Integrated Control of Honey Bee Diseases in Apiculture

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Declaration

I declare that the work carried out in this thesis is entirely done by me, and that any help provided by other individuals with data collection and analysis is fully acknowledged.

I certify that this thesis has not and will not be submitted - in whole or part, to another university for the award of any other degree.

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Summary

The honey bee, *Apis mellifera*, is important both ecologically and economically. Pests and diseases are arguably the greatest current challenge faced by honey bees and beekeeping. This PhD thesis is focused on honey bee disease control including natural resistance by means of hygienic behaviour. It contains eleven independent experiments, ten on honey bee pests and diseases and their control and resistance, and one on stingless bees. Each is written as a separate chapter, Chapters 4 and 14 of this thesis.

Chapter 4: How effective is Apistan® at killing varroa?
This shows that Apistan is not very effective at killing varroa, presumably because of resistance. It also shows that a single Apistan treatment resulted in the next treatment being significantly less effective, indicating strong selection for resistance.

Chapter 5: Towards integrated control of varroa: comparing application methods and doses of oxalic acid on the mortality of phoretic *Varroa destructor* mites and their honey bee hosts.
This shows that oxalic acid can be highly effective at killing varroa mites under beekeeping conditions in broodless hives in winter. However, varroa mortality is affected by application method and dose. In addition, bee and colony mortality and colony performance are also affected by application method and dose. The results of this chapter shows that sublimation is the best method, in that it gives greater varroa mortality at lower doses, and results in no harm to the colonies. In fact, colonies treated via sublimation had significantly more brood in spring that controls, and lower winter mortality, although this difference was not significant.

Chapter 6: Towards integrated control of varroa: varroa mortality from treating broodless winter colonies twice with oxalic acid via sublimation.
This shows that two treatments of 2.25 g oxalic acid via sublimation at an interval of 2 weeks in broodless honey bee colonies in winter result in greater varroa mortality than a single treatment, 99.6% vs. 97%. Making a second oxalic acid treatment was not harmful as the performance (frames of brood, queen and colony survival) of the twice-treated colonies over the next 4 months was not significantly different to the once-treated control colonies.

Chapter 7: Towards integrated control of varroa: Efficacy of early spring trapping in drone brood.
This indicates that trapping in drone brood in spring is probably not sufficiently effective to be able to control varroa populations on its own. It shows that trapping varroa in capped drone cells in early spring is not highly effective at controlling varroa. The first and second test frames of drone foundation removed 44% and 48% of the varroa, respectively.

Chapter 8: Towards integrated control of varroa: Monitoring honey bee brood rearing in winter and the proportion of varroa in small patches of sealed cells.
This shows that December is the month with the least brood. However, winter reduction in brood rearing varied among years and even in December some colonies still had sealed brood. Although the amounts of sealed brood were low, even a small patch of c. 500-600 sealed cells could contain 14% of the varroa in a colony. This will halve the duration of control provided by an oxalic acid treatment.

Chapter 9: Towards integrated control of varroa: effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence.
This shows clearly that hygienic behaviour reduces the one-year population growth of varroa in honey bee colonies by more than 50% and reduces the levels of deformed wing virus by more than 1000 times.

Chapter 10: Hygienic behaviour saves the lives of honey bee colonies.

This shows that hygienic behaviour saves the lives of honey bee colonies with shrivelled wings, a visible symptom of deformed wing virus that is considered a predictor of colony death. Over one year, only 2 of 11 colonies requeened with a non-hygienic queen survived, versus 13 of 15 requeened with a hygienic queen.

Chapter 11: Hygienic behaviour by non-hygienic honey bee colonies: all colonies remove dead brood from open cells.
This shows that all honey bee colonies are highly hygienic in response to dead or diseased brood in open cells. All larvae killed by freezing with liquid nitrogen and larvae with chalkbrood disease were removed. This was true even for colonies with low levels of removal of dead brood from sealed cells, which would be considered as non-hygienic colonies.

Chapter 12: Removal of larvae infected by different strains of chalk brood and other fungi by hygienic and non-hygienic bee colonies.
This shows that hygienic and non-hygienic honey bee colonies are highly hygienic in response to diseased larvae killed with different strains of fungus in open cells.

Chapter 13: Hygienic behaviour in Brazilian stingless bees.
This shows that the three stingless bee species studied (*Melipona scutellaris, Scaptotrigona depilis, Tetragonisca angustula*) all have high levels of hygienic behaviour, quantified as the removal of freeze-killed brood, in comparison to the honey bee *Apis mellifera*. In *S. depilis* there was considerable variation in hygienic behaviour among colonies, and hygienic colonies removed more brood affected by a naturally-occurring disease which we discovered and for which the causative agent remains to be identified.

Chapter 14: First record of small hive beetle, *Aethina tumida* Murray, in South America.
This reports the discovery of adult small hive beetles, *Aethina tumida*, in honey bee, *Apis mellifera*, hives in an apiary in Brazil, in March 2015. This is the first record for South America of this honey bee pest.

Publications arising from this thesis

Al Toufailia, H., Ratnieks, F. L. W. (2016). How effective is Apistan® at killing varroa? Results from a LASI trial. *Bee Craft*, 98 (2): 7-11. **(Chapter 4).**

Al Toufailia, H., Scandian, L., Ratnieks, F. L. W. (2016). Towards integrated control of varroa: 2) comparing application methods and doses of oxalic acid on the mortality of phoretic *Varroa destructor* mites and their honey bee hosts. *Journal of Apicultural Research*, 54(2): 108-120. **(Chapter 5).**

Al Toufailia, H., Scandian, L., Ratnieks, F. L. W. Towards integrated control of varroa: varroa mortality from treating broodless winter colonies twice with oxalic acid via sublimation. Submitted. **(Chapter 6).**

Al Toufailia, H., Scandian, L., Ratnieks, F. L. W. Towards integrated control of varroa: Efficacy of early spring trapping in drone brood. Submitted. **(Chapter 7).**

Al Toufailia, H., Scandian, L., Ratnieks, F. L. W. Towards integrated control of varroa: Monitoring honey bee brood rearing in winter and the proportion of varroa in small patches of sealed cells. Submitted. **(Chapter 8).**

Al Toufailia, H. M., Amiri, E., Scandian, L., Kryger, P., Ratnieks, F. L. W. (2014). Towards integrated control of varroa: effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence. *Journal of Apicultural Research* 53(5): 555-562. **(Chapter 9).**

Al Toufailia, H., Ratnieks, F. L. W. Hygienic behaviour saves the lives of honey bee colonies. Submitted. **(Chapter 10).**

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Al Toufailia, H., Evison, S. E. F., Hughes, W. O. H., Ratnieks, F. L. W. Removal of honey bee larvae infected by different fungal diseases in hygienic and non-hygienic colonies. Submitted. **(Chapter 12).**

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Al Toufailia, H. et al. First Record of Small Hive Beetle, *Aethina tumida* Murray, in South America. Submitted. **(Chapter 14).**

Publications arising outside this thesis during my PhD

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Bigio, G., Al Toufailia, H., Ratnieks, F. W. L. (2014). Honey bee hygienic behaviour does not incur a cost via removal of healthy brood. *Journal of Evolutionary Biology*, 27(1):226-230.

Bigio, G., Al Toufailia, H., Hughes, W.O., Ratnieks, F. W. L. (2014). The effect of one generation of controlled mating on the expression of hygienic behaviour in honey bees. *Journal of Apicultural Research*, 53(5), 563-568.

Couvillon, M. J., Al Toufailia, H., Butterfield, T. M., Schrell, F., Ratnieks, F. W. L., Schürch, R. (2015). Caffeinated Forage Tricks Honeybees into Increasing Foraging and Recruitment Behaviors. *Current Biology*, 25(21), 2815-2818.

Shackleton, K., Al Toufailia, H., Balfour, N. J., Nascimento, F. S., Alves, D. A., Ratnieks, F. W. L. (2015). Appetite for self-destruction: suicidal biting as a nest defense strategy in *Trigona* stingless bees. *Behavioral ecology and socio-biology*, 69(2), 273-281.

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Ratnieks, F. L. W., Scandian, L., Al Toufailia, H. (2016). Using oxalic acid to kill varroa. *LBKA (London Beekeepers Association) News*, 22-26.

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Ratnieks, F. L. W., Al Toufailia, H. (2016). How to apply oxalic acid via sublimation to control varroa. LASI Information Sheet.

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Table of Contents

Chapter 1

General Introduction

1.1 The importance of bees

1.1.1 Honey bees

The honey bee genus, *Apis*, has 9 species native to Africa and Eurasia (Oldroyd and Wongsiri, 2006). The western honey bee (*Apis mellifera)* is native to Europe, Africa and the Middle East and has been introduced into America, Australia and Asia, now with an estimated 81 million managed hives worldwide (FAO, 2015). Between 1961 and 2008 there has been a 45% increase in hive numbers worldwide with large increases in China, Argentina and Brazil but with reductions in North America and Europe (Aizen and Harder, 2009).

Apis mellifera is economically important to humans. It provides 1.6 billion kg honey worldwide annually (FAO, 2011; Moritz and Erler, 2016). In 2013, British honey bees produced about 6400 tones of honey (FAO, 2015). In addition, honey bees provide several other products of value, particularly wax, pollen and propolis.

The main benefit of honey bees to humans is crop pollination (Cuthbertson and Brown, 2006). Worldwide, honey bees are responsible for 50% of the total value of all crop pollination carried out by bees (Kleijn et al, 2015). Honey bee pollination is considered essential to North American agriculture (vanEngelsdorp and Meixner, 2009). The annual value of honey bee crop pollination is estimated to be £230 million in the UK (Mwebaze et al, 2010) and $12 billion in the United States (Calderone, 2012).

1.1.2 Stingless bees

The stingless bees, Meliponini, are a large group of eusocial bees found in the tropics worldwide (Michener, 2000). Like honey bees they live in colonies with a queen and workers, and rear brood individually in cells (Michener, 1974). In Brazil, stingless bees are common with approximately 100 species. They are used for honey production (Nogueira-Neto, 1997), are pollinators of wild plants, and are increasingly being studied and used in crop pollination (del Sarto et al, 2005).

1.2 Pests and Diseases: a major challenge to the honey bee and beekeeping

Honey bees face many challenges and colony mortality seems to have increased in the last decade. For example, there was 30% colony mortality in winter 2006 in the USA (vanEngelsdorp et al, 2009; Steinhauer et al, 2014; vanEngelsdorp et al, 2012). Large scale colony losses in the USA in 2006 led to the term "Colony Collapse Disorder" being invented, a syndrome in which colonies died in such a way that the worker population would rapidly dwindle ("collapse") such that a hive with abundant worker bees would, a few months later, have zero or almost no bees despite having abundant honey stores (vanEngelsdrop et al, 2009; Cox-Foster et al, 2007).

