FINAL REPORT FOR DEFRA FUNDED PROJECT PH0506 – CONTROL AND RISK MANAGEMENT OF HONEY BEE PESTS AND DISEASES

Executive summary

Healthy UK stocks of the Western honey bee (*Apis mellifera*) protect the honey industry and ensure a healthy supply of pollinators, whose activities are key to the successful production of many commercially important crops and wild plants. This project comprised five main modules with different themes in support of honey bee health.

1. Small hive beetle control

- The small hive beetle (SHB) (*Aethina tumida*) is a damaging pest of honey bees, invading the hive and multiplying in huge numbers, eating brood, ruining combs and causing complete colony destruction. SHB is currently exotic to the UK, but represents a major threat to the long-term sustainability and economic prosperity of UK apiculture. There is an urgent need to identify effective, environmentally safe methods for SHB treatment prior to its arrival in the UK.
- A literature review was completed which identified chemical treatments, entomopathogenic fungi, entomopathogenic nematodes, organic acids and diatomaceous earth as potential control agents for SHB.
- An experiment was conducted to determine the efficacy of direct (topically applied to larvae) and indirect (applied to sand prior to pupation) applications of a chemical product (Checkmite++), entomopathogenic nematodes (Nemasys, NemasysL, Capsanem), and entomopathogenic fungi (Mycotal, Naturalis). Checkmite++ and entompathogenic nematodes lead to increased mortality of SHB larvae after direct exposure. Indirect exposure of two entomopathogenic nematode treatments (NemasysL, Capsanem) resulted in high mortality in larvae, and this effect was retained when the sand was treated 7-14 days after larvae had buried themselves to pupate. The entomopathogenic fungi were not effective against SHB in either direct or indirect assays.
- Two insect derived venom toxins were fused into carrier molecules to produce novel SHB control options. However, neither fusion protein affected the survival or development of SHB larvae.
- Prior to this project, it was unclear whether *A. tumida* could successfully survive and reproduce in the sometimes harsh UK climate. Much of the *A. tumida* lifecycle occurs within a thermoregulated honey bee colony. However, beetles pupate in the soil outside the hive and so are exposed to the variability and extremes of localised temperature conditions. We exposed pupating SHB to different temperatures and found that, whilst being susceptible to low temperatures during pupation, SHB can survive to adulthood after 19 days at 5°C. Futhermore, adults surviving up to 9 days at 5°C were able to produce viable offspring. These results clearly indicate that, given the soil temperatures typically observed in the UK, SHB is expected to be able develop and survive under UK conditions.

2. Impact of *Nosema* spp. on UK colonies

- *Nosema apis* and *N. ceranae* are microsporidial fungal pathogens that infect the gut wall of adult bees. *N. ceranae* is an emerging pathogen originally discovered infecting the Asian honey bee *Apis ceranae*, and is now widely distributed in Europe and the United States, where it has been associated with large-scale honey bee colony losses. *N. ceranae* was confirmed in samples from UK apiaries in 2007, but the effect on individual honey bees and colony-level survival in the UK is unknown.
- To identify how long *N. ceranae* has been in the UK, historic samples of adult bees were tested for the presence of both *Nosema* species. *N. apis* was consistently detected more frequently than *N. ceranae*. UK samples collected prior to 1992 tested negative for *N.*

ceranae, but two samples of adult honey bees from 2004 tested positive, suggesting this pathogen has been in the UK for at least 9 years.

- Caged honey bee queens are typically imported with 6 attending workers to maintain queen health during transit. To help to understand how *N. ceranae* may have arrived in the UK, attending workers from caged honey bee queens imported from third countries in 2005, 2006 and 2007 were screened for *N. ceranae*. Attending workers from Hawaii (imported in 2005 and 2006) and New Zealand (imported in 2006) tested positive for both *Nosema* species. The importation of honey bee queens is a common practice in the UK, with up to 14,000 honey bee queens imported annually. Whilst attending workers should not be introduced to colonies, there is evidence that *N. ceranae* can be transmitted via feeding. This evidence suggests that the importation of honey bee queens maybe a viable route for co-importation of *N. ceranae*.
- Studies were competed to gather evidence on the pathology of *N. ceranae* in the UK. Extensive field data demonstrated that whilst adult bees from smaller colonies were more likely to contain *N. apis* than larger colonies, no such relationship was found for *N. ceranae*. In addition, NBU colonies containing both *Nosema* species survived for four years without the need for treatment. Experimental infection with UK strains of *N. apis* and *N. ceranae* demonstrated that both species presented an equal risk of death to adult *A. mellifera* individuals. Taken together, these observations suggest that *N. ceranae* does not currently present a greater risk to UK honey bee stocks than *N. apis*.
- *N. apis* infection is often associated with chronic bee paralysis virus (CBPV), which can cause serious losses on honey bee colonies. However, it is not yet known whether this virus is also associated with *N. ceranae* infection. This project produced preliminary data suggesting that CBPV was associated with *N. ceranae* spores.

3. Literature review to assess efficacy of available methods for IPM of *Varroa*

- The mite *Varroa destructor* is an external parasite of *A. mellifera*, feeding on adult bees and brood. It is present throughout the British Isles. *Varroa* weakens its host, and transmits several very damaging bee viruses. When unchecked, infestation kills affected colonies. A range of chemical varroacides are available, as are several alternative control methods such as drone brood removal, icing sugar application and the use of open mesh floors. However, none of these techniques alone necessarily provides the most effective protection possible and resistance to chemical- (pyrethroid-) based products is widespread and increasing.
- This project produced a literature review covering many aspects of *Varroa* control which was complemented by an excellent publication from Rosenkranz *et al*. in 2010. The development of non-chemical control options is a rapidly developing area of research. However, even with the promising IPM tools suggested within the literature, large-scale uptake of IPM has not been realised in many bee keeping areas of the world. This might suggest a more active training/extension role is required to improve the uptake of *Varroa* IPM.

4. Risk assessment and detection of *Tropilaelaps* mites

- Parasitic *Tropilaelaps* mites are serious pests of honey bees in Asia and a potential threat to UK apiculture. Parasitisation causes abnormal brood development and ultimately colony decline and collapse. The natural geographical range of *Tropilaelaps* spp. is currently largely confined to tropical/sub-tropical zones. None of the four known mite species have yet been found in Europe, but these parasites are statutorily notifiable under EU legislation. If introduced they have the potential to cause major economic damage and losses to beekeeping.
- A full Pest Risk Assessment was completed, describing the threat posed to UK bee stocks by *Tropilaelaps* mites. Five pathways were identified through which *Tropilaelaps* mites could arrive in the UK. These were (i) importation of *A. mellifera* colonies; (ii) importation of live adult *A. mellifera* bees; (iii) movement on beekeeping equipment; (iv) movement with honey bee swarms or other organisms and (v) trade in hive products. Of these pathways *Tropilaelaps* mites are most likely to arrive in the UK if brought in accidentally with infested colonies from the EU. However, *Tropilaelaps* is currently believed to be absent from the EU and levels of imports from this source are very low. Introduction of *Tropilaelaps* mites into

the UK via illicit trade from third countries cannot be ruled out. *Tropilaelaps* mites are reliant on honey bee brood for survival and therefore the establishment of *Tropilaelaps* mites is dependent on the presence of year round honey bee brood. This can occur in some parts of Britain but not all. There is a moderate risk of incursion of *Tropilaelaps* mites but the dependence on brood provides a relatively straight forward means of controlling this pest.

- Molecular diagnostic tests were developed to enable to enable rapid speciation of *Tropilaelaps* mites. However, all assays demonstrated some cross reaction with non-target species, therefore new potentially more variable regions of the mite genome should be explored to further refine the methods.

5. Develop tools to understand AFB transmission

- American foulbrood (AFB) is a damaging notifiable bacterial brood disease of the honey bee. Understanding AFB transmission is important to help refine current control protocols. The project developed a three gene typing system, based on multi-locus sequencing typing. The provisional scheme separated a panel of isolates into many more groups that established methods. The scheme will be further developed to provide a valuable tool to better understand disease movements in the future.

Introduction

The Small Hive Beetle (SHB), *Aethina tumida*, is a parasite and scavenger of the Western honey bee (*Apis mellifera* L.). SHB is native to sub-Saharan Africa where it causes relatively little damage (Brown and Morton 2003). However, SHB has become an invasive species and its global range extends to North America, Egypt and Australia, and has caused considerable damage to European *Apis mellifera* (Neumann and Elzen 2004). The effect on apiculture economics and honey bee colonies in the USA and Australia can be severe (Ellis *et al*. 2002, Hood 2000, Levot and Haque 2006). Increasing international trade of bees and bee products drives a concomitant increase in the risk of SHB arriving and becoming established in the UK (Cuthbertson and Brown, 2009; Cuthbertson *et al*. 2010; Marris *et al*. 2010).

