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The effect of pathogens on honeybee learning and foraging behaviour

By

Emma Wright

A thesis submitted for the degree of Doctor of Philosophy

University of Warwick, School of Life Sciences

And

Rothamsted Research

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Declaration:

I declare that this thesis and the research that is being contained therein is the sole work of the author and that none of this work has been presented for another degree. If the author collaborated with colleagues, used or adapted methodologies originally established by fellow academics, this is fully acknowledged in the relevant part of the text.

Abstract

The European honeybee, *Apis mellifera*, is important economically not just for honey production but also as a pollinator. Bee pollinated plants contribute towards one third of the food eaten worldwide. However, honeybee numbers in some areas are declining. A range of interacting factors are thought to be involved, including pathogens and parasites, loss of forage, pesticide use, bad weather, and limited genetic variability. Pathogens are also known to cause changes in the behaviour of their hosts and these premortality and sublethal effects of disease may well play a role in colony declines and are the focus of this thesis.

For individual bees the fungus *Metarhizium anisopliae* was used as a model pathogen and RT-Q-PCR was used to detect and quantify naturally occurring pathogens. In field colonies the level of infestation of the parasitic mite *Varroa destructor* was modified as a surrogate for disease load as the amounts of many viruses correlate with mite levels.

Survival experiments showed that both disease load and forage availability had an effect on honeybee longevity and feeding the bees pollen increased their survival. Learning experiments showed that both the fungus and some of the bees' naturally occurring pathogens caused changes in the learning ability of young adult and older forager bees. Young adult bees were better able to learn when infected with the fungus, possibly because it made them more responsive to the sucrose stimulus, whilst older forager bees where less able to learn when infected with the fungus. Harmonic radar was used to show that honeybee flight ability was affected by naturally occurring pathogens, especially deformed wing virus which caused bees to fly shorter distances and for shorter amounts of time than uninfected bees. Observation hives were used to study in-hive behaviour showing that bees with more pathogens were likely to start foraging earlier than healthier bees.

Abbreviations

ABPV	Acute Bee Paralysis Virus
AFB	American foul brood
ANOVA	ANalysis Of VAriance
BQCV	Black Queen Cell Virus
CBPV	Chronic Bee Paralysis Virus
CCD	Colony Collapse Disorder
CI	Confidence Interval
CPE	Conditioned Proboscis Extension
CSD allele	Complementary Sex Determining allele
C_t	Cycle Threshold
DEFRA	Department for Environment and Rural Affairs
DWV	Deformed Ring Virus
EFB	European Foul Brood
FERA	Food and Environment Research Agency
FRET	Fluorescence Resonance Energy Transfer
IAPV	Israeli Acute Paralysis Virus
IPA	Industrial Partnership Award
JH	Juvenile Hormone
KBV	Kashmir Bee Virus
LPS	LipoPolySaccharides
LT50	Lethal Time 50
NASS	National Agricultural Statistics Service
NBU	National Bee Unit
PER	Proboscis Extension Reflex
PO	Phenyloxidase
REML	REstricted (residual or reduced) Maximum Likelihood
SBV	Sac Brood Virus
SDA	Sabouraud Dextrose Agar
Q-RT-PCR	Quantitative – Real Time – Polymerase Chain Reaction

Chapter 1: General introduction

1.1 Bees:

1.1.1 Taxonomy:

Bees belong to the order Hymenoptera and honey bees to the family Apidae. The species most commonly used for honey production in Europe is the European Honeybee; *Apis mellifera*, the name coming from the Latin meaning 'honey bearing bee' (Linnaeus, 1758). There are several subspecies of *A. mellifera* including: *A.m. iberica, A.m. mellifera* (native to Britain), *A.m. carnica* (the Carniolan honeybee) and *A.m. scutellata* (the African subspecies used to breed Africanized honeybees). Due to its nature and honey production an Italian subspecies 'ligustica' is currently most favoured although other subspecies are sometimes used. This species was chosen because it is generally a good housekeeper, prolific, a good forager, uses little propolis and shows less tendency to swarm than other subspecies (Hooper, 2008). The honeybee genome sequence was published in 2006 (Weinstock *et al.*, 2006) making it a good model organism for linking experimental results to genetics. The honeybee has been used as a model system for studying immunity, allergic reaction, antibiotic resistance, development, mental health, longevity and diseases of the X chromosome (Reviewed in Dearden *et al.*, 2010).

1.1.2 The Colony:

A typical honeybee colony can contain as many as 80 000 bees at the height of summer although this number decreases dramatically over winter when the bees over winter; relying on the warmth of a cluster of individuals to survive the cold weather. A minimum of about 10 000 bees is required to maintain the temperature required; if brood is present in the hive then the centre of the cluster must be 35^{o}C whilst the edges may be only 6-7^{o}C (Waring, 2006). In cool weather the temperature within the hive is increased by activating the thoracic muscles to produce heat. In hotter weather, however, the bees collect water which they fan with their wings to evaporate, thereby cooling the temperature of the hive (Tautz *et al.*, 2003). Honey must be stored to fuel the colony which usually requires between 200-300 grams per week. 15-20kg of stored honey should last the colony not only through the cold weather but more importantly through the colony's restart the following spring (Waring, 2006).

Honeybees are social insects with a haplo-diploid genome. This means that whilst the females are diploid with two copies of each of their 16 chromosomes the males are haploid;

developing from unfertilised eggs and having only one copy of each chromosome. Sex determination is controlled by complementary sex determining (CSD) alleles. If these are inherited heterozygously a female is produced, if inherited homozygously (or singly as in the case of haploid males) the embryo develops to be male. Diploid males are produced if the queen mates with a male having the same CSD alleles as she does. These offspring do not survive to maturity (Charlesworth, 2008).

It was originally assumed that this haplo-diploidy favoured altruism within the colony. If the queen mates with one male drone then each worker is more closely related to her sister workers (75%) than any nephews (haploid drones produced by other workers) (35%). Hamilton's equations (equations that can be used to predict the likelihood that a certain behaviour or attribute will evolve within a population based on the theory of inclusive fitness) (Hamilton, 1964) show that this close relatedness should favour the evolution of altruism. However, the queen mates with up to 20 different males and therefore workers are only likely to be 30% related to each other (Ratnieks and Wenseleers, 2008). However, workers in the colony continue to act altruistically leaving the queen as the only reproductive female.

Ratnieks (2008) suggests that this is due to coercion rather than altruism. Using Hamilton's equation (Hamilton, 1964) it can be shown that if the queen mates with ten males, then 54% of workers should theoretically benefit from reproducing. However the actual number reproducing is far lower than that, between 0.01 and 0.1%. The coercion in queen-right (ie a colony with an actively laying queen) honeybee colonies takes the form of queen substance, a pheromone produced by the queen that, amongst other things, reduces the worker's propensity to lay and encourages policing. Ratnieks (1993) examined a queen-right colony and found that, on average, one egg was laid by workers per day per 16000 drone cells. Of these 85% were removed within a day and only 2% hatched. In queenless colonies around 40% of workers lay, supporting the idea that it is coercion not altruism that maintains the social structure of the colony (Ratnieks and Wenseleers, 2008).

1.1.3: The Queen:

A honeybee queen is the largest of the bees in the colony being on average 2-3cm in length. She has fewer individual eyes, or ommatidia, in her compound eyes, a short proboscis and toothed mandibles (Waring, 2006). When laid, the queen and worker eggs are indistinguishable, but queen eggs are laid within 'queen cups', shallow cells usually near the base of the comb. Workers then build the cell down around the growing larva. As the larva grows it is fed on 'royal jelly' and it is this rich food source that allows it to develop

into a queen. The queen takes about 16 days from laying to emergence and a week after that is mature enough to mate (table 1.1). The queen may fly one, two or three times and mate with up to 20 males storing their sperm in her spermatheca. The queen then remains in the colony for the rest of her life unless she swarms or is disturbed (Hooper, 2008).

The *A. mellifera* queen produces several pheromones. The pheromone produced by the queens mandibular gland, 'queen substance', prevents the workers from laying their own eggs (Butler *et al.*, 1959). This ensures that every individual in the colony comes from the queen. At the height of summer this means a single queen may lay as many as 3000 eggs a day (Waring, 2006). Another of the queen's pheromones encourages those workers near her to turn to face her. The surrounding workers will then feed, clean and generally care for all her needs allowing the queen's attention to be focused solely on laying (Hooper, 2008). Queen bees have been known to live for up to eight years. These long-lived individuals usually run out of their stored sperm long before this however, and most queens are replaced after three to four years (Waring, 2006). This occurs when a virgin queen is raised to take over from the old queen. In most cases when virgin queens are ready to hatch the colony will swarm. The old queen leaves the hive with several thousand workers to set up a new colony. The hatching virgins can either take workers from the colony, if it is big enough, and swarm to set up a new hive themselves, or kill off any other emerging queens and take over the existing colony. If the old queen is superseded, however, one new virgin queen will emerge, go on her mating flight and return without the colony swarming. Any other queen larvae being raised at the time will either be killed by the emerging virgin queen or by the workers. The old queen usually leaves (or may be killed) but on some occasions the two queens may be seen laying together for some time. Thus the main difference between 'supersedure' and swarming is that no new colony is formed when the queen is superseded (Butler, 1957).

Most of the eggs a queen lays will become workers. These are laid in hexagonal brood cells. Wider cells are used for drones and the queen can differentiate between these with her antennae. Drone production within the colony is likely to be controlled by both workers and queen. The workers having control over the number of larger drone cells produced and the queen having control over the laying of the eggs (Wharton *et al.*, 2007). Queen eggs are laid in queen cups but if the colony's queen is lost the workers may pick a worker egg or young larvae to feed up on royal jelly allowing the brood originally intended to be a worker to develop into a new queen. If the worker larva is too far developed however she will not develop into a good queen (Hooper, 2008).

Table 1.1:

Day	Queen	Worker	Drone
1	Egg laid	Egg laid	Egg laid
3	Egg hatches	Egg hatches	Egg hatches
6		Diet change	Diet change
9	Sealed	Sealed	
11			Sealed
12	5th (final) moult		
14		5th (final) moult	
16	Emerge		
18			5th (final) moult
21	Mature		
	Mates		
	+1 week. Lays		
22		Emerge	
25			Emerge
30		Fly	
36		Mature	
38			Mature
			+3 weeks sexually active
42	Too old to mate		
Life expectancy	2-3 years (+8)	+ 2-3 weeks in summer	
		+ Several months in winter	Driven from colony before winter

Table showing honey bee early development adapted from (Waring, 2006 p20).

Much work has been undertaken to examine reproductive conflicts within the honeybee colony (eg. Trivers and Hare, 1976) and it has been determined, by use of Hamilton's equation (Hamilton, 1964), that for workers, the optimum sex ratio is 3:1 in favour of female (especially young queen) production whilst for the queen the optimum sex ratio is 1:1 (Trivers and Hare, 1976). Trivers and Hare (1976) examined colonies of ants and found

there to be a significant female bias with on average 64% of reproductive offspring produced being female. This is less than the 75% predicted if the workers are in full control but also more than the 50% predicted if the queen is in full control.

Once the larva reaches a certain size the cell is capped, i.e. sealed over with wax. The larva will continue to develop, eating stored food, until ready to pupate. At this point the larva orientates itself by finding the rough end of the cell so that its head is towards the capped end of the cell. The inside of the cell in polished smooth before eggs are laid so that when capped the capped end will be rough. (Hooper, 2008) The timetable for bee development is shown in table 1.1.

1.1.4 The Workers:

The worker caste are the smallest adults within the colony usually being less than 2cm in length. They have smooth mandibles and a long proboscis; on average 6.5mm. These morphological attributes enable the workers to carry out the 'work' of the colony (Waring, 2006). When a worker first emerges she will remain within the hive and carry out tasks there including the cleaning, trimming, capping and polishing of cells, storage of nectar and pollen, feeding larvae and drones and attending to the queen. Some of these young bees may act as undertakers, removing dead bees from the hive. They may also act as guards, stationed at the hive entrance. A guard bee will attack violently if approached by an aggressor, for example by wasps or bees from other colonies attempting to gain entrance to the hive and its stores (robbing).

Sometimes, however, a worker bee from another colony may be confused and try to enter the wrong hive. In these cases the lost bee will not behave aggressively; it acts as if it has entered its own colony. In fact it acts submissively, offering the honey or nectar it carries and dropping its head. The guard bees mob the unfamiliar bee but are not particularly violent. Over time the continued mobbing leaves the lost bee smelling like the new colony and it can soon pass unharmed. The lost bee may now join this new colony. This process is called drifting (Hooper, 2008).

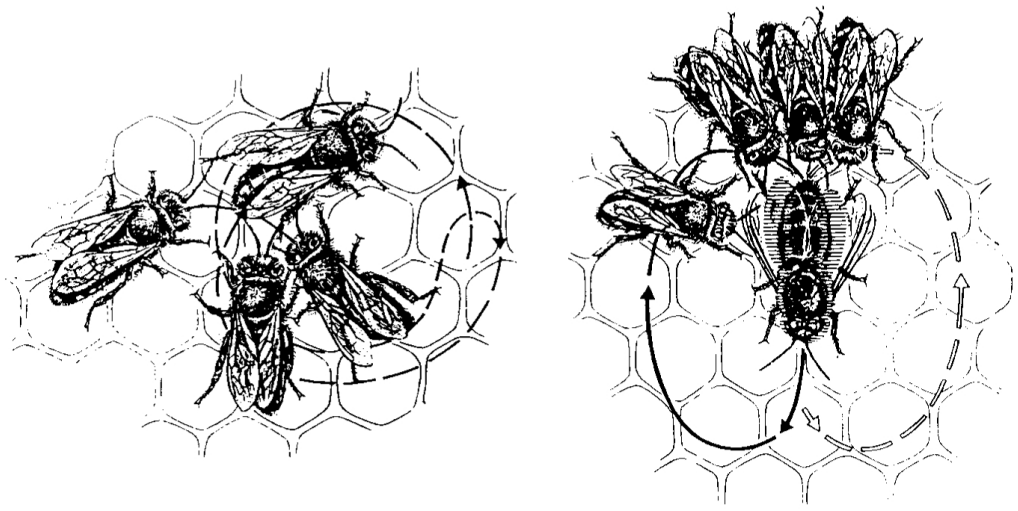
A worker bee will only remain in the hive for the first 2 or 3 weeks of her life. After this she will take a trial or 'play flight' near the hive. This allows the worker to learn the hive's location in preparation for longer flights whilst also testing the bee's wings. The bees use visual cues to identify their hive, beekeepers can paint their hives to make them more recognisable and reduce the chance of bees drifting into other, nearby colonies (Komissar, 1993). Older worker's brood food glands shrivel and cease to function and they become

foragers. These bees collect the resources required by the colony including water, pollen and propolis. The propolis is made from resinous material taken from tree buds and is used as a sealant within the hive. Propolis also has antimicrobial activities. The honeybee hive is warm, humid and provides the perfect conditions for fungal and bacterial growth. Propolis is used to keep microbial growth under control (Silici *et al.*, 2005). The foragers also collect nectar which will be turned into honey. The nectar is mostly comprised of sugars dissolved in water but the high water concentration of nectar (30-40%) means that it cannot be stored as bacteria and fungi would grow within it and contaminate it. If the water content is decreased, however, it can be stored. The bees achieve this by regulating the humidity within the hive by fanning which allows the water in the nectar to evaporate (Hooper, 2008). Honey therefore has a water content of 18% or less. In the UK it is illegal to sell honey containing more than 20% moisture (Waring, 2006). It has been found that the sugar concentration of the nectar is increased during foraging and the return flight to the nest. Nicolson *et al.*, (2008) tested the concentration of nectar in honeybee crops and found that not only was this higher than the nectar from the plant itself but also that the concentration increased between leaving the flower and returning to the hive. It is thought that this is achieved by regurgitating the liquid onto the tongue to evaporate off some of the water (Nicolson and Human, 2008). Further evaporation occurs within the hive where the nectar droplet is placed at the top of the cell and fanned with the wings. The enzyme invertase is added to the nectar and converts the sucrose to fructose and glucose (Gordon, 1980). Ruizargueso *et al.*, (1975) determined that there may be bacterial activity involved in the ripening of honey. They discovered the presence of *Lactobacillus* and *Gluconobacter* within samples of ripening honey. When added to sugar syrup, the bacteria produce lactone and change the pH of the syrup to an acidity similar to natural honey.

Some foraging bees act as scouts to find new sources of these resources. To pass on the information they use several dances the most well-known of which are the round dance and the waggle dance (figure 1.1). If the resources are near to the hive, some spilt honey for example, the scouting bees will rotate in ¾ inch circles stopping every so often to offer a sample of the resource to gathered bees. This dance is only used if the resource is within 50m of the hive and although it has been found to encode directional and distance information this information is not precise to encode directional and distance information this information is not precise (Griffin *et al.*, 2012). If the resource is further away a waggle dance is used. This dance consists of a repetitive figure of 8 motion, the abdomen is 'waggled' rapidly as the bee walks the centre of the pattern. The vigour of the dance indicates how rich the source is whilst the number of waggling runs in a set time determines the distance of the source from the hive. Finally, the direction of the source is also given. The central waggling part of the walk is angled so that the degree of rotation from vertical is

equal to the degree of deviation from the direction of the sun (Couvillon, 2012). For example if the bee walks directly up the face of the hive then the resource can be found directly towards the sun. If the bee walks down the hive then the resource is in the opposite direction and so on. The bees have polarised vision allowing them to accurately detect the direction of the sun even through cloud cover and near its zenith. It was Karl Von Frisch who first determined the meaning of the waggle and round dances (von Frisch, 1974). He even determined that the bees must have polarised vision capabilities long before this was proved physiologically (von Frisch, 1974). He was eventually rewarded with a joint Nobel Prize for his efforts.

Figure 1.1:



a. Round dance

b. Tail-wagging dance

Figure taken from Karl von Frisch's 1973 Nobel lecture showing the 2 forms of dance used by foraging bees to transmit information to other workers. From Nobel Lectures, Physiology or Medicine 1971-1980, Editor Jan Lindsten, World Scientific Publishing Co., Singapore, 1992

In addition to the waggle and round dances Seeley (1992) also identified a 'Tremble dance' in which a returning forager who is unable to pass her load onto another worker shakes her body back and forth, at the same time rotating her body axis by about 50-degrees every second or so, all the while walking slowly across the comb. This can last for 30 minutes and is thought to mean that the forager has found a rich food source but the colony already has enough supplies coming in to keep the workers busy.

Over winter the worker bees are subtly different from their summer sisters. The winter bees are produced in late autumn and unlike other workers their brood food glands remain active for their entire life. Whilst the summer workers may only live for about six weeks on average; the winter bees may live for several months.

1.1.5 The Drones:

Finally the male drones; these are bigger and 'dumpier' than the workers, unable to sting, with very big eyes and long antennae used when searching for virgin queens to mate with. They have only a short proboscis and must be fed by workers (Waring, 2006). many researchers believe that they do little more than mate with the queens. In good weather, the males leave the colony and gather together to wait for passing queens to mate with. It does seem, however, that colonies without drones tend to fare worse than those with. It may be that only better faring colonies can produce drones or the drone may have some as yet unknown function within the hive. It has been suggested that they are important for temperature regulation (Kovac *et al.*, 2009). However at the end of the summer when the colony is preparing to hibernate any remaining drones are evicted from the hive (Hooper, 2008).

1.1.6 Economic value:

The honeybee, *Apis mellifera*, is an ecologically and economically important species across the world. Products taken directly from managed bee colonies include honey, wax and propolis. Honey production in the UK was valued at £10 - 35 million annually (Burr, 2009). However, honeybees are most important as crop pollinators. The honeybee is a good pollinator because of its manageability and large forager populations (Benjamin and McCallum, 2008). In the UK a recent Defra report valued honeybee pollination at almost £200 million annually (Burr, 2009). In the USA honeybee pollination of crops such as almonds and oranges has been valued at over $14 billion per annum (Morse and Calderone, 2000) and worldwide the figure may be as much as $75 billion (~£50 billion) annually (Swinton *et al.*, 2007). In fact, bee pollinated crops could contribute up to 35% of global food production (Klein *et al.*, 2007).

1.2 Colony losses:

Recently there have been several reports suggesting that there has been a massive decline in honeybee numbers especially in the USA and parts of Europe including the UK (Neumann and Carreck, 2010; Potts *et al.*, 2010b; vanEngelsdorp *et al.*, 2011). A lot of media attention has been drawn to dramatic honeybee losses (eg, guardian: http://www.guardian.co.uk/environment/2008/aug/12/conservation.wildlife1). Some honeybee colonies die each year due to parasitism and disease, poor management or starvation if insufficient pollen and nectar are collected over the summer to provide winter food stores

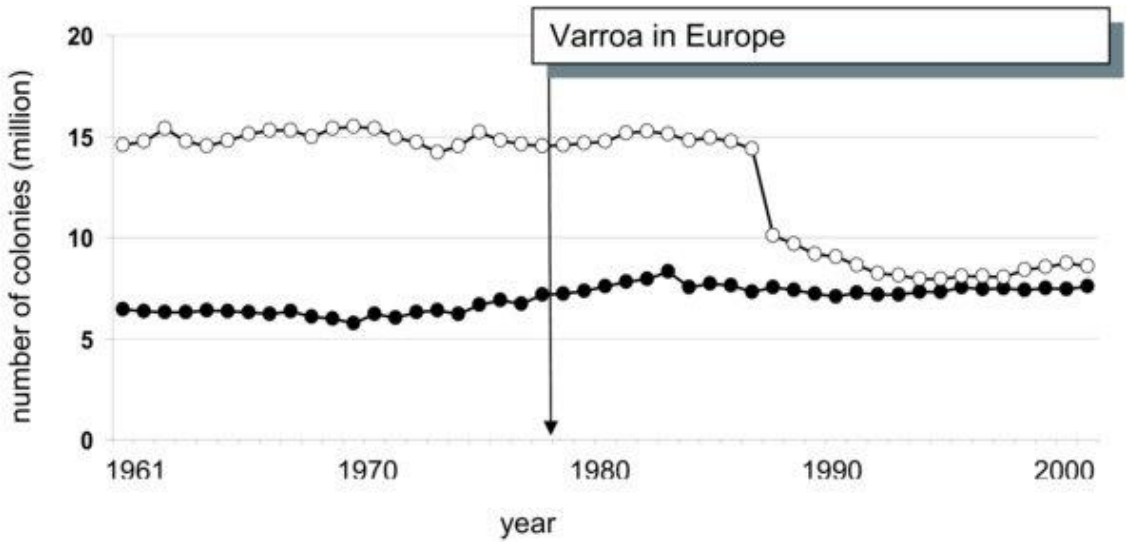
(Hooper, 2008). However, occasionally large numbers of colonies die for apparently unknown reasons.

In the USA honeybee losses have been estimated at around 30% in recent years compared to previous losses of only 5-10% per year (vanEngelsdorp *et al.*, 2008). A long term survey of US honeybee numbers carried out by NASS (National Agricultural Statistics Service) (2008) shows that the trend in declining honeybee numbers is not a new thing as numbers have fallen by more than 50% over the past 50 years. In Europe, a recent paper by Potts *et al.*, (2010b) concluded that honeybee numbers are also declining across much of Europe. Across central European regions, including the UK, honeybee numbers fell by approximately 25% between 1985 and 2005, and in all countries examined the number of beekeepers had also decreased.

The causes behind colony losses in the UK and elsewhere are currently unknown. Various studies have attributed the problem to viruses (especially Israeli Acute Paralysis Virus, IAPV), varroa mites, *Nosema* spp., pesticide use, GM crops, stress, decreased forage, lack of genetic diversity, pollution, climate change, weather… or a combination of several of these and other factors (eg Bromenshaenk *et al.*, 2010; Cox-Foster *et al.*, 2007; Giray *et al.*, 2010; Guzman-Novoa *et al.*, 2010; McFrederick *et al.*, 2008; Oldroyd, 2007; Paxton, 2010; Potts *et al.*, 2010a; Tarpy and Seeley, 2006). It now appears that there is no one cause behind these losses. In fact it is likely that several different factors act together making the problem multifactorial (Oldroyd, 2007). It is also likely that there are different problems in different regions. Varroa mites have been suggested as a cause for honeybee losses in Canada (Guzman-Novoa *et al.*, 2010) but may not be such a problem in Africa (Fazier *et al.*, 2010) and a questionnaire study of colonies in Turkey suggested that Turkish honeybee losses may be more closely related to region and weather than to disease level (Giray *et al.*, 2010). This may be down to differing bee keeping practices, differences in climate and weather conditions, or many other reasons.

There is still much conflict between data, for example whilst several studies point towards varroa mites as a cause (Guzman-Novoa *et al.*, 2010; Le Conte *et al.*, 2010; Yang and Cox-Foster, 2007), data from the food and agricultural organization (FAO) for Europe shows little obvious impact from the introduction of varroa mites (Moritz *et al.*, 2010) (figure 1.2). However there may be a combined effect of politics and varroa such that when the Soviet Union was dissolved in the early 1990s there was less financial support for beekeepers making it harder to replace colonies lost due to pathogens and parasites like varroa. This made it financially unviable for many beekeepers to continue to keep bees in Eastern European countries.

Figure 1.2:



The annual number of hives reported to the FAO in western European countries (the former 15 EU member states, black circles) and the former Warsaw Pact countries in Eastern Europe (including the former USSR, open circles). The dramatic decline in Europe coincides with the political system changes in the in Eastern Europe, whereas the introduction of *V. destructor* had no perceptible impact on the number of hives reported.

Other studies suggest that *Nosema* spp. (microsporidian gut pathogens), especially *N. ceranae*, may be linked to honeybee losses. A review by Paxton (2010) summarises much of the information gathered so far. There is research to suggest that *N. ceranae* can lead to the loss of a colony within 18 months of infection (Higes *et al.*, 2008) it is also thought that *N. ceranae* may be more virulent than *N. apis* (Higes *et al.*, 2007; Paxton *et al.*, 2007), although a more recent study showed no discernible difference in virulence between the two species in caged bee studies (Forsgren and Fries, 2010).

The large metagenomic study by Cox-Foster *et al*. (2007) however showed that although all the colonies identified as having been killed by colony collapse disorder (CCD) studied in the investigation were found to be positive for *N. ceranae*, so too were many of the apparently healthy colonies (47%) studied. This suggests that although *N. ceranae* may be linked to CCD losses it is unlikely to be the sole cause.

It has been suggested that if something is not done to prevent *A. mellifera* colony losses there may be no colonies left by 2035 (Benjamin and McCallum, 2008), but the reality is unlikely to be quite so dramatic. A more recent study suggests that the severest losses may be confined to the USA and some parts of Europe (Aizen and Harder, 2009). A review of the FAO data on the number of commercial bee hives worldwide showed that while the number of honeybees kept commercially has decreased in some areas (like the UK), worldwide numbers have actually been increasing since 1961 (Aizen and Harder, 2009). However, this increase is slower than the increasing demand for pollination (Aizen and Harder, 2009). A

recent UK study suggests that whilst the honeybee contribution to insect pollination is declining there has been no subsequent decline in crop yields. This is likely due to the contributions of wild insect pollinators (Breeze *et al.*, 2011).

1.3 Honeybee pathogens and parasites:

Honeybees are hosts to many different pathogens including viruses, bacteria, fungi, protozoa and microsporidia as well as invertebrate parasites (Bailey and Ball, 1991). It is thought that one of the causes behind the honeybee declines seen in several countries, including the UK, may be pathogens (e.g. Cox-Foster *et al.*, 2007; Oldroyd, 2007). This project focuses on the effect of the honeybee viruses common to the UK, the microsporidia *Nosema* spp. and the parasitic mite *V. destructor*.

1.3.1 Bacteria:

Several bacteria have been isolated from *A. mellifera,* usually from the digestive tract. Dying bees have been found infected with *Pseudomonus apiseptica* and other bacteria. However Bailey and Ball (1991) believe that bacteria act more as agents of septicaemia causing secondary, though potentially fatal infections after previous primary pathogenic or non-infectious disorders are acquired. The two most common and virulent bacteria that infect honeybees are American foul brood (AFB) caused by the rod-shaped Gram-positive species *Paenibacillus larvae* and European foul brood (EFB) caused by the lanceolate, Gram-positive bacterium *Melissococcus pluton.* Both EFB and AFB are 'notifiable' diseases. In the UK this means that if the disease is found in a colony the secretary of state (or more accurately the National Bee Unit acting on his/her behalf) must be informed. This is part of DEFRA's bee health program that was designed to control and limit the spread of bee diseases (DEFRA, 2006).

If AFB is confirmed within a colony that colony, must be destroyed, the frames burnt and the rest of the equipment scorched or fumigated. EFB colonies can be kept but must be quarantined. If the infection is light no treatment is needed other than to reduce the area of brood nest then feed and strengthen the remaining colony. For heavier infections chemical treatments such as Terramycin are used (Tarpy and Keller, 2007).

1.3.2 Fungi:

There are few fungi that affect adult *A. mellifera* naturally. Melanosis affects queens leading to melanization of the tissues surrounding the nurse and egg cells of the ovaries (Bailey and Ball, 1991). There are also pathogenic yeasts which are thought to be *Torulopsis* species

(Giordani, 1952). The two most common fungal pathogens of honeybees only infect the larvae. Chalk brood, *Ascophaera apis,* kills larvae after capping and makes them appear white, fluffy and swollen at first before shrinking and becoming hard. By this stage workers may have removed the cell cap (Bailey and Ball, 1991). Infected combs can be treated with Thymol but cedar oil has been found to be more effective if also more expensive (Mourad *et al*., 2005). Stone brood, *Aspergillus flavus* or *Aspergillus fumigatus*, also affects larvae after capping, first they appear white and fluffy but later become hardened and either a pale brownish or greenish yellow. There are no treatments available for this disease but it does not usually affect strong healthy colonies (Bailey and Ball, 1991).

1.3.3 Protozoa:

Malpighamoeba mellifica is a protozoan of the order Sarcodina and infects the lumen of the malpighian tubules of adult *A. mellifera*. This causes atrophy of the Malpighian tubules but leaves no other sign of infection. To diagnose this disease requires dissection of the bee to reveal the presence of cysts (Bailey and Ball, 1991). It is unclear how serious infection by *M. mellifica* actually is. Heavily infected colonies may show no outward sign of infection and the specific effects of this pathogen are unclear, however it may be linked to shortened worker longevity and is possibly associated with *Nosema* spp. infection (Jordan, 1937).

1.3.4 Parasitic mites:

1.3.4.1 Tracheal mites (*Acarapis woodi*):

This parasite infests the tracheae of the first pair of spiracles of adult honeybees although they have also been found in air sacs in the head and abdomen (Prell, 1927). Injection of the dye congo red into the haemolymph of infested bees causes the mites to change colour indicating that they are likely to be feeding on haemolymph by piercing the tracheal wall (Orosi-Pal, 1934). There are no outward symptoms of parasitism and so dissection is required for diagnosis. Parasitised individuals do, however, have shortened life spans although this does not significantly affect the colony as a whole unless the parasitism level is high. In Britain this occurs only very rarely. In fact, prevalence of this mite in Britain has been falling since records began. This may be due to climate change, treatment or the breeding of resistant strains of bees (Bailey and Ball, 1991).

Acarapis woodi has been found not to infect older bees (of about 9 days or older) or queens, although the reasons for this are unclear (Bailey and Ball, 1991). When foraging activity increases, the older infected bees are separated from younger susceptible ones; the

infestation decreases. In Florida parasitism levels were recorded to drop between 5 and 90% during nectar flows (Taber, 1987).

There are other *Acarapis* species that infect *A. mellifera* including *A. dorsalis* in Britain. This mite is located in the v-shaped groove between the mesoscutum and mesoscutellum on the bee's dorsal region (Morison, 1931). Tracheal mites can be treated with many chemicals including Apiguard and Apilife VAR (Tarpy and Keller, 2007).

1.3.4.2 *Varroa destructor*:

More than 40 species of mite have been associated with honeybees of which six parasitize *A. mellifera*. The most common in UK is *Varroa destructor. Varroa destructor*, hereafter called 'the varroa mite' or simply 'varroa', is a relatively recent parasite of *A. mellifera* having transferred from the Asian honeybee, *Apis cerana,* in the early 1960s probably in the Primorsky region of the former USSR (Dejong *et al.*, 1982). This is likely to have occurred due to mutual robbing and drift between the two bee species as well as the use of *A. cerana* brood to bolster Western honey bee hives. Varroa is therefore co-evolved with *A.cerana* but not with *A. mellifera*, and *A. mellifera* colonies can soon be overrun with mites. Varroa has been linked to reduced adult bee size, weight, flight frequency and life span (Kralj, 2004). Previous studies have suggested that some viruses may be transmitted by the mite (e.g. Bakonyi *et al.*, 2002; Tentcheva *et al.*, 2004a, see also 1.3.5) but a study by Santillan-Galicia *et al*. (2010) was the first to prove that both DWV and slow paralysis virus are transmitted by the mite and mite transmission appears to be more important than bee-to-bee transmission.

The mite is also able to 'activate' viruses. Many bee viruses occur at inapparent (symptomless) levels but varroa appears to trigger overt infections. For example Nazzi *et al* (2012) showed that varroa infestation transformed the usually inapparent virus DWV into a rapidly replicating and lethal infection. This may be due to some biochemical component of the mite's saliva that may suppresshumoral, cellular or genetic immune responses of the individual bees (Bailey and Ball, 1991; Gregory *et al.*, 2005). Yang and Cox-Foster (2005) examined the expression of several immune chemicals in *A. mellifera* infested with varroa both with and without deformed wings. Expression of the antimicrobial peptides hymenoptaecin, defensin and abaecin were all suppressed by the presence of varroa. Hymenoptaecin was not affected by the presence or absence of wing deformity. Defensin and abaecin, however, were suppressed to a greater extent in bees suffering from wing deformity suggesting that suppression of these peptides was not solely due to the mites. Phenoloxidase, lysozyme, glucose dehydrogenase and glucose oxidase are chemicals

important in insect immunity (see 4.5); all of these were also found to be suppressed by varroa (Yang and Cox-Foster, 2005).

Many viruses are more effectively transmitted by injection than by ingestion by the bees and in nature those viruses vectored by varroa are transmitted through the mite's feeding activity and thus effectively injected into the individual (Martin, 2001). Finally varroa transmits viruses between the different life stages of the bees. This is especially important in the case of DWV where the characteristic deformed wings are only seen when infection occurs at the early (white eyed) stage of pupal development (Ribiere *et al.*, 2008).

Zhang *et al.* (2010) examined gene expression in both Asian and European honeybees in response to varroa infestation to determine whether the varroa tolerant Asian honeybees showed different gene expression compared with the more susceptible European bees. Several genes were differentially up or down regulated in response to infestation. Some of these have been pinpointed as potentially linked to varroa mite tolerance; although more work needs to be carried out to confirm this. If these genes are indicators of varroa tolerance they may be useful in selectively breeding varroa tolerant bees.

However, it has been suggested by Fries *et al*. (2006) that if honeybees were left to adapt naturally to varroa infestation without the influence of applied miticides and other treatments, then they may be able to develop natural tolerance to the parasites. Fries *et al*. (2006) studied an isolated island population of honeybee colonies (N=150) each infested initially with 36-89 varroa mites. Mortality increased over the first three years but then over the next three years, mortality decreased, as did varroa levels whilst more colonies began to swarm. A similar study in Louisiana, USA, showed that swarming rates and colony survival decreased in the first three years after the introduction of varroa to the area, but after this swarming rates and colony survival returned to normal (Villa *et al.*, 2008). Similar results were also found in France (Le Conte *et al.*, 2007). A study of the reproductive success of varroa mites in control colonies and colonies that had previously survived varroa infestations showed the average proportion of successfully reproducing varroa mites was significantly lower in surviving colonies compared to control colonies (Locke *et al.*, 2012).

European honeybees in other geographical regions may already be resistant to the varroa mite; for example, in South America (see Rosenkranz, 1999) and in Africa (Fazier *et al.*, 2010) where little or no impact of the mite's introduction to local honeybee populations has been seen, although geographical location and climate may be influential factors here. There is also a Russian honeybee race that may have been affected by varroa for longer than the European honeybee and thus is now better able to withstand attack. Rinderer *et al*., (1997)

examined these colonies in 1995-6 and showed that they had lower mite populations than similar hives in Baton Rouge Louisiana, but could not say for certain that this was not because of other influences such as environment and bee keeping practices. Research is being carried out in the USDA Honey Bee Research Laboratory in Baton Rouge, Louisiana, into the use of Russian stocks to form varroa-tolerant colonies (Harris and Rinderer, 2004).

African honeybees have also been shown to be more hygienic than similar bees found in Europe (Fries and Raina, 2003), and it has been suggested that the bees showing greater hygienic behaviour may be more resistant to the varroa mites. However, a recent study by Çacmak (2010) showed that hygienic colonies of bees, as determined using a liquid nitrogen freeze killed brood assay, were no more likely to survive over winter in the presence of varroa infestations than non-hygienic colonies. This may mean that either this assay is not a good indicator of hygienic behaviour in relation to varroa removal, or it may simply suggest that hygienic bees are no more tolerant to varroa than non-hygienic bees.

Currently, control of varroa is achieved by chemicals (miticides or essential oils), drone trapping or powdered sugar application (Hooper, 2008). Mites are commonly seen crawling on bees and are present in nearly all colonies in the UK, although some island populations have remained varroa-free. Mites can be quantified within colonies by placing sticky varroa boards beneath the mesh floor of the colony and counting the number of mites that fall onto the boards within a week (varroa drop counts). Collecting a known number of adult bees into a container and covering them with icing sugar can induce grooming behaviour and may interfere with how the mites grip onto the bees (Fakhimzadeh, 2000), removing all the mites that can then be counted to provide an estimate of the number of mites living on the adult bees (phoretic mite counts) (Hooper, 2008; Waring, 2006). In Spring, if more than 2-3 mites per bee are detected by these counting methods, or if 40-80 mites are trapped within 24 hours on the sticky trap, then treatment is needed. In Autumn the threshold rises to 5-6 mites per bee or 100-150 mites trapped in 24 hours (FERA, 2010). There are several chemical treatments that can be used from Apiguard and essential oils that encourage mites to leave their hosts, or pesticides such as Apistan or Bayvarol. It is known that resistance to pesticides is widespread, however, so these should be used in rotation (FERA, 2010). Treatment is not recommended in late Spring or Summer during honey flow (Tarpy and Keller, 2007).

1.3.5 Viruses:

As of 2007, a total of 18 viruses had been identified and characterised from bees of the genus *Apis*. With the exception of a single DNA virus (Filamentous virus) all others are single

stranded RNA viruses, usually isometric (with the exception of Chronic bee paralysis virus, CBPV) and forming three separate size classes; 17, 30 and 35nm respectively. Whilst all viruses except filamentous and CBPV are indistinguishable microscopically they do possess differing buoyant densities in CsCl and have different capsid protein profiles (Ribiere *et al.*, 2008). Each virus can also be distinguished using molecular techniques. In this project the viruses were identified using PCR (see chapter 2.6.1).

All the honey bee viruses described below are single stranded, positive sense viruses. This means that each is made up of a single strand of RNA within a protein capsid. The 'positive sense' refers to the fact that it can be read in the same direction as mRNA without need for transcription first. The positive sense RNA can be read as mRNA to produce the protein capsids needed for packaging new virions and to produce RNA dependent RNA polymerase. This enzyme is used to replicate new minus sense RNA from which new positive sense strands can be made thus allowing the virus to replicate (Madigan *et al.*, 2006).

Most bee viruses in nature occur in 'inapparent' or 'covert' forms. This means that whilst the viruses are present within individuals in the colony there are no outward signs of infection. Covert infections are defined as conditions in which there are low levels of the virus which produces no clinical symptoms but the virus can re-emerge at a later date or be passed on vertically to subsequent generations. In contrast inapparent infections are short term, characterised by large levels of virus production and horizontal transmission but again with no obvious symptoms (Yue *et al.*, 2007). Confusingly, 'inapparent' is often used to describe any infection where there are no outward symptoms irrespective of quantity of virus particles present or the transmission route (Ribiere *et al.*, 2008). Virulence is thought to depend on the transmission route to which the virus is adapted. Vertical transmission requires increased longevity of the host compared to horizontal transmission and so vertically transmitted viruses are generally less virulent than horizontally transmitted ones. Thus covert viruses should be less virulent than inapparent ones (Yue *et al.*, 2007). Several viruses may be present in a single colony or individual bee without any obvious signs.

There are six honeybee viruses commonly detected around the world which are routinely checked for in honeybee samples by the National Bee Unit (NBU): acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV) and sac brood virus (SBV). The NBU also routinely test for the presence of one additional virus, Israeli Acute Paralysis Virus (IAPV) which is a new virus and has not yet been detected in the UK (Bee Base: https://secure.fera.defra.gov.uk/beebase/index.cfm?pageid=275). In this project all seven of these viruses were tested for although only BQCV, SBV and DWV were detected.

1.3.5.1 Chronic Bee Paralysis Virus; CBPV:

The symptoms of 'paralysis' may have been witnessed over 2000 years ago as recorded by Aristotle, however, the cause of the disease was not discovered until 1963 (Bailey *et al.*, 1963). There are two sets of symptoms associated with this virus. Firstly, bees may become flightless and trembling, often showing bloated abdomens and 'dislocated' wings. They die within a couple of days. This is the most common outcome in the UK (Bailey and Ball, 1991). Some colonies however show a separate set of symptoms that make them prone to attack by guard bees much as robber bees are. These bees lose their yellow fur and thus appear black and shiny, they also appear smaller with broader abdomens. These bees become flightless within a few days and die soon after (Bailey and Ball, 1991). The mechanism determining which of these symptom sets the bees present is, so far, unclear. It is, however, known that it is not connected to virus titre or serology (Rinderer and Green, 1976). Susceptibility to this virus may have a genetic basis (Bailey, 1965). This virus can also be found as a covert infection with virus particles detectable by molecular methods (section 1.5) (Tentcheva *et al.*, 2004b). There is also some evidence that CBPV can be transmitted vertically (Chen *et al.*, 2006a).

CBPV also has an associative virus (CBPVa) that cannot be transmitted separately from CBPV. It appears to decrease the infectivity of CBPV and may be linked to defence mechanisms of the bees (Ball *et al.*, 1985). There has been no evidence of any influence of varroa on the prevalence or impact of CBPV (Ribiere *et al.*, 2008).

1.3.5.2 Acute Bee Paralysis Virus; ABPV:

This virus was discovered when researchers were searching for the cause of bee paralysis. It presents the same trembling and paralysis symptoms as CBPV but affects individuals much faster. Bees infected with 100 particles of ABPV show symptoms within 2-4 days and are dead 1-2 days later, while bees infected with CBPV show symptoms after 5-6 days and die a few days later (Bailey *et al.*, 1963).

In nature this virus is covert. It was not directly detected by serology in dead bees or brood, or associated with disease or mortality, until combined with the influence of varroa (section 1.3.4.2). The mite appears to transmit the disease (Chantawannakul *et al.*, 2006) although Wiegers (1986) showed that transmission efficiency decreases with time making it unlikely that the virus can replicate within the mite. The virus can be lethal at the colony level, especially when combined with varroa infestation. This virus has been detected in honeybee semen suggesting that it may be vertically transmitted (Yue *et al.*, 2006).

1.3.5.3 Black Queen Cell Virus; BQCV:

This virus was first detected in dead, field-collected queen larvae that came from cells with a blackened appearance (the source of the virus' name) (Bailey and Woods, 1977). BQCV is prevalent and widespread across Europe (Bailey and Ball, 1991). Infected larvae have a pale yellow colouration with a tough sac-like skin. Infection is characterised by the presence of blackened cell walls of sealed cells which contain dead prepupal queen larvae (Bailey and Ball, 1991). The disease sometimes affects worker brood and has been found in drone larvae (Siede and Buchler, 2003). However, it is usually detected as a covert, symptomless infection in adult bees (Chen *et al.*, 2006b). Anderson and Gibbs (1988) found that covert infections in worker larvae could be activated to increase replication to detectable levels by injection of salt solutions followed by incubation of the larvae for 3 days and 35^{o}C. The virus commonly occurs in conjunction with *Nosema apis* (section 1.3.3) and may rely on this microsporidian gut parasite for transmission. In the laboratory, *N. apis* infection was required for BQCV *per os* infection to occur (Bailey *et al.*, 1983). There is some evidence that the virus is vertically transmitted (Chen *et al.*, 2006a). BQCV follows the same seasonal cycles as *N. apis* with peak infection occurring in the spring and early summer (Bailey and Ball, 1991). BQCV was found to worsen the effect of *N. apis* and in combination with *N. apis* was responsible for some colony loses (Bailey *et al.*, 1983). There is no evidence for any association between BQCV and varroa (Tentcheva *et al.*, 2004b).

1.3.5.4 Deformed Wing Virus; DWV:

DWV was first identified in varroa-infested bees from Egypt (Ball, 1983). The virus is one of the causes of bee mortality in varroa-infected colonies (Bailey and Ball, 1991). Individuals infected at an early stage of development (white-eyed pupa stage) show developmental deformities such that, as adults, they are often of reduced size with the characteristically deformed wings. It was first thought that these symptoms were linked specifically to the presence of varroa. However, symptomless bees can emerge from colonies heavily parasitised with varroa and symptomatic bees have been found in colonies with few or no mites suggesting that the symptoms are due solely to DWV (Ball, 1993). Allen & Ball (1996) confirmed this when larvae infected with DWV in the laboratory developed with the wing deformities. More often than not, however, an infected colony will show no outward symptoms and the presence of symptoms is not proportional to the virus titre (Chen *et al.*, 2005; Tentcheva *et al.*, 2006).

Figure 1.3:

A bee with deformed wings caused by DWV. Image taken by Graham Shephard, Rothamsted VCU.

DWV is, however, closely associated with varroa infestation. The introduction of varroa to one of the Hawaiian Islands (Oahu) in 2007, and its later spread to a second island (Big Island) in 2009 allowed researchers to monitor the effect of the mite on naïve honeybee populations. A survey by Nikaido and Villalobos during 2007 and 2008 on Oahu recorded the collapse of 274 of 419 colonies that had not been treated against varroa. Martin *et al.* (2012) found that on the islands still free of varroa there was a high diversity but low prevalence of DWV isolates, however on the islands with the mites the virus diversity decreased and prevalence increased in comparison to the mite free islands. This suggests that the presence of mites was selecting for particular variants of DWV that may give them a competitive advantage. In other studies virus titre in individual bees was proportional to mite infection (eg. Bowen-Walker *et al.*, 1999). The virus can be transmitted by varroa and it has also been suggested that the virus can replicate within the mites (Bowen-Walker *et al.*, 1999). In experiments by Yang and Cox-Foster (2005) it was found that only those larvae parasitized by varroa developed the wing deformities associated with DWV, but not all parasitized larvae were deformed suggesting that varroa may increase the likelihood of bees developing with wing deformities.