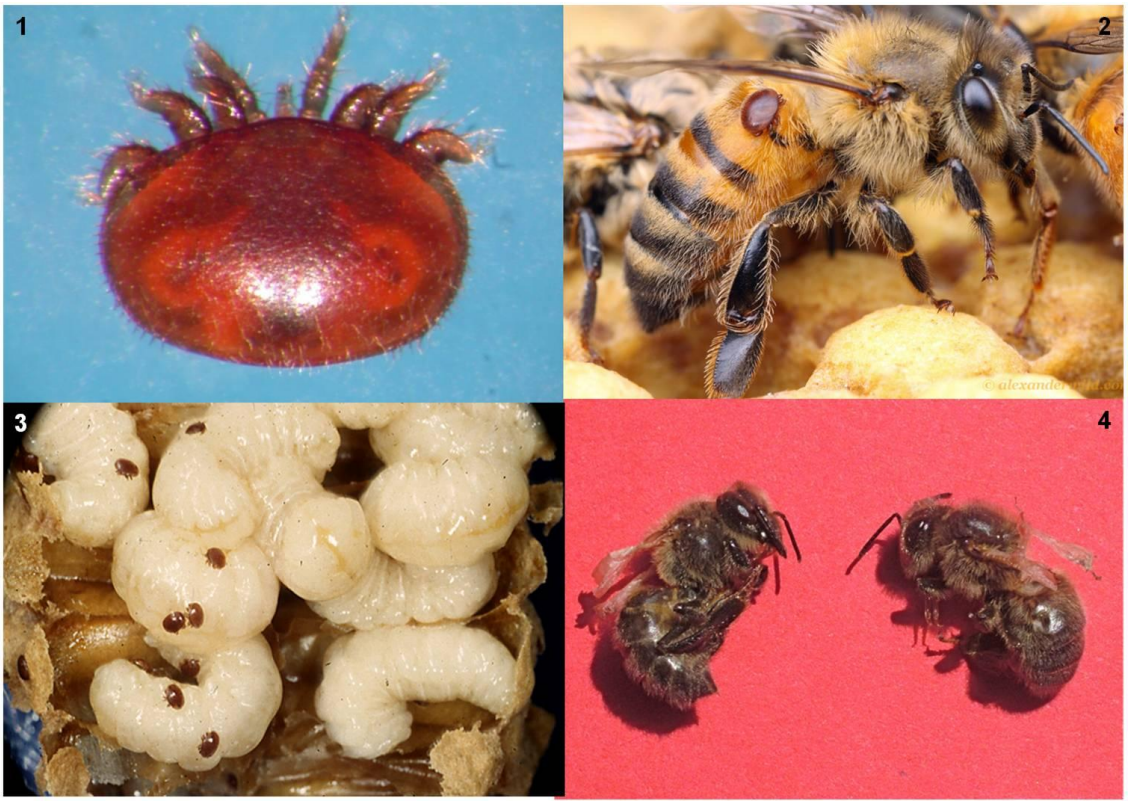

Fig.1.1. 1) adult female *Varroa destructor* mite (Photo, Hdoa.Hawai.gov), 2) phoretic adult female mite on the body of a worker bee (Photo A. Wild), 3) adult female varroa on drone larvae taken from recently sealed cells (Photo F. Ratnieks), 4) worker bees with shrivelled wings, a visible symptom of the disease deformed wing virus (DWV) (Photo F. Ratnieks).

Over 60 factors that may negatively affect honey bee colonies have been listed, not to mention combinations of factors (Anonymous, 2009; vanEngelsdorp et al, 2009; Ratnieks and Carreck, 2010; Sih et al, 2004). Some of these, such as the effects of mobile phones, would seem most unlikely. However, there are several factors which can certainly harm honey bee colonies: 1) reduction in food supply (flowers) (Brown and Paxton, 2009; Couvillon et al, 2014; Howard et al, 2003); 2) insecticides (Paradis et al,

2014; Carson, 1962; Pisa et al, 2014); 3) pests and diseases (Ratnieks and Carreck, 2010; Genersch et al, 2010; Francis et al, 2013; Dainat et al, 2012; Berthoud et al, 2010). This thesis concerns the third of these challenges.

A wide range of pests and pathogens, including insects, mites, protozoa, fungi, bacteria and viruses, can harm honey bee brood and adults (Evans and Schwarz, 2011) (Figure 1). These include a number of species, including the mite *Varroa destructor* and the microsporidian *Nosema ceranae*, that have recently colonized *A. mellifera* from the Asian hive bee *A. cerana*. In addition, many pests and diseases of *A. mellifera* that were previously confined to a part of its worldwide range have increased in range. For example, the small hive beetle, *Aethina tumida*, is a pest of *A. mellifera* and was until recently only found in sub-Saharan Africa. In the past decade it has colonized Europe, Australia, North America and now South America. Its discovery in South America is reported in this thesis (Chapter 14).

The research in this thesis focuses on two areas of honey bee pest and disease control relevant to apiculture: a) Hygienic behaviour (Chapters 9 to 12) in honey bees (and also, Chapter 13, in stingless bees); b) Other control methods (Chapters 4 to 8).

1.2.1 Hygienic behaviour and bee breeding

1.2.1.1 Honey bees

Social insects have many defence mechanisms against pests and pathogens. One of these is hygienic behaviour, which has been studied in detail in the honey bee, *Apis mellifera* (Spivak and Gilliam, 1998; Wilson-Rich et al, 2009; Park, 1937; Rothenbuhler, 1964a,b). In particular, hygienic behaviour is a social defence against diseases of brood (larvae and pupae) in sealed cells. In the honey bee, young larvae are fed in open cells, one per cell. When the larva is fully fed the adult worker bees seal the cell with a wax capping. The larva then metamorphoses into a pupa and then into an adult, which then leaves the cell. The capped brood period lasts for c. 12 days in worker honey bees (Winston, 1991; Gould and Gould, 1988).

There are many pests and diseases that affect brood in capped cells that can be reduced via hygienic behaviour. These include the fungal disease chalkbrood (Spivak and Reuter, 1998a,b), the bacterial disease American foulbrood (Spivak and Reuter, 1998a,b; Spivak and Gilliam, 1989a,b), deformed wing virus (Schöning et al, 2012; Al Toufailia et al, 2014, Chapter 9) and the mite *Varroa jacobsoni* (Wilson-Rich et al,

2009; Rinderer et al, 2010; Spivak and Reuter, 1998a,b; Al Toufailia et al, 2014, Chapter 9).

Fig.1.2. Adult workers honey bees carrying out hygienic behaviour. 1) in a bee hive (Photo G. Bigio); 2 & 3) In an observation hive.

Worker honey bees showing hygienic behaviour detect and uncap cells containing dead and infected brood, and remove the contents from the colony (Rothenbuhler, 1964a,b; Rinderer et al, 2010; Spivak, 1996) (Figure 2).

Fig.1.3. Freeze-killed brood bioassay to test for hygienic behaviour. 1) metal cylinder pushed into a patch of sealed brood, 2) pouring liquid nitrogen into the metal cylinder to kill the circular patch of brood inside. (Photos F. Ratnieks).

In this way they reduce the spread of the disease. Hygienic behaviour is not learned. Rather it is an instinctive heritable trait controlled by multiple genetic loci

(Jones and Rothenbuhler, 1964; Momot and Rothenbuhler, 1971; Rothenbuhler, 1964a,b; Wilson-Rich et al, 2009).

Not all honey bees are hygienic, but it is possible to breed hygienic bees by selecting queens and males from hygienic colonies detected by using the freeze-killed brood (FKB) removal bioassay (Spivak, 1996; Ibrahim et al, 2007; Perez-Sato et al, 2009) or other tests based on killing with cyanide (Jones and Rothenbuhler, 1964), insertion of a pin (Taber, 1982; Spivak and Reuter, 2001), or disease spores of (American foulbrood [Rothenbuhler, 1964b; Spivak and Reuter, 2001], chalkbrood [Gilliam et al, 1983]).

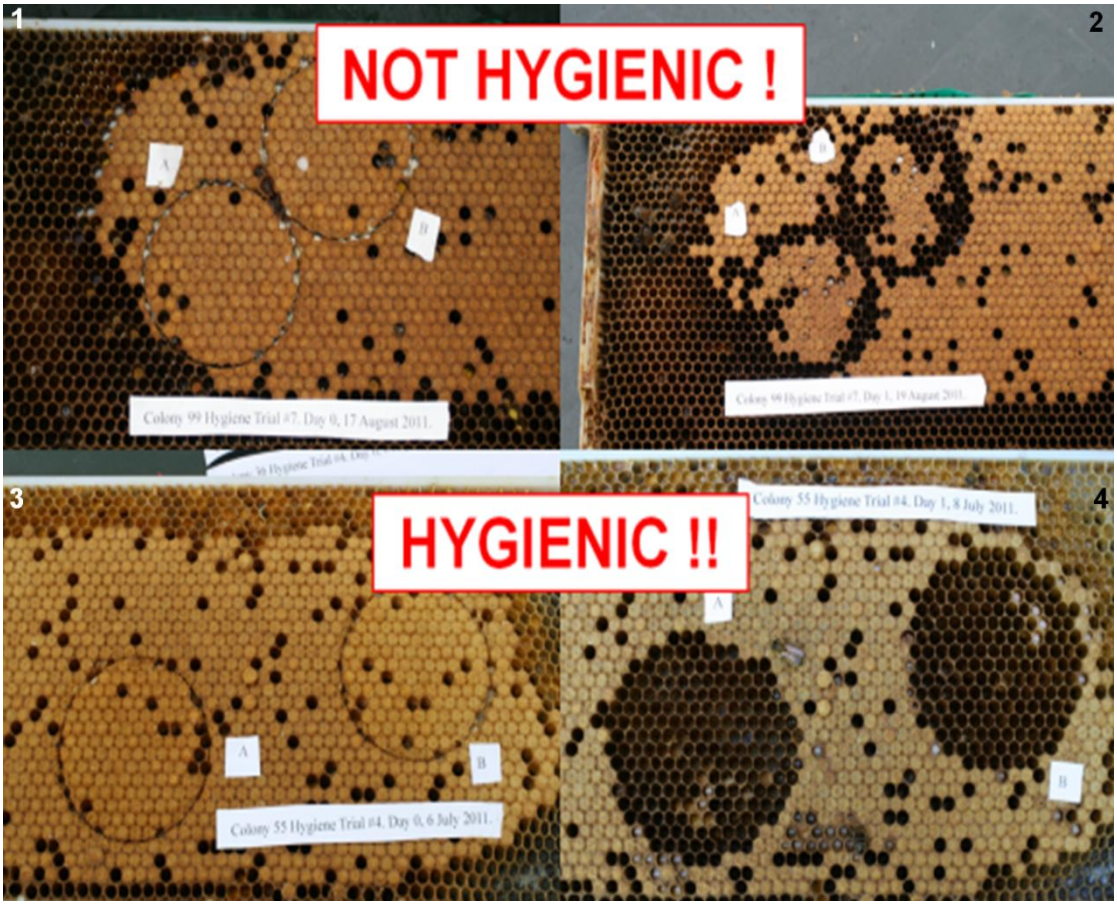

Fig.1.4. Freeze-killed brood bioassay in a hygienic colony and a non-hygienic colony. The left hand photographs show the frame after treatment with liquid nitrogen. Photos 1&3 photographs show the frame after 2 days inside a honey bee colony. It can be seen that the hygienic colony has cleaned out almost all the cells in the circular frozen area (Photo 4), whereas the non-hygienic colony has cleaned out only approximately half (Photo 3) (Photos G. Bigio).

Hygienic behaviour does not result in the excess removal of healthy brood (Bigio et al, 2014b) or reduced honey production (Spivak and Reuter 1998b). To quantify a colony's level of hygienic behaviour using the FKB-removal bioassay

(Figure 3), an area of capped cells is killed with liquid nitrogen (Spivak and Downey, 1998) and photographed. Two days later, the cells with dead brood are checked and photographed to determine the proportion of sealed cells that has been cleaned out (Figure 4).

Unselected populations of honey bees show wide variation among colonies in the level of hygienic behaviour (Perez-Sato et al, 2009). Only a small portion of British colonies in unselected populations normally show high levels of hygiene (1 colony out of 31, Pérez-Sato et al, 2009). By selective breeding it is possible to breed colonies that are fully hygienic, in which 95-100% of dead brood are removed within 2 days (Boecking et al, 2000; Harbo and Harris, 1999; Bigio et al, 2014a; Spivak and Reuter, 1998a,b).