The SHB lifecycle consists of a pupation stage that occurs outside the beehive in the surrounding soil. Both larvae and pupae can be found in the soil. Therefore, there is an opportunity for control measures to be applied at this stage that will unlikely impact the honey bee hosts. Beekeepers in the US have traditionally used pesticides containing permethrin to control larvae and pupae in the soil (Hood 2004). However, continued use of this can give rise to resistance (Hemingway and Ranson 2000) and undesirable side effects on both honey bees and humans (Hassan *et al*. 1983; WHO 1990). Therefore, there is much demand to improve the range of products available for the control of the larval and pupal stages. Such alternative control agents include entomopathogenic nemadodes (EPN) and fungi (EPF), which have successfully been used against other invertebrate pests (Glazer et al. 1999; Cabanillas and Elzen 2006; Cuthbertson *et al*. 2011). Identifying suitable control measures is an important aspect of being prepared for the potential arrival of SHB in the UK.

Many insect-derived polypeptides (e.g. venom toxins) have high potency and specificity of action towards invertebrates. They are therefore ideal candidates for the development of novel insecticides offering improved target specificity and reduced environmental impact compared to conventional insecticides. The novel insecticides are fusion proteins consisting of a toxin fused to a carrier protein. High levels of fusion proteins can be produced by microbial fermentation and subsequently tested for activity against insect pests. Following ingestion of the fusion protein, the carrier component directs transport across the gut of the target insect and thereby delivers the insecticidal component to the circulatory system, where it is active (Fitches et al., 2001). To date, no fusion proteins have been successfully evaluated for the control of SHB.

A. mellifera can be infected by two microsporidial parasites, *Nosema apis* and *Nosema ceranae*. *Nosema* infections occur mostly through ingesting spores with food or water, with the physical and chemical conditions of the midgut triggering the germination of the spores (Chen et al. 2009). *N. apis* was described infecting *A. mellifera* more than one hundred years ago, whereas *N. ceranae* was not discovered until 1994 when it was reported as a parasite of the Asian honey bee (*Apis ceranae*) in China (Fries et al. 1996). In 2005 *N. ceranae* was isolated from diseased European honey bees in Taiwan and found in collapsing *A. mellifera* colonies in Spain (Higes et al. 2006; Chen et al. 2008). This instigated numerous studies on the incidence of *N. ceranae* in *A. mellifera*, revealing a worldwide distribution of *N. ceranae* in honey bee populations (Higes et al. 2006; Chen et al. 2008). *N. ceranae* has been partly attributed to declines in some honey bee populations (Cox-Foster et al. 2007; Higes et al. 2007). As such it is important to understand the risk *N. ceranae* poses to UK honey bee stocks.

Varroa destructor (Anderson & Trueman), the ectoparasitic mite now on *Apis mellifera* L., has caused widespread damage to the beekeeping industry worldwide and if not controlled can cause 100% losses of the bee colonies within months and can increase the prevalence of damaging honey bee viruses (deJong 1997; Martin et al., 2012). Until now, control of this pest has been based on chemical acaricides which increase production costs, toxic residues and the potential for resistance development (Melathopoulos *et al*., 2000). Combining several methods of control within an Integrated Pest Management (IPM) approach may offer the best means of mite control (Delaplane *et al*., 2005; Rosenkranz *et al*., 2010).

Mites in the genus *Tropilaelaps* (Acari: Laelapidae) are damaging ectoparasites of *Apis* honey bees native to Asia (Delfinado and Baker 1961; Anderson and Morgan 2007). *Tropilaelaps* mites infect the brood, suck haemolymoph and can cause abnormal brood development and the death of individual bees, leading to subsequent colony decline or absconding. To-date *Tropilaelaps* spp. have not been found within Europe and there is a legal requirement for all findings to be notified to regulatory bodies under EU legislation. The differentiation of *Tropilaelaps* mites based principally on morphology is difficult and requires taxonomical skills. More recently Anderson and Morgan (2007) showed that nuclear DNA (ITS1-5.8S-IS2) gene sequences and mtDNA (COI) are useful genetic markers in distinguishing four species of *Tropilaelaps* mites. However, there is currently a lack of molecular techniques available for the rapid detection and differentiation of the *Tropilaelaps* spp. Real-time PCR has a number of significant advantages over conventional PCR. This technology provides rapid, sensitive and accurate results and there is no need for post PCR manipulations, such as gel electrophoresis (Boonham et al., 2002).

American foulbrood (AFB) is a notifiable bacterial brood diseases of *A. mellifera* caused by the Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Genersch et al., 2006). AFB is a serious problem in apiculture and causes considerable economic loss to beekeepers all over the world (Genersch, 2010). AFB affects the honey bee larvae and is lethal to the colony if left untreated (Hansen et al, 1999). The incidence of AFB in the UK has fallen in recent decades from over 2,000 cases per annum in the 1950s to typically 100-200 cases per annum today (Wilkins et al., 2007). However, AFB occurrence appears to be sporadic and the reasons for this remain unclear. AFB can be spread by the exchange of hive products, queens and adult bees between colonies (Genersch, 2010). Horizontal transmission is a well documented phenomenon with possible routes including natural movement of adult bees between colonies (drifting) and the behaviour of foragers (robbing). Current typing schemes for *P. larvae* are only able to distinguish four genetically similar groups based on ERIC fingerprinting (Genersch & Otten, 2003) which is of minimal use to identify movement of a global pathogen. Multi-locus typing, which separates species into a higher number of genetically similar groups, has been used to successfully improve the understanding of pathogen epidemiology (Maiden, 2006). Such a scheme could benefit our understanding of how AFB moves within and between countries.

Objectives

1.1. Produce fusion proteins in sufficient quantity for use in laboratory bioassays.

1.2. Investigate the potential of two fusion proteins to act as novel control agents for Small Hive Beetle (SHB).

1.3. Investigate feasibility of using available chemical and biocontrol products (e.g. nematode- or fungal-based) for SHB management in the UK.

1.4. Ascertain the biology (development and behaviour) of SHB under a range of environmental conditions representative of the UK climate.

2.1. Continue to monitor the long-term impact of *Nosema ceranae* and *Nosema apis* on NBU honey bee colonies.

2.2. Investigate the comparative pathology of UK isolates of *Nosema ceranae* and *Nosema apis* and their potential association with honey bee viruses.

3.1. Review literature to assess efficacy of available Integrated Pest Management practices for Varroa.

4.1. Develop a Pest Risk Assessment of exotic threats to UK honey bees, using *Tropilaelaps* species as a model.

4.2. Develop rapid diagnostic methods to speciate *Tropilaelaps* mites.

5.1 Monitor bee movement between hives and between apiaries in the context of AFB transmission.

Methods

1.1 Produce fusion proteins in sufficient quantity for use in laboratory bioassays

Two fusion proteins, designated FP4 and FP5, were produced for bioassays against small hive beetle (SHB) larvae. Each fusion protein contained genes encoding for a venom peptide fused N-terminally to a sequence encoding the carrier snowdrop lectin *Galanthus nivalis* agglutinin (GNA). Clones containing plasmids coding for these fusion proteins were created as part of a LINK programme (09910). Recombinant GNA was produced for use as a control treatment in SHB assays.

For protein production, *P. pastoris* cells containing the FP4, FP5, or GNA constructs were grown in a BioFlo 110 laboratory fermenter (New Brunswick Scientific, www.nbs.com) as previously described (Fitches *et al*., 2004). Recombinant FP4 and FP5 were both purified by nickel affinity chromatography. Following fermentation, culture supernatants were filtered (0.7 μM nitrocellulose), diluted (1:4) in 4X binding buffer (1x BB; 20 mM sodium phosphate, 0.4M NaCl, pH7.4) and loaded onto 5 ml HisTrapFF (Amersham Pharmacia) columns. Recombinant proteins were eluted from columns with elution buffer (BB containing 0.3M imidazole). Peak fractions were analysed for purity by SDS-PAGE. Both purified recombinant proteins were de-salted by dialysis and freeze-dried. Prior to all subsequent bioassays the concentrations of recombinant proteins were estimated by comparison with known amounts of standard proteins by SDS-PAGE. More than 20 mg of each of the recombinant proteins (FP4, FP5, and GNA) were supplied for SHB assays.

1.2 Investigate the potential of two fusion proteins to act as novel control agents for Small Hive Beetle (SHB)

Prior to testing fusion protein efficacy, artificial diets were optimised to produce healthy SHB. The diets tested were pollen, soya flour, yeast and milk powder. For each diet, the substrate was mixed with a quantity of 50% liquid honey. For each replicate, a single weigh boat containing the diet was placed in a 355 ml ventilated, tissue-lined plastic pot (Solo Cup Company, UK). The tissue was dampened with tap water to prevent desiccation of the larvae. Twenty SHB larvae (0-24 h post-emergence) were transferred to the edge of the weigh boat in the plastic pot using a fine paintbrush. The larvae were left to feed for 7 days. At this time all pots were placed in a freezer at -18°C for a minimum of 48 hours. Larvae were carefully removed from the pot and individually rinsed in a small quantity of water to remove traces of the diet. The larvae were counted, air-dried and each individual weighed. The experiment was repeated on four replicate timings using a latin square randomization. A REML analysis was used to compare the mass of the larvae developing on the four different diets.