However other transmission routes do exist as individuals suffering from DWV type deformities were observed in the UK as early as 1963, before varroa was present in this country (Fyg, 1963) and the virus has been found in all developmental stages of bees including eggs, which the mites do not feed on (Chen *et al.*, 2005). Experiments by de

Miranda and Fries (2008) showed that the virus could be transmitted horizontally between drones and queens during mating ('venereal transmission') and then from the queen to her offspring by vertical transmission. In the absence of varroa however the virus is usually covert, having no clinical symptoms but still passed on by both horizontal and vertical routes. Yue *et al.*, (2007) determined that DWV has developed a 'well balanced co-existence' with the honeybee that only leads to colony declines in conjunction with a trigger such as varroa infestation (see 2.3.1). When varroa is present the virus is more likely to become overt leading to much higher virus titres and the deformed wing symptoms (Yang and Cox-Foster, 2005).

1.3.5.5 Kashmir Bee Virus; KBV:

KBV was first detected in 1974 as a contaminant in preparations of Apis Iridescent Virus in the Asian honeybee *Apis cerana* in Northern India (Bailey and Woods, 1977), its name coming from the region in which it was found. Later serologically related strains were found in Australia (Bailey *et al.*, 1979). KBV is serologically, biologically and genetically related to ABPV (Allen and Ball, 1995), although the two viruses are readily distinguishable by RT-PCR (Evans, 2001), and also show differences in capsid protein profiles (Allen and Ball, 1995).

KBV is rapidly lethal to both brood and adults in the laboratory although, despite its close relation to APBV, it does not induce the same paralysis symptoms. In nature however the virus is covert with no clinical symptoms and it is unclear how severe a problem the virus is in nature (Ribiere *et al.*, 2008). Varroa is likely to both transmit (Chen *et al.*, 2004) and activate KBV (Yang and Cox-Foster, 2005). KBV, in association with varroa, has been linked to colony losses in New Zealand (Ball *et al.*, 2004). The virus can, however, persist in the absence of the mites (Carreck *et al.*, 2004) and there is some evidence that it is vertically transmitted (Chen *et al.*, 2006a).

1.3.5.6 Sacbrood Virus; SBV:

SBV was the first honeybee disease attributed to a virus (White, 1917). Infected brood form a thickened skin in which fluid collects, forming the distinctive 'sac' (Bailey and Ball, 1991). Normally the larvae pupate about 4 days after their cell is sealed, infected larvae however do not pupate and fluid accumulates between the larva and its unshed skin. Infected larvae at first appear pale yellow, the colour darkening to brown after death and finally leaving only dried out scales (Ribiere *et al.*, 2008). SBV is primarily a disease of honeybee larvae although it can multiply within adult hosts. However, infection susceptibility appears to be age dependant with only young adults (less than four days old) being experimentally susceptible (Bailey, 1969).

Whilst in the brood SBV is an acute overt infection (short lived with obvious symptoms); as with many other honey bee viruses, SBV can persist as a covert infection (Hails *et al.*, 2008). It is likely that it survives the winter at low levels in adult bees as it is unable to survive outside the host and there is rarely brood within the colony in winter (Ribiere *et al.*, 2008). Bailey found SBV to be common within bee colonies but large numbers of infected larvae were rarely found as workers remove infected individuals from the hive (Bailey, 1967).

SBV has been found in colonies infected with varroa but in a study by Shen *et al.* (2005a) this virus was detected at consistently low levels regardless of mite infection. In this experiment the bees were also infected with DWV and KBV and it is possible that the action of these other viruses suppresses SBV prevalence (Shen *et al.*, 2005b). It is unclear whether the mite is responsible for transmission of the virus (Ribiere *et al.*, 2008). Tentcheva *et al.* (2004b) did find SBV in varroa taken from 14% of the 36 colonies examined, but it has not been determined whether the virus can replicate within the mite so it is likely that it is present only as a consequence of feeding from infected individuals (Ribiere *et al.*, 2008).

1.3.5.7 Israeli Acute Paralysis Virus; IAPV:

IAPV was isolated from honeybees in Israel in 2004. It is similar serologically to ABPV and KBV but was deemed to be unique enough to warrant a separate name (Maori *et al.*, 2007a; Mayo, 2002). However Baker and Schroeder (2008b) believe that all three viruses are merely variants of the same species. The RNA dependent RNA polymerase used by such bee viruses during replication has no proof reading capabilities and thus is error prone. In fact Drake *et al.* (1998) suggests that RNA viruses show the highest mutation rate of any living being. For this reason many virus populations are actually made up of a collection of genetically distinct but related individuals, a quasispecies (Ribiere *et al.*, 2008). With virus particles from different populations showing a high degree of variation it is difficult to define where one species ends and another begins. Baker & Schroeder (2008b) suggest that species identification should be based most strongly on the conserved gene regions as these show the lowest levels of variation both within and between species. These authors also suggest that in previous studies the primers used to identify ABPV and IAPV may not have been specific enough to differentiate between the two viruses, for example in the study of Tentcheva *et al.* (2004b) the primer used to identify the presence of ABPV may also have detected IAPV (Baker and Schroeder, 2008b). For this project primers were chosen that could distinguish between all seven viruses tested for (see chapter 2.6).

Some viruses are known to undergo RNA recombination with other viruses present within a host and also with the host species. IAPV is one such virus and sections of its genome have been found embedded in the host bee genome (Maori *et al.*, 2007c). When this recombination occurs however the bees become immune to subsequent viral infection by IAPV. This immunity may be due to RNAi mechanisms directed towards the viral RNA, disruption of pertinent host gene sequences or reciprocal mobilisation of host gene sequences to the virus (Maori *et al.*, 2007b). The virus can also be transmittable by varroa mites (Di Prisco *et al.*, 2011).

In the study of Cox-Foster *et al.* (2007) into the causes for CCD in the USA it was suggested that one of the main contributing factors was IAPV infection. This paper also suggested that the introduction of this disease to the USA may have been due to importation of bees from Australia; however, later research discovered evidence of the virus in the US as early as 2002, more than 2 years prior to the importation of any bees from Australia (Chen and Evans, 2007). IAPV does correlate closely with CCD occurrence in the study however, it has been suggested that the transposition of viral gene sequences into the host genome may be responsible for the behavioural changes that could cause CCD (Cox-Foster *et al.*, 2007).

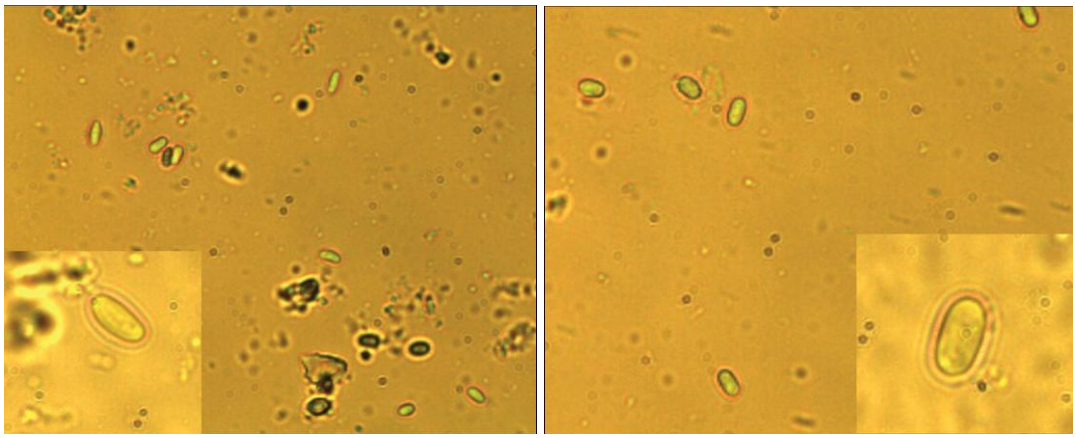
1.3.6 Nosema disease:

Nosema spp. are microsporidia; spore producing, unicellular parasites related to fungi (Texier *et al.*, 2010). There are two species of *Nosema* that are infectious to honeybees: *Nosema apis* and *Nosema ceranae* (Texier *et al.*, 2010). *Nosema apis* was first observed by Zander (1909) and has a worldwide distribution (Matheson, 1996). *Nosema ceranae* was first identified in *A. cerana* and later detected in *A. mellifera*, although it has been suggested that, as the two species are not easily distinguishable, that *N. ceranae* may have been present in *A. mellifera* long before it was first detected (Chen *et al.*, 2008). The parasites are ingested and thereafter reside within the gut tissue of the bees, releasing spores that are transmitted through faeces. The spores can also be found in honey stores, on the comb surface and interior surfaces of the hive (Malone *et al.*, 2001).

Symptoms associated with *Nosema* spp in bees are relatively non-specific making it difficult to distinguish them from other diseases. *Nosema apis*-infected bees usually show symptoms in the spring, the most notable being dysentery, but bees may also be found crawling, with distended abdomens, or dead. *Nosema ceranae*-infected bees show symptoms throughout the year, although the symptoms are often less obvious as bees leave the colony to die (Bourgeois *et al.*, 2010).

As the symptoms of *Nosema* disease are generally non-specific, the best way to diagnose it is microscopically. The abdomens of infected bees are macerated in water and examined under a light microscope. The spores, if present, can be counted using a haemocytometer to determine the level of infection. However, it is very difficult to distinguish microscopically between the two species (Bailey and Ball, 1991, see also figure 1.4). A more accurate diagnostic method to both identify and quantify these two species is by Quantitative polymerase chain reaction (QPCR) which uses the PCR reaction combined with fluorescent dyes to amplify and quantify DNA targets (e.g. Chen *et al.*, 2008). The full genome sequence has been determined for *N. ceranae* but not for *N. apis.* There are data available for sections of the ribosomal RNA genes of *N. apis* from which primers have been designed (e.g. Chen *et al.*, 2009).

Figure 1.4:



Light microscope images of *Nosema caranae* (Left) and *Nosema apis* (right) at x400 with a single spore shown at x1000 magnification taken from Bee Craft January 2008 Page 7: *Nosema ceranae.* Giles Budge, National Bee Unit, Central Science Laboratory, York.

Nosema ceranae, may be linked to honeybee losses although more research is needed to confirm this (Paxton, 2010). Research suggests that *N. ceranae* could cause colony loss within 18 months of infection (Higes *et al.*, 2008) and it is considered more virulent than *N. apis* (Higes *et al.*, 2007; Paxton *et al.*, 2007), although a more recent study (Forsgren and Fries, 2010) showed no discernible difference in virulence between the two species in caged bee studies. The large metagenomic study by Cox-Foster *et al.*, (2007) showed that, although all the CCD colonies studied in the investigation were positive for *N. ceranae*, so too were many of the apparently healthy colonies (47%) studied. This suggests that while *N. ceranae* may be linked to CCD it is not the sole cause.

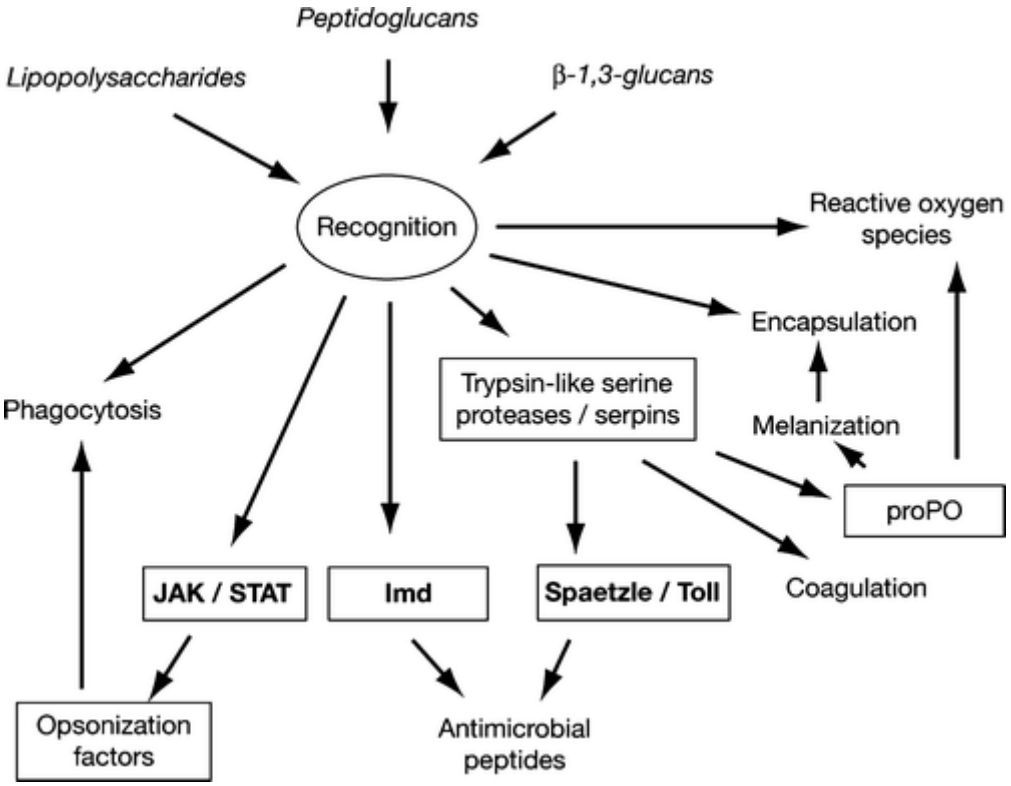
Nosema can be treated with the antibiotic Fumadil B. It is not recommended to use chemical treatments in late Spring-Summer during the honey flow (Tarpy and Keller, 2007).

1.4 Effects of pathogens on hosts:

1.4.1 Immune response:

The honeybee genome was fully sequenced in 2006 (Weinstock *et al.* 2006); analysis of these data revealed that honeybees appeared to have fewer immune related genes than any insect previously sequenced (Evans *et al.*, 2006). Several reasons have been put forward to explain this, for example the immune genes may be present but have diverged far enough away from the known immune genes of other species that they were not identified. But it is more probably that honeybees rely more heavily on behavioural and social adaptations such as grooming, behavioural fever and use of propolis which is known to have antimicrobial properties (Silici *et al.*, 2005) than on innate immune responses (Evans *et al.*, 2006). Homologues for many of the immune related genes previously identified have, however, been found for honeybees (Evans *et al.*, 2006) so it appears that honeybees do have functioning JAK/STAT, Imd and Toll pathways (see figure 1.5).

Figure 1.5:



Typical responses of the insect immune defense after different antigens (lipopolysaccharides, peptidoglucans, and β-1,3-glucans) have been recognized (oval). Rectangles denote major receptors and signaling pathways. Image taken from (Schmid-Hempel, 2005).

The honeybee immune system is made up of cellular responses and induced responses. Cellular responses are always present, non-specific and immediate in effect (Schmid-Hempel, 2005). They include coagulation, phagocytosis, nodule formation and encapsulation, and the humoral prophenoloxidase activating system (ProPO-AS) (Gillespie *et al.*, 1997). Phenoloxydase is responsible for biosynthesis of melanin and is involved in cuticle sclerotisation, wound healing, nodule formation, encapsulation and phagocytosis (Cerenius *et al.*, 2008). It is often used as a measure of immune response (e.g. Alaux *et al.*, 2010b; Yang and Cox-Foster, 2005). Induced responses are activated in response to pathogen recognition and so take longer to take effect; they include antimicrobial peptides and lysozyme-like-activity (Boman and Hultmark, 1987).

The immune response differs between honeybee developmental stages such that honeybee pupae may have higher total haemocyte counts than adults (Wilson-Rich *et al.*, 2008), although Schmid *et al.*, (2008) found contrasting results. Phenoloxydase activity, however, has been shown to increase with age (Zufelato *et al.*, 2004). As adults, honeybee immune responses change again in the transition from in-hive activities to foraging. As in-hive bees become foragers there is a decrease in vitellogenin that causes a decrease in numbers of haemocytes that may then reduce the bee's capacity for cellular-based immune responses (Amdam *et al.*, 2005; Amdam *et al.*, 2004). Honeybee foragers also have lower fat body mass than in-hive bees which could lead to decreased induced immune response, although this may be compensated for by an increase in phenoloxydase activity (Wilson-Rich *et al.*, 2008).

1.4.2 Behavioural responses:

Honeybees also respond behaviourally to pathogen or parasite infection. This may involve adaptive changes, such as grooming, in which the behaviour is modified by the bee in an attempt to combat the pathogen or parasite. Alternatively, it may be a result of 'adaptive host manipulation' as seen when the pathogen/parasite causes changes in the host's behaviour to aid transmission (Poulin, 1995). However, behavioural changes may not be adaptive and may simply be due to the inevitable deleterious effects of invasion as the pathogen/parasite consumes the host's tissue (Poulin, 1998).

1.4.2.1 Adaptive responses:

1.4.2.1.1 Behavioural fever:

Mammals are able to regulate their body temperature such that if infected with a pathogen they can increase that temperature as a defence against the infection (eg. Hart, 1988).

Insects cannot regulate their body temperature in the same way but can change their behaviour to produce an artificial fever by seeking out warmer regions, for example heat lamps in barns or laboratories or sun drenched areas in nature (Roy *et al.*, 2006). This has been recorded in several species including flies; Watson *et al*. (1993), for example, demonstrated that flies infected with the fungus *Entomophthora muscae* could increase their survival rates if heated to 40˚C for several hours at an early stage of infection. However it is difficult to distinguish between active adaptations made by the host to combat infection and normal thermoregulatory basking behaviour (Roy *et al.*, 2006). Honeybees infected with *N. ceranae* have been shown to prefer warmer temperatures, despite the fact that this benefits the pathogen (Campbell *et al.*, 2010). It is possible that the heat seeking behaviour was adopted by the bee to combat other pathogens which may be more heat sensitive than *N. ceranae*. Honeybees also use heat to combat predatory hornets. The Asian honeybee *Apis ceranae*, form a ball around attacking hornets and vibrate their wing muscles to increase the temperature within the ball to almost 47°C killing the hornet (Ono *et al.*, 1995).

1.4.2.1.2 Heat avoidance:

In the reverse of behavioural fever, infected individuals may also attempt to stay cool in the hopes of delaying pathogenic development and increasing their longevity. This was observed in bumble bees, *Bombus terrestris,* parasitised by a parasitic conopid fly (family: Conopidae). Muller and Schmid-Hempel (1993) discovered that infected bees often avoided returning to their nest especially at night. By collecting those bees returning the next morning and collecting bees that had remained in the nest they found that infected bees were significantly more likely to spend the night outside. To continue this work infected bees were kept in warm or cool conditions. Those kept in cool temperatures showed increased longevity as well as decreased parasite development. In a final choice test workers were given the choice between cool ($17.2^{\pm}$ 2.4°C) or warm ($29.2^{\pm}$ 1.7°C). Non-infected workers spent approximately equal time in the warm and cold areas whilst infected workers significantly preferred the cooler area, spending on average 80% of their time there.

Although from the choice experiment it is clear that infected bees intentionally seek out cooler temperatures this may not be the main reason they do not return to the hive at night. Infected bees are known to suffer from hypothermia and thus with the cooler temperatures of evening may become torpid more easily than uninfected bees and, therefore, be unable to return to the nest. Equally it is known that infected bees often have behavioural changes especially with respect to learning (Riddell and Mallon, 2006, see also chapter 4) and nest orientation (Kralj and Fuchs, 2006, section 1.4.2.2.2) thus the infected bees may have simply been unable to find the nest.

1.4.2.1.3 'Suicide hypothesis':

Apis mellifera infested with varroa mites show changes in behaviour and orientation and often fail to return to the hive (Kralj and Fuchs, 2006). This is suggested to be a defence mechanism to remove infected individuals from the colony. Examples of this 'suicide hypothesis' have been found in other organisms including butterflies and aphids (Dill *et al.*, 1990; McAllister *et al.*, 1990; Shapiro, 1976). Smith-Trail (1980) suggests that 'host suicide' should evolve only if 1) the suicide prevents maturation of the parasite, 2) the parasite is unlikely to infect kin after host death or 3) the host's death increases kin fitness more than decreasing its own fitness. In the case of varroa-infested *A. mellifera* these last two reasons may be true as infected honeybees leaving the hive to die will take the parasites with them thus ensuring that their kin are unlikely to be infested and this increases the fitness of the colony as a whole thus increasing kin fitness. It should be noted that the idea of behaviour occurring 'for the good of the species' has now been disproved in many cases as such behaviours that appeared to increase species survival are actually controlled by individual fitness. In social organisms like the honeybees, however, the individual workers do not reproduce so the fitness of the colony has greater importance than their own individual longevity. Their genes cannot be passed on directly and so must continue through sisters, nieces and nephews. Selection therefore acts at the level of the colony, not the individual, with colonies where sick workers are lost from the hive doing better than those where they are not lost from the colony.

In the experiments of Kralj and Fuchs (2006) bees were observed leaving and returning to a hive in June 2001 and 2002. Of 179 varroa-infested bees leaving the hive 39 (21.8%) did not return. However 17% of uninfested bees leaving the hive did not return either. Although these results suggest that infested bees are actively choosing to stay away from the hive, the behavioural change may be non-adaptive, a result of decreased homing ability or energetic stress rather than an active choice (see section 1.4.2.2.2).

1.4.2.1.4 Hygienic behaviour:

The most well studied hygienic activity in bees is removal of infected brood, for example varroa-infested brood are removed by *Apis cerana* workers (Rinderer *et al.*, 1997), *A. mellifera* workers also remove brood infected with fungi, foul brood and sac brood (Bailey and Ball, 1991). There are other activities that I will class as hygienic, for example workers storing nectar in American foul brood-infected colonies are known to avoid storing near cells containing dead larvae (Bailey and Ball, 1991). Furthermore, middle-aged workers may act as undertakers removing dead or dying adult bees from the hive (Waring, 2006).

Apis mellifera can also perform certain behaviours to remove pathogens. For example, ectoparasites can be removed by grooming. Honeybees perform both auto-and allogrooming (ie they groom both themselves and other workers) (Aumeier, 2001). Aumeier (2001) studied the grooming responses of Africanised and Carniolan honeybees when infested with varroa. Africanised honeybees are less susceptible to varroa infestation and thus it was hypothesised that they may show increased or more effective grooming behaviour. In this study only the Africanised honeybees showed allogrooming, they also groomed themselves twice as much as the Carniolan bees did. This was especially obvious for the intense cleaning and shaking activity that actively removed the mites. Africanised honeybees were also quicker to respond to the mite, with 90% showing grooming behaviour within 30 seconds of infestation (only 66% of Carniolan bees reacted this fast). Most importantly, it was shown that this increased grooming activity was more effective at removing the varroa mites than the Carniolan bees' grooming. (Aumeier, 2001)

As larvae develop in individual cells, the spread of pathogens between them is decreased. The young brood are fed on royal jelly which contains glucose oxidase, a chemical with antimicrobial properties (Yang and Cox-Foster, 2005), and finally the propolis used in building and sealing the hive also has antimicrobial activity (Evans *et al.*, 2006; Silici *et al.*, 2005).

1.4.2.2 Host manipulation and deleterious behavioural changes:

It is often difficult to tell whether a behavioural change is adaptive or not. For that reason both manipulative and non-adaptive changes are discussed here together. Some behavioural changes are not discussed in this section because they are covered in later chapters, for example; changes in hunger levels (chapters 3 and 4), changes in learning and memory tested using conditioned proboscis extension (chapter 4), changes in flight behaviour (chapter 5) and division of labour (chapter 6).

1.4.2.2.1 Free flying choice experiments:

Flying choice experiments have been used to study learning ability, with the bees learning to associate colour or scent or position of feeders with a sucrose reward. It is known that results found through free flying experiments correlate to those found using conditioned proboscis extension methods (Laloi *et al.*, 2000, see also chapter 4). In 1914 Karl von Frisch examined the ability of bees to distinguish between coloured cards. He determined that the bees could differentiate yellow and blue from many shades of grey; however they could not distinguish the different grey shades (von Frisch, 1974). For this reason blue, yellow and white are often used for these experiments.

Alghamdi, Dalton *et al.*, (2008) showed that bees injected with lipopolysaccharides, which mimic the cell walls of bacteria thus inducing an immune response without any pathogenic effect, were less able to learn a coloured reward. Injected bees given a choice of yellow and blue coloured flowers with sucrose solution provided only in flowers of one colour found it harder to learn which were the rewarding flowers, compared to uninfected bees.

1.4.2.2.2 Nest orientation:

Bees learn to recognise the position of and entrance to their hives by noting landmarks such as particular plants growing nearby (Cartwright and Collett, 1983; Menzel *et al.*, 2005). Hive entrances can be marked with geometric shapes to help bees recognise their own hive and reduce drifting.

Kralj (2006) marked a hive entrance (reduced in size) with a blue square and marked two circles of similar size at a distance from the real entrance as dummy entrances. One of the dummy entrances was similarly marked to the real entrance, the other was left blank. Individually marked bees were released 1.5m from the entrance and whether they entered the hive directly or visited one of the dummy entrances first was recorded. Of 115 varroa-infested workers released 103 returned to the hive. Of these 73 (70.9%) crossed the circle containing the dummy marker (ie the blue square with no entrance hole) some crossing it several times, before finding the correct entrance. Of 126 uninfested workers, 122 returned with only 43 (35.2%) first crossing the dummy entrance. It should be noted however that very few workers crossed the unmarked circle. (Kralj and Fuchs, 2006). The fact that infected bees found it harder to recognise their own hive entrance may help in the parasite's dissemination as this may increase 'drifting' of infected bees to other colonies.

1.4.2.2.3 Pollination efficiency:

About a third of the food eaten worldwide relies on some form of animal pollination, usually pollination by insects, especially bees (Klein *et al.*, 2007). For this reason the pollination efficiency of bee species in different crops is of great interest. If a nucleus or small hive is placed within a mesh cage with forage plants available to them then the seed/fruit set of the plants can be examined to determine pollination efficiency of the colony. A control cage has to be set up with no bees to determine pollination efficiency with no pollinators present (eg by wind pollination) and further controls can be set up where pollen is dusted from anthers of one flower to stigma of another to show maximum potential pollination efficiency. The pollination efficiency at the individual level can be determined by allowing a bee to make a

single visit to a virgin (previously unvisited) flower that is then bagged to prevent subsequent visits.

Ellis and Delaplane (2008) conducted caged experiments on bees infested with small hive beetle and varroa and determined that there was no significant decrease in the pollination efficiency of the infested colonies compared to controls. They showed that although individual pollination efficiency may be decreased the action of the entire colony compensates for this. There even seemed to be an increase in seed set in some infested treatments compared with the controls. This could be because parasitized and diseased bees have been shown to spend longer time foraging and forage under more adverse weather conditions than healthy bees (Kralj and Fuchs, 2006; Woyciechowski and Kozlowski, 1998).

1.5 Molecular detection and quantification methods:

1.5.1 PCR & electrophoresis:

The Polymerase Chain Reaction (PCR) is used to amplify DNA sequences *in vitro* (Mullis and Faloona, 1987). A polymerase enzyme uses specific primers (short sequences of DNA that complement the target DNA sequence) to produce multiple copies of a target DNA sequence within a sample. The polymerase enzyme used comes from the thermophilic bacteria *Thermus aquaticus* (Taq Polymerase). This enzyme is thermostable so that it is not denatured by the high temperatures used in the reaction. Before the discovery of this enzyme fresh polymerase had to be added for each cycle (Chien *et al.*, 1976).

Each cycle of the reaction starts with a 'denaturation step'. This splits the double stranded DNA into single strands. Although the temperatures and timings used for each reaction may vary to increase sensitivity or specificity, the denaturation step is usually at around 94-98^{o}C for 20-30s (Madigan *et al.*, 2006). An 'annealing step' follows in which the primers bind to their specific regions on the DNA. The temperature for the annealing step is usually about 3-5^{o}C below the T_m (melting temperature) of the primers. This is usually around 30-50^{o}C which is maintained for 20-40s. Higher temperatures can be used to increase the specificity of this step (Madigan *et al.*, 2006).

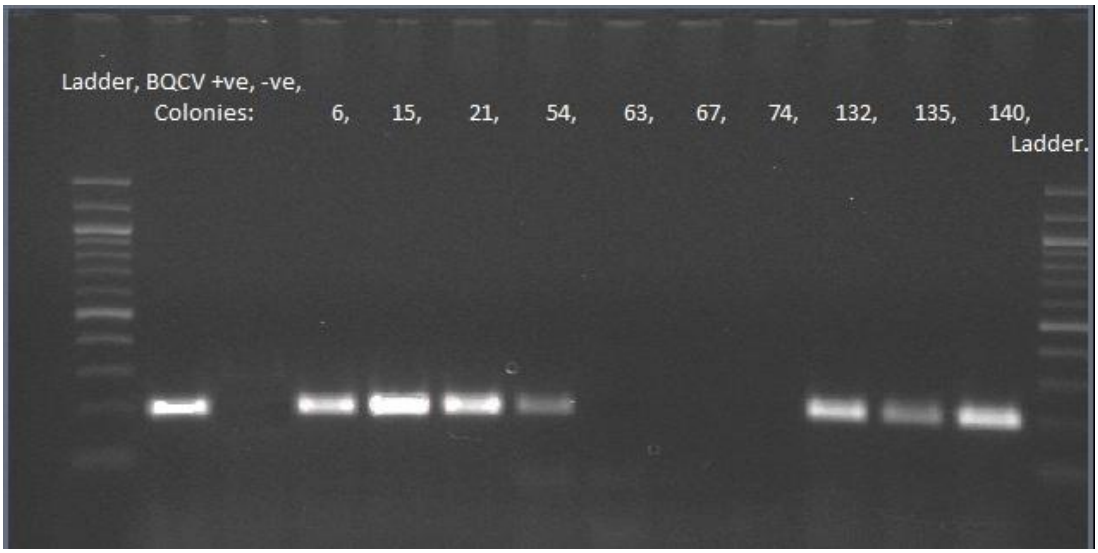
During the 'extension/elongation step' the polymerase enzyme uses the dNTPs to extend from the primers making a copy of the target sequence. This step is usually carried out at 75-80^{o}C for Taq Polymerase and can take a couple of minutes (Madigan *et al.*, 2006).

These three steps (denaturation, annealing and extension/elongation) are repeated to exponentially increase the concentration of the target sequence. Each cycle should double the amount of target sequence present, although this is limited by the amount of reaction material present (Madigan *et al.*, 2006).

30-40 cycles are usually completed although sometimes an initial ~10 cycles can be run at a slightly lower annealing temperature increasing specificity on primer annealing. The remaining ~30 cycles are run at a higher annealing temperature as sufficient template material should be present after the ~10 more specific cycles. There is a final elongation step at 70-74^{o}C for 1-5 minutes to ensure that any remaining single stranded DNA is extended. The reaction can then be 'held' at 4-15^{o}C.

Once the PCR reaction is completed the target sequences should be amplified enough to be visualised using electrophoresis. Each sample is placed in a well of an agarose gel and stained with ethidium bromide. Ethidium bromide binds to double-stranded DNA by intercalation and fluoresces under UV light (Madigan *et al.*, 2006). As ethidium bromide is not particularly sensitive, and the gel electrophoresis is run on the end point product of the PCR reaction which does not depend on the initial amount of DNA; it cannot be used to quantify the amount of DNA present in the sample (Dale and Schantz, 2002).

Figure 1.6:



Sample gel: results for BQCV in 10 honeybee colonies at Rothamsted Research. The ladders are in the first and last well, the positive control is in the second well with the negative control (no band) next followed by each numbered colony. Colonies 4,15,21,54,132,135 and 140 tested positive (bands) colonies 63 and 67 tested negative (no bands). All the bands have travelled the same distance meaning that the PCR fragments are the same size, in this case 294 base pairs.

A current is run through the tank so that the positively charged DNA moves towards the negative electrode. Larger fragments move slower than smaller fragments so that DNA

sequences are separated by size (Madigan *et al.*, 2006). As the lengths of fragments produced for each virus/*Nosema* sp. are known or can be determined, it is then just a case of looking for bands of the appropriate size in each sample. PCR cannot distinguish between two products of the same size but with different sequences so each pathogen is tested for separately. A ladder is run on each gel; this has a range of different sized fragments so that the size of the fragment in each sample can be estimated. A sample gel is shown in figure 1.6.

The PCR method used in this project is given in chapter 2.6.1.

1.5.2 QPCR:

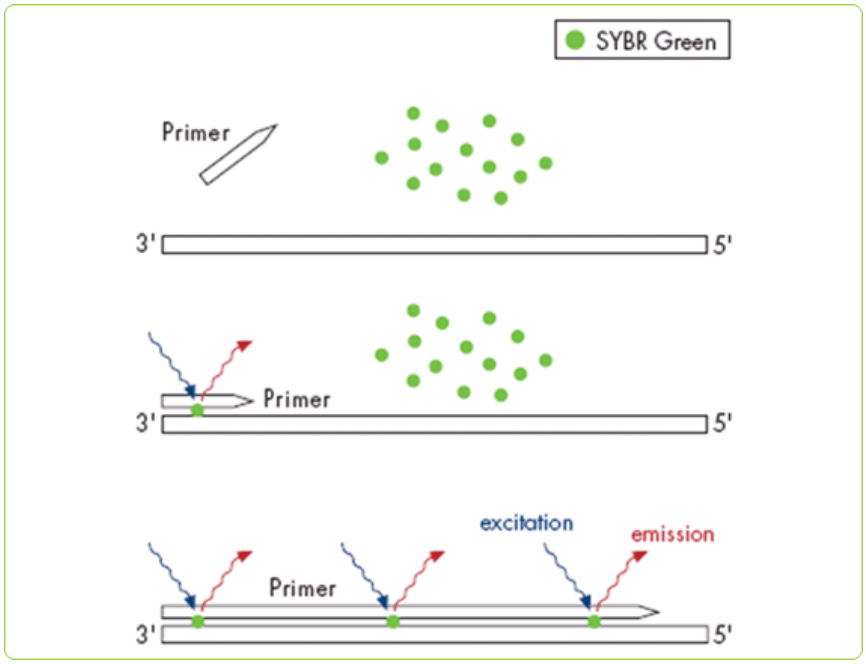
PCR and gel electrophoresis can be used to detect the presence of each pathogen but to determine the quantity or load of that species within an individual sample, quantitative PCR is required. All QPCR relies on the use of fluorescent probes or dyes to follow the production of DNA during the amplification process in real time. The two most commonly used methods are fluorescence resonance energy transfer (FRET) based probes (eg TaqMan) or dsDNA specific dyes (eg SYBR Green I).

FRET based probes are similar to primers in that they bind to a specific sequence within the target DNA. There are several different FRET based methods but the most used is TaqMan. The advantage of TaqMan is that it can be used to follow the amplification of more than one sequence in a single reaction, i.e. multiplex reactions, as different probes can be designed and labelled with different reporters to fluoresce at different wavelengths. Multiplex reactions are useful for the detection of multiple pathogens because it is much quicker; a single reaction can be made to detect several pathogens rather than having to repeat the PCR for each one individually. It also removes any inaccuracies that might be introduced by repeating the PCR for each target (Mackay *et al.*, 2002). The reaction needs to be optimised first, however, to ensure the probes and primers can all act optimally with the same reaction set up and do not cross hybridise. The TaqMan method is also more specific that dsDNA specific dyes as both target specific primers and target specific probes are used.

Multiplex reactions are not possible for dsDNA specific dyes like SYBR Green which bind non-specifically to any dsDNA. However SYBR Green dye is much cheaper than target specific fluorescent probes and is also much more sensitive to lower concentrations of a target sequence as several dyes can bind to a single region of dsDNA (Mackay *et al.*, 2002). For these reasons SYBR green was used for this project.

SYBR Green is the most frequently used dsDNA dye used for QPCR. It is an asymmetrical cyanine dye which binds to the minor groove of dsDNA and fluoresces 1000 times stronger once bound (see figure 1.7).

Figure 1.7:



SYBR Green is a dye that binds to any dsDNA. Image taken from ww1.quiagen.com.

Unlike any of the FRET based probes, SYBR Green is not sequence specific, it binds to any dsDNA. For this reason it is especially important to ensure that the only dsDNA present in the reaction is that of the target sequence and not (for example) primer dimers or non-target sequences (Mackay *et al.*, 2002). Melting curves (figure 1.8) can be used to check if this is the case. The temperature at which the DNA 'melts' (the strands separate) depends on the DNA sequence and length. This can be checked by raising the temperature of the reaction by incremental amounts and recording the fluorescence at each temperature. The fluorescence decreases dramatically as the DNA 'melts' as the SYBR Green dye only binds to dsDNA (Ririe *et al.*, 1997). (Melting curves for one of the pathogens analysed in this project are shown in chapter 2.6.2)

All samples of the same target product should have similar melting curves. If any do not then it is likely that they have some form of contamination. Primer dimers produce melting curves with peaks at a low temperature as they are only short sequences of DNA and thus do not need to be heated by very much to separate; these are easily distinguished from the target sequence (Ririe *et al.*, 1997).

Figure 1.8:

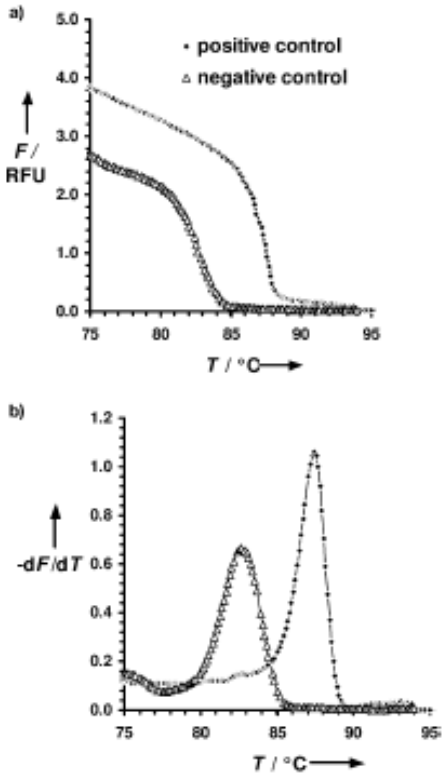


Figure taken from (Wilhelm and Pingoud, 2003). a) Shows the decrease in fluorescence as the reaction is heated for both a positive and negative control. b) Shows the change in fluorescence at each temperature, melting curves. The peak for the positive control occurs at 87.5°C, this is the T_m of the specific PCR product.

As the amount of double stranded DNA increases throughout the reaction the amount of fluorescence also increases. This is recorded with each cycle to produce a plot as in figure 14 which shows time (cycle number) along the x axis and amount of fluorescence on the y axis. There is an initial lag phase where no change in fluorescence or product accumulation can be measured. During this time the background fluorescence is greater than the fluorescence produced by dye. This is followed by an exponential increase in fluorescence as the amount of DNA doubles with each cycle. During this time the fluorescence associated with double stranded DNA becomes greater than the background fluorescence and can be measured. Eventually the amount of dNTPs and primers decreases, limiting the reaction and slowing it down to a plateau phase so that the overall curve is a sigmoid shape (Mackay *et al.*, 2002).

Figure 1.9:

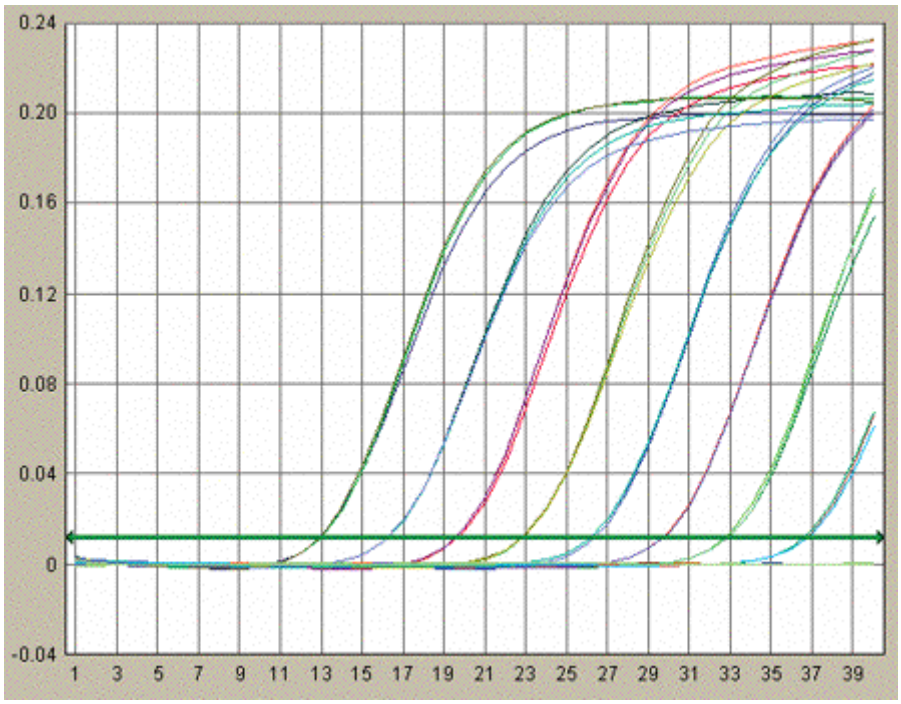


Figure showing repeated QPCR results for a dilution series on an arithmetic scale. The graph shows change in fluorescence over time for 3 technical replicates of 8 samples following a dilution series. The curves nearest the y axis have fluorescence increasing in the earliest cycles because they had the highest concentration of target sequence. Plot taken from New England Biolabs http://www.neb.com/nebecomm/products/productF-450.asp.

The final amount of DNA (and thus fluorescence) produced does not relate directly to the initial concentration of DNA. Instead the relationship is with the speed of the reaction. If there is more DNA to begin with then the exponential phases of the curves begin earlier. The above plot (figure 1.9) shows the results from a sequence of stands produced from a 10 fold dilution. The curves are evenly spaced with those produced by the higher concentrations to the left (Mackay *et al.*, 2002).

The concentration is determined by using the time taken for the curve to cross an arbitrary line (the threshold, green line on figure 13), this time is called the C_T value. The threshold must be within the exponential phase of the curve to be accurate. Outside of this region of the graph the reduction in reaction substrates available make the rate of reaction slower and thus inaccurate to calculate from. Samples with a lower initial volume of DNA will cross the threshold later than those with higher initial concentrations and thus will have a higher C_T value (Mackay *et al.*, 2002).

The QPCR method used in this project is given in chapter 2.6.2.

1.6 Aims:

It is clear from a review of the current literature that honeybees have complex behaviours which can potentially be affected by pathogens and parasites and these behavioural changes may be one of the factors responsible for honeybee declines. The aims of this project were to use new and existing methods to study the effect of pathogens on honeybee behaviour. The effect of forage availability and disease on survival was also investigated.

One technique applied, as explained in chapter 3, was the use of the generalist entomopathogenic fungus, *Metarhizium anisopliae*, as a model pathogen to study the general effect of pathogenic infection on behaviour in honeybees. In order to develop this technique, the germination rate, growth rate and virulence of the fungus had first to be determined to select appropriate abiotic conditions and conidial dose for use in experiments.

The learning ability of honeybees was evaluated in chapter 4 using condition proboscis extension, an existing method that has already been used to look at the effect of some pathogens on learning ability in bees (e.g. Iqbal and Mueller, 2007; Kralj *et al.*, 2007). In this project the method was used to test the suitability of the fungus, *M. anisopliae* as a model pathogen. The null hypothesis in this experiment was that inoculation with the fungus would cause no difference in learning ability for young adult or forager bees. If however the fungus could be used as a model pathogen then learning ability of the bees would be reduced as has been found for other pathogens. CPE experiments were also used to study the effect of the naturally occurring viruses and microsporidia infection with the null hypothesis that these pathogens, detected by Q-RT-PCR, would have no effect on learning ability.

Harmonic radar was used in chapter 5 to study whether pathogen load had any effect on the orientation flights of honeybees with the null hypothesis that the flights of bees from high-varroa colonies, or testing positive for viruses and/or *Nosema* spp. would fly no differently from bees from low-varroa colonies or which did not test positive for the pathogens. Differences in flight could include changes to flight speed or distance, the number of times the bee stopped, the time spent away from the hive or the maximum distance travelled from the hive. This method has not been used previously to study the effect of pathogens on honeybee behaviour.

Finally observation hives were used, as described in chapter 6, to study in-hive behaviour and age at which bees first began foraging. The null hypothesis was that bees would begin foraging at similar times irrespective of the disease load of the colony they came from. If

however one of the pathogens studied had an effect on time to first forage then bees from colonies testing positive for that pathogen should begin foraging earlier on average than those from other colonies. Other aspects of behaviour were also examined including the amount of time spent resting or interacting with other bees.

Again this is an existing method that has been used to study the effect of some pathogens on these behaviours in honeybees (e.g. Bailey and Fernando, 1972; Downey and Winston, 2001; Mattila and Otis, 2006), however, in this project the method was used to study bees from colonies of differing disease status to determine whether the disease status of a bee's colony has an effect on her individual behaviour.

Chapter 2: General Methods

2.1 Maintaining bees in the lab:

In 2009 bioassay boxes modified from those designed by Pain (1966) were made in which small groups of honeybees could be maintained for manipulative experiments (figure 2.1). The boxes were 58mm x 98mm x 88mm. The sliding door design allowed for the removal of dead bees without the live ones escaping. The boxes also allowed for provision of sucrose solution and water in gravity feeders as well as candy or pollen in a feeding draw. The boxes were well ventilated and made from transparent PVC which could be easily and effectively cleaned after experiments.

Figure 2.1:

A) B)

The bioassay cages used for maintaining bees in the lab (Rothamsted VCU: Graham Shephard 2010). A) an empty box with colour coded gravity feeders (red for water, green for 60% sucrose solution). B) Bees inside a box.

Twenty to forty bees could be kept conveniently in each box and transferred either by opening the sliding doors or via a valve that connected the box to the BioQuip® 'insect vac' collection tubes (http://www.bioquip.com) (which were also used as inoculation tubes). Blue roll (Tuddick Mill ltd. Plymouth, UK) was placed in the base of the cage and changed when required (when damp or if covered in fungus in any experiments with *M. anisopliae*). Unless stated otherwise, bees were provided with 60% sucrose solution in one gravity feeder (a 2ml eppendorf tube with a 19G needle hole), water in the other (a 2ml eppendorf tube with a 25G needle hole) and ground pollen in the draw. The pollen came from frozen samples collected from Rothamsted bees using pollen draws fixed to the front of Rothamsted hives.

2.2 Collecting bees for experiments:

Honeybees were collected from the apiaries at Rothamsted Research, Harpenden UK. The Rothamsted colonies are typical to the UK in being a mixture of European subspecies with naturally mated queens and they are maintained according to conventional UK husbandry practice.