1.2.1.2 Stingless bees

Compared to honey bees, little is known about the diseases of stingless bees. There are many books on honey bee pests and diseases (Morse and Flottum, 1997; Bailey and Ball, 2013). By comparison, the main book on beekeeping with stingless bees makes barely any mention of diseases (Nogueira-Neto, 1997). This could be because stingless bees have fewer pathogens and diseases, or are highly effective at controlling diseases so that symptoms are rarely seen, or it may simply reflect a lack of study.

Fig.1.5. Right: Patch of sealed cells in the stingless bees *Scaptotrigona depilis* that has been killed by freezing. Left: The same patch after 2 days in a colony, in which workers have cleaned out all the cells.

Chapter 13 studies hygienic behaviour in 3 species of stingless bees native to Brazil (Figure 5). The results show that stingless bees have high levels of hygienic behaviour (99% FKB-removal in *Melipona scutellaris*, 80% in *Scaptotrigona depilis*

and 62% in *Tetragonisca angustula* compared to unselected honey bee populations (46%; Perez-Sato et al, 2009).

High levels of hygienic behaviour in unselected populations have also been found in two species of stingless bees from Mexico, *Melipona beecheii and Scaptotrigona pectoralis* (Medina et al, 2009). In this study we also detected an as yet unidentified brood disease with symptoms similar to deformed wing virus in the honey bee (Figure 6), and showed that more hygienic colonies of stingless bees were detecting and removing diseased brood prior to adult emergence.

Fig.1.6. Workers of stingless bees *Scaptotrigona depilis* recently emerged from their brood cells. 1) healthy worker with the normal wings, 2) unhealthy worker with shrivelled wings.

1.2.2 Honey bee diseases and control methods

1.2.2.1 Varroa mite

Varroa destructor is now found worldwide with Australia the only remaining varroa-free continent, although it is now found in nearby New Zealand. Adult female varroa are found either in sealed brood cells, where they lay their eggs and where the young mites develop to adulthood and mate, or phoretic on the bodies of adult bees. Only adult females are present outside sealed brood cells.

Varroa can harm *A. mellifera* colonies both directly, by damaging individual worker pupae so that the resulting adult's lifespan and body weight are reduced (van Dooremalen et al, 2012), and indirectly by transmitting virus diseases (Ball and Allen,

1988; Guzmán-Novoa et al, 2010; Boecking and Genersch, 2008; Highfield et al, 2009). Varroa is an important vector for deformed wing virus (DWV), a serious disease than can kill individual bees and colonies. The combined effect of the mite and the virus is considered a major reason for honey bee colony losses in North America and Europe (Nazzi et al, 2012; Dainat et al, 2012; Genersch et al, 2010).

Beekeepers use a wide range of methods to control varroa. These fall into three main categories: a) chemical control, b) mechanical and biotechnical control, c) natural resistance (e.g., hygienic behaviour).

Chemical control using synthetic acaricides has been widely used to control varroa. Several synthetic pesticides that are highly toxic to varroa and less toxic to honey bees have been formulated into commercial products including the organophosphorous chemical coumaphos (Checkmite ®), the pyrethroids tau-fluvalinite (Apistan ®) and flumethrin (Bayvarol ®), and the formamidine (Amitraz) (Santiago et al, 2000). These chemicals are easy to apply in the form of plastic strips impregnated with the active ingredient that are placed in the brood chamber for several weeks or months so that even mites in brood cells are eventually contacted.

Unfortunately, but perhaps inevitably, resistance has developed to these chemicals resulting in reduced efficacy (Floris et al, 2001; Lodesani et al, 2009; Milani, 1995,1999). It seems unlikely that new synthetic chemicals will be developed to control varroa (Dekeyser, 2005) or that they can provide a long term control solution.

A wide range of natural organic chemicals have been used to control varroa, including oxalic acid (Figure 7), lactic acid, formic acid and thymol. Organic acids can cause significant mite mortality if applied in the right way at the appropriate time in relation to brood rearing or outside temperature. For example, oxalic acid is effective when applied to broodless colonies in which all the varroa are phoretic (Chapter 5).

Formic acid is highly volatile and acts as a fumigant and is the only organic acid which kills varroa inside sealed brood cells (Fries, 1991). Much research has been done on the efficacy of these natural chemicals in killing varroa, including studies investigating different doses, times of treatment, and methods of application (Nanetti et al, 2006; Rademacher and Harz, 2006, Chapter 5). One major advantage of these natural compounds is that resistance seems less likely to develop (Nanetti et al, 2006). In addition, they also occur naturally in honey (Bogdanov et al, 1999; Rademacher and Harz, 2006) and are low cost (Chapter 6, Al Toufailia et al, 2016; Imdorf et al 1999).

Fig.1.7. Testing the three methods used by beekeepers to treat honey bee colonies with oxalic acid against varroa. Top left and right: "dribbling or trickling" and "spraying" with oxalic acid solution. Bottom left and right: "sublimation or vaporisation" of oxalic acid dihydrate crystals with an electrically-heated applicator that is inserted into the hive entrance.

There are also a variety of biotechnical method methods for controlling varroa, such as "trapping" in drone cells (Charrière et al, 2003). Adult female varroa have a strong preference for entering drone cells versus worker cells (Grobov, 1977; Fuchs, 1990), probably because they can produce more offspring in a cell with a drone pupa (Ifantidis, 1984). In their native host, *A. cerana*, they are only able to reproduce successfully in drone brood, as the worker pupa develops too rapidly for the varroa offspring to reach adulthood before the adult bee emerges from its cell (Boot et al, 1999; Koeniger et al, 1983).

A female mite can produce approximately three daughers in an *A. mellifera* drone cell versus one in a worker cell (Fuchs and Langenbach, 1989; Martin, 1998). A frame of drone cells can trap 30-70% (Calis et al, 1999; Charrière et al, 2003) of the varroa. Chapter 7 shows that providing colonies with drone foundation in early spring traps 46% of the varroa per treatment (Figure 8).

Fig.1.8. 1) Frame with both worker cell and drone cell foundation as used to introduce into honey bee colonies in early spring to trap varroa in drone cells. 2) the same frame approximately 4 weeks after introduction into a colony and showing many sealed drone cells within which, including the other side of the comb, there are an average of 46% of all the adult female varroa mites in the colony.

In the case of varroa, hygienic bees uncap and clean out cells containing a female mother mite infected with deformed wing virus (DWV) and has transmitted the virus to the honey bee pupa (Schöning et al, 2012). Although this does not kill the mite, it prevents it from reproducing successfully. Cells with uninfected mites were not uncapped. Hygienic bees can reduce varroa population growth and or numbers (Peng et al, 1987; Ibrahim and Spivak, 2006; Al Toufailia et al, 2014, Chapter 9).

1.2.2.2 Deformed wing virus (DWV)

Deformed wing virus (DWV) is one of over 24 viruses known to infect honey bees (Bailey and Ball, 1991; Evans and Schwarz, 2011; McMenamin and Genersch, 2015; Ribière et al, 2008). DWV virus cannot be seen or quantified microscopically but can be identified and quantified using molecular markers (Al Toufailia et al, 2014; Francis et al, 2013, Martin et al, 2012). One symptom of DWV, workers with shrivelled wings (after which the virus takes its name), can be seen by eye during a normal hive inspection (Figure 9).

DWV has been detected in honey bees of all life stages from egg to adult (Yue and Genersch, 2005; Tentcheva et al, 2006; de Miranda and Genersch, 2010). The first detection of this virus was in 1982 in Japan in honey bee workers that did not show visible symptoms (Bailey and Ball, 1991). DWV has become a serious honey bee disease in Europe and North America since the arrival of varroa.

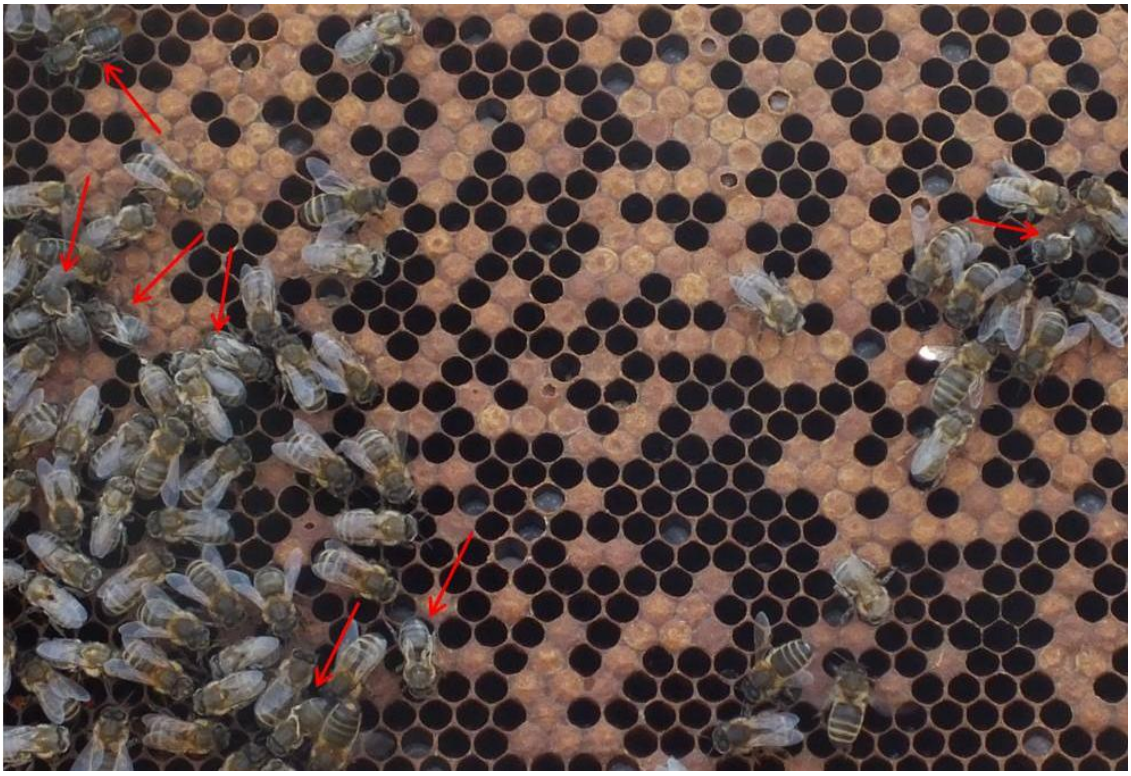

Fig.1.9. Workers honey bees with shrivelled wings (arrows), as seen in a hive inspection in which frames are briefly removed from the hive and checked by eye.

It is not fully understood how DWV transmission occurs. This virus can persist and multiply in all honey bee stages without showing symptoms except the case when the virus is transmitted by varroa mite. The virus is directly linked with the high level of varroa infestation (Bowen-Walker et al, 1999; Martin et al, 2010).The virus mainly replicates in the mite which will feed on the haemolymph of pupae and by doing this the virus will pass into the pupa resulting in shrivelled wings in some emerged adult bees (de Miranda and Genersch, 2010).

This symptom is considered a predictor of colony death (Dainat and Neumann, 2013) and is also something that a beekeeper can easily see in a normal hive inspection. DWV is a harmful disease as it shortens worker life span (Dainat et al, 2012; Francis et al, 2013) and kills colonies due to the collapse of the worker population (Berthoud et al, 2010) including winter collapse (Berthoud et al, 2010; Highfield et al, 2009).