The stability of FP4 and FP5 was assessed in the most effective diet from above using Western blot. Proteins were extracted from diet samples of known weight by homogenisation in PBS. Following agitation for 30 minutes, samples were spun (benchtop centrifuge 8,000 g for 5 mins) and supernatants loaded onto SDS-PAGE gels, alongside standards of known concentration. Proteins were transferred onto nitrocellulose and probed for the presence of intact fusion proteins using anti-GNA antibodies (1:3300 dilution). Finally, the two fusion proteins or recombinant GNA were incorporated into the most appropriate diet from above at concentrations of approximately 270 and 1330 ppm. Replicates were set up as described above using twenty SHB larvae (0-24 h old) in each replicate. Survival and larval mass were assessed as described above, with three replicates at the lower concentration and two at the higher concentration.

1.3 Investigate feasibility of using available chemical and biocontrol products (e.g. nematode- or fungal-based) for SHB management in the UK

A comprehensive review of available chemical and biocontrol methods (entomopathogenic fungi & nematodes) for use against immature stages of Small Hive Beetle (*A. tumida*) in the UK was completed (see Annex 1). *A. tumida* were cultured and maintained as described by Cuthbertson *et al*. (2008) under strict quarantine conditions. Wandering larvae were used for all experimental trials. The control agents trialled were all commercially available products across Europe and comprised 3 EPN's: *Steinernema feltiae* (Nemasys), *S. kraussei* (NemasysL), *S. carpocapsae* (Capsanem) and 2 EPF's: *Lecanicillium muscarium* (Mycotal) and *Beauveria bassiana* (Naturalis) and the chemical product Checkmite+ (Coumaphos). The impact of direct and in-direct exposure to control products was investigated in two separate experiments.

For direct exposure trials, individual wandering larvae were dipped in recommended dose rates of the fungi (10^8 conidia/ml) and nematode products (10,000 infective juveniles/ml) for 3 seconds. They were then placed on moist filter paper within 9 cm diameter Petri dishes and maintained at 20°C, 65% R.H. and 16:8hr Light:Dark regime. Ten larvae were placed in each dish with 10 dishes per treatment. Larvae dipped in water and placed on moist filter paper in Petri dishes acted as controls. For Checkmite+, larvae were exposed to a 2 cm^2 piece of Checkmite+ strip within a 9 cm Petri dish. Mortality for all treatments was assessed after 2 weeks.

For indirect exposure, 7 cm diameter by 15 cm tall plastic containers were filled with sterilised sand (8% moisture content). 50 ml of control product (nematode or fungi) was added over the surface of the sand at the same dose rates as in the direct trials. Once the solution had soaked down into the sand, ten wandering larvae were added to the surface. The containers were then sealed and maintained at the conditions described above. There were ten containers per treatment. Controls consisted of wandering larvae added to containers in which the sand had been treated with 50 ml of water. For Checkmite+, larvae were allowed to wander over Checkmite+ strips for 10 seconds before being placed onto sand. Treatments were then maintained for 6 weeks in order to allow adult beetles to emerge. Mortality was calculated as the number of beetles that failed to emerge. In order to confirm the fate of those individuals that did not emerge as adult beetles, the sand substrate was sieved and searched for insect debris at the end of each trial.

Trials to understand whether delayed application would be effective for SHB control were conducted using two nematode species (*S. kraussei* and *S. carpocapsae*). Containers were prepared as described above. Ten wandering SHB larvae were added to a container. Following 24 hours the first batch of nematode solution was added. Then at weekly intervals, nematode treatments were added (at the same dose rate as before) to larvae infested containers. There were 10 replicates per nematode treatment per application date. Control containers received an equal volume (50 ml) of water. Following treatment all containers were maintained in a CE room (23°C, 65% R.H.) for 6 weeks to allow beetles ample opportunity to emerge. Nematode controls (sand pots with no beetle larvae present) were also maintained to determine their longevity in the sand.

The data was analysed using a Generalized Linear Model (GLM) with a binomial distribution and logit link function. 95% confidence intervals were calculated on the logit scale, then back-transformed (as proportions) and the treatments were grouped, following pairwise testing (at the 5% significance level) using means separating groups on the logit scale.

1.4. Ascertain the biology (development and behaviour) of SHB under a range of environmental conditions representative of the UK climate

Much of *A. tumida*'s life cycle takes place within close proximity of bee colonies and as such it is subject to the modified temperature found within a hive. Since *A. tumida* pupate in the soil outside the hive it is at this life stage that they are most exposed to the variability and extremes of localised temperature conditions. The objective of this study was to investigate *A. tumida* pupa development when exposed to varying periods of time with a temperature lower than those found for published pupation data (typically gathered at 20°C or higher). The choice of 5°C used here for investigation is thought to be closer to those that typically could be encountered by *A. tumida* in UK soils, which according to Met Office data showed an average of 5.08^{o}C for soil temperature at 30 cm depth from 1998-2001.

Culturing commenced by placing 30 – 40 SHB adults in a secure rearing box containing sources of water, honey and pollen (as a source of protein). To exploit the beetle's preference for narrow gaps and crevices within which to lay eggs, pairs of plastic strips (~1 cm x ~3 cm) affixed together with a narrow (~1 mm) gap between them were provided by Jeff Pettis, USDA. The egg strips were monitored daily and, when clutches of eggs were observed (typically 3 days), the plastic strips plus eggs were moved into a fresh secure rearing box. To act as a food source for the hatching larvae, bee brood and comb containing pollen and honey was added to the rearing box. As the larvae reached the final stages of development their behaviour changed and they left the food source and began to 'wander' en masse, signifying their readiness to pupate.

Using the method detailed above, beetles originally sourced from South Africa and North America (Cuthbertson *et al* 2008; Marris *et al*., 2010), were cultured in a CE room at 20°C to produce 2 lots of final instar (wandering) larvae. Equal ratios of these larvae were used to make 90 experimental batches each comprising of 30 individuals. 90 plastic tubs (height 150 mm, radius 40 mm) with secure ventilated lids filled with damp sand (4 ml water : 100 g sand) to a depth of 90 mm were prepared to provide suitable conditions for pupation.

One batch of wandering larvae was placed on the sand surface in each tub. In all cases the larvae were observed to rapidly burrow down into the sand. To allow the larvae to settle all tubs were conditioned in the CE room for 24 hours at 20°C. After conditioning at 20°C, 85 of the tubs were removed and placed in a temperature controlled cabinet at 15°C; the remaining set of 5 tubs remained in the CE room at 20°C to serve as controls. After 24 hours the temperature in the controlled cabinet was reduced to 10°C and then, after a further 24 hours, to 5°C. The time at which the temperature was lowered to 5°C was taken as Day 0 for the experiment.

After 4 days, a set of 5 tubs were removed from the 5°C cabinet and placed in another temperature controlled cabinet set at 10°C for 24 hours. The set was then transferred into an insulated cool box and moved back to the CE room at 20°C. After a further 24 hours the set was removed from the cool box, labelled according to temperature regime, and placed with the controls. Using the same process, further sets of tubs were subsequently returned to 20°C after predefined numbers of days at 5°C. After all the tubs had been returned to the 20°C CE room, a further 4 weeks were allowed to elapse in order to give ample time for the beetles to complete their lifecycle. To check for viability, adults from the same temperature regime sets were collected as they emerged from the sand and aggregated in secure rearing boxes containing sources of water, honey and pollen. These boxes were then monitored over 2 weeks for evidence of egg laying and subsequent hatching.

Finally, all tubs were frozen at -18°C for 1 week to destroy any live beetles. The contents of the tubs were then decanted and a count of the beetles made. An assessment was made of each individual's development and categorised as fully developed to adult, partially developed or undeveloped larvae.

2.1 Continue to monitor the long-term impact of Nosema ceranae and Nosema apis on NBU honey bee colonies

A field trial established in Defra project PH0505 was continued to investigate honey bee colony mortality in the presence of both *Nosema* spp. and pathogen competition between *N. ceranae* and *N. apis*. Adult bee samples were collected from all NBU hives in Autumn 2007 and the DNA extracted tested for the presence of each *Nosema* species using established species-specific real-time PCR. Colonies containing either *Nosema* spp. were selected and moved to a new apiary site and managed using good husbandry practice, with no specific treatments for Nosemosis. Adult bee samples were collected periodically from each colony from April 2008 onwards. The prevalence and concentration of *Nosema* spores from each species were calculated using real-time PCR. In addition, each sample was tested for the presence of honey bee 18S, as an internal control to confirm the quality of DNA extraction.

N. ceranae was first detected in UK honey bee samples in 2007, but it is not clear how long this pathogen has been in the UK. In total, 25 samples of honey bees predating the arrival of *Varroa* in 1992 were gifted by Rothamsted Research. The DNA was extracted from 5 bee pools and tested for the presence of *N. ceranae* and *N. apis* using species specific real-time PCR. In addition, nucleic acid preparations from 5-10 adult bees, collected from random apiaries for virus screening in 2004 (n=110) and 2005 (n=110) were rescreened for *Nosema* spp.. Finally, the DNA was extracted from the attending workers from honey bee imports from 2005 (n=18), 2006 (n=11) and 2007 (n=1), and tested for both *Nosema* spp..