2.2.1 Young adult bees:

Frames that had adult bees beginning to emerge from sealed brood were collected from the chosen hive. These frames were held in observation boxes in an incubator at 35°C overnight.
Newly emerged adult bees were collected from the brood frame using feather forceps as the frame rested on a frame stand. Twenty to forty bees were placed in each bioassay box (see 2.1), usually posted through the holes used for the gravity feeders to prevent other bees from escaping. The boxes were then maintained at 30°C in darkness with water, 60% sucrose solution and pollen, unless otherwise stated.

2.2.2 Forager bees:

Forager bees were collected from around the entrance of chosen hives. The BioQuip® 'insect vac' was used to capture bees as they flew in or out of the hive. Once enough bees were in the collection tube (usually 20-40 depending on experiment) it was removed from the vac and sealed with a plastic lid to prevent escape.

An adapter was used to connect the collection tube to a bioassay box. Bees were encouraged into the bioassay box by blowing into the meshed end of the collection tube. Once all bees were in the box the tube and adapter could be disconnected allowing the bioassay box valve to close.

The boxes were then maintained at 30°C in darkness with water, 60% sucrose solution and pollen, unless otherwise stated.

2.3 Maintaining an *in vitro M. anisopliae* culture

All experiments involving *M. anisopliae* used isolate 445.99 from the Warwick HRI culture collection. This isolate was originally taken from the commercial product Bio-Blast (Eco-Science corporation); its original host and range are unknown (Shaw *et al.*, 2002). The

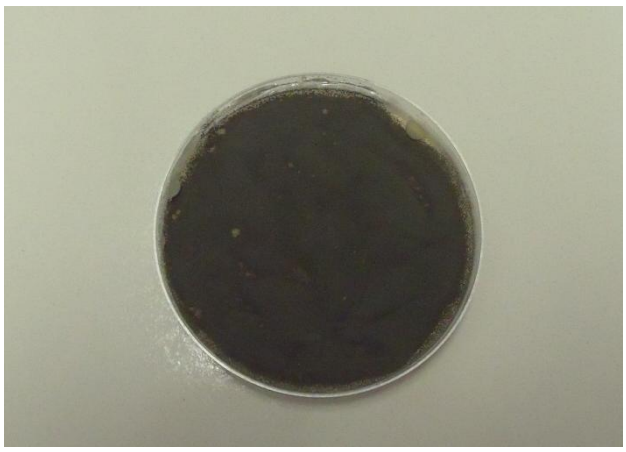
cultures were grown on Sabouraud dextrose agar (SDA) (Sigma-Aldrich, Dorset UK, Mycological Peptone, 10 g/l, Dextrose 40 g/l and Agar, 15 g/l, (Carlier, 1948)). The *M. anisopliae* sample from Warwick was grown for 14 days on SDA (20ml SDA in 90mm diameter, triple vented Petri dishes) at 23°C in darkness. Subcultures were prepared by transfer of a loop of sporulating mycelium onto fresh SDA plates (20ml SDA in 90mm diameter, triple vented Petri dishes) which were then grown for two weeks at 23°C in darkness to ensure the culture grew well. Plugs (cut using a sterile 6mm diameter cork borer) were taken from these subcultures and stored at -80°C in individual sterile 2ml eppendorf tubes (1 plug per tube), in sterile 10% glycerol that had been autoclaved at 122°C for 15 minutes.

For each experiment using *M. anisopliae* an eppendorf tube was removed from storage, thawed at room temperature, shaken thoroughly using a vortex mixer, and 20μl of the conidia-containing glycerol was pipetted onto each of four fresh Petri dishes of SDA (20ml SDA in 90mm diameter, triple vented Petri dishes) spread evenly with a sterile spreader and incubated for 14 days at 23°C in darkness.

2.4 Collecting *M. anisopliae* spores

Once the *M. anisopliae* had been growing for 14 days a layer of dark green conidia was visible (figure 2.2). The plates of fungus could then either be stored at 5°C for up to four weeks before use, or the spores could be harvested immediately for use in experiments. In the lamina flow cabinet a sterile loop was used to gently dislodge the spores from the agar and into a sterile 50ml centrifuge tube. The conidia were then stored in the centrifuge tube at 5°C for no more than one week before use.

Figure 2.2:



***Metarhizium anisopliae* grown on SDA for 14 days at 23°C in darkness.**

2.5 Inoculation of bees with *M. anisopliae* conidia

Young adult bees were inoculated 3 days after emergence (unless otherwise stated); forager bees could be inoculated at any time after collection but were never kept for more than three days before inoculation and were always inoculated on the same day as in-hive bees if both were used in the same experiment (eg the experiment to look at differences in learning between young adult and forager bees infected in Chapter 4, see 4.2.3). Prior to inoculation with *M. anisopliae* each bioassay box was wrapped in blue roll and placed in a cool box with icepacks. The bees were thus chilled to ~5^{o}C. The bees were chilled until they could be moved without any risk of them escaping ; this usually took 30 minutes. After 20 minutes in the cool box the bees were checked at 5 minute intervals and discouraged from clustering by shaking the boxes. Once the bees were chilled to immobility they were removed from the cool box.

Twenty bees were transferred to each collection/inoculation tube and 0.5g of powder, either conidia mixed with sorbitol (treatment) or sorbitol alone (control) were introduced through the meshed end of each tube. The container was sealed with cling-film and turned three times to coat the bees evenly in powder. The tubes were then left to stand for 10 minutes. After 10 minutes the cling-film was removed and any excess powder was shaken into a plastic bag. The bees were then transferred from the tube to a clean bioassay box using feather forceps. Tubes were re-used to inoculate further groups of bees until all replicates were completed. Control tubes were only ever used to inoculate with sorbitol and in the multiple dose bioassay the lowest dose was administered first and then each subsequent dose increasing in concentration after that.

Each bioassay box was lined with blue roll on the base and was resourced with water, 60% sucrose and pollen. Pairs of similarly inoculated bioassay boxes (both *M. anisopliae* or both sorbitol) were placed inside airtight plastic boxes (~12" x 7" x 5"), to ensure an elevated humidity, and placed in an incubator at 30^{o}C in darkness. After 12 hours any bees that had not survived the inoculation process were removed and the food and water replenished. The blue roll was also replaced if it was wet or covered in spores/powder. After a further 12 hours the lids of the airtight boxes were replaced with ventilated lids (6 x 6mm holes in each lid) to reduce the humidity. Thereafter mortality was assessed twice daily, once between 9-10am and once between 4:30-6pm. On every occasion the food and water was replenished and any dead bees recorded and removed.

2.6 Parasite and pathogen detection:

The prevalence of varroa was monitored in all Rothamsted colonies at key time points throughout the year using varroa drop counts. Varroa boards were placed beneath the mesh floor of the colony and the number of dead mites that fell onto the boards within a week were counted (FERA, 2010). Microscopy was used to detect the presence of *Nosema* spp*.* using a standard beekeepers' method based on methods set out by Cantwell (1970). The BioQuip® 'insect vac' was used to capture 30 bees from each colony as they flew in or out of the hive. The bees were killed in a killing jar with ether; their abdomens were removed and ground in a small amount of water (~1ml). A drop of the resulting liquid was viewed at x400 magnification through a light microscope. The spores, if present, were easily visible (eg. Figure 1.4). In addition to this, to determine the colony level infection status of all colonies used in experiments, samples of 30 forager bees were collected from the hive entrance, pooled and analysed using RT-PCR (see section 2.2.2 for forager bee collection and 2.6.1 for PCR analysis). Individual bees from behavioural experiments were killed and stored at -80 to be tested individually using Q-RT-PCR to detect and quantify the load of each pathogen species (see section 2.6.2). The process was made efficient by using RT-PCR to first identify which pathogens were present in a pooled sample of extracts from the individual bees; then Q-RT-PCR could be done on the individual bee samples only for the pathogens identified as being present.

2.6.1 PCR-based analysis of pooled bee samples:

Samples of 30 - 50 bees were collected from the entrances to hives using the BioQuip® 'insect vac' as described previously (section 2.2.2), transferred to 50ml centrifuge tubes and stored at -80°C. The only exception was for the experiment to test learning ability of pollen-starved foragers (chapter 4 section 4.2.4) when the bees were collected from within the hive from the comb above the queen excluder.

Total RNA was extracted from each pooled sample of bees with TRIzol (Sigma-Aldrich) reagent, following the manufacturer's instructions with some alterations. For each sample, 30 bees were ground in liquid nitrogen; half the sample was stored at -80°C and half transferred to another 50ml centrifuge tube for the extraction. The ground bee tissue was homogenized with 10ml of TRIzol and then 1ml of the suspension transferred to a 2ml eppendorf tube. The sample was purified to remove contaminants, such as polysaccharides, using the Qiagen RNeasy plant mini kit (Qiagen, Crawley UK) and following the manufacturer's instructions. To ensure that the RNA extraction process had been successful a NanoDrop spectrophotometer was used to quantify the amount and quality of RNA in each

sample. The absorbance is measured at 260 and 280 nm. The ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has a 260:280 ratio of ~2 whilst likely contaminants have lower 260:280 ratios, phenol for example has an absorbance ratio of 1.2 (Dale and Schantz, 2002). So long as each sample had a 260:280 ratio above 2 it was included in the analysis . No samples had a lower ratio than this but as some of each sample was kept it could be rerun if needed. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration (Commoner and Lipkin, 1949).

All samples were treated with a DNase enzyme to catalyse the hydrolysis and removal of any contaminating DNA from the sample (DNase 1 (RNase free): New England Biolabs, Hertfordshire UK). The manufacturer's instructions were followed to produce 50μl of 0.1μg/μl solution for each sample. Complementary DNA (cDNA) was produced from the RNA by reverse transcription with Superscript II Reverse Transcriptase (Life technologies, Paisley UK) following the manufacturer's instructions and using random hexamers (Life technologies).

Table 2.1:

Stage	Temperature/'C	Duration/seconds
Initial denaturation	94	120
10 cycles:		
Denaturation	94	30
Annealing	50	60
Extension	72	60
30 cycles:		
Denaturation	94	30
Annealing	53	30
Extension	72	45
Final extension	72	420
Cooling	4	

Table showing the thermocycler routine used for all PCR reactions in this project. The protocol was optimised by Dr Ryabov, University of Warwick.

PCR was run using the thermocycling conditions shown in table 2.1, with REDTaq® (Sigma-Aldrich) using primers designed by Yang and Cox-Foster (2005) and De Miranda (2008) (table 2.2). These were chosen as they were SYBR Green primers (the chosen QPCR method for this project) and were cost effective, had good target sites and were designed to discriminate between the two *Nosema* spp. and the seven most commonly occurring viruses (ABPV, BQCV, CBPV, DWV, IAPV, KBV & SBV). PCR product positive controls were acquired

from De Miranda (for *Nosema* spp. ABPV, BQCV, CBPV, IAPV, KBV & SBV) and Ryabov (for DWV) and cloned into *E. coli* plasmids (QIAGEN PCR Cloning Kit) following the manufacturer's instructions. DNase free water (Sigma-Aldrich) was used as a negative control.

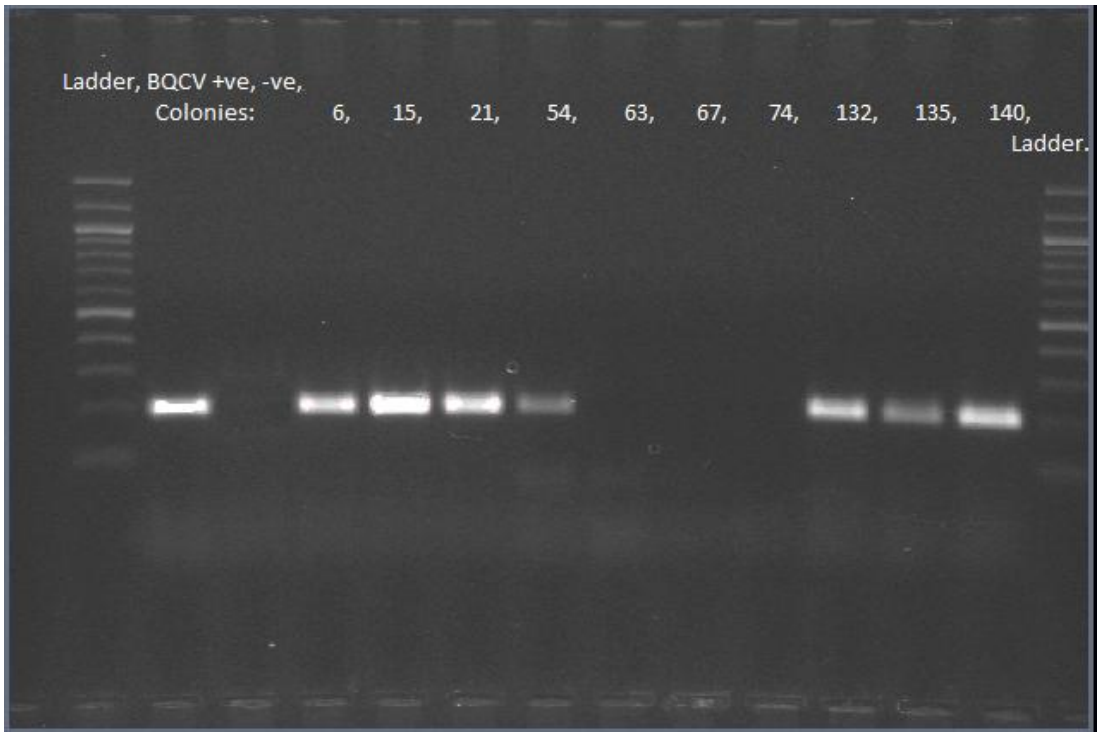
Table 2.2:

Target	Primer sequence	Size	Reference
ABPV	**F -** TCATACCTGCCGATCAAG	197	(De Miranda, 2008)
	R - CTGAATAATACTGTGCGTATC		
KBV	**F -** CCATACCTGCTGATAACC	200	(De Miranda, 2008)
	R - CTGAATAATACTGTGCGTATC		
IAPV	**F -** CCATGCCTGGCGATTCAC	203	(De Miranda, 2008)
	R - CTGAATAATACTGTGCGTATC		
BQCV	**F -** AGTGGCGGAGATGTATGC	294	(De Miranda, 2008)
	R - GGAGGTGAAGTGGCTATATC		
CBPV	**F -** CAACCTGCCTCAACACAG	296	(De Miranda, 2008)
	R - AATCTGGCAAGGTTGACTGG		
SBV	**F -** TTGGAACTACGCATTCTCTG	335	(De Miranda, 2008)
	R - GCTCTAACCTCGCATCAAC		
N. apis	**F -** CTAGTATATTTGAATATTTGTTTACAATGG	277	(De Miranda, 2008)
	R - GCTATGATCGCTTGCC		
N. ceranae	**F -** TATTGTAGAGAGGTGGGAGATT	315	(De Miranda, 2008)
	R - GCTATGATCGCTTGCC		
β-actin mRNA	**F -** CGTGCCGATAGTATTCTTG	271	(De Miranda, 2008)
	R - CTTCGTCACCAACATAGG		
DWV	**F -** CAACTACCTGTAATGTCGTCGTGTT	206	(Yang and Cox-Foster, 2005)
	R - GACAAAATGACGAGGAGATTGTT		

Table showing the primers used for PCR and QPCR analysis. The primer sequences and the size of the fragment amplified are shown. The primers are also shown for β-actin which was used as a housekeeping gene for QPCR analysis (see section 2.6.2)

The results were visualised using electrophoresis (Figure 2.4)(1.5% agarose gel with 0.1μl/ml ethidium bromide in 10% Tris/Borate/Ethylene diamine tetra-acetic acid (EDTA) (TBE) buffer; run at 150 volts for approximately one hour). Each sample, and positive and negative control, was loaded in loading buffer. A UV imager and Quantity One® gel image analysis software (BioRad Laboritories) was used to get photographs of the gel as in figure 2.4.

Figure 2.3:



Typical PCR gel showing presence/absence of honeybee pathogen in pooled bee samples. This gel showed the BQCV results for 10 colonies. The virus was present in colonies 6, 15, 21, 54, 132, 135 & 140 and absent in colonies 63, 67 & 74.

2.6.2 Quantification of pathogens in bees by quantitative reverse transcription–PCR (Q-RT-PCR):

Q-RT-PCR was used to quantify each pathogen, which had been detected in pooled samples by RT-PCR, in the individual bees from the behavioural experiments. Each bee was stored at -80°C prior to RNA extraction. For total RNA extraction each individual frozen bee was ground in liquid nitrogen and half the ground material stored at -80°C whilst the rest was used for the total RNA extraction with TRIzol (Sigma) reagent, following the manufacturer's instructions. For PCR, the total RNA extract was purified using the RNAEasy kit (Qiagen), DNase treated with RNAse-free DNAseI from New England Biolabs and reverse transcribed to produce cDNA using random hexamers (Applied Biosystems) and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturers' instructions (see section 2.6).

QPCR was done for each pathogen that had been detected in pooled sample PCR (as in section 2.6, but using pooled samples of extracts from the individual bees, ~20 bee extracts were pooled in each sample) and also for the housekeeping gene β actin. Platinum SYBR Green Supermix-UDG (Invitrogen) was used following the manufacturer's instructions to the method shown in table 2.3 and with the following reaction mix: per 1μl of sample: 5 μl SYBR

green mix + 0.1 μl of each primer + 3.8 μl RNase free water. The Q-RT-PCR reactions were carried out in an ABI PRISM 7700 sequence detection system (Applied Biosystems).

Table 2.3:

Stage	Temperature/'C	Duration
Hot start	50	2 mins
Initial denaturation	95	10 mins
40 cycles:		
Denaturation	95	15s
Annealing & extension	60	1 min
Dissociation curve:		
	95	15s
	60	15s
	95	15s

Table showing the routine used for all QPCR reactions in this project.

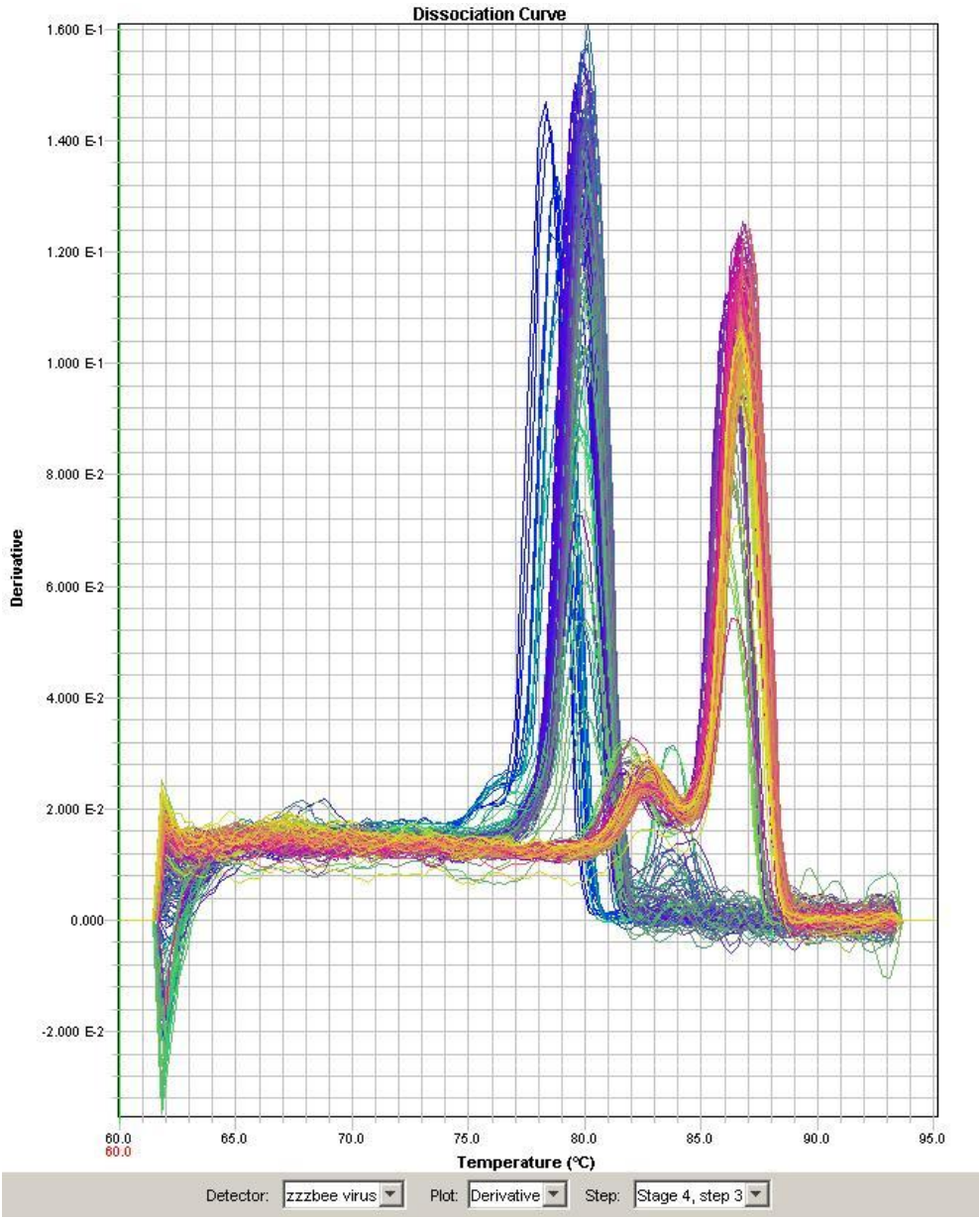
The cycle threshold (C_t see chapter 1.5) was automatically calculated by the SDS software package (version 2.4). Melting curves were produced for each sample and compared to those produced for the positive samples to identify any contaminated samples that were then run again (see figure 2.5). C_t values for the pathogens were normalised to the housekeeping gene β-actin by subtracting the C_t value for each sample.

Relative pathogen load: Delta C_t(pathogen) = C_t(pathogen) –C_t(β-actin)

Because pathogen load is recorded as the number of cycles taken to reach a threshold value (C_t) the lower the value (ie the less cycles it takes to reach the threshold) the higher the amount of pathogen present in the sample. The pathogen load was calculated relative to the amount of the housekeeping gene β-actin to remove the effect of any differences in efficiency of RNA extraction and/or reverse transcription. Therefore, in the case of high virus load delta Ct values were negative and the higher the pathogen load the more negative the relative delta Ct. The water controls gave a relative value of 20 or more so any sample with a relative value of 20 or higher, or with a melting curve that did not match the positive, were given a relative value of 20.

The efficiency for RNA extraction and reverse transcription is not known so the actual amount of pathogen in an insect cannot be accurately calculated from the amount of DNA detected in a sample, but the relative disease loads can be compared between bees tested in this project. As different groups use slightly different methods and all use different equipment it is also impossible to compare relative pathogen loads to the results of others.

Figure 2.4:



Melting curves for 1 plate of samples. The mostly blue/green/purple curves are the curves for the *N apis* positive samples and all the samples of this plate which tested positive for *N. apis*. The mostly yellow/pink/orange curves are the β-actin positive samples and all samples tested for β-actin. This curve has a shoulder (a second smaller peak on the left) which is likely produced by left over primers. There is one green curve at ~83.5°C (between the two sets of curves) this is a negative result, the curve is probable produced by left over primers.

Chapter 3: Characterisation of the germination and radial growth of the fungus *Metarhizium anisopliae* and the effect of fungal infection and forage availability on honeybee survival.

Abstract:

The European honeybee, *Apis mellifera*, is important economically not just for honey production but also as a pollinator. However, honeybee numbers in some areas are declining. A range of interacting factors are thought to be involved, including pathogens and parasites, which are known to cause changes in the behaviour of their hosts.

The purpose of this chapter was to examine potential use of the generalist entomopathogenic fungus, *Metarhizium anisopliae*, as a model pathogen to study the general effect of pathogenic infection on behaviour in honeybees. In order to develop this technique, the germination rate, growth rate and virulence of the fungus had first to be determined to select appropriate abiotic conditions and conidial dose for use in experiments. This dose of fungus was then used to examine the effect of additional stressors on bees from colonies set up to have differing disease loads and forage availability.

The fungus could grow well at 30^{o}C, and was able to kill bees in the multiple dose bioassay with a dose of 1:30 killing 100% of bees in 12 days. There was no effect of different colony disease or forage availability on the bees' reaction to fungal inoculation. There was however an effect of pollen feeding such that bees from high disease and restricted forage colonies were better able to survive when fed pollen although pollen fed bees from these colonies still did not survive as well as those from healthier and free foraging colonies that were also fed pollen. This suggests pollen feeding can aid survival but good forage is required throughout development.

3.1 Introduction

3.1.1: Use of the fungus *Metarhizium anisopliae* for biological control and as a model system:

The taxonomic status of the entomopathogenic fungus *Metarhizium anisopliae* s.l. (Ascomycota: Hypocreales) has recently been revised following a new multilocus phylogeny and three new species names have been included within the genus (Bischoff *et al.*, 2009).

The position of the isolate used in this study is not yet known and so for the purposes of this thesis is referred to simply as *M. anisopliae*. *Metarhizium anisopliae* is a very versatile fungus, being able to infect a broad range of insects, 200 species from over 50 insect families (Roberts and Humber, 1981). Different isolates of the fungus are pathogenic to different insect species (e.g. Chandler *et al.*, 2001) which makes it a good candidate use as a biological control agent against many pests including spider mites (*Tetranychus* spp) (eg. Shi *et al.*, 2008), aphids (eg. Vu *et al.*, 2007), the brown plant hopper (*Nilaparvata lugens*) (eg. Jin *et al.*, 2008) among others (Wang and Ma, 2009). The fungus is also being examined as a possible method for controlling the varroa mite (eg. Chandler *et al.*, 2001; Kanga *et al.*, 2003). Infection occurs by the attachment, germination and growth of conidia through the host cuticle, the fungus then invades the haemocoel before ramifying throughout the host (Tanada and Kaya, 1993). Death usually occurs within 4-7 days of infection, depending on dose and temperature. For use in biological control of varroa the fungus was tested against non-target species and several isolates of *M. anisopliae* were pathogenic to both the target varroa and the non-target *A. mellifera* (e.g. Shaw *et al.*, 2002). *Metarhizium anisopliae* is ubiquitous in the environment, but mainly prevalent in the soil. It has been isolated from overwintered bumblebee queens (Nemec, 1976), but it is unlikely that honeybees could come into contact with it naturally.

Metarhizium anisopliae infection has been used as a model system to study the effect of pathogens on several insect species, especially social insects (e.g. Bos *et al.*, 2012; Gardner and Thomas, 2002; Hughes and Boomsma, 2004; Mburu *et al.*, 2009). The fungus has been used to investigate genetic predisposition to disease resistance and the benefits of polyandry (Hughes and Boomsma, 2004). Polyandrous leaf cutting ants were used to test whether polyandry (the queens mating with multiple males) could benefit the colony by producing a colony with varied genetics with some patrilines less susceptible to infection than others. When inoculated with low concentrations of *M. anisopliae* there was a significant difference in susceptibility between patrilines and mixed groups of ants survived better than groups from a single patriline (Hughes and Boomsma, 2004).

Some insects are able to use behavioural adaptations to avoid or reduce infection. Some insects thermoregulate; sitting in warmer locations so that they heat up, which is thought to have a similar function to fever in mammals (Blanford and Thomas, 2001). *Metarhizium anisopliae* was used to investigate thermoregulation in locusts and it was shown that the effects of a fungal infection are strongly influenced by environmental temperature and host thermoregulation behaviour (Gardner and Thomas, 2002). Another behavioural defence against fungal infection is for insects to groom themselves or each other. Shimizu and Yamaji (2003) showed that termites reared alone were far more susceptible to *M. anisopliae*

infection than those raised in groups so that they could groom each other illustrating the importance of group level defences against infectious disease in eusocial insects, like honeybees. Other insects are able to detect fungal spores and avoid them, for example termites are able to not only detect and avoid *M. anisopliae*, but also seem to be able to determine or recognise the virulence of different isolates of this fungus so that they were more likely to avoid the more virulent isolates than the less virulent ones in choice chamber experiments (Mburu *et al.*, 2009). And ants are able to determine when they have been infected and avoid their nest mates to avoid spreading the fungal infection (Bos *et al.*, 2012).

This fungus has also been used to study the effect of infection on feeding behaviour, showing that infected *Chilo partellus* (Lepidoptera) larvae consume up to 80% less food after infection (Tefera and Pringle, 2003). Blanford *et al.* (2011) used another fungus, *Beauveria bassiana*, to show that inoculated mosquitoes were also less likely to feed than uninoculated controls. The inoculated mosquitos were also less able to fly, presumably because the fungus caused energetic stress so that they did not have the energy to fly as well as uninoculated controls. Other pathogens have been shown to cause energetic stress (e.g. Nosema: Mayack and Naug, 2009).

However, very few studies have used this species on bees. One study used a strain of *M. anisopliae* that was not pathogenic to bees as a control to examine resin collection as self-medication in honeybees (Simone-Finstrom and Spivak, 2012). Bees collected more resin when exposed to the fungus and colonies with more resin showed reduced infection intensity, suggesting that honeybee do use resin successfully to self-medicate.

Metarhizium anisopliae is easy to manipulate so it can be used to test hypotheses that would be difficult to test on the coevolved pathogens of bees that are more difficult to manipulate. It is also absent from honeybee populations so it is possible to be certain that control bees are uninfected whereas many of the co-evolved pathogens can exist as covert or inapparent infections (Hails *et al.*, 2008). Its effect can also be superimposed over those of co-evolved pathogens to understand the generic effects of a pathogen, the differences between coevolved and new association pathogens, and the effect of multiple pathogens on honeybees.

3.1.2: Experimental setup and hypotheses:

It has been suggested that the honeybee declines recorded in the UK, USA and elsewhere may be due to a combination of factors including pathogens and forage availability

(Genersch *et al.*, 2010; Oldroyd, 2007; Potts *et al.*, 2010b). Studies of bees infected with *N. ceranae* show that infected bees become more responsive to lower concentrations of sucrose and become hungry more quickly than uninfected bees (Mayack and Naug, 2009; Naug and Gibbs, 2009). Mayack and Naug (2009) suggest that the reduced longevity of bees infected with *Nosema* spp. was more likely to be due to energetic stress than any direct pathogenic effect. Infected bees, when fed with enough sucrose, were able to survive almost as long as uninfected controls (Naug and Gibbs, 2009). Bees infected with DWV were also more responsive to lower sucrose concentrations, suggesting that this virus may also cause energetic stress (Iqbal and Mueller, 2007). This was not the case for bees infested with varroa mites (Kralj *et al.*, 2007). One of the aims of this project was to examine the effect of pathogens and parasites as well as forage availability on honeybee survival as reduced forage availability may make honeybees more susceptible to the energetic demands associated with pathogen infection.

To examine this bees were used from a project funded by a BBSRC Industrial Partnership Award (IPA) that is currently on-going at Rothamsted (BBSRC project ref: BB/H00114X/1) 'Honeybee population dynamics: Integrating the effects of factors within the hive and in the landscape'. Experiments were designed for the collection of data to parameterise a model capable of predicting how combinations of forage availability and pathogen load affect the structure and survival of a bee colony. In 2011, 20 colonies were established with four treatments of differing disease loads and forage availability (see section 3.2.3). While the IPA project focused on gaining information at the colony level, these colonies could be used, as part of this PhD, to gain information on the effect of forage availability and disease load of the colony on survival at the level of the individual bee.

Newly emerged bees were collected from replicate colonies of each of the treatments and maintained in the laboratory to determine if the longevity of bees was influenced by whether they had been raised in colonies of differing background disease status and forage availability. It was hypothesised that bees from colonies with higher disease loads and with forage restrictions would show decreased longevity as they had developed with less resources and higher immune activity than bees from colonies with lower disease loads and freely available forage.

Another aim of these experiments was to characterise the growth of the fungus *M. anisopliae* and determine the effect of different concentrations on the mortality of honeybees so that the correct dose and timings could be chosen for later behavioural experiments. The chosen dose was then used on the bees from the different disease and forage treatments (project BB/H00114X/1) to see if disease load and forage availability had an effect on the bees' ability to survive additional pathogens. It was hypothesised that bees

from colonies with higher disease loads and restricted in their foraging would be more susceptible to the additional pathogen (*M. anisopliae*) than bees from healthier colonies that could forage freely. It is, however, possible that bees from colonies with higher disease loads, because their immune systems are already activated, might be better able to combat the additional pathogen. For example it has been shown that beetle (*Tenebrio molitor*) larvae injected with Ringer's solution, an isotonic solution relative to the bodily fluids of an animal, or Lipopolysaccharides (LPS) that mimic the cell walls of pathogenic bacteria, showed increased survival compared to uninjected beetle larvae when subsequently infected with the fungus *M. anisopliae* (Moret and Siva-Jothy, 2003). A similar study showed that in (Lepidoptera) immune priming can also be transgenerational with offspring of the individuals previously exposed to a low dose of virus as well as the individuals themselves become less susceptible to later viral infection (Tidbury *et al.*, 2011). In Moret and Siva-Jothy's (2003) study the LPS-injected larvae had increased antimicrobial activity in their haemolymph which may have been responsible for the increased survival. The larvae injected with Ringer's solution, by contrast, had higher levels of phenyloxidase (PO), an enzyme involved in melanisation. One of the purposes of melanisation is to prevent a breach of the cuticle which may be why injection causes increased PO activity and therefore melanisation (Sugumaran, 2002). The biting of varroa mites, which also breaks the cuticle, could also cause increased PO activity. Melanisation is one of the defences used against fungal infections, so, if the honeybees from high varroa colonies have increased antimicrobial activity or PO levels, then they may be better able to survive fungal inoculation.

3.2 Methods:

3.2.1 Experiments to quantify conidia germination and colony growth of *Metarhizium anisopliae* at different temperatures:

Metarhizium anisopliae isolate 445.99 from the Warwick HRI culture collection was used in all experiments (see Chapter 2.3-4 for culturing of *M. anisopliae* and collection of conidia). This isolate was originally taken from the commercial product Bio-Blast (Eco-Science corporation); its original host was unknown but it was known to infect honeybees (Shaw *et al.*, 2002). Mycelial growth rate was measured on Sabouraud Dextrose Agar (SDA) and conidia germination was measured both on SDA and on bee wings. Three temperatures were used: 23^{o}C: the temperature the fungus is usually cultured at, 30^{o}C: the temperature bees were maintained at for the learning experiments (see chapter 4), and 35^{o}C: the average temperature commonly found around the brood within a bee colony (Waring, 2006).

3.2.1.1 Experiment to determine the germination rate of populations of conidia of *Metarhizium anisopliae* at different temperatures:

Metarhizium anisopliae was cultured for 14 days on SDA (20ml SDA in 90mm diameter, triple vented Petri dishes and sealed with Parafilm) at 23°C in darkness (chapter 2.3). The conidia were then collected by adding 20ml of sterile 0.03% Tween 80 to the plate and gently dislodging them into suspension using a sterile loop. The resulting conidial suspension was filtered through muslin to remove any mycelium and then adjusted to 1 x 10^8 conidia ml^{-1} after counting using a Neubauer haemocytometer. Three 20µl drops were inoculated onto replicate SDA plates (20ml SDA in 90mm diameter, triple vented Petri dishes and sealed with Parafilm) or one 10µl drop per bee wing (two wings were set in ~20ml of 1% tap water agar per replicate 90mm diameter, triple vented Petri dish and sealed with Parafilm) and incubated at each of the three temperatures, 23, 30, 35°C. One plate from each temperature was sampled at each of 17 time points over 36 hours; to fit this more manageably into a working day the plates were set up at two time points (8am and 8pm) and then sampled at 1-4 hour intervals (table 3.1) and fixed with a 10% solution of cotton blue in lactophenol beneath a cover slip. A total of 19 plates were set up (one for each time point including 0). This experiment was repeated on three separate occasions.

The time intervals were chosen based on previous experiments (Davidson *et al.*, 2003) such that more measurements were taken when the greatest change in germination rate was predicted to occur. Each sample was examined using a microscope at x400 magnification and the number of germinated and ungerminated conidia were counted in a sample of approximately 100 conidia chosen at random in at least 3 fields of view in each drop. For a conidium to be considered as germinated its germ tube had to be longer than the width of the original conidium. The lengths of ten randomly chosen germ tubes produced by conidia in each drop (30/ replicate dish) were also measured by photographing the image and using video pro software to measure the length of the germ tubes (Deltapix: Infinity X; DPY video pro software).

Table 3.1:

set up 1: 8 am Day 1	
germination time	**recording time**
0	08:00
4	12:00
8	16:00
10	18:00
11	19:00
12	20:00
26	10:00
28	12:00
32	16:00
36	20:00

set up 2: 8 pm Day 1	
germination time	**recording time**
0	20:00
13	09:00
14	10:00
15	11:00
16	12:00
18	14:00
20	16:00
22	18:00
24	20:00

Table showing set up and recording times for the germination experiments. One plate of each type (SDA or tap water agar with bee wings) was set up at each temperature (23, 30 & 35°C) for each recording time. The experiment was repeated on 3 occasions.

These results were analysed in GenStat® (14th edition) (Payne, 2011). The germination data were analysed as proportions of the conidia population that had germinated, which were logit transformed and analysed using ANOVA. For the germ tube length measurements, as the length was only accurately measurable up to 100μm, after which the germ tubes tended to overlap and were impossible to differentiate, the data were censored (i.e. values only recorded to a maximum of 100μm). These data were analysed using ANOVA after replacing censored responses with values estimated using an iterative method tested by Taylor (1973), as implemented in the GenStat procedure CENSOR (Payne, 2011).

This experiment was repeated on three occasions, with the incubator temperatures alternated in a Latin square design such that each incubator had been used at each temperature after the 3 repeats to increase replication and account for any potential

incubator effects. The incubators were monitored using tinytag data loggers (Gemini data loggers UK Ltd.).

3.2.1.2 Experiment to determine the rate of colony growth of *Metarhizium anisopliae* at different temperatures:

20μl of a 1 x 10^8 conidia ml^{-1} suspension of *M. anisopliae* conidia in 0.03% Tween 80, produced as explained earlier (section 3.2.1.1), were evenly spread onto each of three plates of SDA (20ml in 90mm diameter, triple vented Petri dishes sealed with Parafilm) using a sterile spreader and incubated for two days at 23°C in darkness. A flame sterilised cork borer was then used to take three 6mm diameter plugs from each plate. Each plug was placed with the fungal surface down on each of three replicate SDA plates (20ml in 90mm diameter, triple vented Petri dishes sealed with Parafilm) and three replicates were cultured at each of three temperatures (23, 30 & 35°C) for two weeks in darkness. The radius of each fungal colony was recorded every 2-3 days over the two week period. This was achieved by taking four perpendicular radial measurements with vernier callipers accurate to 0.1mm.

This experiment was also repeated on three occasions using the same Latin square design for rotation of incubator temperatures (chapter 2.1.1) and the data were analysed in GenStat® (14th edition) (Payne, 2011) by fitting a linear mixed model, assuming correlated measurements over time within independent subjects, using restricted maximum likelihood (REML).

3.2.2 Multiple dose bioassay to determine the concentration of *M. anisopliae* for use in subsequent experiments:

This experiment used the bioassay boxes modified from those designed by Pain (1966) (chapter 2.1).

Metarhizium anisopliae was grown on SDA (20ml in 90mm diameter, triple vented Petri dishes sealed with Parafilm) at 23°C in darkness for 14 days and the conidia collected into 50ml centrifuge tubes as described previously (chapter 2.3-2.4). The concentration of *M. anisopliae* within 0.5g of power used in the bioassay was adjusted by mixing with sorbitol in different ratios determined by weight (preliminary tests showed that sorbitol did not affect the longevity of honeybees nor the germination of the fungus). The concentrations used were; 1:300, 1:100, 1:30, 1:10, 100% *M. anisopliae* conidia and controls of 100% sorbitol

and no powder at all. The bioassay was run on three occasions and on each occasion there were two replicate groups of approximately 20 bees for each concentration.

On each occasion two frames of brood were collected from Rothamsted hive 146 which had a low varroa count (0.4 mites per day in July 2010) and sealed brood present that were already beginning to emerge as adults. Newly emerged bees were collected as described previously (chapter 2.2.1). Approximately 25 bees were placed into each of fourteen bioassay boxes (two replicates per concentration) and maintained at 30^{o}C in darkness with water, 60% sucrose solution and pollen. The sucrose concentration was determined using a refractometer, a device for the measurement of an index of refraction using Snell's law (Feynman, 1963). Sugar refractometers are calibrated to show the concentration of sugar in a drop of solution. Three days after collection 20 bees from each group were inoculated with *M. anisopliae* as described previously (chapter 2.5).

Mortality was recorded twice daily, once between 9 and 10am and once between 4:30 and 6pm. Dead bees were removed, food and water were replenished and the blue roll replaced where necessary. The date that each dead bee was removed was recorded and survivorship curves produced. Kaplan-Meier plots with 95% confidence intervals (CI) were used to visualise the results and compare treatments. These plots were produced using GenStat® (14th edition) (Payne, 2011) with adaptation to the Kaplan-Meier program to add the CIs written by Suzanne Clark, Rothamsted Research BAB Department.

3.2.3 Experiment to determine the effect of pathogen load and forage availability on the longevity and survival of honeybees when challenged with the fungal pathogen *M. anisopliae*:

Newly emerged bees were taken from each of the project BB/H00114X/1 colonies in July, August and September of 2011 to examine the longevity of bees from each treatment and their susceptibility to additional pathogen challenge in the form of *M. anisopliae*. The design of the BB/H00114X/1 field experiment (executed by others) is described in Box 1 and followed by my experiment on longevity.

Box 1:

Five colonies were maintained in each of four treatments (20 in total): low disease and free foraging, high disease and free foraging, low disease and restricted foraging and high disease and restricted foraging (table 3.2).

Table 3.2:

		Forager availability	
Disease load		Normal	Reduced
	High	Hive 1	Hive 2
	Low	Hive 3	Hive 4

Treatments established for the project BB/H00114X/1 summer 2011 field experiment. Twenty colonies were set up, and of each treatment at each of five field sites.

In this experiment the level of disease was controlled by managing the numbers of varroa within the colonies. This was achieved by treating the low disease colonies against varroa using thymol (FERA, 2010) and choosing colonies with naturally high varroa levels (varroa drop counts of more than 5 mites per day on average (see chapter 1.3.4.2) for the high disease colonies. The high disease colonies were then not treated in any way against varroa. The level of varroa was monitored throughout the experiment using varroa drop counts and phoretic mite counts (FERA, 2010, see also chapter 1.3.4.2). The population size of varroa was used as a surrogate for disease load as the number of varroa in the hive is easier to measure and maintain/ manipulate directly than are the loads of pathogens. Several viruses are transmitted by varroa mites including DWV and ABPV and the loads of these viruses are known to correlate well with varroa loads (Tentcheva *et al.*, 2006; Tentcheva *et al.*, 2004b; Yue and Genersch, 2005). To ensure that the varroa loads did correlate with disease levels five samples of 20 adult bees were taken from inside each hive (section 2.2.2) and tested using PCR and QPCR (section 2.6) to see which other pathogens each colony had. Samples were collected at four time points: 1-3rd June 2011, 11-13th July 2011, 26-28th September 2011 and March/April 2012 for all colonies that survived to the following summer. Samples of adult bees were also taken from each colony that did not survive the winter when they were found to have died.

The colonies were maintained at five apiary sites with one colony from each treatment per site. The colonies were established using frames of bees from Rothamsted colonies between the 20th and 25th May 2011 and mated queens from 2010 were introduced on the 27th May. All colonies were left to acclimatise for one week before foraging restrictions were imposed. The level of forager was controlled by placing each hive within a pollination cage, a mesh cage 3m x 3m and 2m high, that was usually left open. The 'restricted forager' cages could be closed, preventing the bees from foraging. In the

treatments the cages were closed on the first working day of the week (typically Monday with the exception of bank holidays) within half an hour of sunset, when the bees were likely to have finished foraging and all have returned to the hives. The cages were kept closed throughout the following morning and reopened between 12:15 and 13:15 BST. This was repeated each working day such that the colonies were typically closed during mornings from Tuesday-Friday. These restrictions began on the week beginning the 6th June and finished 12 weeks later on Friday 26th August.

On two occasions during the summer of 2011 (14th July and 9th August) brood combs were removed from each of the colonies and newly emerged adult bees were collected and their longevity and susceptibility to a *M. anisopliae* challenge were determined. During the experiment the bees were kept under optimal conditions (with regard to temperature and food availability) so the experiment would examine the effect of the treatments only (disease load and forage availability during development) on survival as adults.

The frames were taken from the colonies and maintained in an incubator in darkness at 30°C whilst the young bees emerged. If a queen was not laying then bees could not be collected from that colony. During this experiment bees could not be collected from one high disease load, free foraging colony and only 20 bees could be collected from one low disease load restricted foraging colony.

Forty bees were collected from each hive/frame less than 24 hours after it had been removed from the colony (thus ensuring that all collected bees had emerged within 24 hours of each other). Of these, 20 bees would be inoculated with *M. anisopliae* and 20 bees sham inoculated with sorbitol. The frames were then returned to their colonies and the bees were maintained in bioassay cages (20 per cage) in incubators at 30°C and in darkness. They were fed on ground pollen (collected from bee colonies using pollen traps and stored at 5°C) 60% sucrose solution and water. The boxes were checked twice daily to ensure a constant food supply was always available.

After 4 days the bees were inoculated with conidia of *M. anisopliae* or sham inoculated with sorbitol as described previously (chapter 2.5). The concentration of *M. anisopliae* used in the bioassay was adjusted to 1:30 (this concentration was determined from the multiple dose bioassays described above (section 3.2.2) to ensure 100% mortality over approximately 8 days). The concentration was achieved by mixing with sorbitol to achieve a final weight of 0.5g.

Mortality was recorded twice daily, once between 7 and 9am and once between 4 and 6pm. Dead bees were removed, food and water was replenished, and the blue roll replaced where necessary. The date that each dead bee was removed was recorded and survivorship curves produced.

Kaplan-Meier plots with 95% CIs were used to visualise the results and compare treatments. These plots were produced using GenStat® (14th edition) (Payne, 2011) with adaptation to the Kaplan-Meier program to add the CIs written by Suzanne Clark.

Survival data for the bees inoculated with *M. anisopliae* were analysed separately from the controls because all inoculated bees died within 15 days whilst the control bees' survival ranged up to 70 days.

3.2.4 Experiment to determine the effect of pathogen load, forage availability during development, and pollen availability as adults, on the longevity and survival of honeybees:

As in 3.2.3, on the 6th September frames of brood were collected from all of the project BB/H00114X/1 colonies, maintained at 35°C overnight and newly emerged brood were collected for survival analysis. If the queen was not laying then bees could not be collected from that colony. For this experiment bees were not collected from one low disease load, restricted forage colony and two low disease load, free foraging colonies.