Honey bee viruses such as DWV cannot be controlled directly, for example with an anti-viral chemical. There are, however, indirect control methods that interfere with DWV transmission. First, is by controlling the varroa vector (Bowen-Walker et al, 1999; Santillan-Galicia et al, 2010; Martin et al, 2010). Second, hygienic behaviour

targets infected pupae DWV (Schöning et al, 2012) and reduces levels of DWV in hygienic colonies (Al Toufailia et al, 2014 Chapter 9).

1.2.2.3 Small hive beetle (SHB)

The small hive beetle, *Aethina tumida,* is a pest of the honey bee *Apis mellifera* and is native to sub-Saharan Africa. It mainly causes harm to weak colonies and can also damage combs in storage or in dead hives. The adult beetle is dark brown to black, 5 x 3 mm (Figure 10). Beetles live and lay eggs within honey bee colonies. Most of the damage is caused by the larvae which feed on honey, pollen and brood (Hood, 2004; Lundie, 1940; Schemolke, 1974).

Fig.1.10. Female adult small hive beetles, *Aethina tumida* . 1) ventral view, 2) dorsal view. Photo taken from samples collected in Piracicaba, Brazil, during the discovery of this pest in South America.

The mature larvae leave the honey bee colony to pupate in the soil. The life cycle from egg to adult takes 4-16 weeks (Neumann et al, 2013). The adult beetle can fly and live and multiply outside the bee hive on rotting fruit. As such, the beetle can easily be transported to new locations via human activity.

The best way to protect colonies from harm is to keep strong colonies. Chemical control is also possible in the hive (Elzen et al, 1999b) or in the soil in front hives where pupae occur (Baxter et al, 1999). African honey bees have behavioural mechanisms to control small hive beetles, such as by entombing the adults in propolis (Neumann et al, 2001).

1.2.2.4 Chalkbrood disease

Two of the chapters in this thesis study the interactions between hygienic behaviour and chalkbrood. Chalkbrood, causative agent *Ascosphaera apis,* is the most

common fungal disease of honey bees and probably the most common of all brood diseases. It normally kills the larva after cell capping (Figure 11).

Fig.1.11. Capped cells in which the larva has been killed by chalkbrood, and in which worker bees have removed the capping to show the dead larva or "mummy" inside. The colour of the mummy ranges from white to black.

The dead larva fills the cell and turns into a solid mass of fungal mycelium called a mummy, which is either white or black (when spores are being produced) in colour (Gilliam et al, 1978). Spores are the cause of larval infection. A larva that is infected when one day old usually dies within 48 hours versus 72 hours in larvae older than 3 days (Aronstein et al 2010). Many larvae die after cell capping. Spores are transmitted via larval food or direct contact with the larval body surface (Spiltoir, 1955). There is no known successful chemical control against chalkbrood. Hygienic behaviour is the only known effective control method for this disease, especially for white mummies (Gilliam et al, 1983; Spivak and Reuter, 1998a).

Chapter 2

General Methodology

This chapter outlines some of the general research methods that I used in my thesis. More specific methods information is given in each of the experimental chapters (Chapters 4 to 14).

2.1 Study organism

The honey bee (*Apis mellifera*) is the study species in ten of the ten experimental chapters. *A. mellifera* lives in colonies with up to 60,000 adult workers, one queen (the mother), up to several thousand adult males (drones), and up to tens of thousands of immature bees known as brood (eggs, larva and pupae).

2.2 Beekeeping

The research used standard beekeeping methods and hives. The honey bee colonies used during my research were kept in three different types of movable frame hives: langstroth, commercial and national. Each hive had one or more boxes of frames, depending on the experiment and the number of bees. Additional boxes were added or removed as needed for honey storage and population changes. Normally, the queen is confined to the lower one or two boxes with a queen excluder. In addition, the research also used queen mating nucleus hives (small hives used in the queen rearing process) to produce some of the mated queens used in experiments on hygienic behaviour.

Beekeeping was the most important experimental method in my PhD (Figure 1). Extensive beekeeping was needed to prepare and manage the colonies used in the various experiments. Sometimes more than one hundred colonies were managed in order to obtain the number needed for a particular experiment (e.g., Chapters 5, 8, and 10).

Generally, in all experiments the colonies were managed in a similar way with certain adjustments or additions depending on the experiment's requirements. Always we ensured that the queen was marked with a paint dot on the thorax and with the wings clipped. This was very important in some experiments (Chapters 10, 11 and 12) to ensure that we were following the same queen in each colony.

During the research it was also necessary to carry out procedures that would not normally be carried out simply in beekeeping. For example, in some experiments on the control of varroa mites (Chapter 4, 5 and 6), we had to remove small patches of brood in preparation for oxalic acid or Apistan treatment in winter. To do this, we had to open the colony for a short time on a warm winter day to check for brood in the middle of the cluster. If brood was present, we moved the worker bees out of the way by touching them gently and then scraped away any brood using a honey fork or hive tool.

Fig.2.1. Some general beekeeping activities. 1) the author inspecting a colony of honey bee and LASI pick-up truck behind. 2) Luciano Scandian is training the author to do beekeeping at the start of the PhD.

To control swarming, and so avoid losing the mother queen with the majority of the workers, we monitored and managed our colonies to prevent swarming. In the case of hygienic colonies that were being used as breeder colonies from which to rear daughter queens, we kept colony populations low to prevent swarming. To do this we located the queen and moved her into a new hive with some of the bees and brood from her colony (c. 2500-3000 worker bees, 1-2 frames of brood) plus food stores. Then the new colony was moved to a different location away from the original apiary to prevent the worker bees going back to the original hive.

2.3 Obtaining hygienic queens and queen rearing

Honey bees naturally raise queens under three conditions: emergency (when the colony's queen dies or is lost); supersedure (when the queen is getting old or infirm);

swarming (colony reproduction). A queen starts her life as a fertilized, female egg. If the resulting larva is in a queen cell, it will be fed royal royal jelly which triggers the larva to develop into a queen instead of a worker. It takes 16 days to rear a queen from egg to adult, and one week (weather permitting) for the queen to make her mating flight or flights and start laying eggs. For more than one hundred years beekeepers have known how to rear queens by transferring young female larvae into artificial queen cells, and placing these cells into a queenless colony to simulate emergency queen rearing conditions.

Fig.2.2. Queen rearing. 1) Larve in worker cells of the correct size for transferring into queen cells; 2) Larva transferred into a queen cell on top of drop of water; 3) Sealed queen cells; 4) small mating nuc (apidea) to introduce the virgin queen in it with few hundred workers. (Photos F. Ratnieks).

In my PhD research I reared many queens, especially hygienic queens, from suitable breeder colonies. To obtain hygienic queens I would rear daughter queens from LASI's tested hygienic colonies. After the queens were reared and were in colonies composed of their own offspring, I would test the colony for hygienic behaviour using the freeze-killed brood (FKB) bioassay (Perez-Sato et al, 2009; Spivak and Reuter, 1998a,b; Spivak and Downey, 1998; Bigio et al, 2014a). To carry out an FKB test on a

colony, a test frame with a large area of sealed brood was removed. Two metal cylinders (6.5 cm diameter × 8 cm height) were pressed into the sealed brood and *c.*300 ml of liquid nitrogen poured into each cylinder to kill the circle of brood inside. A few minutes later the liquid nitrogen had evaporated and we removed the cylinders, took a photograph and returned the frame to the hive. A second photograph was taken again after 48 ± 2 h.

Each colony was tested for FKB-removal at least four times at 7-10 day intervals. From our photographs we were able to determine the proportion of FKB capped brood removed by the hygienic workers. Colonies with more than 95% FKB removal are considered to be fully hygienic. However, in some of the experiments we also needed colonies that were not hygienic, and in some experiments (Chapter 10) we used hygienic colonies with 100% FKB removal.

We used standard queen rearing methods to raise our queens (Laidlaw and Page, 1997) using a Swiss-made grafting tool to transfer young larvae from worker cells to queen cells. Ten days after grafting the ripe queen cells introduced singly into Apidea mating nucleus hives, each containing 700-900 worker bees (Figure 2). A few weeks later, mated and laying queens were taken from the mating hives and introduced using queen cages into larger hives. After 6 weeks, the workers in the colony were the progeny of the new queen and the hive could be tested for FKB removal to determine its level of hygienic behaviour.

In chapter 13, we studied hygienic behaviour in 3 species of stingless bees native to Brazil (*Melipona scutellaris*, *Scaptotrigona depilis* and *Tetragonisca angustula*). To quantify a colony's level of hygienic behaviour we used an FKB-removal bioassay similar to that used in the honey bee (Spivak and Downey 1998), except that instead of using liquid nitrogen we froze brood combs in a refrigerator.

2.4 Oxalic acid control of varroa

In the first experiment on the control of varroa using oxalic acid, Chapter 5, we compared three oxalic acid application methods already being used by beekeepers (trickling, spraying and sublimation).

In the sublimation method, we simply purchased a commercially available applicator used in beekeeping (Varrox M3080) from E H Thorne (Beehives Ltd.) of Wragby, a well known UK supplier of beekeeping equipment. We also bought two large 12 volt lead-acid batteries, of the type used in caravans, from Halfords.

The most challenging aspect in Chapter 5 was having 110 hives available, all without brood. These hives were kept in 10 apiaries, 10 treatment hives and one control hive each. As in much of my PhD research, I was helped by the LASI beekeeping technician Luciano Scandian, who also drove us from apiary to apiary in the LASI's Mitsubishi pick up truck, another vital piece of equipment. The hives also needed to have removable floors and dead bee traps at the entrance so that we could count dead mites and bees.

I also had to learn how to use the various application methods. In the sublimation method, we had to close the hive entrances and the back of the hive with plastic foam to prevent any vapour from escaping, and wear approved respiratory masks (ChapSmith R300 Series, with a filter for organic gases).

2.5 Quantifying varroa in a colony

To estimate varroa infestation levels we used several methods, as appropriate. When we only needed to determine the change in mite numbers over a few weeks in broodless colonies (Chapters 5, 6 and 9) we extracted mites from samples of c. 300 worker bees using a jet of water (Dietemann et al, 2013; Al Toufailia et al, 2014; Al Toufailia et al, 2016).

Fig.2.3. Extracting varroa mites from adult worker bees to determine number per 100 worker bees. 1) Collecting a sample of worker bees from bees shaken from brood combs of one colony into a plastic bucket; 2) Washing mites from the bee sample using a jet of water and a double mesh honey strainer; 3) adult female varroa are collected in the second, fine, mesh and counted; 4) counting the number of bees in the sample.

In this way we could determine, for example, the number of mites per 100 bees before and after an application of oxalic acid (De Jong et al, 1982; Fries et al, 1991) (Figure 3).

When we needed to determine the change in the mite population over one year in a colony (i.e., from one winter to the next, and when the colonies were broodless) we also had to factor in any change in the colony population. For example, if the number of mites per 100 bees had doubled over one year and the colony population (number of worker bees) had also doubled and then the mite population had increased four times.

We measured worker and brood population using a standard method called Liebefeld estimation method (Imdorf and Gerig, 2001) sometimes during a hive inspection and sometimes from photographs of each frame (Chapters 7 and 8; Figure 4). In some cases we counted the number of frames occupied by brood or bees using the "subjective mode" method (Delaplane et al, 2013) (Chapters 5, 6, 9 and 10).

Fig.2.4. Estimating colony strength in bees and brood.1) Using the observation method of Delaplane et al (2013). The frame in the photo is used as a standard of one side of a frame of covered in bees; 2) a frame with wires to monitor colony strength using the method of Imdorf and Gerig (2001).

