty marked bees from each of the six frames were introduced by funnel into each of the two inoculation cages giving a total of 180 marked bees in each cage.

Inoculation Process

The inoculation cages were maintained in a dark incubator at 32°C and 50% RH. After 7 days the dead bees were removed from the bottom of the cage, segregated and counted. The live bees were held on ice for 4-8 hours, and then taking each cage in turn were segregated into host and the six categories of target bees, and counted. The target bees from each cage were also placed in numbered boxes and stored at -30°C to await dissection.

Dissection Technique

The thoracic disk method was initially considered but was discontinued because of its inability to provide accurate estimates of mite intensity. The bees were dissected using a modification to the classical method of removing the head and thoracic collar (Chapter 2, Section 2.6). The number of adult mites, larvae and eggs was counted and recorded.

Statistical Analyses

To test the effects of brood temperature, inoculation cage, colony and their interactions on the likelihood of a target bee becoming infested by tracheal mites (mite prevalence), a binary logistic regression was used. The forward log-likelihood procedure was applied. Brood temperature was coded as an indicator variable, while cage and colony were coded as deviation variables. The mite abundance data tended to have variances in proportion to the sample means and were transformed to $\log_{10}(y + 1)$ to homogenise variances. The mite abundance data were analysed in a 3-way ANOVA, with brood temperature, cage and colony as fixed factors.

3.3 Results

Mite Prevalence

The brood temperature had a significant effect on mite prevalence (Logistic regression: Wald statistic = 46.157, df = 1, $p < 0.001$). There was a two-fold increase in the overall

prevalence from 20% at 34°C to 41% at 30°C. (Tables 3.1, 3.2 and Figure 3.2). There were no significant effects at cage and colony level.

Table 3.1 Summary of infestation levels for host and target bees.

Test	Condition	No. of Bees		Mite Stages				Prevalence
Bees		Total	Infested	Adults	Larvae	Eggs	Total	(%)
Cage 1								
Host:	Before	60	24	129	55	23	207	40
	After	60	26	190	80	58	328	43
Target:								
Colony 1	34°C	28	2	3	2	6	11	7
	30°C	29	9	13	11	27	51	31
Colony 2	34°C	27	6	7	12	18	37	22
	30°C	27	12	14	11	22	47	44
Colony 3	34°C	28	7	7	2	16	25	25
	30°C	25	13	9	16	30	55	52
Cage 2								
Host:	Before	60	16	80	36	20	136	27
	After	60	14	33	12	16	61	23
Target:								
Colony 1	34°C	25	5	7	1	13	21	20
	30°C	26	12	12	5	21	38	46
Colony 2	34°C	30	8	6	9	20	35	27
	30°C	29	8	10	4	26	40	28
Colony 3	34°C	28	6	9	7	12	28	21
	30°C	29	14	9	13	34	56	48

Table 3.2 Summary of mite infestation parameters (mean ± standard errors) for all target bees for brood treatment temperatures of 34°C and 30°C.

Mite Infestation Parameters	Brood Treatment Temperature	
(All bees)	34°C	30°C
Prevalence (%)[1]	20 ± 3	41 ± 4
Abundance (mites per bee)[2]	0.95 ± 0.18	1.74 ± 0.24
Intensity (mites per bee)[3]	4.65 ± 0.51	4.22 ± 0.43
Mean mite fecundity[4]	2.74 ± 0.33	2.73 ± 0.25

The mite infestation terms used are defined as:
[1] Prevalence, number of infested bees in a sample (expressed here as a %).
[2] Abundance, total number of mites per bee in a sample.
[3] Intensity, total number of mites per infested bee in a sample.
[4] Mean fecundity, mite offspring per female mite over a given time period.

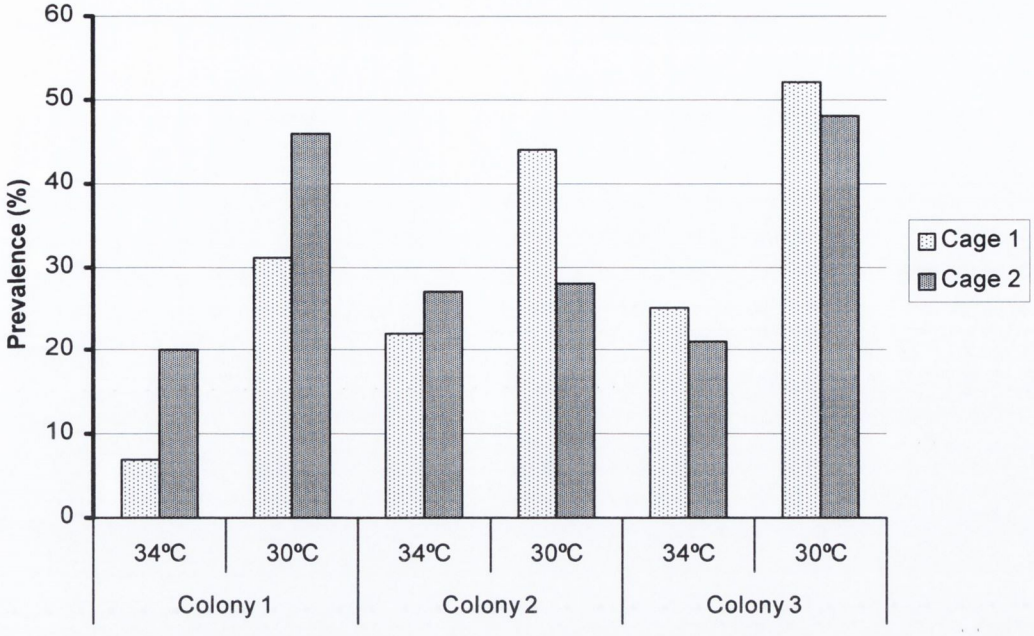


Figure 3.2 Mite prevalence levels (percentage values) for the target bees from the three colonies, after a period of seven days in the two inoculation cages.

Mite Abundance, Intensity and Fecundity

In 17 instances, offspring was found in the tracheae of the target bees but with no associated foundress female present. As in the case of Gary *et al.* (1989) it was assumed that the foundress females had migrated further into the trachea and were not detected during the dissection. Accordingly, in these cases I assigned one female mite to the offspring to give an adjusted female mite abundance (Table 3.3).

Table 3.3 Fecundity analysis for all target bees for the two brood temperature treatments of 34°C and 30°C.

Treatment	Female Mites		Offspring [2]			Adjusted Mean	Overall Ratio
Temp. °C	Actual	Adjusted [1]	Larvae	Eggs	Total	Fecundity [3]	larvae / eggs
34	39	43	34	85	119	2.77 ± 0.32	0.40
30	67	80	60	160	220	2.75 ± 0.22	0.38

[1] Actual mites present plus an allocation of one female mite for each occasion that offspring was present with no female mite.
[2] Includes eggs plus larvae.
[3] Adjusted mean fecundity (mean ± SE).

The mite abundance displayed a significant brood temperature effect ($F_{[1, 319]} = 11.278$, $p = 0.001$) while there were no significant effects for colony ($F_{[2, 319]} = 1.717$, $p = 0.181$), cage ($F_{[1,319]} = 0.001$, $p = 0.971$) or their interactions on mite abundance. The overall mite abundance increased by 82%, from 0.95 at 34°C to 1.74 mites/bee at 30°C (Table 3.2). The adjusted female mite abundance also had a significant increase from 0.98 to 1.82 mites per bee ($F_{[1, 319]} = 13.761$, $p < 0.001$). There were no significant effects for colony ($F_{[2, 319]} = 0.684$, $p = 0.506$), cage ($F_{[1,319]} = 0.056$ $p = 0.813$) or their interactions on female mite abundance. The corresponding mite intensities for treatment at 34°C and 30°C were 4.65 and 4.22 mites per infested bee respectively indicating a similar effect ($F_{[1,90]} = 0.318$, $p = 0.574$). The adjusted mean fecundity (Table 3.3) of 2.77 (34°C) and 2.75 (30°C) mites was similar ($F_{[1,111]} = 0.009$, $p = 0.923$).

Larvae vs. egg ratio

The overall ratio of larvae to eggs was also similar for the two treatments at 0.40 for 34°C and 0.38 for 30°C (Table 3.3). This indicated that the temporal distribution of the foundress mites entering the tracheae was similar (assuming that the time for eggs to hatch was the same). Therefore, since the adjusted mean fecundity and the temporal distribution of entry of foundress mites were similar for both treatments, it indicated that the egg-laying rate (fecundity) of the mites was also similar.

Inoculation pressure (Cages 1 and 2)

It was planned to have a minimum of 300 infested host bees in each inoculation cage. However, using a volumetric measurement to estimate the number of water-misted bees resulted in excess numbers of 20% in cage 1 and over 40% in cages 2 (Table 3.4). The number of infested host bees and the total mites present at all stages were significantly lower in inoculation cage 2, even after allowing for the larger number of host bees initially. The resulting inoculation pressure was about four fifths that of cage 1 yet the number of adult females that migrated to the target bees and the total number of mites at all stages in target bees in cage 2 was similar to that in cage 1; 53 vs. 53 and 218 vs. 227 respectively.

Recovery and mortality rates (Target and host bees)

There was a high recovery rate for the target bees in both cages and mortality rates were similar at 7% for cage 1 and 6% for cages 2 (Table 3.4). This was in contrast to the mortality rate for the older host bees that differed widely at 17% for cage 1 and 32% for cage 2. The food consumption per bee in cage 2 was also much lower during the inoculation period (29.1µl vs. 33.3µl; Table 3.4). The above observations, plus the fact that there were initially 20% more host bees in cage 2 and the presence of dysentery on the sixth day, would indicate that these bees were in a more 'stressed' state.

Table 3.4 Survival, recovery and feeding rates for host and target bees.

Bee	Cage 1			Cage 2		
Colony	Alive (%)	Dead (%)	Total	Alive (%)	Dead (%)	Total
Host:	304 (83%)	63 (17%)	367	296 (68%)	142 (32%)	438
Target:						
Brood at 34°C						
Colony 1	28 (97%)	1 (3%)	29	25 (83%)	5 (17%)	30
Colony 2	27 (96%)	1 (4%)	28	30 (100%)	0 (0%)	30
Colony 3	28 (90%)	3 (10%)	[1]31	28 (97%)	1 (3%)	29
Brood at 30°C						
Colony 1	29 (97%)	1 (3%)	30	26 (90%)	3 (10%)	29
Colony 2	27 (96%)	1 (4%)	28	29 (97%)	1 (3%)	30
Colony 3	25 (83%)	5 (17%)	30	29 (97%)	1 (3%)	30
Total (Target)	164 (93%)	12 (7%)	176	167 (94%)	11 (6%)	178
All Bees: Food / bee /day (µl)		33.3			29.1	

[1]30 target bees introduced into each cage except cage1, colony 3 (34°C) where 31 bees were introduced in error.

Brood development time

The reduction in brood temperature resulted in a considerable lengthening of the time period from sealing of the brood cells to emergence of the callow bees. The callow bees used in the experiment had been pupated at 30°C for a period of 9 days. However, pupation of the three brood combs continued until all of the bees had emerged. The callow bees emerged from the brood raised at 30°C within an average time of 18.1 days (ranging from 17.6 to 18.3 days for the three target colonies). This represents an average delay of 5.1 days from the typical emergence time in normal conditions of 13 days. All the bees appeared in a healthy condition. The proportion of bees that did not emerge (dead in the cell) was similar for the bees raised at 30°C (n = 5366) and 34°C (n = 6492) at 1.0% and 1.1% respectively. The target bee mortality in the inoculation cages was similar for both treatments at 12/177 (30˚C) and 11/177 (34˚C). Hence the treatment had a delaying effect but had no mortality effect.

3.4 Discussion

Reducing brood temperature increased the susceptibility of honeybees to tracheal mite infestation by a factor of two. I believe that this is the first experimental demonstration of variation in intra-colonial resistance of bees to tracheal mites. Furthermore, the analysis of mite abundance showed that this variation relates only to the initial establishment of mites, as mother mites produced the same number of offspring regardless of whether their host had pupated at 30°C or 34°C.

These results could help to explain aspects of the epidemiology of tracheal mite infestation in honeybees. There is a significant increase in colony mortality in winter where > 30% of bees in a hive are infested (Bailey, 1961). Colonies that are infested with tracheal mites have no clinical signs (Bailey, 1964; Adam, 1968) and typically no other pathogens are present (Collison, 2001). This has been the recent experience of mortality in tracheal mite infested colonies in Ireland (pers. obser.). Other studies have shown that winter mortality rates in colonies with tracheal mites are generally higher in cold climates (Eischen, 1987; Otis and Scott-Dupree, 1992). Honeybees in a colony

with a moderate infestation will have reduced capability to use their flight muscles to fly in cold weather (Camazine *et al.*, 2002) and to generate thoracic heat due to mites and associated debris blocking the prothoracic tracheae (Komeili and Ambrose, 1991). The combined effects of the reduced ability to generate thoracic heat and the increased susceptibility to mite infestation of a colony under low temperature conditions (per my findings) are likely to be critical factors mitigating against colony survival in winter. Brood areas in winter/early spring have been negatively correlated with mite infestation (Otis and Scott-Dupree, 1992). A colony during this critical period requires an influx of young healthy bees if it is to survive. My results show that reduced brood temperature will increase the vulnerability of callow bees to tracheal mite infestation and the development of the brood nest will be restricted limiting the ability of the colony to adequately replace the old winter bees. Reduced bee numbers will then contribute to lower brood temperature, increased susceptibility, and the colony viability will spiral downwards. The dynamics involved are explored in detail in the mortality model in Chapter 10.

Variability in host bee mortality between cages indicates that the bees in inoculation cage 2 were more stressed than those in cage 1. It is also noteworthy that while the inoculation pressure in cage 2 (as measured by overall mites per host bee) was 20% less, the overall infestation levels in the target bees in the two cages were similar. It would appear that this cage-specific stress increased either migration of mites from host bees or the overall susceptibility of the target bees to tracheal mite infestation. The increased infestation in cage 2 may be as a result of the increased bee number raising the local temperature as Bailey (1958) has demonstrated that increased temperature in an incubator resulted in an increase in mite transfers between bees.

Overall the results from this experiment concur with the conclusions of Danka and Villa (1996) that differential susceptibility is governed by the success rate of migrating foundress mites entering the tracheae and that mite fecundity does not play a major role. Gary *et al.*, (1989) established that bees were most susceptible up to 4 days old, confirming the findings of Morgenthaler (1930, 1931). In both cases the plot of bee susceptibility vs. bee age ('susceptibility curve') showed the susceptibility to the tracheal mites falling off rapidly from the level at emergence to almost zero at a bee age of 5 days. The much higher susceptibility of the bees pupated at 30°C in the experiment

would indicate a 'susceptibility curve' above that for the higher brood temperature with, for example, a much higher susceptibility at emergence or a slower decline with age of bee. The former is suggested, as an analysis of the data on larvae/eggs ratios from my experiment indicated that the temporal distribution of the mites entering the tracheae was similar for both treatments.

The reason why a lower brood temperature results in an increased susceptibility of honeybees to tracheal mite infestation has not been established in this experiment. Experiments are undertaken in Chapters 4 and 5 to isolate the mechanisms involved.

CHAPTER 4

Autogrooming as a mechanism in honeybee susceptibility to tracheal mites

An extract from this chapter has been published:

McMullan, J.B. and Brown, M.J.F. (2006). The role of autogrooming in the differential susceptibility to tracheal mite (*Acarapis woodi*) infestation of honeybees (*Apis mellifera*) held at both normal and reduced temperatures duration pupation. *Apidologie* **37:** 471-479

4.1 Introduction

The reason for the increased susceptibility of bees reared at a lower temperature, demonstrated in Chapter 3, is not known. One possible reason is that the reduced brood temperature impaired the grooming ability of the callow bees. Sammataro and Needham (1996) showed that mated female mites leave the tracheae of their host bees and migrate to new, young host bees via the pleural hair. Pettis and Pankiw (1998) provided evidence that autogrooming reduced infestation and later it was demonstrated by Danka and Villa (1998) as an important defence mechanism against infestation by the tracheal mites. Since honeybees can use their mesothoracic (middle) legs to groom the thorax, restricting the grooming ability of these legs will affect the success rate of female mites entering the tracheae. Danka and Villa (1998) also showed that removing only the lower segments of the legs (mesotarsi) had a similar influence as the complete removal of the mesothoracic legs.

An experiment was therefore designed to test changes in grooming behaviour and to answer the following questions:
(i) Does grooming ability explain resistance to tracheal mites in normally pupated honeybee colonies? and (ii) Is the increased tracheal mite susceptibility of honeybees pupated at a reduced brood temperature (30°C) caused by a reduced grooming ability?

4.2 Materials and methods

The experiment was undertaken in the April/May period 2004 using test bees from colonies in North County Dublin. The experimental design is shown in Figure 4.1.

Host Bees

On the 7th May 2004 each inoculation cage was filled in turn with an estimated 350 infested worker bees collected from the landing board of the host colony. A separate

sample of sixty bees, thirty before and thirty after filling the inoculation cage, was also taken from the landing board of the host colony, placed in numbered boxes and stored at -30˚C. (The bees were later dissected to give a measure of the initial host-bee infestation level in the cage.) This initial infestation level should give a reasonable measure of the relevant inoculation pressure in the cage as most of the mite transfer takes place in the first 1 - 2 days (Morgenthaler, 1931; Bailey, 1958; Lee, 1963; Giordani, 1977; Gary *et al.*, 1989)). The procedure was repeated for the other two inoculation cages.

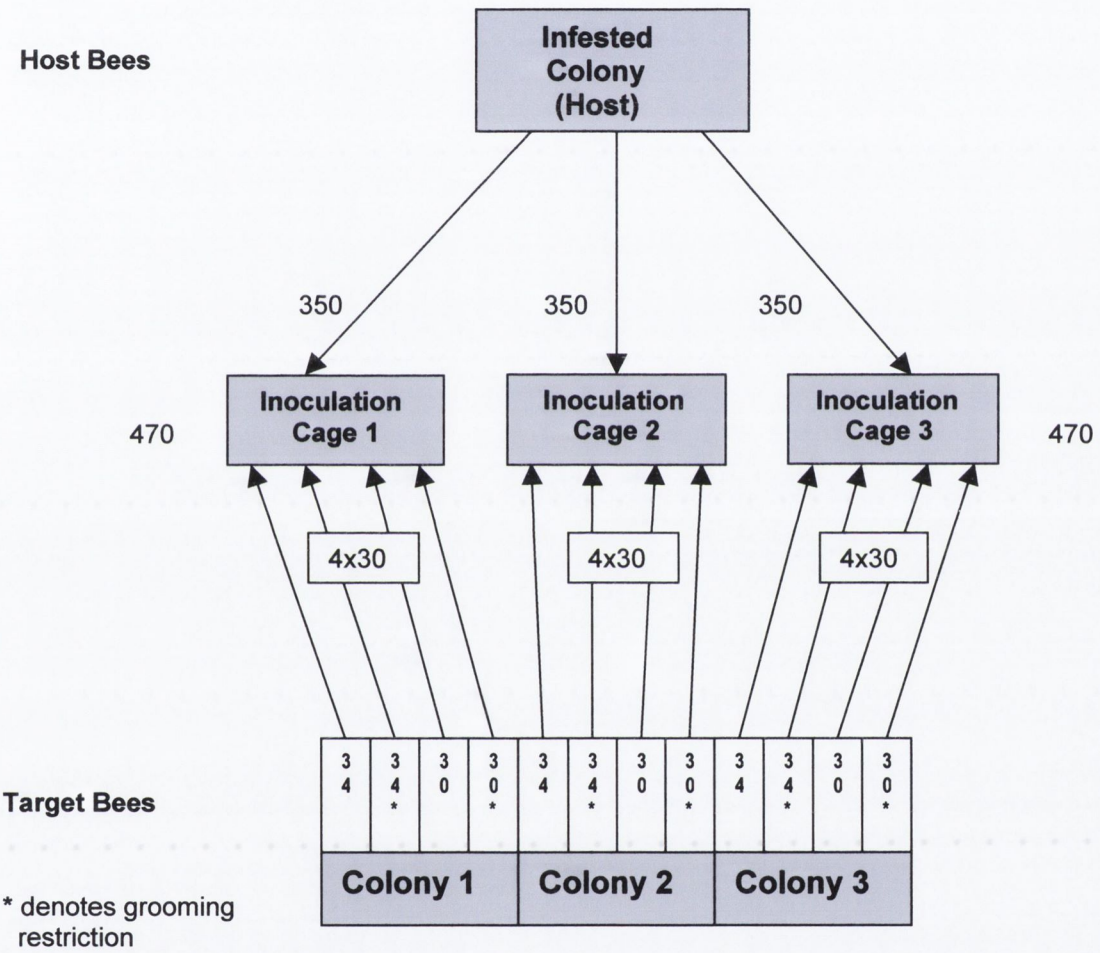


Figure 4.1 Experimental design to evaluate the influence of grooming in the susceptibility of honeybees pupated at 34ºC and 30ºC.

Target Bees

The target bees were obtained from three colonies in three separate apiaries in North County Dublin. The first apiary was located in an urban coastal area, another in a mixed urban/rural area 2 kilometres from the coast and the third in a rural area 7 kilometres

from the coast (Appendix 1). Tracheal mites had not been detected in over thirty-five years in the first apiary while the bees in the other two apiaries were infested on an ongoing basis. Varroa mites were only present in the third apiary and were at a low level after treatment with Bayvarol™ in the previous autumn. A frame of brood with the adhering bees brushed off was taken from each of the three colonies on 28th April 2004 and transferred to a dark incubator at 30°C and 50% RH. For the following 9 days callow bees were removed morning and evening from each of the three frame cages. These bees were not included in the experiment. On 7th May (9 days later) a second frame of brood, adjacent in the hive to the frame already removed, was taken from each of the three target colonies and incubated at 34°C. The brood temperature of the three target colonies had been monitored while the frames were in the hive to ensure that the brood was being pupated at normal hive temperature (34°C) for the preceding 9 days.

On the same day (7th May) callow bees emerging from the six frames (two from each colony) were collected in separate polystyrene cups that had a smear of petroleum jelly along the top. Taking each colony in turn, bees were chosen randomly from the polystyrene cups and (using surgical gloves to handle them) a 1-2 mm spot of Testor's™ gloss enamel paint was applied to the V–VI tergites on the abdomen of the callow bees; i.e. four different colours were used to mark the four target-bee samples (Figure 4.1). One sample was taken from each of the two cups with bees pupated at 34°C and 30°C and one sample was also taken from each of these cups and these bees had the mesotarsus removed (by microdissection spring scissors) from each of their mesothoracic legs (Figure 4.2). All bees were handled in a similar manner. The four samples of bees from each colony were put into the same cage.

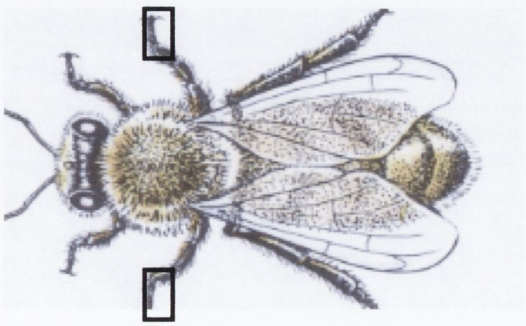


Figure 4.2 The grooming ability of the bee was restricted by removing the mesotarsus of the middle legs as shown boxed.

The four sets of thirty bees per colony were introduced by funnel into each of the three inoculation cages giving a total of 120 marked target bees in each cage. It had been planned to use two different colours (and specific to each cage) to mark the bees in each cage with a double spot of paint on the abdomen to indicate the bees with restricted grooming. While this approach had been given a limited dry run, it became apparent in the first sample to which this was applied in the experiment, viz. colony 2, 34°C*, that there was a risk of the wings of the bees being 'stuck' to their abdomens with the paint and it was decided to stop using this approach and to use four different colours for each cage.

Inoculation Process

The inoculation cages were maintained in a dark incubator at 32°C and 50% RH. The experiment was concluded after 7 days and the dead bees were removed from the bottom of the cage, segregated and counted. The live bees were subdued with CO_2 gas, held at 0°C and, taking each cage in turn, were segregated into host bees and the four categories of target bees and counted. They were then placed in numbered boxes and stored at -30°C to await dissection.

Dissection Technique

The bees were dissected using a modification to the classic technique of removing the head and thoracic collar and after removal each trachea was opened with a dissecting needle and the number of adult mites, larvae and eggs were counted and recorded.

Statistical Analyses

The female mite abundance data were skewed and had variances that were generally in proportion to the sample means. Accordingly, the mite count data were transformed to $\log_{10}(y + 1)$ (Sokal and Rohlf, 1995). Mite abundance data (control vs. test) were analysed as a 1-way ANOVA for the single-colony data, or as a 2-way ANOVA for the all-colonies data with treatment and colony as fixed factors.

4.3 Results

Host bee – mortality and prevalence

The initial numbers of host bees were 382, 387 and 382 for cages 1, to 3 with the proportion of live bees at the end of the experiment at 69%, 77% and 78% respectively (Table 4.1).

Table 4.1 Bee survival / recovery levels in the three inoculation colonies.

Bee	Cage 1			Cage 2			Cage 3		
Colony	Alive (%)	Dead (%)	Total	Alive (%)	Dead (%)	Total	Alive (%)	Dead (%)	Total
Host:	263(69%)	119(31%)	382	297(77%)	90(23%)	387	299(78%)	83(22%)	382
Target:									
Brood at 34°C									
Colony 1	29(97%)	1(3%)	30						
Colony 1 *	28((93%)	2(7%)	30						
Colony 2				24(80%)	6(20%)	30			
Colony 2 *				14 (47%)	16(53%)	30			
Colony 3							29(97%)	1(3%)	30
Colony 3*							28(93%)	2(7%)	30
Target:									
Brood at 30°C									
Colony 1	30(100%)	0(0%)	30						
Colony 1*	25(83%)	5(17%)	30						
Colony 2				28(93%)	2(7%)	30			
Colony 2*				21(70%)	9(30%)	30			
Colony 3							25(83%)	5(17%)	30
Colony 3*							20(67%)	10(23%)	30
Total (Target)	112(93%)	8(7%)	120	87(73%)	33(27%)	120	102(85%)	18(15%)	120
All Bees:									
Food/bee/day (µl)		40.7			40.5			37.5	

The three inoculation cages were filled on the same day from the same host colony and with a similar number of bees. The samples of 60 bees taken at the same time to give a measure of the initial inoculation pressure had similar mite prevalence levels of 16/60, 15/60 and 13/60 for cages 1, 2 and 3 respectively (Table 4.2).

Target bee – mite abundance and fecundity

The infestation data for all colonies and treatments are given in Table 4.2. In instances where mite offspring were present in the trachea and no adult mite found, it was assumed that the foundress female mite had migrated further into the trachea (Gary *et al.* 1989). In these cases (21 out of 78) one female mite was assigned to the mite total to give an adjusted female mite abundance.

Table 4.2 Summary of mite infestation levels for host and target bees in the three inoculation cages and three colonies for all treatments.

Bee	Treatment	No. of bees	Mite stages			
Colony		Total (infested)	Adults (adj.[1])	Larvae	Eggs	Total
Cage 1						
Host:	Before	60(16)	56	160	74	290
Target:						
Colony 1	34°C	29 (6)	6 (6)	11	17	34
	34°C*	28(13)	14(15)	31	46	91
	30°C	30(10)	8(10)	20	26	54
	30°C*	25 (8)	10(11)	24	30	64
Cage 2						
Host:	Before	60(15)	37	176	65	276
Target:						
Colony 2	34°C	24 (9)	9(10)	22	23	54
	34°C*	14 (9)	13(16)	29	37	79
	30°C	28(15)	18(22)	66	67	151
	30°C*	21(12)	9(16)	40	35	84
Cage 3						
Host:	Before	60(13)	25	96	54	175
Target:						
Colony 3	34°C	29(15)	14(18)	62	42	118
	34°C*	28(14)	12(17)	48	37	97
	30°C	25(15)	17(24)	65	58	140
	30°C*	20(12)	14(19)	46	46	106

* grooming restriction.
[1] adjusted by adding to the actual female mites present in the target bees an allocation of one female mite for each occasion that offspring was present without a corresponding female mite.

There were no cases of foundress mites without offspring and 40 bees (out of 301 infested) were multiply infested. The overall fecundity data (offspring/female mite) were similar for all treatments with means (± s.e.) of 5.21 (± 0.31), 5.06 (± 0.24), 5.41 (± 0.21) and 4.86 (± 0.25) for treatments 34°C, 34°C (restricted grooming), 30°C and 30°C (restricted grooming) respectively ($F_{[3,155]} = 0.527$, $p = 0.664$). This is consistent with the findings of Danka and Villa (1996) and the findings in Chapter 3 that mite reproduction in the honeybee trachea is similar across treatments and that differential mite abundance is determined by the success rate of the female mites migrating to the new hosts. Accordingly, the adjusted female mite abundance is used in the analysis and this is given for all colonies in Figure 4.3.

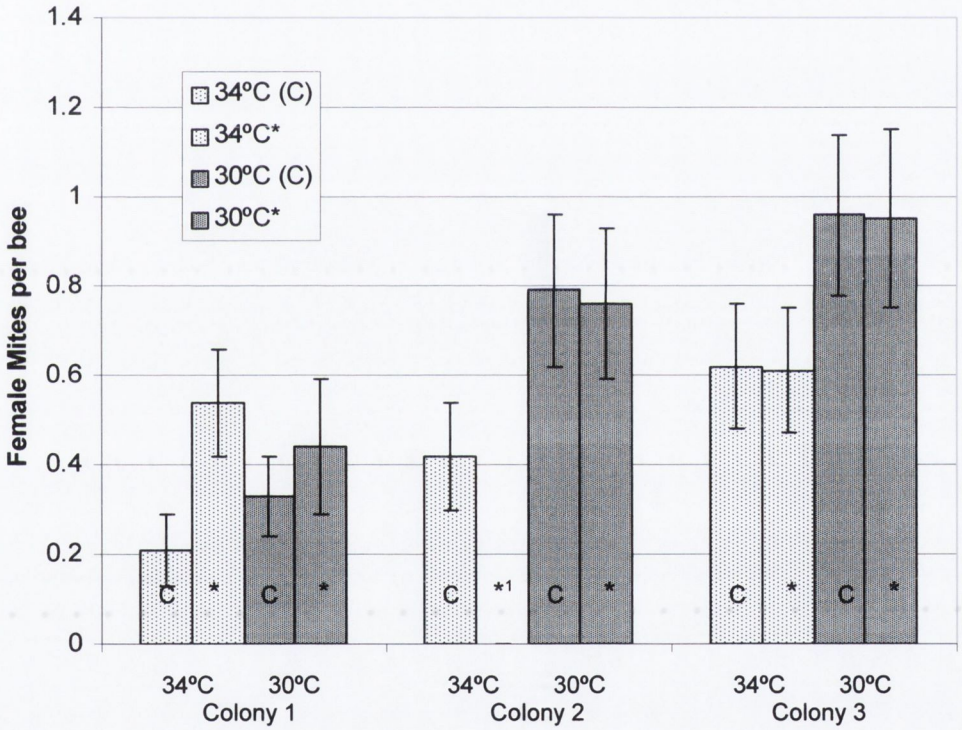


Figure 4.3 Female mite abundance (mean mites per bee ± s.e.) for the target bees from the three colonies and treatments. The control data bars are denoted by 'C', treated bee (restricted grooming) bars are donoted by '*'. The data from colony 2, 34°C* were unreliable and are not shown.[1]

Unrestricted grooming (34°C and 30°C)

The brood pupation temperature had a significant effect on female mite abundance ($F_{[1,159]} = 5.613$, $p = 0.019$). There was a two-thirds increase in overall abundance (mean

± s.e.) from 0.41 ± 0.07 mites/bee at 34°C to 0.67 ± 0.09 mites/bee at 30°C (Table 4.2, Figure 4.3). There was also a significant colony effect on the mite abundance ($F_{[2,159]}$ = 7.702, p = 0.001) but no significant interaction between treatment and colony ($F_{[2, 159]}$ = 0.256, p = 0.774).

A measure of the relative susceptibility (or resistance) of a colony to infestation by the tracheal mite is its infestation performance at normal pupation temperature (34°C) compared to another colony when exposed to a similar inoculation pressure. There was a significant difference between the mean female mite abundance of colony 1 and colony 3 at 34°C (Bonferroni post hoc multicomparison test p = 0.028). However, neither colony differed significantly from colony 2 (1 vs. 2, p = 0.554; 3 vs. 2, p = 0.702). The abundance varied widely from a mean (± s.e.) of 0.21 ± 0.08 mites/bee in colony 1 ('low susceptibility' colony) to 0.62 ± 0.14 mites/bee in colony 3 ('high susceptibility' colony). The designations of low and high susceptibilities are also consistent with the colony disease history as tracheal mites had never been detected in colony 1 while tracheal mite infestation was ongoing in colonies 2 and 3.

Evaluating the grooming capability of bees raised at 34°C

As already mentioned the marking difficulty with the sample of control bees (34°C) for colony 2 (restricted grooming) rendered these data unreliable. The abundance data for this sample was out of line with the other eleven sets of data (Table 4.2). A large proportion of these bees was observed to reside at the bottom of the inoculation cage. At the end of the experiment the wings of many of the bees in this sample were stuck to their abdomens and the mortality rate was > 50%. In fact the recovery of live bees was just 14/30 compared to 28/30 for each of the 34°C (restricted grooming) samples for colonies 1 and 3 (Table 4.1).

There is evidence that the grooming ability of normally pupated bees is colony dependent (Table 4.3, Figure 4.3). In the case of colony 1, a low susceptibility colony, there was a significant increase in susceptibility when grooming was restricted ($F_{[1, 55]}$ = 5.152, p = 0.027). The female mite abundance (mean ± s.e.) increased from 0.21 ± 0.08 to 0.54 ± 0.09 mites per bee. This was in contrast to colony 3, a high susceptibility colony, where the response was minimal ($F_{[1, 55]}$ = 0.009, p = 0.924) and the mean

varied from 0.62 ± 0.14 to 0.61 ± 0.14 mites per bee. The mite abundance in the low susceptibility colony (colony 1) was just 13% lower than that in the high susceptibility colony (colony 3) when grooming was restricted i.e. 0.54 mites / bee vs. 0.62 mites / bee.

It should be noted, however, that since the treatment effect was colony dependent further replication is required to test the effects in detail (Discussion 4.4, page 52).

Evaluating the contribution that changes in grooming make to the increased susceptibility of bees raised at 30°C

It is assumed that the grooming restriction applied to the bees has the effect of removing all their grooming capability. Hence the change in susceptibility (due to grooming restriction) in bees pupated at 30°C when compared to the change in bees pupated at 34°C is a measure of the contribution that grooming makes to the increased susceptibility of unconstrained bees raised at 30°C. The three samples of test bees pupated at 30°C had only a small response to restricted grooming, with changes in susceptibility of 0.11, 0.03 and 0.01 respectively for colonies 1, 2 and 3, none of which were statistically significant (Table 4.3, Figure 4.3). The two samples of test bees pupated at 34°C (colony 2 data unreliable, see above) had a colony dependent response to restricted grooming as described above.

The changes in susceptibility were 0.33 and 0.02 mites per bee respectively for colonies 1 and 3, with the former being a statistically significant difference (Table 4.3). There are therefore indications that in the high susceptibility colony (colony 3) reduced grooming makes very little contribution to the increased susceptibility of bees pupated at 30°C. In contrast, in the low susceptibility colony (colony 1) there are indications that reduced grooming may have made a contribution to the increased susceptibility of bees pupated at 30°C.

Table 4.3 Summary of significance tests on adjusted female mite abundance data from the three colonies and for all treatments. A log transformation was used on the abundance data which were then analysed as a 1-way ANOVA.