2.2. Investigate the comparative pathology of UK isolates of Nosema ceranae and Nosema apis and their potential association with honey bee viruses.

The final analyses were completed on the colony loss projects which ran from 2007-2009, to determine if there was evidence that *N. ceranae* was impacting the health of UK honey bee colonies. In these studies, samples of adult bees were collected from dead, dying and apparently healthy colonies in all three years. The numbers of frames (combs) of adult bees present in each hive was recorded as a measure of colony health. The nucleic acid was recovered from bulk samples of adult bees (n=30) from each colony in the study. Nucleic acid extracts were tested for the presence of *N. apis* and *N. ceranae* using real-time PCR. "Healthy" and "unhealthy" classes of colony were derived according to whether they contained more or less than 5 combs of adult bees/hive. To determine whether the presence of either *Nosema* spp. might predict colony health, multiple logistic regression analyses were completed for samples of larvae and adult bees independently, using colony health (healthy or unhealthy) as the response and pathogen presence/absence as predictors. In addition, multiple linear regression analyses were completed for samples of larvae and adult bees using the square root of the number of combs of bees in each colony as the response and pathogen presence/absence as predictors. All analyses were completed using Genstat version 10.2.

N. apis infection is often associated with black queen cell virus (BQCV) and chronic bee paralysis virus (CBPV), but it is not yet known whether these viruses are also associated with *N. ceranae infection*. Guts were removed from 20 adult honey bees from a batch that have previously tested positive for *N. ceranae*, BQCV and CBPV. Macerated guts were overlayed on a decreasing discontinuous Percoll gradient (25%, 50%, 75% and 100%; Sigma-Aldrich,St. Louis, MO) and spun at 8000 g for 10 mins at 4^{o}C. The spore pellet was resuspended in 1 ml of sterile water and divided into 2 aliquots. One aliquot was exposed to

an RNase and DNase (Fermentas) and incubated at 37°C for 15 minutes. Aliquots were then placed in a 2 ml tube containing 0.5 ml 0.5 mm zirconia silica beads and the spores disrupted on a mini-bead beater (Biospec). RNA was extracted using RNeasy spin columns (Qiagen) following the manfacturer's instructions. The resulting RNA preparations were tested for the presence of *N. ceranae*, CBPV and BQCV using real-time RT PCR.

N. ceranae has been reported to express a different pathology to *N. apis* (Paxton et al. 2007) and colony infection has been linked to high levels of mortality in Spain (Higes et al. 2008). To understand the comparative pathology of UK *N. apis* and *N. ceranae*, single species cultures were established in eclosed adult honey bees, and the resulting spores purified using a Percoll (GE healthcare) gradient. Spore inocula for each *Nosema* species were diluted to 50,000 spores/µl with sterile distilled water, and 2 µl fed to caged cohorts of 10 adult *A. m. mellifera* honey bees. In addition, a 50% sucrose solution was fed to a cohort to act as a control. The experiment was repeated 8 times. Dosed cohorts were fed 50% sugar syrup as required and held an in an incubator at 33ºC. The mortality in each cage was checked daily for a period of 35 days, with dead bees being removed upon discovery. The bee mortality data were analysed using survival analysis. First, Kaplan-Meier estimates of the survival functions were used to produce estimates of the time 50% mortality. Then, non-parametric tests were used to assess whether there was evidence that the survival curves differed between treatments. The final approach was to look at a parametric test, using a proportional hazard model. This method assumes a baseline hazard function (the probability to die at a given time point given that the individual has survived up to that time point) which is modified proportionally between the treatments.

3.1.Review literature to assess efficacy of available Integrated Pest Management practices for Varroa

A literature review was completed assessing the available chemical and biological *Varroa* control methods (see Annex 2). During the preparation of this review, an excellent digest of the current literature was produced by Rosenkranz et al. (2010).

4.1.Develop a Pest Risk Assessment of exotic threats to UK honey bees, using Tropilaelaps species as a model

A full pest risk assessment for *Tropilaelaps* mites was completed. This has now been published on Bee Base, www.nationalbeeunit.com/ under the Contingency Planning section.

4.2.Develop rapid diagnostic methods to speciate Tropilaelaps mites

Rapid diagnostic methods were developed to improve the speed and versatility of mite detection. Adult *Tropilaelaps* mites were received from Thailand and identified based on morphology according to Anderson and Morgan (2007). Samples were preserved in 70% ethanol and kept at -20°C until required. The DNA was extracted from individual mites using the methods described by Boonham et al. (2002). Briefly, individual mites were ground in 0.5 ml microcentrifuge tubes with 50 µl of molecular grade water and held on ice. A slurry of 50% w/v of Chelex ®100 resin (Bio-Rad) was added to each tube and heated at 95°C for 5 min on a thermocycler. The extract was centrifuged at 8,000 g for 5 min, the supernatant removed and stored at -20°C until use.

To confirm the identity of potential positive control material, the ITS1-5.8S-ITS2 gene region was amplified by PCR using primers and thermal profiling outline by Anderson and Morgan (2007). The products were sequenced on an ABI Prism 3130xl genetic analyser, using data collection v 3.0 software (Applied Biosystems). Sequences were edited in *MEGA* version 5 (Tamura et al., 2011) and compared with published sequences in the GenBank database using a BLAST search.

Real-time PCR assays were designed to *T. mercedesae*, *T. clareae, T. koenigerum* and *T. thaii* using the Primer Express™ software (PE-Biosystems) (Mumford et al., 2000). The real-time PCR assays were designed within the CO1 gene sequence and a nucleotide alignment of all available *Tropilaelaps* CO1 gene sequences in GenBank database revealed a number of single nucleotide polymorphisms (SNP) that discriminates between these four species. Real-time PCR was carried out in a volume of 25 µl on 96-well reaction plate. Each 25 µl reaction comprised of 1 µl of DNA extract, 10X Buffer A, 5.5 mM of $MgCl_2$, 0.025U AmpliTaq Gold® (Applied Biosystems), 0.2 mM of each dNTP (Web Scientific) and 300 nM of each primer and 100 nM of the common probe, with the remaining volume being made up of molecular grade water. Reactions were run on the ABI 7500 or 7900 HT Sequence Detection System using generic cycling conditions of 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Real-time PCR data is expressed as C_t (threshold cycle) values. This is the PCR cycle at which PCR products are first detected above a baseline threshold. Positive control constituted validated control material when available, and synthetic single stranded nucleotides complimentary to the respective amplicon when unavailable. Assays were also tested for cross-reaction against DNA extracted from *Acarapis externus, Acarapis dorsalis* and *V. destructor*.

5.1 Monitor bee movement between hives and between apiaries in the context of AFB transmission

Multi locus sequence typing (MLST) schemes, based on comparing sequences of 6-10 housekeeping genes between isolates, have proved useful to understand the epidemiology of human pathogens (Maiden, 2006). Putative MLST loci were identified using genomic comparisons between single isolates of *P. larvae* from ERIC I and ERIC IV using the online programme doubleACT. Putative loci contained 85-99% sequence similarity over 300-1500 nts. Primers were designed to amplify between 300-800 nt of each locus using the online program Primer 3, version 4.0 (Rozen and Skaletsky, 2000). Suitable primer sequences were then tested for self complementarity using the website Oligocalc (Kibbe, 2007).

DNA was extracted after growing cultures on brain heart infusion plus thiamine and incubated at 35^{o}C for two days. 300 µl 6% Chelex ®100, heated to 56°C for 20 minutes, then heated to 100°C for 8 minutes before being placed on ice. Each sample was subjected to a specific test for *P. larvae* using real-time PCR. PCR products from putative MLST loci were purified using Qiagen® PCR purification and sent to Eurofins-MWG-Operon for sequencing on ABI 3730xl 96-capillary DNA Analysers.

Primers for each putative MLST locus were validated on seven *P. larvae* isolates with diverse biogeography. An allele was identified as a sequence or sequences with one or more genuine nucleotide difference from previously assigned sequences. The combination of allele numbers for each of the target genes provided the sequence type (ST) of a sample. Samples with the same ST have the same allele numbers for all loci and can be considered genetically similar.

Results

1.1 Produce fusion proteins in sufficient quantity for use in laboratory bioassays

FP4 and FP5 constructs were successfully produced in sufficient quantities to complete project objectives.

1.2 Investigate the potential of two fusion proteins to act as novel control agents for Small Hive Beetle (SHB)

Larvae that developed on the yeast and honey diet had a significantly greater mass than those that developed on any other diet, whilst larvae that developed on the soya flour and honey diet had a significantly lower mass than those that developed on the other diets (Table 1.2.1). The yeast and honey diet also had the greatest survival of SHB larvae and was therefore chosen for all future studies.

Table 1.2.1 Mean (± SE) mass of SHB larvae after 7 days development on different diets. Means followed by the same letter are not significantly different (P>0.05).