In some experiments we also had to determine the varroa population in a colony with brood, or to estimate the proportion of varroa that were phoretic on adult bees. To do this we also had to estimate the number of varroa in sealed brood cells. In one experiment we did this in autumn when there were sealed brood cells but no sealed drone cells (chapter 8). In another experiment carried out in spring there were also sealed drone cells which were sampled separately as they are more attractive to varroa (Fuchs, 1990; Chapter 7).

Fig.2.5. Procedure for extracting varroa mites from sealed worker and drone brood cells. 1) cutting a piece of sealed brood from a frame for freezing and later varroa counting; , 2) A frame with distinct areas of worker and drone brood; 3) Inspecting brood cells for varroa, removing a drone pupa; 4) Inspecting brood cells for varroa using a cotton swab to remove any varroa in the cell after the larva or pupa has been removed from the cell.

To quantify the number of adult female varroa is brood cells; we took a sample of sealed brood from the hive. The cells were uncapped and forceps were used to remove the larva or pupa (Figure 5). The number of adult mother varroa mites in each cell was then counted. In this way we calculated the number of adult female mites per 100 sealed brood cells, and from the number of all sealed worker or drone cells.

Chapter 3

How the thesis evolved

After graduation from the University of Damascus in Syria with a BSc degree (1st class) in Agricultural Engineering, I was given a scholarship to carry out a full-time graduate degree course in the UK on my favourite subject: honey bees. However, I was not sure how to do this. I then started looking for a suitable UK university. After the first click of a Google search about honey bee research in the UK, it became evident that the most active laboratory was that of Professor Francis Ratnieks who had recently moved to the University of Sussex. I was also attracted to Sussex because of its good reputation in research in general. Professor Ratnieks accepted me. I came to the University of Sussex in August 2010 to do an English course and then joined the Laboratory of Apiculture and Social Insects in October 2010.

I wanted to obtain broad training in honey bee biology, both basic and applied. I started out by doing a research Master's degree on honey bee foraging and dancing behaviour, with the title "Honey bee foraging: persistence to non-rewarding feeding locations and waggle dance communication". The Masters degree worked out well and resulted in two research publications, one in the journal *Behavioural Ecology and Sociobiology* and the other in *Ethology*. As I was doing my Masters degree I was thinking of what to study in my Doctoral degree. After learning about the challenges faced by honey bees, I thought that the control of honey bee diseases would be a good topic.

In one of my meetings with my supervisor, he mentioned that one part of Sussex Plan for Honey Bee Health and Well Being, the name given to the applied research being done in the Laboratory of Apiculture and Social Insects, was the control of honey bees diseases. After discussions with him about ideas and possibilities of research in this area, he agreed and asked me to apply to Sussex University for a PhD focused on methods for controlling varroa mites and also to build on the research on hygienic behaviour that was already underway at LASI and which was the main PhD topic of another LASI student, Gainluigi Bigio. On submitting my Master's thesis in April 2012, I registered as a PhD student at Sussex University. Even before my Masters was defended, I started to work with the LASI beekeeping technician Mr Luciano Scandian and to help Gianluigi in one of his projects by helping him quantify the levels of

hygienic behaviour in hives using the freeze-killed brood bioassay. I learnt how to manage honey bee colonies and to study hygienic behaviour, often working closely with Luciano in the LASI apiaries. The various research projects were carried out as follows.

Chapter 4. The data collection for this project was carried out between August 2014 and January 2015. Francis suggested that it would be a good idea to test the efficacy of Apistan to determine if it was still effective at controlling varroa. It was a simple project but provided useful data. In this project I received help in practical beekeeping and transport between apiaries from LASI technician Mr Luciano Scandian.

Chapter 5. This project was carried out between December 2012 and January 2013 and was actually the first research project in my PhD. The first plans for this first project arose in July 2012 when I had a meeting with Francis. He asked me to look at the literature to determine what had been done, and more importantly, what had not been done in the area of controlling varroa using oxalic acid. The initial plan was to use 30-40 colonies to test the sublimation method using different doses. However, after reading and further discussions, Francis and I decided to run an experiment to compare all three application methods being used by beekeepers (sublimation, spraying, trickling) each at several doses. I ended up managing a large experiment using 110 colonies in 10 apiaries.

I also decided to quantify the proportion of varroa killed by taking samples from worker bees before and after treatment, not just to count the number of dead varroa falling down to the hive floor. I ran a preliminary experiment to test my methods for determining the proportion of varroa that were phoretic on worker bees and bee mortality using a trap net (dead bee trap) at the hive entrance. I also ran a second trial in the next year to confirm the most promising method, sublimation of 2.25g oxalic acid shown in year 1. An interesting aspect of this experiment was that all the field work was done in the winter, a time at which no field work on honey bees is usually carried out. I did the project in the winter as we needed to work with broodless hives. During this project I worked closely with LASI technician Mr. Luciano Scandian and received help in practical beekeeping and driving between apiaries.

Chapter 6. This project was carried out between December 2014 and January 2015. It was also a follow up project to Chapter 5. It showed the efficacy of two treatments of 2.25 g oxalic acid via sublimation at an interval of 2 weeks in broodless honey bee

colonies in winter. The idea of this project was raised by one of the beekeepers who attended a summer workshop on varroa control in LASI in 2014. Francis and I discussed this possible project later on and developed our methodology. I had to run a preliminary experiment to test the short term effect on bees as this was one of my worries. In this project I worked and received help from LASI technician Mr. Luciano Scandian in practical beekeeping and driving between apiaries.

Chapter 7. This project was carried out between April 2013 and June 2013. This project was already in the Sussex Plan, and was also a logical follow up project to Chapter 5 as it would provide varroa control by a different method and a different time of year and, if successful, could be combined with varroa control using oxalic acid or other methods. The idea was to test the efficacy of trapping varroa in drone brood in early spring when hives were small and it would be simple to do. In addition, no previous research on trapping varroa in drone brood had been done before in early spring, when the honey bee colonies are rearing their first batch of males. I received help from LASI technician Mr. Luciano Scandian in beekeeping and driving between apiaries.

Chapter 8. This project was carried out from September 2012 to January 2016. This project came also linked in to Chapter 5 and varroa control using oxalic acid. I already knew from published research that oxalic acid is only effective at killing varroa phoretic on adult bees, not varroa in sealed brood cells, which meant that it should be used in broodless hives to ensure maximum efficacy.

After looking at the literature we found little information on seasonal brood rearing. This led to a project monitoring brood rearing in honey bee colonies from autumn to spring, September to March. I also wanted to determine the degree to which small patches of brood would reduce the mortality of varroa mites when a colony was treated with oxalic acid, and this led to the second part of this chapter. Mr. Luciano Scandian helped me run this project over three years of monitoring bee hives, and driving between apiaries.

I also wanted to work with beekeepers in different parts of the UK to monitor brood. I contacted more than 20 beekeeping associations and 20 individual beekeepers, but none of them would risk opening their hives in winter to make the necessary inspections. It was a shame that the beekeepers were not more cooperative as it would

have enabled use to gather a lot more data and to compare autumn and winter brood rearing in different areas of the UK. As it turned out, our winter inspections of hives caused no increase in colony mortality.

Chapter 9. This project was carried out from January 2013 to December 2013. By January 2013, we had finished the oxalic acid experiment and after reading the literature, especially the paper by Schöning et al (2012), I suggested to Francis that I might do a project to test the effect of hygienic behaviour on varroa populations. As always, I came to Francis's office with one idea and left with a better one. I had to use over 70 colonies in this project, and carry out a massive amount of beekeeping to test the colonies for hygienic behaviour, to manage them, to control swarming, to check the queens, and so on.

I received help from Luciano Scandian in practical beekeeping. All the hard work was forgotten when I got some amazing results to show that hygienic behaviour not only helps control varroa, but also reduces levels of deformed wing virus (DWV) by 10,000 fold. In this project I also collaborated with colleagues, Per Kryger and Esmaeil Amiri from the University of Aarhus in Denmark, who quantified levels of DWV in samples of workers bees and varroa mites that I had collected during the field work.

Chapter 10. This project was carried out from May 2014 to April 2016 and followed on from Chapter 9 by determining the effect of hygienic behaviour on the survival of colonies with symptoms of (DWV). I had the idea of testing the effect of hygienic behaviour on colony survival using only hygienic colonies and keeping them without treatment for a longer time. Again, after discussion with Francis and his great ideas the project became more interesting as what he suggested resulted in data that much more clearly showed the benefit of hygienic behaviour in terms of colony survival.

This project was challenging and entailed much hard work in two ways. First I had to obtain 15 colonies with DWV symptoms to start the experiment. Second I had the challenge of obtaining and maintaining queens of the right type, hygienic or non-hygienic, in the study hives over the nearly two years of the experiment. In total, I carried out over three hundred freeze-killed brood tests. I received help testing large numbers of colonies for hygienic behaviour using the freeze-killed brood bioassay from Luciano Scandian, from Sussex colleague Dr. Steve Pearce, and from two summer volunteers from France, Ludovic Dubuisson and Valentin Duflo. As in all of the

experiemnets with honey bee colonies carried out at LASI, LASI technician Mr. Luciano Scandian helped with practical beekeeping and driving between apiaries.

Chapter 11. This project was carried out from July 2013 to September 2013. As the bacterial pathogens (EFB, AFB) are notifiable diseases in Britain, scientists are not allowed to study them without special permission, which is hard to obtain. Therefore, I studied fungal pathogens including the pathogen, *Ascosphaera apis*, which causes chalk brood. In this project I received some help in microbiological methods from Dr. Sophie Evison, who trained me to make the fungal suspensions, and Sussex colleague Professor Bill Hughes, who allowed me to use his lab facilities and helped in planning the project.

Chapter 12. I carried out this project between August 2014 to September 2014. It followed on from chapter 11. I tested whether hygienic behaviour was similar when faced with fungal pathogens with different levels of virulence. As in Chapter 11, I received training from Dr. Sophie Evison, who trained me to make the fungal suspensions, advice and facilities from Professor Bill Hughes, who also helped in designing the project. In addition, Dr. Annette Jensen of the University of Copenhagen, Denmark, who provided me with some pathogen cultures.

Chapter 13. I carried out this project during my visit with Francis to Brazil in 2015 at a Brazilian University, ESALQ, located in Piracicaba, São Paulo State. The plan was to test three species of Brazilian stingless bees for hygienic behaviour using both freeze-killed brood and a natural pest, the larvae of phorid flies.

After I started testing 8 colonies of each of the 3 different species of stingless bees, Francis and I saw lots of dead bees underneath one colony of *Scaptotrigona depilis.* I took a sample inside the lab and examined them under the microscope and saw symptoms similar to deformed wing virus in honey bees. As a result of this discovery, I was also able to test the *S. depilis* colonies against this, as yet unidentified, brood disease. During this project I worked closely with Brazilian bee biologist and colleague Dr. Denise Alves, who trained me to inspect and manipulate stingless bees colonies.

Chapter 14. During the same visit to Brazil in 2015, I was helping fellow LASI students and temporary roommates Kyle Shackleton and Nick Balfour on a project studying honey bee foraging on fallen fruit. This also required me to check some honey

bee colonies in a nearby apiary. While I was checking I noticed that insects looking like the small hive beetle were running over the frames and on the hive bottom boards. I collected a few and examined them under a microscope. After a discussion with my supervisor and looking at the samples under the microscope I was almost sure that I had found the small hive beetle, *Aethina tumida*. Francis and I took photos and sent them to specialists for identification, and also sent samples to a specialist working in Brazil. This project involved working with a large number of colleagues in both Brazil and the USA who helped in identification and in registering the disease with the Brazilian Government.