Treatment	Mite abundance - untransformed data per bee (mean ±s.e.)	Significance tests on transformed data			
		Control/Treatment	d.f.	*F*-value	*p*
Colony 1					
34°C	0.21 ±0.08	Control	-	-	-
34°C*	0.54 ±0.09	Treatment	1, 55	5.152	0.027
30°C	0.33 ±0.09	Control	-	-	-
30°C*	0.44 ±0.15	Treatment	1, 53	0.117	0.743
Colony 2					
34°C	0.42 ±0.12	Control	-	-	-
34°C*	n.a.	Treatment	n.a.	n.a.	n.a.
30°C	0.79 ±0.17	Control	-	-	-
30°C*	0.76 ±0.17	Treatment	1, 47	0.001	0.971
Colony 3					
34°C	0.62 ±0.14	Control	-	-	-
34°C*	0.61 ±0.14	Treatment	1, 55	0.009	0.924
30°C	0.96 ±0.18	Control	-	-	-
30°C*	0.95 ±0.20	Treatment	1, 43	0.001	0.978

* restricted grooming.

4.4 Discussion

Bees pupated at the lower brood temperature of 30°C (with unrestricted grooming) had a two-thirds higher susceptibility to tracheal mite infestation than those raised at the more typical brood temperature of 34°C. This concurs with the results of the experiment in Chapter 3 and reaffirms that a low pupation temperature considerably increases the likelihood of honeybees being infested. The mite fecundity was also shown to be similar for all colonies and treatments. This is in line with the findings of Danka and Villa (1996) and Chapter 3 that mite reproduction in the honeybee trachea is similar across treatments and that differential mite abundance is determined by the success rate of the female mites migrating to the new hosts.

It was demonstrated that in the case of bees pupated at a normal temperature, grooming is an important element in the reduced infestation in a colony with low mite susceptibility (e.g. colony 1), while in a colony with high susceptibility (e.g. colony 3) the ability to groom had no influence on susceptibility. Also, the low susceptibility colony with grooming restriction (colony 1, 34°C*) had a susceptibility close to that of the high susceptibility colony (colony 3, 34°C). Hence there is evidence that the differential susceptibility of colonies to tracheal mite infestation in normally pupated bees is explained to a large extent by the autogrooming capability of the colonies. This is consistent with the findings of Danka and Villa (1998).

The experiment explored whether grooming could explain the increased susceptibility to tracheal mite infestation of bees pupated at a low temperature (30°C). In the case of a high susceptibility colony (colony 3) there was no evidence that grooming made a contribution to its increased susceptibility. If there were no interaction between treatment (grooming restriction) and colony (genetically based susceptibility) then we would expect the data from colony 1 to show a similar pattern, with the bees in the restricted grooming samples (34°C* and 30°C*) exhibiting similar mite abundances to their respective control samples (34°C and 30°C). However, this was not the case, as the restricted-grooming bees in colony 1 had a considerably increased abundance. The change (increase) in abundance was much greater with the 34°C treated bees (34°C to 34°C*) than with the 30°C treated bees (30°C to 30°C*) indicating that reduction in grooming ability made a contribution to the increased susceptibility of 30°C pupated bees. In high susceptibility colonies raised at a reduced pupation temperature it is therefore indicated that mechanisms other than grooming are responsible for the increased susceptibility.

The reason why some bees are better groomers than others is not understood. Danka and Villa, (2003) demonstrated that bees with low susceptibility make more grooming attempts but grooming efficiency was similar for both low and high susceptibility bees. This increased grooming frequency of good groomers is likely to be due to increased sensitivity in the sense organs (Snodgrass, 1956). "The essential element of an insect sense organ is a sensory cell of the ectoderm with a sense process extending into the central nervous system". The relevant sense organ is likely to be the *sensilla trichodea* where the cuticular part of the sensillum is a small hair or seta. According to Snodgrass

(1956) these sense organs occur all over the body and probably respond to movements of the seta caused by contact with some external body or surface and are therefore organs of touch. Why are some bees more sensitive than others to the presence of tracheal mites? This may be due to their nervous system having a much lower 'pick-up' threshold and responding to movements of hairs more readily. It may, on the other hand, be due to the structure of the hair providing a greater stimulus at the sense organ in the cuticle due to the thickness or length of the hair. The hair length may not only influence the grooming efficiency of the bees but may also facilitate the transfer of the mite to the new host bee in the first place. The potential effect of hair length will be discussed further in Chapter 9 when the possible influence of honeybee subspecies on mite susceptibility is considered.

The hair effect may also help to explain the reduced grooming ability, discussed above, of bees with low susceptibility (colony 1) when pupated at a reduced temperature. These bees that developed slower may not have a fully developed hair structure and this may affect their ability to detect and groom-off migrating mites. This may be further compounded by the underdevelopment of their nervous system as manifested by considerably lower forage-dance activity and reduced cognitive ability (Tautz, *et al.*, 2003). Hence physiological traits (other than grooming) or the influence of cuticular hydrocarbons may be accounting for the increased mite susceptibility. The other possible mechanisms involved will be considered in Chapter 5.

This experiment involved three colonies that had a wide spread in their measured susceptibility to tracheal mites. Since the results of this experiment indicated that the characteristics of susceptibility in both normally and reduced-temperature pupated bees are colony dependent, further work is required to analyse in detail such colony-level variation. Future test colonies should be replicated on the basis of high/low susceptibility to mite infestation.

CHAPTER 5

Chemical cues as a mechanism in honeybee susceptibility to tracheal mites

5.1 Introduction

In previous work it was shown that honeybees pupated at a lower than normal temperature had a significantly increased susceptibility to infestation by tracheal mites. The overall increase in susceptibility was over twice in the experiment undertaken in March/April 2003 (Chapter 3) while the corresponding increase in the experiment of April/May 2004 (Chapter 4) was two thirds. In Chapter 4 it was demonstrated that changes in autogrooming only had a limited role to play in this increased susceptibility. In fact in the case of a colony with a high susceptibility (low resistance) to infestation by the tracheal mite, changes in autogrooming made no contribution to the increased susceptibility. In a colony with a low susceptibility there was evidence that changes in autogrooming did make a contribution to the increased susceptibility.

Since autogrooming was shown to have only played a limited role, the question remains as to what was the principal mechanism behind this increased susceptibility. What causes honeybees pupated at a lower than normal temperature to have a higher number of foundress female mites migrate into their tracheae? One possible explanation for this differential susceptibility may be the influence of chemical cues. Hydrocarbons make up the major part of the lipid layer on the outer cuticular surface of honeybees (Phelan *et al.*, 1991). Branched and saturated hydrocarbons decrease rapidly from the time of emergence of new callow bees (Francis *et al.*, 1989) and this may be used by mites to identify young bees. Phelan *et al.* (1991) concluded that the specific attractiveness of young callow bees to tracheal mites was their cuticular compounds, and demonstrated a significant effect in three out of four honeybee test colonies. Using a similar methodology, Van Engelsdorf and Otis (2001) undertook an experiment to identify if cuticular compounds had a role in the differential resistance of different bee strains to infestation by the tracheal mite. While the results demonstrated that the mites had a preference for some bee colonies as opposed to others there was no consistency based on colony susceptibility/resistance.

Phelan *et al.* (1991) used hexane to extract the cuticular compounds from test bees and employed a two-choice bioassay to identify mite preference. The hexane extracts were

applied to two filter papers (1 cm diameter), 4 mm apart on a plastic slide and the female mite placed in the centre. The location of the mite after a period of 5 minutes was used to indicate a preference for one extract over the other. If mites are attracted to the cuticular extract of bees it must also be possible to elicit a response from a direct 'whole bee' approach. By presenting mites with treated live or dead bees it would be expected that a mite should express in a two-choice bioassay any preference that was present. Sammataro and Needham (1996) showed that mites placed on the thoraces of treated bees demonstrated treatment-dependent responses. In the transfer process from old to new host bees two of the mite's critical behavioural modes are 'questing' and 'seeking'. Hirschfelder and Sachs (1952) identified 'questing' as the behaviour when the mite crawls up on a bee hair (seta), holds on with a leg and tries to make contact with any adjacent objects. In the experiments that I carried out the mites normally held on by their rear legs (fourth legs) and became highly animated behaving like trapeze artists (Figure 5.1). 'Seeking' on the other hand describes niche-location behaviour on the bee thorax including finding the entrance to the spiracle.

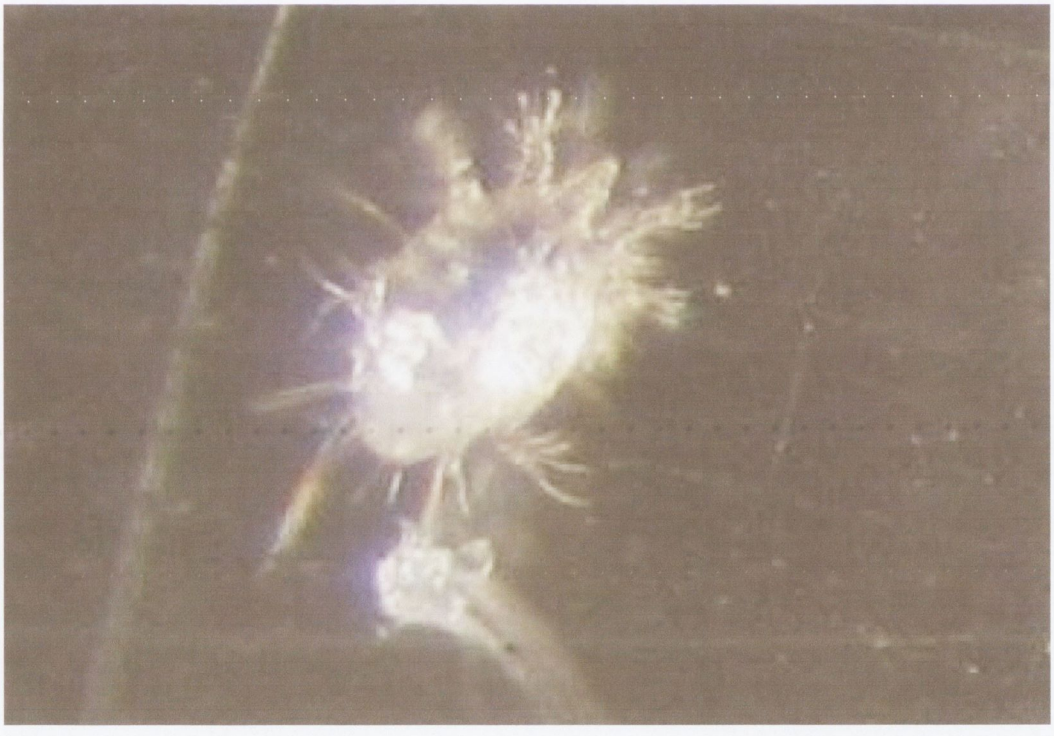


Figure 5.1. Photograph of mite in 'questing' mode, with its right rear leg attached to a bee hair (seta).

On the basis of these results, I undertook an experiment to establish if the increased susceptibility to tracheal mite infestation of honeybees pupated at a lower than normal

temperature was influenced by cuticular compounds on the honeybee. A two-choice bioassay using a whole bee approach was employed.

5.2 Materials and methods

A two-choice experiment was originally set up along the same lines as that in Phelan *et al.* (1991), and Van Engelsdorf and Otis (2001) with the exception that a whole bee was used in place of cuticular extract.

Preparation of the test bees

The source honeybees for the experiment were the control (34°C) and treated bees (30°C) from the three colonies used in the autogrooming experiment in Chapter 4 (Appendix 1). These callow bees, less than 8 hours old, were held in a freezer at -30°C. Bees pupated at 34°C and 30°C were removed from the freezer and held at room temperature for 20 minutes to allow them to dry off. The bees to be hexane washed were put in a glass container with a generous covering of hexane and the stopper inserted. The container was agitated for a period of 10 minutes after which the bees were removed and allowed to dry-off at room temperature.

Initial two-choice arrangement

A plastic slide cover had the centre line marked and a 4 mm diameter hole (similar in diameter to bee thorax) was punched on each side of the centre line, the holes being 4 mm apart (Figure 5.2). The plastic slide cover was held horizontally and the control and treated bees inserted from below and wedged to the level of the wing root. The bees were in a vertical axis, aligned laterally to each other and facing in opposite directions. The tests were undertaken blind. Giordani (1967) demonstrated that the highest survival rate for tracheal mites on bee pupae was at a temperature of 28°C and relative humidity (RH) of 70%. The experiment was conducted in an ambient temperature of 28 (± 1)°C and (RH) of 68 (± 2)%. These conditions were maintained in a confined laboratory using a humidifier and meant that the removal of mites from the infested bees, their

transfer and the experiment itself were undertaken in stable conditions causing the minimum environmental shock to the mites.

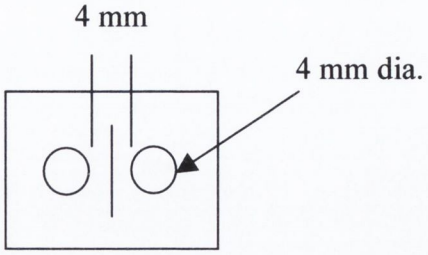


Figure 5.2. Line drawing of the initial two-choice test arrangement similar to that used by Phelan *et al.* (1991) with the exception that a 'whole' bee is used in place of extract of cuticular compounds.

Selecting female test mites

The mites were harvested from a host colony with a mite prevalence of >70%. Flying bees in groups of about 40 were collected from the landing board of the hive using a vacuum collector. They were held, awaiting dissection, in wire cages in an incubator and fed water and 50% (*w/w*) sugar syrup. The live female mites for testing were obtained by removing the bee's head and pronotum thereby exposing the tracheae. To avoid injuring the mites a piece of cuticle was cut around the spiracle end of the trachea and this was removed with the infested trachea itself under a stereomicroscope at 60-100X magnification and placed on a glass slide. Some salivary gland tissue was also extracted to enable the trachea to adhere to the glass slide. No more than five infested tracheae were removed simultaneously to minimise the time that the mites were outside of the dissected bee. An eyelash attached to a thin wooden probe was used to lift the mites. The female mites were lifted from a questing position on the hair attached to the cuticle at the spiracle end of the trachea or by gently opening the trachea with a dissecting needle and removing a mite. Only young (lightly bronzed) female mites and lightly pregnant mites were used. Old heavily pregnant mites tended to lack mobility and stayed fixed in one position in the test area.

Initial two-choice test

The experiments of Phelan *et al.* (1991) had significant responses in all cases where cuticular hydrocarbon extract from a bee of any age was tested against a hexane control. In order to test for a similar response, the 34°C pupated bees as a control were tested against 34°C pupated bees that had been washed in hexane for 10 minutes. The test was repeated for the 30°C (unwashed vs. washed) as well as 34°C vs. 30°C (both unwashed). The test was carried out on one colony, colony 2, as this colony showed the greatest change in susceptibility when pupated at 30°C in the autogrooming experiment, Chapter 4 (Figure 5.3). The selected female mite was deposited in the centre position between the test bees and its location was scored after 5 minutes as being on the control or treated bee or on the slide cover as a no choice. A total of 16 pairs were tested for each case and the data are given in the Results. Since this approach did not elicit a meaningful response (see Results and Discussion), the alternative approach below was employed.

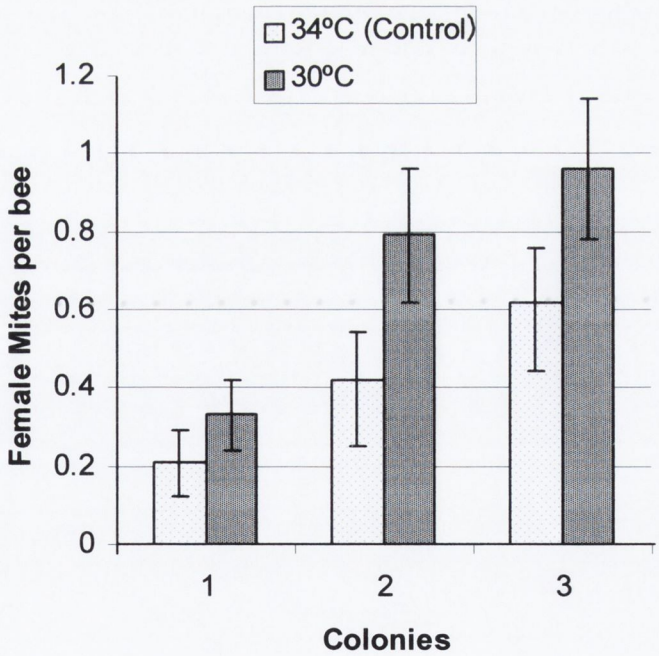


Figure 5.3 Mite abundance levels (mean female mites per bee) for the bees pupated at 34°C and 30°C, from the three colonies, after a period of seven days in the inoculation cages (autogrooming experiment, Chapter 4).

Modified two-choice test

An alternative two-choice approach was designed to enable the mites to be in contact simultaneously with cuticular compounds from the control and treated bees, and to make their choice using the gustatory sensors on their legs (Waladde and Rice, 1982). The head was removed from each bee to improve the visibility of the thorax and excess body fluid was removed with blotting paper to prevent it staining the hairs on the thorax and affecting their condition. The bees were mounted in the vertical axis within short lengths of 4mm diameter straws spaced 2mm apart (Figures 5.4 and 5.5).

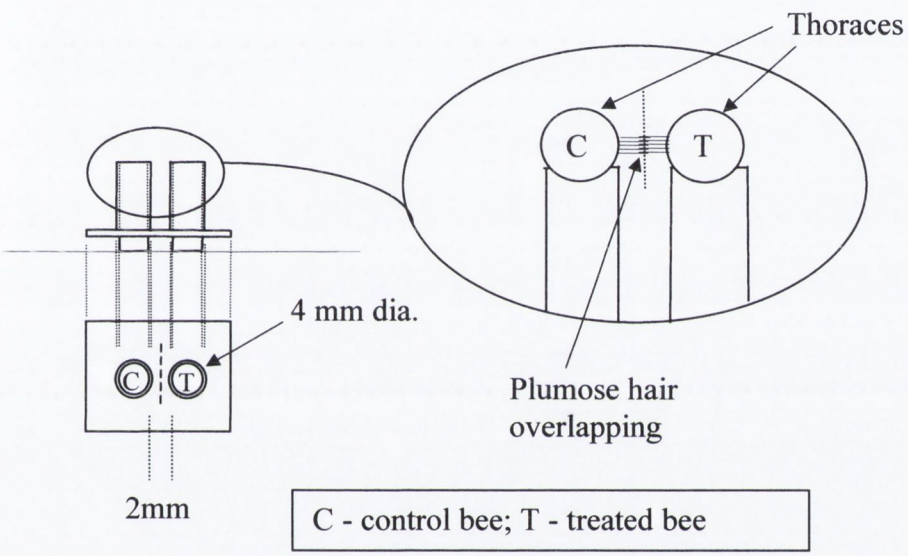


Figure 5.4. Line drawing of modified two-choice arrangement with bees mounted using coaxial straws allowing the plumose hair on the thoraces to overlap.

The bees were pushed abdomen first into the straws as far as the root of the wings, with wings and legs on the outside. They were then held in position with short lengths of wider straws that went over the inside straw and trapped the wings and legs in position. The bees were in a vertical axis, aligned laterally to each other and facing in opposite directions, and were tilted slightly towards each other to enable the plumose hair on the thoraces to interlock. The plumose hair was 'combed' with an eyelash probe to enhance the interlocking of the hair. Separate probes were dedicated to either the control or treated bees to avoid cross-contamination of the cuticular compounds. The hair was allowed to overlap by about a quarter of its length.

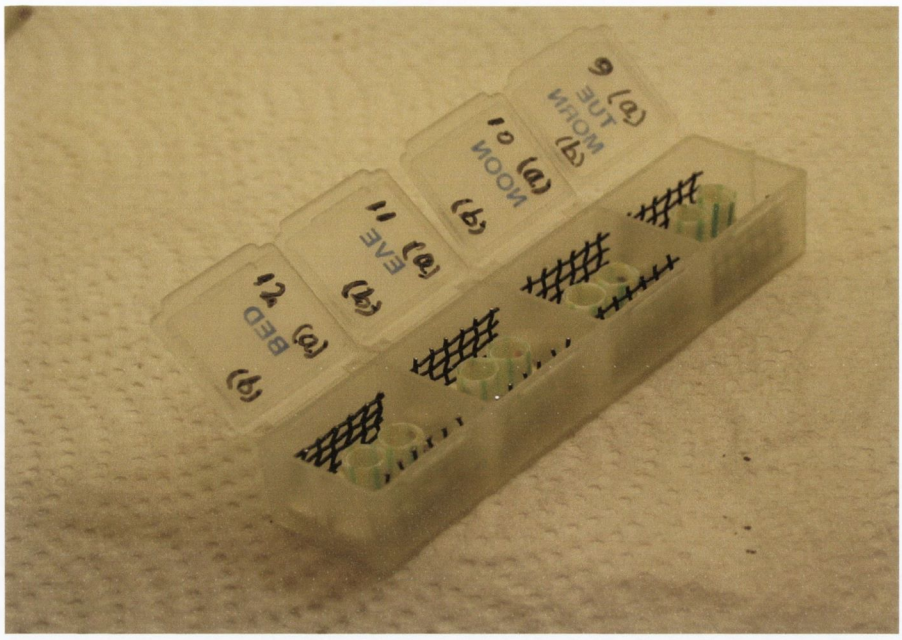


Figure 5.5. Photograph of modified two-choice arrangement for 4 pairs of bees and showing the straw-fixing approach.

A selected female mite was deposited at the centre of the overlapping plumose hair. Since the female mites are large relative to the thickness of the plumose hair they were simultaneously in contact with the hair of both test bees. The mite choice was scored after 5 minutes by noting the location of the mite. A no score was registered if the mite had not located on one of the two bees. The behavioural mode of the mites (questing / seeking) was noted after 5 minutes and also after a period of about 3 hours. It had been hoped to dissect bees after the longer period to establish how many of the mites had migrated into the tracheae. However, after this period dissection was not possible as dark body fluid had gathered in the tracheae.

It was of some concern that the dead bees would lose their 'freshness'. To test the experimental protocol 16 pairs of samples of bees from each pupation temperature in colony 2, were presented as an unwashed vs. washed bee choice. Eight pairs were tested within three hours of the bees being removed from freezer storage and the second batch was tested in the 4 to 6 hour period. The mite preference for unwashed vs. washed in the first 3 hours was 13 : 2 respectively and the corresponding choices in the 4 to 6 hour period 8 : 7. This would indicate that the aging of the bees cloaked any difference in bee cuticular compounds in the two-choice test, as a significant difference in choices was

demonstrated between the two periods (χ^2 =3.96, $p < 0.05$). As a result it was decided to test the bees in batches of 4 pairs and the tests were undertaken within 2 hours of the bees being removed from storage.

The permutations used to obtain the initial two-choice test for response above were repeated but this time for all three colonies as opposed to only colony 2. For each colony 32 two-pair tests were undertaken for the 34°C (unwashed vs. washed) and 30°C (unwashed vs. washed). In the case of 34°C vs. 30°C (both unwashed), 48 pairs were tested for each colony.

Statistical analyses

All of the data in the two-choice tests were analysed using chi-square tests.

5.3. Results

The initial two-choice arrangement with the test bees 4 mm apart did not elicit a meaningful response (Table 5.1).

Table 5.1 Initial two-choice arrangement. Female tracheal mite choices when presented with 16 pairs of bees, made up of unwashed (uw) and hexane washed (w) bees, pupated at 34°C and 30°C from colony 2. The thoraces of bee-pairs protruded through holes spaced at 4 mm in a cover slip and the mites placed in the centre.

Choice	Outcome uw : w (no choice)	χ^2 test (p value) choice : no choice
34°C (uw) : 34°C (w)	1 : 2 (13)	6.25 (p = 0.01)
30°C (uw) : 30°C (w)	2 : 0 (14)	9.0 ($p < 0.01$)
34°C (uw) : 30°C (uw)	1 : 2 (13)	6.25 (p = 0.01)
All choices	4 : 4 (40)	21.3 ($p < 0.01$)

The mites when released generally moved in circles relatively close to the centre or moved, at what appeared to be at random, in a particular direction. On occasions when a

mite moved close to one of the bees it was observed that there did not appear to be any awareness on the part of the mite of the proximity of the bee. The number of individual choices made by the mites was so low as to yield no worthwhile comparisons. In all only 8 choices were made out of 48 two-pair tests representing a significant no-choice preference (χ^2 =21.3, $p < 0.01$). The experiment demonstrated that the mites were quite mobile in this new environment and those mites that did not find one of the two bees could still be seen moving on the slide cover for up to three hours.

When the choices above were presented to the mites in the modified two-choice arrangement (with the interlocking plumose hair) the choice (vs. no choice) option was significant in all cases, with most mites actively choosing a potential host bee (Tables 5.2 and 5.3). In the unwashed vs. washed choice the outcome was largely dependent on colony (Table 5.2). Colony 1 expressed no significant preference for an unwashed as opposed to a washed bee while for colonies 2 and 3 a significant preference ($p < 0.05$) was expressed for the unwashed bee in 3 out of 4 cases. When the data for colonies 2 and 3 were combined, the mites expressed a significant preference ($p < 0.01$) for the unwashed as opposed to the hexane washed bees for both pupation temperatures.

Table 5.2 Modified two-choice arrangement. Female tracheal mite choices when presented with 32 pairs of unwashed (uw) and hexane washed bees (w), pupated at 34°C and 30°C from the three colonies. The plumose hair of the bee-pairs was overlapping and the mites placed in the centre.

Colony	Choice uw : w (no choice)	χ^2 test (*p* value)	
		choice : no choice	uw : w
Colony 1			
34°C	12 : 17 (3)	21.2 (*p* <0.01)	0.86 (*p* = 0.36)
30°C	16 : 13 (3)	21.2 (*p* <0.01)	0.31 (*p* = 0.58)
Colony 2			
34°C	21 : 8 (3)	21.3 (p <0.01)	*5.80 (p = 0.02)*
30°C	21 : 9 (2)	24.5 (p <0.01)	4.80 (*p* = 0.03)
Colony 3			
34°C	17 : 10 (5)	15.1 (p <0.01)	1.81 (*p* = 0.17)
30°C	19 : 9 (4)	22.2 (p <0.01)	3.57 (*p* = 0.05)
All Colonies			
34°C	50 : 35 (11)	57.0(p <0.01)	2.65 (*p* = 0.10)
30C	56 : 31 (9)	63.3 (p <0.01)	7.18 (*p* < 0.01)
Col 2 & 3			
34C	38 : 18 (8)	36.0 (p <0.01)	7.14 (*p* < 0.01)
30C	40 : 18 (6)	42.2 (p <0.01)	8.34 (*p* < 0.01)

The mites did not have a significant preference for 30°C as opposed to 34°C pupated bees (Table 5.3). While the mites did choose bees pupated at 30°C 26 times compared to 17 times for 34°C pupated bees for colony 1 (χ^2 =1.88, p <0.17) the combined data for colonies 2 and 3 shows a 46 : 43 non significant preference for 30°C compared to 34°C pupated bees (χ^2 =0.10, p <0.75).

In 81% (271/336) of the bee-pairs undertaken in the modified two-choice arrangement, the mites were in seeking mode when scoring was undertaken at the end of 5 minutes. After three hours less than a quarter of the mites remained on the bees, these being fairly equally divided between seeking and questing (39 : 43). In a few cases the mites could be seen in a feeding position on the thorax cuticle or in the thorax dorsal groove.

Table 5.3 Modified two-choice arrangement. Female tracheal mite choices when presented with 48 pairs of bees pupated at 34°C and 30°C, from the three colonies. The plumose hair of the bee-pairs was overlapping and the mites placed in the centre.

Colony	Choice 34°C : 30°C (no choice)	χ^2 test (*p* value)	
		choice : no choice	34°C : 30°C
Colony 1	17 : 26 (5)	30.1 (p <0.01)	1.88 (*p* = 0.17)
Colony 2	19 : 25 (4)	33.3 (p <0.01)	0.82 (*p* = 0.37)
Colony 3	24 : 21 (3)	36.7 (p <0.01)	0.20 (*p* = 0.66)
All Colonies	60 : 72 (12)	100.0 (p <0.01)	1.09 (*p* = 0.30)
Col 2 & 3	43 : 46 (7)	169.0 (p <0.01)	0.10 (*p* = 0.75)

5.4. Discussion

There was no evidence that cuticular compounds played any role in the increased susceptibility to tracheal mite infestation of honeybees raised at a reduced pupation temperature (Tables 5.2 and 5.3). For colonies 2 and 3 the mites did have a significantly higher preference for bees with their cuticular compounds intact (unwashed) for both pupation temperatures while no preference was demonstrated in the case of colony 1. This colony dependent effect coincided with the differential susceptibility to tracheal

mites demonstrated in Chapter 4 where colonies 2 and 3 were designated 'high susceptibility' colonies and colony 1 a 'low susceptibility' colony. The high susceptibility colonies were also identified by morphometry as *Apis mellifera mellifera*, a subspecies of *Apis mellifera* that had evolved in the damp and cooler north European region (Chapter 9). The results demonstrated that for the low susceptibility colony (colony 1) the mites were not able to differentiate between their cuticular compounds and that of hexane washed bees, which would indicate that this subspecies, that fell outside the criteria for *Apis mellifera mellifera* designation, had little cuticular hydrocarbon or that it was not attractive. The former is more likely as Phelan *et al.* (1991) and Van Engelsdorf and Otis (2001) demonstrated in all their tests that mites had a preference for any bee extract in preference to no extract (hexane only). Also it is speculated that the *Apis mellifera mellifera* subspecies that has evolved in the damp and cooler Northern regions of Europe may have a greater level of cuticular compounds than non *Apis mellifera mellifera* subspecies in order to aid waterproofing of the bee (Gullan and Cranston, 2001). An analysis of the profiles of cuticular compounds from the three colonies should give an improved insight into the nature of the relative attractiveness of the cuticular hydrocarbons. This should also include an analysis of the compounds on the plumose hair separately from the cuticle.

There is no evidence that cuticular compounds have played any role in the increased susceptibility to tracheal mite infestation of honeybees pupated at 30°C compared to 34°C. Mites that transferred to a new host bee could not differentiate between a bee pupated at 34°C as opposed to one pupated at 30°C. It could be argued, however, that the cuticular compounds from the 30°C pupated bees may stay attractive for longer as the callow bees get older and establish their differential attraction in this way. However this is not supported by the evidence from Chapter 3 where it was shown that the 'susceptibility curves' for the two pupation temperatures were similar in shape and duration but with the 30°C pupated bees having a much higher initial attraction.

In Chapter 4 it was demonstrated that autogrooming was only implicated to some extent in the increased susceptibility of bees from colony 1, the low susceptibility colony and not at all for the high susceptibility colonies. Since the mite is only aware of the presence of the new host once it is in physical contact with it, it follows that the increased susceptibility is almost exclusively determined during the actual transfer

process of the mite from old to new host. (Autogrooming only played a part in the case of the low susceptibility, colony 1). This would indicate that the relative physiology of the bees was the determining factor that allowed more founding female mites to make the transfer. This could include length or texture of plumose hair or relative movement of the old and new host bees. Recent research has shown that bees pupated at a low temperature are less active and have a significantly reduced dance and cognitive ability (Tautz *et al.* 2003). They also suggested, "that the temperature at which pupae are raised will influence their behavioural performance as adults and may determine the tasks they carry out best inside and outside the hive". Also Jones *et al.* (2005) demonstrated that reduced pupation temperature affected the short term learning and memory of the bee. This evidence when taken in conjunction with the findings of Pettis *et al.* (1992), that mite dispersal takes place almost exclusively at night time when the movement of bees is substantially reduced, could explain the role of pupation temperature in increased bee susceptibility.

The experiments give some insights into the nature of mite migration. The mites did not respond to bees even when they were in close proximity to them. It was only when they came in contact with the bees that there was any response. Thus there is evidence that the mites are not stimulated through their olfactory senses but through their gustatory senses requiring direct contact between the mite and the potential host bee (Tables 5.1, 5.2). Hirschfelder and Sachs (1952) had shown that bees responded to contact and not the scent of old or young bees while Sammataro and Needham (1996) had demonstrated that mites detect the different thoracic conditions only if placed on the host. Thus while the findings of the present experiments are consistent with the work of Hirschfelder and Sachs (1952) and Sammataro and Needham (1996) it is not clear whether the findings of Phelan *et al.* (1991) and Van Engelsdorf and Otis (2001) demonstrate a response based on an olfactory or a gustatory sensory reaction. The mites were put at the centre between two treated areas (and some distance from each) and their locations noted after a 5-minute period. This would indicate that the response of an olfactory sense was being tested. However, Phelan *et al.* (1991) stated that, "Preliminary observations of female mites walking in the vicinity of honeybee extract revealed no orientation of the mites towards treated areas; however, when placed in contact with a zone treated with young-bee extract, the locomotory tracts of the mite were dramatically different from those of mites placed in hexane treated zones." This would indicate that only a gustatory

response was present and raises the questions as to why Phelan *et al.* (1991) used the particular test methodology.

However, the results from the experiments undertaken in this chapter also raises the question of how the rapid reduction in bee infestation with age (Morgenthaler, 1930; Bailey, 1958; Lee, 1963; Giordani, 1977; Gary *et al.*, 1989) arises in practice. It can be postulated that there are essentially two (rather than one) modes of mite transfer. The first would be the generally accepted approach whereby the mite would transfer to a neighbouring moving bee, test for 'suitability' and either accept or continue to quest for a suitable bee (Sammataro and Needham, 1996). Assuming contact between the old and the new bees happens at random it would be expected that an equal number of bees of all ages, and therefore also in the age range 0 to 5 days, are visited by the mites. Hence the mites may be seeking a particular cuticular compound that identifies a very young bee and must be leaving, for example, almost all of the 5-day bees that they visit. This would appear to represent a high-risk strategy for such a small animal that is in danger of desiccation or being groomed off by the bees. It would indicate that the mite would normally make several bee transfers before it can accomplish its task and the success rate is likely to be low. External feeding by the mites on unsuitable hosts as observed in the experiments may therefore be routinely practiced as an interim measure to reduce the risk of dessication but the risk of being groomed off during transfer is ever present. The alternative mode is less risky and given the rapid increase in mite infestation that can take place in a colony (and hence high transfer success rate) and the findings of Pettis *et al.* (1992) that 85% of mite transfers take place at night, this is likely to be the selected mode of transfer. Pettis *et al.* (1992) considered that mites might detect movement of the bees and wait to initiate dispersal until the host is motionless. This alternative mode would occur during the bees' inactive period when their plumose hair would be in close contact enabling the mites to 'taste' the new potential host before transferring.

A further aspect of migration is the distribution of mites between the two tracheae of an infested bee. It has been demonstrated that bees are more likely to be bilaterally infested or uninfested (rather than unilaterally infested) than expected under the assumption that the probability of a trachea being infested is the same for all tracheae (Gary and Page, 1987; Fries and Morse, 1992; Calderone and Shimanuki, 1993). Fries and Morse (1992)

suggested that different infestation rates within the brood nest could cause this distortion in the distribution of infestation of tracheae. While Calderone and Shimanuki (1993) concurred with this hypothesis they also stated that, "this phenomenon cannot be attributed solely to high infestation rates". The phenomenon may also be influenced by the mites' migration strategy. Female mites in their migration to a new host bee and finding a mite already in a trachea, rather than risking further questing and migration to another bee, may preferentially seek the second trachea of the bee. This would have the effect of increasing bilateral and uninfested bee proportions. It would align with a mite strategy of reducing the risk of mite host finding.

The methodology described in the modified two-choice bioassay ('hair-contact' approach) would provide a simple approach to investigate the cuticular compound basis for the age-related attractiveness of honeybees to tracheal mites.