Forty bees were collected from each hive/frame, and split between two bioassay boxes. Half of the boxes were provisioned with 60% sucrose, water and pollen as before, the rest received 60% sucrose and water but no pollen. The boxes were checked twice daily to ensure a constant food supply was available.

Mortality was recorded twice daily, once between 7 and 9am and once between 4 and 6pm. Dead bees were removed, food and water was replenished, and the blue roll replaced where necessary. The date that each dead bee was removed was recorded and survivorship curves produced.

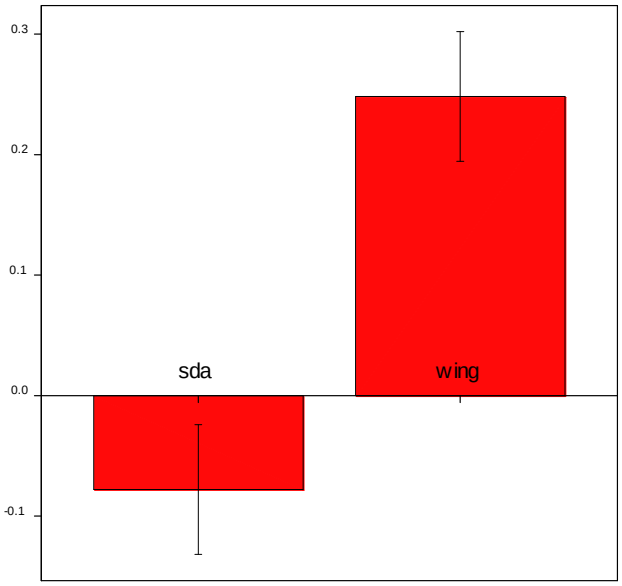
Kaplan-Meier plots with 95% CIs were used to visualise the results and compare treatments. These plots were produced using GenStat® (14th edition) (Payne, 2011) with adaptation to the Kaplan-Meier program to add the CIs written by Suzanne Clark.

3.3 Results:

3.3.1 Experiments to determine germination rate of *Metarhizium anisopliae* at different temperatures:

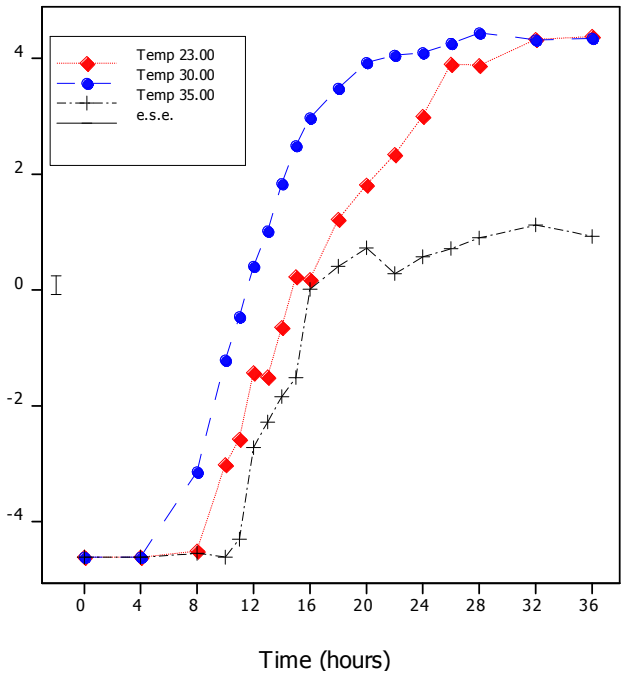
The germination rate of *M. anisopliae* conidia was significantly faster on bee wings than on SDA (ANOVAL: H_0: SDA mean = wing mean. SDA mean = -0.078, wing mean = 0.248 standard error of difference (SED) = 0.0761, $F_{1,210}$ = 18.38, P = <0.001), especially at 23°C (Figure 3.1). There was also a significant effect of temperature (ANOVA: : H_0: 23°C mean = 30°C mean = 35°C mean. 23°C mean = 0.131, 30°C mean = 1.535, 35°C mean = -1.410. SED = 0.2495, $F_{2,2}$ = 69.72, P = 0.014) with the fungus growing fastest at 30°C; at 35°C germination did not reach 100% within the course of the experiment (36 hours) (Figure 3.2).

Figure 3.1:



Graph showing the mean logit proportion of conidia that had germinated for *M. anisopliae* (445.99) when grown on SDA and bees wings. The germination rate was highest on bee wings. The error bars show the standard error of the mean (SEM) for this data.

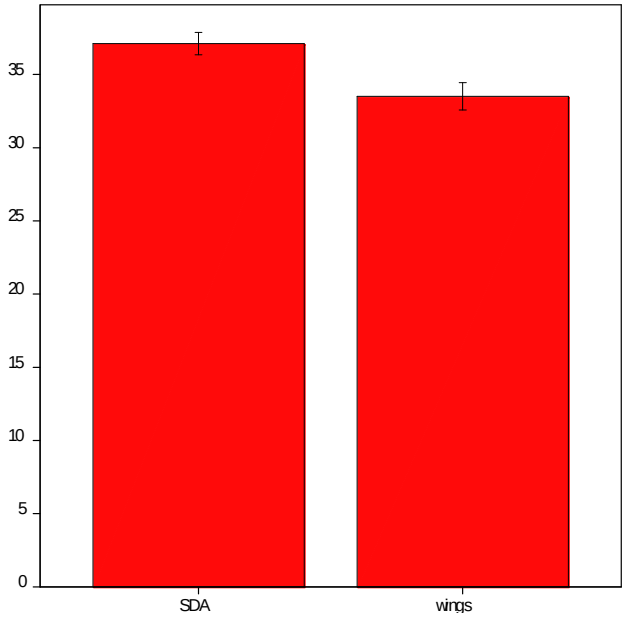
Figure 3.2:



Graph showing the mean logit proportion of conidia that had germinated over time for M. anisopliae (445.99). The germination rate was highest at 30°C. At 35°C 100% germination was not achieved within the time of the experiment (36 hours). The error bar (0,0 on the plot) is the average SEM for the data.

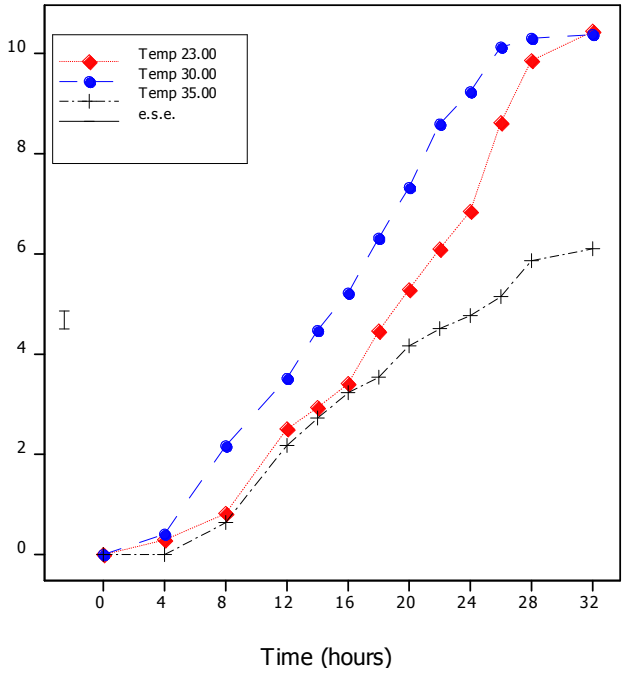
The rate of extension of germ tubes, however, was significantly faster on SDA compared to on bee wings (ANOVA using censor estimated values: H_0: agar mean = SDA mean. Agar mean = 4.79, SDA mean = 4.52 SED = 0.10, $F1_{,150}$ = 7.18, P = 0.008) (figure 3.3). There was also a significant effect of temperature (ANOVA using censor estimated values: H_0: 23°C mean = 30°C mean = 35°C mean. 23°C mean = 4.74, 30°C mean = 6.01, 35°C mean = 3.30 SED = 0.27 $F_{2,40}$ = 50.19, P = 0.002) with the extension rate being greatest at 30°C; at 35°C the germ tubes did not reach 100μm within the course of the experiment (36 hours) (figure 3.4)

Figure 3.3:



Graph showing the square root of the mean germ tube length for *M. anisopliae* (445.99) when grown on SDA and bees wings. The germ tubes grew best on agar. The error bars show the SEM for this data.

Figure 3.4:



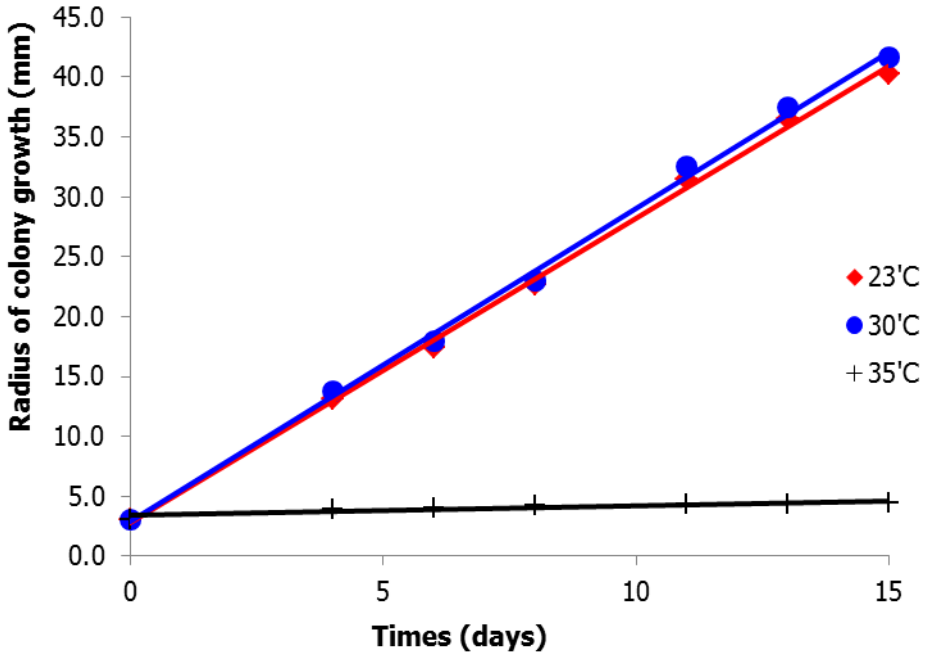
Graph showing the square root of the length of germ tube growth over time for *M. anisopliae* (445.99) incubated at 3 temperatures. The germination length grew fastest at 30°C and slowest at 35°C. The error bar shows and average SEM for the data set.

3.3.1 Experiments to determine colony growth of *Metarhizium anisopliae* at different temperatures:

There was very little difference in colony growth at 30°C compared with 23°C, but there was a dramatic decrease in colony growth at 35°C such that at this temperature the fungus was

barely able to grow at all (figure 3.5). There was a significant effect of both temperature and time on colony growth (REML: Temperature: H_0: 23°C mean = 30°C mean = 35°C mean. 23°C mean = 3.22, 30°C mean = 3.25, 35°C mean = 1.44 SED = 0.018 $F_{2,4}$ = 10040.89, P < 0.001. Time: H_0 = all time means equal. Means for each time point increase from 2.19 at 4 days to 2.97 at 15 days SED = 0.0069 $F_{5,109.8}$ = 2041.46, P < 0.001.) and a significant interaction between temperature and time (Interaction: $F_{10,\ 111.3}$ = 367.28, P < 0.001).

Figure 3.5:



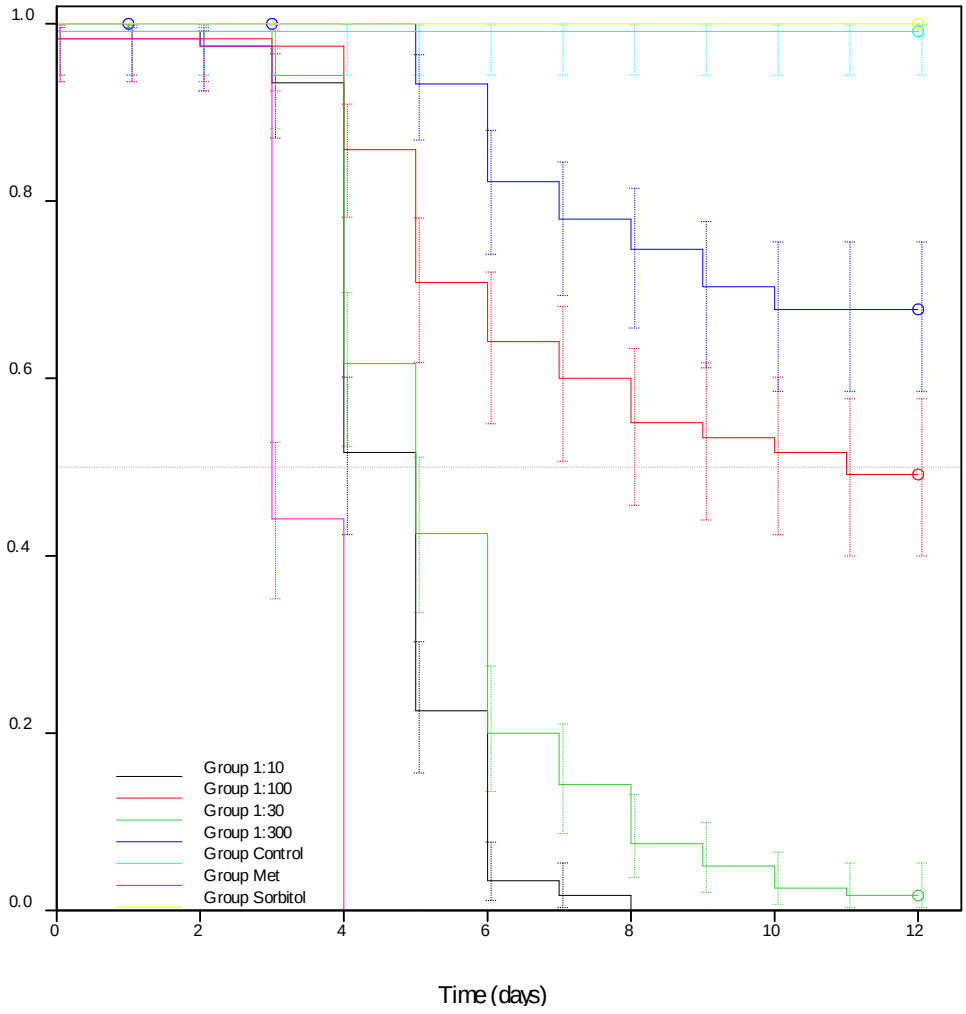
Colony growth of the fungus *M. anisopliae* recorded over a fifteen day period. There was a significant reduction in growth at 35°C compared to 23 and 30°C.

3.2.2 Multiple dose bioassay to determine the concentration of *M. anisopliae* to use in experiments:

There was an increase in the number of bees that died and a decrease in the time until death with increasing concentration of the fungus *M. anisopliae*; bees inoculated with 100% *M. anisopliae* conidia all died within four days, bees inoculated with a 1:10 to 1:30 concentration of fungal conidia had all died within two weeks (nb figure shows only up until day twelve as one repeat of this experiment could only be carried out for this long. In the other two repeats all bees inoculated with these concentrations had died within two weeks). Some bees survived the course of the experiment when inoculated with the lower concentrations (1:100 to 1:300) and in the control groups, only two of the bees inoculated with sorbitol died.

From the Kaplan-Meier plot with 95% CIs (figure 3.6) it is clear that there is a significant difference between the CIs for 4 groups of concentrations: 100% fungus, 1:10-1:30, 1:100-1:300 and the controls.

Figure 3.6:



Kaplan-Meier plot showing survival of bees after inoculation with different concentrations of the fungus *M. anisopliae* . There were only two deaths in the control treatment inoculated with sorbitol and no deaths in the uninoculated control group. There was an increase in the number of bees that died and a decrease in the time until death with increasing concentration of the fungus.

3.2.3 Experiment to determine the effect of background pathogen load and forage availability on the longevity and survival of honeybees when challenged with the fungal pathogen *M. anisopliae*:

Varroa drop counts and phoretic mite counts (see chapter 2.6) in July-September (when bees were taken for these experiments) confirmed that high disease colonies had, on average, higher numbers of varroa than the low disease colonies (ANOVA, H_0 = low varroa colonies mean = high varroa colonies mean. Mean values in table 3.3, SED = 1.81, $F_{1,79}$ = 145.51, $P < 0.001$).

Q-RT-PCR analysis of bees sampled from the Rothamsted colonies studied in this thesis, and in the BBSRC IPA project, showed that bees from the colonies with high average levels of varroa also had higher DWV loads compared to bees from the low varroa colonies (S. Hilton, Warwick University, unpublished). At the time of writing this thesis, other diseases have not yet been tested for.

Table 3.3:

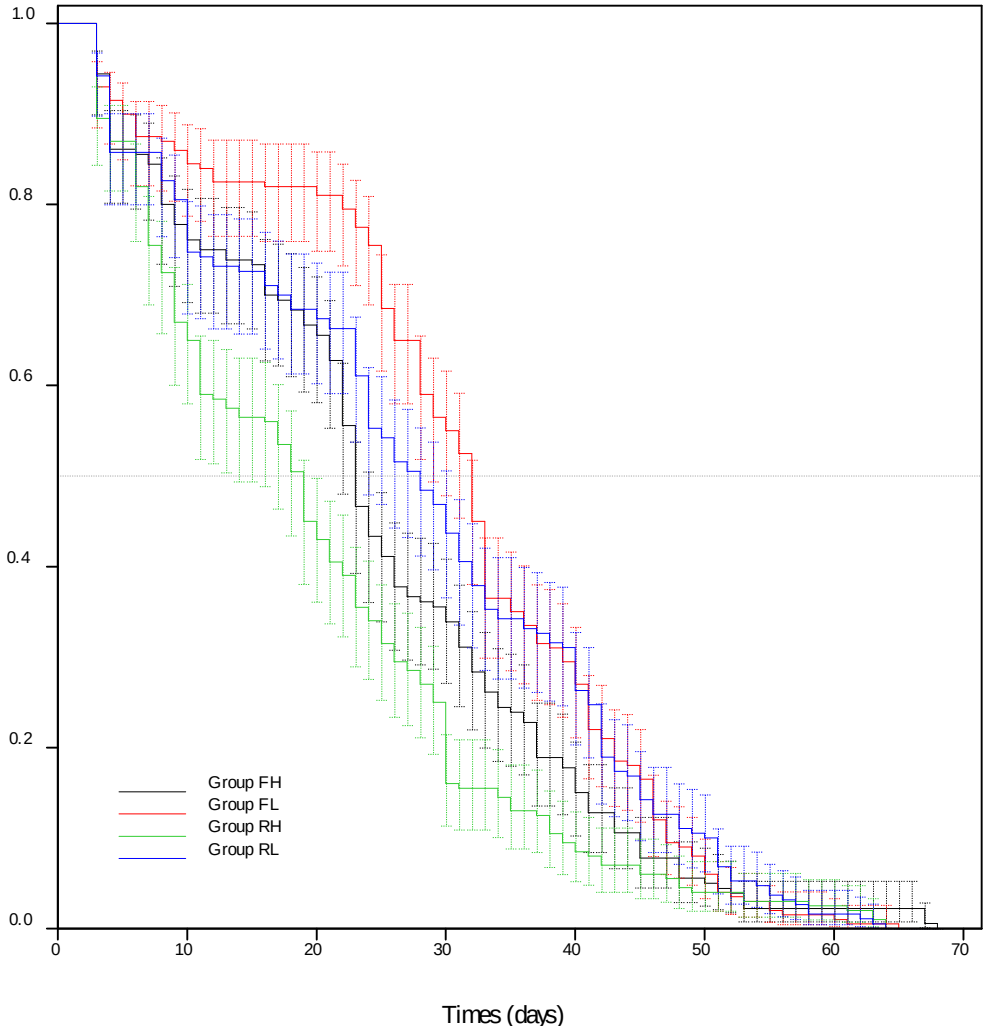
	Varroa drop counts mean (SEM)		phoretic mite counts mean (SEM)	
	July-Aug	**Aug-Sept**	**July-Aug**	**Aug-Sept**
FL	5.8 (2.4)	12.0 (3.1)	6.3 (2.6)	25.4 (6.3)
RL	7.1 (3.4)	9.0 (6.6)	8.0 (3.4)	28.6 (6.5)
FH	20.6 (6.9)	39.3 (19.7)	14.3 (1.6)	54.8 (9.2)
RH	24.4 (11.5)	29.7 (8.9)	21.6 (8.2)	54.1 (10.9)

Table showing the varroa counts for different treatments of colonies used in the survival experiments which took place between July and September 2011. FL (free forage, low disease) and RL (restricted forage, low disease) colonies have lower average varroa numbers by both counting methods than FH (free forage, high disease) and RH (restricted forage, high disease) colonies.

Both disease load and forage availability during development had an effect on survival of the adults. The bees from the low challenge, best case scenario colonies, those from colonies with low disease loads and able to forage freely, survived for the longest time and the those from high challenge colonies, with high disease loads and restricted in their foraging, showed the shortest survival and intermediate survival was seen in the intermediate treatment colonies. The Kaplan-Meier plot (figure 3.7) shows no significant difference between CIs at the start and end of the experiment suggesting that the difference in survival between treatments is only significant in the middle of the experiment, most obviously between 10 and 30 days. The plot also shows no significant difference between the CIs of the two intermediate treatments at all time points, suggesting that these treatments do not significantly differ from each other.

When the LT50s (median lethal times) are compared for each treatment, the bees from the colonies with restricted forage and high varroa loads had a shorter LT50 than any other treatment. The bees from these colonies died faster than any other treatment (confidence intervals do not overlap, table 3.4). The means for the other treatments were not significantly different from each other however.

Figure 3.7:



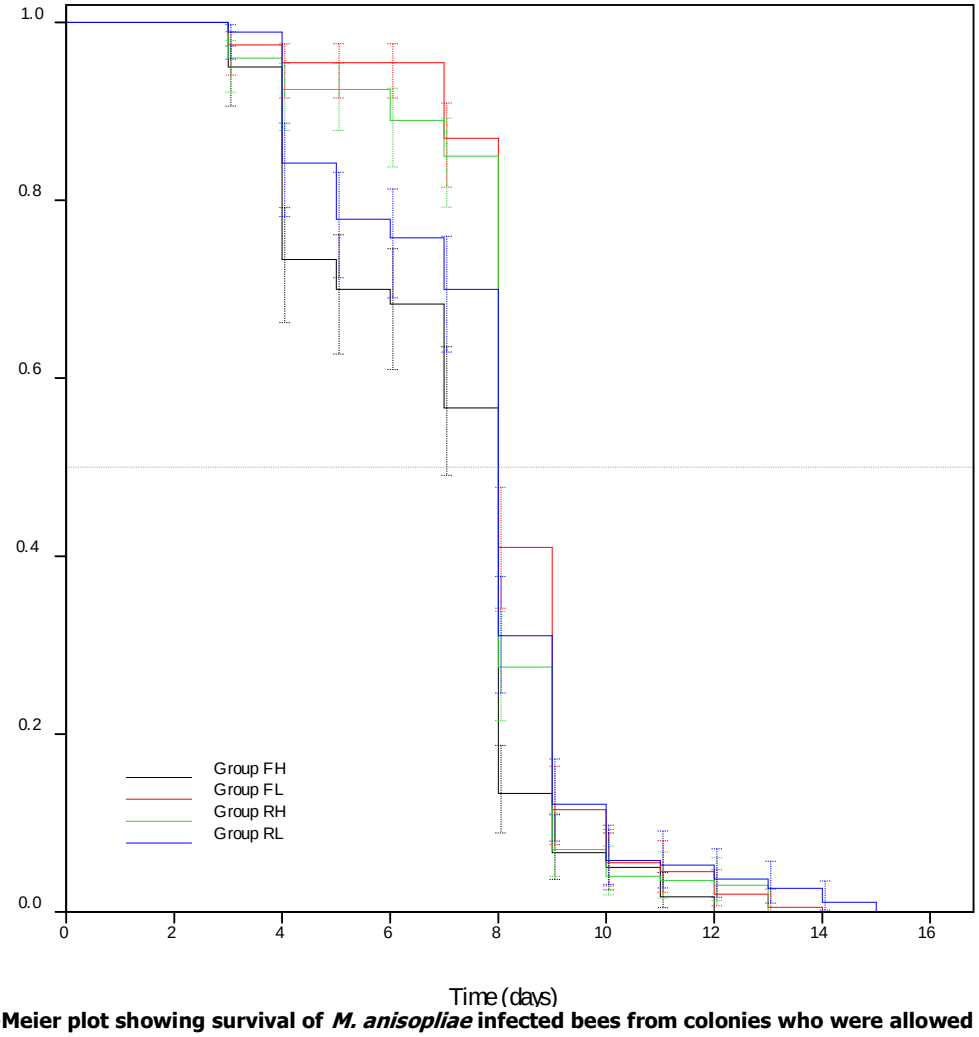
Kaplan-Meier plot showing survival of bees from colonies that were allowed to forage freely (F) or were restricted (R) in their foraging and with high (H) or low (L) varroa loads. The best survival was seen in bees from FL (Free forage, low varroa) colonies and the worst in RH (Restricted forage, high varroa) colonies. (FH N = 180, FL N = 200, RH N = 200, RL N = 190).

Table 3.4:

	LT50 (day)	upper CI	lower CI
Restricted forage, low varroa	27.2	30.2	23.6
Free forage, Low varroa	31.3	32.2	28.7
Free forage, High varroa	22.6	24.2	21.7
Restricted forage, High varroa	18.1	19.9	13.3

Table of LT50s (time until half the bees were dead) for each treatment when not inoculated with fungus. The bees from the colonies with restricted forage and high varroa loads had a significantly shorter LT50 (died significantly earlier) than any other treatment.

Figure 3.8:



Kaplan-Meier plot showing survival of *M. anisopliae* infected bees from colonies who were allowed to forage freely (F) or were restricted (R) in their foraging and with high (H) or low (L) varroa loads. The best survival was seen in bees from RH (Restricted forage, high varroa) and FL (Free forage, low varroa) colonies, and the worst in RL (Restricted forage, low varroa) and FH (Free forage, high varroa) colonies. (FH N = 180, FL N = 200, RH N = 200, RL N = 190).

When challenged with *M. anisopliae*, all bees died within sixteen days. There was only a significant difference in survival between 4 and 8 days when bees from the low challenge colonies: low disease load, free foraging colonies and the high challenge colonies: high disease load, restricted foraging colonies survived significantly better than those from the intermediate colonies: high disease load, free foraging, and the low disease load, restricted foraging (Figure 3.8).

When the LT50s are compared the bees from the free forage, low varroa colonies have the longest LT50s, whilst the bees from the colonies with free forage and high varroa had the shortest LT50s (CI for these colonies do not overlap with the others, table 3.4). The bees from both the restricted forage treatments have intermediate LT50s.

Table 3.5:

	LT50 (days)	upper CI	lower CI
Restricted forage, low varroa	7.5	7.6	7.4
Free forage, Low varroa	7.9	8.1	7.7
Free forage, High varroa	7.2	7.3	6.9
Restricted forage, High varroa	7.6	7.7	7.5

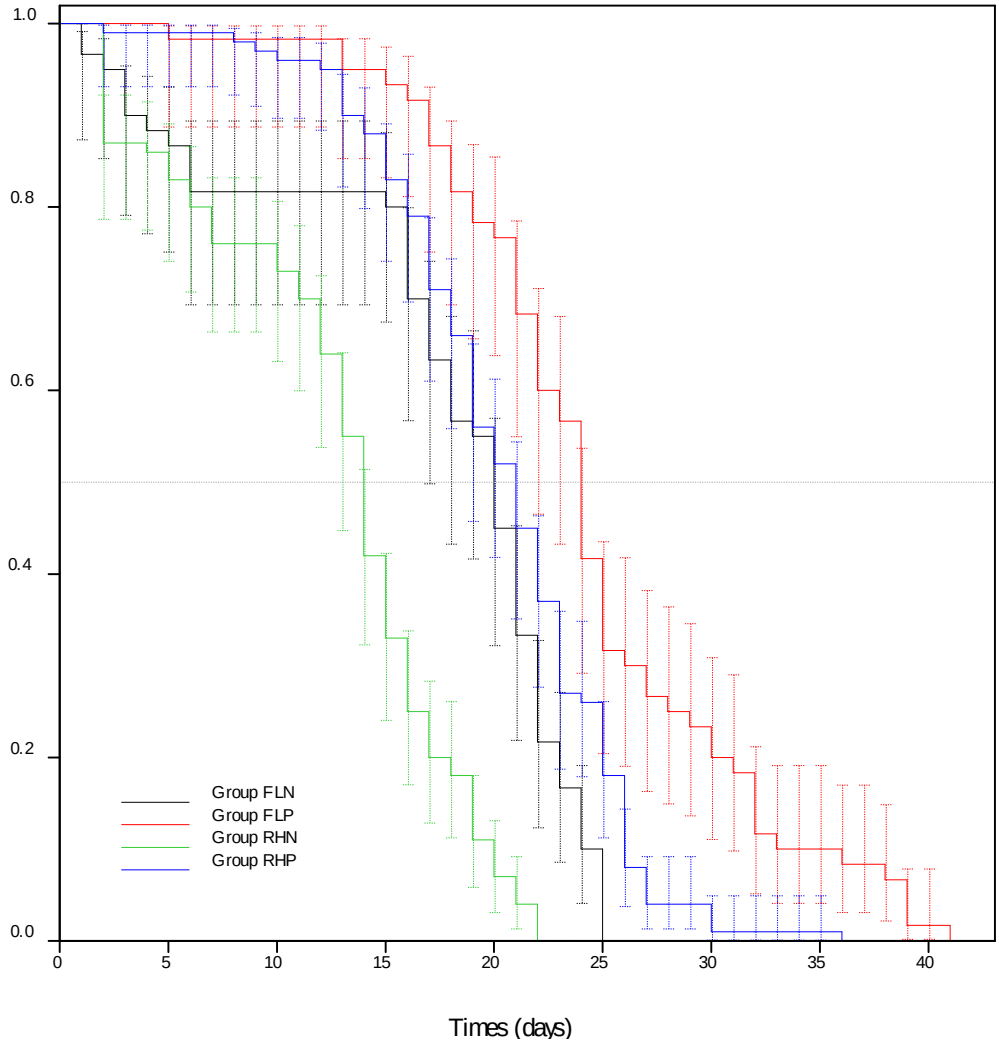
Table of LT50s (time until half the bees were dead) for each treatment when inoculated with the fungus *M. anisopliae*. The bees from the colonies with free forage and high varroa has significantly shorter LT50s than the bees from the restricted forage colonies who in turn had significantly shorter LT50s than the bees from the colonies with free forage and low varroa.

3.3.4 Experiment to determine the effect of pathogen load and forage availability during development and pollen availability as adults on the longevity and survival of honeybees:

When bees from these colonies were maintained with and without pollen, the bees from the low challenge colonies, those with low disease loads and able to forage freely, survived for the longest. The high challenge colonies, with high disease loads and restricted in their foraging, showed the worst survival. But when the low challenge colony bees where starved of pollen their survival was as bad as for those bees from the high challenge colonies who had been given pollen (Figure 3.9). The increased longevity seen in the high challenge colonies when provided with pollen is more than the decrease in survival seen in the low challenge colonies when starved of pollen.

Similar results were found when comparing the LT50s. The bees from the low challenge colonies have the longest LT50 and the bees from the high challenge colonies have the shortest LT50s. However, bees from the high challenge colonies that had been given pollen and bees from the low challenge colonies but deprived of pollen were not significantly different (table 3.5).

Figure 3.9:



Kaplan-Meier plot showing survival of bees with (P) and without (N) pollen, from colonies who were allowed to forage freely (F) or were restricted (R) in their foraging and with high (H) or low (L) varroa loads. This plot shows the lowest and highest challenged colonies, bees from high varroa, restricted forager colonies and bees from low varroa, free foraging colonies. (FLP, N=60. FLN, N=60. FHP, N=100. FHN, N=100. RLP, N=80. RLN, N=80. RFP, N=100. RHN, N=100).

Table 3.6:

	LT50 (days)	upper CI	lower CI
Free forage, Low varroa, Pollen	23.4	24.4	21.6
Free forage, Low varroa, No pollen	19.5	20.6	17.0
Restricted forage, High varroa, Pollen	20.3	21.6	18.6
Restricted forage, High varroa, No pollen	13.4	14.2	12.4

Table of LT50s (time until half the bees were dead) for the low and high challenge colonies when fed or deprived of pollen. The low challenge colonies bees had the longest LT50 and the high challenge colonies bees had the shortest. When bees from the low challenge colonies were deprived of pollen or bees from the high challenge colonies were fed pollen their LT50s were not significantly different from each other.

3.4 Discussion:

Metarhizium anisopliae has a broad range of invertebrate hosts, (Roberts and Humber, 1981) and different isolates are pathogenic to different species (e.g. Chandler *et al.*, 2001) which makes it a good candidate use as a biological control agent against many pests and also as a model system for studying the effect of pathogens on their hosts.

None co-evolved pathogens like *M. anisopliae* can provide insights into the potential effects of newly introduced parasites (Boughton *et al.*, 2011) or for understanding the consequences of an invasion by a closely related host carrying parasites (Dhondt *et al.*, 2008), and the global phenomenon of increases in emergent diseases (Marra *et al.*, 2004). They are also often used as biological pesticides and so effects on potential, unintended, hosts require study (Chandler *et al.*, 2001; Shaw *et al.*, 2002). But they can also be used to show changes in behaviour or physiology that would be difficult to do with co-evolved species. For example, Joop *et al.* (2006) assessed survival of wild-caught damselflies after infection with a novel entomopathogenic fungus to show that survival depended on colour morph and sex.

An artificial immune insult could be used in place of a model pathogen to examine the effect of activation of the immune system on behaviour because all hosts will be naïve to it, it can be highly controlled and it removes potential host-parasite interactions. Examples include Phytohemagglutinin (Biard *et al.*, 2009) and sheep red blood cells (Snoeijs *et al.*, 2007) in vertebrates and lipopolysaccharides (Riddell and Mallon, 2006) in invertebrates. However, using an artificial immune insult does not reflect how the immune response will react to a living pathogen (Hanssen *et al.*, 2004).

It is difficult to inoculate bees with many of their co-evolved pathogens, especially the viruses which often need to be transmitted by injection. But merely injected the bee can cause immunological and behavioural responses (Mallon *et al.*, 2003). *Metarhizium anisopliae* infection was chosen as a model system for studying the effect of pathogens on honeybee survival and learning behaviour (see chapter 4) because of its ease of use (the bees could be inoculated with a known dose of a powder with no need for injection or feeding by hand) and because it was unlikely to be present in any of the bees used in these experiments so that all individuals would be equally naïve to it.

The main aim of these experiments was to characterise the growth of the fungus *M. anisopliae* for use in later experiments and then to use the fungus in a model system to

analyse the effect of multiple factors (forage availability, co-evolved pathogens and additional pathogens) on honeybee survival.

There was a significant difference in the germination rate of the fungus *M. anisopliae* when grown on different media (SDA or honeybee wings set in tap water agar) and at different temperatures. This was determined using both germination rate (% conidia that had germinated) and germ tube elongation. Germination rate was fastest on bee wings but then germ tubes grew faster on the SDA. It has been shown that for an isolate of *M.* anisopliae specific to the locust species *Schistocerca gregaria*, only host cuticle stimulated the full developmental program of germination, cuticles of other insects caused reduced or even no germination (Wang and Leger, 2005). The isolate used in these experiments, whilst pathogenic to honeybees, is not co-evolved with them and so there may be a similar reduction in germination or in growth. This may be due to some defence mechanism, for example honeybees are known to secrete Metalloproteases, enzymes containing metallic ions, predominantly zinc ions, which help prevent infection (Strachecka *et al.*, 2012) and leaf cutter ants have been found to have symbiotic bacteria on their cuticles which protect them from entomopathogenic fungi (Mattoso *et al.*, 2012). And antifungal chemicals were found on flea beetles inoculated with *M. anisopliae* in an experiment by Butt *et al.* (1995), but in that experiment the fungus grew better on dead than live beetles, suggesting that the antifungal chemicals are only present on live insect cuticle. However other isolates of this fungus have been germinated and grown on several growth media including SDA (Ibrahim *et al.*, 2002).

An alternative explanation for the difference in growth and germination rate on the different media is that the fungus can detect when it is on a potential host species and so germinates faster when it detects the bee wings than it does on the SDA. Studies have shown that *M. anisopliae* changes its gene expression profile dramatically depending on whether it was growing in a nutrient rich medium or on a host cuticle (Freimoser *et al.*, 2005; Wang *et al.*, 2005). The genes up-regulated in response to host cuticle are those associated with cuticle degradation and combatting the host's immune system, suggesting that the fungus is able to detect its host and alter its growth patterns accordingly. It has also been shown that an isolate of this fungus which targets Lepidoptera has cell-bound enzymes which aid cuticle degradation and these enzymes were more abundant on spores taken from host cuticle than SDA, again suggesting that the fungus can adapt to its environment (St Leger *et al.*, 1991). The fact that the fungus then probably grew germ tubes faster on the SDA could be because this is a more nutritious medium providing more nutrients for growth, as has been shown for other isolated of *M. anisopliae* which grew fastest on nutrient rich media than nutrient poor media (Ibrahim *et al.*, 2002).

There was reduced germ tube growth and germination at 35°C so that there was not 100% germination and nor had germ tubes grown to 100μm within the course of the experiment (36 hours). The radial growth experiment showed similar results with very little radial growth at 35°C. This suggests that within the hive, where bees can easily maintain a temperature of 35°C for their brood (Hooper, 2008), the fungus is unlikely to grow well. For this reason all inoculated bees were maintained at 30°C for these experiments. Some isolates of *M. anisopliae* have been shown to grow at 35°C, but the optimal temperature range for most isolates studied is lower than this (Ouedraogo *et al.*, 1997).

In the multiple dose bioassay 100% mortality was only achieved with doses of 1:30 and above with some bees surviving at lower doses. These bees may have overcome infection, or there may have been individuals that were not exposed to the conidia. The results of this experiment were used to choose the dose appropriate for future experiments. It was decided that 100% mortality was required to ensure that all bees had actually been infected. Sufficient time was also required between inoculation and death for behavioural experiments to take place (see chapter 4) so a dose of 1:30 was chosen as this dose caused 100% mortality over the longest period of time; the majority of inoculated bees survived for up to six days.

This dose of *M. anisopliae*, when used on bees from colonies of differing varroa load and forage availability, killed all inoculated bees within sixteen days. There was no significant effect of either treatment (disease load or forage availability) on survival, but there was a significant interaction. This is because both the low and high challenge colonies bees survived longest when compared with the intermediate treatments. This suggests that neither factor contributed to survival when challenged with the fungus which may be because the dose was too high for any benefit of a primed immune system or good forage availability during development to be seen. It is possible that if a lower concentration of *M. anisopliae* had been used, 1:100 or 1:300 where 100% mortality was not achieved, that there might have been a difference between treatments in the proportion of bees that survived as bees from the low challenge colonies are better able to fight off the fungus or bees from the high varroa colonies have primed immune systems which help them fight off the fungus. For example in Moret and Siva-Jothy's (2003) study on immune primed beetle larvae, the larvae injected with Ringer's solution or LPS to stimulate an immune response were then better able to survive subsequent challenge with *M. anisopliae* and some inoculated beetles survived for over fifty days.

There was a significant effect of colony treatment on survival without the added fungal pathogen. In the lab, the bees from colonies with low disease loads and able to forage freely survived best whilst those from colonies with high disease loads that were restricted in their foraging survived the worst. The intermediate treatments showed intermediate survival with neither combination significantly different from the other. This suggests that disease load and forage availability during development are equally important in determining survival. Kunert and Crailsheim (1988) did a two year study recording the forage availability and quality of colonies and the weight and survival of newly emerged bees. They found no correlation between larval feeding and longevity. In fact that study found that life span seemed to be linked with productivity of the colony such that when there were the most resources (pollen and nectar) being brought into the hive, bees had the shortest lives. Life span has been linked with flight distance (Neukirch, 1982) and wing damage (which increases with increased foraging activity) (Higginson *et al.*, 2011). My results, however, measure the longevity of adult bees kept under ideal conditions without the need to forage or care for brood, similar to studies used to look at survival of bees when infected with *Nosema* spp. (Mayack and Naug, 2009; Rinderer and Elliott, 1977; Rutrecht and Brown, 2009). It should be remembered that laboratory experiments are simplified compared with the real world and any conclusions taken from them should be considered with respect to factors that are not accounted for in the experiment. For example the bees in cages couldn't fly more than a few centimetres and did not have brood to care for.

My results suggest that bees from the high disease load colonies who were restricted in their foraging did not live as long as the healthier bees so that, within the high disease colonies, there were fewer bees in total and thus fewer forager bees to bring back pollen and nectar. This would mean that colonies with high disease loads are less able to bring in forage than healthy colonies so they would have fewer stores for the winter. This may be one of the reasons, along with reduced worker longevity, that pathogen infected colonies are less likely to survive over winter (e.g. Bach Kim *et al.*, 2011; van Dooremalen *et al.*, 2012).

When bees from the high challenge colonies (high varroa and restricted foraging), that usually survive the least well under ideal conditions, were fed with pollen their survival increased. Equally, when bees from the low challenge colonies (low varroa and free foraging) were starved of pollen their survival decreased. But bees from the high challenge colonies that were fed pollen still survived less well than bees from the best colonies that were fed with pollen. These results suggest that bees require adequate resources throughout their development and whilst feeding bees after the colony has gone through a period of sparse forage or when they are suffering from high disease loads will help them

improve their survival, they really need a constant supply or stores of both nectar and pollen resources. For example Schmidt and Buchmann (1985) determined the average requirement for a small colony within a flight cage to be 3.07 mg N per bee per twenty-eight days. This corresponds to 19.2 mg protein. This also suggests that the immune system is energetically costly to the bees (Chown and Nicolson, 2004). It has been shown that a high pollen diet can aid in survival of bees infected with *Nosema apis* in caged trials (Rinderer and Elliott, 1977), but under similar conditions in the field the added pollen did not aid survival (Mattila and Otis, 2006). This result highlights the fact that field conditions can be very different from the controlled environment of the laboratory. For example, although feeding the bees pollen in this experiment, and in Rinderer and Elliott's (Rinderer and Elliott, 1977) experiment, aided the survival of infected bees, in the field excess pollen may be used preferentially to feed young or bees may be under more energetic stress as they forage so the added pollen may not actually aid survival to the extent that it does in the caged experiments.

In conclusion *M. anisopliae* infection could work well as a model system for investigating the effect of pathogens on honeybees because of its ease of use. It was however too fast acting at the dose used here to show any significant difference in the survival of bees from colonies with differing disease and forage treatments. It was clear however that both forage availability and disease load have an effect on honeybee survival in the laboratory and pollen feeding can aid in survival under laboratory conditions.

Chapter 4: The effect of pathogens on honeybee learning and sucrose sensitivity.

Abstract:

The European honeybee, *Apis mellifera*, is important economically not just for honey production but also as a pollinator. However, honeybee numbers in some areas are declining. A range of interacting factors are thought to be involved, including pathogens and parasites, which are known to cause changes in the behaviour of their hosts.

The learning ability of honeybees was evaluated in this chapter using condition proboscis extension, an existing method that has already been used to look at the effect of some pathogens on learning ability in bees (e.g. Iqbal and Mueller, 2007; Kralj *et al.*, 2007). Here the method was used to test the suitability of the fungus, *M. anisopliae* as a model pathogen as well as studying the effect of naturally occurring viruses and microsporidia.

Metarhizium anisopliae infected nurse bees were less able to learn than controls however infected forage bees seemed to learn better than controls. This may be due to different immune competence of different aged bees. The increased learning ability may be due to increased hunger leading to increased responsiveness to the sucrose stimulus. The results also suggest that *Nosema* spp. and sac brood virus cause decreases in learning ability.

4.1 Introduction

4.1.1 Behavioural effects of pathogens and parasites on honeybees:

Many species of pathogens and parasites are thought to influence their hosts' behaviour. These behavioural effects can be diverse but one form they can take involves changes in learning ability (e.g. Iqbal and Mueller, 2007; Kralj *et al.*, 2007; Mallon *et al.*, 2003). This is especially important in honeybees, which need to navigate large areas of the landscape to find nest sites and resources, use the position of the sun and landmarks to remember where these sites and resources are, and communicate that information within the hive via the waggle dance - all of which require both learning and memory (Menzel and Giurfa, 2001). Other behavioural changes brought about by pathogens have been seen in several insect species including changes in movement, grooming, thermoregulation and appetite (e.g. Roy *et al.*, 2006).

These behavioural effects may be as a result of 'adaptive host manipulation' as seen when the pathogen/parasite causes changes in the host's behaviour to aid transmission (Poulin, 1995). One of the best examples of this are the so-called 'zombie-ants'. Ants infected with fungi of the *Ophiocordyceps* clade leave their nests and bite onto leaves at the tops of plants before they die due to infection. This ensures that the infected ant is in the best elevated position for the fungus to complete its lifecycle and distribute its spores (Lefèvre *et al.*, 2009). Hosts can also demonstrate adaptations in the form of defensive behaviours, such as grooming, in response to infection/attack. Ants inoculated with the fungus *Metarhizium anisopliae,* for example, show an increase in self-grooming behaviour. Ants also groom nest mates that return to the colony after foraging irrespective of whether they have been experimentally inoculated with the fungus or not. These behavioural adaptations decrease the number of spores on inoculated individuals and thus decrease the likelihood of infection and transmission of the fungus through the colony (Reber *et al.*, 2011). However, behavioural changes may not be adaptive and may simply be due to the inevitable deleterious effects of invasion as the pathogen/parasite consumes the host's tissue (Poulin, 1998).

It is often difficult to differentiate between effects caused by the pathogen/parasite and effects caused by the host because of a lack of information about causal mechanisms of behavioural change or evidence of effects on fitness. For this reason, experiments done to elucidate relationships between disease and behavioural changes caused by altered learning ability in both honeybees and bumblebees have used injected lipopolysaccharides (LPS) as a surrogate for infection (Mallon *et al.*, 2003; Riddell and Mallon, 2006). LPS mimics bacterial cell walls thereby activating the bee's immune system without any pathogenic effect. These experiments showed that bees injected with LPS were less able to learn to associate a scent with a food reward than bees that were injected with a control of Ringer's solution (Mallon *et al.*, 2003; Riddell and Mallon, 2006). This was especially evident in pollen-starved bees (Riddell and Mallon, 2006). As pollen is the only source of protein for bees, this suggests that there may be a protein involved in both the immune system and in learning that is used preferentially by the immune system. Riddell and Mallon (2006) suggested some potential candidate proteins (octopamine or eicosanoids), but more research is needed in this area. It is also known that mounting an immune response is energetically expensive; wound healing and encapsulation in insects can raise the metabolic rate by up to 28% (Ardia *et al.*, 2012). So lack of appropriate nutrition, both nectar and pollen, would likely have a negative effect on immunity. For example if mosquito larvae are raised with suboptimal nutrition or completely starved then they become more susceptible than controls to viral infection (Muturi *et al.*, 2011). This was seen in chapter 3 where bees raised in colonies with

restricted forage survived for less time as adults on average than those raised in freely foraging colonies.