Diet	Soya flour	Milk powder	Pollen	Yeast
Mean larval mass (± SE) (mg)	4.22 ± 1.12 a	7.89 ± 1.12 b	8.58 ± 1.14 b	12.89 ± 1.13 c
% survival	88.75	88.75	72.5	98.75

FP4 and FP5 were produced in sufficient quantity for future studies and appeared to be stable following incorporation into a yeast and honey diet, with no evidence of significant degradation of either recombinant protein as compared to fusion protein standards run on the same gel (Figure 1.2.2).

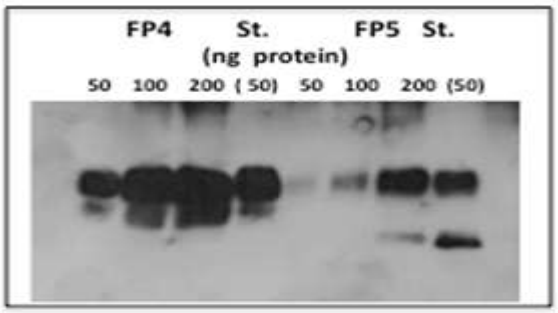

Figure 1.2.2 Western blot analysis of FP4 and FP5 samples extracted from small hive beetle larval diets. Three loadings of either FP4 or FP5, based on estimated concentrations of proteins in prepared diets are shown (50, 100, & 200 ng) and a standard of known concentration is depicted in brackets. Blots were probed with anti-GNA antibodies.

Each fusion protein was added to the yeast and honey diet. Unfortunately, at the concentrations tested, no diet reduced the survival or development of SHB larvae (Table 1.2.3).

Table 1.2.3 Mean (± SE) mass of SHB larvae after 7 days development on diets incorporating FP4, FP5 or recombinant GNA at two concentrations.

(a) Protein concentration approx. 270 ppm

	Control	GNA	FP4	FP5
Mean larval mass (± SE) (mg)	12.6 ± 0.9	11.8 ± 0.9	14.8 ± 0.6	12.9 ± 0.8
% survival	96.7	96.7	96.7	95

(b) Protein concentration approx. 1330 ppm

	Control	GNA	FP4	FP5
Mean larval mass (± SE) (mg)	15.8 ± 0.8	17.0 ± 0.9	16.5 ± 0.8	14.9 ± 1.3
% survival	97.5	92.5	100	87.5

1.3 Investigate feasibility of using available chemical and biocontrol products (e.g. nematode- or fungal-based) for SHB management in the UK

Direct exposure demonstrated a significant treatment effect on the wandering larvae when compared to the control ($P<0.001$), however neither entomopathogenic fungus caused mortality above that seen in the water controls (Figure 1.3.1). The entomopathogenic nematodes showed more promise; *S. carpocapsae* achieved significantly higher mortality than *S. kraussei* and *S. feltiae* ($P<0.05$), which in turn achieved significantly higher mortality than the control after 2 weeks. Upon dissecting the larvae, nematodes freely emerged from the body cavity confirming their ability to infect the larvae (Figure 1.3.2). Direct exposure to Checkmite+ resulted in total mortality of larvae following 6 days (Figure 1.3.3).

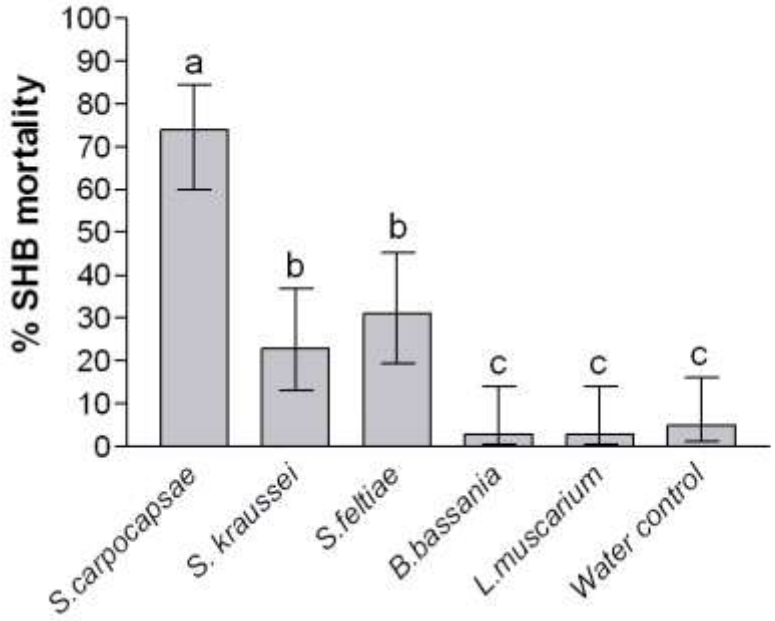

Figure 1.3.1 Impact of direct exposure of control agents on *A. tumida* wandering larvae after 2 weeks. Error bars represent the 95% confidence intervals. Means were separated using least significant differences after adjusting for multiple comparisons. Significantly different means (5% significant level) are represented by suffixes a-c.

Figure 1.3.2 Dissection of infected wandering Small Hive Beetle larvae releasing nematodes.

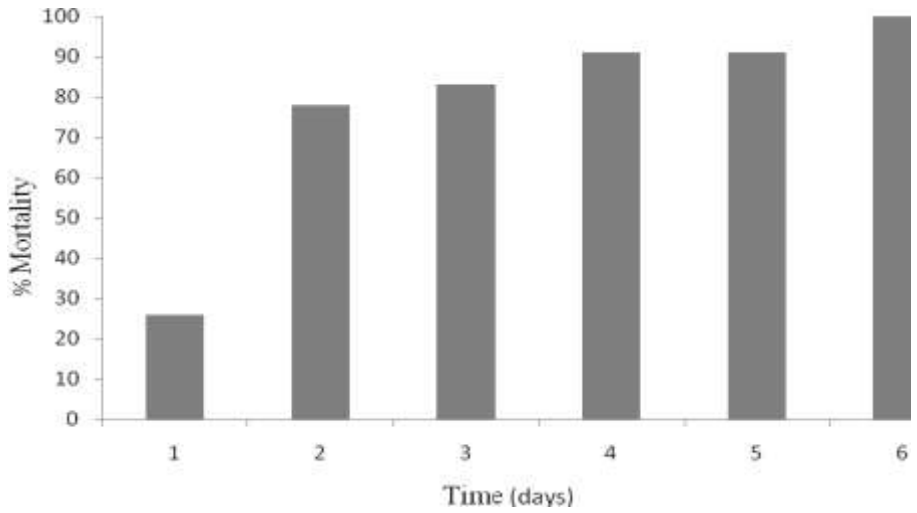

Figure 1.3.3 Impact of direct exposure to Checkmite+ on Small Hive Beetle larvae.

Treating the sand before adding the larvae exposes the SHB to the biocontrol agents during pupation (indirect exposure), and more closely simulates how beekeepers might apply such products in the field. Indirect exposure demonstrated a significant treatment effect on SHB mortality when compared to the control ($P<0.001$). However, once again neither entomopathogenic fungus caused mortality significantly above that seen in the water controls (Figure 1.3.4). Treating the sand produced excellent results for *S. kraussei* and *S. carpocapsae* where total mortality of *A. tumidia* was achieved. No adults emerged from either of these two treatments and sieving the sand did not yield any remains of developing beetles, suggesting the larvae became infected, died and their remains dissolved during the 6 week test. Also, *S. feltiae* achieved significantly higher mortality than the control ($P<0.05$; Figure 1.3.4). Conversely, indirect exposure of wandering larvae to Checkmite+ strips had no effect on beetle survival, with 94% of larvae indirectly exposed to Checkmite+ proceeding to develop successfully to the adult stage.

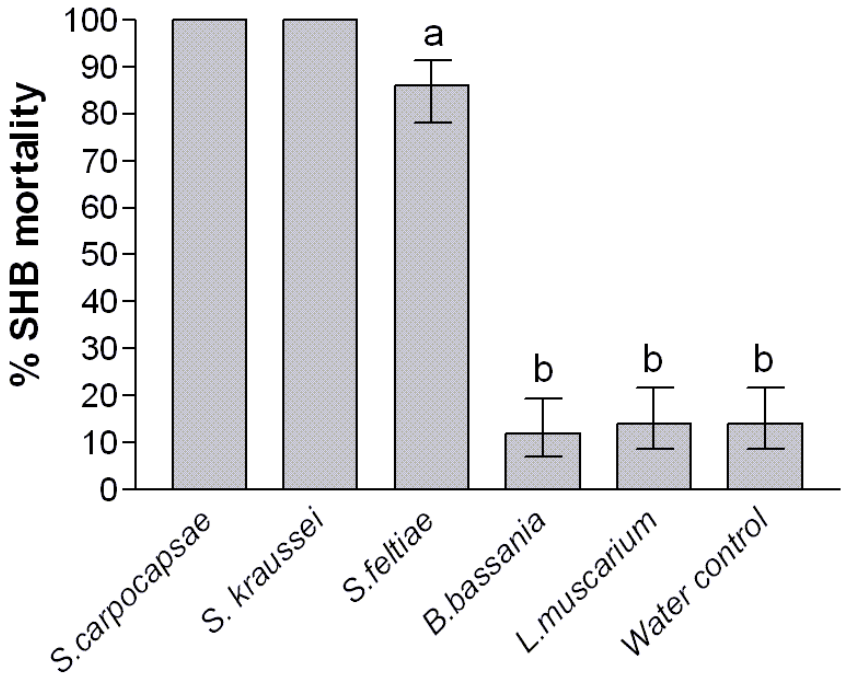

Figure. 1.3.4 Impact of in-direct exposure of control agents on *A. tumida* wandering larvae. Error bars represent the 95% confidence intervals. Means were separated using least significant differences after adjusting for multiple comparisons. Significantly different means (5% significant level) are represented by suffixes a & b.