CHAPTER 6

Influence of relative humidity on honeybee susceptibility to tracheal mites

6.1 Introduction

The enigmatic nature of mite infestation has been reported for a long time (Rennie, 1921). Giordani (1977) who studied the effect of the tracheal mite over a long period in Italy concluded, "On the basis of collected data, though numerous and detailed (150,000 bees analysed in 5 years), it seems difficult to explain the apparently capricious behaviour of the disease, the sudden and wide fluctuations from year to year and even from one month to another, the irregular annual cycle in the same apiary, without definite seasonal peaks". While acknowledging the well-known influences such as a good or bad season or even management of the colonies, she felt that "something else is involved" such as temperature or humidity. Also Arizona, a region with very low relative humidity (RH), has only very low levels of mite infestation, much lower than adjacent states (Sammataro, pers. comm.).

Mites are small animals and because of their high body surface area to volume ratio are constantly in danger of dehydration. Ticks and mites can conserve water reserves in many different ways; waterproofing cuticles, spiracle closing devices, absorption of vapour from surrounding atmosphere or by adopting behaviour that reduces exposure to harsh environments (Knulle and Rudolf, 1982). *A. woodi* mites have a pair of spiracle openings at the base of the gnathosoma (Rennie, 1921). They feed on the haemolymph of the bee and hence their fluid levels are normally regulated when *in situ* in the tracheae. However, when transferring from the old to the new host, mites are vulnerable and prolonged exposure particularly in a low RH environment presents a high risk. Also, the locomotor effort in the host-questing mode results in frequent spiracle opening further depleting moisture levels (Knulle and Rudolph, 1982).

Consequently, an experiment was undertaken to test the hypothesis that relative humidity affects the susceptibility of honeybees to tracheal mite infestation. To my knowledge there is no published experimental work on the affect of relative humidity on tracheal mite infestation in honeybees. In order to get a better understanding of the possible reasons for any differences in susceptibility it is proposed to evaluate some of

the elements of the biology of mite reproduction such as the egg hatch time and delay time before the first egg is laid.

6.2 Materials and methods

Colony preparation and inoculation:

The experiment was undertaken during October 2004 using three medium/strong colonies (Appendix 1). All three colonies had a low daily varroa dead mite drop (natural) on their hive-floor screens (< 2 per day) and no other diseases were detected except for a low prevalence of tracheal mite infestation in colony 2. The colonies had between 4 and 6 frames of brood. A frame of brood with a good spread of eggs and 6 - 7 day old larvae was identified in each colony on 29th September. This was undertaken to ensure that callow worker bees would emerge on 12th and 19th of October and permit the inoculation of the test bees in Part 1 at 60% RH (nominal) on 12th October and at 40% RH (nominal) on 19th October in Part 2. As the presence of brood odour/pheromone may influence susceptibility it was planned, in keeping with the approach in the other experiments, to have two frames of brood from each colony (six frames in all) in the incubator during inoculation. The general procedure in Chapter 2 was followed.

Part 1: High relative humidity (60%)

On 12th October the marked frame of brood plus one other were removed from each of the three colonies and put into the incubator at 50% RH and 34°C. Thirty callow bees issuing from each of the marked frames were introduced to inoculation cage 1 and 400 bees (nominal) added from the host colony (Figure 6.1). The planned inoculation conditions were 60% RH and 32°C. The marked frame was replaced in the incubator with another frame of brood and the marked frame returned to its parent hive. On 19th October the bees in the inoculation cage were subdued with CO_2, held at 0°C, counted, labelled and stored at -30°C to await dissection.

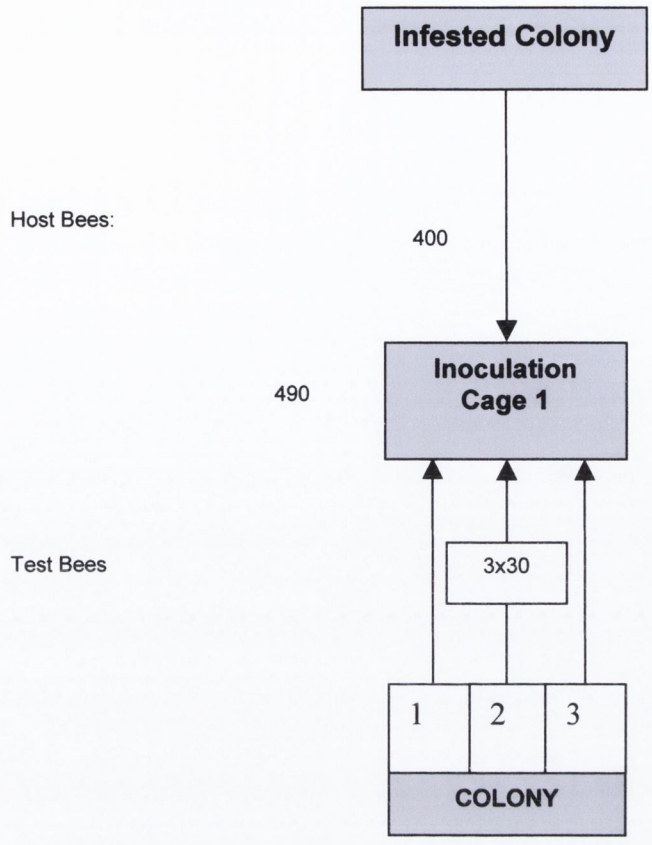


Figure 6.1 Experimental design.

Part 2: Low relative humidity (40%)

On the 19th October the original brood frames were returned to the incubator at 50% RH and 34°C. The callow bees were collected and the host bees introduced into inoculation cage 2 as before. The inoculation conditions were 40% RH and 32°C with six frames of brood present in the incubator.

On 26th October, when the bees had inoculated for the same length of time as in Part 1, the bees were subdued with CO_2 and prepared for dissection as before.

Dissection and bee morphometry

The bees were dissected using the technique developed for the previous experiments. The comparison of the ratio of mite offspring stages between the treatments was of particular interest in the evaluation of possible changes in mite reproductive biology and

hence to explain possible treatment effects. Accordingly, specific attention was given to cutting and removing a piece of adjacent cuticle from the thorax with the spiracle/trachea to avoid bursting eggs or damaging larvae.

Before dissecting the bees a right forewing was removed. The cubital index and discoidal shift were measured using Beemorph© program as before to determine the genetic strain (Ruttner, 1988a).

Mite reproduction biology

Mite reproductive rate can be described in terms of fecundity (egg laying rate), egg hatch time and delay time before egg laying cycle commences (Figure 6.2). The time taken for mites to mature is outside the short timescale of the inoculation experiments in this research. It is assumed that reproduction is described by a linear model of the form;

$$\text{Offspring} = (t - D) \times \text{Egg laying rate} \qquad (1)$$

where 't' = inoculation time and 'D' = the time delay (days) before the mite starts its laying cycle.

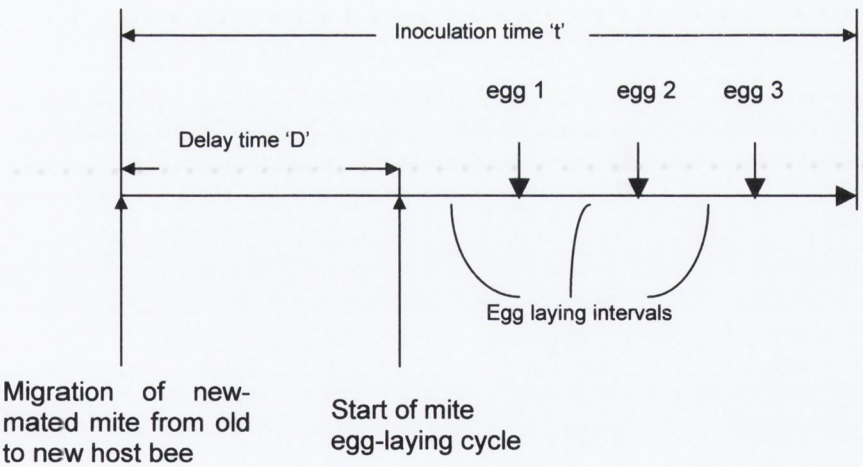


Figure 6.2 Schematic of mite reproduction.

(a) Fecundity. In the experiments to date the fecundity has been similar for all treatments and this is analysed in the mite biology model in Chapter 8. The egg-laying rate derived from the regression analysis gave a significant value of 1.38 eggs per day. It is proposed to use this value in the analysis that follows.

(b) Mite egg-hatch time. There is no absolute time base during the experiment that will measure the time to hatch. However, the number of eggs in the offspring of a family of mites which also has larva(e) will give a measure of the time to hatch in terms of the number of "egg laying intervals" (Figure 6.2). There must be uninterrupted egg laying so that steady-state conditions exist. A data set can be made up from all the mite infestation data by compiling it on a per trachea basis and only retaining data where the founding female mite is not dead, where there is only one female mite, and where there is at least one larva present. The egg abundance (eggs/mite) is calculated from this data and this represents the egg-hatch time in terms of number of "egg-laying intervals". Using the egg lay rate the hatch time can be calculated.

(c) Initial egg-laying delay time. Using the maximum brood produced by single foundress female mites in the inoculation period (days) and the egg-laying rate (1.38 eggs per day as above) the initial egg delay time can be derived. This derived delay time assumes that the mites in these cases transfer at the start of the inoculation process. Since this will not necessarily happen the calculated delay time is therefore the maximum value. Using the data set compiled above, the offspring abundance (mean mite fecundity) can be analysed. The difference in offspring abundance for the two treatments will be due to the difference in initial delay times (since egg laying by all mites in all treatments at the end of the inoculation period will have been delayed). This difference in abundance can be converted to delay time using the egg laying rate.

Statistical Analyses

The mite abundance, egg abundance and offspring abundance data were analysed using a 2-way ANOVA with treatment and colony as fixed factors. A chi-square test was used to compare the target bee and mite mortality data from the 60% RH and 40% RH treatments.

6.3 Results

Relative humidity in the cages

It had been planned to have the RH in cage 2 considerably lower (30% nominal) to obtain a good spread in mean RH values between the two cages. However, maintaining six frames of brood (much of it open brood) in the incubator made it difficult to achieve low values. In fact the RH level achieved was 43% which is close to the nominal 50% RH used in the other experiments. On the other hand, the mean RH in cage 1 during inoculation was 65% which was much higher than the nominal 50% RH.

Recovery and mortality (Target and host bees)

The inoculation process ran for a similar length of time for each cage, 6.49 days and 6.48 days for cage 1 and cage 2 respectively. The food consumption was 3.3 % higher in the 43% RH cage at 43.51 µl /day/bee compared to 42.14 µl/day/bee in the 65% RH cage. All of the 90 target bees in each cage were recovered (Table 6.1). The mortality data for bees and mites in the two cages are given in Table 6.2. The mortality for all target bees in cage 1 at 11 bees was significantly higher than the 3 bees in cage 2 (χ^2 = 4.96, $p < 0.05$) and this higher mortality was found across all colonies. There was also a significant three-fold higher mite mortality (identified as shrivelled mites in the trachea) in cage 1 (χ^2 =4.75, $p < 0.05$) and this was also found across all colonies (Table 6.2).

The host bee mortality during the short inoculation period was almost two thirds at 65% and 66% for cages 1 and 2 respectively (Table 6.1).

Bee Morphometry

The cubital indices mean (± s.e.) were 1.71 ± 0.04, 1.73 ± 0.05 and 1.63 ± 0.04 for colonies 1, 2 and 3 respectively. The corresponding values for discoidal shift (± s.e.) were -1.79 ± 0.21, -2.74 ± 0.22 and -4.36 ± 0.22. These values indicate colonies that are of the subspecies *Apis mellifera mellifera,* per the German Breeding Regulations (D.I.T.), with colony 3 being strongly of this subspecies (see Figure 9.1). Colonies 1

and 2 displayed similar morphometry which was to be expected as they were from the same *A. m. mellifera* breeding stock.

Table 6.1 Survival and recovery levels of host and target bees in the two inoculation cages.

Bee	Inoculation Cage 1 Humidity ~ 65%			Inoculation Cage 2 Humidity ~ 43%		
Colony	Alive (%)	Dead (%)	Total	Alive (%)	Dead (%)	Total
Host:	149 (35%)	279 (65%)	428	157 (34%)	304 (66%)	461
Target						
Colony 1	28 (93%)	2 (7%)	30	29 (97%)	1 (3%)	30
Colony 2	24 (80%)	6 (20%)	30	29 (97%)	1 (3%)	30
Colony 3	27 (90%)	3 (10%)	30	29 (97%)	1 (3%)	30
Total (Target)	79 (88%)	11 (12%)	90	87 (97%)	3 (3%)	90
All Bees: Food/bee/day (µl)		42.14			43.51	

Table 6.2 Mortality of the target bees and female mites for each colony and in each inoculation cage.

Bee	Inoculation Cage 1 Humidity ~ 65%		Inoculation Cage 2 Humidity ~ 43%	
Colony	Dead Bees (total)	Dead Mites (total)	Dead Bees (total)	Dead Mites (total)
Colony 1	2 (30)	2 (13)	1 (30)	0 (9)
Colony 2	6 (30)	4 (13)	1 (30)	3 (13)
Colony 3	3 (30)	3 (6)	1 (30)	0 (15)
Total	11 (90)	9 (32)	3 (90)	3 (37)

Female mite abundance

The infestation data for all host and target bees are given in Table 6.3. The adult mite and larval numbers in the 60-bee host samples are about 25% less in cage 2. However, when allowance is made for the greater number of host bees in cage 2 and the fact that the host bees were taken from the same host colony within a period of one week, a similar inoculation pressure is assumed in each cage.

Table 6.3 Infestation data for all host and target bees in the two inoculation cages.

Bee	Number of Bees		Mite Stages				Abundance (mites/bee)			
Colony	Total	Infested	Adults	Larvae	Eggs	Total	Adults(Adj)[1]	Larvae	Eggs	Total
Cage No. 1										
R H ~ 65%										
Host; Before	60	13	30	102	58	190	0.5	1.7	0.97	3.17
Target:										
Colony 1	28	12	12(13)	20	32	64	0.43 (0.46)	0.71	1.14	2.32
Colony 2	24	11	12(13)	18	22	52	0.50 (0.54)	0.75	0.92	2.21
Colony 3	27	5	6(6)	5	8	19	0.22 (0.22)	0.19	0.3	0.7
Total	79	28	30(32)	43	62	135	0.38 (0.41)	0.54	0.78	1.73
Cage No. 2										
R H ~ 43%										
Host; Before	60	9	23	65	18	106	0.38	1.08	0.3	1.76
Target:										
Colony 1	29	8	7(9)	3	23	33	0.24(0.31)	0.38	0.79	1.21
Colony 2	29	11	12(13)	11	24	47	0.42 (0.45)	0.38	0.83	1.66
Colony 3	29	11	13(15)	21	44	78	0.45 (0.52)	0.72	1.52	2.76
Total	87	30	32(37)	35	91	158	0.38 (0.43)	0.4	1.05	1.87

[1] Female mites present plus an allocation of one female mite for each occasion that offspring was present with no corresponding female mite.

The overall female mite abundance (mean ± s.e.) was similar for the two treatments at 0.41 ± 0.07 and 0.43 ± 0.07 mites per bee for the high and low RH treatments respectively (Figure 6.3). There was no treatment effect ($F_{[1, 160]} = 0.027$, $p = 0.870$) or colony effect ($F_{[2, 160]} = 0.635$, $p = 0.531$) on the overall data. However, the individual colony infestation patterns showed contrasting susceptibility characteristics (Figure 6.3). The female mite abundance rose with increased RH in colonies 1 and 2 but was considerably reduced in colony 3. However, there was no individual colony treatment effect; colony 1, ($F_{[1, 55]} = 0.727$, $p = 0.398$); colony 2, ($F_{[1, 51]} = 0.105$, $p = 0.747$); colony3, ($F_{[1, 54]} = 0.3.162, p = 0.081$).

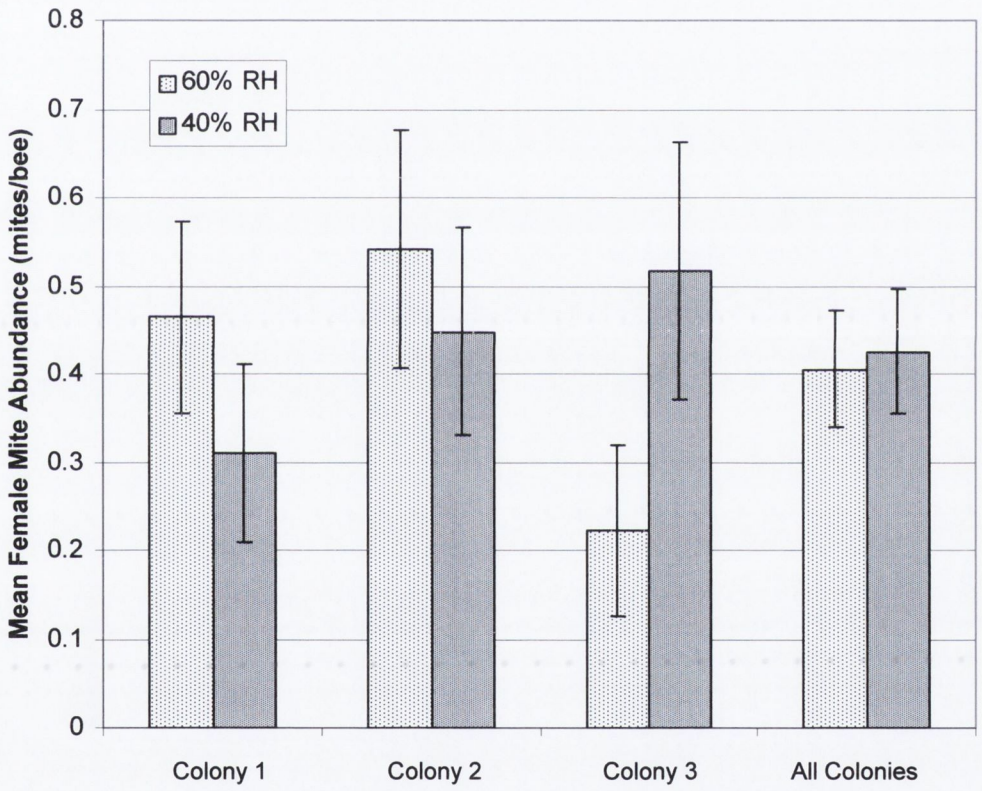


Figure 6.3 Female mite abundance (mean ± s.e.) for target bees in cage 1 (60% RH) and cage 2 (40% RH).

Mite egg hatch time

The mite infestation data set (Table 6.3) was modified by removing data for dead mites, organising data on a per trachea per adult mite basis and only retaining data where

larval load was at least 1. The resulting adjusted mite egg abundance data had no significant treatment effect ($F_{[1,22]} = 0.042$, $p = 0.839$) but had a significant colony effect ($F_{[2, 22]} = 3.324$, $p = 0.05$) and a significant interaction between colony and treatment ($F_{[2, 22]} = 4.603$, $p = 0.02$). The mean adjusted egg abundance ± s.e. for colonies 1, 2 and 3 were 2.90 ± 0.18; 3.00 ± 0.27 and 3.40 ± 0.16 respectively. The mean adjusted egg abundance for all colonies was 3.1 ± 0.19. This suggests a mite egg hatch time of 3.1 times the egg laying interval and this was similar for both treatments. Using the egg laying rate of 1.38 per day results in an egg hatch time of 2.24 days.

Mite larvae vs. eggs

The overall larvae to egg ratio in the 65% RH cage was over 80% higher than in the 43% RH cage at 0.69 vs. 0.38. This higher ratio was repeated across the three colonies. When the data for the mites that had died are removed the new ratio is 0.52 (30 larvae / 58 eggs) for the 60% RH cage and 0.35 (32 larvae / 90 eggs) for the 40% RH cage. Given that the inoculation period was the same for each RH treatment (6.49 vs. 6.48 days), this increased larval content may be due to reduced egg hatch time and/or a shorter time delay before the first egg is laid in the case of the higher (60%) RH cage. As the egg hatch time was similar for the two treatments, a shorter time delay before laying is indicated in the 60% RH case.

Egg-laying time delay

The frequency of the maximum numbers of offspring for a single female mite over the inoculation period is given in Table 6.4. Using this maximum value of 6 mite offspring and the egg laying rate of 1.38 eggs per day and substituting into equation (1) above gives a delay time of 2.13 days.

The mite infestation data set (Table 6.3.) was modified by removing data for dead mites, organising data on a per trachea basis and only retaining data where adult mites are equal to 1. The mean mite fecundity (offspring abundance) for the two treatments was not significantly different ($F_{[1,41]} = 1.844$, $p = 0.182$). However, there was a difference of 0.6 at 4.35 ± 0.32 and 3.74 ± 0.26 offspring per female mite for the 65% RH and 43% RH cages respectively. As indicated above, colony 3 was out of step with the other two

colonies in the high RH treatment – low level of mite infestation and half of the mites dead at the end of the inoculation period (Table 6.4). If colony 3 is excluded, colonies 1 and 2 together had a significant treatment effect in fecundity ($F_{[1,29]} = 5.998$, $p = 0.021$) of 1.19 offspring per female mite at 4.39 ± 0.34 for 65% RH and 3.20 ± 0.35 for 43% RH. At an egg laying rate of 1.38 eggs per day this represents a lower initial delay time before laying of 0.86 days in the case of the high RH treatment. This gives delay times of 2.13 days and 1.27 for the 43% and 65% treatments respectively.

Table 6.4 Frequency of 6 and 5 offspring per foundress female mite for each treatment and colony along with the corresponding number of female mites.

Bee Colony	Inoculation Cage 1 Humidity ~ 65%			Inoculation Cage 2 Humidity ~ 43%		
	Offspring X 6	Offspring X 5	Total mites ( )[1]	Offspring X 6	Offspring X 5	Total Mites ( )[1]
Colony 1	3	3	13 (11)	0	1	9 (9)
Colony 2	2	3	13 (9)	2	0	13 (10)
Colony 3	0	1	6 (3)	4	2	15 (15)
Total	5	7	32 (23)	6	3	37 (34)

[1] The figures in ( ) are the numbers of mites alive at the end of the inoculation period.

6.4 Discussion

There was evidence that the mites in the lower humidity cage (43% RH) took longer to start laying after migrating to the new host trachea than those in the 65% RH cage. There was also evidence that the target bees and mites from colony 3 were exposed to an abnormal ambience in the high RH cage resulting in stress as indicated by the significantly higher levels of mortality. When the data from colonies 1 and 2 only were used there was a significant increase of about one day (0.86) in the delay time in the

lower compared to the higher RH conditions. This would indicate that the foundress female mites were under greater danger of desiccation in the lower RH cage and required additional time to restore their body fluid levels after initial migration. A total delay time of 2.13 days was estimated for the lower humidity cage and this compares with a delay time of 1.76 days from the model in Chapter 8 and the estimate by Pettis (2001) of an overall ovipositing delay of 1-2 days. An egg hatch time of 2.24 days was estimated for both treatments and this compares with a hatch rate in the biology model in Chapter 8 of 2.12 days.

The delayed egg-laying behaviour indicated by these results is consistent with known mite biology and behaviour. The *A. woodi* mite in the trachea of a honeybee is in a habitat of relative security. The open circulatory system of the bee enables the mite to have ready access to its blood meal by simply piercing the wall of the trachea and sucking the haemolymph of its bee host. In this habitat of plenty the mite can regularly replenish body fluids and hence the danger of dehydration, which is normally critical for a small animal, is removed. In contrast, during its short period of migration the mite is separated from its source of sustenance and faces real danger. The movement of the mite from bee to bee during migration and in particular the highly physical behaviour involved in questing puts a high demand on the mite's energy resources. This requires frequent opening of the spiracles resulting in moisture loss (Knulle and Rudolph, 1982). An environment of low relative humidity places further demands on the fluid level of the mite, hence the increased delay between migration and egg laying identified in the experiment.

The overall susceptibility was similar for the two RH treatments but this masked differences within individual colonies. Colonies 1 and 2, from the same breeding stock, behaved in a consistent manner between treatments in contrast to the response of colony 3, a more strongly *A. m. mellifera* strain. However, without replication within colonies it was not possible to show significance in the RH treatments for each colony. The susceptibility of the three colonies in the low RH inoculation cage behaved in a manner consistent with that predicted from their wing venation characteristics – a high negative discoidal shift corresponding with a high susceptibility (Chapter 9). If the same relationship had obtained for the three colonies at 65% RH, the susceptibility of colony 3 would have been higher than that of the other two. In fact, the susceptibility of colony

3 was much lower. In temperate climates such as in Ireland, with ambient temperature normally well below brood nest temperature it would be expected that the RH would be comparatively low (Velthuis and Kraus, 2002). Could the comparatively high RH (65%) have created an ambience that was not tolerable as demonstrated by the significantly higher callow bee and female mite mortality? It would appear as though the mites and the callow bees in this cage had been under more stress and in the case of the mites in colony 3 have not been able to freely migrate as evidenced by the low mite migration numbers, half of which were dead at the end of the inoculation period. The morphometry of this colony would indicate that it has low grooming ability (Chapter 4) and therefore the mites are unlikely to have been groomed off. It may be that these mites have been less able, due to the high moisture level in the cage, to use their gustatory senses to identify the age of the new host and may have tended to quest for longer periods (Chapter 5).

It is clear from this experiment that many aspects of the dynamics involved need to be further studied. A further experiment would be required using colonies from a similar subspecies to colony 3, replicated by three, to obtain more representative data on the high RH response.

CHAPTER 7

Influence of comb-cell size on honeybee susceptibility to tracheal mites

Two extracts from this chapter have been accepted for publication:

McMullan, J.B. and Brown, M.J.F. (in press). The influence of small-cell brood combs on the morphometry of honeybees (*Apis mellifera* L.). *Apidologie* (accepted 31/01/06).

McMullan, J.B., Brown, M.J.F. (2006). Brood-cell size does not influence the susceptibility of honeybees (*Apis mellifera*) to infestation by tracheal mites (*Acarapis woodi*). *Experimental and Applied Acarology* **39:** 273-280.

7.1 Introduction

The honeybee species *Apis mellifera* is made up of a number of geographical strains or subspecies that have developed largely as a result of natural selection, unhindered by man, in their native regions (Ruttner, 1988a). This changed with Langstroth's discovery of 'bee-space' and the subsequent use of large-cell foundation. The apparent reason for this increase (~ 10%) was to compensate for the cocoon build-up in the cell as well as the culture at the time that "big is good" in agriculture (Baudoux, 1933).

In the 1930s experiments were undertaken by Grout (1937) to identify the influence of even larger brood cells on the size and variability of the emerging bees. Grout concluded that while there was some conflict between the results they did show that an increase in the size of the brood cells is accompanied by a corresponding increase in a range of physical measurements. This belief in a corresponding relationship between cell size and bee size is still widespread. Ruttner (1988a) stated "If bees are forced to raise brood in cells of a size other than the specific one – smaller or larger – then the size of emerging bees is changed correspondingly". A quotation from Erickson *et al.* (1990) also illustrates the position: "These data clearly demonstrate the ease with which a beekeeper can effectively reduce comb cell width in colonies. A corresponding reduction in bee size should follow without selection and breeding". This assumption has also appeared in more recent work (Martin and Kryger, 2002).

After the discovery of 'bee-space' by Langstroth in 1851 moveable hive frames and wax foundation came into common usage. The brood-cell size of the Northern European dark bee, *Apis mellifera mellifera,* indigenous to Britain and Ireland up to the early part of the twentieth century, was in the range of 4.9 to 5.1 mm (Cowan, 1904; brood cell size was measured as the distance between the opposite faces of the hexagonal cell Figure 7.1). The period from the late 1800s to the 1920s saw a major change in cell size with the introduction of commercially produced foundation of about 5.5mm width (Erickson, 1990).

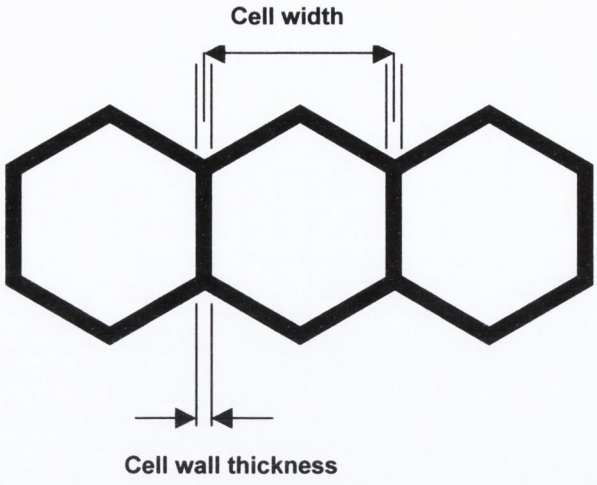


Figure 7.1 Brood comb showing the measurement of the external cell width across opposite faces of the hexagonal cell. The cell width includes half the width of the cell walls on each side.

Erickson suggested that brood comb cell widths larger (or smaller) than normal might stress colonies making them more susceptible to parasites. It has also been suggested that *A. woodi* may have evolved prior to 1900 from one of the closely related external mites in the *Acarapis* genus (De Jong *et al.*, 1982), possibly related to this change in cell size.

A morphometric study of the Greek subspecies, *Apis mellifera macedonica*, considered that the reduced dimensions of certain thoracic features relative to those in other subspecies might help to explain its resistance to tracheal mite infestation (Hatjina *et al.*, 2004). The increase in cell size in Britain and Ireland did coincide with the outbreak of the 'Isle of Wight' disease at the early part of the last century and there is a possibility that the larger cell size triggered the change in host location and heralded the onset of tracheal mite infestation.

The research in this chapter covers both the bee morphometry and susceptibility elements. First, a study investigates the ability of colonies of *A. m. mellifera* to draw out and raise workers in the brood-cell size of the 1800s (circa 5.0mm) and undertakes a series of measurements of key morphometric characteristics of honeybees. The results are discussed in the context of other published work that examined changes in bee

morphometry resulting from changes in cell size, and possible implications for tracheal mite parasitic infestation are considered. Second, an experiment investigates the effect of cell size on tracheal mite infestation. To my knowledge, no experimental work has been undertaken to identify the impact of cell size on the susceptibility of honeybees to tracheal mite infestation. As honeybees are susceptible to tracheal mite infestation almost exclusively in their first 4 to 5 days after emergence from the cell (Morgenthaler, 1930; Bailey, 1958; Lee, 1963; Giordani, 1977; Gary *et al.*, 1989), an experiment was conducted on callow bees. The experiment tested the hypothesis that callow bees from small-brood cells (the historical size of *circa* 5.0 mm) are less susceptible to tracheal mite infestation than callow bees raised in the increased brood cells (standard size of *circa* 5.5 mm).

7.2 Materials and methods

Preparation of test colonies

The experiment was undertaken during the last week in April 2005 using honeybees from colonies in North County Dublin (Appendix 1). In spring 2003, three colonies of bees that had over-wintered on standard-sized brood cells (new combs in the previous summer/autumn) in 5 frame nucs were transferred to full sized hives, fed 50% sugar syrup and had small-cell foundation progressively added over a period of four weeks. The foundation was 4.9/5.0 mm wired Langstroth bees wax (E.H. Thorne Ltd.) sourced in the U.S., and was fitted into Modified Commercial hive frames. All three colonies had fully drawn out the six frames of small-cell foundation in the four-week period. The frames in each hive were arranged alternately small-cell/standard-cell (5.5 mm nominal) brood comb. The hives were in two apiaries ten kilometres apart. The mite *Varroa destructor* was first identified in these apiaries in 2003 and Bayvarol™ treatment was given in the autumn of 2003 and 2004.

A period of 21 days (time from egg to emergence for worker bees) before the start of the experiment, two adjacent frames in the centre of the brood nest, one of each cell

size, were assessed for the presence and quantity of eggs to ensure that bees would be emerging at the start of the experiment. These frames were marked in each of the three hives. Hence the bees developing in these two marked frames were exposed to the same hive conditions, the only variable being the size of the brood cell.

Laboratory bioassay

Inoculation cages of infested bees were set up and the newly emerged worker bees to be tested were introduced into them. A schematic of the experimental design is given in Figure 7.2. The inoculation cages were filled in turn with an estimated 360 infested worker bees collected from the landing board of the host colony. A sample of sixty bees was also taken from the landing board of the host colony, placed in numbered boxes and stored at -30°C. These bees were later dissected to give a measure of the initial host-bee infestation levels in the cages.

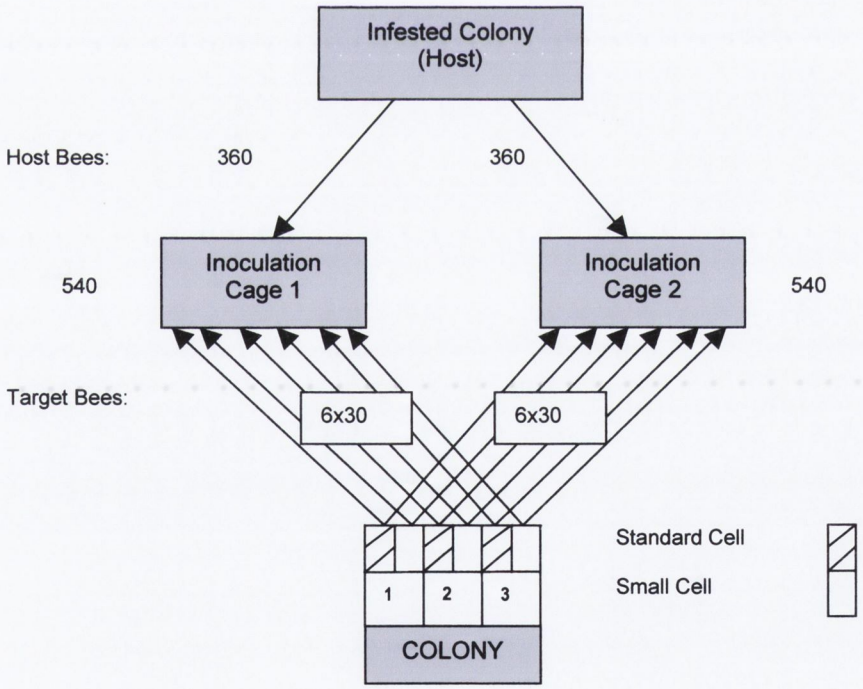


Figure 7.2 Schematic of the experimental design.

Target bees

The two marked frames from the three target bee colonies had the adhering bees brushed off on 20th April 2005 and each was inserted into separate stainless steel (perforated) frame cages and transferred to a dark incubator at 34°C and a relative humidity of 50%.

At eight-hour intervals over the next 24 hours callow bees emerging from the six frames were collected in separate polystyrene cups that had a smear of petroleum jelly along the top to prevent the callow bees from climbing out. Over a 24-hour period, thirty marked bees from each of the six frames were introduced by funnel into each of the two inoculation cages giving a total of 180 marked target bees in each cage. The inoculation cages were maintained in a dark incubator at 32°C and 50% RH. The experiment was concluded after 7 days. The dead bees were removed from the bottom of the cage, segregated and counted. The live bees were subdued with CO_2 gas, held at 0°C and then, taking each cage in turn, were segregated into host bees and the six categories of target bees and counted. The target bees from each cage were then placed in numbered boxes and stored at -30°C to await dissection.

Measurement of brood combs cell size

The cell sizes (width) of the brood combs from which the bees emerged were measured. The width was taken as the distance between opposite faces of the hexagonal cell and included half the thickness of the cell wall on each side (Figure 7.1). Five samples of 30 cells each were taken from each of the three axes (at 60° spacing) from both sides of each of the six target brood combs. The cell depth was also measured using twenty samples from each of the six target brood combs.

Target bee morphometric measurements

Prior to and during the dissection of the target bees a number of physical measurements were made:

(i) before dissection the width of the head was measured using calipers (± 0.01mm).

(ii) the diameter of the prothoracic trachea was measured under a stereo microscope (± 2μm) using an ocular grid. The diameter, mid-way between the spiracle and the first branch, was calculated by converting from the measurement of the circumference of the flattened trachea on the sticky tape.

(iii) the size of the radial cell on the right forewing was calculated using digitized wing venation data from Beemorph©, the bee wing morphometric analysis program.