Similar experiments have shown that both varroa mites and DWV decrease learning ability in honeybees (Iqbal and Mueller, 2007; Kralj *et al.*, 2007). Bees injected with DWV-containing lysate (an extract from bees) were less able to learn to associate a scent with a sucrose reward and were also less likely to remember the association after two, and especially after 24 hours, than bees injected with DWV-free lysate (Iqbal and Mueller, 2007). Bees infested with varroa mites, however, showed decreased learning ability when tested after 1 minute but were not significantly different to uninfested controls after 12 minutes (Kralj *et al.*, 2007). These decreases in learning ability may be due to activation of the immune system as shown in Riddell and Mallon's experiments (2003; 2006) or due to the direct influence of the pathogen/parasite, or a combination of the two.

Studies of bees infected with *Nosema ceranae* showed that they became more responsive to lower concentrations of sucrose and became hungry more quickly than uninfected bees (Mayack and Naug, 2009; Naug and Gibbs, 2009). Mayack and Naug (2009) suggested that the reduced longevity observed in bees infected with *Nosema* spp. was more likely to be due to energetic stress than any other pathogenic effect. Infected bees, when fed with enough sucrose, were able to survive almost as long as uninfected controls. Infected bees were also less likely to share food with nest mates (Naug and Gibbs, 2009). This is important because *Nosema* spp. can be transmitted during feeding and it was originally thought that the increased hunger associated with infection might lead to increased food sharing which would have aided transmission (Mayack and Naug, 2009). Bees infected with DWV were also more responsive to lower sucrose concentrations, suggesting that energetic stress may also be caused by this virus, although this was not the case for bees infested with varroa mites (Iqbal and Mueller, 2007; Kralj *et al.*, 2007).

The experiments in this chapter use classical conditioning to examine the effect of honeybee pathogens on learning by using the fungus *Metarhizium anisopliae* (see chapter 3) as a model, generalist pathogen.

4.1.2 Conditioned Proboscis Extension (CPE):

CPE is a common behavioural tool used to measure learning and memory in insects (El Hassani *et al.*, 2008; Farooqui, 2008; Ramirez-Romero *et al.*, 2008). It relies on the proboscis extension reflex (PER), a reflex found in many insects eg Diptera (e.g. Nakamura *et al.*, 2008) Lepidoptera (e.g. Omura *et al.*, 2000), Hymenoptera (e.g. Abramson *et al.*,

2008), and was first described by Minnich (1921). When presented with a food source, sucrose for example, the insect extends its proboscis. If a scent is presented at the same time as the food source the insect will associate the scent with the food such that the scent alone will ultimately stimulate the extension reflex. This is an example of classical conditioning as first demonstrated by Ivan Pavlov who received a Nobel Prize for his research in 1904 (Pavlov, 1927). Frings (1944) was the first to condition the proboscis extension reflex and experiments using it have since been refined based on further research (Bitterman *et al.*, 1983). It was the use of this method which demonstrated that both DWV and varroa infestation affected honeybee learning (Iqbal and Mueller, 2007; Kralj *et al.*, 2007) and that the immune system was involved (Mallon *et al.*, 2003; Riddell and Mallon, 2006).

Some studies have shown that CPE was best done using foraging bees rather than the younger hive bees (Behrends *et al.*, 2007) as foraging bees were considered more responsive to gustatory stimuli. However, young 6 day old adult bees can be conditioned and 10 day old bees show results comparable to forager bees (Ray and Ferneyhough, 1997). As part of this project, preliminary experiments were done using Rothamsted bees to verify this information and to better determine what age of bees would be most appropriate for each behavioural experiment.

In 4.2.2 CPE was used to study the effect of a pathogen, the fungus *Metarhizium anisopliae*, on honeybee learning throughout the infection period. This was accomplished by testing the learning ability of infected bees 2, 4 and 6 days after inoculation. It was hypothesised that learning ability would decrease in infected bees and that the decrease in learning ability would become more pronounced over time.

The method was also used in 4.2.3 to study *M. anisopliae*-infected bees that were either young adult bees collected after emergence, or older forager bees to determine whether age had an effect on the bees' response to infection. It was hypothesised that young adult bees would respond differently to forager bees. All bees were killed after experimentation and stored at -80^{o}C for QPCR to detect and quantify co-evolved pathogens. It was hypothesised that, within each age group, bees that were also infected with other pathogens (potentially multiple pathogens), or with higher pathogen loads would show decreased learning ability compared to bees with fewer pathogens or lower pathogen loads.

CPE was then used in 4.2.4 to test the effect of *M. anisopliae* infection on forager bee learning when the bees were also pollen-starved. It was hypothesised that any potential decreases in learning associated with infection would be exacerbated by pollen starvation

because pollen is the bees' only source of protein and several proteins are required for both learning and immunity (Riddell and Mallon, 2006).

Furthermore, in 4.2.5 *M. anisopliae*-infected and uninfected forager bees were examined in a sucrose sensitivity experiment to determine whether *M. anisopliae*-infected bees were more responsive to lower concentrations of sucrose and thus hungrier than uninfected bees. These experiments were also done on bees two, four and six days after infection. It was hypothesised that infected bees would be energetically stressed by the fungal infection and would thus be hungrier and respond to lower concentrations of sucrose. It was further hypothesised that the difference in responsiveness would increase over the course of the infection such that the bees tested six days after inoculation would respond to lower concentrations of sucrose then those tested four days after inoculation.

4.2 Methods

4.2.1 General methods for CPE:

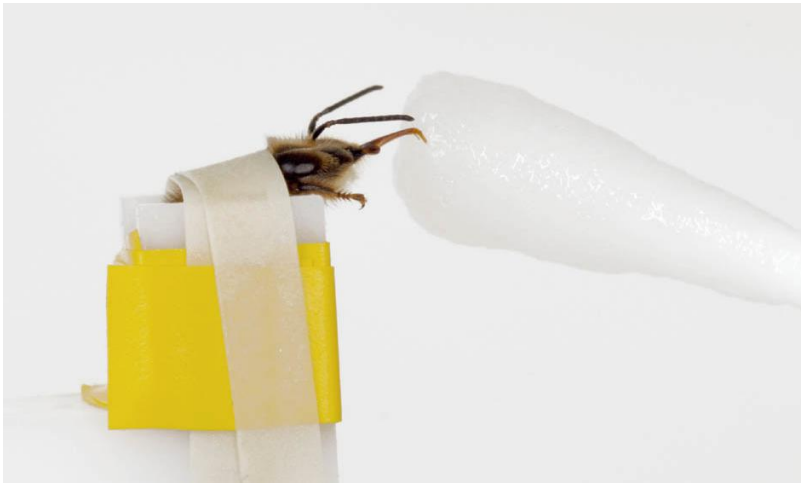
Classical conditioning in the form of CPE was used to test the bees' ability to learn and retain information. The techniques used were modified from those used by Dr Mathilde Briens of Inscentinel (http://www.inscentinel.com/) and adapted from a technique similar to Bitterman *et al*. (1983).

Bees were fed with 60% sucrose and then starved for 3-4 hours before experimentation began so that they would all be equally responsive to the sucrose stimulant. Bees were immobilised by chilling at 5˚C, as described previously (chapter 2.5), and then secured in glass tubes with PVC tape (Figure 4.1). This held the bee secure whilst leaving its antennae and proboscis free. The bees were left for 30 minutes to habituate under ambient conditions (22˚C, in light).

All CPE experiments were done in a controlled environment room at 22^{o}C with an air extractor to remove odours. Each bee was submitted to six training trials. For the first training trial, each bee in turn was placed in an air stream set at 2l/min and exposed to clean air for 15s. The conditioned stimulus (citronella) was then introduced for 5s without changing the overall air flow. After the first 3s of odour stimulation the bee was presented with 30% sucrose on a cotton wool bud that was touched to the bee's antennae until the bee extended her proboscis. This allowed the bee to associate the conditioned stimulus (citronella) with the unconditioned response (extending the proboscis for food). The bee

was then allowed to feed for the final 2s of conditioned stimulus, reinforcing the association, then left for a further 15s in clean air with no sucrose stimulation.

Figure 4.1:



A bee secured for CPE experimentation. The bee is secured in such a way that the antennae are free and it can extend its proboscis. It is held in a glass tube by a band of electrical tape (yellow) and by thinner tape (lining tape) to hold its head in place (grey).

This process was repeated for each bee in turn, taking about 1 minute per bee. As there were 32 bees tested on each day of this experiment, this meant that each bee's subsequent training trial occurred after 30 minutes. In the subsequent trials each bee was expected to extend its proboscis within the first 3s of citronella stimulation without need for sucrose, indicating it had learnt to associate the scent with a food reward and had responded to the scent alone. If the bee extended her proboscis in response to the citronella she was rewarded by being fed sucrose, if not then the sucrose was touched to her antennae again as in the first training trial to reinforce the association.

In the sixth trial there was no sucrose stimulation, giving an extra 2s for the bee to respond to the citronella. On each occasion the bee's response was recorded; either the bee responded to the odour alone, responded only to the sucrose or responded to neither. For each bee, a blank test was run after the third trial without any odour and thus no reward to ensure that the bees were learning to associate the odour and nothing else. None of the bees responded in this blank test, but should they have done so, they would have been removed from the analysis.

Each bee was recorded as either having not responded to the reward (not extending proboscis on three or more of the six trials), not learned the response (responding to the

sugar, but not to the scent alone) or learned (responding to the scent alone in the final test trial).

4.2.2 Testing for differences in learning behaviour in young adult bees over the course of infection with *M. anisopliae*:

Newly-emerged young adult bees were collected as described previously (Chapter 2.2.1) then maintained at 30°C in darkness and provided with pollen, 60% sucrose solution and water. After three days the bees were inoculated with 0.5g of a 1:30 concentration of *M. anisopliae* in sorbitol or sham inoculated with 0.5g of sorbitol alone and maintained under conditions to ensure infection as described previously (chapter 2.5) and then tested two, four and six days after inoculation. These times and the concentration of *M. anisopliae* used were chosen based on the results of a virulence bioassay and represented the lowest concentration of conidia to achieve 100% infection and mortality within 12 days (chapter 3.3.2).

The bees used in this experiment came from Rothamsted colonies 30 and 12. Both colonies were similar in respect to their varroa loads (Colony 30: high varroa levels, 33.4 mites per day counted in July 2010; Colony 12: high varroa levels, 25.7 mites per day counted in July 2010) and had been foraging in the same area. These colonies were chosen specifically for their high levels of varroa as they were expected to also have high levels of other diseases, such as DWV (Yue and Genersch, 2005). After experimentation all bees were stored at -80°C for Q-RT-PCR analysis to examine the interaction between these natural diseases and the *M. anisopliae*. Although the intention was to use bees from the same colony, sufficient brood of the correct age was not available from one colony and so two were used. For that reason, bees tested six days after inoculation on the 21/07/2010 came from colony 30 and the rest came from 12 (Table 4).

The experiment was designed and blocked to allow each treatment to be compared whilst taking time and date effects into account. On each experimental day 32 bees were tested; eight from each of two of the six treatment groups. This was repeated over six non-consecutive days until 32 repeats of each treatment were achieved (Table 4.1).

Table 4.1:

Experimental day	Treatments (days after inoculation (d) and + *M. anisopliae* (M) or sorbitol (S))		No bees in each treatment		Colony
1	+2d + M	+2d + S	8	8	12
21/07/2010	+6d + M	+6d + S	8	8	30
2	+2d + M	+2d + S	8	8	12
23/07/2010	+4d +M	+4d + S	8	8	12
3	+4d +M	+4d + S	8	8	12
26/072010	+6d + M	+6d + S	8	8	12
4	+2d + M	+2d + S	8	8	12
28/07/2010	+6d + M	+6d + S	8	8	12
5	+2d + M	+2d + S	8	8	12
30/07/2010	+4d +M	+4d + S	8	8	12
6	+4d +M	+4d + S	8	8	12
02/08/2010	+6d + M	+6d + S	8	8	12

Table showing the experimental design employed to examine changes in learning ability of honeybees over the course of infection with *M. anisopliae.*

Each bee was tested as described above (4.2.1) and recorded as either having not responded to the reward (not extending proboscis on three or more of the six trials), not learned the response (responding to the sugar, but not to the scent alone) or learned (responding to the scent alone in the final test trial).

These data were analysed in GenStat® (14th edition) using repeated measures ANOVA after logit transformation with the treatment structure to test the effect of time since inoculation (two, four or six days) and inoculation treatment (inoculated with *M. anisopliae* or sorbitol), and a blocking structure to take account of experimental day.

4.2.3 Testing for differences in learning behaviour in young adult and forager bees infected with *M. anisopliae*:

Newly emerged young adult bees were collected from Rothamsted colony 12 (which had high varroa levels in July 2010, 25.7 mites per day) and maintained as described previously (chapter 2.2.1) for three days. They were then inoculated with 0.5g of a 1:30 concentration

of *M. anisopliae* in sorbitol or sham inoculated with 0.5g of sorbitol and maintained at 30°C in darkness and provided with pollen, 60% sucrose solution and water. Forager bees were collected from the entrance of the same colony using the BioQuip® 'insect vac' as described previously (chapter 2.2.2) on the same day as the frames of brood were collected. The forager bees were maintained for three days prior to inoculation with *M. anisopliae* in the same manner and at the same time as the young adult bees.

This experiment was also designed and blocked to allow each treatment to be compared whilst taking time and date effects into account (Table 4.2).

Table 4.2:

Experimental day	Treatments		No bees tested per treatment	
	+ *M. anisopliae*	**+ sorbitol**		
1 09/08/2010	Young adult Forager	Young adult Forager	8 8	8 8
2 10/08/2010	Young adult Forager	Young adult Forager	8 8	8 8
3 13/08/2010	Young adult Forager	Young adult Forager	8 8	8 8
4 16/08/2010	Young adult Forager	Young adult Forager	8 8	8 8

Table showing the experimental design employed to examine differences in learning behaviour in young adult and forager bees after infection with *M. anisopliae*.

Each bee was tested as described above (4.2.1) and recorded as either having not responded to the reward (not extending proboscis on three or more of the six trials), not learning the response (responding to the sugar, but not to the scent alone) or learned (responded to the scent alone in the final test trial). These data were analysed in GenStat® (14th edition) using repeated measures ANOVA after logit transformation with the treatment structure to test the effect of age (young adult or forager bees) and inoculation treatment (inoculated with *M. anisopliae* or sorbitol), and a blocking structure to take account of experimental day. All experimental bees were then killed by freezing and stored at -80°C for later molecular analysis to detect and quantify the background level of natural infection of viruses and *Nosema* spp. in each individual (chapter 2.6). A form of multivariate analysis, canonical variance analysis, was done using both the behavioural data and the molecular

analysis to determine whether differences in learning ability could be accounted for differences in disease load.

4.2.4 Testing for differences in learning behaviour of pollen starved forager bees infected with *M. anisopliae*:

Forager bees were collected from hive entrances using the BioQuip® 'insect vac' as described previously (chapter 2.2.2). These bees came from colonies 40 and 41 which had similar varroa levels (Colony 40: low varroa levels, 0.7 mites per day counted in July 2010. Colony 41: low varroa levels, 0 mites per day counted in July 2010) and had been foraging in the same area. Any pollen sacs the bees had were removed to ensure the bees had no access to pollen. The bees were inoculated with 0.5g of a 1:30 concentration of *M. anisopliae* in sorbitol or sham inoculated with 0.5g of sorbitol and then maintained at 30°C in darkness and provided with 60% sucrose solution and water (but without pollen). These pollen-starved forager bees were tested at four and six days after inoculation. Three repetitions of 15-16 bees for each treatment were run on each of six non-consecutive days for each time since inoculation (Table 4.3).

Table 4.3:

Experimental day	Treatments (days after inoculation (d) and + *M. anisopliae* (M) or sorbitol (S))	No bees in each treatment	Colony
1	+4d + M	16	40
20/08/2010	+4d + S	16	40
2	+4d + M	15	40
25/08/2010	+4d + S	15	40
3	+4d + M	15	41
26/08/2010	+4d + S	15	41
4	+6d + M	15	40
30/08/2010	+6d + S	15	40
5	+6d + M	15	41
31/08/2010	+6d + S	15	41
6	+6d + M	15	41
03/09/2010	+6d + S	15	41

Table showing experimental design employed to test changes in learning ability after infection with *M. anisopliae* in pollen-starved bees.

Each bee was tested as described above (4.2.1) and recorded as either having not responded to the reward (not extending proboscis on three or more of the six trials), not learning the response (responding to the sugar, but not to the scent alone) or learned (responded to the scent alone in the final test trial). These data were analysed in GenStat® (14th edition) using repeated measures ANOVA after logit transformation with the treatment structure to test the effect of inoculation treatment (inoculated with *M. anisopliae* or sorbitol), and a blocking structure to take account of experimental day. As the bees tested four days after inoculation were all tested on separate days to those tested six days after inoculation the two time points could not be statistically compared and were analysed separately.

4.2.5 Testing for differences in sucrose sensitivity in young adult bees infected with *M. anisopliae*:

The sucrose sensitivity of *M. anisopliae*-infected bees was tested for pollen-starved bees two, four and six days after inoculation with *M. anisopliae*. A frame of sealed brood was collected from colony 150 (low varroa loads: 0.1 mites/day drop count and 0 phoretic mites 28/06/12, see chapter 2.6) and newly emerged adult bees were collected as described previously (chapter 2.2.1). Bees were inoculated over the course of a week such that on the experimental day the same aged bees would either have been inoculated two, four or six days earlier (timings for the experiment in table 4.4). On each inoculation day, three groups of 40 bees were chilled and split into two groups of 20. One group was inoculated with 0.5g of a 1:30 concentration of *M. anisopliae* in sorbitol, the second was sham inoculated with 0.5g of sorbitol. This meant on each inoculation day, three groups of 20 *M. anisopliae* inoculated bee and three groups of 20 sorbitol inoculated bees were produced. Only ten bees of each treatment in each group were needed, but twice this number were inoculated in case some bees died. The bees were then maintained at 30°C in darkness and provided with pollen, 60% sucrose and water. Bees were starved of all food, but not water, for 12 hours prior to experimentation and secured in glass tubes with PVC tape as for the CPE experiments (see 4.2.1). Ten bees from each treatment (two, four and six days after inoculation/sham inoculation) were tested on each run of the experiment which was repeated three times. This meant that 60 bees were tested in each of the three runs, 180 bees in total.

Table 4.4:

Day	Experimental procedure:
1	Brood frame collected from colony 150
2	Newly emerged adult bees collected
5	First group of 120 bees inoculated/sham inoculated
7	Second group of 120 bees inoculated/sham inoculated
9	Third group of 120 bees inoculated/sham inoculated
11	Bees tested for sucrose sensitivity in three runs

Table showing timings for the sucrose sensitivity experiment.

Each bee in turn was stimulated with a drop of water on a cotton wool bud touched to the antennae. Whether the bee extended her proboscis or not was recorded. This was then repeated with increasing concentrations of sucrose (0.1%, 0.3%, 1%, 3%, 10% & 30%) with 10 minute intervals between each concentration. The number of concentrations each bee responded to was counted as used as the 'gustatory response', this could range from 0 (the bee responded to none of the concentrations) to 7 (the bee responded to all six concentrations and water).

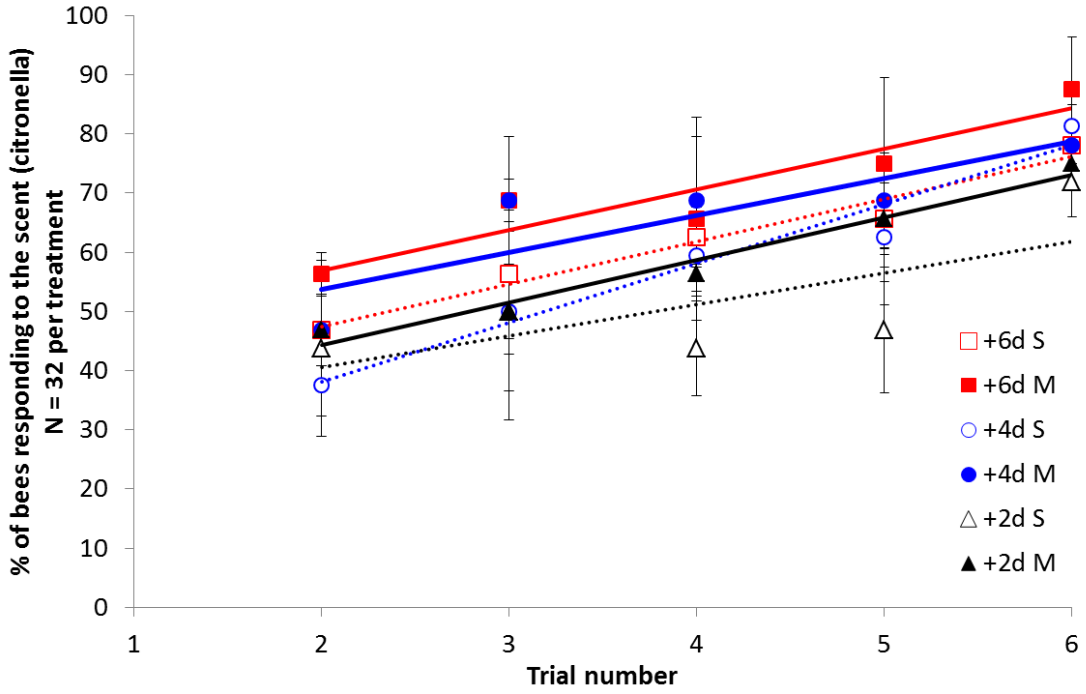
These data were analysed in GenStat® (14th edition) using ANOVA analysis with the treatment structure to test the effect of infection with the fungus *M. anisopliae* and time since inoculation on gustatory response. The blocking structure took account of when each group of bees was tested and which groups of bees were inoculated together. The data did not require transformation.

4.3 Results:

4.3.1 Testing for differences in learning behaviour in young adult bees over the course of infection with *M. anisopliae*:

Learning in *M. anisopliae*-infected and sorbitol-inoculated (control) young adult bees was evaluated two, four and six days after inoculation. In each successive training trial more bees learnt the association, irrespective of treatment, so there was a positive trend in learning ability over time for all treatments (Repeated measures ANOVA: H_0 = equal means for each time point. The logit transformed means ranged from -0.048 for trial 2 to 0.45 for trial 6. SED = 0.072. $F_{2.67, 48.0} = 12.76$, $P<0.001$, see also Figure 4.2). This is to be expected as each training trial reinforced the association allowing more bees to learn each time.

Figure 4.2:



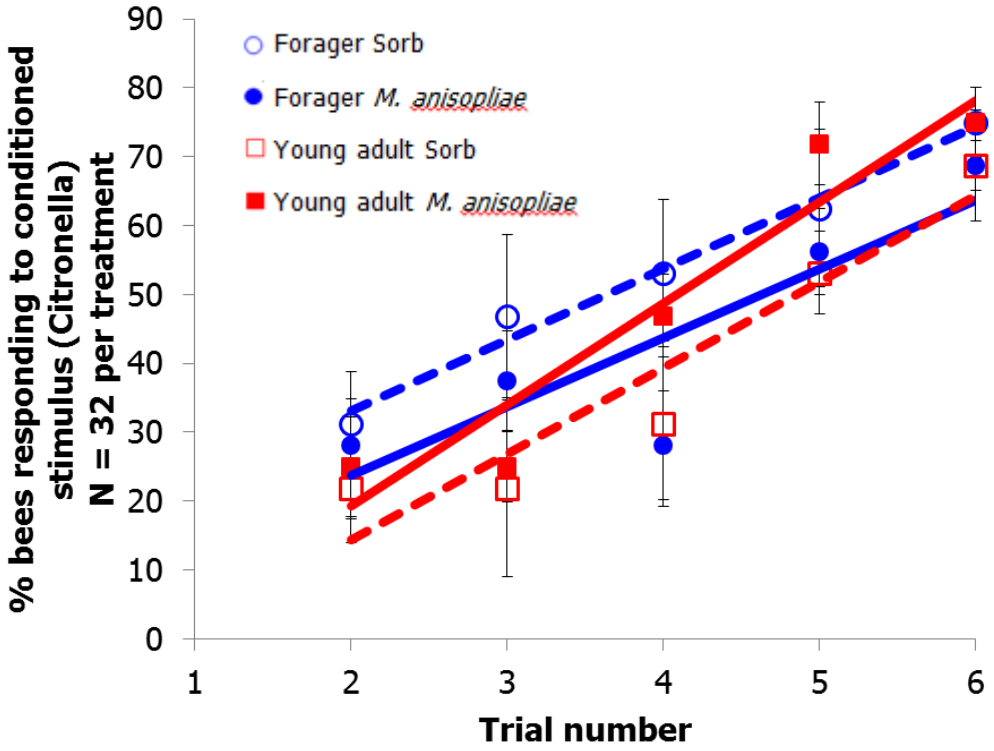
Learning ability of young adult bees infected with *M. anisopliae* at 2, 4, and 6 days after inoculation. The proportion of bees that learnt the CPE response is shown for each of the six training trials except the first in which no bees responded to the odour stimulus. Open shapes and dotted trend lines are *M. anisopliae* inoculated bees, filled shapes and continuous trend lines are sham (sorbitol) inoculated bees. Error bars show +/- standard error.

There was a significant effect of *M. anisopliae* infection; infected bees were more able to learn the association than uninfected bees (Repeated measures ANOVA: H_0 = infected mean = control mean. Infected mean = 0.25, control mean = 0.11. SED = 0.063. $F_{0.67, 8.67} = 4.86$, P = 0.046). There was no significant effect of the time since infection, or any interaction between treatments over the course of the experiment.

4.3.2 Testing for differences in learning behaviour in young adult and forager bees infected with *M. anisopliae*:

Learning in *M. anisopliae*-infected and sorbitol-inoculated (control) forager and young adult bees was evaluated four days after inoculation and again, with each successive trial more of the bees had learned the association, irrespective of treatment (Repeated measures ANOVA: H_0 = equal means for each trial. The logit transformed means ranged from -0.38 for trial 2 to 0.33 for trial 6. SED = 0.08. $F_{2.42, 29.07} = 28.16$, P<0.001, see also Figure 4.3).

Figure 4.3:



Learning ability of forager and young adult bees infected with the fungus *M. anisopliae* 4 days after inoculation. Open shapes and dotted trend lines are *M. anisopliae* inoculated bees, filled shapes and continuous trend lines are sham (sorbitol) inoculated bees. Error bars show +/- standard error.

There was a suggestion that the forager bees were better able to learn than the young adult bees, although this was only significant at the 10% level (Repeated measures ANOVA: H_0 = forager mean = young adult mean. Forager mean = -0.023, control mean = -0.11. SED = 0.039. $F_{0.61, 5.45} = 5.05$, P = 0.051). There was also a significant interaction between age and *M. anisopliae* infection; whilst infected young adult bees were better able to learn the association than uninfected controls (as in the previous experiment) (as in 4.3.3) the infected forager bees were less able to learn than uninfected controls (Repeated measures ANOVA: H_0 = infected mean = control mean. Infected mean = -0.062, control mean = -0.072. SED = 0.039. $F_{0.61, 5.45} = 19.28$, P = 0.002).

The bees from this experiment were analysed using Q-RT-PCR (see chapter 2.6). Five common bee diseases were detected; DWV, BQCV, SBV, *Nosema apis* and *Nosema ceranae* (table 4.5). Most of the bees tested positive for both *Nosema* species. This is likely to be because the bees were not treated against these pathogens. Several colonies failed in the winter following these experiments, potentially because of the high levels of *Nosema* spp. infection.

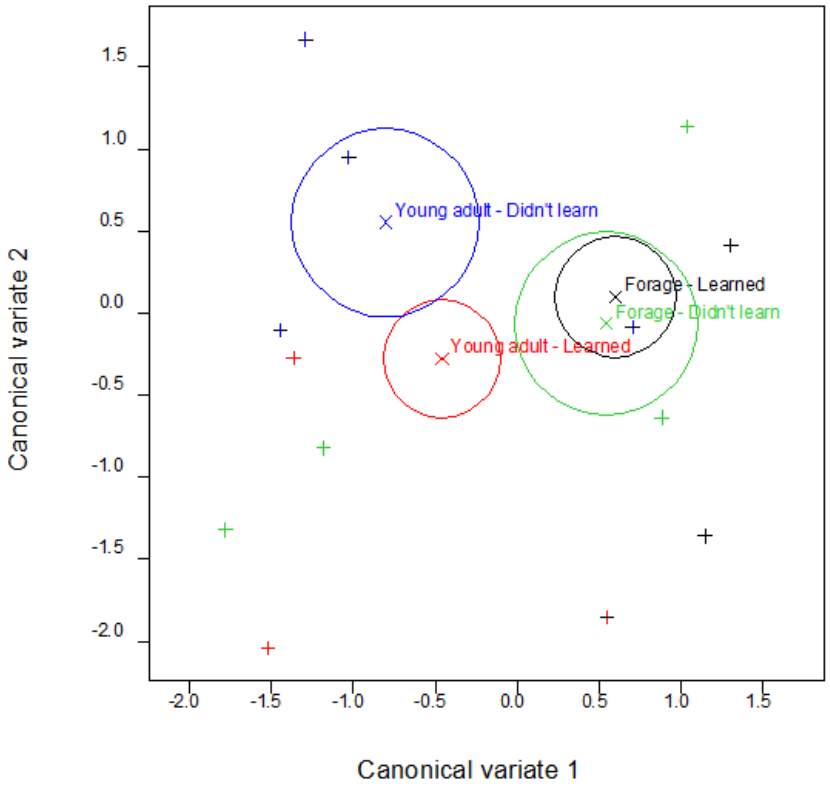
Table 4.5:

	BQCV	DWV	SBV	*N.apis*	*N.ceranae*
Total tested	128	128	128	128	128
No. positive	75	126	49	105	106
No. negative	51	0	78	20	19
Inconclusive	2	2	1	3	3

Summary of PCR analysis. 128 bees from experiment 4.2.2 were analysed using RT-PCR based detection to identify the viral diseases and *Nosema* spp. present. Five diseases were detected, the viruses BQCV, DWV & SBV and both *N.apis* and *N.ceranae*. A few samples were inconclusive.

The multivariate analysis accounted for 95.04% of the variance within the data. There are four groups of bees that can be represented as circles equating to a 95% CIs (figure 4.4). Those four groups are: Forager bees that learned, forager bees that did not learn, young adult bees that learned and young adult bees that did not learn. The CIs around the groups that were unable to learn are larger than around those that did learn because fewer bees were unable to learn the association than those that did. The forager bees are separated from the young adult bees and the young adult bees that learned are separated from the young adult bees that did not.

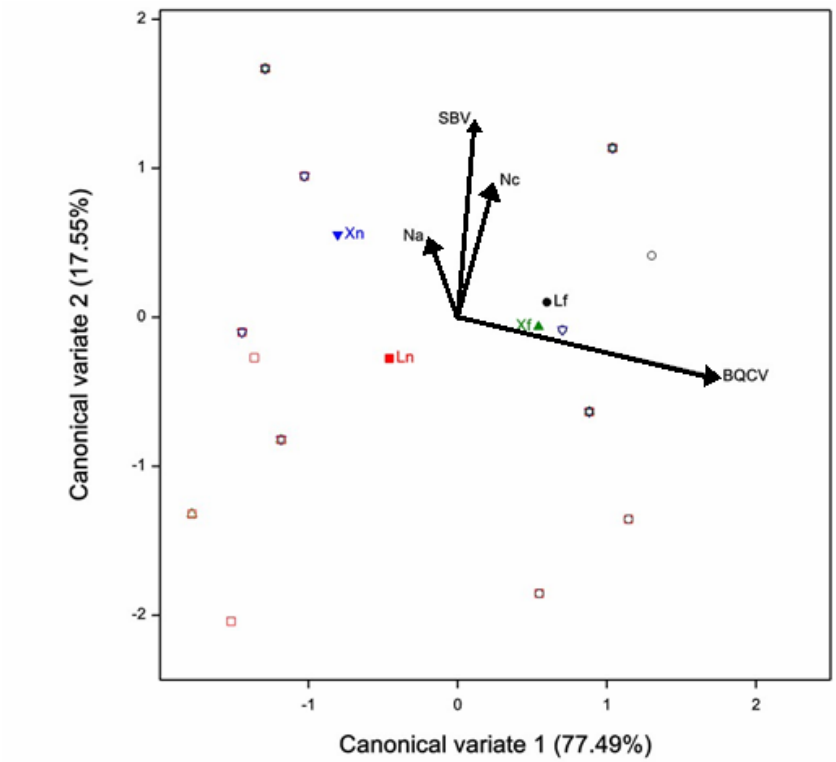
Figure 4.4:



Canonical variance analysis including presence or absence of BQCV, SBV, *N.apis* & *N.ceranae*, age and whether the bees had learned the association in the sixth and final trial.

A bi-plot was produced to determine which factors were contributing to the separation of the data (figure 4.5). The direction of the lines produced on the biplot show the direction of data separation produced by each factor. The position and direction of the arrow point along the line is indicative of the strength and direction of the effect. The analysis suggests that the horizontal separation of the data was caused by BQCV, which was more likely to be found in the forager bees than in the young adult bees. The vertical separation, between the young adult bees that learned and those that did not, seems to be caused by a combination of SBV and *N. ceranae*, SBV having the greatest effect.

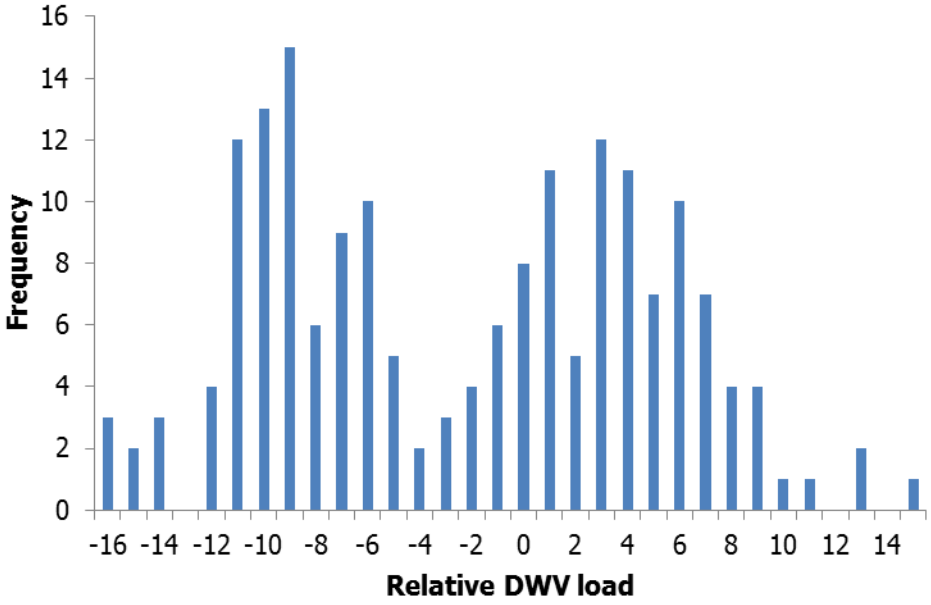
Figure 4.5:



Biplot from the canonical variance analysis including presence or absence of BQCV, SBV, *N.apis* & *N.ceranae*, age and whether the bees had learned the association in the sixth and final trial.

Quantitative PCR analysis was done to quantify the absolute load of each pathogen in each bee. The DWV load for each bee, when plotted as a histogram, has a bimodal distribution such that there appear to be bees with a relatively high DWV load (centred around -9) or a relatively low DWV load (centred around 3) (see figure 4.6). This was not found for any of the other diseases detected (BQCV, SBV, *N. ceranae* or *N. apis*).

Figure 4.6:



Histogram showing the distribution of DWV load relative to the housekeeping gene β-actin within the bees from experiment 4.2.3. The lower (or more negative) the relative load the more virus was present, see chapter 2.6.2.

REML analysis of the Q-RT-PCR and behavioural data (Table 4.6) showed that for some diseases there was a significant correlation between the disease load and age of the bee; the forager bees had higher levels of BQCV and DWV than the young adult bees. Also, for some diseases there was a significant interaction between age and disease (DWV and *N. apis*) but there was no effect of disease load on learning.

Table 4.6:

Disease	DWV	BQCV	SBV	*N.ceranae*	*N.apis*
Learning	$F_{3,110.7}$ = 2.06 P = 0.109	$F_{3,63}$ = 0.6 P = 0.616	$F_{3,38}$ = 0.23 P = 0.877	$F_{3,88.9}$ = 0.25 P = 0.861	$F_{3,86.2}$ = 0.12 P = 0.948
Age	**$F_{1,109.1}$ = 14.98** **P = <0.001**	**$F_{1,63}$ = 9.05** **P = 0.004**	$F_{1,38}$ = 0.64 P = 0.43	$F_{1,87.7}$ = 0.38 P = 0.541	$F_{1,82.6}$ = 0.13 P = 0.723
Interaction	**$F_{2,111}$ = 3.18** **P = 0.045**	$F_{1,63}$ = 0.25 P = 0.621	$F_{2,38}$ = 0.01 P = 0.991	$F_{2,86.8}$ = 1.6 P = 0.207	**$F_{2,83}$ = 4.32** **P = 0.016**

Table showing REML analysis combining molecular and behavioural data for bees whose learning behaviour was tested after infection with the fungus *M anisopliae*. The relationships amongst each pathogen, the learning behaviour and the age of the bee were analysed. The only significant effects were on age (for DWV and BQCV) and the interaction between age and learning (for DWV and *N. apis*).

4.3.3 Learning behaviour of pollen-starved forager bees infected with *M. anisopliae*:

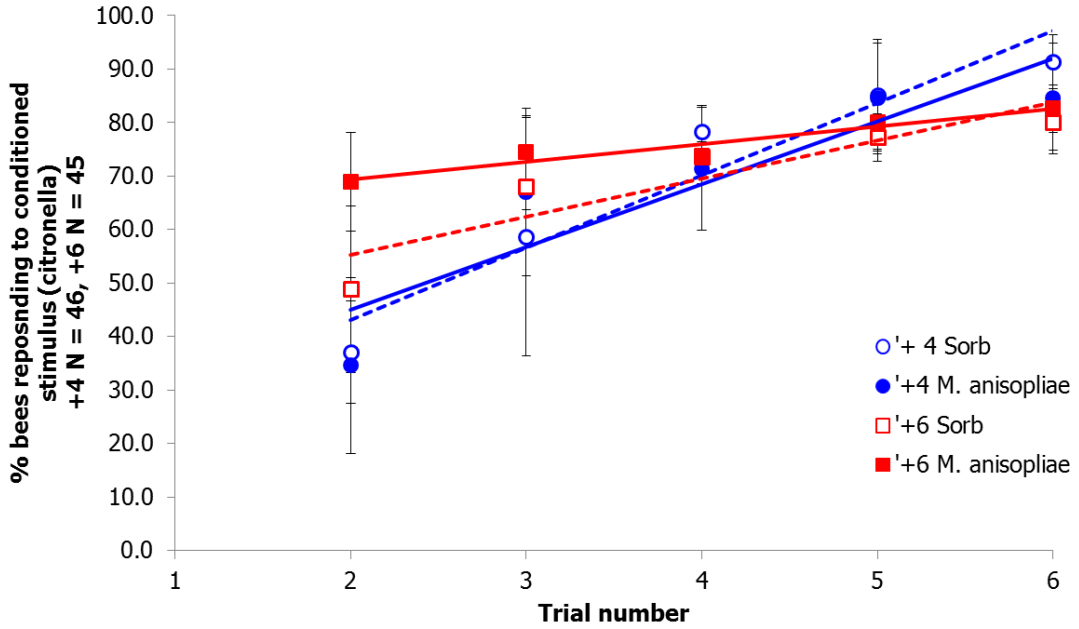
Learning in *M. anisopliae*-infected and sorbitol-inoculated (control) forager bees that had been starved of pollen was evaluated four and six days after inoculation. There was a

significant effect of trial for the bees tested four days after infection, but not for those tested six days after infection (Repeated measures ANOVA, H_0 = equal means for each trial. +4 days: The logit transformed means ranged from -0.26 for trial 2 to 0.77 for trial 6. SED = 0.10. $F_{1.42, 10.30} = 32.06$, P = 0.001. +6 days: The logit transformed means ranged from 0.15 for trial 2 to 0.35 for trial 6. SED = 0.09. $F_{1.21, 4.85} = 1.40$, P = 0.304 (Figure 4.7).

There was no significant difference in the learning behaviour of pollen-starved forager bees infected with *M. anisopliae* compared to uninfected controls at either time point (repeated measures ANOVA, H_0 = infected mean = control mean. +4 days: Infected mean = 0.35, control mean = 0.41. SED = 0.41. $F_{0.36, 0.71} = 0.02$ P = 0.899. +6 days: Infected mean = 0.34, control mean = 0.23. SED = 0.12. $F_{0.61, 0.30} = 0.84$ P = 0.455).

Bees tested 6 days after inoculation were more likely to die during the experiment than bees tested 4 days after inoculation; eight of the 45 fungus-infected and four of the 45 sorbitol-inoculated bees tested 6 days after inoculation died during the experiment compared to no deaths in the bees tested 4 days after inoculation.

Figure 4.7:



Learning ability of pollen- starved forager bees infected with *M. anisopliae* at 4 and 6 days after inoculation. There was no significant effect of wither *M. anisopliae* infection or time since inoculation on learning ability in this experiment.

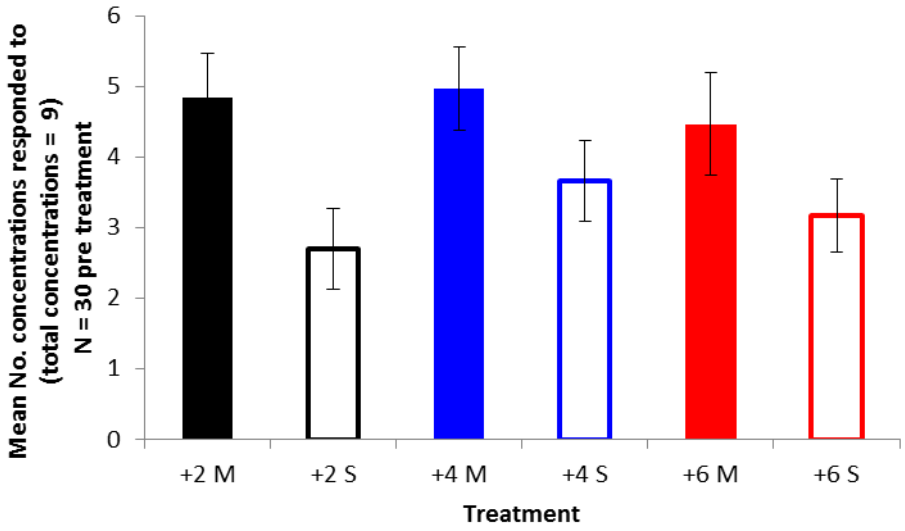
4.3.4 Sucrose sensitivity in young adult bees inoculated with *M. anisopliae*:

Each bee that responded to a concentration of sucrose then continued responding to each subsequent, increasing, concentration. Thus the number of concentrations a bee responded

to could be used as the gustatory response with 0 = responded to no concentrations, 1 = responded only to the most concentrated solution…7 = responded to all concentrations including water (0%).

The bees that had been inoculated with *M. anisopliae* where significantly more likely to respond to more concentrations of sucrose and thus lower concentrations of sucrose and therefore were hungrier than uninoculated bees (ANOVA: H_0 = inoculated mean = unincoluated mean number of concentrations responded to. Inoculated mean = 4.76, uninoculated mean = 3.18. SED = 0.16. $F_{1,12,}$= 94.00, P <0.001, see also figure 4.8). There was also a significant effect of the time since inoculation such that bees tested 2 days after inoculation responded to less concentrations of sucrose than those tested 4 or 6 days after inoculation. Thus bees tested 4 or 6 days after inoculation responded to lower concentrations of sucrose and were therefore hungrier than those tested 2 days after inoculation (ANOVA: H_0 = equal means for each testing time (2, 4 or 6 days after inoculation). +2 days mean = 3.77, +4 days mean = 4.32, +6 days mean = 3.82. SED = 0.28. $F_{2,12}$ = 4.66, P = 0.032, see also figure 4.8).

Figure 4.8:



Graph showing mean number of concentrations each treatment of bees responded to. *Metarhizium anisopliae* infected bees (filled bars) were significantly more likely to respond to more sucrose concentrations than uninfected bees (open bars).

4.4 Discussion:

Previous studies have shown that several factors can cause differences in learning ability in honeybees, such as time of day (Lehmann *et al.*, 2011) and temperature during development (Tautz *et al.*, 2003), but these were standardised in all the experiments described here. It has also been shown that time of year can affect results (Frost *et al.*, 2011) but, although experiments had to be carried out over several months, the timing of each experiment was accounted for in the statistical analysis of the results.

In experiment 4.2.2 there was a significant effect of *M. anisopliae* infection; young adult bees infected with the fungus were more able to learn the association than uninfected bees. This is surprising given that the work of others has shown decreases in learning with other parasitic/ pathogenic organisms including varroa and DWV (Iqbal and Mueller, 2007; Kralj *et al.*, 2007) and when the bee's immune system was stimulated (Riddell and Mallon, 2006). Varroa and DWV are not fast acting, lethal infections as *M. anisopliae* is and so bees should not be expected to respond in the same way. It has, however, been shown that young adult bees infected with *M. anisopliae* show up regulation of immune pathways, including the Toll pathway (Bull *et al*, in press). This should lead to decreased learning ability, given Riddell and Mallon's (2006) results, so the increase in learning ability of the inoculated bees in these experiments cannot be explained by lack of immune response.

Previous experiments have also shown that forager bees that are more responsive to lower concentrations of sucrose are better able to learn the CPE response (Mujagic *et al.*, 2010). *Nosema ceranae*-infected bees were shown to be energetically stressed which led them to be more responsive to lower concentrations of sucrose (Naug, 2009). Although CPE tests have not been carried out on *N.ceranae*-infected bees it could be predicted that the change in sucrose sensitivity might mask any decreases in learning caused by infection. The results to experiment 4.2.4 suggest that young adult bees infected with *M. anisopliae* may also have become more responsive to lower sucrose concentrations and this could explain why they were better able to learn the CPE response.