PART 1: VARROA CONTROL

Chapter 4

How effective is Apistan at killing varroa in honey bees colonies?

4.1 Abstract

Varroa mites are a serious problem for honey bee colony health. They can kill colonies on their own. More importantly, they exacerbate viral diseases. Over the past two decades varroa has become harder to control because of resistance to fluvalinate, the active ingredient in Apistan, which had previously been highly effective. This study determined the efficacy of Apistan ® (a registered trade mark of Vita (Europe) Ltd), two strips per hive, 35 days, at killing varroa mites phoretic on adult worker bees in broodless hives in winter in Sussex, UK. Two groups, each of 20 hives, were studied. In one group, Apistan had not been used for five years. In the other, one treatment of Apistan had been made four months previously. Varroa mortality was determined by extracting mites from samples of c. 300 worker bees per hive, collected both before and after treatment, using a water jet. Our results show that the proportions of varroa killed were 58% and 33%, with the 33% kill being significantly lower than the 58% kill ($P<0.001$). The results show that Apistan is not very effective at killing varroa, presumably because of resistance. They also show that a single Apistan treatment resulted in the next treatment being significantly less effective, indicating strong selection for resistance.

4.2 Introduction

The varroa mite, *Varroa destructor*, is native to Asia where it is a pest of the honey bee *Apis cerana* (Oudemans, 1904). Through human intervention, varroa has been allowed to colonise *Apis mellifera* (Anderson and Trueman, 2000; Rosenkranz et al, 2010) and is now spread worldwide, except Australia (Rosenkranz et al, 2010). Varroa was first detected in Britain, in Devon, in 1992.

One method that beekeepers used to control varroa successfully is the use of synthetic acaricide chemicals.These include the organophosphate coumaphos (Checkmite®), the pyrethroids tau-fluvalinite (Apistan ®) and Flumethrin (Bayvarol®), and the formamidine amitraz (Alonso et al, 1990; Fries et al, 1991; Milani and

Lob,1998; Santiago et al, 2000). Several of these are commercially-available as easy to use plastic strips impregnated with the active ingredient. For example, the recommended Apistan treatment is to place 2 strips in each hive for six weeks, positioned in a gap between frames. Over this period all the varroa are contacted, including those temporarily in brood cells.

Fluvalinate, the active ingredient in Apistan, is highly toxic to varroa but has low toxicity to honey bees and humans. However, when pests are controlled with a specific synthetic chemical, resistance usually develops. Resistance to fluvalinate is now widespread and has been known for over 15 years (Elzen et al, 2000; Floris et al, 2001; Milani, 1994; Miozes-Koch et al, 2000).

Fluvalinate-resistant varroa were first reported in 1992 in Italy (Lodesani et al, 1995) and in 2001 in Britain (Thompson et al, 2002). When varroa are not resistant to fluvalinate, hive treatment with Apistan can kill almost 100% (Borneck and Merle, 1990; Ferrer-Dufol et al, 1991). However, when varroa are resistant, the kill is only approximately 30% (Faucon et al, 1995). Laboratory studies have shown that resistant varroa can withstand 10–20 times more fluvalinate (Thompson et al, 2002). The occurrence of resistant varroa can lead to increased colony mortality (Milani, 1999) because varroa can spread virus diseases that kill colonies (Dainat et al, 2012; Francis et al, 2013).

The aim of this study was to determine the efficacy of Apistan in the hives in our laboratory in Sussex, UK. We had previously used Apistan but for the past 5 years had not. We wondered, therefore, if resistance to Apistan might have reduced. Our results show that Apistan was not effective at killing varroa, indicating strong resistance. In addition, we found that a single Apistan treatment resulted in the next treatment being significantly less effective (58 v 33%), indicating strong and rapid selection for resistance.

4.3 Materials and Methods

4.3.1 Study colonies and data collection

We studied 40 colonies located in two apiaries, one at Sussex University campus (hives not treated with Apistan in the summer) and the second 3km to the east at Ashcombe Farm (hives treated with Apistan in the summer). The two apiaries were under very similar conditions in the South Downs area. Each colony had a marked queen and was housed in a hive consisting of two medium depth Langstroth hive bodies (20 frames in

total, volume 58.9 litres), bottom board, inner cover and telescopic cover. All 40 colonies had substantial numbers of varroa as they had not been treated against varroa, using oxalic acid (Al Toufailia et al, 2016) since 16 December 2013. We also observed a high level of varroa fall onto the tray above the bottom board.

On 12 August 2014, 20 colonies were treated with Apistan, 2 strips per hive in the brood area. The other 20 colonies were left without any treatment. On 22 September 2014 the strips were removed from the colonies. All colonies were inspected in October 2014 and prepared for winter. On 6 December 2014 all colonies were treated with 2 strips of Apistan in the area of the hive where the bees were clustered. All colonies were broodless.

On 6 December 2014, we took samples of approximately 300 worker bees (mean ± standard deviation: 310.3 ± 40.4 bees per hive) from all 40 hives. The colonies had an average of 6.4 frames of worker bees, range 5–8.5 frames, with no significant difference between the two groups of hives.

At the same time as we collected the worker bees, we treated each hive with two Apistan strips. On 10 January 2015, 35 days later, we took a second sample of worker bees from each hive (mean ± standard deviation: 495.3 ± 53.7 bees per hive) and removed the Apistan strips. At this time all colonies had small patches of brood. Therefore, a few days before collecting the worker bee samples, any capped brood cells were uncapped and other brood removed to allow any adult female varroa to leave their cells. This ensured that all varroa were phoretic on adult bees.

4.3.2 Determining varroa numbers on worker bees

The worker bee samples were frozen after collection. Later, we extracted and counted the varroa mites from each sample using a jet of water and a strainer to catch the mites, as used in previous research (Al Toufailia et al, 2014; Al Toufailia et al, 2016; Dietemann et al, 2013). In this way we were able to determine the proportion of varroa killed by the Apistan. For example, if the first sample had 10 mites per 100 bees and the second had six mites per 100 bees, then the mortality was (10 - 6)/100 = 0.04 or 40%.

4.3.3 Statistical Analysis

Data were analysed using the IBM SPSS statistical program version 20. If necessary, we log or arcsine transformed the response variable to meet the assumptions of ANOVA (Grafen et al, 2002; Zuur et al, 2010). We then used ANOVA to test the effect of the

Apistan in winter, when the colonies are broodless, on colonies treated and non-treated with Apistan the previous summer. P<0.05 is defined as significant. Descriptive statistics are given as mean ± SE (standard error).

4.4 Results

Our first winter samples of worker bees contained many varroa mites. In the samples taken from hives that had not been treated with Apistan in August, the average was 17.1 ±1.6 mites per 100 bees (mean ± standard error), versus 8.8 ± 0.6 mites per 100 bees in the hives that had been treated. In the second sample, the proportion of varroa was reduced to 7.3 ± 0.8 per 100 bees in the hives previously not treated versus 5.9 ± 0.4 per 100 bees in the previously treated hives.

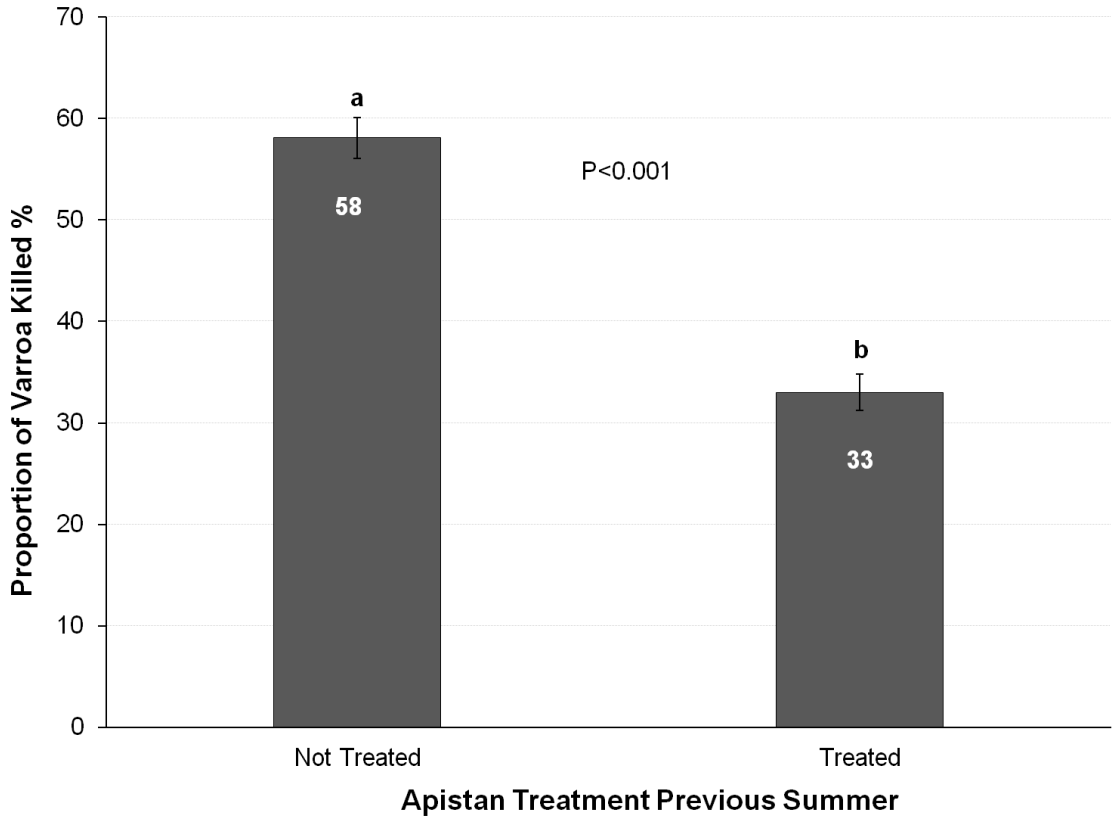

Fig.4.1. Proportions of *Varroa destructor* mites killed via a 35-day Apistan treatment (6 December 2014 to 10 January 2015). Treated hives (n = 20 hives) had received a previous Apistan treatment from 12 August to 22 September 2014. Other than that, hives had not been treated with Apistan for five years or more.

We determined the proportion of mites killed in each hive, and from this worked out that, on average, Apistan killed 58.1% ± 2.1 (range 40–69%) of the varroa in the previously untreated hives versus 33.0% ±1.8 (range 20–46%) in the hives treated with

Apistan the previous summer. This difference is highly significant ($F = 82.8$; $P < 0.001$). In other words, Apistan killed a lower proportion of varroa in colonies that had been treated with Apistan four months previously. Colony strength had no effect on the proportion of varroa killed by Apistan ($F= 0.3$; $P=0.6$).

4.5 Discussion

Our results showed that Apistan was not effective at killing the varroa in our colonies, presumably because of high levels of resistance to fluvalinate. The kill of 33% in the colonies we had treated the previous summer is very similar to that reported previously, 30%, for resistant varroa (Faucon et al, 1995). It is likely that similar results would be found by other beekeepers in Britain, given that resistant varroa have been present for 15 years. The results indicate a high level of Apistan resistance and also showed that the single previous Apistan treatment a few months before caused a large and significant drop in varroa mortality, from 58% to 33%, indicating that the previous treatment had selected for resistant mites within the study hives.