(iv) the cubital index (Figure 2.9) was also measured using the Beemorph© program to give an assessment of the genetic strain of the bee colony (Ruttner, 1988a, b). A mean value less than 1.9 and individual bees under 2.2 are taken as indicative of *A. m. mellifera*. in the German Breeding Regulations (D.I.B., 1986).

(v) the discoidal shift (Figure 2.9) was also calculated using the Beemorph© program. A negative value is indicative of *A. m. mellifera* while in the case of *A. m. ligustica* it is positive, and positive or zero for *A. m. carnica* (Ruttner, 1988a, b).

(vi) the callow bees emerging from the brood frames in the incubator were collected every eight hours and the average mass was obtained on a daily basis for each of the two cell sizes for a period of ten days.

(vii) the colouration of the plates of the abdomen (tergites) and the width of the tomentum were noted using the approach of Ruttner (1988b).

In September 2003, when the brood combs had been in the hives for about 6 months, a similar morphometric study was carried out to the one described above. Because bees may gradually adapt to new nesting conditions, comparing the 2005 and 2003 data would allow behavioural adaption and plasticity trends to be identified.

Dissection of target bees

The bees were dissected as in previous experiments using a modification to the classic technique of removing the head and thoracic collar as described by Shimanuki and Knox (2000) and removing the tracheae. Using a stereomicroscope at 60-100X magnification, each trachea was opened using a dissecting needle. The number of adult mites, larvae and eggs were counted and recorded.

Statistical analyses

Morphometric data:

Differences between small-cell and standard-cell bees in biometric traits (i) to (v) (see above) were analysed using a 2-way MANOVA for overall and individual effects, with cell-size and colony as fixed factors. The bee mass and cell-size data were analysed using one-way and two-way ANOVA respectively, the latter being log transformed to $\log_{10}(y + 1)$ as the variances were not homogeneous. A chi-square test was used to compare the abdominal colouration of the bees from the two cell sizes.

Abundance data:

The mite abundance data were skewed and had variances that were generally in proportion to the sample means. Accordingly, the mite count data were transformed to $\log_{10}(y + 1)$ (Sokal and Rohlf, 1995). Mite abundance data were analysed as a 3-way ANOVA with treatment, cage and colony as fixed factors.

7.3 Results

The morphometric data from this experiment and also that of September 2003 are given in Table 7.1.

Drawing wax and rearing brood

The colonies constructed small-cell comb and reared brood without any apparent difficulty. The small-cell comb was drawn out in a regular manner and had a much smaller variance in cell size than the standard comb (Table 7.1).

Brood cell sizes

Comparing the small-cell comb to the standard-cell comb, there was a significant overall reduction of 7% & 8% in the cell width (mean ± s.d.) for 2005 and 2003 respectively. The average cell depth (mean ± s.d.) was similar at 11.09 ± 0.61 & 11.03 ± 0.64 mm for the standard cells and 11.03 ± 0.62 & 10.98 ± 0.49 mm for the small-cell comb. There was no significant difference in the cell sizes between the three colonies ($F_{[2, 174]} = 2.71$, p = 0.07).

Morphometric comparisons

Multivariate analysis:

There was an overall cell-size effect, (2005: $F_{[5, 344]} = 37.66$, $p < 0.001$); (2003: $F_{[5, 338]} = 21.61$, $p < 0.001$), and colony effect, (2005: $F_{[10, 690]} = 15.42$, $p < 0.001$); (2003: $F_{[10, 678]} = 19.21$, $p < 0.001$) on the five biometric measurements; head width, radial cell length, trachea diameter, cubital index and discoidal shift.

Table 7.1 Summary of morphometric measurements for all the test honeybees raised from the standard and the small brood cells in (a) April 2005 and (b) September 2003.

(a) April 2005 Study

Measurements	Standard Cell (mean ±s.d.)	Small Cell (mean ±s.d.)	Size ratio[1]	Significance[2]
Cell size (mm)	5.44 ±0.13 (n = 90)	5.07 ±0.08 (n = 90)	0.931	***
Head width (mm)	3.75 ±0.04 (n = 179)	3.71 ±0.04 (n = 175)	0.989	***
Radial cell length (mm)	3.40 ±0.08 (n = 179)	3.35 ±0.07 (n = 175)	0.986	***
Trachea dia (µm)	185 ± 3.8 (n = 179)	182 ±3.4 (n = 175)	0.983	***
Cubital index	1.82 ±0.22 (n = 179)	1.81 ±0.24 (n = 175)	0.999	n.s.
Discoidal shift (°)	- 2.05 ±2.20 (n = 179)	- 2.35 ±1.81 (n = 175)	1.150	n.s.
Bee mass (grams)	0.116 ±0.004 (n = 13)	0.107 ±0.003 (n = 13)	0.922	***

(b) September 2003 Study

Cell size (mm)	5.48 ±0.12 (n = 90)	5.04 ±0.03 (n = 90)	0.920	***
Head width (mm)	3.80 ±0.06 (n=173)	3.77 ±0.05 (n=175)	0.992	***
Radial cell length (mm)	3.46 ±0.06 (n = 173)	3.40 ±0.06 (n = 175)	0.982	***
Trachea dia (µm)	191 ± 5.7 (n = 173)	189 ±5.6 (n = 175)	0.993	*
Cubital index	1.62 ±0.24 (n = 173)	1.64 ±0.24 (n = 175)	1.010	n.s.
Discoidal shift (°)	- 2.35 ±2.31 (n = 173)	- 1.67 ±2.63 (n = 175)	0.710	**
Bee mass (grams)	0.113 ±0.003 (n = 10)	0.101 ±0.004 (n = 10)	0.894	***

[1] Measurements of bees raised in small cells divided by corresponding measurements in standard cells.
[2] Significance of comparison between morphometric measurements of bees raised in small and standard brood cells; *** significance at 0.001, ** significance at 0.01. * significance at 0.05, n.s. not significant.

Univariate analysis:

Small cell size caused significant reductions in head width (~1%), radial cell length (1-2%) and tracheal diameter (~1%). There was no effect on cubital index but discoidal shift was significantly greater in 2003 (see Table 7.1 for statistics). The cubital indices for the three colonies indicated dominant *A. m. mellifera* strains as did the high negative values of discoidal shift for the three colonies.

Other measurements

There were significant overall reductions of 8 and 11% in the mass per bee for the callow bees that emerged in 2005 and 2003 respectively. All the bees examined had a 'narrow' tomentum width that is consistent with an *A. m. mellifera.* strain (Ruttner, 1988b). The bees from all the colonies, except colony 3 (2003 study), had the tergites either 'all black' or with very 'small brown/yellow spots' on the second tergite. In the case of colony 3 (2003 study) 27 of 58 bees from standard cells had a narrow yellow ring on the second tergite while the corresponding data for small-cell bees was 24 of 58 bees, representing no statistical difference (d.f. = 1, $\chi^2 = 0.315$, $p = 0.58$).

Published data

Data from other studies in the published literature were reviewed to enable a comparison with different honeybee strains in the different parts of the world. Within each of the experiments reviewed there has been a similar percentage change in linear bee measurements across all measurement categories e.g. forewing length, thorax width etc. Hence change in thorax size would be representative of the other measurement categories (Table 7.2). It can be seen from the table that the *A. m. mellifera* strain has a different morphometry. The changes in thorax width in the 'Italian' or 'American' bee strains in papers 1, 2 and 3 reflected strongly the changes in cell size (80%, 67% and 120% respectively). This compared with a weak (10%) response in *A. m. mellifera.* The thorax width is about one third larger in *A. m. mellifera* at *circa* 4 mm versus *circa* 3 mm. The thorax width to cell width ratio ('fill factor') varied from 53% to 57% for the 'American' strains compared to 73% to 79% for *A. m. mellifera.*

Table 7.2. Comparison of measurements of brood-cell width and thorax width from published papers.

Published Data	Cell Width (mm)	Thorax Width (mm)	Fill Factor[1] (%)
Paper 1. Spivak M. *et al.* (1992)			
Apis mellifera (European strain[2])			
Tucson, Arizona			
Commercial comb	5.37	3.02	56
Small comb	5.08	2.89	57
Change (%)	-5.04	-4.3	+1
Paper 2. Jagannadham G. *et al.* (1980)			
Apis mellifera (Californian strain[2])			
Ladhiana, India			
Large comb	6.00	3.20	53
Normal comb	5.24	2.93	55
Change (%)	-12.7	-8.5	+2
Paper 3. Baudoux U. (1933)			
Apis mellifera ligustica (Belgian Congo strain[2])			
Large comb	5.55	4.15	75
Normal comb	4.95	3.59	73
Change (%)	-11.2	-13.5	-2
Paper 4. Grout R.A. (1937)			
Apis mellifera (Strain not stated)			
Ames, Iowa			
Large comb	5.50	9.66[3]	n.a.
Normal comb	5.19	9.61[3]	n.a.
Change (%)	-5.6	-0.60	
Paper 5. Current experiment			
Apis mellifera mellifera			
County Dublin, Ireland			
Standard comb	5.48	4.00	73
Small comb	5.04	3.97	79
Change (%)	-8.0	-0.8	+6

[1] Fill factor is thorax width / cell width expressed as a percentage.
[2] Strain designations as given in the source papers.
[3] Forewing lengths used as a representative measurement as thorax width not available.

Recovery and mortality levels (per inoculation)

There was a total of 356 and 362 host bees in inoculation cages 1 and 2 respectively and corresponding mortality of 26% and 19% at the end of the inoculation period (Table 7.3). The recovery rate for the target bees was 178/180 and 179/180 for cages 1 and 2 respectively with a mortality rate of less than 1%. The mean food consumption (per bee per day) for target and host bees over the inoculation period was similar at 45.3µl and 45.6µl for cages 1 and 2 respectively.

Table 7.3 Recovery and mortality levels for the host and target bees in the two inoculation cages.

Bee Colonies	Cage 1			Cage 2		
	Alive No.(%)	Dead No.(%)	Total No.	Alive No.(%)	Dead No.(%)	Total No.
Host:	262(74%)	94(26%)	356	295(81%)	67(19%)	362
Target:						
Standard cell						
Colony 1	30	0	30	30	0	30
Colony 2	30	0	30	29	0	29
Colony 3	30	0	30	30	0	30
Small cell						
Colony 1	30	0	30	30	0	30
Colony 2	29	0	29	29	1	30
Colony 3	27	2	29	30	0	30
Total	176	2	178	178	1	179
All Bees: Food/bee/day (µl)		45.3			45.6	

Mite abundance and fecundity

The infestation data for all host and target bees are given in Table 7.4. Where mite offspring were present in the trachea and no adult mite found, it was assumed that the foundress female mite had migrated further into the trachea (Gary *et al.* 1989). In these cases one female mite was allocated to the actual female mite total to give an adjusted female mite abundance. The adjusted mean fecundity (± s.e.) for the target bees from the standard and small brood cells was similar at 4.33 ± 0.21 and 4.22 ± 0.20 respectively (Table 7.4). There was no treatment (cell-size) effect on fecundity ($F_{[1, 166]} = 0.026$, $p = 0.873$). This is in line with the findings of Danka and Villa (1996) and the experiments in Chapters 3 and 4 that mite reproduction in the honeybee trachea is similar for different treatments and that differential mite abundance is determined by the success rate of the female mites migrating to the new hosts. Accordingly, the adjusted female mite abundance is used in the following analysis.

Table 7.4 Fecundity analysis for all target bees for the two brood cell sizes. The adjusted mean fecundity ± s.e. is the mite offspring per adjusted female mite over the inoculation period.

Treatment	Female Mites		Offspring			Adjusted[2] Mean
Cell Size	Actual	Adjusted[1]	Larvae	Eggs	Total	Fecundity
Standard	72	87	179	198	377	4.33 ± 0.21
Small	81	91	189	195	384	4.22 ± 0.20

[1]Actual mites present plus an allocation of one female mite for each occasion that offspring was present with no corresponding female mite.
[2]The adjusted mean fecundity ± s.e. is the mite offspring per adjusted female mite over the inoculation period.

Female mite abundance

The overall adjusted female mite abundance (mean ± s.e.) was similar for the standard and small brood cell sizes at 0.49 ± 0.06 and 0.52 ± 0.06 respectively (Table 7.5, Figure 7.3). There was no brood cell size effect ($F_{[1, 342]} = 0.139$, $p = 0.710$). There were also

Table 7.5 Summary of mite infestation levels for host and target bees for the three colonies in the two inoculation cages.

Bee	Treatment	No. of Bees		Mite Stages			
Colony		Total	Infested	Adults (Adj.)[1]	Larvae	Eggs	Total
Cage 1							
Host:	Before	60	21	41	110	47	198
Target:							
Colony 1	Standard	30	8	7 (10)	18	22	47
	Small	30	8	9 (10)	24	25	58
Colony 2	Standard	30	12	11 (15)	39	30	80
	Small	29	9	10 (10)	18	20	48
Colony 3	Standard	30	11	12 (14)	24	35	71
	Small	27	12	12 (17)	44	34	90
Total	Standard	90	31	30 (39)	81	87	198
(Cage 1)	Small	86	29	31 (37)	86	79	196
Cage 2							
Host:	Before	60	20	27	105	25	157
Target:							
Colony 1	Standard	30	12	20 (21)	44	56	120
	Small	30	15	20 (21)	30	38	88
Colony 2	Standard	29	9	10 (11)	16	19	45
	Small	29	9	14 (15)	34	34	82
Colony 3	Standard	30	13	12 (16)	38	36	86
	Small	30	12	16 (18)	39	44	99
Total	Standard	89	34	42 (48)	98	111	251
(Cage 2)	Small	89	36	50 (54)	103	116	269
Total	Standard	179	65	72 (87)	179	198	449
(All Cages)	Small	175	65	81 (91)	189	195	465

[1]Adjusted female mites in ( ) are the adult female mites present plus an allocation of one female mite for each occasion that offspring were present with no corresponding female mite.

no significant effects of cage or colony ($F_{[1, 342]} = 2.297$, $p = 0.131$; $F_{[2, 342]} = 0.838$, $p = 0.433$ respectively) or any of the interactions; colony * cell size ($F_{[2, 342]} = 0.172$, $p = 0.842$); cage * cell size ($F_{[1, 342]} = 0.143$, $p = 0.706$); cage * colony ($F_{[2, 342]} = 2.048$, $p = 0.131$).

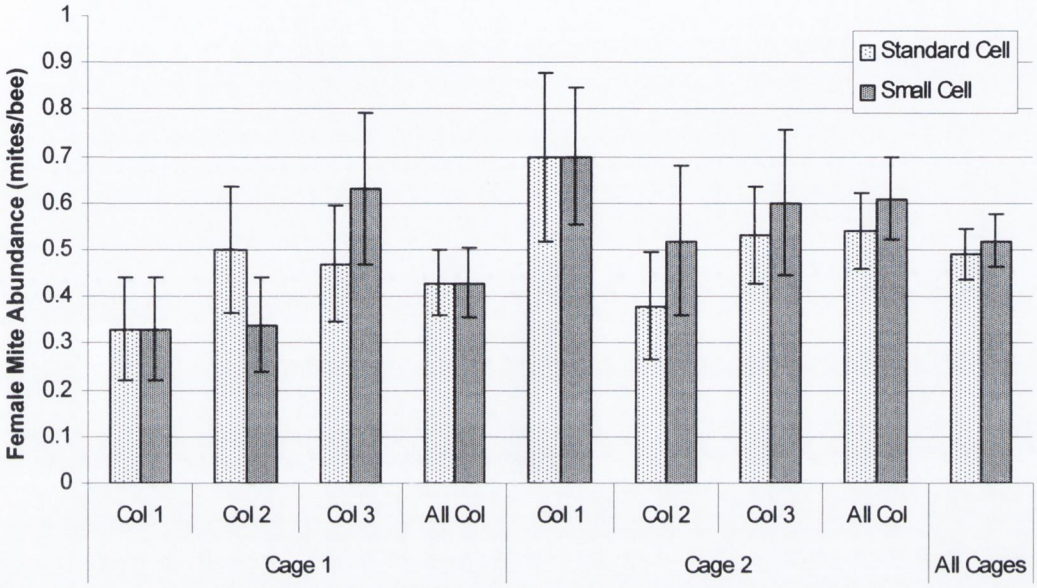


Figure 7.3 Female mite abundance (mean ± s.e.) for three colonies in the two inoculation cages.

7.4 Discussion

The morphometric data showed that the honeybee colonies used in the experiment were predominantly of the Northern European dark bee strain, *A. m. mellifera*, demonstrating that I was working with the same sub-species of *A. mellifera* in which tracheal mites were first discovered in Britain and Ireland. These colonies constructed small-cell comb and reared brood in the cell size of the mid 1800s without any apparent difficulty and this suggests that the cell size of the mid 1800s may still be the 'natural' size for these *A. m. mellifera* colonies. The ease with which the bees adapted to the new comb is in contrast to the experience in other parts of Europe where bees were moved onto wax

foundation with small-sized cells. There are reports of colonies of honeybees in Britain that are thought to have been on large cells (> 5.5 mm) for many decades, drawing out small-cell foundation in a series of rosettes across the face of the comb (Dave Cushman, pers. comm.).

There was no evidence that the susceptibility to tracheal mite infestation of bees raised in small-sized brood cells was any different to that of those raised in standard-sized brood cells and thus the hypothesis that callow bees from small-brood cells (*circa* 5.0 mm) are less susceptible to tracheal mite infestation than callow bees raised in the increased brood cells (standard size of *circa* 5.5 mm) is rejected. The female mite abundance and fecundity in bees from the two cell sizes were similar. Hence, there is no reason to believe that the increase in cell size in the late 1800s – early 1900s period influenced the onset of tracheal mite infestation in the indigenous honeybee in Britain and Ireland, *A. m. mellifera*.

These findings are consistent with the honeybee morphometric data that showed that the change in cell size (7 – 8%) only contributed to small changes in bee dimensions; head width (~1%), radial cell length (1-2%), tracheal diameter (~1%) and these small changes in tracheal diameter (~2 μm) are unlikely in themselves to affect access or reproduction of female tracheal mites (*Acarapis. woodi* width 70μm) in the trachea. Similar percentage changes in linear bee dimensions would be expected for the spiracle and spiracle access. In an interesting comparative study of honeybee morphometry as a possible explanation for tracheal mite resistance, measurements were made of the tracheae of three *A. mellifera* subspecies in a Mediterranean climate; *A. m. macedonica*, *A. m. ligustica* and *A. m. carnica* (Hatjina *et al.*, 2004). The measured tracheal diameters of all three subspecies were much larger than the diameter of the *A. m. mellifera* subspecies studied here. The smallest diameter (mean ± s.d.) was in *A. m. macedonica* and this was still over 10% larger (198 ± 14 to 218 ± 17 μm vs. 185 ± 4μm) than the measurements for *A. m. mellifera,* the Northern European dark bee. This appears contrary to Bergmann's rule where a smaller size in the south would be expected. One reason for the difference may be the method of measurement of tracheal diameter. Hatjina *et al.* (2004) mounted the trachea on a slide and measured the diameter while the diameter in the case of *A. m. mellifera* was calculated from a measurement of the

tracheal circumference. The former method of measuring the diameter is likely to be less accurate as it is subject to deformation of the tracheae and less consistent; the latter evidenced by a much greater standard deviation (14 to 17μm vs. 4 μm).

The results are in direct contrast to the changes in bee measurements resulting from changes in brood cell size in 'American' honeybee strains which show a proportional response. In my experiment the small-cell brood combs also resulted in smaller bees, but this reduction in size was clearly not in proportion to the reduction in cell size. While Grout (1937) concluded that changes in brood-cell size and bee dimensions are proportional, this is not consistent with his data. In fact his data show a similar pattern to that in the present study and would indicate that the strain of bees used in Grout's experiments was likely to have been *A. m. mellifera* as this strain was widespread prior to 1940 (Ruttner, 1988a). The results from the experiment were consistent between September 2003 and April 2005, indicating not only that the conclusions are reliable, but also that the reduction in bee size caused by smaller cell size is a step change rather than a response that changes over time.

It is clear from this study that the morphometry of *A. m. mellifera* honeybees is significantly different in many respects from other *A. mellifera* strains. The thorax width was about one third larger than that of the 'American' strains probably reflecting Bergmann's rule for the North European strain. Furthermore, the 'fill factor' (thorax width to cell width ratio) varied from 53% to 57% for the 'American' strains compared to 73% to 79% for the *A. m. mellifera* strain in Europe (Table 7.2). The latter represents a relatively restricted condition for the developing bee in the brood cell and this is further intensified with the change to small brood cells.

These results may have implications for parasitism by mites. Since the size of the bee does not reduce *pro rata* with the cell reduction there is considerably less space for the developing bee in the small cell for the *A. m. mellifera* honeybees. There are indications from other honeybee species that reproduction of *Varroa destructor* is reduced where the developing bee is restricted in the cell (Martin and Kryger, 2002; Piccirillo and De Jong, 2003).

While there is no evidence that raising bees in small brood combs has any direct bearing on their susceptibility to tracheal mite infestation, there may be an indirect colony effect. Infested honeybees have a reduced ability to heat the winter cluster (Komeili and Ambrose, 1991). Reduced brood pupation temperature in this period would increase susceptibility of the bees to tracheal mites (Chapter 3). Hives with small cell brood combs, as used in the experiment, would have an increased (+16%) density of bees in the winter cluster. Hence, tracheal mite infested honeybee colonies in the temperature stressed late winter – early spring may be better able to thermoregulate in hives with small-cell brood combs and this may provide a specific advantage to these colonies in this critical period.

CHAPTER 8

Biology models of mites and honeybees
(using data from inoculation experiments)

8.1 Introduction

The previous chapters described experiments that were undertaken to improve understanding of the factors that influence the susceptibility of the honeybee host to the tracheal mite parasite *Acarapis woodi.* The experiments have accumulated a large data set on aspects of the biology of both the tracheal mite and the honeybee. In this chapter I have analysed the data and extracted basic biological parameters that describe the life cycle of the tracheal mite. Changes in the mites' external morphometry and reproduction since the mite was discovered in Scotland in 1919 and also with respect to the more recent data from the US will be identified. As the inoculation experiments were limited to about 7 days duration the life cycle data will be accordingly constrained. The influence of brood pupation temperature on honeybee development will also be analysed to identify relationships and to compare with biological parameters from published work.

Tracheal mite biology

There is limited published data on the life cycle of the tracheal mite. Some of the earliest work was undertaken by Morgenthaler (1931) in southern Germany. He showed that foundress female mites migrate preferentially to the youngest bees and start laying eggs after 3 to 4 days. He also concluded that the female lays on average 5 to 7 eggs and these develop into larvae after 3 to 4 days with the male and female offspring being fully developed in 11 and 14 days respectively. In some contrasting estimates on the reproductive cycle of the tracheal mite in the US Gary *et al.* (1989) estimated that the mite starts laying after 1 to 2 days and the eggs hatch in 2 to 3 days. Still later Pettis and Wilson (1996) estimated a 2 to 3 day delay period prior to laying and a 5-day egg hatch time. They also estimated an egg laying rate of 0.85 eggs per day.

These wide variations in some of the basic biology of mite reproduction may of course be due to different strains of the mite being studied (although this is unlikely in the case of the recent US estimates of mite reproduction where the research was undertaken a short time after the mite was first introduced). However, it is more likely to be partly due to methodology and interpretation of results as will be discussed later. There are no

published data on mite reproduction in bees from Ireland or Britain, where the mite has been associated with honeybees for over 100 years. It is therefore proposed to compile similar parameters from data specific to conditions in Ireland and with the same subspecies of honeybee (*Apis mellifera mellifera*) that was indigenous to Britain and Ireland when the mite was first discovered.

Honeybee pupation time vs. temperature

There is little basic biological data available on the influence of temperature on the pupation development time of the honeybee in the brood cell. The honeybee (*Apis mellifera*) is unique among insects in northern and temperate regions in its ability to reside all year round in a heated habitat. Even during severe winter conditions in Ireland a healthy colony of honeybees can maintain a differential temperature between the brood nest in the hive and the outside of 40°C, in order to raise brood (Appendix 2). When brood is present this temperature is normally held in the range 32-37°C (Kronenberg and Heller, 1982). However under adverse conditions the pupation temperature in the brood nest can range from 29.5 to 34.5°C (Owens, 1971).

Here I use data from the experiments to identify the parameters that describe the development time response to changes in pupation temperature. This analysis would be with respect to the North European Dark bee, *A. m. mellifera*, that is still widespread in Ireland and which has a reputation for foraging in low ambient conditions.

8.2 Materials and methods

Data from the experiments described in Chapters 3, 4, 6 and 7 were used to evaluate the mite morphometry, mite reproduction in the honeybee tracheae and the temperature response of the pupating bees in the brood cells.

Tracheal mite biology

Data on the mite's appearance and physical dimensions (Figure 8.1) were compiled during the experiments.

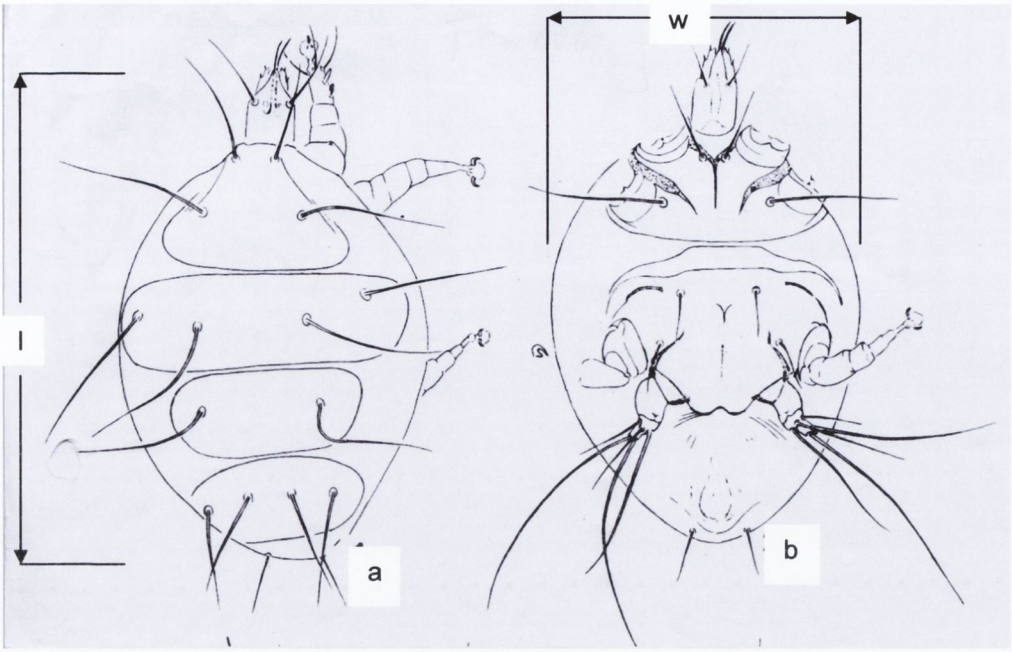


Figure 8.1 Drawings of female Acarapis woodi mite: a, dorsal view; b ventral view. Mite length 'l' and width 'w' shown. *(Delfinado-Baker and Baker, 1982).*

The delay time before the mite starts laying in the trachea, the egg laying rate and the egg hatch time are three parameters that describe the reproductive capacity of the mites. The time taken for the mites to mature is outside the short timescale of my experiments. I assume that reproduction is governed by the linear model below, which considers that after the foundress female mite enters the trachea it commences to lay eggs at a constant interval with the exception of a possible initial delay (see Chapter 6, Figure 6.2).

$$Ot = (t - D) * r \qquad (1)$$

Where Ot is the maximum number of offspring; t is the time of infestation (days) of the bees with the tracheal mites, D is the time delay (days) before the mite starts its laying cycle in the trachea and r the rate of egg laying (eggs/day).

The four inoculation experiments were undertaken in cages in similar ambient conditions of 32°C and relative humidity of 50%, with the exception of part 1 of the relative humidity experiment (Chapter 6) which was at 65%. Data from the latter were not used. The only known mite development times during these experiments were the inoculation times, namely the time periods from the introduction of the target bees into

the inoculation cage up to the time that these bees were killed at the end of the experiment. This would represent the full reproductive period for a mite that migrated to the new host bee (target bee) at the exact time that the target bees entered the inoculation cage. These early foundress female mites would be those that had the greatest number of offspring and where the larvae present displayed a progression of development stages. This would distinguish these families from those that had more than one mother mite in the trachea. Using these maximum offspring values (Ot) and their corresponding inoculation periods, assumed to be the infestation time (t), a linear regression was undertaken to obtain a best fit model and to provide values for the egg laying rate (r) and the initial lay-delay time (D). This egg laying rate (r) will be a minimum value as the maximum offspring (Ot) will be a minimum value and the infestation time (t) will be a maximum value.

There was no absolute time base during the experiments that will measure the time required for an egg to hatch (Chapter 6). However, the number of eggs (egg abundance) in the offspring of a family of mites, which also has larva(e), where only one live adult mite is present, will give a measure of the time to hatch in terms of the number of "egg laying intervals". This can be converted to an egg hatch time in days using the egg laying rate (r) estimated from the model above.

Honeybee pupation time vs. temperature

The time taken for insects to develop varies depending on the temperature during pupation (Daly *et. al.*, 1978). This physiological time varies from species to species and is a measure of the amount of heat required over time for an insect to complete its development. It is governed by the relationship that the time to develop (Dt) is proportional to the inverse of the difference between the pupation temperature (T^t) and a critical temperature (T^o) at which the insect ceases to develop. This can be represented by the expression:

$$Dt = k / (T^t - T^o)$$

Where k is a constant for the insect and measured in degree days.

$$\text{Hence } T^t = (k / Dt) + T^o \qquad (2)$$

The parameters above will be derived using the data from three of the experiments (Chapters 3, 4 and 7). In these experiments the three brood combs for each treatment remained in the incubator after the target bees were removed and were allowed to pupate until all the callow bees had emerged. The mean temperature over the period and the time for the last callow bees to emerge were noted. Since these data were being logged from the time that each brood frame was inserted into the incubator, the time for the last bees to emerge represents the full pupation period. This assumes that these bees had just been sealed in their brood cells prior to the removal of the brood comb from the hive and that the callow bees emerge in the order in which the cells were sealed.

Statistical analyses

I evaluated the mite reproductive data using regression analysis on a linear model and the honeybee pupation temperature response data using regression analysis on an inverse relationship model. The mite egg abundance data were analysed using a two-way ANOVA with colony and treatment as fixed factors.

8.3 Results

Tracheal mite morphometry

The two castes of the tracheal mite (female and male) are similar in appearance but the female is substantially larger. During dissection, mite size is a good method of identification and as a standard practice I logged the dimensions of the female. The mean length (± s.e.) and mean width (± s.e.) for the female mites were 146 ± 0.05 μm (n = 260) and 74 ± 0.05 μm (n = 206) respectively. The males were much smaller (n = 5) at ~100 μm in length and ~55 μm in width. However, there was a slight variation in the female width depending on the stage of pregnancy. The young female mites in Ireland were ivory in colour with a distinctive broad yellow band across their backs. As they got older their appearance darkened eventually becoming bronze. The males were also ivory with a thin yellow dorsal stripe but retained a much lighter colour with age.

Estimating 'egg-laying time delay' and 'egg-laying rate'

The maximum offspring and corresponding inoculation times for each experiment (and treatment/colony as appropriate) are listed in Table 8.1. In the experiments in Chapters 3, 4 and 7, as the maximum offspring #9 was only present once for each experiment, data for the #8 offspring were also included to give a broader representation. The offspring data (Ot) were plotted against the inoculation time (t) and a straight line of the form in equation (1) was fitted (Figure 8.2).

Table 8.1 Maximum offspring from four inoculation experiments with the corresponding inoculation period.

Experiment Chapter	Maximum Offspring (Ot)	Inoculation Time (days)
Chapter 3	9	7.44
	8	7.29
	8	7.29
Chapter 4	9	7.69
	3x8	7.69
	2x8	8.11
Chapter 6	6x6	6.48
Chapter 7	9	8.10
	8	8.10
	8	7.37
	8	6.52
	8	7.11
	2x8	7.48

Using equation (1); $Ot = (t - D).\ r$, where slope = r (is the egg laying rate) and intercept on t axis is D (delay time)

A linear regression analysis gave a slope (mean ±s.e.) of 1.38 ± 0.23 (corresponding to the laying rate r) and was significant ($p < 0.001$). The intercept on the vertical axis (Ot) was –2.41 ± 1.66 (corresponding to -D. r) but was not significant ($p = 0.162$). [R = 0.803, $R^2 = 0.628$, s.e. of estimate = 0.643. ANOVA ($F_{[1,20]} = 15.048, p < 001$)].

Hence laying rate r = 1.38 eggs per day and delay time D = 1.76 days (see Figure 8.2).

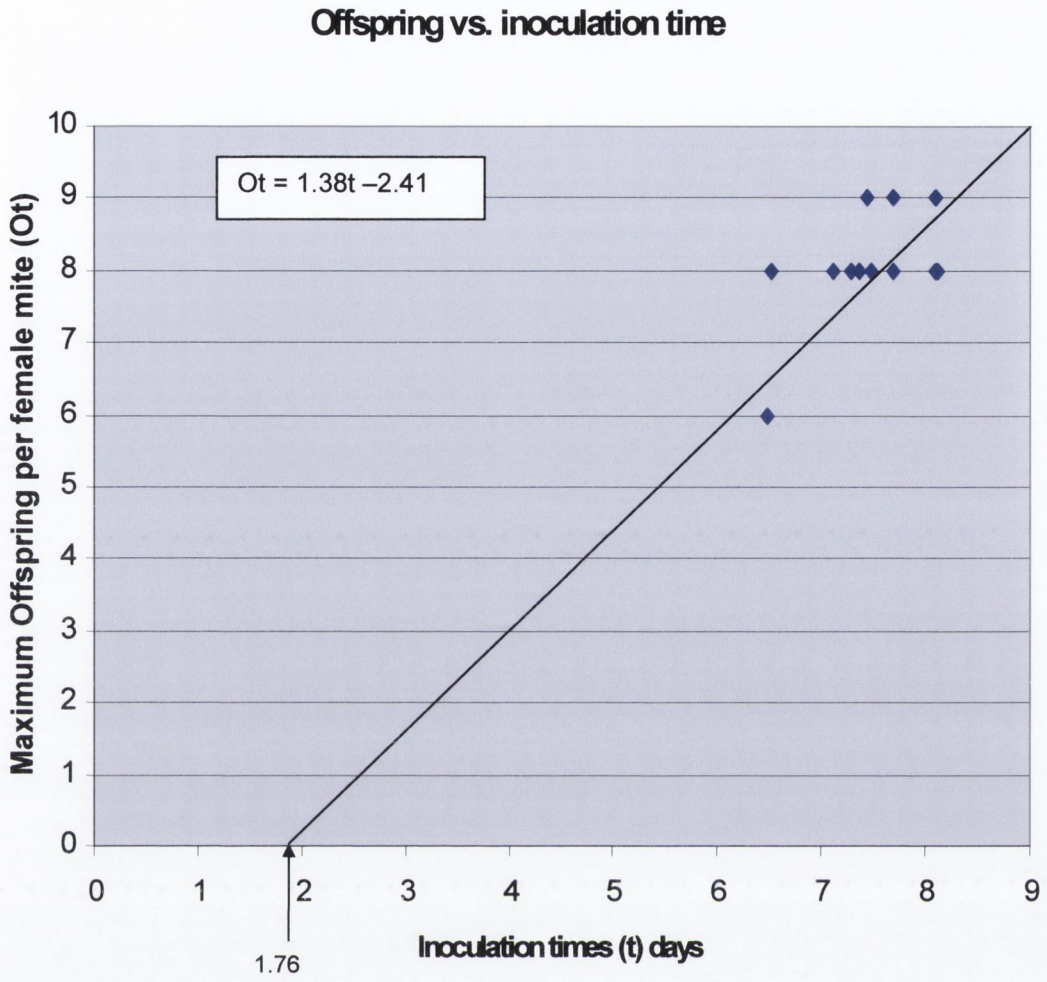


Figure 8.2 Graph of maximum offspring from the experiments vs. inoculation time. The slope of the regression line represents the egg laying rate and the intercept on the inoculation time chart is the egg-laying delay time D.