It is also possible that learning in young adult bee is not affected by infection in the same way that forager bees are. For example a recent study on the response of newly emerged and 23 day old forager bees' response to fungal infection showed that the younger bees were more susceptible to the fungus (died faster and allowed more fungal growth) but showed a greater immune response than the forager bees (Bull *et al*, in press). This study also showed that several immune related genes seem to become activated as honeybees age and this may be the cause for the increased resistance to *M. anisopliae* found in the

older forager bees. All previous research on the effect of pathogens on learning has used forager bees (Iqbal and Mueller, 2007; Kralj *et al.*, 2007; Mallon *et al.*, 2003) without looking at younger bees. It is possible that the forager bees' learning behaviour may be more susceptible to the influence of infection than young adults'. This is supported by the results of the second experiment (section 4.3.2) in which there was a significant interaction between the fungus treatment and age; the young adult bees were better able to learn after infection whilst the forager bees were less able to learn after infection (figure 4.3).

The PCR and QPCR results suggest that forager bees have higher levels of naturally occurring infective agents (such as *Nosema spp.* or viruses) than young adult bees. This may be because the nurse bees are exposed to DWV and *Nosema* sp., and potentially other pathogens, during hygienic activities, especially if they catabolise infected larvae (Mockel *et al.*, 2011) or when cleaning faecal matter (Bailey, 1955) so older, forager bees are more likely to have come into contact with pathogens and parasites and, being older, have had more time for them to accumulate (Wilson-Rich *et al.*, 2008). This would lead to an accumulation of effects if the diseases act synergistically such that bees with higher disease-loads or infected with a greater number of diseases show greater changes in learning.

It is known, for example, that some diseases and parasites can act synergistically or additively, causing greater overall negative effects on the colony. Colonies infested with both varroa mites and tracheal mites (*Acarapis woodi*) are more likely to die (Downey and Winston, 2001) than those infested with either mite alone. There is also evidence to suggest that varroa mites transmit and activate the replication of some viruses, making their effects more severe than they would otherwise be (Bailey and Ball, 1991). Combinations of diseases, parasites and other factors can also affect aspects of behaviour. For example whilst varroa-infested bees began foraging earlier and tracheal mite-infested bees began foraging later, dual infections led to an intermediate foraging time (Downey *et al.*, 2000). Pollen-limited bees also begin foraging earlier than controls whilst pollen-limited bees with varroa infestation begin foraging even earlier, again showing an additive effect (Janmaat and Winston, 2000). Recent studies have also shown interactions between pesticides and pathogens. For example, Alaux *et al.* (2010a) showed an interaction between *N. ceranae* and a neonicitinoid pesticide that caused higher mortality in affected bees than in bees affected with either pathogen or pesticide alone and could also help explain some colony losses.

In addition to this, forager bees are older and might be less able to respond to and recover from fungal infection than younger bees. It has been shown that nurse bees (comparable to my young adult bees) have greater fat body mass than forager bees, which means that

they may be better able to respond to infection via the production of anti-pathogenic proteins than older forager bees (Wilson-Rich *et al.*, 2008). Forager bees also have a decreased haemocyte count compared to nurse bees, although phenyloxidase activity was greater in forager bees compared with nurse bees (Schmid *et al.*, 2008), and the average level of encapsulation, a method used by honeybees to combat pathogenic infection, remained stable across all life stages (Wilson-Rich *et al.*, 2008).
If this were the case I would have expected to find decreased learning ability in pollen-starved bees that were challenged with a pathogen as the immune response is costly and lack of nutrition could lead to a trade-off between learning and immune response (as seen in Riddell and Mallon's (2006) experiments. However, the pollen-starved infected bees showed no difference in learning compared to the pollen-starved uninfected bees (see 4.3.3). This may be because the bees used for this experiment could compensate because they came from a large, healthy colony and had been provided with plenty of pollen during development and until just before the experiment started. The fat bodies of bees are important in energy metabolism and as a store for excess nutrients. The fat body is also involved in synthesizing most haemolymph proteins (Law and Wells, 1989). Forager bees generally have lower concentrations of protein in their fat bodies than younger bees, although winter bees (those bees who will survive in the colony over the winter) have far greater protein stores (Shehata *et al.*, 1981). This experiment was run late in the season, in September, when the bees tested may also have been winter bees with higher protein stores and thus less likely to be affected by pollen limitation. In addition to this Frost (2011) found that bees tested in June responded to the sucrose stimulus significantly less often that bees tested in July, showing that there are seasonal changes in sucrose sensitivity which could lead to changes in learning ability. The bees tested after pollen starving were tested later in the year than any others (late August, early September, experiment 4.2.2 was run in July and experiment 4.2.3 finished by mid-August) so the pollen starved bees may also have been more responsive to the sucrose stimuli which would affect the results of the CPE experiment.

I was expecting to find a more pronounced effect of the fungus on learning ability at both ages, but it is possible that learning is a robust trait that the bees only lose very near death if the immune system has not been activated. This could be tested by doing similar experiments but testing the bees much closer to the point of death after fungal-inoculation and infection.

It is also possible that whilst the aspect of learning that I was examining was not greatly affected by the fungus, other aspects may have been. The method I used in these experiments was developed for the company, Inscentinel (http://www.inscentinel.com/),

whose main purpose is to ensure bees learn novel odours so that they can be used for detection purposes. The method is therefore designed to get the highest proportion of learning bees as possible. There does not appear to be a standardised method for testing honeybee learning using the CPE method. Kralj *et al.* (2007) gave bees a single training trial then tested after one and four minutes, finding that varroa-infested bees showed a significant decrease in learnt response for the 1 minute test. Mallon *et al*. (2003) carried out a similar test on bees injected with LPS which activated the bees' immune systems; however they tested after one and 12 minutes and found significant differences only for the 12 minute test. Finally Iqbal and Mueller (2007) tested DWV-infected bees this time giving three training trials at two minutes intervals and then testing after two and 24 hours. The virus-infected bees showed significant decreases in learning ability on the third training trial and at both testing trials (after two and 24 hours).

Frost (2011) states that there is 'substantial inconsistency in PER (proboscis extension reflex) experimental design'. In that paper the focus was on the handling methods because chilling the bees to allow them to be secured for experiments can affect their learning behaviour (Frost *et al.*, 2011). However, the fact that different experiments focus on different aspects of learning behaviour is also an issue. It is also of concern that negative results are published less readily than positive ones (Callaham *et al.*, 1998) such that if two pathogens are examined by different groups, but using the same techniques and looking at the same aspect of learning, but one experiment shows a negative result, this negative result is less likely to be published.

It is possible that different methods could produce different results. The method I used tested each bee at 30 minute intervals over six training trials and so was testing relatively long-term memory. This does not test the short or mid-term memory (tested one-12 minutes after the training trial) so my results are not directly comparable to those of Kralj *et al.*, (2007) or Mallon *et al.*, (2003). Nor did I test the bees after a two or 24 hour lag as was done by Iqbal and Mueller (2007). It is also possible that the fungus genuinely has little or no deleterious effect on learning behaviour of either age of bees and changing the method may not influence this.

I also looked at the effect any naturally occurring pathogens might have had on the bees' learning ability. The presence/absence data showed a correlation between learning ability of the young adult bees and both *Nosema* spp. and SBV, but BQCV only differed with age such that the older forager bees were more likely to be infected with this virus. Although neither SBV nor either species of Nosema have been shown to effect learning in honeybees it has been shown that *N. ceranae*-infected bees are more responsive to lower concentrations of

sucrose which may affect the results of PER experiments which rely on the bees' response to a sucrose reward.

There was a bimodal distribution to the quantity of DWV detected in these bees suggesting that the bees are separated into two groups with regard to DWV load; those with a high or a low dose of the virus. This may be because the bees became infected at different times and had longer or shorter periods of time for the virus to replicate within them, but the clear peaks in quantity suggest that if this were the case then there are two separate times when the bees are most likely to be infected. This could mean that one set of bees were infected as larvae or even earlier, as eggs via vertical transmission, whilst the other set were infected as adults. There is evidence that DWV can be transmitted vertically (Chen *et al.*, 2006a) and the quantity of DWV correlated with age such that the older forager bees were more likely to have higher level of virus which suggests that the amount of virus does increase with age.

An alternative explanation for this distribution is that the virus has two different strategies, either rapid replication throughout all tissues, or slower replication, potentially in a limited number of tissues. It would be possible to test this by using lysate from infected bees identified as having either low or high levels of DWV to infect healthy bees and then test the quantity of virus at set time points and in different tissues.

The quantitative data also showed an interaction with age and learning for both DWV and *N. apis,* suggesting that, as with the presence/absence data, the pathogens affect the young adult bees' learning differently from the forager bees. DWV is known to affect the learning ability of forager bees (Iqbal and Mueller, 2007) but to the best of my knowledge the effect of *Nosema* spp. has not been tested in this way, although as stated above, changes in learning behaviour detected using this method may be complicated because *Nosema* spp. also cause changes in sucrose sensitivity. There was no effect of *N. ceranae* on learning in this experiment. *Nosema ceranae* is thought to be more virulent that *Nosema apis*, although these results are contentious (Martin-Hernandez *et al.*, 2011) as it may be that *N. ceranae* is only more virulent at higher temperatures (Forsgren and Fries, 2010).

It would be interesting to do further work to examine the effect of these pathogens on learning in a more controlled manner, i.e. by actively infecting bees with the diseases rather than merely looking for them *post hoc*. This would allow the effects of each individual pathogen or interaction between pathogens to be studied and potentially lead to useful information for managing bee diseases. However this is difficult to do as to really work it requires bees clean of all viruses which are unlikely or even impossible to find in nature.

Colonies can be found in some areas (Australia and some island populations) which have no varroa mites, but to the best of my knowledge no colony has been found without a single pathogen present.

Chapter 5: The effect of disease load on the orientation flights of honeybees.

Abstract:

The European honeybee, *Apis mellifera*, is important economically not just for honey production but also as a pollinator. However, honeybee numbers in some areas are declining. A range of interacting factors are thought to be involved, including pathogens and parasites, which are known to cause changes in the behaviour of their hosts.

Harmonic radar was used in this chapter to study whether pathogen load had any effect on the orientation flights of honeybees. This was achieved by following the orientation flights of bees from high and low varroa colonies exploring a novel area. Q-RT-PCR was also used to detect and quantify naturally occurring viruses and microsporidia.

It was found that bees with higher levels of DWV infection flew shorter distances and for shorter periods of time than those will lower levels of infection whilst bees with higher levels of *N. Apis* or BQCV flew faster than those with lower levels of infection. This may have knock on effects to colony survival by effecting how well foragers exploit their landscape.

5.1 Introduction

5.1.1 Honeybee navigation, memory and exploration of novel environments:

Honeybees often need to be able to travel over great distances, explore novel environments for potential food sources and then must be able to return to their colony. Honeybees are able to find their hives when they have been moved up to 11km away from their original location (Pahl *et al.*, 2011). Southwick and Buchmann (1995) showed that honeybees were able to find their way back from greater distances in mountainous terrain (~9km) than in flat terrain (~5km) suggesting that they used horizon landmarks. Pahl (2011) found that bees released from points at different directions, but the same distance from the hive, had differing success in finding their way back. Bees are believed to use visual cues to find their way home so it is possible that this difference in homing success is because some landmarks work better than others, but it is equally possible that the bees merely knew certain areas around their colony better than others, especially if the better known regions had better forager available.

Bees may learn the route they took as a sequence of landmarks and then follow the same route back (Collett, 2005). In order to do this bees need sequential memory. It has been shown that bees can easily learn to associate a single stimulus with a reward, (Horridge, 2003), but bees can also learn sequences of stimuli. Giurfa *et al*. (2001) used Y tube choice experiments to show that honeybees can learn to match similar or different stimuli to reach a reward. To start with, the bees were taught to match similar stimuli. If the bee was first shown one colour, blue for example, then she should later choose the same colour in a y tube choice test. Next bees were taught to match different stimuli; a blue stimulus with a vertical line pattern and a green stimulus with a horizontal line pattern. The bees were able to learn these simple sequences.

Honeybees can also learn more complex sequences. Collett *et al*. (1993) set up mazes which honeybees learned routes through. In one experiment coloured patches were used at each point where the bee's flight direction changed. When the order of the colours was changed the bee's flight changed to match the colours rather than the original route suggesting that the bees learn to match the changes in flight with the coloured landmarks.

When honeybees first begin foraging they perform flights known as orientation flights. These begin with the bee hovering in front of the hive, presumably learning what the hive looks like so that it can be recognised when the bee returns (Vollbehr, 1975). The bee then explores the landscape, usually taking several flights to do so (Capaldi *et al*., 2000). This allows bees to view the landscape around their hive and possibly learn the cues they later use as landmarks.

Worker honeybees are able to fly over long distances allowing them to take advantage of resources across a large area and support colonies of tens of thousands of individuals. The distance covered by foraging bees is variable depending on colony size, season and landscape (Steffan-Dewenter and Kuhn, 2003). For example Waddington *et al*., (1994) found a foraging range of 534-1138m in suburban areas whilst Visscher and Seeley (1982) found a mean foraging range of 2260m in temperate deciduous forest and Beekman and Ratnieks (2000) observed foraging distances of over 5km when distant but immense patches of heather were in bloom. Under extreme conditions of food shortage, honeybees have been found foraging up to 13km from their hives (Eckert, 1931). Other aspects of bee flight that have been studied include speed and duration of flight, ability to explore and navigate through different environments, foraging duration, and pollination efficiency (e.g. Capaldi *et al*., 2000; HanauerThieser and Nachtigall, 1995; Rader *et al*., 2012; Wenner, 1963).

5.1.2 Effect of pathogens on honeybee flight:

Some bee pathogens have obvious effects on honeybee flight capabilities. Deformed wing virus, for example, can cause wing deformities that prevent flight entirely (see figure 1.3) (Ribiere *et al.*, 2008). Chronic and acute bee paralysis viruses cause bees to tremble and become paralysed, again preventing flight entirely (Bailey, 1965). Other diseases have less dramatic effects. Mayack, Naug and Gibbs have done studies on bees infected with *N. ceranae* (Mayack and Naug, 2009; Mayack and Naug, 2010; Naug and Gibbs, 2009), which showed that infected bees were more sensitive to lower concentrations of sugar, suggesting that they were more hungry than uninfected bees. They also had lower levels of sugars in the haemolymph, which suggests that *N. ceranae* is energetically stressing the bees.

This could mean that *N. ceranae* infected honeybee forager bees don't have enough energy for long flights. This could lead to them failing to return to the hive and may be the reason why colonies infected with *N. ceranae* often show reduction in the numbers of forager bees that is also one characteristic of colony collapse disorder (CCD) (Mayack and Naug, 2009; Mayack and Naug, 2010; Naug and Gibbs, 2009).

In the work of Duay *et al.* (2002), healthy honeybee forager bees were flown to exhaustion in wind tunnels and then fed differing amounts of glucose solution before their flight ability was tested, again in a wind tunnel. This experiment allowed information on flight energetics to be gained. Bees fed 5μl of a 1.28mol/L solution of glucose monohydrate could only fly for an average of 9.28 mins ($\pm$2.00) whereas when fed 20μl of the solution they could fly for 27.13 ($\pm$3.13) mins on average.

Duay *et al.*, (2002) then measured the duration of flight in drones that came from cells infested with zero, one, or two mites and found that those infested with two mites flew for significantly less time than those infested with one or no mites. Kralj (2006) also showed that varroa-infested bees spent more time foraging than uninfested bees and were also less likely to return to the colony. When infested and uninfested bees were released 5 and 400m from their colonies, the infested bees took longer to return and were more likely not to return at all (Kralj and Fuchs, 2006). This suggests that varroa mites, or the diseases that they spread, may also be energetically stressing bees or otherwise affecting their flight capabilities. For instance both DWV and varroa mite infestation has been shown to affect honeybee learning ability (Iqbal and Mueller, 2007; Kralj *et al.*, 2007; see also chapter 4) so infected/ infested bees may find it harder to remember how to get back to their colonies. It

has also been shown that varroa infested bees are less capable of finding the correct entrance to their hives than uninfested bees (Kralj and Fuchs, 2006).

5.1.3 The Harmonic Radar:

Radar has been used to study high flying insect migration patterns for over 40 years (Riley, 1989). The harmonic radar differs from other radar techniques in that it uses a re-radiated signal from a transponder attached to the experimental insect. This means that only the insect of interest is seen and not reflections from other objects in the environment (Riley *et al.*, 1996). In contrast to radio tracking, the harmonic radar also uses a much lighter transponder (typically ~0.012g) as it does not require a power source (Osborne *et al.*, 1999). This is approximately 6-7% of the bee's own weight and less than the pollen loads that foraging bees are capable of carrying, which may reach 90% of the bee's body weight (Osborne *et al.*, 1999). Tests were initially made with bumblebees and showed that bees with transponders were able to collect comparable pollen and nectar loads, but spent slightly longer outside of the nest than bees without transponders (Osborne *et al.*, 1999). The harmonic radar can be used to follow an individual bee's flight in real time.

This method has been used to follow butterflies, bumblebees and honeybees (e.g.Cresswell *et al.*, 2002; Osborne *et al.*, 1999; Ovaskainen *et al.*, 2008; Reynolds *et al.*, 2009; Riley *et al.*, 1999). For honeybees it has been used to study the orientation flights of young forager bees (Capaldi *et al.*, 2000) and the search patterns of forager bees when a known food source was removed (Reynolds *et al.*, 2007). The harmonic radar is uniquely suited to these studies as the actual pattern of the bees' flight paths can be analysed. To the best of my knowledge this technique has not been used to study changes in behaviour brought about by pathogens but it is clearly a valuable tool for comparing the ability of infected and uninfected forager bees to explore their environment.

5.1.4 Experimental background and hypotheses:

The purpose of this experiment was to examine the effect of disease on honeybee flight capability, especially on how honeybees explore novel environments. To do this, honeybee colonies were moved to a new area and the harmonic radar was used to track the first explorative flights of the foraging bees. The tracks of individual bee flights from colonies infested with high and low levels of varroa were recorded. Bees from colonies with high levels of varroa are also likely to have high loads of the viruses associated with these mites (see chapter 1.3.4), especially DWV (Genersch, 2005), and so it was predicted that within these colonies there would be individuals representing a wide range of virus species and

disease loads. Disease identity and absolute load within tracked bees were measured at the molecular level using Q-RT-PCR (chapter 2.6.2) to determine whether the individual disease load of the bee affected its flight behaviour.

It was hypothesised that the scouting honeybees would firstly orientate themselves to the new hive location and then search the landscape in a looping pattern until they found the resources they were looking for (e.g. nectar and pollen producing flowers or water) (Capaldi *et al.*, 2000; Reynolds *et al.*, 2007). It was further hypothesised that bees with higher disease loads, or infected with several diseases, would fly shorter distances from the colony or need to stop more often due to the deleterious effects of disease progression and/or because heightened immune response took an energetic toll on the bees. As some diseases can affect honeybee learning ability and memory (Iqbal and Mueller, 2007; Kralj *et al.*, 2007; Mallon *et al.*, 2003; Riddell and Mallon, 2006, see also chapter 4) it was also hypothesised that bees with higher disease loads would be more likely to get lost, either taking longer to return to the hive or not returning at all.

5.2 Methods

5.2.1 Experimental setup:

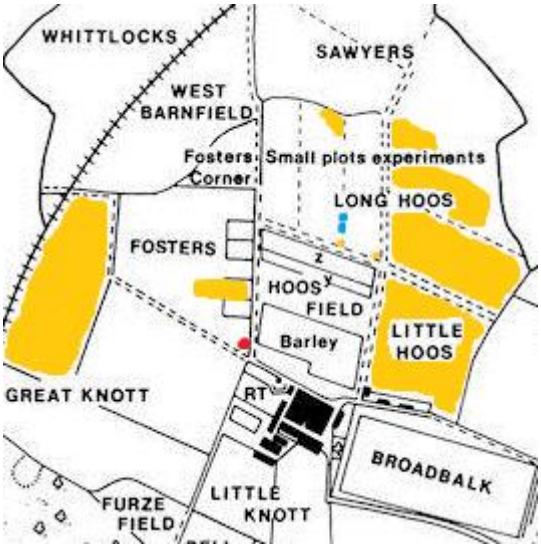
The radar experiment was run on Rothamsted Farm (Hertfordshire, UK: open farmland, mixed crops) between June and August 2009. Two experimental bee hives were moved from an out apiary over 3km from the farm and positioned approximately 250m away from the radar (see figure 5.1). This made it unlikely for any of the foraging bees to have already visited the area so that their initial explorative flights could be followed. During the experiment each hive was placed within a pollination cage; a mesh cage 3m x 3m and 2m high was used to allow the foraging bees to fly outside of the hive without getting out into the environment (figure 5.1c).

The hives were adapted from standard national bee hives and were made up of two brood boxes stacked one on top of the other. The bottom box contained ten frames of bees with their queen, and was open, via a Perspex tunnel, into the pollination cage. Sucrose syrup (30%), water and pollen were provided within the cage for the bees to feed on. The top box was separated from the bottom by a wire mesh which prohibited bees moving between boxes but allowed for physical and chemical contact between the bees. At any time during experimentation the top box would contain only one frame of bees with a Perspex cover to aid in temperature regulation whilst allowing the frame to be observed for waggle dancing

and other behaviour. The top box had its own Perspex tunnel which led out of the pollination cage and was opened on experimental days, allowing foraging bees to exit the hive and explore the local landscape. It was these explorative flights that were tracked with the radar.

Figure 5.1:

A)



B)

C)

The experimental set up. A) Map of Rothamsted farm (Black boxes are farm buildings). The position of the radar is shown as a red circle; the positions of the hives used in the experiment are shown as blue circles. Potential forage at the time of the experiment (oilseed rape fields, bean fields and wild flowers) are shown in yellow. B) The harmonic radar dishes. C) The external view of experimental hive V125. A second tunnel opens into the mesh pollination cage.

Brood combs of bees including the queen and some forager bees were collected from colonies placed more than three miles from the experimental area so that the bees would not have prior knowledge of the area. The collected bees came from a colony with low numbers of varroa (H132: Daily varroa drop count 0.4 in June), and a colony with high

numbers of varroa (first V125: Daily varroa drop count 64.7 in June, and later V91: Daily varroa drop count 6.6 in June) and were placed in the experimental hives. Bees visiting the syrup feeder within the pollination cage were deemed to be forager bees and were marked by painting the thorax with queen marking paint (E. H. Thorne Ltd., U.K.). In the day before the experiment began, a frame of bees was moved from the lower box to the top box, ensuring that the queen was left behind. As relatively few marked bees could be moved into the top box a different colour was used to mark more bees on this frame. This allowed the bees to get used to having something on their thorax so that when the transponder was attached they did not become distracted and begin grooming themselves.

On experimental days the top-box tunnel of one hive was opened allowing the bees in that top box to explore their new environment and begin to forage outside of the pollination cage. Initially, individuals from the two colonies were tracked on alternate days (first H132, then V125 the next day, then H132 the day after that etc). However, after two weeks both colonies were tracked on the same day with individual bees from each colony tracked alternately. As tracks from different bees cannot be distinguished from each other, only one bee was tracked at any one time to avoid confusion between flight tracks. Each bee could be detected within a range of 700m of the radar and from just above ground level up to 6-7m, although if the bee landed or moved behind a solid object, such as a tree, it was temporarily lost from view. Where possible, marked bees were captured on the way out of the hive and transponders were attached to their thorax (section 5.2.2). If marked bees were not seen then unmarked bees were tracked.

When the bee returned to the entrance/exit tunnel it was captured, the transponder was removed and could be used again whilst the bee was frozen and stored for later molecular analysis. If the bee did not return or had not been recorded by the radar in more than 30 minutes, then the bee was deemed lost and a new marked bee was captured and tracked. If the bee did not fly within 30 minutes of release it was recorded as 'did not fly' and stored for later molecular analysis (section 5.2.3). Some bees made very short flights, <1m, these bees were given a chance to perform a longer orientation flight, but again, if they had not done so within 30 minutes then they were recorded as 'flew but no tracks' and stored for molecular analysis (section 5.2.3).

Each frame of bees was monitored over several days until the explorative orientation flights had become foraging flights (indicated by straightened tracks directly to and from resources such as flower patches). At this point the frame was removed to a colony more than 3km away so that the bees could not return to pass on their knowledge to the next frame of bees. A new frame of marked bees was then moved up into the top box, ensuring that the queen was left in the bottom box and new forager bees were tracked.

5.2.2 Attaching the transponders to the bees:

The transponder consists of a 16mm vertical diode aerial with a small low-barrier Schottky diode and an inductive loop weighing about 13mg.

Marked bees emerging into the tunnel were captured in a queen marking cage and manipulated until their thorax was framed in the mesh. The paint was then removed with a blunt cocktail stick and the transponder attached using double sided sticky foam (Self-adhesive 'sticky fixers®', Sellotape®). The bee was then returned to the tunnel.

5.2.3 Molecular analysis:

All returning bees were captured when they entered the entrance tunnel. Forceps were used to hold the bee by the transponder. Once captured the transponder was removed and the bee placed in individual sterile 2ml eppendorf tubes and stored at -80^{o}C for later molecular analysis to detect and quantify their disease load as described previously (chapter 2.6). PCR analysis was done on pooled bee samples to determine which pathogens to run QPCR for. For the QPCR, 56 bees were selected for individual analysis that included individuals making all the different flight types (didn't fly, flew but no track, flew less than 250m from hive, flew more than 250m from hive) and all hives (H132, V125 and V91) were represented.

5.2.4 Track analysis:

Any bees that did not return to the hive could not be analysed using molecular techniques and so were excluded from the analysis. For those bees that did return, the flights were recorded as either 'did not fly', 'flew but no radar track', 'flew less than 250m' or 'flew more than 250m'. If the bee flew but no radar track was produced then the bee's flight had to be less than 3m from the hive as the radar cannot discriminate between two positions less than 3m apart.

The radar data was recorded as the position of the bee on each revolution of the radar (every 3s) as range (distance from the radar) and theta (angle of the bee from north). These data were then converted into X, Y coordinates from the hive using simple trigonometry.

From the radar tracks several flight parameters were quantified (table 5.1). The total flight distance was taken as the complete length of track measured for each bee. This was calculated by summing the distance between each point recorded. It should be noted

however that if the bee's flight was obscured, by a hedge or tree for example, then the distance the bee flew might be longer than seen in in radar track. The total flight duration was the sum of all the points multiplied by three (as the bee's position was recorded every 3s the sum of these recordings multiplied by three is the flight duration in seconds). The average groundspeed of the flight was calculated by dividing the total flight distance by the total flight duration. The maximum distance of the bee from the hive was recorded as the furthest signal from the hive.

The bees may land within a flight. If they do this then they are lost from the radar. This leaves gaps in the recording which may be as little as 3s (one radar rotation) or may last for several minutes. Short gaps may be caused by the bee flying behind an obstruction such as a tree so only gaps of greater than 15s are recorded as such. It should be noted however that some of the longer gaps might be caused by the bee flying behind bigger obstructions such as hedgerows. As the number of stops that can be taken is relative to the length of flight (ie a bee that only flew for a short time is not able to stop as many times as a bee that flew for a longer time), the gaps were calculated as the number of gaps relative to the total number of fixes (number of gaps divided by total number of recordings) and the number of gaps per 100s.

Table 5.1:

Flight parameter (units)	**Description**
Total flight distance (m)	The total distance travelled by the bee
Total flight duration (s)	The time taken for the flight
Average flight speed (m/s)	The average speed of the flight (distance/duration)
Maximum distance from hive (m)	The furthest signal from the hive
Gaps relative to total points	The number of stops relative to the total flight duration (in terms of number of fixes)
Gaps per 100s	The number of stops relative to the total flight duration (in terms of time)

Table showing the flight parameters calculated from the radar track data.

5.2.5: Statistical analysis:

Where the population of bees tested had a good mix of individuals that were either infected or uninfected or had a bimodal distribution of high and low DWV (see chapter 4) the presence/ absence and high/low load was analysed separately from the relative disease loads.

All statistical analysis was carried out in GenStat® (14th edition) (Payne, 2011).

5.2.5.1: Chi Square analysis of flight types:

Chi square analysis was used to determine whether high or low loads of DWV or presence/ absence of disease was related to flight types (didn't fly, flew but no track, <250m, >250m). The null hypothesis was that there should be an equal distribution of bees with each disease load (high/low) across the flight types and an equal distribution of bees for which each disease was either present or absent across the flight types. This analysis could be done on all bees that returned to the hive even if there was no radar track produced.

5.2.5.2: Mann-Whitney U tests on flight parameters:

Mann-Whitney U tests were used to determine whether the high or low loads of DWV or the presence/ absence of disease had an effect on any of the flight parameters (see table 5.1). This analysis could only be carried out on bees that were successfully tracked.

5.2.5.3: Simple linear regression analysis on flight parameters:

Because the QPCR analysis also provided the disease load for each virus relative to the housekeeping gene β actin; these could also be used for analysis. Simple linear regression analysis was carried out to look for the relationship between the relative loads of each disease in individual bees and aspects of their flights. The log values were taken for some of the flight data to normalise them (total flight duration, total flight distance, average flight speed and the maximum distance from the hive).

5.3 Results

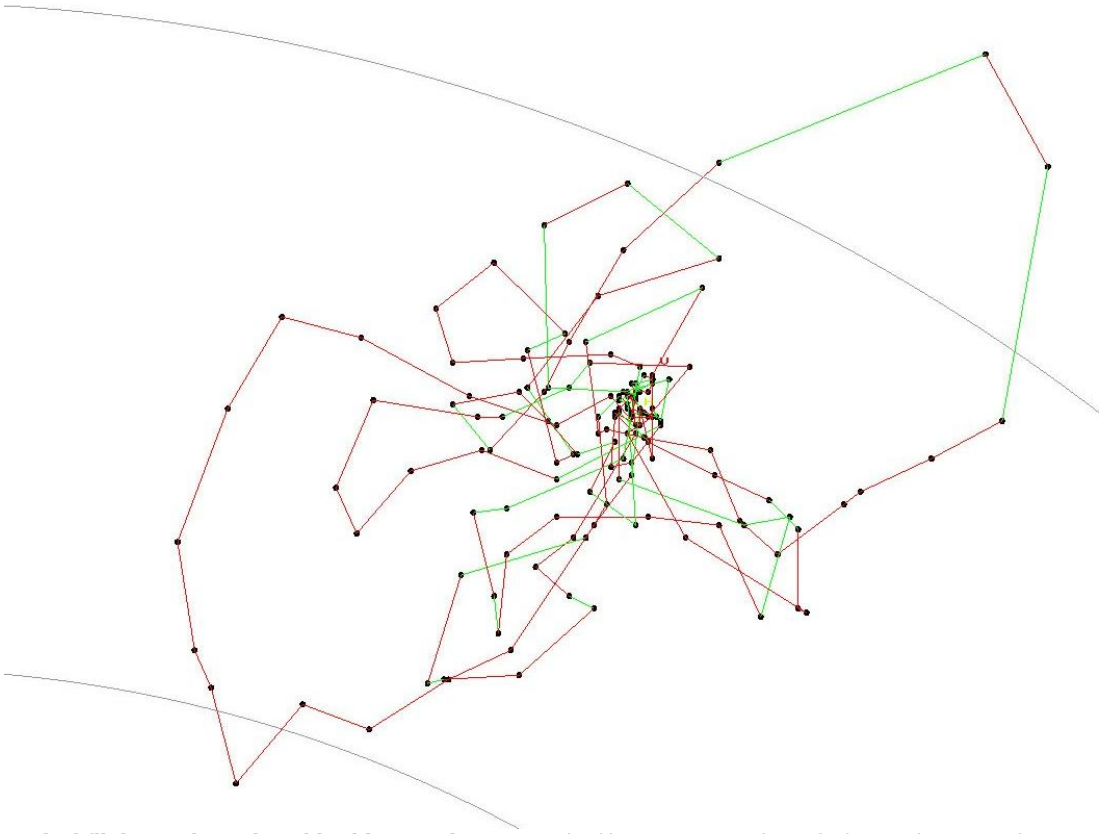
5.3.1: Radar summary:

A total of 80 bees were used in this experiment, 36 from the healthy colony (H132), 21 from one of the high varroa colonies (V125) and 23 from the second high varroa colony (V91) (a total of 44 from high varroa colonies). Of these, nine bees did not fly (six from the healthy colony, three from the high varroa colonies) and, of those that did fly, 32 bees flew far enough to produce radar tracks (14 from the healthy colony and 18 from the high varroa colonies). There were eight bees that either did not return to the hive, five from high varroa colonies and three from the low varroa colony. A chi square test showed that the number of bees that did not return was not significantly affected by the varroa load of the colony (χ^2 (1, N = 80) = 0.202, P = 0.35.)

Many of the tracks produced look similar to those found in similar studies of honeybee search patterns (Reynolds *et al.*, 2007), with looping flights that in a previous investigation were shown to be Levy flight patterns, an optimal search pattern (Reynolds *et al.*, 2009) (figure 5.2).

To determine whether the looping flights found in this study were the optimal search patterns, or Levy flight patterns, shown in Reynolds' work (2009), further analysis of these tracks is being carried out by Dr Reynolds. This analysis will also examine whether there is an effect of disease status on the likelihood of bees performing optimal search patters.

Figure 5.2:



Typical flight track produced in this experiment. Track of bee H132-14, a bee which tested negative for BQCV, had low DWV load and relatively low levels of *n. apis* and *N. ceranae*. This bee shows a looping flight pattern as it explores the landscape. The range lines are 100m apart. The red lines connect consecutive points, if there was a gap, even of only one rotation, the points are connected by green lines.

5.3.2: Disease loads:

Although all of the tested bees had DWV, because the quantitative molecular analysis always show a bi-modal distribution for DWV load (4.3.2; figure 4.6), the data could be analysed as high (relative DWV load <-4) and low (relative DWV load >-4). As before the lower/more negative the relative disease load, the more disease was present (see chapter 2.6.2). There were a good number of bees testing positive and negative for BQCV so the

effect of the presence/absence of BQCV could also be tested. There were only very few bees that tested negative for the other diseases detected (*N. apis* and *N.* ceranae) so the effect of the presence/absence of these diseases could not be tested for (table 5.2).

For all diseases the relative disease load could be used for analysis. The amount of disease was calculated relative to the housekeeping gene β actin. The negative control (water) produced a result of 20 and so any bees testing negative for any disease were also recorded as 20. Bees with higher disease loads had lower relative values (chapter 2.6).

Table 5.2:

Pathogen	No. bees
Low DWV	37
High DWV	18
*	1
- BQCV	21
+ BQCV	35
*	0
- Na	4
+ Na	49
*	3
- Nc	2
+ Nc	53
*	1

Table of disease presence for bees tracked using the harmonic radar. DWV results are given as high (relative DWV load <-4) and low (relative DWV load >-4), all other diseases (BQCV, *N. apis* (Na) and *N. ceranae* (Nc)) are shown as presence (+)/absence(-). Inconclusive results are shown as ' * '. 56 bees were tested in total.

5.3.3: Chi square analysis of flight types:

A Chi-Square test showed no significant deviation from the null hypothesis for either disease (DWV: χ^2 (3, N = 56) = 5.37, P = 0.147. BQCV: χ^2 (3, N = 56) = 1.65, P = 0.647.). This suggests that the load of DWV (high or low) and the presence/absence of BQCV had no effect on flight type (Tables 5.3 & 5.4).

Table 5.3:

Flight type	**Didn't fly**	**No track**	**<250m**	**>250m**
Low DWV load	6	11	6	14
High DWV load	2	9	5	2

Table showing the number of bees with low/ high DWV load for each flight type.

Table 5.4:

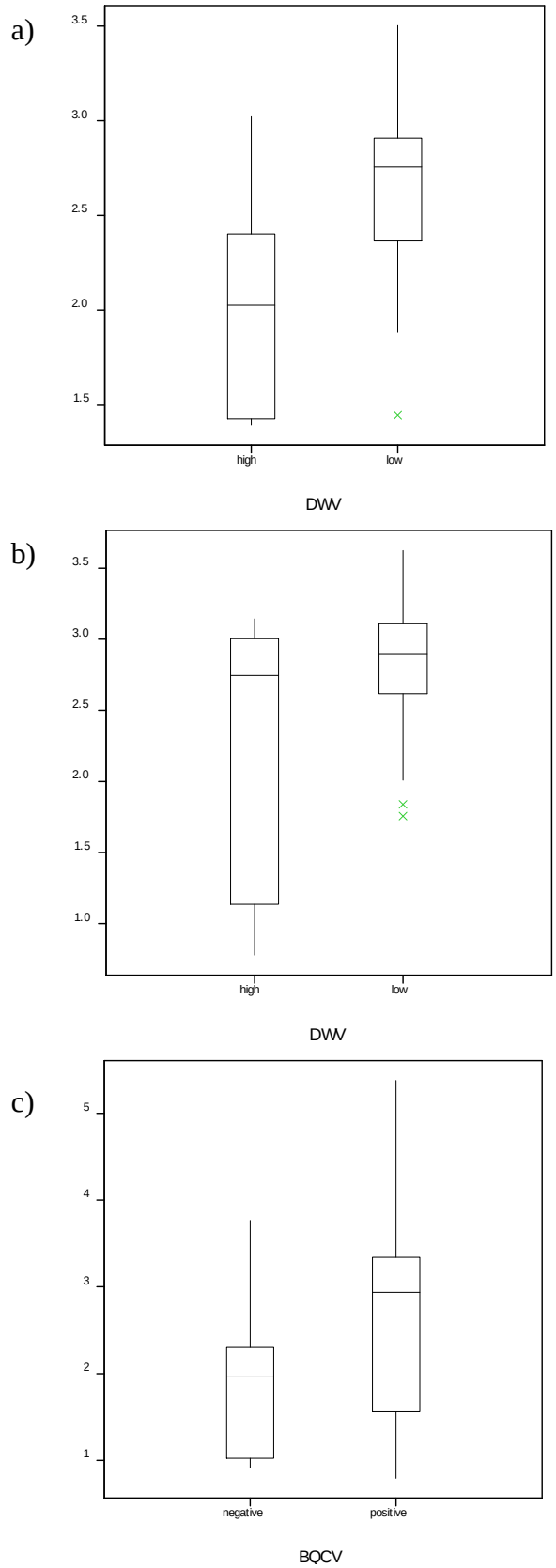
Flight type	Didn't fly	No track	<250m	>250m
Negative for BQCV	2	8	6	5
Positive for BQCV	6	12	6	11

Table showing the number of bees tested positive/ negative for BQCV for each flight type.

5.3.4: Mann-Whitney U test on flight parameters:

The Mann-Whitney U tests on the flight parameters calculated from the radar tracks showed that the load of DWV (high or low) had a significant effect on total flight distance (U = 29.0, P = 0.022. See also Figure 5.3a), it also had an effect on maximum distance travelled from the hive (U=35.0, P = 0.055. See also Figure 5.3b) although this was only significant at the 10% level. Bees categorized as having high DWV loads flew shorter distances and tended to stay closer to their hives than bees with low DWV loads. DWV had no effect on mean flight speed however (U = 65, P = 0.808). The presence/absence of BQCV had an effect at the 10% level on mean flight speed (U = 52.0, P = 0.053. See also Figure 5.3c); infected bees tended to fly faster than uninfected bees. BQCV had no effect on total flight distance (U = 80, P = 0.547) or maximum distance travelled from the hive (U = 66, P = 0.208). Neither virus had a significant effect on any of the other measures of flight performance including duration of flight (DWV: U = 42, P = 0.13. BQCV: U = 79, P = 0.517), and number of gaps in recording (representing the bee either being hidden from the radar or having landed) (DWV: U = 64, P = 0.766. BQCV: U = 70, P = 0.285).

Figure 5.3:

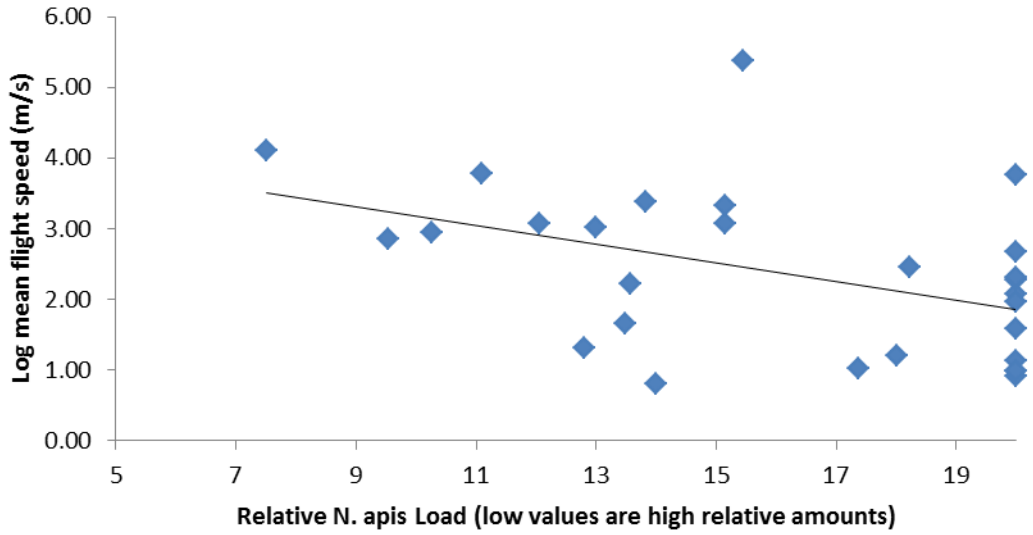


Box plots showing the effect of DWV load (high/low) on flight distance (a) and duration (b) and BQCV (presence/absence) on speed (c). Box plots show the median, interquartile range (box) and the range (sticks) with any outliers shows as Xs. Bees with high DWV flew for shorter distances and times and bees infected with BQCV flew faster.

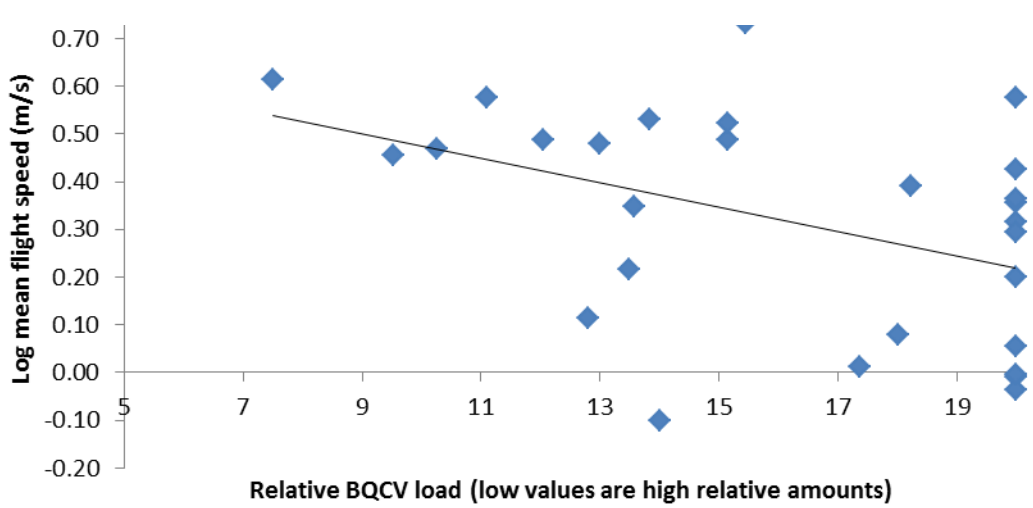
5.3.5: Simple linear regression analysis on flight parameters:

Figure 5.4:

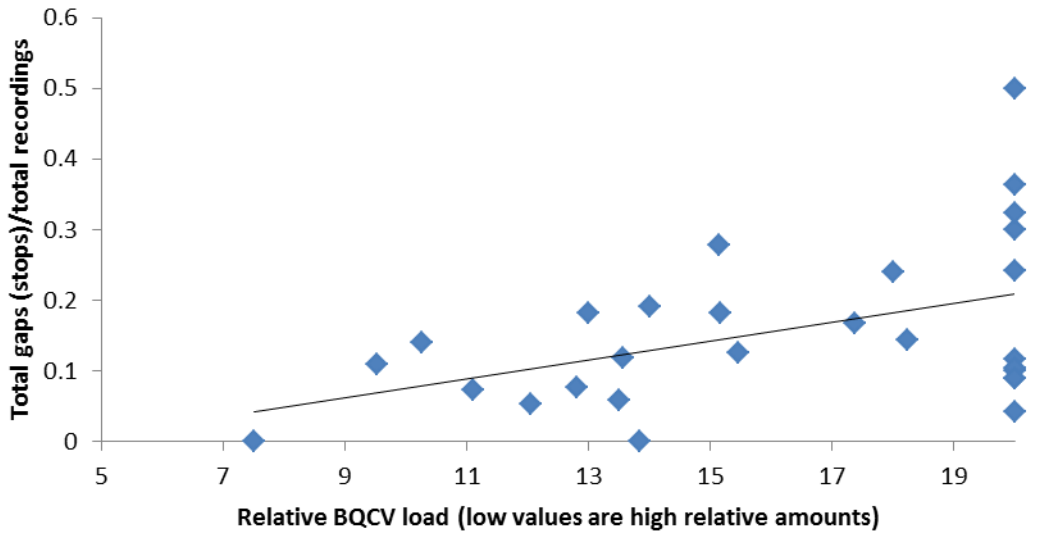
a)



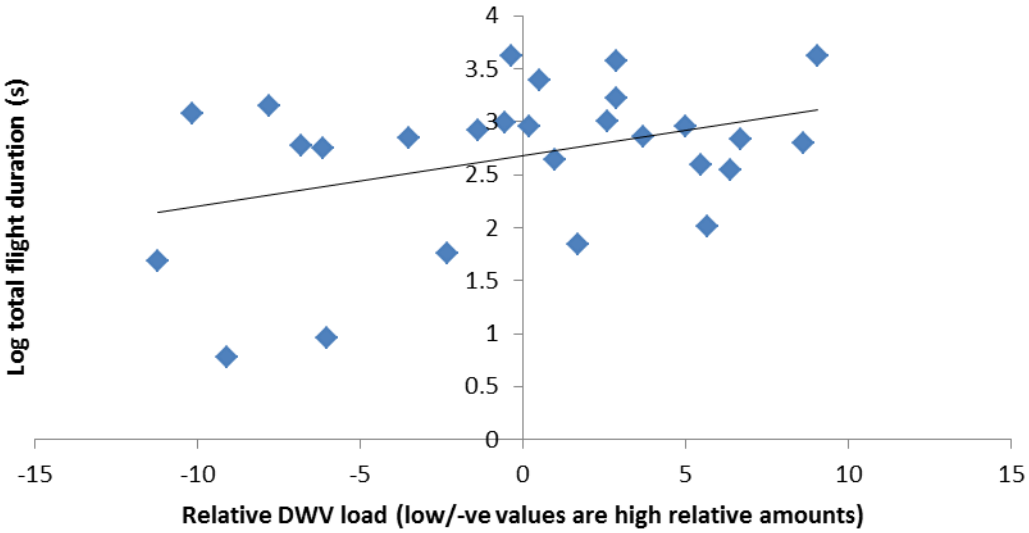
b)



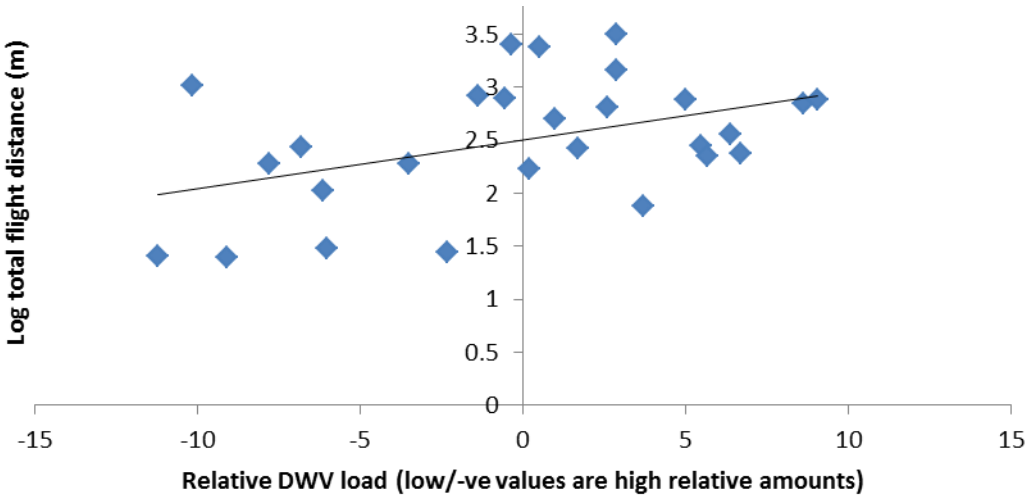
c)



d)



e)



Plots showing relationship between disease load relative to β actin and flight analysis. For all plots a relative disease load of 20 is a negative result, and lower values are higher disease loads. Bees with higher *N. apis* loads tended to fly faster (a) than bees with lower *N apis* loads. Bees with higher BQCV loads tended to fly faster (b) and stop less often (c) than bees with lower BQCV loads. Bees with higher DWV loads tended to have shorter flights in terms of distance (d) and duration (e) than bees with low DWV loads.