Following delayed application of the nematodes, significant reduction in adult beetle emergence was obtained (P<0.001; Figure 1.3.5) for up to 3 weeks following larvae entering the sand to pupate. However, later applications (21 days) showed reduced efficacy when compared to earlier applications (7 and 14 days). Under laboratory conditions the nematodes were deemed viable in the sand following 1 week after application. Nematodes were observed under a light microscope moving between the sand grains.

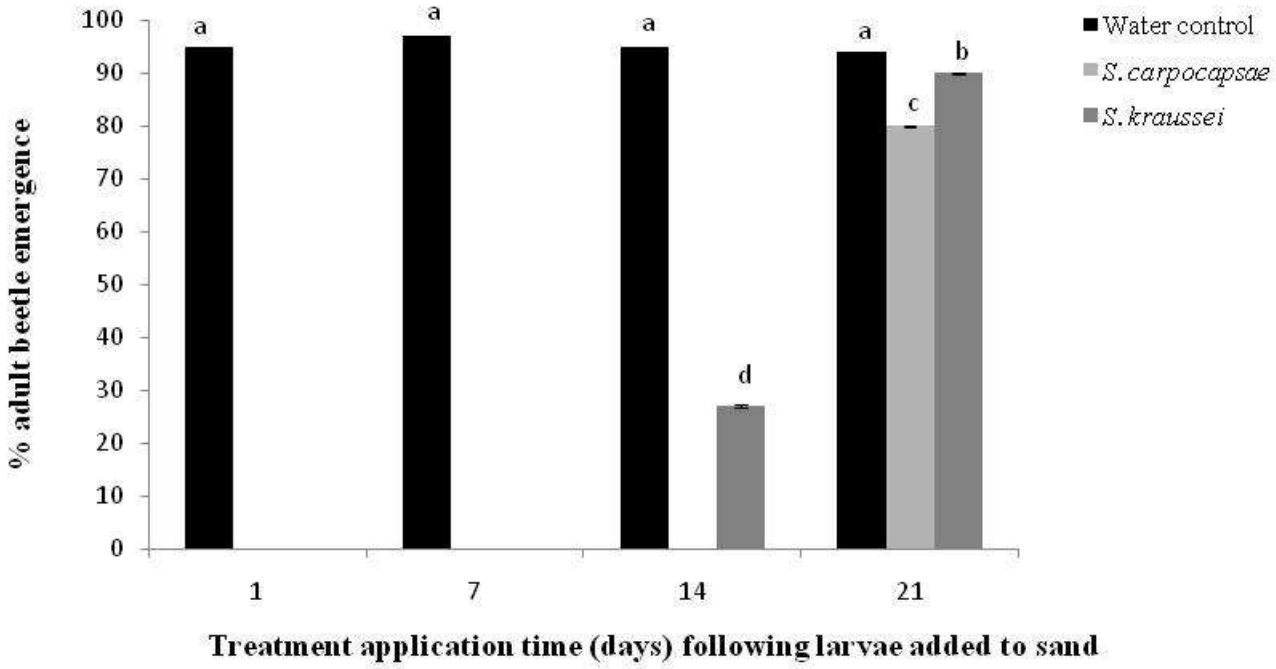

Figure 1.3.5 Impact of delayed applications of entompathogenic nematodes on *A. tumida* wandering larvae following their submergence in sand. Beetle emergence assessed after 6 weeks following larvae being added to sand. Means were separated using least significant differences after adjusting for multiple comparisons. Significantly different means (5% significant level) are represented by suffixes a-d.

1.4. Ascertain the biology (development and behaviour) of SHB under a range of environmental conditions representative of the UK climate.

The assessments made for individual beetle development (Table 1.4.1) and percentage beetle emergence (Figure 1.4.2) after different periods of exposure to incubation at 5°C demonstrated that beetles were able to survive to adulthood after 19 days exposure.

Table 1.4.1 Beetle development following incubation at 5°C.

	Combined Sets (n=150; 5 x 30)			
Days @ 5°C	**Adult**	**Partial development**	**Larvae**	**Std. Dev. (Adult)**
0	150	0	0	0.0
4	125	10	15	3.9
5	116	17	17	3.6
6	108	2	40	3.4
7	90	0	60	4.5
8	71	0	79	8.8
9	77	1	72	4.9
10	39	2	109	5.5
11	20	0	130	3.4
12	15	0	135	1.9
13	7	0	143	1.1
14	11	1	138	1.6
15	7	0	143	1.5
16	4	1	145	0.4
17	2	0	148	0.5
18	0	0	150	0.0
19	1	0	149	0.4
20	0	1	149	0.0

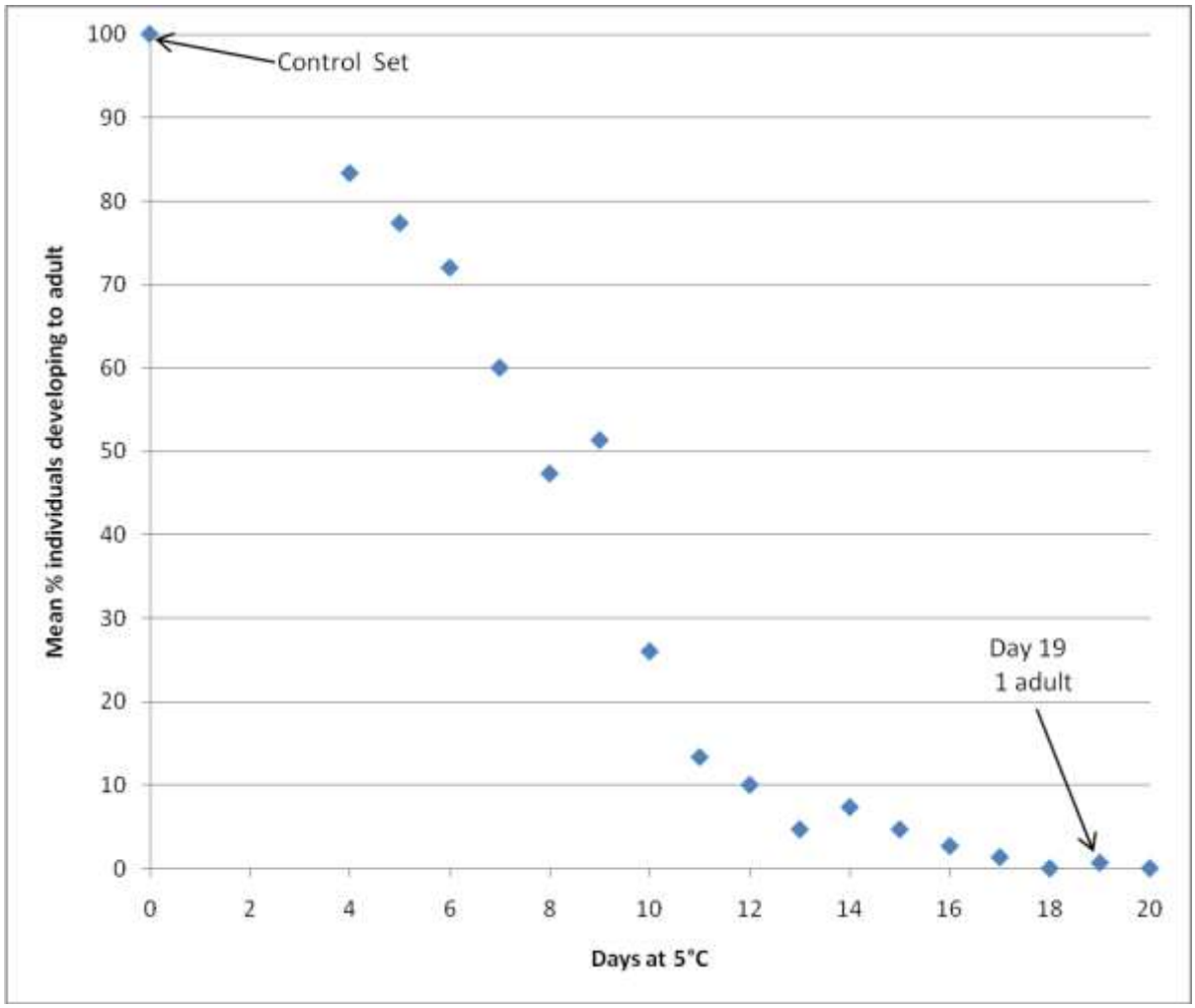

Figure 1.4.2 Percentage beetle emergence following incubation at 5°C.