Estimating 'egg hatch time'

There was no significant difference in the egg abundance for the four experiments either on the basis of treatment or colony. [Treatment ($F_{[1,13]} = 0.139$, $p = 715$; $F_{[3,83]} = 1.331$, $p = 0.27$; $F_{[1,22]} = 0.042$, $p = 0.839$; $F_{[1,65]} = 1.969$, $p = 0.165$), Colony ($F_{[2,13]} = 0.536$, $p = 0.598$; $F_{[2,83]} = 1.719$, $p = 0.186$; $F_{[2,22]} = 3.324$, $p = 0.055$; $F_{[2,65]} = 2.415$, $p = 0.097$) for data from Chapters 3, 4, 6 and 7]. The overall mean egg abundance from each experiment is listed in Table 8.2. The mean egg abundance over the four experiments was 2.92 (range 2.61 to 3.29) eggs per mite and using an egg laying rate of 1.38 eggs per day corresponds to an egg hatch rate of 2.12 (1.90 to 2.39) days.

Table 8.2 Egg abundance (mean ±s.e.) from four inoculation experiments and showing sample size.

Experiment Chapter	Egg Abundance[1] (mean ±s.e.)	Sample Size
Chapter 3	3.29 ± 0.17	21
Chapter 4	3.05 ± 0.06	95
Chapter 6	3.11 ± 0.12	28
Chapter 7	2.61 ± 0.08	77
Mean	2.92 (range 2.61 to 3.29)	221

[1] Overall egg abundance from the data set where: data for all dead mites removed; larva(e) > 0; adults = 1

Honeybee pupation time vs. temperature

In all of the experiments where low pupation temperature was used there was no temperature effect on the emergence level from the sealed cells. In fact, 99% of the bees emerged from brood comb pupated at 30°C. From the three experiments (Chapters 3, 4 and 7) the mean pupation temperatures and the corresponding pupation times for the last callow bees to emerge were established (Table 8.3).

A plot of mean pupation temperature vs. pupation time is shown in Figure 8.3. Using a linear regression analysis on this data a best fit for the expression in (2) above was obtained.

$$T^t = (170.0 / Dt) + 20.8, [R^2 = 0.942; F_{[1,4]} = 64.71, p = 001].$$

or $Dt = 170.0 / (T^t - 20.8)$. (This regression line is shown in Figure 8.3)

The estimate of the critical temperature (T^o) where no development of the pupae takes place is 20.8°C from above. Using this expression the pupation time at a normal hive brood temperatures of 34°C is 12.9 days.

Table 8.3 Mean pupation temperature ±S.D. of brood combs from three experiments with the corresponding pupation development times.

Experiment Chapter	Mean Pupation Temperature[1] ± SD °C (T^t)	Pupation Development Time[2] days (Dt)
Chapter 3	32.0°C ± 0.15	14.8
	30.1°C ± 0.21	18.2
Chapter 4	31.9°C ± 0.31	16.4
	29.7°C ± 0.44	19.1
Chapter 7	33.3°C ± 0.41	13.9
	32.8°C ± 0.42	14.2

[1]The mean temperature of the brood combs during their pupation in the incubator.
[2]The development time for the bees to pupate is the average time for the last bees to emerge in three brood combs in the incubator frame cages.

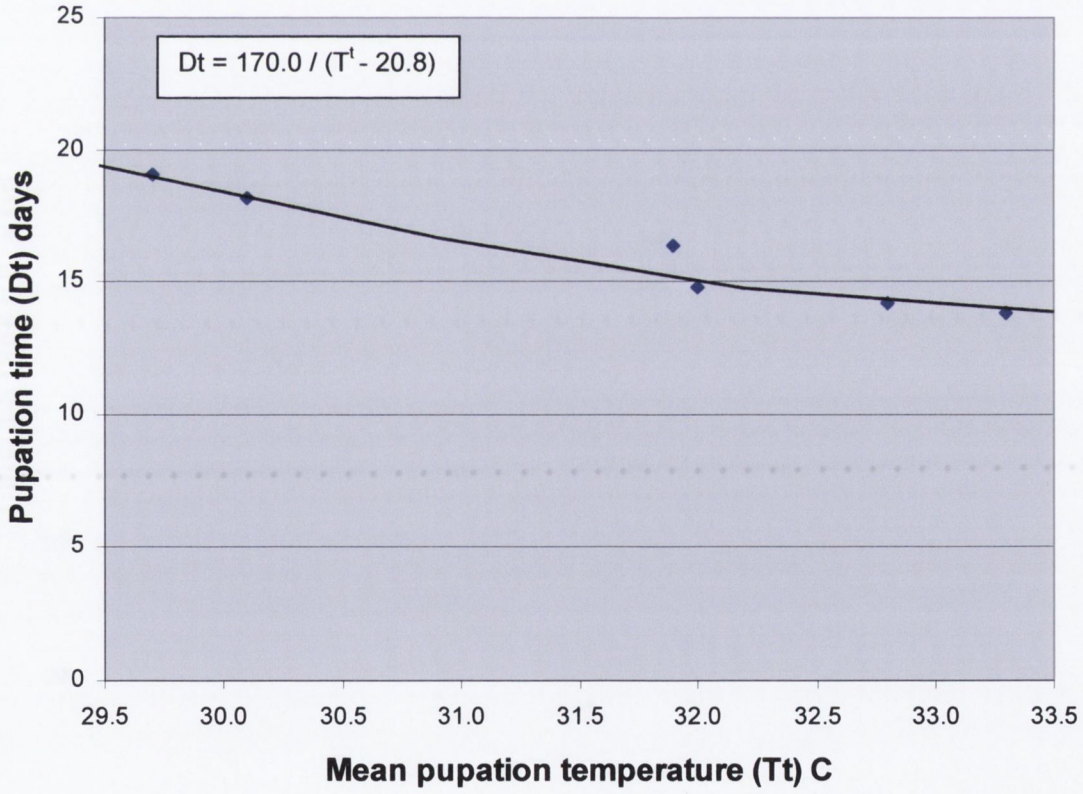


Figure 8.3 Graph of mean pupation temperature (T^t) in the incubator vs. the pupation time (Dt) for the last bees to emerge from the brood cells.

8.4 Discussion

The morphometry data for the mites indicated that they were much smaller than those described by Rennie in 1919 (Rennie, 1921). The estimated length and width were 146 μm and 74 μm respectively compared to Rennie's estimates of 190 μm and 100 μm. The corresponding estimates for the male mites were 100 μm and 55 μm compared to Rennie's 110-150 μm and 68-80 μm. The mites in my experiments were initially ivory with a yellow dorsal band that darkened to bronze with age while Rennie's mites were 'greyish' in colour (It is unlikely that the accuracy of the microscopes accounted for the difference in physical dimensions of mites, however, it is possible that the quality of 'white' light illumination in the 1920s may have affected mite coloration). The mites that Rennie's team discovered in 1919 are likely to have been the same strain that appeared in Ireland in 1912 (see Section 1.4) and hence we are potentially looking at an evolution of the mite in a period of almost 100 years. In contrast, mites were studied by Delfinado-Baker and Baker (1982) from samples that were obtained from around the world, including museum specimens, prior to the mite arriving in the US (there does not appear to be any recent US data on size). These mites, while closer in size to the Irish ones were still substantially larger; the female mites were 143-174 μm in length and 77-86 μm in width. The males were 125-136 μm and 60-77 μm in width. A recent report of mites in US described the young female mites as light amber (Danka and Villa, 2003).

The overall egg laying rate for the foundress female mites in the four experiments was estimated at 1.38 ± 0.23 eggs per day during the reproductive cycle up to day seven and is a minimum value as explained in the Materials and Methods. This compares with an estimated rate of 0.85 by Pettis and Wilson (1996) in the US. While the large difference may be due in part to a different strain of mite, it is likely that different methodologies contributed to some of this difference. The estimate in the present study was based on the offspring produced by single foundress female mites. In the Pettis and Wilson (1996) study, the estimated rate was made over a twelve-day period and represented the mean fecundity over the period. The number of female mites would be increasing over this period, at least for the first few days, and this would have reduced the derived egg laying rate. Also no allowance seems to have been made for an initial delay time before egg laying commenced.

Previous studies had delay times before the first egg was laid ranging from 1 to 2 days (Gary *et al.*, 1989) up to 3 to 4 days (Morgenthaler, 1931). The estimate from this study was 1.76 days before the commencement of the reproductive cycle. The first egg would appear 0.72 days later (at the laying rate of 1.38 eggs per day) or 2.48 days after the migration to the new host, which is in the mid range of cited work above. It should be noted that the delay time value of 1.76 days was estimated as a result of extrapolating outside the area of observations.

The estimated egg hatch rate in the study was 2.12 days compared with a range in other studies of 2 to 3 days (Gary *et al.*, 1989), 4 days (Royce *et al,* 1988) to 5 days (Pettis and Wilson, 1996). There can be some variation due to judgement in assessing the time of transition from the egg to larval state. In the present study all the assessments were made by the author which should increase consistency across treatments and experiments.

This study indicates that the reproductive activity of the mites in *A. m. mellifera* bees in Ireland is high as illustrated particularly by the egg laying rate and egg hatch time. The other three studies cited were undertaken only a short time (2 to 3 years) after the tracheal mites were first discovered in their regions whereas the Irish infestations are much older. There may, of course, have been other factors influencing the outcome such as strain of bees or climatic conditions. However, in the experiments there was no evidence that different bee strains or different treatments, including relative humidity, had an effect on egg laying rate or egg hatch time.

Pupation temperatures down to 30°C did not affect the level of emergence of the callow bees from the brood combs or the subsequent mortality level of the target bees in the inoculation cages when compared with those pupated at normal temperatures. It would be expected that the *A. m. mellifera* subspecies selected in the cool North West of Europe would perform well in this regard. However, there is little published data with which to make comparisons. Himmer (1927) found that few bees emerged at < 26°C, those that emerged at 28-30°C had a high proportion of malformed wings and those in the range 32-36°C were normal.

The model of the development response to pupation temperature indicated an inverse relationship with a development time of 12.9 days at 34°C. Given a cell capping age of 8 days, an emergence time of ~21 days is indicated. Milum (1930) showed that within a temperature range of 33.5 – 34.5°C, 93.5% of bees had completed their development in less than 21 days. He also illustrated that brood held at 31°C took over 24 days to complete development which compares with 24.9 days predicted by this study. My study also estimated a critical temperature of 20.8°C where no development takes place. Degrande-Hoffman *et al.* (1993) estimated a critical temperature for development of 20.9°C for queens in Arizona from mixed lines.

This study has indicated that the mites found in Ireland today are about one quarter smaller in linear dimensions than those described from the early part of the 1900s. This would represent a reduction of about half in volume and weight. The mites found recently in the US are also substantially larger than the Irish mites today. Are smaller mites being selected? Smaller sizes and weight are likely to be more difficult for the honeybee to detect on the pleural hairs and less easily groomed off. They would also have more space in which to reproduce. Further, the mite reproduction biology in this study compared with that in the US studies, as discussed above, would suggest that more aggressive mite reproduction is also being selected.

Hence, the data would suggest that evolutionary changes may be taking place in the tracheal mites. Since the time of Rennie at the beginning of the 1900s in excess of 2500 mite generations would have occurred. Is it realistic to expect that the changes in mite physical dimensions, reproduction and physical appearance indicated in this study occurred within this time period? In other taxa there is evidence of rapid evolutionary change. In the case of the Darwin finches, for example, there has been significant change in their body and beak sizes in the comparatively short time of 30 years (Grant and Grant, 2002). Further analysis of the evolutionary implications of this work remains to be done using additional samples with both geographical and historical spreads.

CHAPTER 9

Infestation models of honeybees infested with tracheal mites

9.1 Introduction

This chapter investigates the elements that influence the infestation of honeybee colonies when exposed to tracheal mites and evaluates their relative importance. I am not aware of any models of tracheal mite infestation in published work with the exception of the fecundity aspects that have already been discussed in Chapter 8. An understanding of the epidemiology of tracheal mites, and in particular the factors that determine infestation levels, is important if the biology of the system is to be understood and if colonies with tracheal mites are to be managed in the field.

A mated female mite leaves the trachea of an old bee where it has been raised and seeks a new young host bee in which to reproduce. The mite must transfer via the bee's pleural hair (Hirschfelder and Sachs, 1952). Only young bees are infested (Morgenthaler, 1930; Lee, 1963) which gives the mite the maximum time to complete its life cycle before the bee dies. This can be short in the case of a summer bee and hence for maximum reproductive advantage the mite offspring should be able to mature and migrate themselves before its host dies. This dispersal can take place either between individuals of a single generation such as within the colony or between colonies through drifting and robbing, horizontal transmission, or between individuals from different generations through swarming, vertical transmission (Fries and Camazine, 2001). The vertical transmission built into the bee reproductive system enables adaptation to take place at the colony level through swarming and drone mating, including feral drones. All of the three bee castes can become infested although it is much less common in the case of the queen. Drones do become heavily infested but their potential to transfer the mite is uncertain (Royce and Rossignol, 1991). The individual mite's success in migrating to the new bee is largely determined by the new host's ability to groom which is a heritable trait (Pettis and Pankiw, 1998; Danka and Villa, 1998; Chapter 4). Once inside the trachea the mite feeds on the haemolymph of the bee and lays eggs. The offspring mature in about 12-14 days, the males maturing earlier, and these males fertilise the young adult females (Pettis, 2001). A young mated female leaves the trachea and the cycle continues.

This is not a static situation. The honeybee and the mite have coevolved for at least 100 years. The result of this interaction between host and parasite is largely determined by the resistance of the host and the virulence of the parasite. The latter can be defined as an increase in the host mortality as a result of the parasite's presence (Schmid-Hempel, 1998) or as reduced host fecundity or parasite replication rate within the host (Bull, 1994). The co-evolution of the honeybee and tracheal mite over the last 100 years has involved global horizontal transmission and initial high levels of honeybee mortality. This is common in the case of new pathogens where high initial virulence reduces in time (Fenner and Ratcliff, 1965) and a trade-off between virulence and reproduction is established. High initial virulence on the part of the tracheal mite, if sustained, could wipe out honeybees while, on the other hand, a virulence that is too low may result in the mite not being able to establish itself. Changes in parasite virulence may be due to adaptation of the parasite to a lower rate of reproduction or to changes in the host's response. The former is more likely as the shorter generation time of the parasite will enable it to evolve faster (Hafner *et al,* 1994). The changes in appearance and physical size (~half) of the present mite in Ireland compared to Rennie's 1920s mites indicates an adaptation on the part of the mite (Chapter 8). However, this adaptation may be a response to the reduced incidence of tracheal mite infestation (due to selection of increased bee resistance) in Britain and Ireland since the 1960s after the high bee mortality in the period up to 1925 and again in the 1940-1960 period (Watson, 1981). Also in Chapter 8 there was evidence of the present-day mites in Ireland having a more aggressive rate of reproduction than those in US in the 1980s and 1990s, shortly after the initial infestation, again identifying possible adaptation on the part of the mites in Ireland.

It is against this background that the present honeybee - mite relationship has developed and has shaped today's susceptibility in bees and virulence in mites. This chapter will attempt to identify the factors at the individual bee level that influence the present day infestation of the honeybees to tracheal mites.

My research has shown that the fecundity in all control and treatment colonies has been similar and hence infestation has been determined by the number of foundress female mites that have migrated to the new host bee. The infestation models will therefore have the female mite abundance as the dependent variable.

Previous experiments on honeybee susceptibility have shown a good correlation between the results from short-term bioassays, as in the inoculation experiments in this research, and from field colonies (Page and Gary, 1990; Danka and Villa, 1996; Nasr *et al.*, 2001). Hence the results from the analysis of the experiments are applicable to hive bees in production colonies. The experiments in the previous chapters were undertaken to identify different aspects of honeybee susceptibility and therefore contain data on infestation in honeybees under different conditions; bee strain, pupation temperature, cell size, relative humidity, season and host bee infestation level (inoculation pressure). Two models will be developed to explain the dynamics of the interactions between the host honeybees and the tracheal mites.

(i) A composite infestation model that includes both control and relevant treatment data (such as pupation temperature) having a predictable relationship to bee susceptibility (Part 1).

(ii) A mite population model to give indicative projection of mite levels (Part 2).

Making some indicative projections based on measured parameters and stated assumptions can give a useful insight into the dynamics of tracheal mite infestation.

9.2 Materials and methods

Part 1

Developing a theoretical model

It would be expected that the likelihood of a target bee in an inoculation cage being infested with a female tracheal mite from a host bee would in the first instance be influenced by the number of migrating female mites available. The number of these 'free' host female mites in relation to the number of receiving or target bees would be a quantifiable measure of the number of migration attempts or pressure that the target bees would be under. The inoculation pressure (Ip) in the cage could be considered as

the number of 'free' host female mites (Hf)[1] per target bee (Tn). The probability that the host and target bees will come in contact (Pc)[2] would also influence the likelihood of infestation as would the efficiency of the mite transfer (Et)[3].

[1]'Free' female mites (Hf) can be considered as the new mated female mites that are available to migrate to a new host (target) bee. In the model the 'free' mites during the first few days (up to ~ 3 days) of the inoculation process were used.

[2] Probability of contact (Pc) can be considered as the likelihood that a host (donor) and target (receiver) bee come into physical contact.

[3] The transfer efficiency (Et) is stated as the proportion of the free female mites, that come in contact with the target bees, that are successful in entering the trachea of the new host (target) bee.

In all of the experiments the total number of bees in the inoculation cage and the ratio of host to target bees has been relatively small, ranging from 1.7 to 5. Given also that there is a high mobility and rapid mixing of the bees in the inoculation cages it would be expected that probability (Pc) would be fairly constant and be close to unity (unity is where contact is certain).

Therefore based on above:

Total mite transfer to target bees (Mn)	= *Fn*(Hf, Pc, Et)	*Fn* denotes function
	= *Fn*(Hf, Et)	when Pc = 1
Target bee abundance (Ta) = Mn/Tn	= *Fn*(Hf/Tn, Et)	Tn is the target bees
	= *Fn*(Ip, Et)	where Ip is the inoculation pressure

Note: *Et*, the efficiency of transfer of free female mites to the target bees, is effectively a measure of the susceptibility of the bees to tracheal mite infestation.

Therefore the target bee female mite abundance is a function of the inoculation pressure and the susceptibility of the target bees to tracheal mite infestation. The likely elements

of this honeybee susceptibility for control bees are the bee strain (which can be described by their cubital index (Ci) and their discoidal shift (Ds)) and the season (S). A composite infestation model should also include the treatment element, the temperature (Tp) at which the bees have been pupated as this had a significant effect on susceptibility. However, the data from cell-size treatment colonies had a similar susceptibility to the control colonies and all this data has been included in the model as control data. The other treatment element, relative humidity, had no consistent effect on susceptibility and this data have not been included in the composite model.

Measurement of the inoculation pressure in the cage

The sample of 60 host bees collected at the same time as the host bees for the inoculation cages (30 bees collected before and 30 bees after collecting the host bees for the inoculation cage) was used as the basis for estimating the free female mites in the experiments. The total number of female mites in the sample was reduced by the number of laying mites present, which was estimated from the egg and larval load in each infested bee. This gives the number of 'free' adult female mites in the sample at the beginning of the inoculation process (It is assumed that the only migration out of the tracheae is undertaken by newly mated daughter mites).

During the first few days of inoculation a certain number of larvae will mature, mate and leave the trachea to migrate to a new host. This estimated number of maturing mites was added to the initial number of 'free' adult female mites to give the total 'free' mites in the 60-host bee sample. Female mites can only migrate in the first few days (~ 3 days) of the inoculation process. Then assuming one day to mate and migrate, only females maturing from the 'larval' stage in a two-day period will be able to migrate. This would be two days in a total 'larval' period of ~10 days or 20% (0.2) of the larvae present. This proportion will be referred to as the maturing factor (Mf). The model can be tested with different maturing factors.

The number of free mites in the sample of 60 was scaled up in the ratio of the total host bees to sample host bees (60). The resulting figure is the 'total free mites' in the inoculation cage.

Testing model - Regression analysis

The data from the experiments will be used to develop a model of the infestation of the target bees in the inoculation cages. The high humidity (60-65%) in cage 1 of the humidity experiment (Chapter 5, part 1) has not been included in the data set as previously discussed.

A linear form of the expression in Box 1. below will be tested

BOX 1. Model to be tested:

Ta = *Fn*(Ip, Tp, Ci, Ds, S) where Ta = female mite abundance
Ip - inoculation pressure in the cage
Tp = pupation temperature
Ci - cubital index of target bees
Ds - discoidal shift of target bees
S - seasonal effect (and set at S= 0 in spring and S = 1 in autumn)

Part 2

Mite population projection

There are few data on the number of days during which a female mite lays. While figures of up to 21 days have been reported by Pettis and Wilson (1996) a more conservative period of 14 days will be assumed, which is the median of the published range. A female mite reaches maturity in ~ 14 days (Delfinado-Baker and Baker, 1982; Royce *et al.,* 1988; Pettis and Wilson, 1996). The number of mated female offspring per female foundress mite (Om) is determined by the number of female offspring per female foundress mite (Of) in the 14-day period, scaled down by the mortality/fertility rate (Ms). Therefore at the end of 28 days (4 weeks) one female mite would have produced the product of *P*, *Su*, *Of* and *Ms* mated female mites, where *P* is the

probability that a young mated female mite will make contact with a young bee and *Su* is the proportion of mites that make contact with a young bee that in fact successfully migrate. Hence, starting with *Hn* fertilised female mites, Table 9.1 shows the progression through five phases and in 14 days the first of *Hn.P.Su.Of.Ms* mated female mites are ready to migrate out of the tracheae and all will have left in 28 days.

Table 9.1 Five phases of infestation. The table shows the progression of Hn young mated female mites for the first 28 days. The last row gives the number of female mites at each phase.

Infestation phases				
1 (day 1-3)	2 (day 1-3)	3 (day 1-3)	4 (day 14)	5 (day 28)
Old host	New host	New host	New host	Old / New host
Initial Infestation Young mated females Hn	Contact Probability P[1]	Migration Susceptibility Su[2]	Reproduction Offspring Of[3]	Mated Fertility/mortality Ms[4]
Hn →	Hn.P →	Hn.P.Su →	Hn.P.Su.Of →	Hn.P.Su.Of.Ms

[1] P - the probability that a young mated female mite will make contact with a young (< 3 day) bee.
[2] Su - the proportion of mites, making contact with young bees, that successfully migrate.
[3] Of - the number of female offspring per foundress female mite.
[4] Ms - the proportion of female offspring that are alive and mated.

In an ideal situation where the number of young bees is sufficient to absorb all the migrating mites (P = 1), the susceptibility of the bees is high (Su = 1) and mortality of female mite offspring low and fertility high (Ms = 1) then one mated female mite would produce in 4 weeks (28 days) *Of* mated female mites.

If r is the number of 2-week periods and *Of* the number of female offspring in the 2-week period, then after any time period r:

$$\text{Potential number of mated female mites} = Of\,[(Of + 1) / 2]^{r-2} \quad (1)$$

where r >1

A mite population will only increase if after a 28-day cycle (Table 9.1) the number of mated female mites is greater than the original number (Hn).

For population increase $Hn < Hn.P.Su.Of.Ms$

or $1 < P.Su.Of.Ms$ (2)

Statistical analyses

The composite infestation model was developed using linear regression analysis.

9.3 Results

Part 1 Inoculation model

The data from four experiments were used to test the model in Box 1 (Table 9.2). This table includes the data for the temperature-treated bees in Chapters 3 and 4. The data were compiled using a maturing factor of 0.2 (20%) for the larvae. The inoculation pressure was calculated on an overall experiment basis rather than a per cage basis as the host bees were taken from the same hive of host bees, on the same day. Averaging the 60-bee samples over two or three cages (120 to 180 bees) gives a better prediction of inoculation pressure. The proportion of free host mites, using a maturing factor of 0.2, that transferred to target bees varied from 0.17 for the humidity experiment (40% RH) to 0.58 for the pupation temperature experiment. The corresponding figures for maturing factors of 0.1 and 0.3 are 0.22 and 0.13 for the humidity experiment and 0.76 and 0.46 for the pupation temperature experiment respectively. The value of 0.76 (ie. 76% of available female mites transferring to a new host) would appear high given that some of the colonies in the experiment had a relatively low susceptibility to tracheal mites. This transfer ratio corresponded to a maturing factor of 0.1. The larval maturing factor of 0.3 represents about three days for the development of larvae into adult mites and adding additional time for mating and host transfer would appear an excessive time given that most mites transfer in the first 2 days. A transfer ratio of 0.2 has therefore been chosen for the model.

Table 9.2 Control and treatment data from four experiments used to test the infestation model. The maturing factor (Mf) is 0.2[1].

Chapter Experiment	Cage	Colony	Target female abundance (Ta)	Temper. °C (Tp)	Inoculation Pressure (Ip)	Cubital Index (Ci)	Discoidal Shift (Ds)	Season (S)
Chapter 3	0	1	0.11	34.5	0.68	1.58	-0.23	0
Pupation	0	2	0.26	34.5	0.68	1.7	-1.13	0
Temperature	0	3	0.29	34.5	0.68	1.53	-2.21	0
	1	1	0.29	34.5	0.68	1.58	-0.23	0
	1	2	0.33	34.5	0.68	1.7	-1.13	0
	1	3	0.33	34.5	0.68	1.53	-2.21	0
	0	1	0.45	30.1	0.68	1.58	-0.23	0
	0	2	0.52	30.1	0.68	1.7	-1.13	0
	0	3	0.45	30.1	0.68	1.53	-2.21	0
	1	1	0.48	30.1	0.68	1.58	-0.23	0
	1	2	0.38	30.1	0.68	1.7	-1.13	0
	1	3	0.48	30.1	0.68	1.53	-2.21	0
Chapter 4	0	1	0.21	34.5	2.66	2.06	0.51	0
Grooming	1	2	0.42	34.5	2.66	1.64	-1.04	0
	2	3	0.62	34.5	2.66	1.7	-2.17	0
	0	1	0.33	29.7	2.66	2.06	0.51	0
	1	2	0.78	29.7	2.66	1.64	-1.04	0
	2	3	0.96	29.7	2.66	1.7	-2.17	0
Chapter 6	1	1	0.31	34.5	2.38	1.72	-1.85	1
Humidity	1	2	0.45	34.5	2.38	1.72	-2.75	1
	1	3	0.52	34.5	2.38	1.65	-4.45	1
Chapter 7	1	1	0.33	34.5	1.39	1.787	-1.402	0
Cell-size	1	2	0.42	34.5	1.39	1.768	-2.372	0
	1	3	0.54	34.5	1.39	1.861	-2.287	0
	2	1	0.70	34.5	1.39	1.803	-2.219	0
	2	2	0.45	34.5	1.39	1.806	-2.166	0
	2	3	0.57	34.5	1.39	1.85	-1.843	0

[1]The maturing factor (Mf) is the proportion of the mite larvae in the infested host bees that mature during an initial period of inoculation, mate, leave the trachea and migrate to a new host. A 2-day period in a total larval development time of ~10 days would represent a maturing factor of 0.2.

Test 1

A linear regression of the data in Table 9.2 gave the relationship:

Mite abundance (Ta) = + 0.113Ip – 0.042Tp – 0.038Ci – 0.115Ds – 0.258S + 1.592

[$R = 0.829$, $R^2 = 0.688$; s.e. = 0.112; ANOVA ($F_{[5,21]} = 9.261$, $p < 0.001$)]
with p values for the coefficients, Ip = 0.004; Tp = 0.001; Ci = 0.851; Ds < 0.001; S = 0.008 and constant = 0.001.

Test for contribution of individual variables

Using the coefficients above the contributions of the variables, over their typical ranges, to the female mite abundance are: -

Inoculation pressure (Ip) – (range 0.68 to 2.66 = 1.98)	|0.22|
Temperature (Tp) – (range 29.7 to 34.5°C = 4.8)	|0.21|
Cubital index (Ci) – (range 1.53 to 2.06 = 0.53)	|0.02|
Discoidal shift (Ds) – (range – 4.45 to 0.51 = 4.96)	|0.57|
Season (S) – (range 0 to 1 = 1)	|0.26|

The above demonstrates that the discoidal shift is the major significant determinant of female mite abundance. The inoculation pressure (Ip), pupation temperature (Tp), and season (S) also make a large contribution and are significant ($p < 0.01$). The cubital index only varies over a short range and its contribution is small. This indicates that female mite abundance is not sensitive to changes in *Ci* and this is reflected in the significance value (p) = 0.851. Figure 9.1 gives the range of wing venation values for the main bee strains along with the actual range in the study (Cooper, 1986; Ruttner, 1988b)

Test 2

Removing cubital index as a variable and testing the model with the data in Table 2 gives the expression:

Mite abundance (Ta) = + 0.109Ip – 0.043Tp – 0.116Ds – 0.256S + 1.549 (3)

[$R = 0.829$, $R^2 = 0.687$; s.e. = 0.109; ANOVA ($F_{[4,22]} = 12.097$, $p < 0.001$)] with p values for the coefficients, Ip = 0.001; Tp < 0.001; Ds < 0.001; S = 0.006 and constant < 0.001.

Test for contribution of individual variables

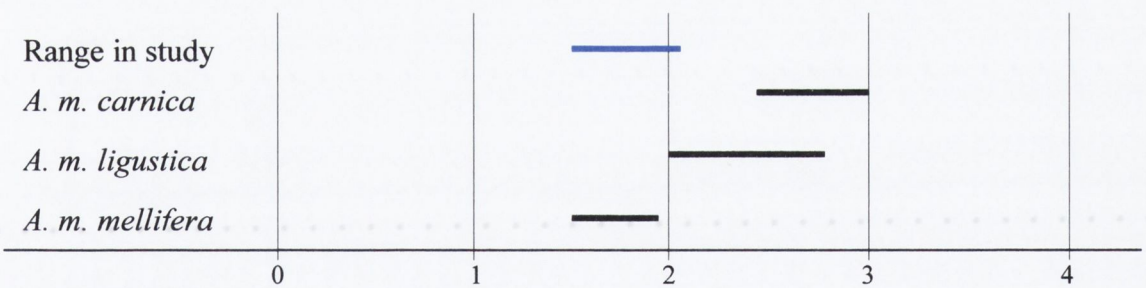
Using the coefficients above, the contributions of the variables (over their typical ranges) to the female mite adundance are:

Inoculation pressure (Ip) – (range 0.68 to 2.66 = 1.98)	|0.22|
Temperature (Tp) – (range 29.7 to 34.5°C = 4.8)	|0.21|
Discoidal shift (Ds) – (range – 4.45 to 0.51 = 4.96)	|0.58|
Season (S) – (range 0 to 1 = 1)	|0.26|

The discoidal shift is still the major determinant of female mite abundance and is significant ($p < 0.001$). All the other factors are significant ($p < 0.01$).

Regression lines for abundance vs. discoidal shift, abundance vs. inoculation pressure and abundance vs. pupation temperature with all other factors fixed, as appropriate, are given in Figures 9.2, 9.3 and 9.4 respectively. The fixed values in the three graphs are the mean values for the inoculation pressure (Ip), 1.47; pupation temperature (Tp), 32.9°C and discoidal shift (Ds), -1.52°.

(a) Cubital index



(b) Discoidal shift

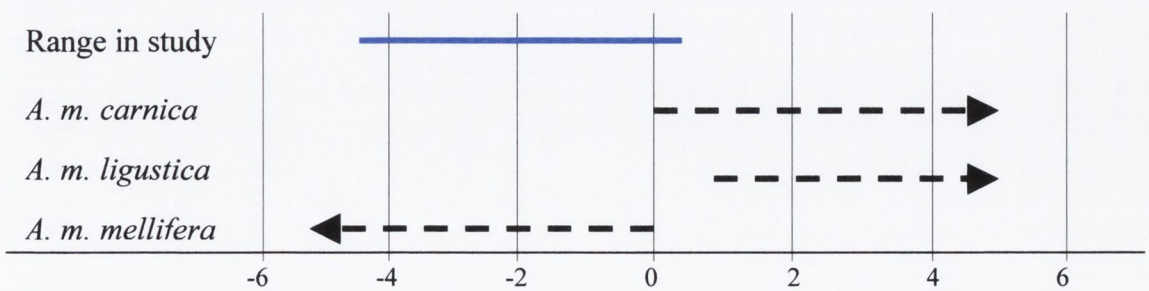


Figure 9.1 Wing venation data comparing values for the bee strains; *A. m. mellifera, A. m. ligustica* and *A. m. carnica* along with the actual range of values in the study. (a) cubital index and (b) discoidal shift (Ruttner, 1988b).

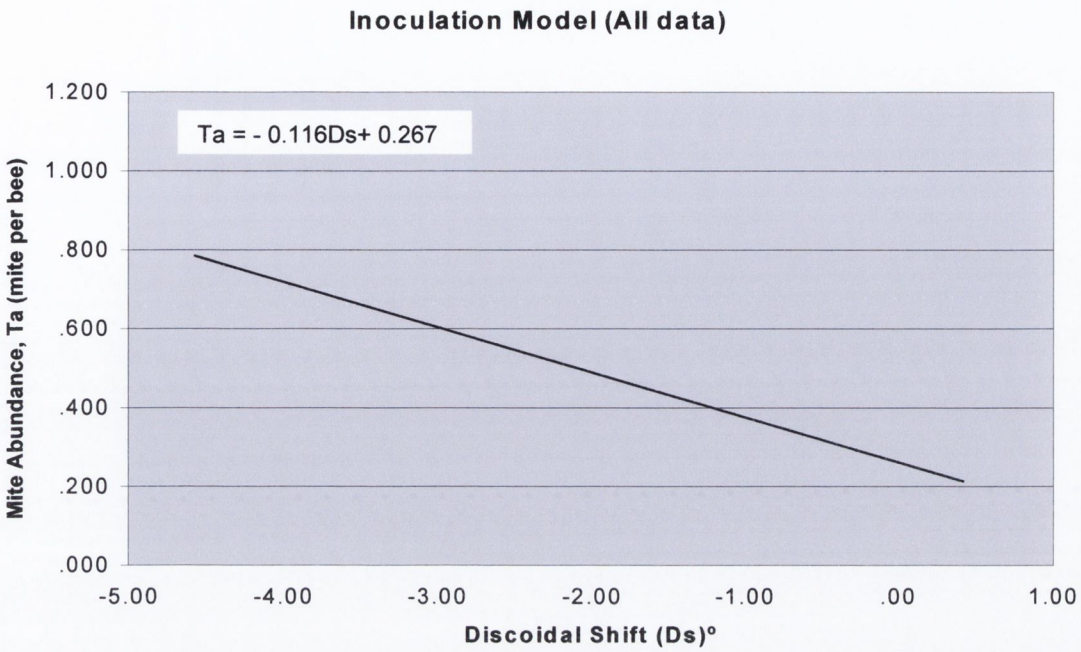


Figure 9.2 Regression line of female mite abundance vs. the discoidal shift based on equation (3) with all other factors fixed. Range of discoidal shift values in data tested was –4.45° to 0.51°.