There was no effect of the load of *N. ceranae* on any flight parameters. There was a significant effect of BQCV and *N. apis* loads on the log of speed of flight (Figure 5.4 a & b). (BQCV: $F_{1,26}$ = 6.13 P = 0.02, intercept = 18.47 SE = 1.18, slope = -7.46 SE = 3.01. *N. apis*: $F_{1,26}$ = 4.46 P = 0.04, intercept = 0.661 SE = 0.167, slope = -0.0258, SE= 0.0122.)

There was also a significant effect of BQCV load on the number gaps relative to the total number of recordings ($F_{1,26}$ = 6.86 P = 0.015 intercept = 13.64 SE = 1.15, slope = 15.56 SE = 5.94. See also Figure 5.4 c) suggesting that bees with high BQCV loads and/or *N. apis* loads flew faster and bees with high BQCV loads also stopped less often than bees with low loads of these pathogens. There was a significant effect of the relative DWV load on the log of duration of flight ($F_{1,26}$ = 4.24 P = 0.05, intercept = -8.23 SE = 4.08, slope = 3.04 SE = 1.47. See also Figure 5.4 d) and the log of flight distance ($F_{1,26}$ = 6.08 P = 0.021, intercept

= -10.72 SE = 4.4.2, slope = 4.25 SE = 1.72. See also Figure 5.4 e) suggesting that bees with higher DWV loads took shorter flights, in terms of both flight time and distance, than bees with lower DWV loads.

5.4 Discussion

Many of the tracks produced in this experiment by bees exploring a novel landscape had the appearance of the looping tracks seen in previous research (Reynolds *et al.*, 2007), (see figure 5.2). These flight patterns were previously seen when bees were searching for a feeder which had been removed. Similar loops were produced by bees when they did their first orientation flights (Capaldi *et al.*, 2000), although on these flights the bees only did one loop whilst the bees tracked here often did several loops. This suggests that a similar searching flight is used to explore new areas and, on a smaller scale, to search for missing items. The initial orientation flights of new forager bees may only have single loops whilst experienced forager bees explore with several loops because the older experienced forager bees may have more energy to fly over greater distances or have better memories to remember more of the landscape.

The results of this experiment suggest that the presence of DWV reduces honeybees' flight capability by causing them to take shorter flights both in terms of distance covered and time spent outside of the colony. The wings of all the bees used in this experiment appeared normal and so the virus had this effect even in the absence of the classic wing deformities that give it its name. The bees may not show deformity because they became infected after their wings had developed or the virus may have been at a low enough level during development that their wings developed normally. Despite this the bees' flight was still affected. The Chi-Square analysis however showed that DWV load didn't affect whether the bees were likely to fly or not. This is possibly because the majority of bees tracked in this experiment were of unknown age or task so that the tracked bees might not have all been forager bees; although every effort was made to choose bees that seemed eager to leave the tunnel there is the possibility that some could have been guard bees or middle aged bees performing fanning duties in the tunnel. Therefore, some of the bees may not have flown due to age and task rather than disease presence or load.

A study on bees artificially infected with DWV showed increased sensitivity to sucrose in the infected bees compared with controls (Iqbal and Mueller, 2007) suggesting that the virus was energetically stressing the bees' and making them more hungry. This could also mean that they have less energy for sustained flight which would explain why the bees with higher DWV loads flew for shorter distances than bees with lower DWV loads.

Like DWV, it has been suggested that *N. ceranae* causes energetic stress in honeybees as bees with *N. ceranae* infection are more responsive to sucrose than uninfected bees (Mayack and Naug, 2009). Bees with *Nosema* spp. infection also take longer to return to their colonies and are more likely to get lost than uninfected bees when released 6 or 10 m away from the hive (Kralj and Fuchs, 2010). It is surprising therefore that, in my study, there was no significant effect of *N. ceranae* load on flight behaviour and *N. apis* only affected flight speed. However, the QPCR analysis detected only very low levels of either species of *Nosema* in any individual bee. Of all the bees tested as part of this project only two had relatively high levels of *N. apis* (Copy number in QPCR sample: $3.28x10^8$ and $9.64x10^8$) whilst all the rest had much lower loads (Average copy number for rest of samples: $9.72x10^3$). Three bees had relatively high levels of *N. ceranae* (Copy number in QPCR sample:$6.50x10^7$, $3.19x10^9$ & $3.95x10^{10}$) compared to the rest (Average copy number for rest of samples: $1.08x10^4$). All the bees tested as part of this experiment had low *N. apis* loads and only one bee had high levels of *N. ceranae* (Copy number in QPCR sample: $3.95x10^{10}$). In both Mayack & Naug (2009), and Kralj & Fuchs' (2010) experiments the bees were artificially infected and probably had higher *Nosema* spp. loads than I detected in this experiment. It is possible therefore that the energetic stress and changes in flight behaviour are only seen with higher *Nosema* spp. loads.

There was a trend for bees infected with BQCV and/or *N. apis* to fly faster than uninfected bees, and bees with higher BQCV and/or *N. apis* loads flew faster than bees with low BQCV or *N. apis* loads although this was only significant at the 10% level. It is known that BQCV and *N. apis* often co-occur (Bailey *et al.*, 1983), so although both correlated with flight speed it is possible that only one of the two actually caused the effect. Little is known about any effects of BQCV on honeybee behaviour. However, *N. ceranae* is known to energetically stress bees and it is likely that *N. apis* has a similar effect. It was thought that this was due to increased immune defence (Mayack and Naug, 2009) but it could also be due to the pathogen causing the bees to overexert themselves; it is possible that infected bees lose their ability to judge the speed at which they are flying and so fly faster thus using up more energy than they otherwise would.

It should be noted that the experience of the bee has been shown to have an effect on flight speed, with more experienced bees flying faster (Capaldi *et al.*, 2000). The results of Q-RT-PCR analysis in chapter 4 showed that older bees were likely to have higher levels of BQCV (see chapter 4.3.2) so although the results suggest that bees with higher loads of BQCV flew faster this may in fact be because the bees with higher loads of BQCV were older

than those with lower loads and older bees were more experienced fliers and flew faster than younger bees.

It is also possible that the effect of BQCV and *N. apis* on flight speed was only found to be significant due to the low level of replication. Much of this analysis was carried out on a fairly small number of bees as the analysis could only be carried out on bees which had been tracked by the radar, recaptured and then undergone Q-RT-PCR analysis (N=28). If a similar study could be carried out on a larger number of bees then the statistical analysis would be more robust.

The data do however clearly show that bee pathogens can affect honeybee flight behaviour. My analysis shows that bees infected with common honeybee diseases are able to fly shorter distances and for shorter lengths of time than healthier bees; in this instance that meant that the foraging bees could explore less of the novel environment which could mean that they would have less chance of finding good food sources for the colony. In general it could also mean that bees from sicker colonies would be less able to take advantage of mass flowing crops at a distance from the colony than healthier colonies. This could have knock-on effects to foraging potential and colony strength and therefore colony survival.

Chapter 6: In-hive behaviour and time to first forage for honeybees from colonies of differing disease status in observation hives.

Abstract:

The European honeybee, *Apis mellifera*, is important economically not just for honey production but also as a pollinator. However, honeybee numbers in some areas are declining. A range of interacting factors are thought to be involved, including pathogens and parasites, which are known to cause changes in the behaviour of their hosts.

In this chapter observation hives were used to study whether the disease load of the colony a bee develops in has an effect on in-hive behaviour and age at which bees first began foraging. The aspects of behaviour which were examined were the amount of time spent resting or interacting with other bees.

Bees taken from colonies infected with deformed wing virus, black queen cell virus and *Nosema* spp. appeared to begin foraging earlier than bees taken from colonies with only one or two of these pathogens; however, this was not statistically significant. There was also no statistically significant effect of colony disease status on the interacting or resting behaviour of individuals within the observation hives. This may mean that the disease status of the colony a bee develops within has no effect on that bee's behaviour, or the replication used in this experiment may have been too low to pick up statistically significant changes to behaviour.

6.1 Introduction

6.1.1: Use of observation hives to study honeybee in-hive behaviour:

In nature bees usually nest in dark cavities with small entrance holes, inside hollow trees for example. This environment is simulated in a hive but makes it very difficult to learn anything about the behaviour of the bees within (Gary, 1975). Observation hives are the solution to this problem. An observation hive is a transparent walled hive into which 2 or more frames of bees can be placed and observed (Dadant, 1975). Observation hives can be used to study the in-hive behaviour of honeybees and their progression from in-hive to foraging activity (e.g. Bailey and Fernando, 1972; Mattila and Otis, 2006). The first documented use of observation hives to study honeybee behaviour was in the eighteenth century, when the

French scientist René-Antoine Ferchault de Réaumur used such hives to study, amongst other things, the egg laying behaviour of honeybee queens (Ratcliff, 2005).

More recently observation hives have been used to study a range of behaviours including hygienic behaviour in honeybees. Hygienic behaviour is the recognition and removal of dead or diseased brood from the hive (Rothenbuhler, 1964). Panasiuk *et al.*, (2010) showed that bees of all ages were involved in hygienic behaviour, but that these tasks were mostly carried out by bees aged 6-10 days. Some colonies are also better at this hygienic behaviour than others (e.g. Palacio *et al.*, 2000; Spivak and Gilliam, 1993). Palacio et al., (2005) observed hygienic and non-hygienic bees over several hours to show that the hygienic bees were able to detect pin-killed brood within an hour of their death; these hygienic bees then uncapped and removed the affected brood much more quickly than the non-hygienic bees.

Survival can be monitored in observation hives. Higginson *et al.*, (2011) examined the effect of wing damage on survival to show that mortality rate increased with wing damage and that bees with damaged wings were less able to forage. Observation hives have also been used to investigate survival and behaviour of bees infected with different pathogens (section 6.1.3).

Observation hives can also be used to study the behaviour of other species within hives. Atkinson and Ellis (2011) used observation hives to study the hiding behaviour of six beetle species living within honeybee colonies and demonstrated that species coevolved with honeybees were better at finding confinement sites to hide in than other species. Observation hives also facilitated the study of the division of labour within the hive (e.g. Seeley and Kolmes, 1991; Trumbo *et al.*, 1997).

6.1.2: Division of labour in bees:

There is a division of labour within social insect colonies such that different groups, or castes, of individuals, perform different tasks. The division of labour in honeybees was first recorded by Dönhoff in 1855 (see Calderone, 1998) who requeened a colony of black honeybees with a yellow queen and observed that the new yellow worker bees did not begin foraging for up to seventeen days. This age-related division of labour in honeybees is often called age-polyethism because workers tend to perform different tasks at different ages (Calderone, 1998). Younger bees attend to in-hive tasks such as caring for brood and attending the queen and older bees undertake foraging for resources such as nectar and pollen (Hooper, 2008). Age polyethism is not fully understood in honeybees. For example,

Ribbands (1952) found that honeybee workers could alter the duration of tasks or omit them entirely resulting in variation in the time to first forage of between nine and 35 days. He suggested that this variation demonstrated that division of labour was controlled more by the needs of the colony than by the age of the workers. Many subsequent experiments have shown that there is a significant association between age and task (e.g. Calderone, 1995; Seeley and Kolmes, 1991) but the mechanisms behind when bees move from one task to the next are still under debate. Several models have been put forward, many of which are reviewed by Calderone (1998) and modelled by Johnson (2003).

Division of labour in honeybees does not rely on behavioural changes alone; forager bees are physiologically different from in-hive bees. For example, in-hive honeybees have larger hypopharyngeal glands than forager bees (Huang *et al.*, 1994). Higher levels of dopamine, serotonin, and octopamine were found in the antennal lobes of forager bees when compared to in-hive bees, regardless of age. The difference was most pronounced for octopamine (Schulz and Robinson, 1999).

It is thought that juvenile hormone (JH) is responsible for the switch from in-hive to foraging tasks. Juvenile hormone increases with age in worker honeybees (Fluri *et al.*, 1982; Jassim *et al.*, 2000); forager bees typically have higher JH levels than in-hive bees (Elekonich *et al.*, 2001), and the transition from in-hive to foraging tasks can be initiated by treating bees with juvenile hormone or chemicals that mimic it (Jaycox, 1976; Jaycox *et al.*, 1974). However, Sullivan *et al.*, (2000) showed that JH did not activate the transition from in-hive to forage duties but merely controlled the rate at which the transition occurred. Bees that had their corpora allata (the gland which produces JH) removed still became forager bees, just much more slowly than sham treated bees.

Bees generally move from in-hive to foraging activity after about three weeks (Hooper, 2008), but some factors can accelerate or slow down this progression. Janmaat and Winston (2000) found that bees with low pollen stores began foraging earlier than normal, but a similar study by Mattila and Otis (2006) found no such effect. Bees began foraging earlier than normal when treated with CO_2 (Woyciechowski and Moron, 2009) and when raised at a constant 36^{o}C, the highest temperature normally found in bee hives, rather than the usual 34^{o}C (Becher *et al.*, 2009).

6.1.3: Effect of bee diseases on honeybee in-hive behaviour and polyethism:

Several experiments have shown that honeybees infected with *Nosema* spp. and SBV are less likely to feed brood or attend the queen than uninfected honeybees (Wang and Moeller,

1970Bailey and Fernando, 1972). Varroa infestation and SBV infection caused honeybee workers to begin foraging earlier than controls (Bailey and Fernando, 1972; Downey *et al.*, 2000; Janmaat and Winston, 2000), whilst honeybee workers infested with tracheal mites began foraging later than uninfested controls (Downey *et al.*, 2000). There is conflicting evidence when it comes to *Nosema* spp. however. Whilst both Wang & Moeller (1970) and Woyciechowski & Moron (2009) found that *Nosema* spp. infection caused honeybee workers to begin foraging earlier than uninfected individuals, Mattila & Otis (2006) found no effect of *Nosema apis* infection on age at first forage. To the best of my knowledge there have been no such experiments on bees infected with BQCV.

6.1.4: Experimental setup and hypotheses:

For this experiment bees were taken from colonies with differing combinations of pathogen species (as determined using molecular techniques) and transferred to observation hives where their in-hive behaviour and foraging behaviour could be monitored. It was hypothesised that bees from colonies with more pathogen species present would begin foraging earlier than bees from colonies with fewer pathogen species present. The pathogens studied were DWV, *Nosema* spp. and BQCV. DWV was found in all colonies used in this experiment and so its effects cannot be determined with these results. *Nosema* spp. infected bees have been shown to avoid feeding brood and begin foraging earlier than uninfected bees (Wang and Moeller, 1970; Woyciechowski and Moron, 2009) although other studies showed no effect on age to first forage of *Nosema spp.* (Mattila and Otis, 2006). BQCV has not been studied before for its potential effects on behaviour but it is known to interact with *Nosema spp.* to decrease honeybee survival (Bailey *et al.*, 1983) and so it is possible that the reason no effect of *Nosema* spp. was found in the work of Mattila and Otis (2006) was because the behavioural change was only found when the two diseases interacted. It was, therefore, hypothesised that the bees from colonies with both *Nosema* spp. and BQCV would begin foraging earlier and be less likely to attend brood or the queen than bees from colonies without these pathogens.

The survival of the bees was also monitored and it was hypothesised that bees from colonies with more pathogen species present would die faster than bees from colonies with fewer pathogen species present as, individually, all three pathogens have been shown to decrease the survival time of bees (e.g. Bailey *et al.*, 1983; Forsgren and Fries, 2010; Yang and Cox-Foster, 2007).

6.2 Methods

6.2.1: Observation hive set up:

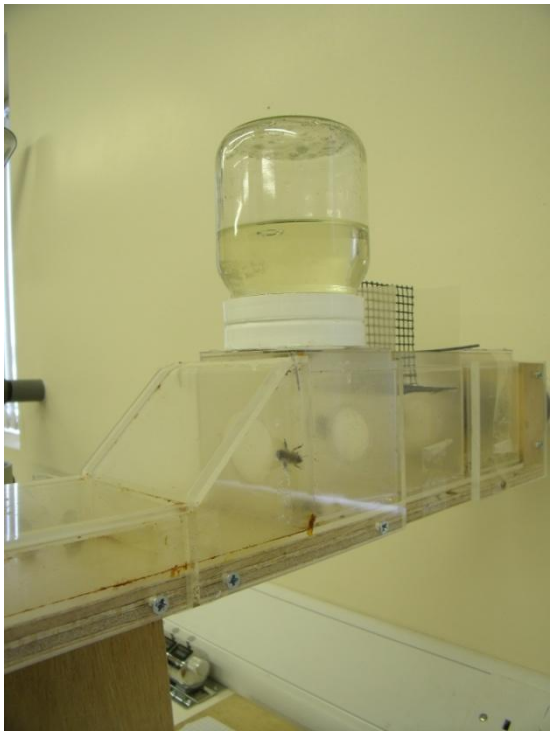
Observation hives were used to examine the in-hive behaviour, time to first forage and longevity of bees from colonies with differing numbers of diseases present. Two observation hives were set up in the field lab on Rothamsted farm (figure 6.1). Each section of the observation hive was 460 x 227 x 45mm and could hold a single national frame (the size of frames used in the Rothamsted hives). The entire set up was approximately 1m tall (when three sections were in place) and the base rested on the work surface. The room was maintained at 26˚C. A tunnel connecting the base of the hive with the outside allowed bees to enter and exit for foraging. The top and sides were made from clear Perspex to allow the foraging bees to be observed and their behaviour recorded. To select colonies to establish in the observation hives, pooled bee samples from 32 Rothamsted colonies were evaluated using PCR and of these, 21 tested positive for *Nosema* spp*.* and 17 tested positive for DWV, and of those 12 colonies were positive for both pathogens (chapter 2.6). The colonies that were chosen for the observation hive experiment were selected from those that remained. The observation hive colonies were selected to be as similar as possible with regard to disease profile.

Observation hive A originated from field colony 134, a colony based at Cheapside that had low varroa levels (1.1 daily mite drop in May 2011) but tested positive for DWV in PCR analysis in the same month. Observation hive B originated from field colony 90, a colony also based at Cheapside that also had low varroa levels (0.4 daily mite drop in May 2011) and also tested positive for DWV. It would have been preferable to use colonies with no diseases, however all colonies available tested positive for some pathogen so colonies used to establish the observation hives were chosen to be as similar as possible and to have as little disease as possible. Three frames of bees were placed in each observation hive, one on top of the next, with space remaining so that a fourth, empty frame could be added if the colony became large and needed more space (figure 6.1). Having additional space for expansion also helped prevent them from swarming. If the colony began to dwindle the extra frame could be removed or a frame of new bees added as necessary. Food stores within each observation hive were monitored and if supplies grew too low a jar of ~200ml of 60% sucrose solution was fed to the colony (figure 6.2).

Figure 6.1:

One of the observation hives at the Rothamsted Bee Field Lab. Each section is 460 x 227 x 45mm.

Figure 6.2:



The tunnel of the observation hive with the feeder attached. The feeder has a honey jar which can be filled with sucrose solution. The bees can get to the sucrose via a gauzed hole.

The aim of this experiment was to study the in-hive behaviour of individual bees taken from source colonies of differing disease status. To this end, six colonies were selected from the Rothamsted apiaries; two colonies testing positive for DWV only, two for DWV and *Nosema* spp. and two for DWV, *Nosema* spp. and BQCV.

Brood frames were collected from the source colonies and maintained in an incubator in darkness at 35°C overnight. Newly emerged adults were collected within 24 hours of emergence and were marked with coloured, numbered queen marking tags (E. H. Thorne Ltd., U.K.) to indicate the source colony, receiving observation hive and replicate number for every individual bee. Orientation of the marking tags was used to differentiate between which observation hive the bees went into so that it was possible to tell if bees had drifted between the hives. Bees going into observation hive A had the tags aligned so that they were the right way up if the bees head was facing downwards (head down) whilst bees going into hive B had the tags aligned so that they could be read if the bee's head was upwards (head up) (see figure 6.3). Ninety four bees from each source colony were added to each observation hive via a small hole at the top and monitored over a period of 67 days until all marked bees had died. The source colonies used, methods used for marking individual bees and the date and observation hive that the bees were added to, are shown in table 6.1.

Table 6.1:

Source colony	No of bees	Disease status of source colony	Receiving Observation hive	Tag colour	Tag orientation	Date added to observation hive
15	94	DWV	A	yellow	Head up	06/08/2011
	94		B	yellow	Head down	07/08/2011
88	94	DWV, BQCV & *Nosema* spp.	A	red	Head up	06/08/2011
	94		B	red	Head down	07/08/2011
98	94	DWV	A	green	Head up	06/08/2011
	94		B	green	Head down	07/08/2011
146	94	DWV, BQCV & *Nosema* spp.	A	white	Head up	06/08/2011
	94		B	white	Head down	07/08/2011
64	94	DWV & *Nosema* spp.	A	dark blue	Head up	09/08/2011
	94		B	dark blue	Head down	09/08/2011
132	94	DWV & *Nosema* spp.	A	light blue	Head up	08/08/2011
	94		B	light blue	Head down	08/08/2011

Table showing the set up for both runs of the observation hive experiment to monitor the in hive and foraging behaviour of bees from colonies of differing disease statuses.

Figure 6.3:

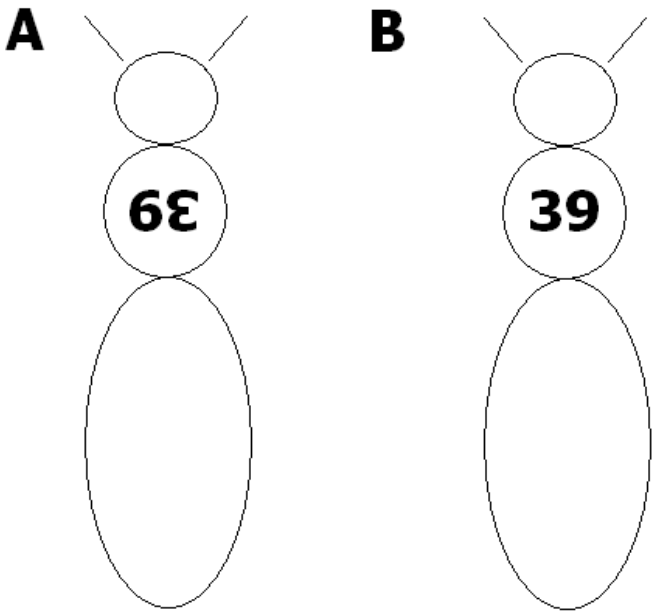


Figure showing the orientation of marking tags on the bees for run 2 of the observation hive experiment. A: bees added to observation hive A with the tags readable when the bee's head is down. B: bees added to observation hive B with the tags readable when the bee's head is up. Both bees are shown with the number 39 and their heads up.

6.2.2: Monitoring of behaviour within the observation hives:

Over the six weeks of this experiment, in-hive behaviour of individual bees in both observation hives was monitored twice a day; once in the morning between 8 and 10am and once in the evening usually between 5 and 7pm. The position and behaviour of each marked bee that could be seen was recorded in a scan sample. The position of the bee was recorded in relation to a 6 by 4 grid for each frame, and its behaviour was chosen from the list of behaviours in table 6.2.

Not all the marked bees in each hive were recorded on each day as some of the bees were not seen. This may have been because they were outside of the hive, foraging, or because they were hidden within cells. However, some measure of longevity could be achieved by recording when marked bees were last seen.

Table 6.2:

Solo behaviour	
Self-grooming	Rubbing of legs against body for the purpose of grooming
Cleaning	Actively checking/cleaning the comb surface
Manipulating Wax	Manipulating wax either to cover/uncover stores or draw out comb
Head in cell	Cleaning in cell or tending egg (if brood is visible 'tending brood')
Head in nectar/pollen cell	Storing or moving pollen or nectar
In cell	In cell with only tip of abdomen visible (possibly sleeping)
Walking	Moving purposefully across the comb
Sitting	Still, not interacting with others or the comb
Shaking	Shaking to show a need to be groomed by another bee
Fanning	Fanning wings to increase air flow within the hive
Inter-individual behaviour	
Grooming worker	Touching surface of a worker for the purpose of grooming
Grooming drone	Touching surface of a drone for the purpose of grooming
Being groomed	Being touched by another worker for the purpose of grooming
Antennal touching	Face to face touching antennae
Giving food	Transferring liquid food by exuding a drop of liquid between mandibles
Receiving food	Receiving liquid food from another worker
Waggle dancing	Waggle dancing to pass on information about food source
Watching waggle dance	Touching or following a dancing worker
Shaking bee	Shaking a worker bee
Being shaken	Being shaken by a worker bee
Tending Queen	In the queen's retinue, facing queen and touching with antennae
Tending brood	Head in cell with larvae

Table showing a list of behaviours recorded in the observation hive experiment. Behaviours recorded are based on those used by Mattila and Otis (2006).

On days when the weather was good (i.e. not raining or too windy) foraging from each hive was also monitored. The tunnel of one hive was watched for 1 hour and every marked bee that exited or entered the tunnel was recorded along with the time that she left and returned. From this the time spent foraging could be calculated. The presence of any pollen or propolis loads was also recorded. The second hive tunnel was also monitored for an hour per day. On each day that foraging was monitored the hive monitored first was alternated so that on day one hive A was recorded for 1 hour followed by hive B and on day 2 hive B was monitored first followed by hive A etc. Before and after each tunnel was monitored, 5 minute counts were taken at the entrance to each hive of the total number of bees exiting and entering. This was used to estimate the amount of 'traffic' for each hive during the observation period.

6.2.3 Statistical analysis of results:

All statistical analysis was carried out in GenStat® (14th edition) (Payne, 2011).

6.2.3.1 Survival analysis:

The last time each marked bee was seen within an observation hive was recorded as the extent of its life. It is possible that bees may have lived longer than this and just not been seen in the observation hive but the exact time of death could not be determined in this experiment. Bees often die away from the colony and those that did die within the observation hive and were carried outside were often removed by wasps.

Kaplan-Meier plots with 95% CIs were used to visualise the results and compare treatments. These plots were adapted by Suzanne Clark to add CIs to the Kaplan-Meier program.

6.2.3.2 Time to first forage:

The day that each bee was first seen flying for more than five minutes, or seen returning carrying pollen, was taken as the day of first forage. Again, it is possible that bees might have foraged at times when the hive was not being observed.

analysis linear mixed model was fitted using REML; no transformation was required to normalise the data which were analysed with a treatment structure to test the effect of number of pathogens in the source colony (1, 2 or 3) on time to first forage, and a blocking structure to take account of the source colony and observation hive for each individual bee.

6.2.3.3 Behavioural analysis:

The behavioural data were examined for only the first 20 days of the experiment. After this time the number of bees seen on each day decreased to a point where the proportions were often out of 2 or 3 bees (figure 6.4). The statistical analysis used (see below) does not take sample size into account such that 1/1 is treated the same as 50/50. To avoid low sample sizes only the first 20 days were analysed as, on the plot below, this looked like the period when good numbers of bees observed. The mean number of bees observed in the first 20 days was 29.07 whilst the mean number of bees observed in the remaining 46 days was only 8.63.

Figure 6.4:

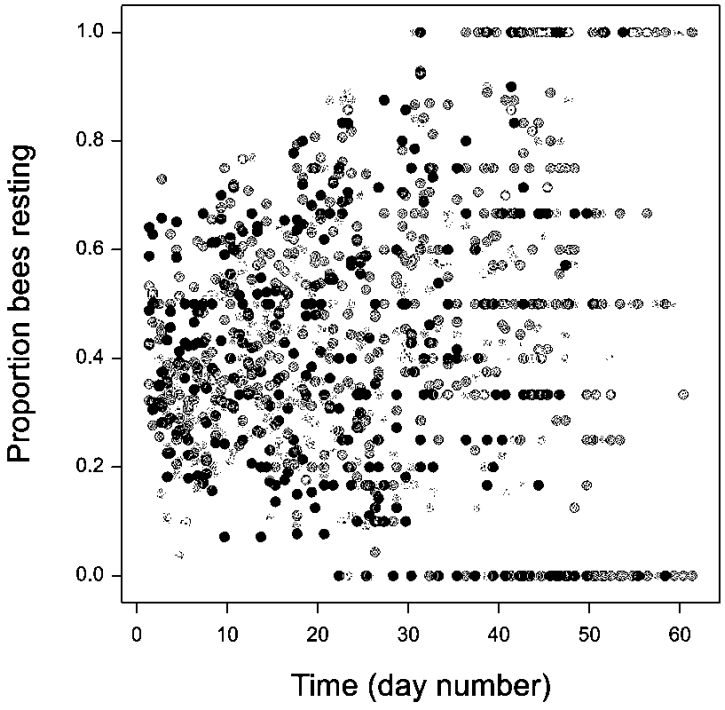


Figure showing the proportion of bees, from all treatments, resting over time for the observation hive experiment. The number of bees recorded in this experiment decreased over time.

Biologically it has been suggested that by about three weeks of age, the majority of bees have started foraging (Hooper, 2008), so this time period should cover when most of the bees are focusing on in-hive activities.

The proportion of bees resting and interacting with other bees were analysed because these were the behaviours most often affected by pathogens in previous studies (e.g. Bailey and Fernando, 1972; Wang and Moeller, 1970). Other behaviours were recorded but only at low frequencies through the course of the experiment, making them more difficult to analyse. A linear mixed model was fitted using REML to examine the effect of time and number of diseases on the proportion of bees resting and interacting, whilst taking into account the source colony and receiving observation hive. Splines were fitted to produce smooth curves for the data and a power correlation model used to test for an effect of autocorrelation (whether there was any pattern over time within the data).

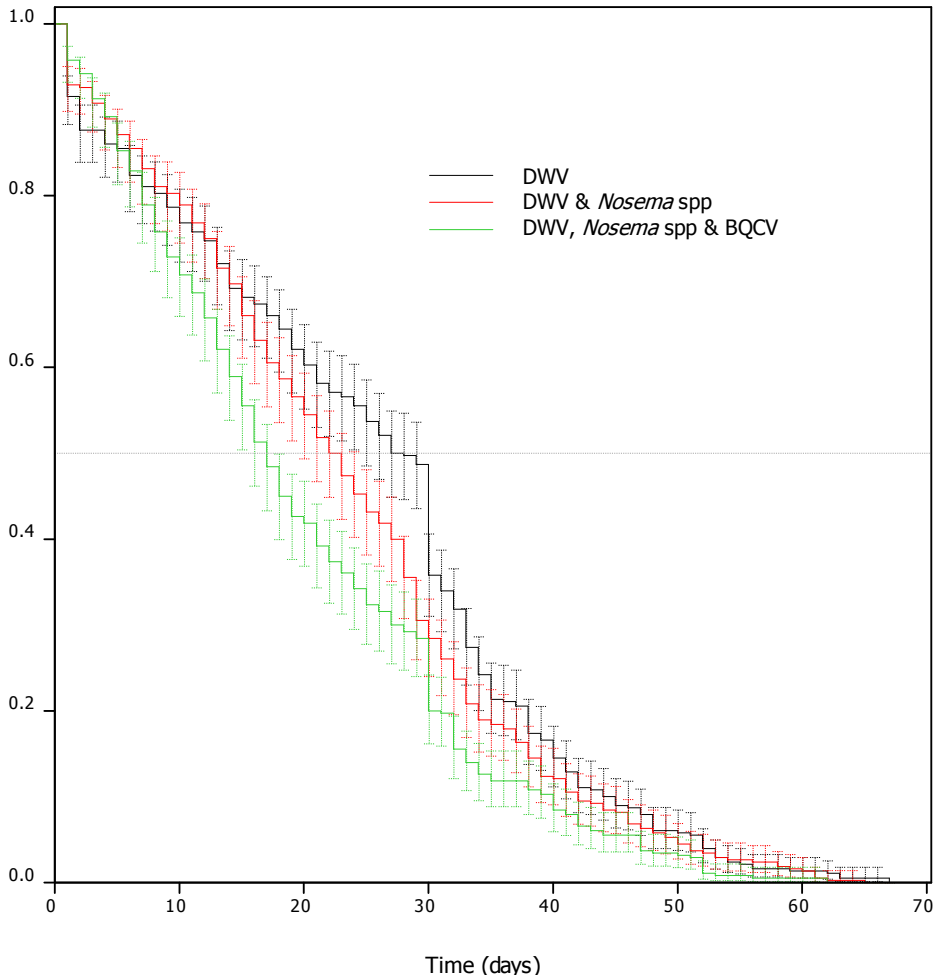
Barycentric triangles (Aitchison, 1986) were produced to visualise the proportion of bees either resting, cleaning or doing other behaviours (see table 6.2) over the first 20 days of the experiment.

6.3 Results

6.3.1 Survival results:

The last bee remaining alive was recorded 66 days after introduction to the observation hives. The Kaplan-Meier plot of survival for each disease treatment shows that, although there is no significant difference between the CIs across all three treatments, the bees from colonies with only DWV appeared to survive longer than those from the other colonies. There was a significant difference amongst the CIs for the bees from colonies with only DWV and the bees from colonies with all three diseases between about 20-30 days into the experiment (figure 6.5). The mean average survival for each treatment was: DWV: 24.81 days, DWV and *Nosema* spp.: 23.10 days and DWV, *Nosema* spp. and BQCV: 19.83 days.

Figure 6.5:



Kaplan Meier plot of survival for each treatment of bees in the observation hives. For most of the experiment there is no significant difference in the CIs for each treatment, however, between 20-30 days the DWV only bees and the bees from colonies with all three diseases differed significantly such that the DWV bees survived better than those from colonies with all three diseases.

There was a sharp decrease in the proportion of each group of bees still alive at around day 30 (figure 6.4). This decrease in bee numbers occurred between the 5th and 6th of September. Table 6.3 shows the Rothamsted meteorological data for that period of time.

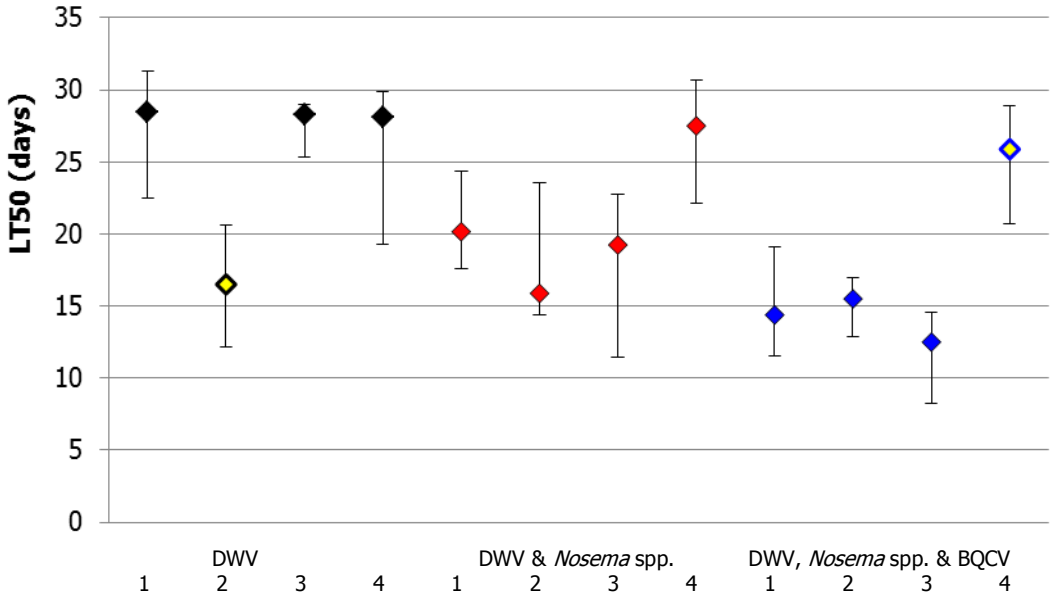
Table 6.3:

Date	Average temperature [min-max] (°C)	Sun light (hrs)	Precipitation (mm)	Wind speed (knots at 10m)
04/09/2011	13.9 [14-18]	2.0	12.2	6
05/09/2011	14.9 [9.3-18]	5.8	3.0	12
06/09/2011	13.9 [13.1-17.6]	0.0	3.2	10
07/09/2011	13.6 [11.3-16.2]	1.2	0.4	9
Average	[8.6-11.3]	4.8	1.7	7.2

Table showing meteorological data for Rothamsted farm from 4th-7th of September 2011. All values were recorded from 0900-0900 the following day. The average temperatures are the mean average of hourly recordings taken from 0900-0900 the following day.

Figure 6.6 shows the LT50s for all the bees used in this experiment. Three of the groups of bees from colonies infected with only DWV (black) show similar LT50s (~28 days) but one group has a much lower value (16.5 days) its CIs only overlapping with one of the other DWV only groups. Similarly most of the groups of bees from colonies with all three diseases (blue) have similar LT50s (~14 days) but one has a much higher LT50 (25.88 days) and its CIs don't overlap with any of the other groups with all three diseases. It is not clear why these two groups should be so different from the rest. Ignoring the two anomalies the DWV bees and the bees from colonies with all three diseases have different LT50s with CIs that are significantly different such that, as with the Kaplan-Meier results, the bees from colonies with just DWV survived longer than those from colonies with all three diseases whilst the bees from colonies with DWV and *Nosema* spp. (red) are intermediate, overlapping with both of the other groups.

Figure 6.6:



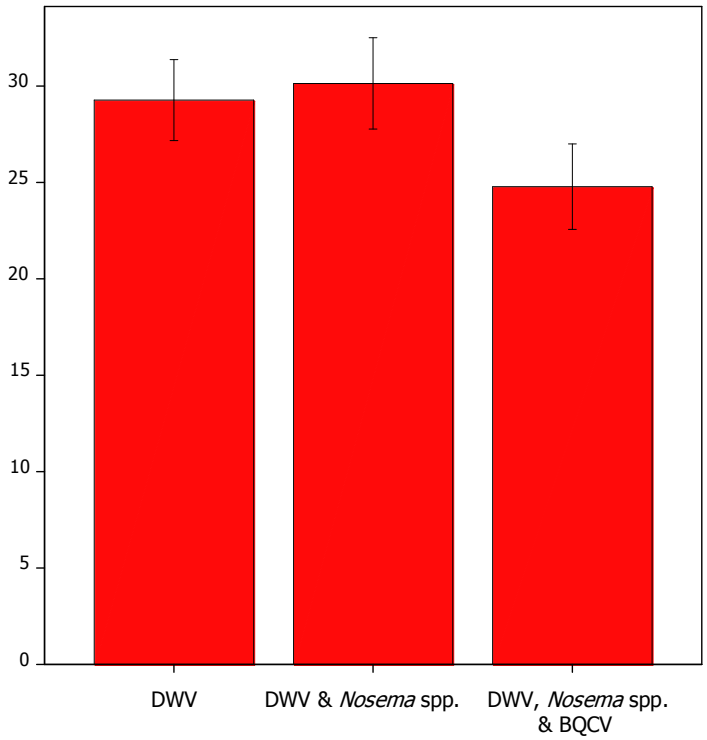
Graph showing LT50s calculated from the Kaplan Meier results for each group of 95 bees from colonies with 1 (DWV), 2 (DWV and *Nosema* spp.) or 3 (DWV, *Nosema* spp. and BQCV) diseases. 1 and 2 for each treatment went into observation hive A and 3 and 4 went into observation hive B.

6.3.2 Time to first forage:

When all three treatments were compared there was no significant effect of the number of diseases in the source colony on the time to first forage of individual bees (REML: H_0 = equal means for each disease treatment. DWV mean = 29.26, DWV & *Nosema* spp. mean = 31.13, DWV, *Nosema* spp. & BQCV mean = 24.78. SED = 3.156. $F_{2,8.6} = 1.64$, P = 0.25). However, a graph of the average time to first forage for each treatment shows that the bees from colonies with all three diseases appeared to have an earlier average time to first forage than bees from the other two treatments (figure 6.7).

On average the bees from colonies with only DWV began foraging 29.25 ($\pm$1.13) days after being introduced to the receiving observation hive, bees from colonies with both *Nosema* spp. and DWV began foraging 30.48 ($\pm$1.83) days after introduction, but bees from colonies with all three diseases began foraging only 24.68 ($\pm$1.18) days after introduction.

Figure 6.7:

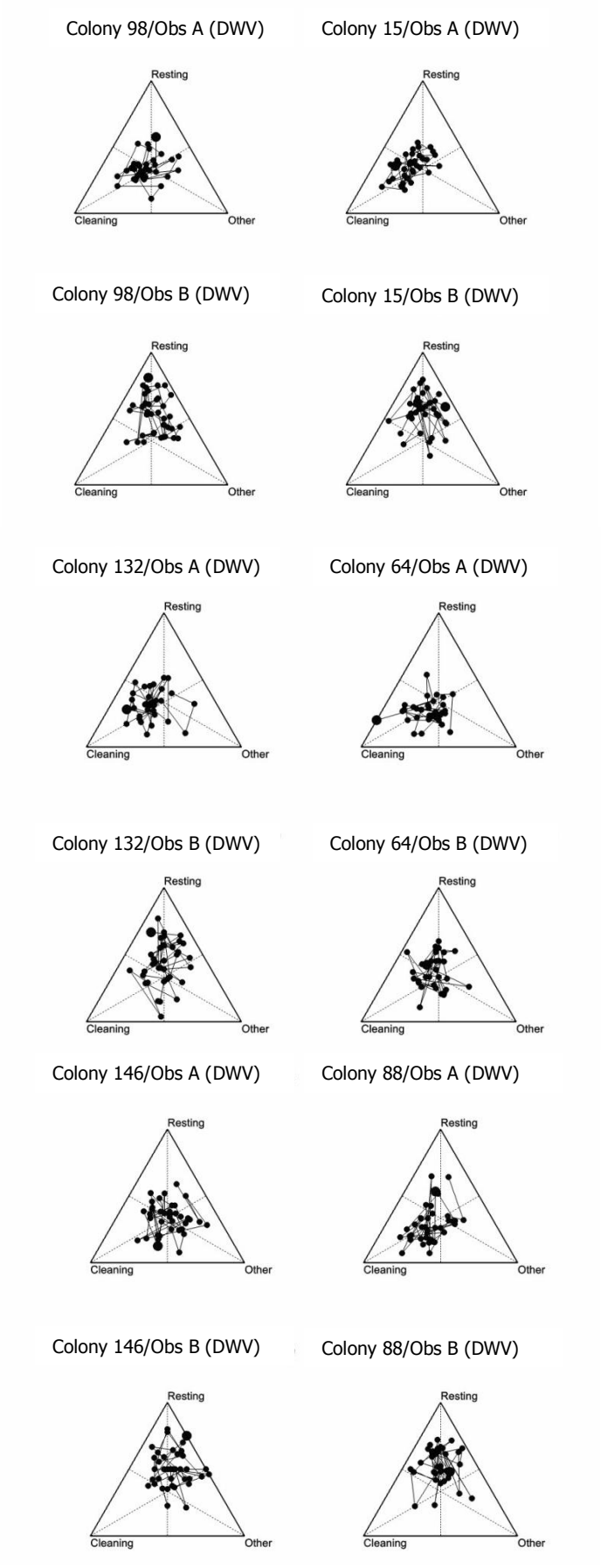


Graph showing the average time to first forage against number of diseases in the source colonies. The error bars show the standard error of the mean (SEM) for these data.

6.3.3 Behavioural analysis:

Barycentric triangles were produced to show the proportion of bees from each treatment and in each observation hive that were recorded either resting, cleaning or performing any other behaviour over the course of the experiment (figure 6.8). Each point on the plot was a different recording time with the final record shown as a larger point. For the most part, pairs of treatments are similar, however the bees from colonies with only one disease (DWV) or with all three diseases (DWV, *Nosema* spp. and BQCV) in observation hive B seemed to be more likely to be resting than those in observation hive A. Although this was not the case for those bees from colonies with two diseases (DWV and *Nosema* spp.), this suggests that bees were more likely to rest in observation hive B.