The viability of individuals surviving to adulthood up to maximum of 14 days exposure at 5°C was assessed. Viable eggs were obtained from adults exposed for up to and including 9 days at 5°C. Individuals exposed to more than 9 days at 5°C produced no eggs.

2.1 Continue to monitor the long-term impact of Nosema ceranae and Nosema apis on NBU honey bee colonies

After 4 years of observation and treatment only using good husbandry practice, 5 of 21 colonies had been lost to CBPV and wasps and a single colony to wet Nosemosis. Samples from the latter colony tested positive for both *N. apis* and *N. ceranae*. *N. ceranae* has been reported to be more pathogenic than *N. apis*, and some researchers have indicated *N. ceranae* may be replacing *N. apis* on the continent. The results of this long-term study suggest both species survive after 4 years coexistence, and aside from a single colony, neither species showed overt pathology despite leaving colonies untreated for Nosemosis.

In total, 23 of 25 historic UK samples collected prior to 1992 tested positive for *N. apis* using species-specific real-time PCR. However, all 25 samples tested negative for *N. ceranae*, suggesting *N. ceranae* was not widespread in the early 1990's. A small number samples of adult bees collected from random apiaries for virus screening in 2004 and 2005 tested positive for *N. apis* (2004: 9 of 110; 2005:11 of 110) and *N. ceranae* (2004: 2 of 110; 2005

1 of 110). Finally, DNA from the attending workers from honey bee imports from 2005 (Hawaii) and 2006 (Hawaii and New Zealand) tested positive for both *Nosema* species.

2.2. Investigate the comparative pathology of UK isolates of Nosema ceranae and Nosema apis and their potential association with honey bee viruses.

Colonies from between 2007 and 2009 that tested positive for *N. apis* on adult honey bees were likely to be smaller than those testing negative for *N. apis* (Figure 2.2.1). However, *N. ceranae* was not associated with weak colonies.

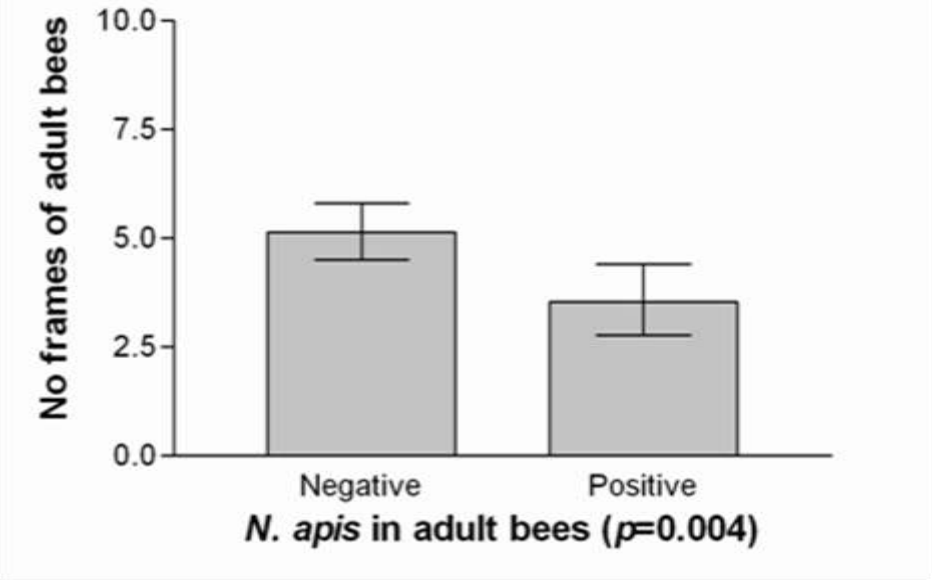

Figure 2.2.1 The predicted number of adult frames of bees present in colonies that tested positive or negative for *N. apis* using real-time PCR. Error bars represent the 95% confidence intervals.

Prior to purification, the spore suspension tested strongly positive for *N. ceranae* (C_t 20.4) and CBPV (C_t 21.5), and weakly positive for BQCV (C_t 34.6). After purification of the spores the samples remained positive for *N. ceranae* (C_t 32.3) and CBPV (C_t 28.0), but testing failed to detect BQCV (C_t 40). Interestingly, external RNase/DNase treatment of the spores resulted in a failure to detect CBPV (C_t 40), without affecting the ability to detect *N. ceranae* (C_t 29.9). This result suggested that CBPV was associated with *N. ceranae* spores, and that the location was likely external rather than internal.

Kaplan-Meier estimators indicating the time taken to reach 50% mortality demonstrated that honey bees dosed with *N. apis* (22±1 day) and *N. ceranae* (22±1 day) died significantly quicker than those dosed with sugar syrup (26±1 day). The proportional hazard function, which indicated estimated risk of death, was expressed as a ratio to the control, such that a ratio more than 1 indicated more rapid death than the control. Honey bees treated with both *Nosema* species had higher ratios than the control (Table 2.2.2).

Table 2.2.2 The hazard function presented as a ratio to the control group. Means followed by the same letter are not significantly different (P<0.05)

Treatment	**Hazard ratio (means separator)**
Sugar syrup control	1.00 (a)
UK *N. ceranae*	2.30 (b)
UK *N. apis*	2.24 (b)

4.2. Develop rapid diagnostic methods to speciate Tropilaelaps mites

Four forward primers, four reverse primers and a common probe labelled with 6-FAM (6-carboxyfluorescein) at the 5´end and a minor groove binding (MGB) group at the 3´ end were designed to detect *T. mercedesae*, *T. clareae, T. koenigerum* and *T. thaii*, respectively. All the mites received from Thailand and The United States (courtesy Jeff Pettis USDA) were identified by morphometry and sequencing as *T. mercedesae.* Therefore positive control material was only available for this species and four long single-stranded, complimentary to the respective assay amplicon, were used to represent the other mite species. Given the variation in the available GenBank sequences for *T. clareae*, two oligonucleotides were designed for this species (Table 4.2.1).

Table 4.2.1 Sequence of the single-stranded oligonucleotides

Name	Sequence 5'-3'
T. clareae (most common)	GGAGGAGGAGATCCAATCTTATACCAACATTTATTTTGATTTTTTGGTCACCCTGAAGTATATATCCTAATCCTACCAGGATTTGGTATCATCTCACAT
T. clareae (most different)	GGAGGAGGAGATCCAATCTTATATCAACATTTATTTTGATTTTTTGGTCACCCTGAAGTATATATCTTAATTCTACCAGGATTTGGTATCATCTCACAT
T. koenigerum	GGAGGAGGTGACCCAATTTTATATCAACATTTATTCTGATTTTTTGGTCACCCTGAAGTTTATATTTTAATTTTACCAGGATTTGGTATTATCTCTCACACAT
T. thaii	GGAGGTGGAGACCCAATTCTTTACCAACACTTATTTTGATTTTTTGGTCACCCTGAAGTTTATATTTTAATCCTACCAGGATTCGGTATTATTTCACACAT

Each primer set amplified the targeted *Tropilaelaps* spp. with C_ts of between 19.88-22.33. *T. mercedesae* and *T. clareae* primers were found to cross react with non-target *Tropilaelaps* spp. (C_t 32.88-35.20) and other unrelated mite species (C_t 39.77-39.33). The primers for *T. koenigerum* and *T. thaii* assays showed no cross reaction with unrelated mite species (C_t 40) but both showed cross reactions with non-target *Tropilaelaps* spp. (C_t 32.87-39.58).

5.1 Monitor bee movement between hives and between apiaries in the context of AFB transmission

Three potential MLST loci were identified: *Natrans*, a gene involved in sodium transportation; glpT, a housekeeping gene; and 86A, a putative protein. Despite this pilot work only assessing 9 isolates of *P. larvae* for 3 loci, this crude MLST scheme identified five genetically different STs (Table 5.1.1), demonstrating potential for this method to yield a useful 6-10 gene typing scheme for *P. larvae*.

Table 5.1.1 Summary of allele numbers and strain types (STs) of samples

Sample no.	ERIC type /origin	Allele number (86A *glpT, Natrans*)	ST
6254*	ERIC1/Unknown	1-1-2	1
6993*	ERIC4/Unknown	3-2-1	2
6815	ERIC1/Unknown	1-1-2	1
6260	ERIC2/Unknown	3-2-1	2
6261	ERIC3/Unknown	3-2-1	2
6264	ERIC4/Unknown	3-2-1	2
7370	ERIC1/Sweden	3-3-2	3
7371	ERIC2/Sweden	2-3-2	4
L1/1	ERIC??/South Africa	3-3-2	5

*Samples 6254 and 6993 are original isolates used for genome comparison

Conclusions/Future Research

1. Small hive beetle control

- Our trials demonstrate that entomopathogenic nematodes, commercially available in the UK, can infest and kill *A. tumida* wandering larvae. Furthermore, these products are available across Europe, and so have the potential to be used as control agents should the SHB expand its range to this continent. The sequential application of the nematodes also provided excellent control of the beetle larvae. No beetles emerged following weekly treatment of *S. kraussei* for 3 weeks. After this period the nematodes would appear to be ineffective at penetrating the developing beetles. One area of further work now required is to determine economic dose rates that provide maximum control of *A. tumida*. The information gathered supports the development of contingency plans to deal with *A. tumida* should there be an outbreak in the UK.