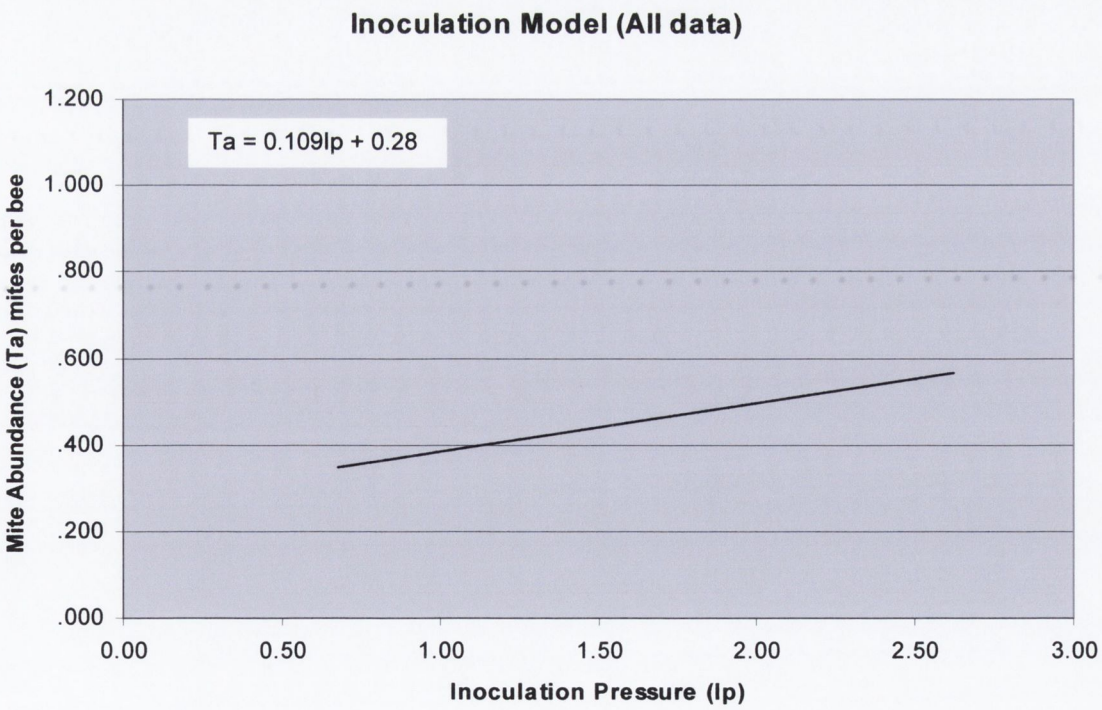


Figure 9.3 Regression line of female mite abundance vs. inoculation pressure based on equation (3) with all other factors fixed. Range of inoculation pressure values in the data tested was 0.68 to 2.66.

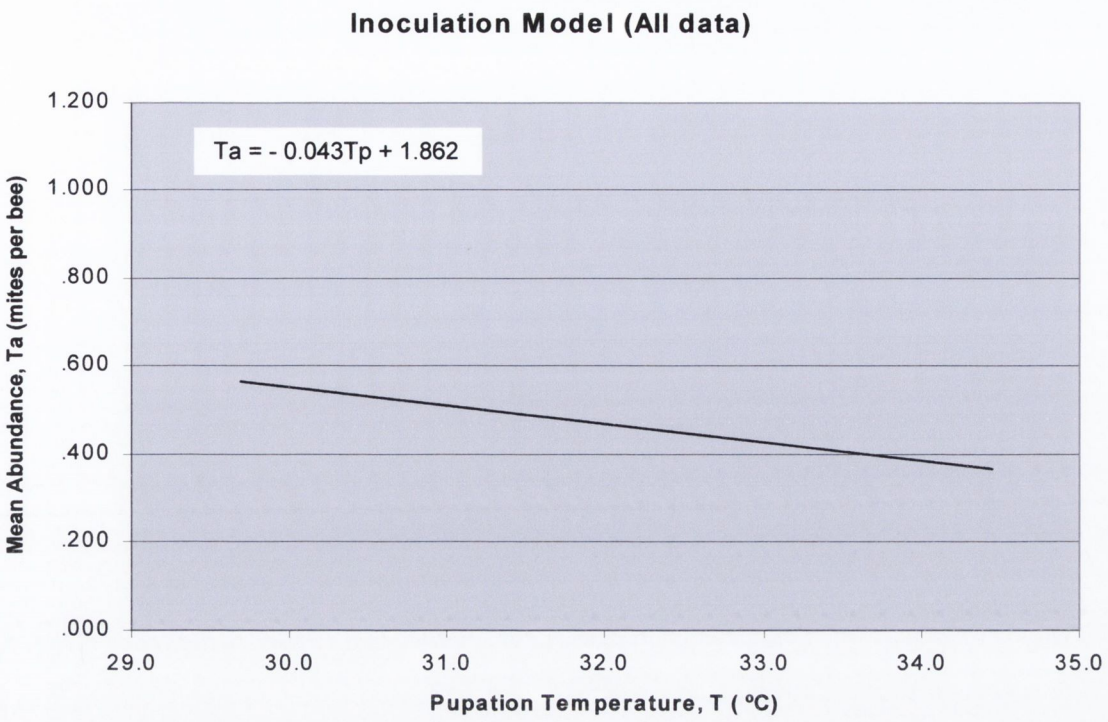


Figure 9.4 Regression line of female mite abundance vs. the pupation temperature based on equation (3) with all other factors fixed. Range of pupation temperature values in the data tested was 29.7 to 34.5°C.

Part 2. Mite population projections

From Chapter 8, the egg-laying pattern has been estimated at 1.38 eggs per day with a delay of 1.76 days before the egg-laying cycle begins. This represents 17 offspring in the 14-day period. The female : male ratio has been reported as 3:1 or 4:1 (Delfinado-Baker and Baker, 1982) and would suggest ~12 females developing in a 14-day period. Note that the egg-laying rate (fecundity) has been similar for control and treated bees in all the experiments.

Using equation (1) above

Potential number of mated female mites = $Of\,[(Of + 1)/2]^{r-2}$ where $Of = 12$

Based on above and starting with one fertilised female mite, at the end of a 12-week period the adult female mite population will have increased to ~21,000 and will increase at a rate of 6.5 times every two weeks.

Using equation (2) above

For population increase $P.Su.Om.Ms > 1$

Typically

$P < 1$ and will reduce as the mite population rises and migrating female mites have fewer receptive young hosts

Su will be < 1

Ms, female mite mortality and mite fertilisation will be < 1

Hence the infestation will only progress if at least one of the 12 female offspring survives, is mated and successfully migrates to the trachea of a new young host bee. This means that the product of *P*, *Su* and *Ms* must be > 0.08 (1/12) for the population to increase.

9.4 Discussion

Four factors had a significant influence on the infestation level of honeybees when exposed to tracheal mites. The major determinant of female mite abundance was the discoidal shift and this had an influence over its typical range of two to three times that of the other factors. The other three factors, inoculation pressure, pupation temperature and season of the year made similar contributions and were significant.

As would be expected the higher the level of inoculation pressure the higher the female mite abundance. It would also be expected that the full regression line would go through the zero coordinate (0,0) and that the slope of the line would decrease with increased inoculation pressure as a reducing proportion of the free mites would find a new host. The calculated regression line, which assumed a linear relationship and was derived from inoculation pressure values from 0.68 to 2.66, is consistent with the expectation above.

In line with the findings from the two experiments in Chapters 3 and 4 (temperature treatment) reduced pupation temperature resulted in a large and significant increase in susceptibility. The linear model predicted an increase in infestation of over 50% when the pupation temperature drops from normal to 30°C. However, the reduced pupation temperature in the experiments was only applied for 9 days at the end of the pupation period. If the reduced temperature had extended over the full pupation period of ~18 days (pupation development time at 30°C, Chapter 3) the differential susceptibility is likely to have been even more pronounced.

The time of the year had a significant effect with susceptibility in the springtime predicted to be 0.26 mites per bee higher than in the autumn. The reason for this may lie in the different life span of bees in spring compared to the autumn. The tracheal mite has a limited time period in which to transfer to its new host. A mated female mite leaving its old host bee must find a new host where it can raise a family and have its offspring migrate before the host dies. The honeybee in spring and early summer is a short-lived host and rapid development (high virulence) would be selected (Lipsitch *et al.* 1996). This may help to explain the increased susceptibility displayed by the bees in the spring where a greater urgency may be applied by the mites to identifying a young host than in the autumn where long-lived bees are more available.

Honeybees with large negative values of discoidal shift are predicted by the model to have high susceptibility to tracheal mite infestation. This is indicative of the North European dark bee *A. m. mellifera* where large negative values are present in typical examples of this subspecies while in the case of *A. m. ligustica* it is positive, and positive or zero for *A. m. carnica* (Ruttner, 1988a). It is interesting that at the time of the outbreak of the so-called 'Isle of Wight disease' in Britain and Ireland in the first two decades of the 20th Century, and in fact post 1920 in North Western Europe, the indigenous subspecies in these areas was *A. m. mellifera.* Why should this be so? In Chapter 4 (autogrooming) the 'low susceptibility' bees (colony 1) were the good groomers and these bees had values of discoidal shift outside of the criteria for *A. m. mellifera.* It was also concluded in Chapter 4 that the bee hair structure may be the critical element in determining the grooming ability of bees. One of the unique characteristics of the *A. m. mellifera* subspecies is its length of body hair (Ruttner, 1988a). "..length of abdominal hair, up to 0.5mm in the north (= double the length of

bees from a warmer climate)." It would be expected that the hair of the thorax would also be correspondingly longer given that this hair cover had evolved in cool temperate regions. Hence these bees may be poor groomers by virtue of their long-hair and this long hair would also be more likely to facilitate the transfer of questing mites onto their plueral hair in the first place. It may therefore be that by 'definition' the *A. m. mellifera* subspecies is destined to have a high susceptibility to tracheal mites. Follow-on research is required to test the hypothesis that long pleural hair increases the susceptibility of honeybees to tracheal mite susceptibility.

The dynamics of the infestation are illustrated by the projected potential mite population increase from a single foundress mite to over 21,000 female mites after only twelve weeks. While accepting that this projection is made under specific assumptions, it does represent over 2 female adult mites for each honeybee in a winter colony and this level of infestation is common in colonies over the winter period that eventually die out in early spring (Chapter 10). This projection of infestation is typical of the experience described by Morgenthaler (1931) where colonies that have remained at low levels for a few years can suddenly escalate to high levels of infestation and perish. The model suggests that these large mite population explosions can take place when conditions are favourable.

This work is a first attempt in the development of models to explain tracheal mite infestation in the field. Further work is required using much larger data sets to refine the approach.

CHAPTER 10

Mortality model of honeybees infested with tracheal mites

10.1 Introduction

It is over 100 years since the condition that became known as 'the Isle of Wight disease' first appeared and with which the tracheal mite, *Acarapis woodi,* has been associated. Yet there has never been any consensus on what was the cause of this condition (Bailey, 1985; Adam, 1987). The tracheal mite was discovered for the first time in 1919 (Rennie *et al.*, 1921) and doubt about its role in colony mortality has remained to this day.

The more pertinent issue today is, however, whether tracheal mite infestation can cause honeybee colonies to die. The mite was first discovered in the US in 1984 and since then widespread colony mortality has occurred. There is evidence that the mite moved from South and Central America into the US. Concerted sampling in the Southern States prior to 1984 had failed to find any mites (Mussen, 2001). This adds to the direct relationship between the presence of tracheal mites and colony mortality. The subsequent research into tracheal mite infestation in the US, while generally acknowledging its role in the mortality, has not been able to establish an actual cause of death. Many possible reasons have been raised including degenerative changes in the trachea, flight muscles, nerve ganglia and hypopharyngeal glands (Collison, 2001). Other pathogens such as bacteria and viruses have also been implicated (Bailey, 1999).

Here I develop a model in three stages to attempt to explain the mortality of honeybee colonies infested with tracheal mites:

First, I evaluated a honeybee colony (to be referred to as the 'test colony') with a high mite infestation from autumn 2005 through to its demise in the spring of 2006. Second, the evidence from the experiments described in the previous chapters and other published research was considered and critical findings identified. In the last stage I developed a qualitative mortality model to explain colony mortality in the field.

10.2 Materials and methods

Study of test colony mortality

A colony of bees with a high prevalence level (53%) of tracheal mite infestation in September 2005 was identified in North County Dublin. The colony in a Modified Commercial hive had a large bee population which at the end of September 2005 had ten frames of bees and five frames of brood. The 2005 season was one of the best in the locality in a decade with honey yields of about twice average (35 kg vs. 18 kg). The colony behaviour was normal and apart from tracheal mite infestation no other adult bee diseases were detected (discussed in detail later).

The colony was transferred into a standard Modified Commercial hive that had the facility of an observation hive and which also had temperature probes for installing in the brood combs. The transfer took place by moving all the brood combs, with accompanying bees, into the new hive and brushing in the balance of the bees. This colony was subsequently observed and evaluated from the end of September 2005 until its demise at the end of March 2006. During this period the following activities were undertaken.

: Evaluation of test colony behaviour. This involved a general observation of colony behaviour and a detailed post mortem.

: Disease Status. Tests were made on the bees to identify any pathogens that were present, including viruses (see Results).

: Assessment of honeybee cluster profile. The side of the test hive had a window that was exposed when a thick insulated shutter was removed. At intervals during the winter the profile of the bees in the cluster was traced onto a transparent film.

: Weighing (4) of the test hive to obtain the extent of stores. This was undertaken to allow an assessment of stores consumption by the bees during the winter period. The

weight readings were taken using a simple lever method and an allowance was made for the weight of the hive hardware and bees (McMullan, 2000).

: Daily readings of temperature inside the test hive and ambient temperature. Each morning the instantaneous, minimum and maximum temperatures on four probes inside the cluster and outside the hive were taken.

: Sampling for mite prevalence. Samples of 30 live (12) and 30 dead (7) bees were dissected for the presence of tracheal mites and the prevalence logged. The live bees were collected at the hive entrance and were mostly of flying bees. The dead samples were taken from the bees that had not been able to fly and that had crawled away from the hive and clung onto stalks of grass.

: Caging grass-bound bees and evaluating their half-life, ie. time (days) taken for the number of bees in the sample to reduce to half. On four occasions from January to March 2006, bees that had crawled out of the hives and were clutching the grass were collected in late afternoon and transferred to a cage in an incubator at 30°C and fed 50% sugar syrup and water. The dead bees were logged on a daily basis until all the bees in the cage had died.

: Daily collection of dead bees on grass outside the hive. Each day (late afternoon) any bees that had died on the grass were counted, logged and stored in a freezer at -30°C. The maximum mid-day temperature (in the shade) was also logged as well as the sun-status of the day (> an hour of direct sunlight at mid day, 11 to 15.00hrs).

Statistical analysis

A 3-way ANOVA was undertaken on the daily bee deaths with sun taken as a fixed factor, and temperature & date as covariates.

Other considerations

The critical findings from the experiments in the earlier chapters are identified and listed (Results section) and these are used in conjunction with the findings from the

colony mortality study above and other published work to provide the basis for the development of the qualitative mortality model (Discussion section).

10.3 Results

The test colony was monitored from September 2005 to March 2006 and ongoing readings and observations were made.

Evaluation of colony behaviour

The test colony was in a hive that had an 'observation' facility and also the facility to measure cluster temperature (Figure 10.1).

Figure 10.1 Photograph of hive during an ivy honey flow in Autumn 2005. The south facing hive has an 'observation' facility on the west side and a facility for temperature measurement on the east side.

The colony in autumn 2005 had a large population of bees (ten frames of bees and five of brood) and this is demonstrated by the large number of foragers outside the hive during the ivy flow (Figure 10.1). The behaviour of the bees was normal during the autumn and early winter; flying bees with pollen, normal physical appearance and no dead or deformed bees outside the hive. Apart from tracheal mite infestation no other significant conditions were detected (detailed summary later). Bee deaths up to mid-January 2006 were small. From the last half of January the number of bees crawling on the grass in the afternoons increased with large numbers being recorded on sunny days particularly after a few cool sunless days. The following descriptions of colony activity in two individual days in March 2006 gives a picture of the typical colony behaviour.

4th March 2006 (7.2°C, sunny). It was a cool morning. The sun was shining and the bees moved out of the hive from mid-day and were still moving out at 3.00 pm. In most cases they moved from the landing board in a short 'hop-flight' or 'fell-off' landing on the grass about a half metre from the hive. They moved away from the hive towards the sun, crawling over the grass and eventually resting on top of blades of grass. The impression was given that they were trying to warm themselves in the sun with the hope of eventually flying. Occasionally some of the bees on the landing board could be seen vibrating their wings in a vain attempt to warm up before flight, sometimes with only one wing vibrating while the other one was stuck out 'K-wing' style (Figure 10.2). The movement out of the hive seemed to be in panic to defecate and slight bloating of their abdomens was evident. The lucky ones would fly in a short trip around the hive and could be seen defecating in 5 to 15 stages. By late afternoon many were still coming out of the hive and falling from the landing board. Others would walk along the landing board wriggling their abdomens in an effort to defecate or managed to defecate as they went along leaving a trail of up to 10 centimetres long on the landing board (Figure 10.3). In the process the bees could be seen making a great effort to keep their abdomens clear of the faecal matter. Some others would rush out of the hive in a palpable panic with excreta stains on their wings or abdomens and throw themselves off the landing board as though driven by a strong instinct that dictated that they must not empty their rectums in the hive.

Figure 10.2 Photograph of crawling bee on grass. The 'K- wing' effect can be seen on the left side where the hindwing has become unhooked from the forewing and sticks out (white arrow).

Figure 10.3 A bee excretes on the landing board of the hive during the final days of the colony.

6th March 2006 (10.1°C, sunny). The bees started to move out of the hive from early afternoon. Some bees were falling off the landing board, while others were flying a short distance to the ground. They then crawled over the grass, moving away from the hive and moving southwards towards the sun. Wings were occasionally 'K-winged' but not always. Apart from the appearance of bloated abdomens there were no other signs of abnormality; no trembling, no hairlessness. A few dozen bees collected on the landing board and along the front of the hive and remained at rest in the sun. Another small group gathered in a clump below the roof with the odd one dropping away to fall to the ground. In the evening the crawling had stopped and the bees were stuck, motionless on grass stalks.

The bees came out of the hive and crawled on the grass for the last time on 23rd March. At this stage there was a lot of dysentery staining on the landing board and on front of the hive. The bees died out on 25th March 2006. During the post mortem the following was established:

Total dead bees on frames	494
Total dead bees on floor	259
Total dead bees	753
Number of frames with bees	7 (3 inner frames had 97% of bees)
Total brood cells (sealed)	338
Number of frames with brood	4

The cluster had contracted to essentially 3 frames of bees and these bees were dispersed in a few small clusters. The queen was in one of the largest clusters surrounded by over 100 bees (Figures 10.4, 10.5). The queen was not infested with tracheal mites. There were a total of 338 sealed brood cells spread over 4 frames (Figure 10.6). These four frames did not coincide with the frames with most dead bees indicating that the bees had moved one or two frames forward towards the warmer front (south side) of the hive leaving brood uncovered.

Figure 10.4 Photograph, after the death of the colony, showing dead bees on a frame with the queen in the main cluster. Queen with a white spot on the thorax indicated with arrow.

Figure 10.5 Photograph showing close-up of the queen, with white thorax, in a small cluster of dead bees. The wings of the bees are heavily soiled with dysentery.

Figure 10.6 Photograph of a small cluster of dead bees on a comb with a small patch of sealed brood cells.

Disease status (Summary)

Apart from a tracheal mite prevalence of over 50% in October 2005 and rising to ~100% at the time of colony death at the end of March 2006, no other significant pathogens were detected.

- *Varroa destructor* - A natural varroa 'mite drop' of only two dead mites occurred during the winter period.
- *Nosema apis* - No presence of spores detected from samples in October 2005 and March 2006. These samples were tested by Teagasc, the national agriculture and food development authority.
- *Paenbacillus larvae and Melissococcus plutonius* - Neither American nor European foulbrood was identified by symptoms. There is no history of either foulbrood in the locality.

- *Ascosphaera apis* - No extensive patches of chalk brood present. During the post mortem a few infected cells were discovered but this would be normal for any colony that had experienced temperature stress (Bailey and Ball, 1991).
- *Chronic paralysis virus* (CPV) - Neither syndrome of the virus was detected on any of the bees from clinical symptoms. Testing of bee samples using molecular biology techniques, quantitative RT-PCR, for chronic paralysis virus had negative results. (Table 10.1).
- *Deformed wing virus* (DWV) – No clinical symptoms found. Using RT-PCR techniques, low levels of the virus were found (Table 10.1).
- *Acute paralysis virus* (APV) – Negative RT-PCR results (Table 10.1).

Table 10.1 Results of tests for chronic paralysis virus, deformed wing virus and acute paralysis virus in 6 samples using 40 cycle RT-PCR

No.	Date	Sample	Chronic paralysis Virus (CPV)	Deformed wing Virus (DWV)[1]	Acute paralysis Virus (DWV)
1	09/03/06	Test colony[2]	negative	37.75	negative
2	05/05/06	Colony 1[3]	negative	30.25	negative
3	17/02/06	Test colony[2]	negative	35.25	negative
4	01/05/06	Colony 2[3]	negative	38.5	negative
5	30/01/06	Test colony[2]	negative	34.5	negative
6	01/05/06	Colony 3[3]	negative	36.75	negative

[1]The results for the RT-PCR 40-cycle tests for DWV showing the average CT value for the two sub-samples and the duplicates of each sample. The negative results are recorded as CT 40.
[2]Three samples of the test colony, samples 1, 3, 5, taken at different times.
[3]Samples of three reference colonies, samples 2, 4 and 6 were taken. Sample 2 had about 5% of its nurse bees with wing deformity. None of the other samples had any clinical symptoms.

Samples of bees were analysed by quantitative RT-PCR techniques in the National Bee Unit in the UK using 40-cycle tests. A CT level of 40 indicates no virus detected, with levels below 40 indicating increasing virus loads in descending order. The CT levels were 37.75, 35.25 and 34.5 for the three samples from the test colony taken in March, February and January 2006 (Table 10.1). No bees with deformed wings were ever detected in the test colony. Three reference samples were also tested. Two of these samples (no. 4 and no. 6) were from colonies with strong bee populations and where no

deformed wings detected. The third reference colony (no. 2) was from an adjacent apiary and had about 5% of the nurse bees with slight wing deformation. All of the bees tested were from colonies that have low levels of *Varroa destructor* mites that have been controlled by treatment.

Caging grass-bound bees

The half-lives for the four samples of caged bees collected from the grass are given in Table 10.2. The bees were collected after the temperature had dropped in the afternoon and were put into the cages in a fairly lifeless state. While most of the bees initially fell to the bottom of the cage, within 15 minutes most of them had revived and crawled to the top of the cage and became animated. In general none of these bees was able to vibrate their wings again but otherwise behaved in a normal manner in the cage. Presumably because of their inability to defecate outside the hive, traces of dysentery did appear from time to time.

Table 10.2 Mortality of bees collected from the grass (crawling) and caged at 30°C.

Date bees introduced	Number of bees in cage	Half-life (days)
21/01/06	35	25
31/01/06	40	22
13/02/06	100	21
08/03/06[1]	100	13

[1]Parent colony died out on 25/03/06. The last bee to die, of the bees introduced into the cage on 08/03/06, died on 08/04/06 14 days later than the parent colony in the hive.

Mite prevalence

The mite prevalence was tested at intervals during the year and particularly during the winter period using bees collected from the landing board of the hive. The prevalence at the beginning of May 2005 was 33%, increased to 53% by the end of September and increased progressively during the winter/early spring period to 90/100% (Figure 10.7).

This sampled prevalence at all times lagged behind the prevalence of the dead bees on the grass, initially ~40% lower in the autumn but almost merging by the middle of February. Over 85% of the dead bees were bilaterally infested and this ratio was fairly constant over the period, while the corresponding average was 50% for the sampled bees and this proportion rose steadily for the later samples. The typical mite intensity (mite load) for the worker bees crawling on the grass was 78 mites (at all mite life-stages) per bee at the end (Table 10.3).

Drones were also highly infested with a prevalence of 100% for the crawling bees and a typical intensity of 291 mites (at all life-stages) per bee at the end (Table 10.3).

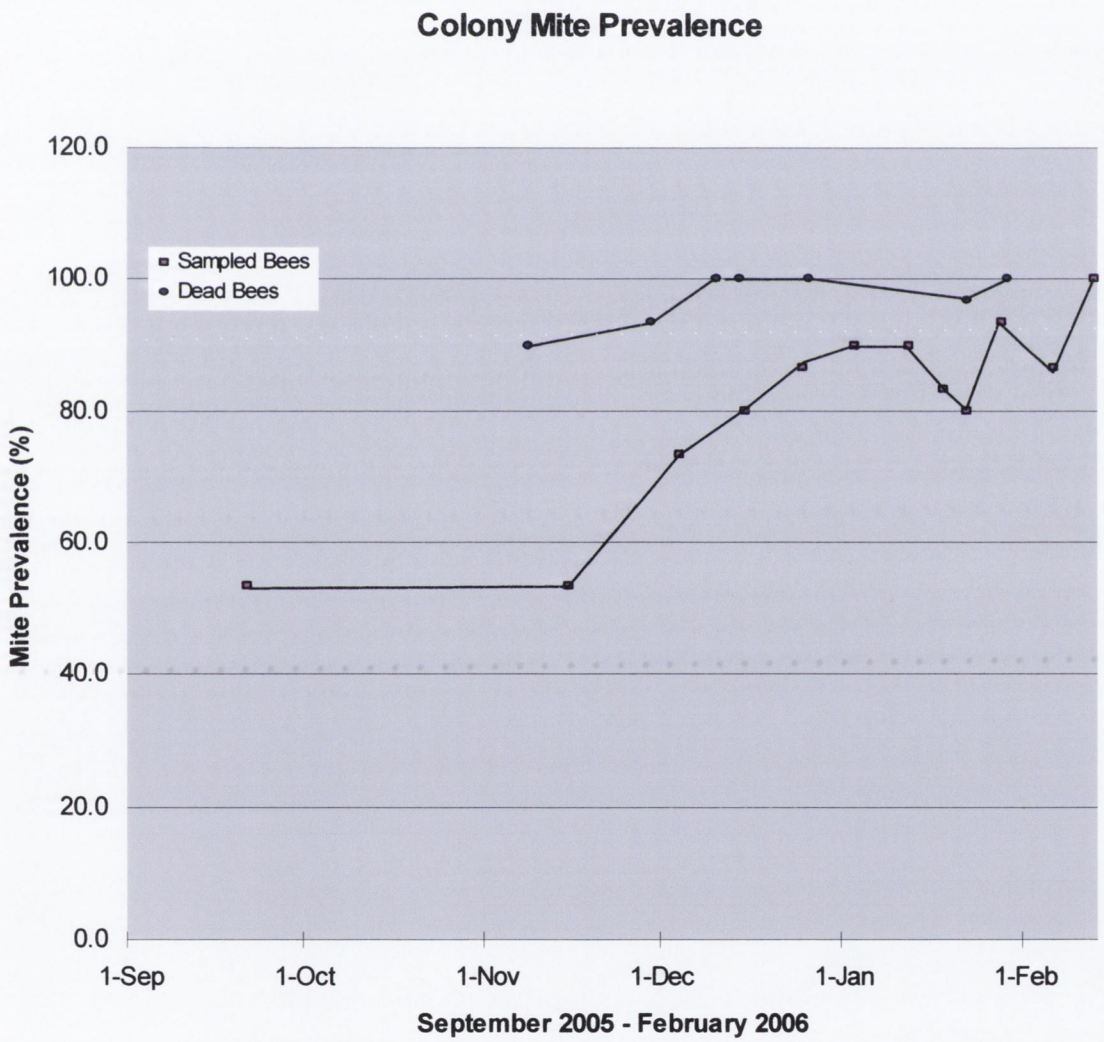


Figure 10.7 Mite prevalence in the infested colony being monitored from sampled bees (flying bees at the entrance) and dead bees collected from the grass. The prevalence in each case was measured using a total of 30 bees.

Table 10.3 Summary of analysis of dissection of 40 drones collected from the grass (crawling).

Level of infestation estimated (Mites per trachea)	Trachea		
	Left	Right	Average[2]
100+	28	30	29
50+	7	2	4.5
< 50	-	2	1
Black (no mites)	5	5	5.0
None[1]	-	1	0.5
Total	40	40	40

[1] Mite prevalence in the crawling drones was 100% with 39 out of 40 bilaterally infested

	Adult females	All mites
[2] Detailed dissection of 5 drones at random (per bee)	49	291
The corresponding mite intensity for workers (per bee)	12	78

Profile of winter cluster

The test colony was in a hive with a 'window' in the side that provided an end view of eight frames, shown shaded, and numbered 2 to 9 (Figure 10.8). The frames were in the hive the 'warm way' around (parallel to the front of the hive, the frames being numbered from the back) and the hive faced almost due south. During the winter period 7 profiles of the cluster were drawn. These were undertaken by marking on a transparency the lowest point that the bees in the cluster extended down between all of the frames and joining these points by a curve. In September and October the brood nest was to the back of the hive but as the winter progressed the cluster moved progressively forward towards the front. Eventually by 23rd March the cluster had dwindled to occupy a small space at the front of the hive and no movement was detected in the bees for the first time signifying the death of the colony. There was only a small variation in the outside ambient temperature at the times in the morning when the profiles were being drawn, median (range) 5.6°C (± 2.5°C).

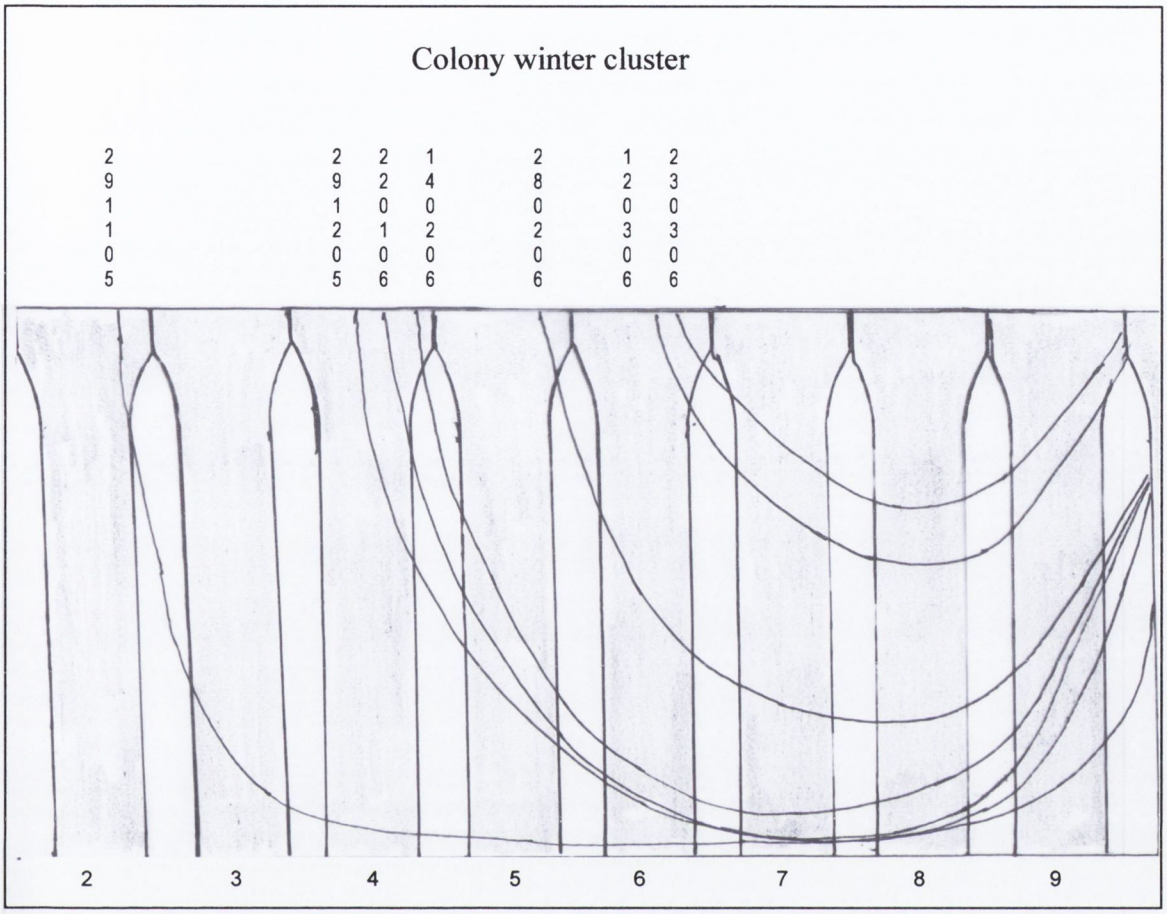


Figure 10.8 This illustrates the depletion of the winter cluster from 29/11/05 to 23/03/06. End frames 2 to 9 are shown in the 'window' of the hive. N.B. When the cluster profiles were being drawn the hive ambient temperatures were fairly similar - within the limits, median (range) 5.6°C (± 2.5°C).

Colony stores

The stored honey was monitored during the winter period and showed a steady decline from ~20 kilograms to ~12 kilograms (Figure 10.9). During this period the ivy produced a strong flow and this is reflected in the increased stores for similar strength colonies (#1 and #2) in the autumn. However, the infested colony that had already produced a good main honey crop in the summer, failed to show any increase during the autumn ivy flow. During the period January to March there was a similar consumption of stores by the three colonies (denoted by the similar slopes of the graphs for the three colonies) even though the test colony had a lot less brood and a much depleted bee population.

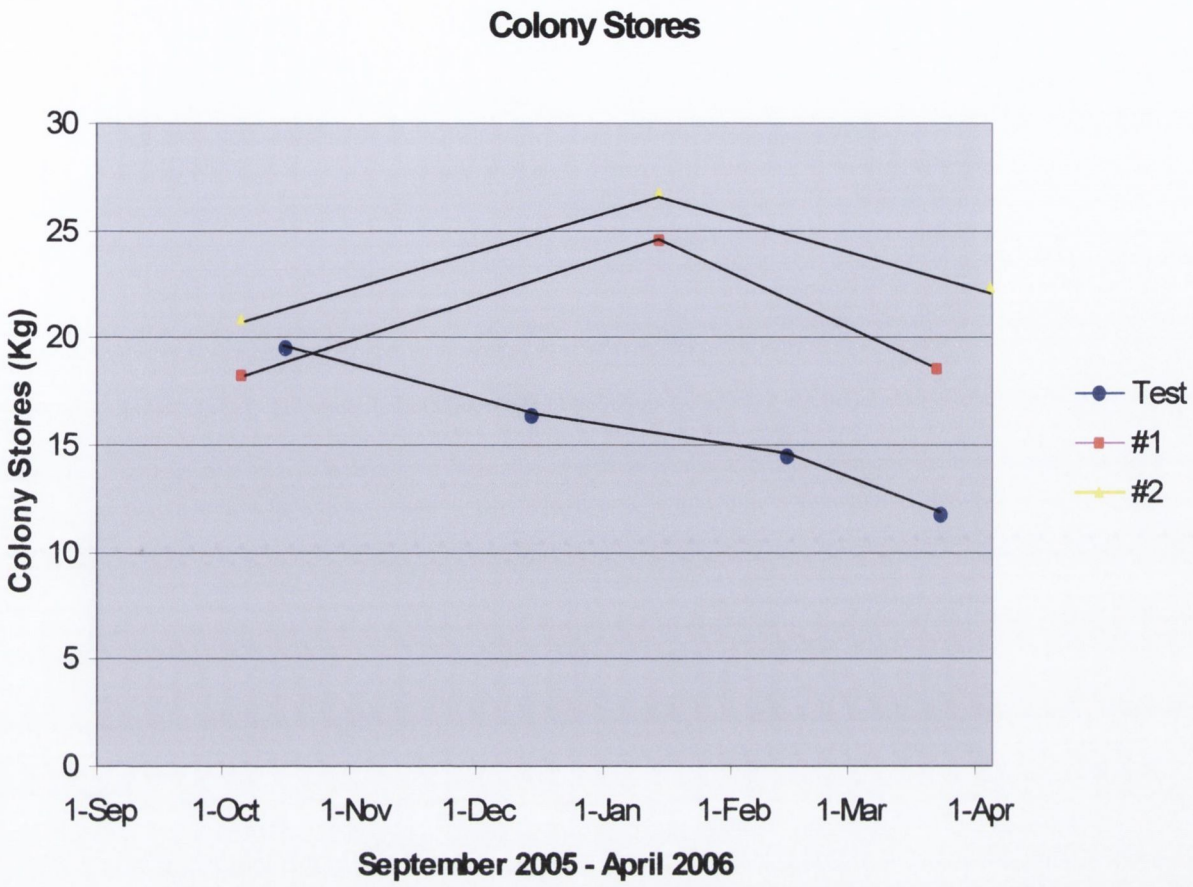


Figure 10.9 Weight of honey stores in infested test colony compared to uninfested colonies of similar strength in the autumn (denoted by #1 and #2).