How useful is Apistan if it kills 33% of the varroa in treated colonies? A single treatment of oxalic acid can kill 97% (Al Toufailia et al, 2016) versus 33%. It seems that the oxalic acid is three times as effective as 97% is about three times of 33%. However, when we look at the surviving proportions, 3% versus 67%, it is clear that oxalic acid is much more than three times as effective. With 3% survival the varroa population would have to double slightly more than five times (3 to 6, 6 to 12, 12 to 24, 24 to 48, 48 to 96) to get back to where it was but for 67% survival it has to double slightly less than once. In the study area, varroa populations increase on average by 40 times per hive over one year, equivalent to slightly more than five doublings (Al Toufailia et al, 2014). This means that killing half or one third of the varroa using Apistan slows population growth by the equivalent of approximately one-fifth of a year, on average.

Our results confirmed what we strongly suspected. Apistan is not very effective in killing varroa in our hives. Overall, our results suggest that Apistan is of limited value in controlling varroa in honey bee colonies in Britain and other areas where resistance has been established, even in beekeeping operations that have not used Apistan for a number of years. The evolution of resistance to synthetic chemicals is almost inevitable and it is unrealistic to expect that varroa can be controlled by these

chemicals in the future. Alternative approaches, including the use of oxalic acid and hygienic behaviour (Al Toufailia et al, 2014), should be used instead.

Chapter 5

Towards integrated control of varroa: Comparing application methods and doses of oxalic acid on the mortality of phoretic *Varroa destructor* mites and their honey bee hosts

5.1 Abstract

In the past two decades, the parasitic mite *Varroa destructor* has become harder to control with synthetic acaricide chemicals due to genetic resistance. We determined the efficacy of the natural chemical oxalic acid (OA) in killing phoretic mites on adult worker bees under field conditions in southern England. We compared three OA application methods (trickling, spraying, and sublimation) at three or four (sublimation) doses, using 110 broodless colonies in early January 2013. Treatment efficacy was assessed by extracting mites from samples of c. 270 worker bees collected immediately before and 10 days after treatment. All three methods could give high varroa mortality, c. 93–95%, using 2.25 g OA per colony. However, sublimation was superior as it gave higher mortality at lower doses (0.56 or 1.125 g per colony: trickling 20, 57% mortality; spraying 25, 86%; sublimation 81, 97%). Sublimation using 2.25 g of OA also resulted in 3 and 12 times less worker bee mortality in the 10 days after application than either trickling or spraying, respectively, and lower colony mortality four months later in mid spring. Colonies treated via sublimation also had greater brood area four months later than colonies treated via trickling, spraying, or control colonies. A second trial in December 2013 treated 89 broodless colonies with 2.25 g OA via sublimation to confirm the previous results. Varroa mortality was 97.6% and 87 (98%) of the colonies survived until spring. This confirms that applying OA via sublimation in broodless honey bee colonies in winter is a highly effective way of controlling *V. destructor* and causes no apparent harm to the colonies.

5.2 Introduction

The mite *Varroa destructor* is native to Asia where it is a parasite of the eastern honey bee *Apis cerana* (Oudemans, 1904). Through human intervention (Anderson and Trueman, 2000; Delfinado, 1963), it has been transferred to *Apis mellifera*, and is now found on *A. mellifera* worldwide except Australia (Anderson and Trueman, 2000).

Varroa is a serious pest of *A. mellifera*. It can harm colonies and bees both directly, for example, by damaging individual worker bees during the pupal stage so that their adult lifespan and body weight (Amdam et al, 2004; De Jong et al, 1982) are reduced, and indirectly, by exacerbating virus diseases (Boecking and Genersch, 2008; Brødsgaard et al, 2000; Tentcheva et al., 2004) and causing colony mortality (Boecking and Genersch, 2008; Genersch et al, 2010; Hayes et al, 2008; Potts et al, 2010).

Varroa has been successfully controlled using synthetic acaricides (Alonso De Vega et al, 1990; Milani and Barbattini, 1988; Milani and Iob, 1998). However, resistance to the most effective compound, fluvalinate (the active ingredient in Apistan) is now widespread (Elzen et al, 2000; Floris et al, 2001; Milani, 1999; Milani and Barbattini, 1988; Mozes-Koch et al, 2000; Patti et al, 2000). Resistance to coumaphos and flumethrin also occurs (Elzen et al, 2000; Eizen et al, 2001; Elzen and Westevelt, 2002; Milani, 1999). Many other varroa control compounds have also been tried (Rosenkranz et al, 2010; Wallner, 1999), including natural organic acids such as oxalic acid (Aliano and Ellis, 2008; Bacandritsos et al, 2007; Gregorc and Poklukar, 2003; Marinelli et al, 2006; Nanetti et al, 2003; Rademacher and Imdorf, 2004; Takeuchi and Sakai, 1985), formic acid (Althen, 1979; Eizen et al, 2001; Fries et al, 1991; Mahmood et al, 2012; Satta et al, 2005), and lactic acid (Emsen and Dodologlu, 2009; Koeniger et al, 1983; Kraus and Berg, 1994) and essential oils such as thymol (Emsen and Dodologlu, 2009; Floris et al, 2004; Imdorf et al, 1999).

Beekeepers have been using OA (oxalic acid) against varroa for several decades (Popov et al, 1989) and research has shown that it can be effective. OA kills varroa phoretic on the bodies of adult bees, but not those in brood cells (Charriére and Imdorf, 2002; Gregorc and Planinc, 2001, 2002; Nanetti et al, 1995). As a result, it is more effective when applied to broodless colonies, such as in winter (Bacandritsos et al, 2007; Marinelli et al, 2000; Nanetti and Stradi, 1997) or with a queen that has been caged for long enough to allow existing brood to reach the adult stage (Wagnitz and Ellis, 2010). OA appears to combat varroa in two ways. It damages varroa mouthparts and also causes increased bee to bee contact and grooming (Aliano and Ellis, 2008; Aliano et al, 2006; Fries et al, 1996; Schneider et al, 2012). Grooming dislodges mites which then fall onto the hive floor where many die from starvation (Aliano et al, 2006; Stevanovic et al, 2012). OA may potentially harm the bees (Higes et al 1999). It can penetrate into the body after topical or oral application, which resulted in detectable OA

concentrations in different organs of caged worker bees although mortality was not measured in the honey bee colony (Nozal et al, 2003).

Beekeepers apply OA to the bees in a hive either in water solution, usually combined with sucrose, or in pure form via sublimation. Two methods of applying OA solution are widely used. In the spraying method, the hive is opened, the frames are briefly removed one at a time, and the exposed bees are misted with the OA solution (Imdorf et al, 1997; Nanetti et al, 2003; Rademacher and Harz, 2006). In the trickling method, the hive is opened and the solution is poured onto the exposed bees and into the gaps between the frame top bars without removing the frames. In the sublimation method, pure OA is applied using a heated metal tool that causes the crystals to sublimate (Marinelli et al, 2004; Rademacher and Harz, 2006; Radetzki, 2001). The hive does not need to be opened as the tool is inserted via the entrance. This saves time and can also be carried out more easily in winter or under conditions unsuited to opening a hive.

Sublimation is a more recent method (Marinelli et al, 2004; Radetzki and Bärmann, 2001b). Surprisingly, previous research has not directly compared these three application methods to determine which of them, and which dose, is best. Previous research typically compared a few doses (Gregorc and Poklukar, 2003; Mahmood et al, 2012) or one or two methods (Bacandritsos et al, 2007; Imdorf et al, 1997; Marinelli et al, 2004; Nanetti et al, 1995), but no study has systematically compared all three methods across a range of doses. In addition, most previous research did not determine the proportion of phoretic mites in the colony that were killed. More typically, the number of mites killed, but not the number surviving, was determined by counting dead mites on the hive floor (Calderone and Lin, 2003; Fries et al, 1991; Mahmood et al, 2012). In addition, most previous studies did not determine possible harmful effects on the bees and colony, such as the numbers of worker bees killed at the time of application and longer term colony and queen survival or colony performance.

The aim of this study was therefore to compare the effect of winter application of OA to broodless honey bee colonies on the mortality of phoretic varroa mites and their host bees and colonies. We compared the three main methods (trickling, spraying, and sublimation) using the same three doses with one additional higher dose for sublimation. The study was carried out in southern England, which has a temperate climate with little or no foraging during winter, December to February, and a natural

reduction in brood rearing to a low level in December and January (personal observations).

5.3 Materials and Methods

5.3.1 Experimental setup and OA treatment

5.3.1.1 Setup of colonies

Trials were carried out in winter, January 2013, using 110 honey bee colonies located in 10 apiaries at or within 20 km of the University of Sussex in southern England. The colonies were all in hives consisting of a single "commercial" brood chamber (11 frames each 43.8 x 25.4 cm, vol. 56.4 l), wooden bottom board with mesh floor, inner cover, and telescopic outer cover. Hive inspections approximately four weeks before the trials were used to exclude any queenless colonies.

Subsequent hive inspections approximately three weeks before the experimental trials showed that c. 90% of the colonies did not have any brood. The other 10% had small amounts of sealed or open brood, which was removed 1–2 days later using a honey fork. As a result, all varroa mites in all colonies were phoretic on the adult bees. This is the situation in which varroa can most effectively be killed by OA, and also makes it possible to quantify changes in varroa numbers by extracting mites from samples of worker bees. In particular, in broodless colonies changes in the numbers of phoretic mites on the worker bees are unaffected by mites either emerging from or entering brood cells.

5.3.1.2 Monitoring mite mortality on the hive floor

The fall of mites from the colony was monitored at two-day intervals for 8 days before, and 10 days after OA treatment (Figure 1). Each hive had a mesh floor with a sticky white plastic sheet underneath. Mites would fall through the mesh onto the plastic. Worker bees did not have access to this part of the hive and so could not clean away dead mites. We stopped counting fallen mites after 10 days, as by then the number had been at low levels, less than three mites per day, for 4 days (see Results).

5.3.1.3 Monitoring bee fall

Dead worker bees were also monitored for 8 days prior to treatment and for 10 days after. Dead bees that had fallen onto the mesh floor were removed and counted. Dead bees that were removed from the hive by undertaker bees were caught in a dead bee

trap. This was a 50 x 30 cm fine-mesh net attached to the bottom board at the two corners on either side of the hive entrance and to bamboo canes pressed into the soil below the hive stand at the other two corners. As the study was carried out in winter during cold weather (day time high temperatures before and after OA treatment were 9 °C (average of 8 days), 5 °C (day of treatment), and 3 °C (average of 10 days after treatment), respectively), the bees were not flying, so that undertaker bees (Visscher, 1983) did not fly away with the dead bees. Temperature and humidity are considered important when applying OA (Aliano and Ellis, 2009), and outdoor temperatures in the range 4–16 °C are recommended, depending on application method (Rademacher and Harz, 2006).

5.3.1.4 OA application methods

The three methods we used (trickling, spraying, and sublimation) are widely used by beekeepers. We followed standard application procedures as used by beekeepers and previous researchers (Imdorf et al, 1997; Mahmood et al, 2012; Marinelli et al, 2004; Nanetti et al, 2006; Rademacher and Harz, 2006). In the trickling method, we followed existing protocols (Brødsgaard et al, 1999; Imdorf et al, 1997) using a plastic bottle connected to a syringe pump (Vacc 5 ml V grip syringe- M3090) to dispense 50 ml of a water solution of OA and sucrose per colony in a narrow stream. Approximately, half was applied in an equal layer onto the exposed bees on the top bars of the frames, and half into the gaps between the top bars of the frames where the cluster of bees was located. In the spraying method, we used an applicator of the type used to spray plants by hand to apply 50 ml of solution in a fine mist directly onto the bees on both sides of each frame, which were removed one at a time from the hive. In this way, most of the bees were directly contacted by the solution.