- Much effort was spent developing novel approaches to SHB control using fusion proteins. However, neither fusion protein demonstrated any significant effect on the development or survival of SHB larvae. The toxins used in the fusion proteins have been demonstrated to be effective against other invertebrates including Lepidopteran, Coleopteran and Hymenopteran species (E. Fitches personal communication), so it is most likely that the carrier protein failed to deliver the toxin beyond the beetle's gut wall, therefore not allowing the insecticidal component to enter the circulatory system as required to produce a toxic effect.

- Pupating beetles were exposed to a range of temperatures commonly observed in UK soils to gather important data on the likely survival and spread upon arrival. Our data showed that wandering larvae are capable of pupating and surviving through to adulthood after being exposed to 5^{o}C for as long as 19 days. This result is at variance with data presented by Meikle and Patt (2011) who determined a minimum temperature of 10^{o}C for larvae and pupae development. Stedman (2006) suggested that chilling for 8 days at 4^{o}C could be method of killing larvae. However, our data clearly show SHB can survive to adulthood after exposure to temperatures only 1^{o}C above this temperature. Furthermore, adults that had been subject to 9 days at 5°C during development were capable of producing viable offspring. The lack of breeding success after this indicates a reduction in viability but the lower numbers of adults available may make this inconclusive. These data demonstrate that SHB is more cold tolerant than previously thought and likely capable of developing and surviving under UK climatic conditions.

2. Impact of *Nosema* spp. on UK colonies

- *N. ceranae* is an emergent honey bee pathogen which has become a serious threat to the global beekeeping industry after spreading rapidly throughout most of the world (Forsgen & Fries, 2010; Smith, 2012). Our data indicate that *N. ceranae* was not widespread in the early 1990's, but has been present in the UK since at least 2004. Chen et al. (2008) suggest *N. ceranae* is widely distributed in the US and has been resident since at least 1995. However, unlike the US, where *N. ceranae* has been linked to losses (Cox-Foster et al., 2007), no field pathology has been reported associated with this pathogen in the UK.

- The mechanisms for such swift pathogen proliferation are not clear but could have been facilitated by international trade in honey bee queens. Attending workers from honey bee queens imported from Hawaii and New Zealand tested positive for *N. ceranae*. Historical honey bees collected from Hawaii have previously been reported to test positive for *N. ceranae* (Chen et al., 2008). The importation of honey bee queens is a common practice in the UK, and more than 20,000 queens have been imported from Hawaii and New Zealand over the last 10 years. Whilst the attending workers are not placed in the colony, *N. ceranae* is known to be transmitted by food (Smith, 2012), and so queens caged with attending workers carrying *N. ceranae* are highly likely to become infected. Such a transmission route

would explain the rapid spread of *N. ceranae*. Routes of transmission could be further illuminated by developing a typing scheme capable of identifying genetically similar *Nosema* groups which likely share a common history.

- Experimental infection of individual adult honey bees has suggested that *N. ceranae* may be more virulent than *N. apis* (Paxton et al., 2007) and may be linked to large-scale honey bee colony losses (Higes et al, 2008). Our data demonstrated that whilst adult bees from smaller colonies were more likely to contain *N. apis* than larger colonies, no such relationship was found for *N. ceranae*. In addition, NBU colonies containing both *Nosema* species, and receiving no control for Nosemosis aside from good husbandry practice, survived for four years. Experimental infection with UK strains of *N. apis* and *N. ceranae* demonstrated that both species presented an equal risk of death to adult *A. mellifera* individuals. Taken together, these observations suggest that *N. ceranae* does not currently present a greater risk to UK honey bee stocks than *N. apis*. These observations are in support of Forsgren & Fries (2010), who suggested that *N. ceranae* does not cause significantly higher mortality compared to *N. apis*.

- *N. apis* infection is often associated with BQCV and CBPV, but it is not yet known whether these viruses are also associated with *N. ceranae* infection. A preliminary experiment suggested that CBPV was associated with the external surface of *N. ceranae* spores. If correct, this work indicates that *N. ceranae* could also be associated with CBPV, which has been shown to cause honey bee colony losses in the UK. Future work could repeat the experiment described in this project on many more samples containing *N. apis*, *N. ceranae* and mixed infections.

3. Literature review to assess efficacy of available IPM for *Varroa* control

- This module produced a literature review covering many aspects of *Varroa* control and was complemented by an excellent publication from Rosenkranz *et al*. (2010). In the development of IPM schemes against *V. destructor*, the main aim is to reduce or eliminate beekeepers' reliance on synthetic acaricides. The development of non-chemical means is a rapidly developing area of research. However, even with the promising IPM tools suggested within the literature, large-scale uptake of IPM has not been realised in many bee keeping areas of the world. This might suggest a more active training/extension role is required to improve the uptake of *Varroa* IPM.

4. Risk assessment and detection of *Tropilaelaps* mites

- A full PRA was completed for *Tropilaelaps* mites including a review of the biology and distribution and detailed assessments of entry pathways. A comprehensive list of findings are available in the pest risk assessment which has been published on Bee Base , www.nationalbeeunit.com/ under the Contingency Planning section.

- Rapid detection tools were designed to four species of *Tropilaelaps* mite. However, all assays showed low-level cross reactivity with non-target species. Nevertheless, these assays could be applied to rapidly speciate individual mites, where the level of detection would easily discriminate between target species and false positives. However, these assays would not be appropriate to screen for the presence of mites in samples such as hive debris. Despite contacting many published authors, only two samples of mites were received and both were identified as *T. mercedesae*. The absence of reference samples precludes generating sequence information from alternative regions of the mite genome, and presents a major problem to improving assay design. Nevertheless, future work should continue to develop these potentially valuable rapid assays.

5. Develop tools to understand AFB transmission

- AFB is a serious problem in apiculture and causes considerable economic loss to beekeepers all over the world (Genersch, 2010). AFB occurrence appears to be sporadic and the reasons for this remain unclear. We demonstrated that a primitive MLST scheme was able to infer a greater number of genetically similar strain types in a test panel of 9 isolates than had been found globally using current ERIC typing methods (Genersch & Otten, 2003). Such methods are becoming the gold standard for epidemiological studies and can provide the necessary resolution to study both global and local bacterial disease outbreaks, whilst improving the reproducibility between laboratories (Francisco et al., 2012). Further development of the MLST scheme should be prioritised as it has the potential to track AFB movements and help understand when the disease occurs from endemic and exotic sources.

Benefits

- The literature review on control options for SHB control raised the possibility of using readily available products containing entomopathogenic nematodes. These off-the-shelf products proved to be highly effective at killing pupating SHB larvae. Furthermore the treatment application was still effective several weeks after pupation had begun, potentially improving the chances of eradication, and also providing bee keepers with a control option.

- The improved understanding of the cold tolerance of pupating beetles demonstrated quite clearly that SHB larvae would be able to survive the temperate UK climate, providing valuable information for the contingency plan.

- The arrival of *N. ceranae* caused much consternation through the UK beekeeping community and came only a few years before the only registered treatment for Nosemosis, Fumidil B, was withdrawn from use. This project has produced several lines of evidence that suggest that *N. ceranae* is currently no more of a risk to UK honey bee stocks than *N. apis*. Furthermore, the successful management of both *Nosema* species in NBU honey bee colonies, simply by following good husbandry practices, provides evidence that skilled beekeepers can manage the disease without chemical intervention.

- The PRA for *Tropilaelaps* mites has proved immensely valuable in developing an updated contingency response, to include *Tropilaelaps* mites, and minimise the chance of these damaging mites arriving and establishing in the UK. The PRA included future risk management strategies for *Tropilaelaps* spp., detailing the relative significance of entry pathways. One such pathway (importation of honey bee colonies with associated brood) was identified as a likely route with a high degree of confidence, and justifies the recent increase in checks for within-EU trade imports to 50% documentary checks and 30% physical checks (from 40% documentary and 10% physical). However, it should be noted that *Tropilaelaps* is currently believed to be absent from the EU.

- The rapid diagnostic tests have reduced the time to result for suspect *Tropilaelaps* mites from 1-2 days to 3-4 hours. This could make a big difference during the initial stages of an incursion, and should contribute towards the successful eradication of these pests should they arrive.

- The proof of principle that an MLST scheme could improve our understanding of AFB epidemiology led to industry funding a three-year PhD studentship entitled “Epidemiology of American foulbrood”. The tools developed in this PhD will be used to inform the future inspections programme, ensure the impact of notifiable brood disease is minimised and the biosecurity advice provided to beekeepers and the hive product importation industry is appropriate.

- Finally, many aspects of the evidence presented in this report were used to inform the Bee Health Policy Review process from 2011 to 2012.

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