Cluster temperature

The daily readings over the period from a temperature probe in the brood nest are given in Figure 10.10. There are two plateaux of temperature, one during the late autumn brood rearing and the second during the late winter/early spring period. The mean autumn temperature in the cluster over a 14-day period was 34.6°C while the corresponding temperature at the start of the year was 33.2°C but this temperature was not maintained and declined rapidly with the demise of the colony. In the winter broodless period there were wide fluctuations in temperature with a minimum temperature of ~27°C (except when it dropped to ~24°C during a short cold period) indicative of restlessness amongst the bees and fragmentation in the cluster. This temperature profile contrasts with a typical profile for a healthy colony from temperature measurements that I took in a previous year during the same winter/spring period (Figure 10.11). The temperatures of the two plateaux in this profile were similar

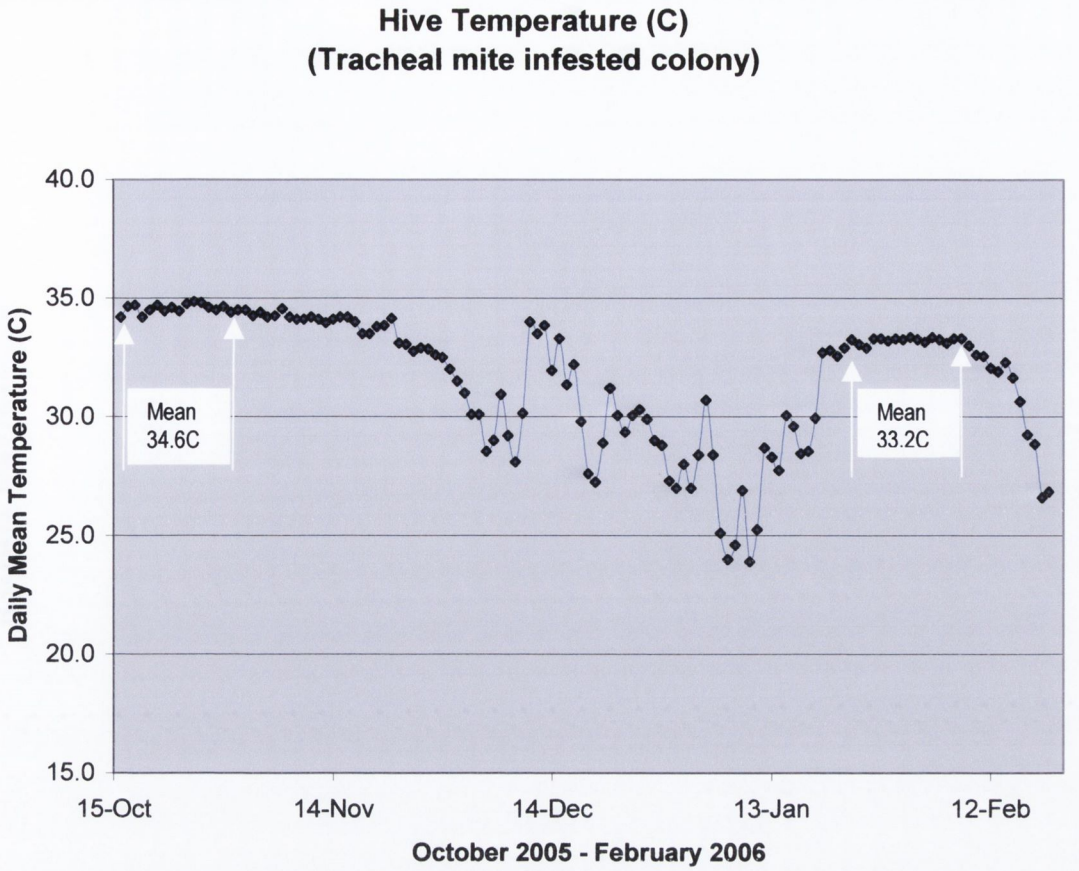


Figure 10.10 Daily temperature in infested-colony cluster over the winter period.

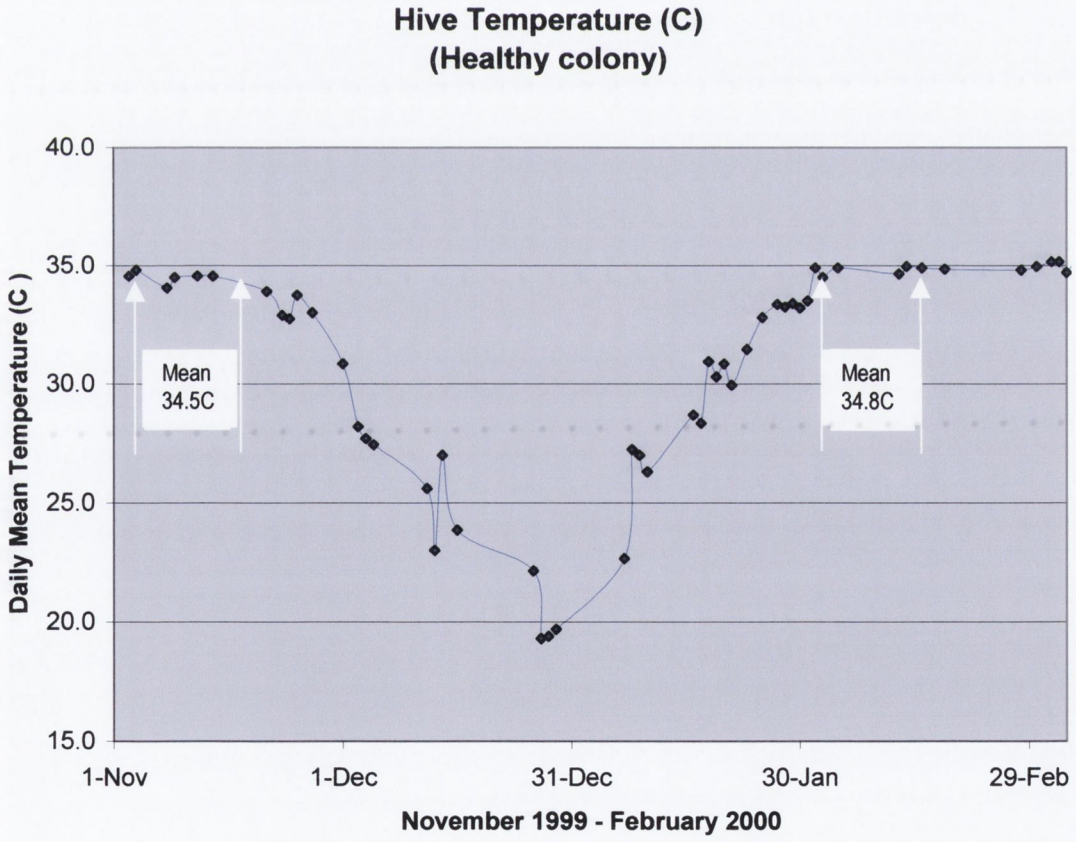


Figure 10.11 Daily temperature in a typical healthy-colony cluster over the winter period.

with means of 34.5°C and 34.8°C over a 14 day period. It also illustrates a smooth decline into a mid-winter valley where for a large proportion of the time the cluster temperature lies between 20 and 25°C. While the temperature profiles (Figures 10.10; 10.11) were for different years the ambient temperature was similar in the two periods.

Winter bee mortality

Any bees that had died were gathered from the grass each day in late afternoon (Figure 10.12). There was no bird activity to remove dead bees during this period. In the three months up to 20th January 2006 a total of 116 bees were found dead/crawling on the grass outside the hive. In the next two months until the colony's demise the number collected rose to 3,243 with an average daily rate of 51 bees (Figures 10.13, 10.14). Including 753 bees dead in the hive and analysed in the post mortem (below) the total number of dead bees collected was 4,112.

Figure 10.12 Crawling bees (circled) on grass on a sunny afternoon in early spring.

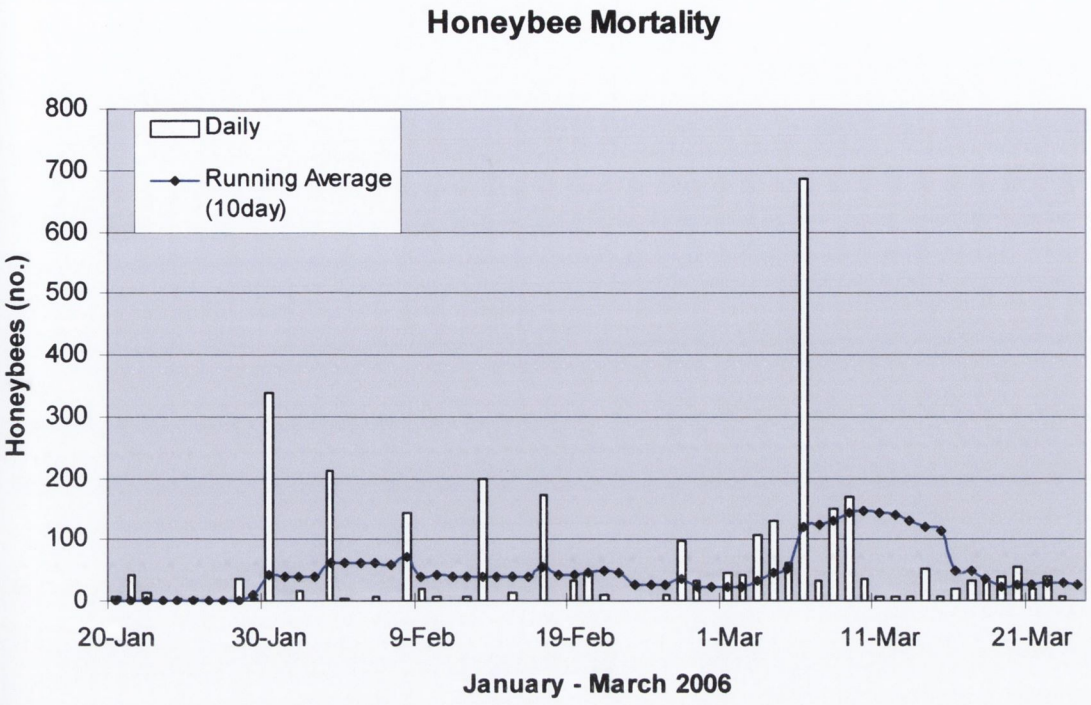


Figure 10.13 Daily and 10-day running average mortality of honeybees during January – March 2006 that died crawling on the grass.

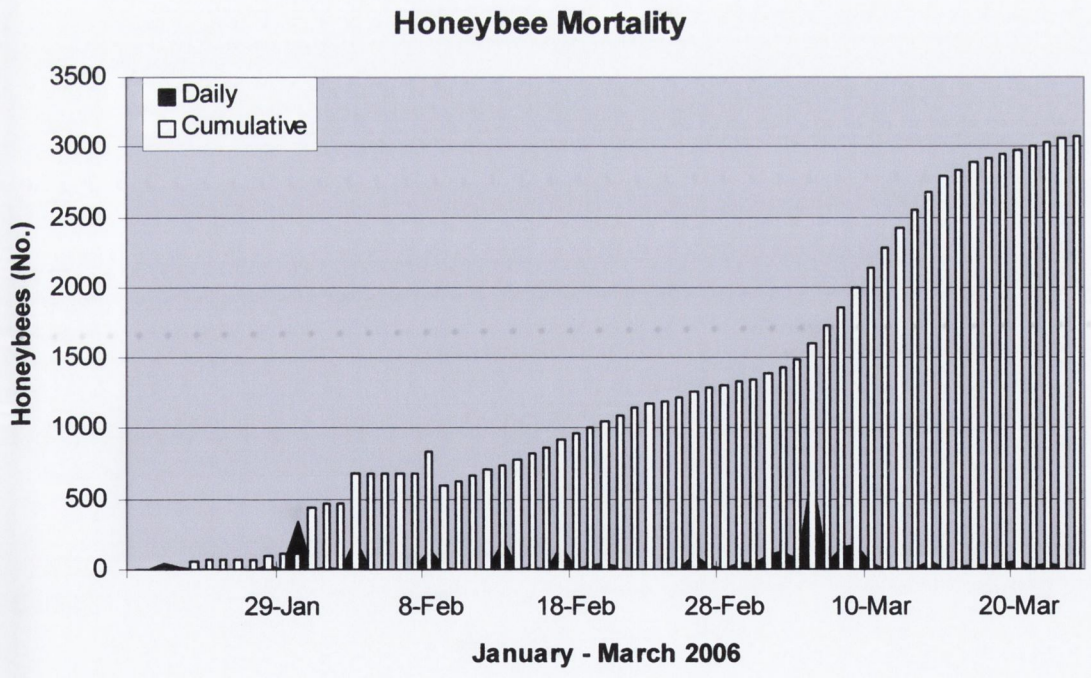


Figure 10.14 Daily and cumulative mortality of honeybees during January – March 2006 that died crawling on the grass.

The colony had 10 frames of bees at the end of September, ~ 10,000 bees. At the end of November the number had reduced to 8 frames, ~ 8,000 bees (see Figure 10.8) most likely due to the high death rate of the older bees during ivy foraging in the autumn. A comparison of cluster profiles (Figure 10.8) would indicate a reduction in colony population to 5 / 6,000 bees by the end of January 2006. The total of 4,112 bees collected from the end of January 2006 is therefore likely to represent a large proportion of the bee population at that time. Almost all the grass crawlers are likely to have been accounted for as over 99% of these bees were found within 8 metres of the hive and in prominent positions, invariably clutching to stalks of grass. Any deficit in the number recovered is likely to be made up of flying bees not returning to the hive.

It was apparent that the numbers of crawling bees was much higher on sunny days. Representing the sun-status of the days as 'sun' - '1' or 'no sun' - '0' it was shown that the sun-status had a significant effect on the number of crawling bees ($F_{[1, 53]} = 11.332$, $p = 0.001$). In order to differentiate this effect from the other possible associated effects of sunlight such as 'temperature' or 'time of year', the maximum (in the shade) daily temperature and the calendar date were included as covariates of sunlight. A 3-way ANOVA showed a significant effect for sun ($F_{[1, 51]} = 10.007$, $p = 0.003$) but not significant for temperature ($F_{[1, 51]} = 3.117$, $p = 0.083$) or date ($F_{[1, 47]} = 0.429$, $p = 0.515$).

On two occasions when in sunny conditions > 100 bees crawled on the grass outside the test colony, another apiary with 5 colonies was visited. Less than 2 bees / colony were located on the grass in this apiary and some of these had pollen and were 'resting' in the sun prior to taking off again. No tracheal mites were found in these colonies in the autumn testing and none of the bees on the grass had any tracheal mites.

Other considerations

The experiments in Chapters 3 and 4 demonstrated that bees developed under reduced pupation temperature had a much higher susceptibility to tracheal mite infestation. Also in limited trials I have been able to show that wrapping (lagging) beehives containing colonies with high mite prevalence levels can reduce colony mortality. In these trials,

with four colonies in double-brood box hives and with similar prevalence levels going into the winter, the two wrapped colonies survived while the two unwrapped colonies died out.

10.4 Discussion

Association of tracheal mites with colony mortality

The test colony went into the autumn with a high tracheal mite prevalence (53%) and died out during the early spring in line with normal expectations (Bailey, 1958; Furgala *et al.,* 1989). The honeybee population became depleted over the winter period largely accounted for by bees dying on the grass. There is evidence that the bees are propelled away from the hive in an attempt to defecate on the wing. The presence of sunshine, not temperature or the time of year, had a significant effect on the numbers of bees that left the hive. This need to defecate may be exacerbated in infested colonies as a result of the apparent need to consume large quantities of stores to heat the hive, thereby accumulating a lot of residual matter in the rectum. This will be discussed later.

Deformed wing virus (DWV) was detected in the test colony. It is likely that DWV is endemic in Ireland as in other countries where *Varroa destructor* is present (Nordstrom, 2003; Tentcheva *et al.*, 2004). DWV is claimed to be responsible for wing deformation when infection occurs during the white-eyed pupation stage of bee development (Bailey and Ball, 1991). Its pathogenicity is not clear. In one study DWV has been considered as potentially responsible for colony collapse (Nordstrom *et al.,* 1999), however, other studies have stated that the virus might be considered poorly pathogenic (Bowen-Walker *et al.,* 1999; Tentcheva *et al.,* 2004). Asymptomatic bees have been found to have much lower virus loads than those that were deformed or had died during pupation (Tentcheva *et al.,* 2006) and it has been suggested that DWV must be present in pupae above a certain concentration to be pathogenic (Bowen-Walker *et al.,* 1999). The bees in the test colony would appear to have DWV at relatively low levels (Table 10.1) and no deformed wings were ever detected while the bees were under daily scrutiny. The reference bees (sample 2) from an adjacent apiary that had about 5% of the house bees

with some wing deformation showed a much higher virus load count (Table 10.1) indicating that the virus load in the test colony was not pathologically significant.

Apart from tracheal mites and very low levels of *Varroa destructor* no other pathogens were detected. The bees from the test colony crawled on the grass in the commonly reported manner but there were no clinical signs of other conditions. Chronic paralysis virus is considered by some as the reason why infested colonies die and the crawling effect has been attributed to this virus (Bailey, 1999). However, no CPV was detected in the molecular virus testing. The two syndromes exhibited by this virus have clear clinical signs (Bailey, 1975; Bailey and Ball, 1991); "This disease has two distinct symptoms or syndromes. One of these (Type 1)..... includes an abnormal trembling motion of the wings and bodies of affected bees. These fail to fly but crawl on the ground and up grass stems, and sometimes in masses of thousands of individuals. Frequently they huddle on top of the cluster in the hive. They often have bloated abdomens and partially spread, dislocated wings. ... The other syndrome (Type 2) ..... At first the affected bees can fly, but they become almost hairless, appearing dark or almost black which makes them smaller than usual but with a relatively broad abdomen; they are shiny, appearing greasy in bright light." Neither of these symptoms was present in the monitored colony. There was no trembling of wings nor abdomens and no hairless, black greasy appearance. I am familiar with both of these syndromes as my first colony of bees died out in the month of February over ten years ago having displayed both of these syndromes and had a 60% prevalence of tracheal mite infestation in the previous autumn. The previous two summers had been particularly dull and wet compared to the brighter, drier and warmer weather over the past decade. Since that time I have experienced the deaths of many colonies in North County Dublin with tracheal mite infestation but with no clinical symptoms of other diseases. The absence of other pathogens has typically been the experience with tracheal mites infested colony deaths in the US (Collison, 2001).

The account given by H. M. Cooper, writing in 1906 about a visit he paid to the Isle of Wight, could well have been written about my recent experience with tracheal mite infested colonies. Cooper produced a report in the *British Bee Journal,* entitled 'Bee paralysis: is the cause known?' that included the following description.

> During a bright, sunny day, recently, the bees in coming out of the hive dropped on to the grass by the dozen, and seemed quite powerless, so far as using their wings… . The abdomen of the bee was not distended in every case, while the wings were often twisted back, having the appearance of being dislocated… .They kept crawling up the blades of grass and on to the alighting board only to tumble off again, and when the sun went down, and the day became colder, the bees collected in little bunches of a dozen or so in each, and soon perished. Another hive since becoming affected by *paralysis* has developed dysentery, and the bees are rapidly dwindling.

Chronic paralysis virus causes the rapid death of bees (Bailey, 1975, 1985 and 2002). If the bees in the test colony had been suffering from a normal terminal illness they would have been expected to die in a short time in normal hive conditions. However, the four samples of bees that were caged at 30°C did not die rapidly and had a half-life of 13 to 25 days. The 13 day half-life was for old winter bees at the end of their normal life span, after initially spending a few hours on the grass in cold conditions and even some of these lived for a month, outliving those bees in the colony itself by two weeks.

The comparison of the prevalence of sampled bees vs. dead bees showed that the sampled bees had initially a much lower mite prevalence and the prevalence level rose with later sampling. It would be expected that that the older bees would have a much lower prevalence yet the dead bees had a much higher prevalence which would indicate that infested bees have a much greater likelihood of dying. This concurs with the findings of Harrison *et al.,* (2001) who demonstrated that in cool weather bees that were unable to return to the hive during late winter flights had significantly higher levels of mite infestation than those that returned safely. Very high levels of mite intensity were found in the dying bees crawling on the grass with typical numbers of mites (at all stages) of 78 and 291 for workers and drones respectively (Table 10.2). There is little published data on mite intensity/abundance, mite prevalence being the common expression of infestation. Royce and Rossignol (1990) did relate that in a study in Oregon State, 30-40 mites at all stages were often found in a single infested trachea which is somewhat less than that reported in this study.

The foregoing demonstrates that tracheal mite infestation can result in honeybee mortality at the higher levels of infestation. We must look elsewhere for the cause of death.

Colony thermoregulation

It was concluded by Bailey (1958), "that infestation with *A. woodi* shortens the lives of bees only slightly" It was also concluded that, "Increased mortality in winter of colonies with over 30% bees infested was statistically significant" (Bailey, 1961). Therefore tracheal mite infestation appears to cause no individual bee effect but does cause a colony effect in the winter. One possible colony effect may be the inability of a highly infested colony to thermoregulate while an infested individual bee in a lowly infested colony or in the summertime requires less heat or can depend on other bees to take care of thermoregulation.

The test colony had a normal temperature profile during brood rearing in the autumn but failed to maintain a normal temperature for brood rearing in the late winter/early spring period. During brood rearing the temperature in the brood nest would be maintained within 30-35°C, the range for summer brood rearing being much narrower (Winston, 1987). With local bees in Ireland the temperature is normally 34.5°C with a range of about 0.5°C (Appendix 2). The reduced pupation temperature in the test colony will increase the susceptibility of bees to mite infestation in accordance with the findings in Chapters 3 and 4. There were also abnormally high and fluctuating temperatures in the non-brood valley period of mid winter indicating high levels of bee movement and contributing to excessive heat loss. Loose winter clusters have been reported by others (Adam, 1968). These loose clusters and the continuous agitation of individual bees can be detected by 'listening' (ear to brood box) to tracheal mite infested bees during the winter (pers. obser.). This inability to keep the colony warm was accompanied by an excessive consumption of stores in the test colony. Eischen (1987) has demonstrated a significant relationship between stores consumption and infestation level. The bees may be making a vain attempt to heat the hive and an inefficient energy conversion may be taking place due to the bees' limited ability to 'shiver' and produce heat with their thoracic flight muscles (Komeili and Ambrose, 1991). This increased stores consumption will increase the pressure on their rectum capacity, increases the need for

cleansing flights and contributes to dysentery in and around the hive. In requiring more cleansing flights infested bees are more exposed to flying in cold winter conditions than healthy colonies. The winter cluster profile in the test colony showed a colony that was rapidly losing its bee population and hence its ability to heat the hive. It also displayed a level of restlessness most likely motivated by heat deprivation resulting in the need to move the cluster forward towards the warm south-facing front of the hive.

Reduced bee populations and brood levels in infested colonies have also been identified (Eischen, 1987). In addition, analysing the data in Eischen's paper shows that the brood cover (bee population to brood size) in infested colonies is lower. The brood cover in November and February for three stages of colony infestation, uninfested, moderately infested and heavily infested were 4.4 and 3.4; 3.6 and 3.7; and 2.6 and 2.2 respectively.

Jeffree (1959), based on the experience at that time of tracheal mite infestation, suggested that mite infestation would only occur globally within particular latitudes where specific climatic conditions occurred, particularly those of temperature and rainfall. His predictions were largely based on reported colony infestations which, particularly in the mid twentieth century, would have closely corresponded with colony mortality. In the event his theory did not hold up. If Jeffree had stated his theory as predicting colony mortality he would, in my opinion, have been largely correct. In many sub-tropical locations tracheal mite infestation can be high and also widespread but with colony deaths remaining at a low level. This is the case in locations such as the Southern States of America, while further north colony deaths are commonplace (Eischen, 1987; Otis and Scott-Dupree, 1992; Sammataro, pers. comm.). This would appear to further strengthen the link between the demands of thermoregulation and colony mortality. The limited trials that I undertook of wrapping the hives of highly infested colonies in winter is further evidence supporting this conclusion.

Mortality model

The foregoing is used to develop a qualitative model for colony mortality. The model is illustrated in Figure 10.15 as a spiral demonstrating the continuous, progressive and cumulative effects of the different influences on a colony infested with tracheal mites. Ten nodes are highlighted in the model.

Node 1. Infested colony in winter

This is the starting position for an infested colony of tracheal-mite susceptible bees in the late autumn/winter/early spring period. A climate with low ambient temperature accompanied by wet/humid conditions would put increased demands on a colony.

Node 2. Reduced brood

An infested colony has reduced brood rearing in the late winter/early spring period (Otis and Scott-Dupree, 1992). The reduced brood and corresponding reduced emergence of callow bees will increase the infestation pressure on the existing callow bees and drive up the mite prevalence of the colony.

Node 3. Reduced bee population and brood cover

The reduced brood will have the critical effect of contributing to a reduced bee population and particularly of young bees on which the future of the colony depends (Eischen, 1987). The analysis above of Eischen's paper shows a large reduction in the brood cover with increased infestation in the late autumn and also in late winter periods. This has the effect of increasing thermal stress and reducing brood temperature in the colony.

Node 4. Loose cluster

Infested colonies have looser winter clusters (Adam, 1968). The test colony also displayed a loose and restless cluster. I have also confirmed this feature by 'listening' to infested colonies in winter. The effect of loose clusters is to reduce the level of cluster insulation and combined with the continuous bee movement, to increase the energy needs of the bees. It is interesting that in the test colony the cluster temperature during the non-brood period was higher than in a typical healthy colony, further increasing the energy needs of the bees.

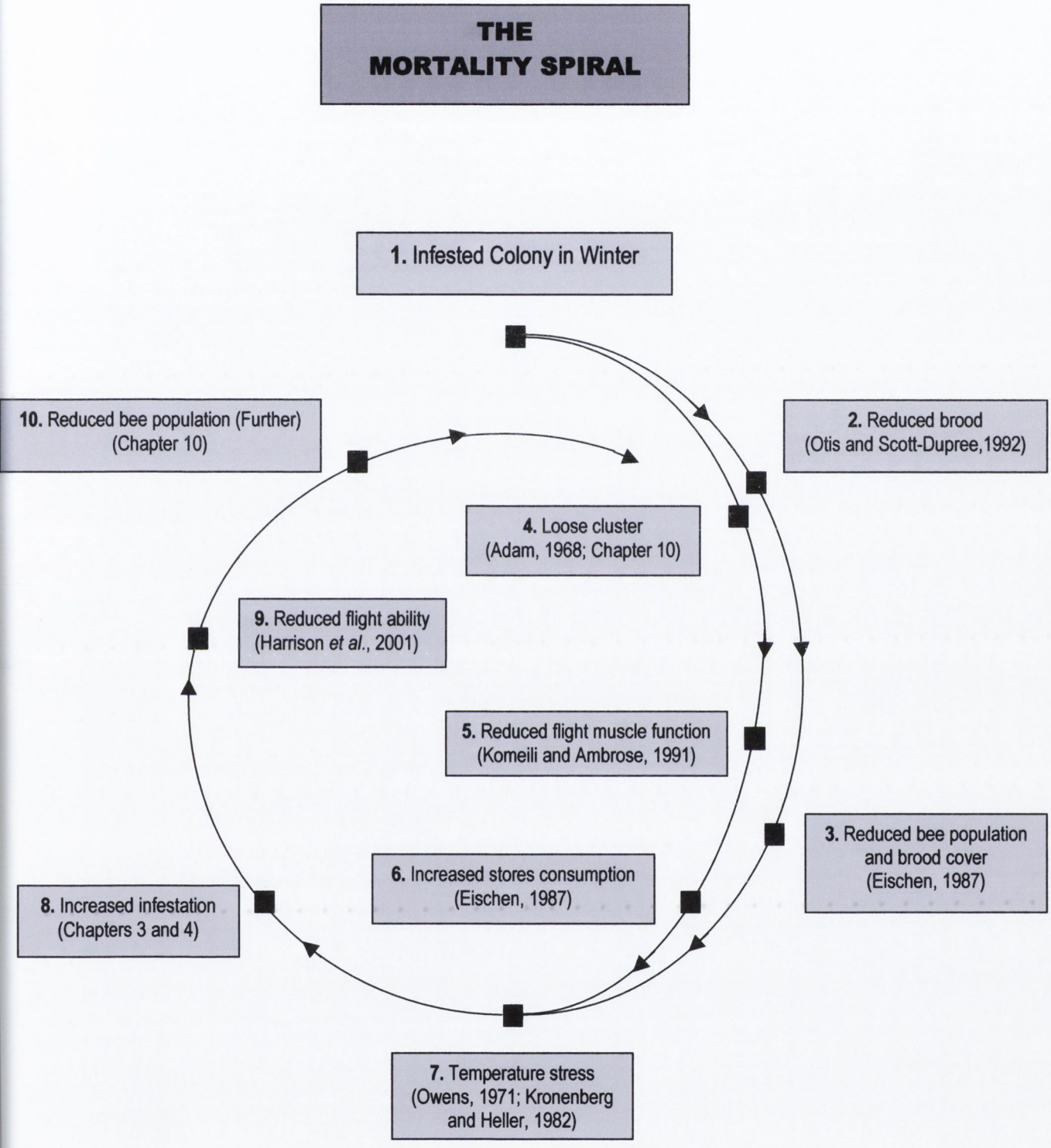


Figure 10.15 Mortality Spiral showing the dynamic influences on a moderately infested colony during the winter/early spring period.

Node 5. Reduced flight muscle function

Honeybees use their thoracic muscles to generate heat in the winter cluster by 'shivering' (Heinrich, 1996). Tracheal mite infested bees have a reduced ability to use their thoracic muscles to generate heat (Komeili and Ambrose, 1991). This will put further pressure on the colony's ability to maintain the cluster temperature.

Node 6. Increased stores consumption

Infested colonies consume more honey stores than healthy colonies during winter (Eischen, 1987). This was also apparent in the test colony. This increased consumption may be due to the infested bees having a low efficiency in metabolising the honey stores and converting it to heat energy since Komeili and Ambrose (1991) have identified that infested bees have flight muscle degeneration. If the heat energy produced per bee by shivering is less than the heat energy lost per bee which has increased by the poor cluster insulation (due to the loose cluster) and the reducing bee population, the bees will tend in the short term to keep the temperature close to the brood cluster minimum level. In the longer term towards colony collapse it would slip continuously lower. The increased honey consumption would increase the need for cleansing flights and increase the risk of not returning to the hive

Node 7. Temperature Stress

While healthy colonies of *Apis mellifera* have good regulation of brood temperature, it is also known that in adverse conditions the brood temperature held by the bees can be well below typical levels; as low as 29.5-34.5°C within the brood area (Kronenberg and Heller, 1982; Owens, 1971). Conversely, in normal conditions bees that are not healthy may have difficulty regulating brood temperature. The compound effect of the influences in the outer spiral arc from nodes 2 and 3 and the inner arc from nodes 4, 5 and 6 may reach a stage where the brood temperature will have to drop. The reduced brood temperature in the test colony during late winter corroborates this position (Table 10.10).

Node 8. Increased infestation

Reduced temperature in the brood nest increases the susceptibility of all strains of honeybees to tracheal mite infestation (Chapters 3 and 4). This will have the effect of increasing further the infestation in an already highly infested colony and result in the

bees towards their terminal stage having 100% prevalence and multiple female mites in bilaterally infested bees.

Node 9. Reduced flight ability

Honeybees with moderate mite infestation have reduced flight metabolic rate in severe atmospheric conditions and this reduces further with increased mite infestation (Harrison *et al.*, 2001). Thus infested bees in cold weather conditions will have difficulty in returning to the hive. Also highly infested bees are not able to fly whether due to severe oxygen delivery restriction or damage to their thoracic wing muscles. This does not appear to appreciably reduce their life span in normal conditions but their ability to fly does not return (study of grass-bound bees transferred to cages in incubator, Table 10.2).

Node 10. Further reduced bee population

The need to take cleansing flights in winter, triggered by the presence of sunshine, results in highly infested bees leaving the hive with an inability to defecate on the wing (Test colony, Figure 10.12). These bees perish on the grass in late afternoon in the winter/early spring period. This results in a dramatic reduction in the colony population.

The model describes the interaction of many dynamic variables and whether or not the infested colony actually dies out will primarily depend on a number of factors such as severity of original infestation, strain of bee, hive insulation and winter climate. The research demonstrates that a colony with a high level of tracheal mite infestation in the autumn can die out in the early winter period with no other significant pathogen present. Of course, honeybee colonies infested with tracheal mites may also occasionally be afflicted with other pathogens. In such cases a synergistic effect has been demonstrated to be present where more than one significant pathogen is present (Downey and Winston, 2001). The colony studied in my research had all the characteristics of those described by Rennie and his contemporaries at the time, indicating that tracheal mite infestation was most likely to have been the cause of the 'Isle of Wight disease'. Additionally the honeybee subspecies used in my experiments and studies was *Apis mellifera mellifera,* the indigenous bee at the time of the outbreak of the 'Isle of Wight disease' in Britain and Ireland. In particular, there were no symptoms of chronic

paralysis virus which has been widely held as the cause of the clinical symptoms and mortality of colonies with tracheal mite infestation. Furthermore, measurements and observations made during the colony study illustrate that the lack of thermoregulation is likely to be the cause of colony mortality. While only one colony was used in this experiment without replication, the evidence from this colony taken in conjunction with the earlier experiments and the findings from previous published work provides a consistent argument for the conclusions drawn.

CHAPTER 11

Implications for colony management

11.1 Introduction

The previous chapters investigated the relationship between tracheal mites and honeybees. This relationship entailed high levels of colony deaths initially in Britain and Ireland in the early 1900s, later in mainland Europe, Asia and Africa and eventually in the 1980s in the United States and Canada (See previous references). During the initial outbreaks the beekeepers had no knowledge of how to deal with the situation. In the early stages there was no known cause and no management approach to apply. A bacterium and a fungus, *Nosema apis* (previously ranked among protozoa; Sina *et al.*, 2005), were at first considered as the cause. The tracheal mite was later considered to be responsible after its discovery in 1919.

Over time techniques and treatments were developed to manage infested colonies. The early control in Europe was mainly through the use of chemicals and this approach is still the dominant method of management of tracheal mite infestation. In the 1920s smouldering sulphur fumes were used (Bailey, 1963). Cardboard impregnated with saltpetre was burned in a smoker and smoke puffed into the hive entrance. The earliest treatment in widespread use was developed by R. W. Frow using a mixture of petrol, nitrobenzene and safrol to produce vapours (Frow, 1940). Vapours from the 'Frow' treatment also killed brood and shortened the life of bees (Bailey, 1963). Wintergreen oil (menthyl salicylate) was also used and is still in use as a winter treatment. From the 1950s Folbex (chlorobenzilate) strips burning in the hive were widely used in Europe. Later menthol (peppermint oil) was developed in Italy and today is the main method of control in the US (Wilson *et al.*, 1988). It is however only suitable for warm climates. Other botanical oils such as thymol have also been used in Europe with some effect on tracheal mites. Currently the only registered treatment in Ireland for tracheal mites is a thymol gel (Apiguard™). To be effective the treatment with thymol gel needs to be given when the bees are active which normally requires an air temperature of not less than 15°C which is difficult to obtain at the time of treatment in early spring or autumn. Vegetable oil made up with shortening in the form of patties has been used extensively in the US as an effective treatment (Delaplane, 1992). The mode of operation of these treatments, it would appear, was to confuse the questing female mite as to the age of its

new host bee after migration and to keep it in a permanent questing behaviour which eventually resulted in it desiccating in the manner described in Chapter 5.
Formic acid is an effective miticide against tracheal mites (Hoppe *et al.,* 1989) and is also effective against *Varroa destructor* mites which makes it particularly attractive as most varroa treatments are ineffective against tracheal mites. Formic acid is hazardous to handle and can occasionally cause the loss of the queen and worker bees (Nelson *et al.,* 1994).