Figure 6.8:

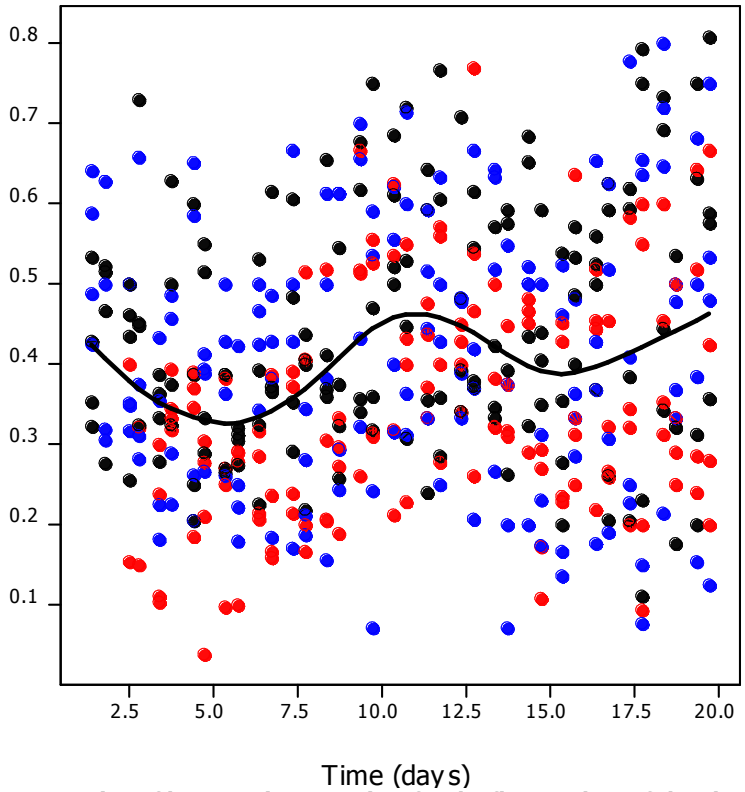


Barycentric triangle plots showing the proportion of bees resting, cleaning or doing other activities over time (20 days) for each treatment in each observation hive.

Analysis of the proportion of bees resting over time showed no evidence of autocorrelation and so no need for the power correlation model to be used. There was also no effect of disease treatment on the results and so the final model looked at the effect of time on the proportion of bees resting and took the source colony and receiving observation hive into account, using a spline to produce smooth curves (figure 6.9).

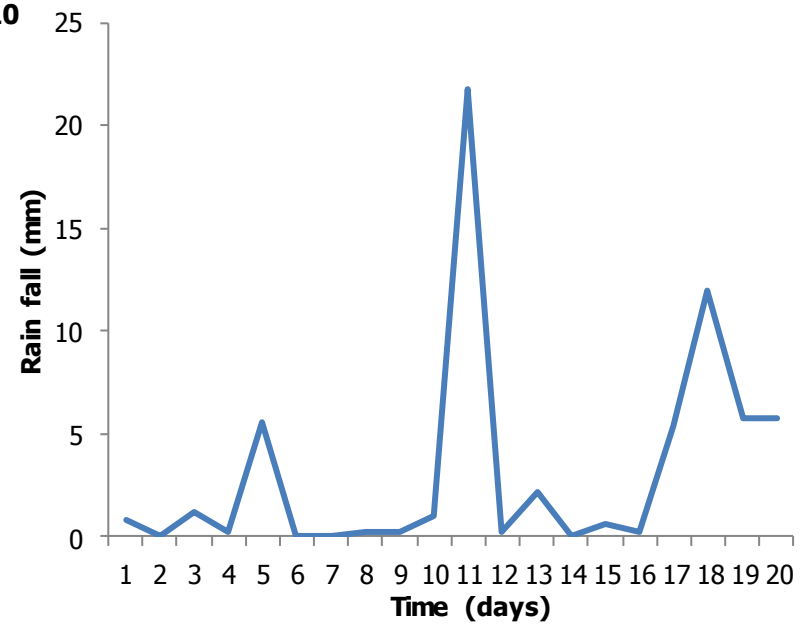
There was no significant effect of disease treatment on the proportion of bees resting but there was a change over time shown by the spline on the plot (figure 6.9, black line). The spline suggests that the proportion of bees followed an oscillating pattern. To determine whether this pattern was caused by the weather, Rothamsted meteorological data was examined. Only the rainfall data seemed to match the pattern seen in the proportion of bees resting over time (figure 6.10).

Figure 6.9:



Plot of the proportion of bees resting over time for the first 20 days of the observation hive experiment. Black spots are bees from colonies with one disease (DWV), red spots are bees from colonies with two diseases (DWV & *Nosema* spp.) and blue spots are bees from colonies with all three diseases (DWV, *Nosema* spp. and BQCV). The fitted mixed model is shown as the black line; this was found to be the same for all disease treatments.

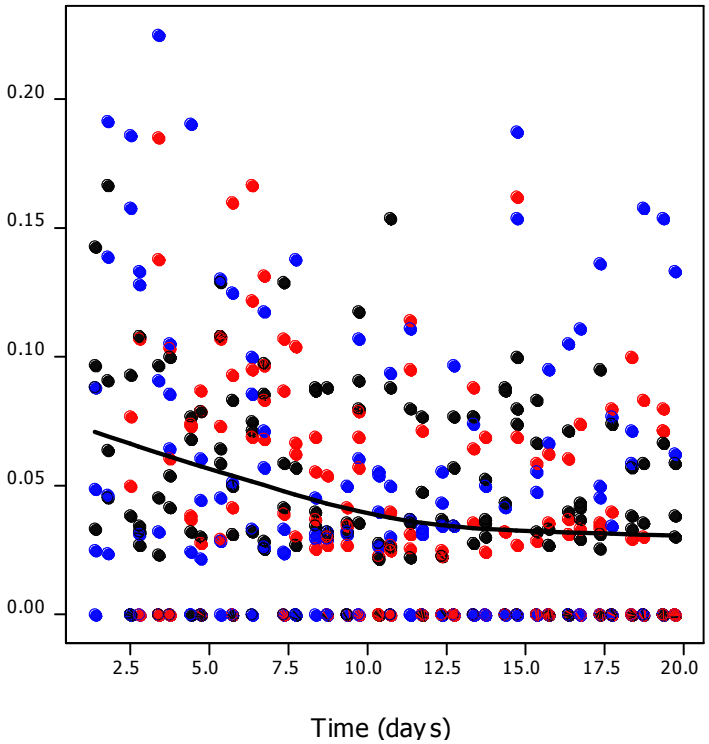
Figure 6.10



Graph of the rainfall data for Rothamsted farm over the first 20 days of the observation hive experiment. All values were recorded from 0900-0900 the following day.

Analysis of the proportion of bees interacting with other bees over time also showed no evidence of autocorrelation and no effect of disease treatment on the results and so the final model examined the effect of time on the proportion of bees interacting and took the source colony and receiving observation hive into account, using a spline to produce a smooth curve (figure 6.11).

Figure 6.11:



Plot of the proportion of bees interacting with other bees over time for the first 20 days of the observation hive experiment. Black spots are bees from colonies with one disease (DWV), red spots are bees from colonies with two diseases (DWV & *Nosema* spp.) and blue spots are bees from colonies with all three diseases (DWV, *Nosema* spp. and BQCV). The fitted mixed model is shown as the black line; this was found to be the same for all disease treatments.

There was no significant effect of disease treatment on the proportion of bees interacting. The proportion of bees interacting decreased over the first ten days and then remained steady for the following ten days.

6.4 Discussion:

Observation hives were the perfect tool for examining the behaviour of honeybees within the hive; it also proved possible to record honeybee longevity. The survival times were determined in this experiment by recording the last time that each individual bee was recorded alive in the behavioural and foraging observations. This is not completely accurate as a bee may have survived for an unknown time, anything from minutes to days, after it was last seen. The original plan for the experiment was to collect dead bees that were removed from the observation hive and left on a sheet outside. However the majority of bees either died away from the hives or were taken from the sheet by scavenging wasps. This meant that only 19 bees were actually recovered which was not enough to use for survival analysis. This also meant that disease analysis could not be carried out on individual bees, as has been done for previous chapters (see chapters 4 and 5).

However, despite the limitations of the method, the survival times for the bees in this experiment were not very different from previous studies. In this experiment the bees survived for a mean average of 19.8-24.8 days depending on treatment; other studies recorded survival times of between 11 and 36 (table 6.4).

Table 6.4:

Reference	Factor	Average survival time (days)
(Janmaat and Winston, 2000)	Control	12-15
	Varroa	11-13
(Mattila and Otis, 2006)	Control	31
	Pollen supplemented	36
(Woyciechowski and Moron, 2009)	Control	31.0
	N.apis infection at one day old	15.5
	CO_2 treated	25.4

Table showing survival time results for observation hive experiments.

There was, however, a sharp decrease in the proportion of each group of bees still alive at around day 30 (figure 6.4). This decrease in bee numbers occurred between the 5thand 6th of September. There was no significant temperature change for either of these days, although the 6th was more overcast and both days had higher wind speeds (table 6.3). The average wind speed over the course of the experiment was 7.2 knots at 10m, whilst on the

5-6th of September the wind speed was recorded at 10 and 12 knots respectively at 10m. However, several other days during the experiment had similar wind speeds, so this is unlikely to be the cause of the sharp decline in honeybee numbers. Equally there were several days that were just as overcast and none of the other meteorological data was sufficiently different on those days to explain the decline in honeybee numbers. A nearby field of field beans was combined on the 5th of September, but I am unaware of any evidence of combining having a negative effect on honeybees so the sharp decrease in proportion of bees alive remains a mystery. It is possible that another farm in the area may have been spraying pesticides. Although usually pesticide spraying leads to much higher numbers of dead bees (Girolami *et al.*, 2012), if the field being sprayed was far enough away then smaller numbers of bees would have flown that distance and been affected by it.

Bees from colonies with three pathogens, DWV, *Nosema* spp. and BQCV, survived for less time on average than bees from colonies with only one pathogen, DWV, whilst bees from colonies with two pathogens, DWV and *Nosema* spp., survived for an intermediate amount of time. This suggests that the number of pathogens present has an effect on survival; bees from colonies with more diseases survive for shorter amounts of time on average than bees from colonies with fewer pathogens. Similar results are discussed in chapter three where the effect of a combination of pathogens and forage availability on survival was examined.

However, it is also likely that it is the identity of the pathogens present, rather than merely their number, that is important in determining whether there are any negative effects on honeybee survival. Previous studies have shown a deleterious effect of *Nosema spp.* on survival (e.g. Higes *et al.*, 2007; Mayack and Naug, 2009; Woyciechowski and Moron, 2009) although Mattila and Otis found no effect of *N. apis* on honeybee survival in their observation hive experiment. There is also controversy when it comes to the relative virulence of *N. apis* versus *N. ceranae*. For example *N. ceranae* it is considered more virulent than *N. apis* (Higes *et al.*, 2007; Paxton *et al.*, 2007), although a more recent study (Forsgren and Fries, 2010) showed no discernible difference in virulence between the two species in caged bee studies. This may be accounted for by temperature effects as, at higher temperatures, *N. ceranae* is more virulent than *N. apis*. For example, at 33°C *N. ceranae* was shown to produce spores faster than *N. apis* (Martin-Hernandez *et al.*, 2009). It is possible that the experiments that suggested *N. ceranae* was more virulent than *N. apis* were carried out in warmer regions than those that found no difference. This is supported by the fact than the significant results described were found in Spanish bees (e.g. Higes *et al.*, 2007) whilst those that found no effect of *Nosema spp.* or no greater virulence of *N. ceranae* compared with *N. apis* took place in Canada (Mattila and Otis, 2006) and Sweden

(Forsgren and Fries, 2010). Colonies in this experiment were infected by a mixture of the two species.

BQCV has also been shown to have deleterious effects on honeybee survival but only in combination with *N. apis* (Bailey *et al.*, 1983). In the laboratory, prior infection by *N. apis* was required for BQCV *per os* infection to occur, coinfection increased the deleterious effects of *N. apis* on bees and was responsible for some colony loses (Bailey *et al.*, 1983). This is supported by the results of this experiment where the colonies with both *Nosema spp.* and BQCV did not survive as long on average as those without BQCV. However, there was no BQCV-only treatment to confirm the effect of the virus alone.

When time to first forage was investigated there was no significant difference amongst treatments. The bees began foraging on average 24-30 days after introduction to the observation hives. This is slightly later than other studies have reported (table 6.5)

Table 6.5:

Reference	Factor	Average time to first forage (days)
(Downey *et al.*, 2000)	Control	18.0
	Tracheal mites	20.5
	Varroa mites	15.9
(Janmaat and Winston, 2000)	Control	13-14
	Varroa	9
(Mattila and Otis, 2006)	Control	21
	N. apis	17
(Woyciechowski and Moron, 2009)	Control	28
	N. apis infected at one day old	17.4
	N. apis infected at six days old	19.4
	N. apis infected at 11 days old	24.9

Table showing time to first forage results for observation hive experiments.

For all of these results, with the exception of Woyciechowski and Moron (2009), bees began foraging at an earlier average time than those in this experiment. This might be because of the way the time to first forage was determined in this experiment. Because the bees could not be monitored continuously, each hive was monitored for an hour each morning to record which bees were foraging and the first time a bee was seen making a trip longer than five minutes, or returning with propolis or pollen, was counted as her first foraging trip. This means that some bees could have begun foraging at a time when their hive was not being

monitored and then have been seen and recorded a day or more later. One of the best current methods for monitoring bees in this sort of experiment is to use RFID tags to monitor each marked bee as she leaves and returns to the hive (e.g. Schneider *et al.*, 2012). This can be carried out 24 hours a day when physically watching the bees for that time is impractical. However this technology was not available to me at the time of this experiment.

There was no significant effect of the pathogens in the source colonies on time to first forage, although the average time to first forage was slightly lower for bees from colonies with all three diseases when compared to the other two treatments. It was expected that the bees from colonies with more diseases would begin foraging earlier as this has been seen in previous studies; for example Mattila and Otis (2006) found that *N. apis* inoculated bees began foraging earlier than uninoculated controls. Woyciechowski and Moron (2009) also found that the earlier bees were inoculated with *N.* apis, the earlier they began foraging. To the best of my knowledge the effect of DWV and BQCV on time to first forage has not been previously examined. However, varroa infested bees begin foraging earlier than uninfested controls (Downey *et al.*, 2000; Janmaat and Winston, 2000), and varroa infestation is often associated with DWV infection (Yang and Cox-Foster, 2007). Earlier foraging ages with more disease may not have been detected in the statistical analysis of this experiment due to the low level of replication at the colony level. Only six colonies were used for this experiment, two with DWV, two with DWV and *Nosema spp.* and two with DWV, *Nosema spp.* and BQCV. This makes it difficult for any statistical analysis to determine whether an effect is caused by the disease in the colony or just due to random chance. To determine whether colony disease level affects individuals' behaviour, more colonies are needed.

It is also possible to look at individual disease level by inoculating individual bees with each pathogen treatment. This would require fewer colonies of bees. However as there are genetic effects on behaviour, such that different colonies may behave differently regardless of pathogen treatment (e.g. Laloi and Pham-Delegue, 2010; Mattila and Seeley, 2011), the experiment should still need to be repeated at the colony level. Individual bees should be taken from at least three colonies and then individually inoculated with the different pathogen treatments. However, it is difficult to find bees that are completely disease free to use as a control group. All colonies tested in this experiment tested positive for at least one virus. The other way to look at individual disease load is to use *post hoc* molecular analysis to determine exactly what each individual bee was infected with. Unfortunately this was not possible in this experiment because each bee was followed until death and the majority of

bees either died away from the observation hives or, when removed from the hives, were scavenged by wasps.

It has also been shown that some honeybee forager bees prefer to forage in the morning whilst others prefer foraging later in the day and this preference has a genetic basis(Kraus *et al.*, 2011), although no one has looked at whether pathogens might also influence it. It is therefore possible that by only watching bees in the morning, to avoid recording orientation flights that usually take place in the early afternoon (Vollbehr, 1975), bees who preferred to forage later in the day were excluded from analysis.

Previous studies of in-hive behaviour have identified several factors that influence honeybee behaviour. For example, Wang and Moeller (1970) used observation hives to show that honeybees infected with *Nosema spp.* were less likely to attend the queen or feed brood than healthy controls. Bailey and Fernando (1972) showed a similar effect for bees infected with SBV that were also less likely to attend the queen than healthy controls. This is likely to be an adaptation to reduce pathogen spread. However Mattila and Otis (2006) found no effect of *N. apis* on behaviour in their experiments. They did show that pollen-limited bees rested more than control bees ,although pollen availability did not affect brood or food related activities. Pesticides have also been shown to affect behaviour. Cox and Wilson (1984) showed that bees treated with permethrin, a pyrethroid neurotoxin used against a wide range of insects and mites, spent significantly more time cleaning than controls and significantly less time walking, in the cells, sharing food, or touching their antennae to other bees when compared with control bees. Treated bees were also more likely to make abnormal dances (rotation, tremble, abdomen curling). Again some of these changes may be adaptations to reduce spreading the pesticide to other bees (reduced food sharing and increased cleaning for example) while others may be due to deleterious effects of the pesticide (decreased time spent walking for example).

In this experiment, however, there was no effect of the disease treatment on in-hive behaviour. Neither the proportion of bees resting over time nor the proportion of bees interacting with other bees over time differed with the number of diseases present in the first 20 days of this experiment. These were the two most commonly affected behaviours in previous studies (e.g. Cox and Wilson, 1984; Mattila and Otis, 2006; Wang and Moeller, 1970) and appear to have two different drivers for behavioural change. The increased time resting caused by some factors may be a result of the deleterious impact of that factor; infected or treated bees have less energy and so spend more time resting. The decreased time interacting with other bees (feeding, grooming, caring for brood or attending the queen for example) may be an adaptation to reduce spread of the pathogen or pesticide.

The lack of effect found in this study may again be due to the low level of replication at the source colony level (see above).

There was, however, an effect of time on both behaviours studied. The proportion of bees resting increased and decreased in an oscillating manner. A previous study by Seeley and Kolmes (Seeley and Kolmes, 1991) showed no such changes in amount of time spent resting, although Kolmes' method found that in the first few days newly emerged bees were more likely to be seen inside cells (something it was not possible to see in this experiment because only the bees' thoraxes were marked and only the tip of the abdomen is visible when the bee is in a cell) whilst Seeley's method showed that in the first few days newly emerged bees were more likely to be cleaning. It has also been shown that there is no difference between in-hive and forager bees in the time spent sleeping (Eban-Rothschild and Bloch, 2008).

When compared with the amount of rainfall over time, it appears that the proportion of bees resting increased with increased rainfall. Previous studies have shown that adverse weather, especially rain, leads to a reduction in foraging activity, as the bees do not fly in the rain, and that leads to reduced activity within the hive (Riessberger and Crailsheim, 1997). With less forage brought in, the bees would spend less time collecting and storing pollen and nectar from the forager bees or following waggle dances. There would also be more bees in the colony to share tasks.

Bees in observation hive B were more likely to be resting than those in hive A. This is likely to be because hive B had more bees in it through the course of the experiment. With more bees to share jobs, bees without work would be more likely to be seen resting. It has been suggested the bee colonies have reserve workers, nurse bees that can be called upon when there is a sudden increase in work, for example if a profitable food source is found and more bees are needed to collect and store to food (Robinson, 1992). If there are more than enough bees in the colony then there may be more bees acting as reserves with no work to do.

The proportion of bees interacting with other bees decreased over the first ten days and then remained at a constant low level of about 0.05 for the next ten days. It has been suggested that honeybees may reduce the amount of time they spend interacting with other bees if they are infected with a pathogen to prevent the spread of that pathogen (Bailey and Fernando, 1972; Wang and Moeller, 1970). It has also been shown, as part of this PhD, that the quantity, or load, of some pathogens that bees carry increase with age (see chapter 4), so it is possible that, as the bees grow older, and the number and load of pathogens

increases, that they interact with other bees less frequently. Also as bees age they move from in-hive tasks to foraging (Calderone, 1998). Many of the in-hive tasks, such as attending the queen, involve interacting with other bees whilst forager bees only interact whilst dancing or giving food to other bees to store.

In conclusion, this experiment showed no statistically significant effect of the diseases present in the source colony on in-hive behaviour or time to first forage for individual bees, although bees from colonies with more diseases were likely to live for less time than those from healthier colonies. The lack of significance in these data may have been down to the low level of replication at the colony level or because the disease status of the source colony was less important than the disease status of the individual. Mattila and Otis (2006) showed that bees taken from their colony and tested in another colony showed fewer behavioural changes caused by pollen and *N. apis* treatment than those raised in their own colonies, suggesting that the colony bees live in as adults may be more influential on their behaviour as adults than the colony that they are raised in as larvae.

Chapter 7: General Discussion

The aim of this project was to use new and existing methods to study the effect of pathogens on honeybee behaviour. To this end several different methods were used to study learning behaviour, foraging behaviour, flight and survival. This type of research, combining laboratory and field based techniques, is important because information gained only at the laboratory level do not always reflect what happens in the real world where there are many additional and interacting factors that cannot always be included or controlled for in laboratory based experiments (Pedersen and Babayan, 2011).

In the learning experiments in chapter 4 forager bees inoculated with a fungal pathogen were less able to learn than uninoculated forager bees, suggesting that the fungus had a negative effect on the bees' learning ability. This has been shown previously for the honeybee pathogen DWV (Iqbal and Mueller, 2007) the parasitic varroa mites (Kralj *et al.*, 2007), and for the bees' own immune system (Mallon *et al.*, 2003; Riddell and Mallon, 2006). What is interesting is that there was no difference in the effect of time since inoculation (two, four or six days) on learning ability; although it was hypothesised that over time, as the fungus did more damage, the negative effect on learning should increase. For example several pathogens have been shown to decrease survival of their hosts over time as it usually takes days or even weeks for the pathogen to cause enough damage to kill the host (e.g. Bos *et al.*, 2012; Forsgren and Fries, 2010; Yang and Cox-Foster, 2007). Very few experiments have looked at changes to behaviour over the course of infection, although it has been shown that fungal infection in ants leads to changes in behaviour over time such that infected ants initially received more grooming from nest mates and were more likely to groom themselves than uninoculated individuals, but after two days the amount of grooming they received and the amount of self-grooming decreased (Bos *et al.*, 2012). The lack of effect of time since inoculation in these experiments may simply be because the fungus was too fast acting. At the dose used in these experiments *M. anisopliae* killed its host within about six days on average. This may not have been enough time to see any change in behavioural effects in the honeybees.

Although it has been shown that younger bees are less able to learn than forager bees (Behrends and Scheiner, 2009), this is, to the best of my knowledge, the first experiment to test the effect of a pathogen on the learning ability of different ages of honeybees. I showed that young adult bees were better able to learn after inoculation than uninoculated bees of the same age; whilst the inoculated forager bees were less able to learn than uninoculated bees. This is likely to be because the fungus was affecting the different aged bees' hunger differently. As with some other pathogens, like *Nosema* spp. (Naug and Gibbs,

2009), the fungus may be energetically stressing the bees and making them more responsive to the sucrose stimulus that was used in the learning experiment. This makes it appear that they are better able to learn (Mujagic *et al.*, 2010). A sucrose sensitivity test was used to show that the fungus was indeed causing increased responsiveness in the young adult bees but a similar test was not performed on forager bees. It would be useful to test the effect of the fungus on sucrose responsiveness in forager bees to see whether there is less effect than was seen in the young adult bees. But this does highlight the fact that the age of bees used in experiments is an important factor when examining behaviour.

Age is known to be an important factor in the immune response as well. For example young adult bees have greater fat body mass than forager bees, which means that they may be better able to respond to infection via the production of anti-pathogenic proteins than older forager bees (Wilson-Rich *et al.*, 2008). Forager bees also have a decreased haemocyte count than young adult bees, although phenyloxidase activity was greater in forager bees compared with young adult bees, (Schmid *et al.*, 2008) and the average level of encapsulation, a method used by honeybees to combat pathogen invasion, remained stable across all life stages (Wilson-Rich *et al.*, 2008).

The learning experiments were also used to look at the effect of naturally occurring pathogens. Although none of the bees tested showed any obvious symptoms of disease, they were tested using Q-RT-PCR to see exactly how much covert and inapparent virus and *Nosema* sp. infection was present. There are several methods used to detect pathogens in honeybees (reviewed in De Miranda, 2008) but since RT-PCR was shown as a good method for detecting and distinguishing KBV from closely related viruses (Stoltz *et al.*, 1995), the majority of groups now use RT-PCR detection (e.g. Baker and Schroeder, 2008a; Blanchard *et al.*, 2008; Chen *et al.*, 2006b; Kukielka *et al.*, 2008a; Tentcheva *et al.*, 2004a). Unlike the serological methods previously used to detect honeybee viruses (eg Ouchterlony gel diffusion, indirect fluorescent antibody (IFA) and enzyme-linked immunosorbent assay (ELISA) tests, (Allen and Ball, 1995; Allen *et al.*, 1986; Anderson, 1984)), RT-PCR methods can be highly specific and sensitive. For example primers have been designed that can differentiate between even closely related honeybee viruses like Kashmir bee virus (KBV) and acute bee paralysis virus (ABPV) (De Miranda, 2008) and SYBR green Q-RT-PCR is sensitive enough to detect viruses at 10^{-7} in a 10-fold serial dilution (Kukielka *et al.*, 2008b). However, this does raise the question: is it biologically relevant to be detecting pathogens at such low levels?

The results of this project showed that even at the low, covert levels, with no obvious symptoms, some of these pathogens still had an effect on behaviour. For example young

adult bees infected with sac brood virus (SBV) and *Nosema* spp. were less able to learn than uninfected young adult bees. There was, however, no significant effect of the presence of any pathogen on forager bee learning ability or of the quantity of each pathogen on the learning behaviour at either age.

Harmonic radar was used in chapter 5 to examine the effect of naturally occurring pathogens on the bees' behaviour, this time looking at their ability to perform orientation flights. The effect of parasites, pathogens and pesticides on honeybee flight behaviour has been studied in the lab (Duay *et al.*, 2002; Harrison *et al.*, 2001) and in the field (Kralj and Fuchs, 2010; Kralj and Fuchs, 2006; Schneider *et al.*, 2012). However, with the harmonic radar, more detailed information could be gathered, for example the speed of flight in the field, the total distance travelled and the number of times the bee stopped, all of which are difficult if not impossible to record using other methods.

This experiment showed that low levels of pathogens also affected the flight behaviour of honeybees; for example the amount of BQCV and *N. apis* seemed to affect how fast the bees were likely to fly and the amount of BQCV affected how often the bees stopped. These results were only, however, significant at the 10% level. DWV was detected at higher levels than the other pathogens; although all bees tested had normal wings. The amount of DWV detected had an effect on flight duration such that bees with higher loads of DWV tended to fly for shorter distances and times. This could be because the pathogens, especially DWV, cause energetic stress to the honeybees reducing the distance or time they can fly for. However, as this was the first time this method has been used to study the effect of pathogens on honeybee orientation flight, the level of replication was quite low (N = 56 but only 28 were tracked with the radar, the rest either did not fly or flew too short a distance to be detected). The experiment allowed the method to be refined and it is currently being used to look at the effect of *Nosema* sp. infection on orientation flights in honeybees (Wolf, unpublished).

Observation hives were also used, as described in chapter 6, to look at the effect of naturally occurring pathogens on honeybee in-hive behaviour and time until first forage. Observation hives have been used since the eighteenth century (Ratcliff, 2005) and are still the best way to study the in-hive behaviour of bees. However, as the bees in this study could not be retrieved after death, the individual disease loads of these bees could not be determined. This meant that only the effect of the disease load of the source colony could be tested statistically and, due to low replication at the colony level, the results of this experiment were mostly inconclusive. The results did suggest, however, that bees from colonies with more diseases were likely to begin foraging earlier; a result that has been

found for several diseases including *Nosema* sp. (e.g. Wang and Moeller, 1970), varroa mites (Downey *et al.*, 2000) and SBV (Bailey and Fernando, 1972).

One of the main issues with bee research is knowing whether replication should occur at the level of the individual bee or at the level of the colony. For example in the learning behaviour experiments in chapater 4 32 bees of each treatment were tested to see whether the fungus *M. anisopliae* had any effect on their learning ability. All those bees came from the same colony. It has been shown that bees from different patrilines (bees with different fathers) have different learning abilities (Laloi and Pham-Delegue, 2010). In other species, genetics can have an effect on susceptibility to disease; for example bumblebees from different sire groups (with different fathers) have different susceptibility to parasites (Baer and Schmid-Hempel, 2003) and leaf cutter ants with different fathers, or from colonies where the queen has mated with multiple males, are less susceptible to fungal infection (Hughes and Boomsma, 2004). So it is likely that bees from different colonies, with different queens who will have mated with different drones, will respond differently. This could mean that bees from one colony could show different behavioural changes in response to pathogen infection than bees from another colony. For this reason replication should be carried out at the level of the colony as well as the individual, taking a good number of bees from several different colonies.

However, this is not always feasible. In the observation hive experiment, chapter 6, bees were taken from six separate colonies, marked and monitored until death. The colonies had different levels of disease; one, two or three pathogens were present. This meant that there were only two replicates of each treatment despite the fact that over 1000 individual bees were monitored. However, finding enough colonies with each disease level, and then collecting, marking and monitoring all the individual bees was not practical for a single person.

Another major challenge in honeybee research is that many of the methods used to study honeybee pathogens and behaviour are not standardised. When Seeley and Kolmes were studying age polyethism in in-hive bees they came up with contrasting results, Seeley (1982) finding age related changes in in-hive behaviour but Kolmes (1985) finding no changes. The two decided to experiment on sister bees within the same colony at the same time, each using their own method for recording and analysing the behaviour, and they still found contrasting results (Seeley and Kolmes, 1991).

I used CPE to study the learning behaviour of honeybees. However there are several different methods in use, making the results difficult to compare. Different groups use

different methods in order to restrain the bees for testing and then test them at different times of day, use different odours for conditioning, reward the bees with different concentrations of sucrose, and test after different numbers of training trials and after differing lengths of time (e.g. Iqbal and Mueller, 2007; Kralj *et al.*, 2007; Laloi *et al.*, 2000; Mallon *et al.*, 2003; Mujagic *et al.*, 2010). Some of these factors do affect how well bees learn. For example time of day; honeybees learn best in the morning (Lehmann *et al.*, 2011). Also restraining technique; honeybee are usually chilled to immobilise them, allowing them to be restrained for the experiment. However, the length of time and the temperature at which bees are chilled can itself have an effect on learning ability (Frost *et al.*, 2011).

In addition to this, the molecular identification and quantification of honeybee viruses is carried out using a variety of different methods and different primers. Most groups now use RT-PCR to detect the different pathogens, especially viruses that often have ambiguous or no symptoms (see chapter 1.3), but often use different primers. Partially this may be down to the fact that all but one honeybee virus detected to date are RNA viruses (Ribiere *et al.*, 2008) which have very high mutation rates (one mutation per 2200 bases or 4 mutations per transcript) (Drake *et al.*, 1998). This is largely because the RNA dependant RNA polymerase (RdRp) has no 3'-5' endonuclease activity (proof reading capability) (Dale and Schantz, 2002). This high mutation rate is at the threshold for genetic maintenance such that even a slight increase in mutation rate would exceed the maximum viable mutation rate leading to extinction (Drake and Holland, 1999). However it also allows these viruses to adapt more easily to new situations as they have huge genetic plasticity (Carter & Genersch, 2008). This high mutation rate means that genetically related virons may show high levels of variation, which makes it difficult to classify viruses in terms of species, thus the term quasispecies was used to describe the population of phylogenetically related variants that may be present in a single infected organism (Carter & Genersch 2008).

Primers have to be designed for conserved regions (eg sequences coding for important proteins such as the RdRp) that are less likely to vary between isolates. However, there is still some variation between variants and different groups use different primer sequences (De Miranda, 2008). When quantification is needed, some groups use TaqMan Q-RT-PCR (e.g. Blanchard *et al.*, 2007; Chantawannakul *et al.*, 2006; Chen *et al.*, 2005) whilst others use SYBR Green (e.g. de Miranda and Fries, 2008; Tentcheva *et al.*, 2006), as was used in these experiments. All this variation makes it difficult to compare results between groups, especially as some of the viruses are so closely related that some of the primers used may not differentiate between them (de Miranda *et al.*, 2010).

Animal and human cell cultures and cell lines have been used to study the properties of animal viruses and to reveal the mechanisms by which viruses cause disease; but currently there is no cell culture system to easily propagate honeybee viruses (Hails *et al.*, 2008). It is possible to extract a lysate from infected bees that can then be used to infect healthy individuals (e.g. Iqbal and Mueller, 2007), but merely injecting the bee can cause behavioural changes as the bee's immune system responds to the physical damage (Mallon *et al.*, 2003) and many of the honeybee viruses, like deformed wing virus (DWV), are not easily transmitted by feeding (Ribiere *et al.*, 2008). Bees can be taken from colonies infected with a particular pathogen, as in the observation hive experiments (see chapter 6), but each individual bee will have its own disease profile; some will be infected with the pathogen whilst others will not, as was seen in the individual disease analysis used in chapters 4 and 5.

Until cell lines are produced, an easier way to examine the effect of pathogens on honeybee behaviour is by using a model system. Krogh's principle states that 'for a large number of problems, there will be some animal of choice, or a few such animals on which it can most conveniently be studied' (Krogh, 1929). These model organisms and systems are specially selected as research materials because they are viewed as easy and relatively inexpensive to gather, transport, maintain, and manipulate experimentally. There are many examples of model organisms including the honeybee which, since its genome was sequenced in 2006 (Weinstock *et al.*, 2006), has been used as a model organism. For example, Abramson *et al.* (2000) used honeybees as a model to study the effect of ethanol on various aspects of behaviour and physiology. Other model organisms include the sea urchin for the study of a variety of developmental phenomena (e.g. Maienschein, 1991), the sea slug *Aplysia* sp. for neurobiological studies (e.g. Rajasethupathy *et al.*, 2012) and the mouse in many different fields, for example for medical research (e.g. Bouchard *et al.*, 2012; Manga and Orlow, 2012; Norgett *et al.*, 2012).

I used the generalist entomopathogenic fungus, *Metarhizium anisopliae*, as a model pathogen to study the general effect of pathogenic infection on survival (chapter 3) and behaviour (chapter 4) in honeybees. *Metarhizium anisopliae* is easily administered in known doses in a powdered form without any physical damage to the bees. In these experiments a dose of 1:30 was able to kill 100% of inoculated bees within two weeks. This ensured that the dose was enough to definitely kill the bee whilst leaving it alive long enough for behavioural experiments to take place, meaning that bees could be tested two, four and six days after inoculation with the fungus to see whether the effect of infection changed over time.

There is also the problem that in nature it is hard to find individuals without any pathogen species present. This means that any behavioural results observed after infection with a test pathogen may be caused by the test pathogen or as a result of interactions between the test pathogen and any natural pathogens the host was already infected with. Although this is still a problem when using *M. anisopliae*, it is at least possible to be sure that the bees were not infected with this fungus. As many of the honeybee viruses can occur as inapparent or covert infections, with no obvious symptoms, molecular analysis is required to be certain a colony is not infected (Ribiere *et al.*, 2008).

The fungus was used to examine both survival (chapter 3) and learning (chapter 4) in honeybees. Unfortunately the *M. anisopliae* killed the bees too quickly to determine the effect of pre-existing pathogens or forage restriction on the bees' ability to defend against subsequent pathogen challenge. A repeat of this experiment using a lower dose of *M. anisopliae* might give the bees a chance to defend themselves against the fungus and determine whether bees infected by more than one pathogen or suffering restricted forage are less able to survive subsequent fungal attack.

Survival analysis was also used in chapter 3 to investigate the effect of a combination of forage restriction and disease on honeybees. It has been suggested that combinations of factors that negatively affect bees may be more important than single factors. For example whilst the evidence for the effect of pathogens on honeybee survival is contentious (Cresswell *et al.*, 2012), recent studies have shown the combined effect of pathogens and pesticides is much worse than any single factor alone (Pettis *et al.*, 2012; Vidau *et al.*, 2011; Wu *et al.*, 2012). My results showed that forage availability and disease both had an effect on honeybee survival and that the combined effect of restricted forage and high disease levels was worse than either factor alone. I also showed that, by feeding bees that had been raised in colonies with restricted forage, their survival could be improved. However these experiments were carried out in cages under optimal conditions and so the results may not be exactly the same in the field. For example Mattila and Otis' (2006)observation hive and colony experiments showed no effect of additional pollen feeding on survival of *N. apis* infected bees despite previous cage studies having shown this (Rinderer and Elliott, 1977). This highlights the difference between laboratory and field-based experiments. Laboratory-based experiments often simplify things, removing factors like weather that may affect the results. Field experiments may take more factors into account, but can be influenced by so many factors that interpreting results becomes difficult or even impossible.

The results of my experiments suggest that pathogens can have an effect on honeybee behaviours. This is true for low levels of viruses even when no obvious symptoms are seen

and for the fungus *M. anisopliae* which can be used as a model system for studying infection in honeybees. These effects, which include decreased learning ability, increased energetic stress and hunger and decreased flight ability, will all have indirect effects on honeybee survival. For example if honeybees are less able to learn then they may be less able to return to their colony when foraging, as has been shown for varroa infested (Kralj and Fuchs, 2006) and *Nosema* sp. infected (Kralj and Fuchs, 2010) bees. Energetic stress has been suggested as the main reason *Nosema* sp. kills honeybees (Mayack and Naug, 2009).

Honeybee decline is the focus for many current studies and these results add to the increasing evidence that pathogens may be responsible. However there are several other factors that are also likely to be important, Each factor may be important in different situations or geographical regions and multiple factors often act together (Brown and Paxton, 2009; Oldroyd, 2007; Potts *et al.*, 2010a; Potts *et al.*, 2010b; Wu *et al.*, 2012).

Future work needs to focus on standardising the methods used to study honeybees and their pathogens so that work from different groups can be more easily compared. One of the main goals of the prevention of colony losses network (COLOSS) is to produce a standardised protocol for monitoring honeybee colony losses (Bach Kim *et al.*, 2010). However, other methods for studying both behaviour and pathology also need standardising. Cell cultures for virus propagation are needed to advance research on viruses substantially. One of the goals of an insect pollinator initiative funded project; 'Impact and mitigation of emergent diseases on major UK insect pollinators' is to produce cell cultures for DWV and *Nosema* spp. (Paxton *et al.*, unpublished). However, cultures for the other viruses are also needed. Finally a thorough review of the current literature is required to ensure that work done or in progress is well known and not repeated. Such a review should also identify hypotheses that still need testing, thereby targeting future research.

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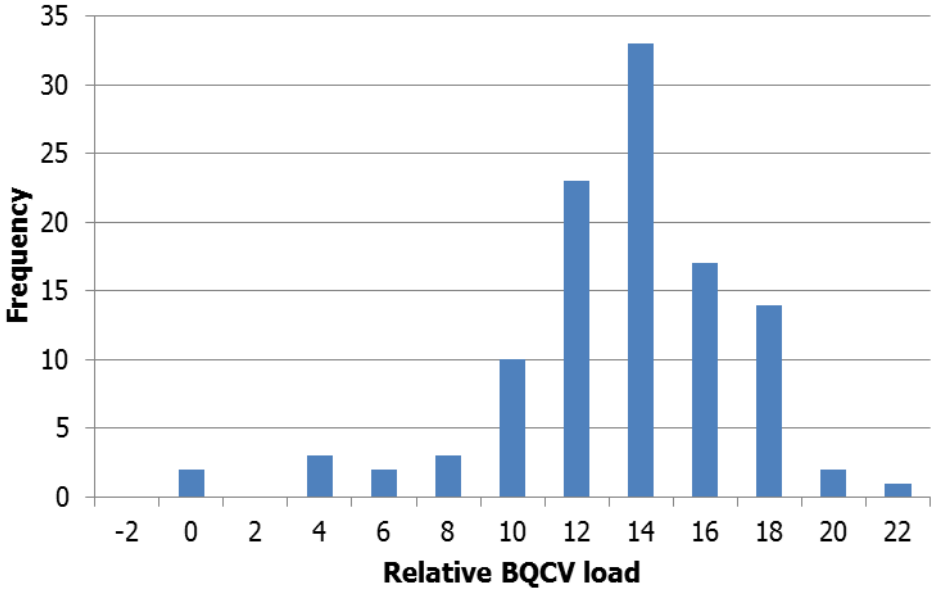
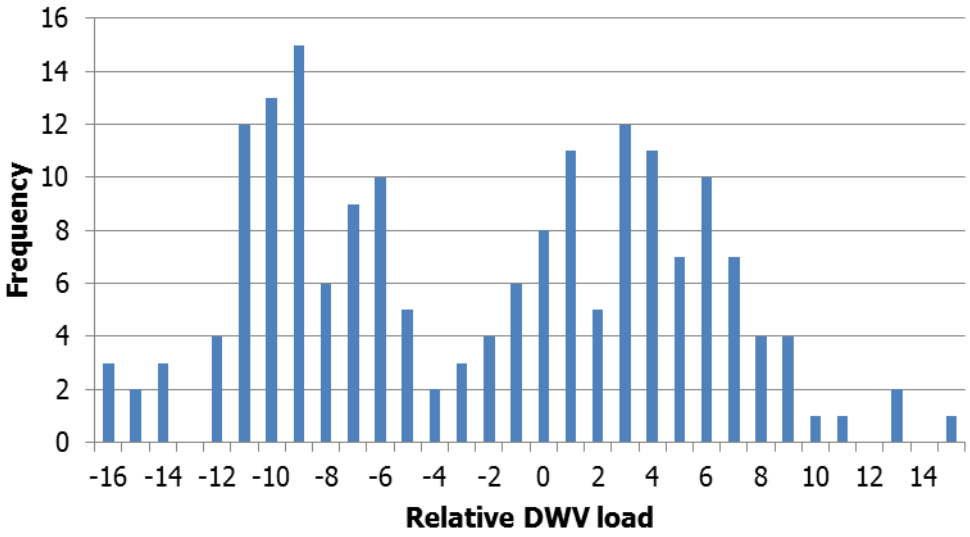
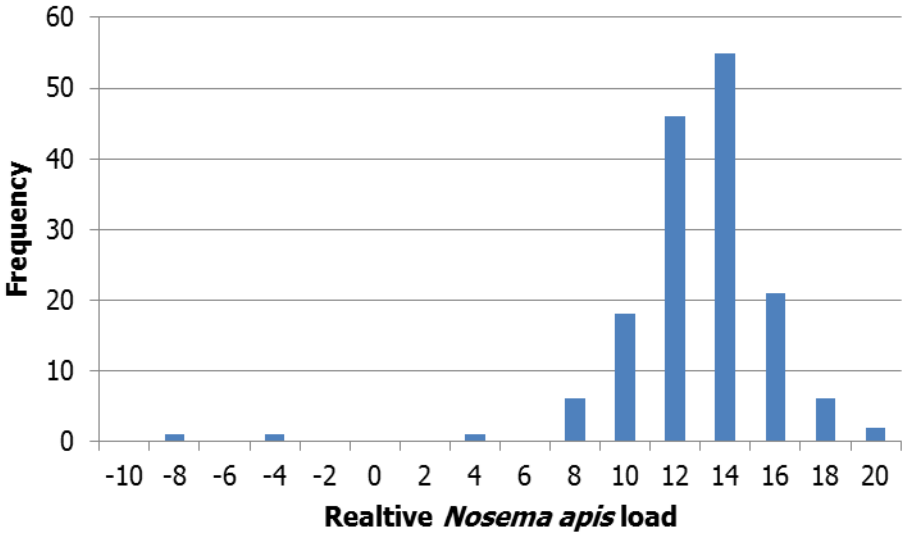
Appendix I

Histograms showing the distribution of DWV load relative to the housekeeping gene β-actin within the bees from the learning experiment in chapter 4.2.3. The lower (or more negative) the relative load the more virus was present, see chapter 2.6.2. Only DWV shows a bimodal distribution.






Frequency
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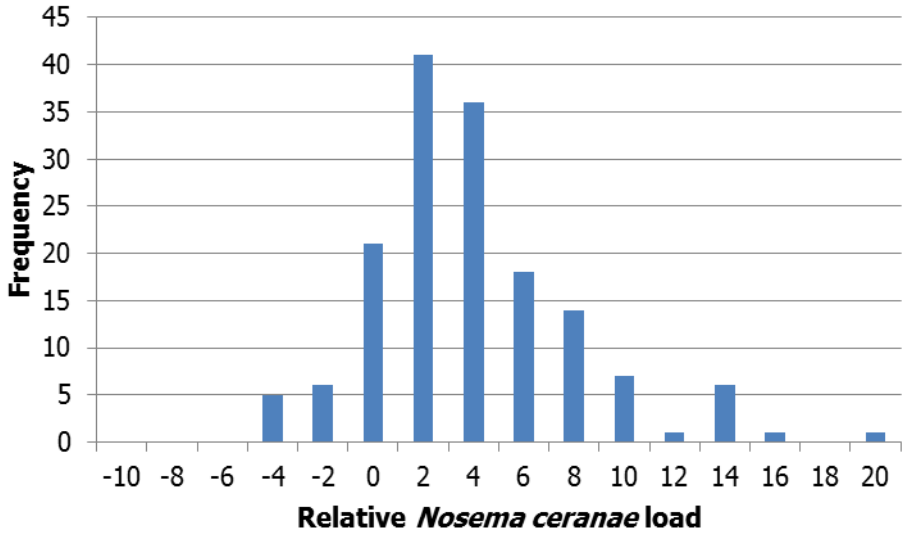
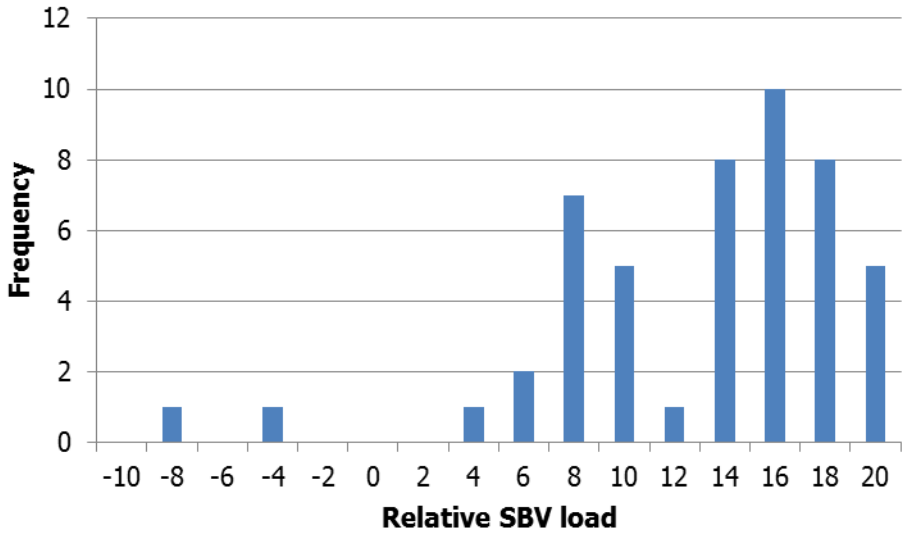
50
40
30
20
10
0
-10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16 18 20
Realtive *Nosema apis* load


Frequency
45
40
35
30
25
20
15
10
5
0
-10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16 18 20
Relative *Nosema ceranae* load


Frequency
12
10
8
6
4
2
0
-10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16 18 20
Relative SBV load