The limited efficacy of most of the treatments in a temperate climate, the hazards in use, their effect on the bees and the leaving of residual traces in honey highlight the importance of non-chemical controls. This chapter will look at what the beekeeper can do based on the findings in this research.

11.2 Colony management

In this research I have tried to obtain a greater understanding of the relationship between the mite and the honeybee and this should allow action to be taken that will minimise the use of chemicals. A non-chemical approach has many attractions. It is cheaper, can be less labour intensive and does not expose the bees or the beekeeper to pesticides as well as removing the risk of contaminating the products of the hive. A structured approach (having a long-term effect) and approaches involving colony manipulations (having short-term effects) are proposed.

Structured approach (Long term)

Re-queening the colony with a queen of a strain that has improved resistance to the tracheal mites gives a solution that provides long term benefits by ensuring control for at least the lifetime of the queen.

There has been a long history of claims of genetic resistance in bees to tracheal mite infestation. In 1911 Anderson claimed to have identified resistance among colonies (Anderson, 1930). Brother Adam in Devon devoted seventy years to developing a

hybrid, the Buckfast bee, that displayed resistance having first observed resistance among colonies in his apiary in the early 1920s (Adam, 1968). The merit of Buckfast bees was pronounced by Calvert, in Northern Ireland, who conducted trials with the stock in the 1950s and claimed in one year to have overcome tracheal mite infestation (Calvert, 1957). More recently in the US and Canada trials on different strains have further confirmed the Buckfast strain as having high resistance to the mite (Danka *et al.*, 1995; Lin *et al.,* 1996). It is now generally accepted that honeybee genetics have a major influence on bee susceptibility to tracheal mites (Gary and Page, 1987; Page and Gary, 1990; Guzman *et al.*, 1998, 2005; Villa and Danka, 2005).

In Chapter 9 it was demonstrated that particular honeybee strains had a marked influence on the susceptibility to infestation. Specifically it was shown that those bees exhibiting morphometric characteristics of the Northern European dark bee, *A. m. mellifera,* had a high susceptibility to the mite. This was the indigenous bee of Britain and Ireland at the time of the 'Isle of Wight' disease and is still the dominant strain in Ireland. There is evidence that the critical morphometric characteristic from Chapter 9 that determined tracheal mite susceptibility was the value of the discoidal shift. Choosing a queen from a strain with a discoidal shift of say > - 1.0, would be expected to increase the resistance of the colony to tracheal mites. This would in effect move the chosen strain away from *A. m. mellifera.*

A. m. mellifera has evolved over time in the Northern European region and has many qualities that are suited to this region which are largely related to their winter hardiness (Ruttner, 1988b). They are capable of foraging at low temperatures and are frugal in the use of their honey stores in winter. If the strain is to be retained then short-term colony manipulative approaches will be required.

Colony manipulation (short term)

Artificial swarm

Manipulating colonies to reduce tracheal mite infestation has been practiced in the past (Svoboda, 1947). This has normally been done by removing 2 or 3 frames of brood with nurse bees into a nuclei and providing a sealed queen cell (Eischen, 2001). This action

will produce a gap of 1 to 2 weeks (2 to 3 weeks if sealed brood is used) in the availability of young vulnerable bees (up to 5 days old) and break the infestation cycle.

Swarming may also have an effect on infestation levels. It has been shown that feral colonies have lower levels of mite prevalence than the hive bees in the same locality (Royce *et al.*, 1993). The lower infestation is explained as follows; first, the swarms are more likely to have fewer mites and second, new swarms will have no new worker bees for 21 days and the mites will consequently have no young bees in which to migrate. The modern beekeeper's preoccupation with ensuring that colonies do not swarm may well be maintaining infestation levels higher than they would otherwise be by keeping old bees and young bees in continuous contact. I have used this approach to maintain high infestation levels in host colonies for the inoculation experiments.

Undertaking an artificial swarm in the spring/summer period as a swarm control method has the added benefit of reducing mite infestation but, in contrast to the experience of Royce *et al.* (1993) in naturally occurring swarms, it is my experience with the artificial swarm that it is normally the remaining colony in the parent (old) hive with the young bees that achieves the greatest reduction in infestation level. This may be explained by the worker age composition of the naturally occurring swarm where younger bees have a higher probability of issuing with a swarm than older bees (Winston, 1987), leaving a high proportion of the older infested bees in the parent hive. In contrast, the parent hive in the case of the artificial swarm will have few old bees with which to infest the emerging brood and the number of old bees can be further reduced by undertaking the 'enhancement' one week later as described below.

As a method to reduce tracheal mite infestation, the artificial swarm contains the principle of separating the old and young bees inherent in the approach of Svoboda (1947). The artificial swarm requires the colony to be manipulated as in Figure 11.1. The old hive is moved from its original site location and a new hive with new comb foundation is placed in the old location and the old queen installed. The old mite-infested bees will fly back to the old site and the new hive will now contain the old queen and the old bees. The new hive will still have a lot of mites available to enter the new bees that will emerge from 21 days later and will result in these new bees having a high prevalence level that will in turn ensure high mite levels later. On the other hand

the old hive, which in these swarming circumstances will have queen cells and a lot of brood, will not have many old bees with migrating mites, hence most of this brood will emerge mite free. This large population of bees that are virtually mite free will break the infestation cycle and get the new colony off to a good start.

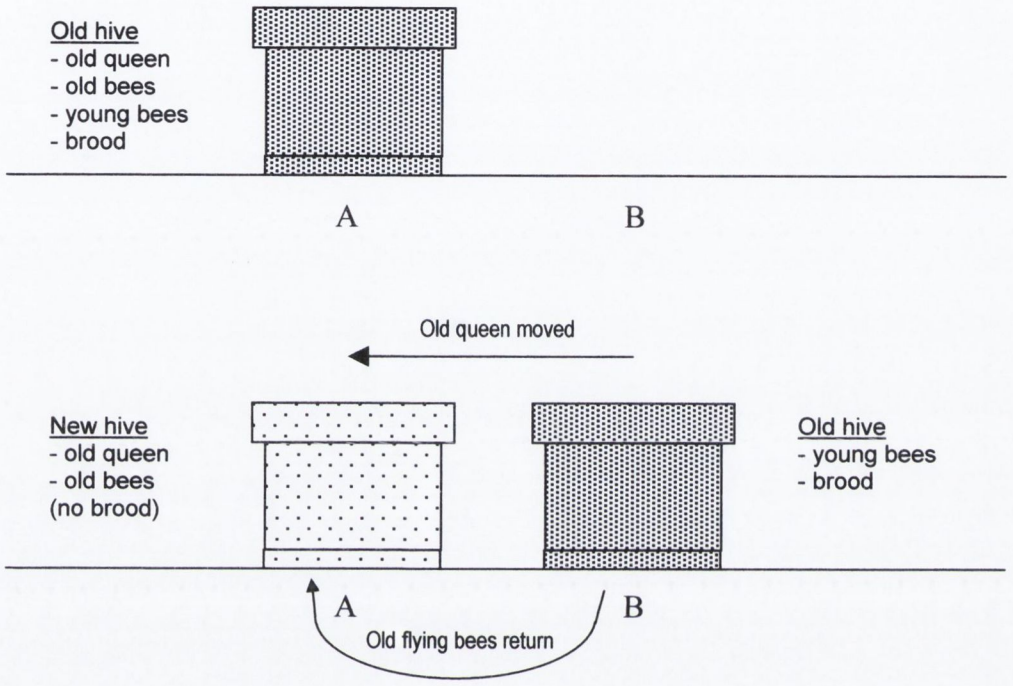


Figure 11.1 Artificial swarm. Top figure shows an infested colony in old hive in position 'A'. Bottom figure shows a new hive in position 'A' with old queen transferred and old flying bees. Old hive moved to position 'B' with young nurse bees.

The artificial swarm method can be 'enhanced' by choosing an 'open' (uncapped) queen cell and moving the old hive after 1 week to the other side of the new hive (by choosing an open queen cell the maximum number of flying bees will be available before the young queen has emerged). This will have the effect of 'bleeding-off' the older (now flying) bees to the new hive and in this way there will be fewer infested bees in the old hive. Hence there will be fewer mites to migrate to the young bees in the old hive that will emerge about 5 weeks later, the offspring of the new mated queen.

Winter wrapping (lagging)

Chapter 10 detailed the association between tracheal mites and temperature and highlighted the role of thermoregulation in the mortality of honeybees. The limited trial that I have undertaken demonstrated that winter wrapping of hives can reduce mortality in colonies with high mite infestation. The wrapping will considerably reduce heat energy loss, thereby enabling a higher late winter/early spring brood temperature with reduced callow bee mite susceptibility and increased spring brood. In addition, the reduced heat loss will require the bees to consume less honey stores and consequently undertake fewer winter cleansing flights resulting in reduced bee losses (Chapter 10). A method of achieving effective wrapping is shown in Figure 11.2. Insulation, such as 25 mm of polystyrene, is cut to size (Figure 11.2, left photograph) and waterproofed by putting inside black polythene bags and sealing / fixing to the hive by adhesive tape (Figure 11.2, right photograph).

Figure 11.2 The photograph on the left shows the wrapping insulation around the hive while the one on the right shows it waterproofed and sealed.

This should be carried out in the autumn and can include insulating inside the hive between the crown board and the roof in addition to insulation of the external walls as described. Winter wrapping is not undertaken in Britain or Ireland but is common in winter climates with severe cold conditions, such as in Canada, as a standard practice for normal healthy colonies. From my limited trials of wrapping I have found a rapid colony buildup in spring, reversing the downward population spiral described in Chapter 10.

The colony manipulation methods described will enable beekeepers to maintain colonies of the native *A. m. mellifera* strain which are indigenous to Ireland while taking specific steps to manage colonies that are susceptible to tracheal mite infestation. Carrying out an artificial swarm will reduce colony infestation while winter wrapping will reduce the incidence of colony mortality of infested colonies. Both methods should be applied on an annual basis as an integrated control approach. Further evaluation of the winter wrapping method is required to perfect the approach.

CHAPTER 12

General discussion and future research

12.1 General discussion

My research investigated the susceptibility and mortality of honeybees infested with tracheal mites. It also considered the basic biology of the parasite and proposed improved beekeeping methods for the management of infested honeybee colonies in the field.

A female tracheal mite (*Acarapis woodi*) leaves the trachea of its old honeybee host and starts on a precarious journey. The outcome of this journey for the young mated female mite will be determined by chance and the ability of its new bee host to resist its migration. To the honeybee *A. woodi* is a parasite, bestows no known benefit and hence it is not in the host's interest to facilitate this migration. The mite is a small animal, with the female having a length of 146 μm and width 74 μm (Chapter 8) and weight of 5.10^{-4} mg (Sammataro and Needham, 1996), about $1/200{,}000^{th}$ the weight of a honeybee. Due to its high surface area to volume ratio the mite is exposed to the dangers of desiccation and this constrains the duration of the migratory journey. The mite will transfer from bee to bee by climbing onto the hair of its old host (Lee, 1963) and then making contact with and transferring to the hair of the new host (Morgenthaler, 1931; Hirschfelder and Sachs, 1952). Only young bees can be infested (Morgenthaler, 1930). This has been the cause of much speculation over the years. At one time it was considered that the age effect was governed by the stiffening of the bee setae surrounding the spiracle permitting mites to enter only in the first few days of a new bee's life. This has since been discounted (Lee, 1963). More recently it has been proposed that migrating female mites use cuticular hydrocarbons to identify young bees (Phelan *et al.,* 1991).

The honeybee's ability to interrupt the hair-to-hair transfer of the mite largely establishes its susceptibility. I demonstrated that the ability to autogroom largely accounts for low susceptibility in honeybees pupated at normal temperature. Bees with a high susceptibility to tracheal mites were correspondingly shown to have no grooming ability. The high susceptibility colonies were of the Northern European dark bee subspecies, *Apis mellifera mellifera,* the predominant subspecies in Ireland. The question arises as to why these bees are poor autogroomers. A distinguishing feature of

this subspecies is its long plumose hair and it is possible that the hair length and texture diminishes its sensitivity to the presence of mites and/or facilitates the transfer of foundress female mites from old to new bee hosts.

The research identified that a reduced pupation temperature had a large and significant effect on susceptibility. The mechanisms that caused a reduction in pupation temperature to effect an increase in the number of mites that successfully transfer to new bee hosts were considered. There was no evidence that the increased susceptibility of bees pupated at a low temperature, in high susceptibility colonies, was influenced by changes in autogrooming while grooming was shown to make a partial contribution to the increased susceptibility in low susceptibility colonies. Hence the increased susceptibility in bees pupated at a low temperature was largely unaccounted for by the bees' autogrooming ability.

I found no evidence that chemical cues acted as a mechanism in the increased susceptibility due to reduced pupation temperature. In two-choice tests the mites had a significantly lower preference for bees washed in hexane compared to unwashed bees (cuticular compounds intact) from high susceptibility colonies raised at both low and normal pupation temperatures, while no preference was expressed in the case of bees from the low susceptibility colony. This may indicate that the bees from low susceptibility colonies (that were less strongly of the *A. m. mellifera* strain and that would have evolved in drier warmer climates) had low levels of cuticular compounds. It was demonstrated that mites must come in physical contact with bees and use gustatory rather than olfactory senses and a new 'hair-contact' method for comparative testing, that is simple to carry out, was identified. This alternative method was adopted after an attempt to use a similar method to that used by Phelan *et al.* (1991) to demonstrate olfactory senses of bees, had failed to elicit any response. This outcome also raised questions about the nature of mite migration. The generally accepted transfer strategy of a new foundress female mite moving from an old to a new bee host, whereby the mite transfers randomly to a passing bee and then tests for suitability, is contested in this research as risky and unnecessary. Pettis *et al.* (1992) demonstrated that most (85%) of the daily mite transfers take place at night. The evidence in this research would support an alternative strategy where the mite would transfer during the inactive period in the

hive after using its gustatory senses to taste the pleural hair of its potential new host bee prior to transferring.

Since cuticular compounds are not implicated in the increased susceptibility of bees pupated at a reduced temperature, it is concluded that the physiology of the bees (other than limited autogrooming as above) is likely to be the critical factor. This would include the texture of the hair, developmental state of the sensory system (Tautz *et al.*, 2003) and the relative mobility of the old and new bee hosts.

The mated female mite in its migratory journey must contend with more variable conditions than the genetic characteristics of its honeybee host. Changes in climate, honey flows and broodlessness can affect conditions in the brood nest particularly the relative humidity. I was interested to investigate the susceptibility response to changes in humidity as I was aware of the concerns of Giordani (1977) in this regard and of low mite infestation level in arid areas (Sammataro, pers. comm.). It was demonstrated that high relative humidity had the effect of reducing the delay time (after mite migration and before egg laying) for the three colonies tested, by about one day relative to the delay time at normal humidity. This was to be expected as there would be less time required for the mites to restore their body fluids in high humidity conditions. However, there was also evidence that a high relative humidity (~65%) produces a strain-related response. Two of the three colonies, from the same breeding stock, had increased susceptibility to the mites with increased relative humidity. The more strongly *A. m. mellifera* colony had a reduction in tracheal mite infestation with increased relative humidity. This colony also appeared to have had a higher stress level as indicated by increased mortality of mites and bees. It is noteworthy that in my research bee strain not only influenced honeybee infestation due to relative humidity, but also the bee's grooming ability and the response of mites to honeybee cuticular compounds.

It is not known when tracheal mites first arrived in Britain and Ireland. We do know that the mite was discovered for the first time in 1919 and there was speculation that changes in brood-cell size in the late 1800s may have influenced the first migration of the mite into the trachea of the honeybee (Erickson, 1990). I showed that honeybee colonies, *A. m. mellifera,* had no difficulty in constructing brood comb and rearing brood in the small-cell brood combs used in the late 1800s. It was demonstrated

experimentally for the first time that there was no evidence that the tracheal-mite susceptibility of bees reared in small-cell brood combs was any different to that of bees reared in standard-sized combs. While giving no direct advantage to honeybees in resistance to tracheal mites, the possibility of a higher bee density in the winter cluster may give it an increased ability to thermoregulate and reduce mortality in the critical winter period. In a comparative study of the morphometry of *A. m. mellifera* bees raised in standard and small cell-sized comb it was demonstrated that, contrary to common belief, the bee size did not change in proportion to the change in cell size. In fact the bee size only changed by a small proportion (10-20%) of the change in cell size compared to an almost proportional change in 'American' honeybee strains reviewed in published papers. The study showed that *A. m. mellifera* is different in many respects to that of other *Apis mellifera* bee strains. The thorax of the *A. m. mellifera* strain was about one third larger than the thorax of the 'American' strains which probably reflects Bergman's rule for the larger North European dark bee raised in the cooler northern climate. It was also demonstrated that the 'fill factor' (thorax width to cell width ratio) for this strain was much higher and this reduced space for the developing bee may give it an advantage in resisting *Varroa destructor* infestation by restricting the reproduction of the mite in the brood cell (Martin and Kryger, 2002; Piccirillo and De Jong, 2003).

If the female mite has been able to navigate the treacherous journey it will eventually arrive at its new habitat, the trachea of the honeybee. If delayed along the way or exposed to low humidity conditions the mite may have had to make an intermediate feeding stop near the wing base of the bee (Hirschfelder and Sachs, 1952). Once safely inside the trachea it will raise its family and eventually die in the same trachea either before or at the same time as its host. There will be a delay time before laying its first egg. In the experiments that were undertaken the mites in the control bees under normal conditions took 1.8 days from migration to the start of the egg-laying cycle and the estimated time from mite migration to the first egg being laid was 2.5 days. The latter compares with a German study (Morgenthaler, 1931) of 3 to 4 days and a US study (Gary *et al.*, 1989) of 1 to 2 days. The mite eggs hatched in 2.1 days which compares to recent US estimates of 4 days (1988), 2 to 3 days (1989) and 5 days (1996). This analysis in Chapter 8 (biology model) also estimated a mite fecundity (egg laying rate) of 1.4 eggs per day, which contrasted with 0.85 eggs per day in a recent US study (1996). I believe that the methodology used in the US study would partly account for

some of the difference as an allowance does not appear to have been made for the initial delay time when calculating the offspring per female mite. Notwithstanding this, the data above indicates a more aggressive mite reproductive rate for the tracheal mites in Ireland than the mites in the other studies. These other studies were undertaken a short time after the mites were first discovered in the study regions which may indicate that virulence has been selected for in the mites in Ireland. There may also be a host effect due to bee strain.

Furthermore, the physical size of the mites in my research were about half that of the mites during Rennie's research in 1920. Reduced physical size is likely to have given the mites an advantage by reducing the likelihood of the honeybee detecting the mite on its hair in the first place as well as making it more difficult to subsequently groom-off a migrating mite. The physical appearance of the mites has also changed since the 1920s. It may be that size has been selected for in the mite as a possible strategy to redress the balance in the host–parasite relationship as levels of mite infestation have reduced in Britain and Ireland since 1906 and particularly from the 1960s to the present day.

It was established that the female mites, after arriving in their new host bee, had similar egg laying rates (fecundity) for all the experiments irrespective of the treatments that were applied. Hence, susceptibility of the bees to tracheal mite infestation was determined by the success rate of migrating foundress female mites entering the tracheae. While this confirmed recent findings by others in relation to grooming treatments, I confirmed the similarity of fecundity across a number of treatments and applied the approach of measuring susceptibility in terms of female mite abundance rather that by a total-mite (all stages) abundance. The approach also used an 'adjusted' female mite abundance by including additional female mites, as appropriate, where offspring was present without accompanying mother mites. By concentrating on establishing the presence of the primary agent (female mites) and making an allowance for female mites that were obviously present but not detected, the approach improved accuracy and reduced the volume of data to be analysed to measure honeybee susceptibility to mite infestation.

The experiments offered the opportunity to study honeybee biology at a reduced incubation temperature. Pupation at a reduced temperature (30°C) did not affect the

level of emergence of the callow bees from the brood combs or the subsequent mortality of these target bees in the inoculation cages when compared with those pupated at a normal temperature. Hardiness at this lower temperature would be expected from honeybees that were predominantly of the *A. m. mellifera* strain, that had evolved in the cool damp climate of North West Europe. Modelling emergence time vs. pupation temperature gave a critical pupation temperature of 24.9°C for worker bees, below which no development of the brood takes place. The model also predicted a pupation period of 12.9 days for brood pupated at the normal temperature of 34°C, suggesting a full development time for the worker bees of 20.9 days, which is in line with expectations.

The infestation model (Chapter 9) identified four factors that had a significant effect on female mite abundance. Three of these factors made similar contributions over their range of values. The female mite abundance was predicted to increase (i) with increased inoculation pressure, as defined in the chapter, (ii) with reduced bee pupation temperature and (iii) in springtime relative to autumn. However, the dominant influence on female mite abundance was the strain of bee as determined by the value of discoidal shift from venation analysis of the bee's forewing, where increasing negative values were associated with increasing abundance. Thus the predominant strain in Ireland, *A. m. mellifera,* which was the indigenous honeybee in North West Europe at the time of the 'Isle of Wight' disease has been identified as susceptible to tracheal mites. It was shown in an idealised model that mite populations are capable of rapid increases if suitable conditions prevail. A single mated female mite in a colony can potentially result in a female mite population of over 20,000 at the end of a 12-week period.

So far the journey of a foundress female mite from its old to its new host and raising a family within the honeybee trachea has been considered. But the tracheal mite would most likely have never been discovered if it had not been associated with honeybee damage, specifically the widespread deaths of bees in Britain and Ireland in the early 1900s. There has never been a consensus on the cause of the Isle of Wight disease and conflicting opinion is rife (Adam, 1968; Bailey, 1964). There is still little insight or theories on whether colonies die due to infestation by tracheal mites. The exception is the often expressed view of Dr Bailey that tracheal mites do not kill bees but that chronic paralysis virus (CPV) provides the clinical symptoms and caused the death of

bees during the period of the 'Isle of Wight' disease and is responsible for the death of colonies infested with tracheal mites today (Bailey, 1964, 1975, 1985, 1999 and 2002). Bailey and Ball (1991) conclude that in relation to tracheal mite infestation in colonies, "its only significant pathological effect being to shorten very slightly the life of bees, but usually causing no obvious sickness in spite of the abnormal appearance of infested tracheae". This contrasts with the experience of the tracheal mite infestation in the US. The mite had been detected in Central America in the 1970s and, after intensive sampling in the US over an extended period, tracheal mites were discovered for the first time in the US in 1984, followed by widespread colony losses (Delfinado-Baker, 1988). The research into mortality in Chapter 10 demonstrated how a colony with a strong tracheal mite infestation can die out in the late winter/early spring period. This is the first time in a published work, to my knowledge, that a tracheal-mite infested colony has been studied on a daily basis over the last 6 months of its life. This was a strong colony that had foraged well throughout the year and during the autumn ivy flow and had no clinical signs of disease. There was evidence that infested bees in the winter cluster were restless, consumed above average levels of honey stores, had wider fluctuating temperature levels and maintained higher minimum temperature levels in the broodless hive than a non-infested colony. There was an inability to keep the temperature of the hive at the normal level at the start of the new brood season in late winter. It was also demonstrated that bees die outside the hive when attempting to defecate on the wing and it was also established for the first time that the presence of sunshine (and not other factors such as temperature) triggered the bees to attempt these cleansing flights. The bees that were crawling on the grass and dying in the late winter afternoons, when collected and caged in an incubator, lived for many weeks and were shown to eventually outlive their sisters in the hive. I concluded that bees can die in the late spring/early summer period as a result of high levels of tracheal mite infestation. The research suggests that mortality is due to infested colonies' inability to thermoregulate and a comprehensive model to explain this process was developed.

The evolutionary history of the tracheal mite, *Acarapis. woodi* is still unknown. Eickwort (1988) speculated that the mite evolved from predatory mites that had originated in the honeybee-nest detritus and eventually transferred to the bees. It was further speculated that the tracheal mite evolved around 1900, in England, from one of the closely related external species (De Jong, *et al.,* 1982). Jeffree (1955, 1959)

considered that temperature limited the distribution of *A. woodi* and that it could survive in only a few places outside Britain and Ireland. I believe that his conclusions came from the fact that the mite was only detected in these locations as they were found as a result of the follow-up to mortality in honeybee colonies in these regions. In fact it is likely that Jeffree was monitoring colony mortality due to mite infestation rather than colony infestation. Colony mortality would not be expected in regions with tropical climates where thermoregulation is not a critical task. It is known that tracheal mite infestation can persist in bee colonies in regions with warm climates without killing the bees (Otis and Scott-Dupree, 1992). The apparent tolerance of honeybee colonies in Africa to mites may be due to lower mite infestation (resulting from good grooming ability that evolved from exposure to many mite types over time, and reduced contact between young and old bees through reduced clustering) and reduced colony deaths where clustering is not required to thermoregulate. Could the 'Isle of Wight' disease have resulted from exotic mites transferring to the Isle of Wight and living for the first time in vulnerable colonies exposed to damp, cold wintering conditions? Several colonies were reported to have been imported from France or Switzerland a few years prior to the outbreak on the island (Adam, 1968). Could these bees have originated in one of France's African colonies?

Finally, my research identified non-chemical techniques that the beekeeper can apply in the field to manage tracheal mite infested colonies. Requeening the colonies with a local queen selected from a strain with a high discoidal shift (> -1.0) will provide a long-term solution. This would, however, mean that the beekeeper would move away from the *A. m. mellifera* native strain that has desirable qualities that have been selected for in a damp temperate climate such as in Ireland. Two techniques are proposed that can be used on an annual basis to give control while retaining the native strain. First, an artificial swarm method will reduce susceptibility and second, winter wrapping the colonies will reduce mortality. The combination of these two techniques should provide a good level of protection to native Irish bees from infestation.

12.2 Future research

- My research identified autogrooming as the dominant factor in reducing honeybee susceptibility to tracheal mite infestation in colonies that are normally pupated. However, it did not identify the specific reason for high or low grooming ability in honeybees. The role of pleural hair length and of hair-sensory sensitivity should be investigated. The former can be done using the frozen target bee samples stored from the experiments already undertaken. Since autogrooming ability in the bees is colony dependent, repeating the experiment in Chapter 4 with the test colonies segregated on the basis of high and low susceptibility would provide additional information. This would offer a much better statistical basis on which to evaluate behaviour, particularly in the case of low susceptibility colonies.

- An analysis of the comparative levels of the cuticular compounds in the bees from high and low susceptibility colonies and from normal and reduced temperature pupated bees should be undertaken. This would enable the establishment of any chemical basis that may be present for the behavioural traits in the mite preferences and give greater certainty to the conclusions that are being drawn. It may also open up further lines of enquiry. Analysing the chemical compounds on the hair and cuticle separately would help to strengthen the formulation of an alternative mite migration strategy. The new 'hair-contact' two-choice approach to the study of relative attractiveness of bees to tracheal mites should be used to investigate the role of cuticular compounds in the preference of mites to migrate to young callow bees and these should also be related to the chemical profiles of the cuticular compounds as above.

- The tracheal mites that I was working with had a different appearance and different physical dimensions than those that were discovered by Rennie's team in 1919 and to those that infested colonies in the US from 1984 onwards. The reproductive characteristics of the Irish and US mites were also different. (No mite reproductive data is available from Rennie's period). An interesting evolutionary question arises as to the changes in the indigenous mites in Ireland

and Britain over the last 100 years. A separate evolutionary question arises in the case of the US mites which, it would appear, left Britain and Ireland in the early 1900s and, moving globally in a clockwise direction via mainland Europe, Asia, Africa and South America, eventually reached North America in the late 1900s. These mites present an interesting subject for the study of evolution in a species and it would be proposed that the study includes the phenology of *Acarapis woodi* using samples from the US.

- The impact of relative humidity on the behaviour of infested colonies has never previously been addressed to my knowledge. Repeating the experiment with test colonies segregated on the basis of high and low susceptibility strains should redress the limited data in the experiment, particularly in the case of the high susceptibility colony.

- The North European dark bee, *A. m. mellifera*, which was globally one of the major honeybee strains in the nineteenth and early part of the twentieth century has since lost much of its popularity as a commercial strain. It was also one of the most studied strains in this period, particularly in Germany. Further work should be undertaken to analyse its special morphometric characteristics particularly its potential to restrict *Varroa destructor* mite reproduction in the brood cell. Work by Piccirillo and De Jong (2003) indicated that small cells reduce *Varroa destructor* mite production in Africanised colonies. Also Martin and Kryger, (2002) showed that restricted space for the developing new bee in the brood cells of African honeybees resulted in reduced *Varroa destructor* mite reproduction. The 'fill factor' (thorax width to cell width ratio) was much higher in the *A. m. mellifera* strain than in other strains studied and higher still when small-cell brood comb was used. Further work should be undertaken to confirm if this potential benefit of using small- cell brood combs to control Varroa mite infestation obtains in the field.

- The limited investigation of the non-chemical techniques to control tracheal mites requires further evaluation. Trials should be undertaken on the artificial swarm and winter-wrapping methods over a large number of colonies to confirm their efficacy and to refine the application.

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Publications and presentations

Publications

McMullan J.B. and Brown M.J.F. (2005). Brood pupation temperature affects the susceptibility of honeybees (*Apis mellifera*) to infestation by tracheal mites (*Acarapis woodi*). *Apidologie* **36:** 97-105.

McMullan, J.B. and Brown, M.J.F. (2006). The role of autogrooming in the differential susceptibility to tracheal mite (*Acarapis woodi*) infestation of honeybees (*Apis mellifera*) held at both normal and reduced temperatures duration pupation. *Apidologie* **37:** 471-479.

McMullan, J.B. and Brown, M.J.F. (in press). The influence of small-cell brood combs on the morphometry of honeybees (*Apis mellifera* L.). *Apidologie* (accepted 31/01/06).

McMullan, J.B. and Brown, M.J.F. (2006). Brood-cell size does not influence the susceptibility of honeybees (*Apis mellifera*) to infestation by tracheal mites (*Acarapis woodi*). *Experimental and Applied Acarology* **39**: 273-280.

Presentations

- An oral presenation to the winter meeting of the British Section of IUSSI in Trinity College Dublin, December 2003 entitled, "Interactions between the honeybee, *Apis mellifera*, and the tracheal mite, *Acarapis woodi*".

- A poster presentation at the European Conference of Apidology 'EurBee' in Udine, Italy in September 2004 entitled, "Infestation dynamics of the tracheal mite *Acarapis woodi* in honeybees".

- An oral presentation at the 39th Apimondia International Congress Dublin in August, 2005 entitled, "The influence of small-cell brood combs on the morphometry of honeybees (*Apis mellifera*)".

- A poster presentation at the 39th Apimondia International Congress Dublin in August, 2005 entitled, "Mortality of honeybee colonies infested with tracheal mites (*Acarapis woodi*)".

- A paper presented at the 2005 Annual Conference of the Central Association of Bee-Keepers in Stratford upon Avon in November, 2005 entitled, "Tracheal mite (*Acarapis woodi*) infestation in honeybees - some recent research".

Appendix 1: Summary of source of bees used in the experiments

The experiments were undertaken over the period March 2003 until February 2006 with bees taken from colonies in North County Dublin (Table A1.1).

Table A1.1 Summary of source of bees used in the experiments

Chapter No. Experiment	Year (Month)	Colony	Apiary ( )[1]
3. Pupation temperature	2003 (March/April)	1	EA[2] (2)
		2	AA[3] (1)
		3	ST[4] (1)
4. Grooming	2004 (April/May)	1	EA (1)
		2	PE[5] (1)
		3	ST (8)
5. Chemical cues[6]	2005 (November)/	1	EA (1)
	2006 (February)	2	PE (1)
		3	ST (8)
6. Humidity	2004 (October)	1	AA (1)
		2	ST (8)
		3	ST (1)
7. Cell size	2005 (April)	1	ST (4)
		2	ST (9)
		3	AA (2)
	2003 (September)	1	ST (4)
		2	ST (9)
		3	AA (2)

Note:

[1]Colony number in apiary.
[2]Apiary EA: Urban area, coastal.
[3]Apiary AA: Mixed urban/rural area 3 kilometres from the coast.
[4]Apiary ST: Rural area 7 kilometres from the coast.
[5]Apiary PE: Mixed urban/rural area 2 kilometres from the coast.
[6]Stored target bees were used from the grooming experiment in Chapter 4.

Appendix 2: Thermoregulation in a healthy colony during winter

Healthy colonies of *Apis mellifera mellifera* honeybees thermoregulate the brood nest in the late winter/early spring period and maintain high minimum temperatures even during harsh weather conditions. A typical example of this regulation is given below for temperature readings taken in one of my colonies during the year 2000.

Daily readings of minimum temperature in the hive are shown in Figure A2.1 along with the corresponding readings of minimum ambient temperature. The temperature differential being maintained by the colony in the brood nest is shown and it can be seen that this reaches 40°C during a frosty period in late February. During this period the minimum hive temperature is kept at over 34°C.

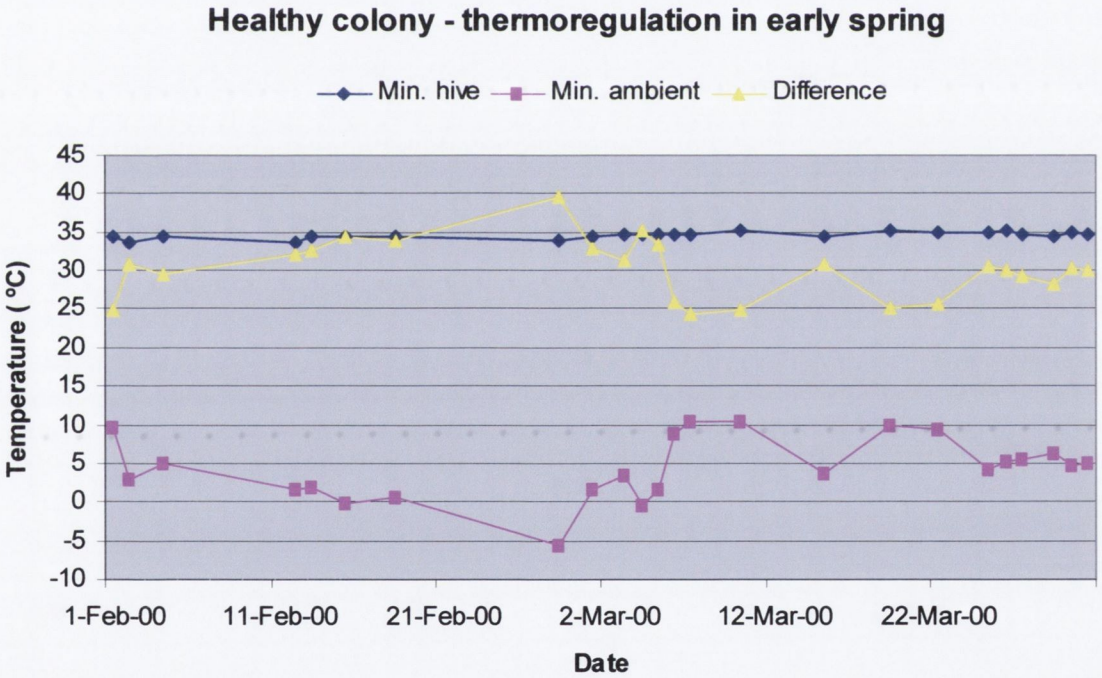

Figure A2.1 Thermoregulation in a healthy colony during winter conditions showing high temperature differential between brood nest and ambient.