In the sublimation method, we used a commercially available applicator used in beekeeping (Varrox M3080) obtained from a UK beekeeping equipment supplier (E H Thorne (Beehives Ltd.); Wragby) powered by a 12 V car battery to heat a metal dish, diameter 3.5 cm, containing OA to cause sublimation. This part of the applicator was inserted into the hive entrance. Previous trials of the applicator had shown us how long it would take to sublimate different amounts of OA, and that it was possible to insert the tool into the hive before any vapor was produced. During application, the hive entrance was closed with plastic foam to prevent any vapor from escaping. As an additional

safety precaution, the operators wore approved respiratory masks (ChapSmith R300 Series, with a filter for organic gases).

5.3.1.5 OA doses

Based on previous research, we chose three OA doses that would cover the critical range from low to high varroa mortality (Gregorc and Poklukar, 2003; Martín-Hernández et al., 2007, Nanetti et al, 2003; Radetzki, 1994). We used dihydrate OA, purity 99.6% (Sigma-Aldrich obtained from Riedel-de Haën, Enologia Apicoltura). The solutions of OA used in the trickling and spraying methods were the same, 0.8, 1.6, and 3.2% (3.2% means that 4.5 g of OA crystals were dissolved and added to sugar solution and made up to 100 ml, and is a 0.5 M solution of OA). By applying 50 ml of solution, the actual dose per colony was thus 0.56, 1.125, and 2.25 g.

The sucrose solution was itself made up using one kilogram sucrose per one liter water. It is standard beekeeping procedure to apply OA in strong sucrose solution (50%W/W). The solution was prepared 12–18 h before application. The sublimation method used pure OA. We applied the three equivalent doses (0.56, 1.125, and 2.25 g) plus an additional higher dose (4.5 g). We decided to use a fourth higher dose because there was less background information on varroa mortality using sublimation. In addition, our pilot research had shown that this high dose appeared not to cause high bee mortality, meaning that it could, if necessary, be used to control varroa in hives.

5.3.1.6 Experimental design

We used 10 apiaries within 20 km of the University of Sussex, each of which had 11 experimental colonies. At each site one colony was a control, untreated with OA or syrup but opened and inspected and used to recover dead bees and mites in the same way as the treatment colonies. Each of the other 10 colonies at each apiary was used for 1 of the 10 experimental treatments (dose x application). The 10 treatments and control were applied at random to the colonies within an apiary. Colonies were also inspected one day before treatment and collection of the first and second worker bee sample to verify that none had sealed brood. Colony strength, in terms of number of frames of bees, was also quantified during this final inspection.

5.3.1.7 Time taken to apply OA

The time taken for two people to treat each colony was noted, including the time needed to open the hive in the trickling and spraying methods.

5.3.2 Estimating varroa and bee mortality at the time of application, and colony survival and strength in spring

5.3.2.1 Determining varroa mortality by extraction from samples of worker bees

To estimate the proportion of varroa mites killed, we collected one sample of worker bees per colony immediately before treatment and another 10 days after treatment. Samples were sufficiently large (mean = 266.9 worker bees, range 256–302) to contain sufficient mites for meaningful analysis (Dietemann et al, 2013). The samples were frozen. Subsequently, the mites were washed off the bees using a jet of water (warm water for about 5 min) and caught in a fine metal screen. A pilot study that checked three samples of 300 worker bees under a microscope after washing had shown that 100% of the mites were extracted. The number of bees per sample was also counted. The proportion of varroa killed was then calculated as $1 - d/e$, where e is the number of mites per 100 worker bees before treatment and d the number after.

5.3.2.2 Determining varroa mortality from mite fall onto hive floor

Most previous studies used mite fall onto the hive floor or bottom board to measure varroa mortality (Dietemann et al, 2013) using the formula: $(a - b)\ 100/(a + b)$, where a= number of mites falling per day after using OA and b = number of mites falling per day before using OA (bottom board) (Calderone and Lin, 2003; Fries et al, 1991; Gregorc and Jelenc, 1996; Ritter, 1981). However, this method does not determine the proportion of varroa that have been killed. We used this method to link our study to previous research and to show the relationship between absolute varroa mortality and mortality estimated from the increase in mite fall.

5.3.2.3 Quantifying worker bee mortality

Dead bees were collected from the dead bee trap and from the mesh above the bottom board every 2 days for 8 days before and 10 days after OA treatment.

5.3.2.4 Quantifying effects on colonies after 4 months

Each colony was inspected in mid-spring, 3 May 2013, 111 days after treatment with OA on 12 January, to determine whether it was still alive and if alive, whether it had a queen. The number of frames (counting 0.5 per side with brood) of sealed and unsealed brood was also determined in the colonies with a queen.

5.3.3 Confirming high varroa mortality, colony survival and strength

A second trial was carried out in the period 12–21 December 2013 using 89 bee colonies located in nine apiaries within 20 km of the University of Sussex, most being colonies and apiaries also used in the first trial. As before, the colonies were all broodless or made broodless and in hives consisting of a single "commercial" brood chamber. All colonies were treated with 2.25 g OA via sublimation.

The aim was to test this particular dose and method, which was the best combination as shown by the results of the first trial, to verify that the results were replicable and to firmly establish the result using a large number of colonies, rather than the 10 in the first trial. The 89 bee colonies treated in December 2013 with OA were inspected on 31 March 2014 for survival and brood amount, to compare with results of the efficacy, strength, and colony mortality from the previous year. Colony inspections were carried out earlier than in the previous year because spring 2014 was approximately one month in advance of spring 2013.

5.3.4 Statistical analysis

Data were analyzed using the SPSS statistical program version 20. If necessary, we log or arcsine transformed the response variable to meet the assumptions of ANOVA (Grafen and Hails, 2002; Zuur et al, 2010). We then used two-way ANOVA to test for differences between the effects of OA application method (treatments) and dose efficacy against varroa and side effects on acute bee mortality. We then used Tukey's post hoc tests to compare varroa mortality and effects on bees between OA application methods or doses. $P < 0.05$ is defined as significant. Descriptive statistics are given as mean ± standard error.

5.4 Results

The initial varroa level was 9.8 (range: 2-29) / 100 bees (110 hives).

5.4.1 Varroa fall before and after treatment

After OA treatment, the number of dead mites on the hive bottom board increased greatly in comparison to pre-treatment levels, and then decreased to a low level six days after treatment (Figure 1). This shows that the killing effect was considerable, and occurred at or soon after treatment. It also shows that extracting surviving mites from a sample of worker bees collected 10 days after treatment was appropriate to measure the

number of surviving varroa, and from this to calculate the proportion of mites killed by OA treatment.

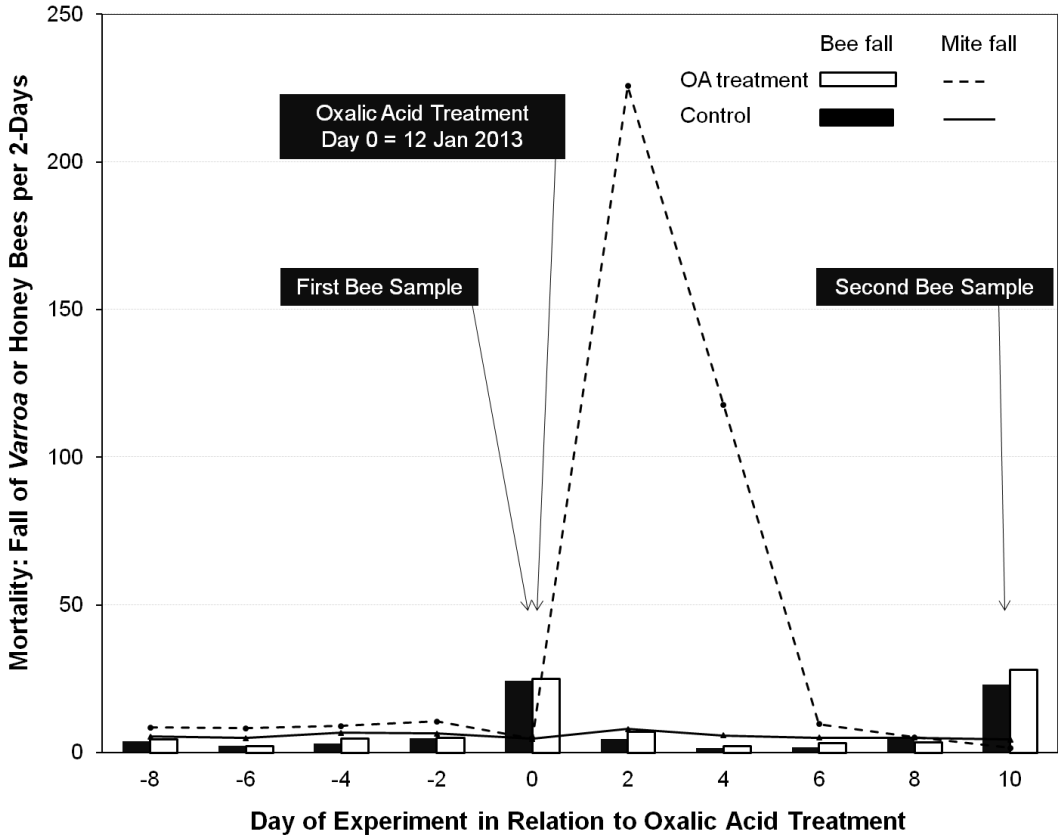

Fig.5.1. Time course of experiment showing the average number of dead varroa on the hive floors and the number of dead worker bees on hive floors and dead bee traps combined for 8 days before treatment and 10 days after, per 2 days. OA treatments were made on day 0. Control colonies (untreated) N = 10. Treatment colonies combined (N = 10 colonies for each of 10 dose x application methods).

5.4.2 Effect of OA on varroa and bee mortality

5.4.2.1 Determining varroa mortality from samples of worker bees

We first tested whether varroa mortality depended on colony strength (number of frames of adult bees). As this had no significant effect ($F = 1.027$; $p = 0.44$), we removed it from the model. We found a significant effect of application method ($F = 22.53$, $p < 0.001$), dose ($F = 38.13$, $p < 0.001$), and their interaction ($F = 9.59$, $p < 0.001$). We then compared the effect of each dose and method post hoc with Tukey's test (Table 1, Figure 2a). All treatments gave significantly greater varroa mortality than the control, except for the lowest dose (0.56 g) via both spraying and trickling (Figure 2a). All three methods gave high (c. 93% or above) and statistically similar levels of mortality at the 2.25 g dose.

Table.1.5. P values for pairwise post hoc Tukey tests comparing varroa mortality based on numbers of mites in samples of worker bees from hives with different OA application methods and doses before and after treatment. Bold values are significant ($p < 0.05$). T, Sp, and Su refer to OA application via trickling, spraying, and Sublimation, respectively. Numbers, such as 2.25 g, refer to the amount of OA in the 50 ml of syrup applied or to the weight of OA applied directly via sublimation, per hive.

	T 0.56g	T 1.125g	T 2.25g	Sp 0.56g	Sp 1.125g	Sp 2.25g	Su 0.56g	Su 1.125g	Su 2.25g	Su 4.5g
Control	0.81	**<0.001**	**<0.001**	0.48	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
T 0.56g		**0.007**	**<0.001**	1.00	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
T 1.125g			**0.003**	**0.03**	**0.025**	**<0.001**	0.15	**<0.001**	**0.005**	**<0.001**
T 2.25g				**<0.001**	1.00	1.00	0.97	1.00	1.00	0.92
Sp 0.56g					**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Sp 1.125g						0.98	1.00	0.97	1.00	0.58
Sp 2.25g							0.72	1.00	1.00	0.99
Su 0.56g								0.66	0.98	0.18
Su 1.125g									0.99	0.99
Su 2.25g										0.88

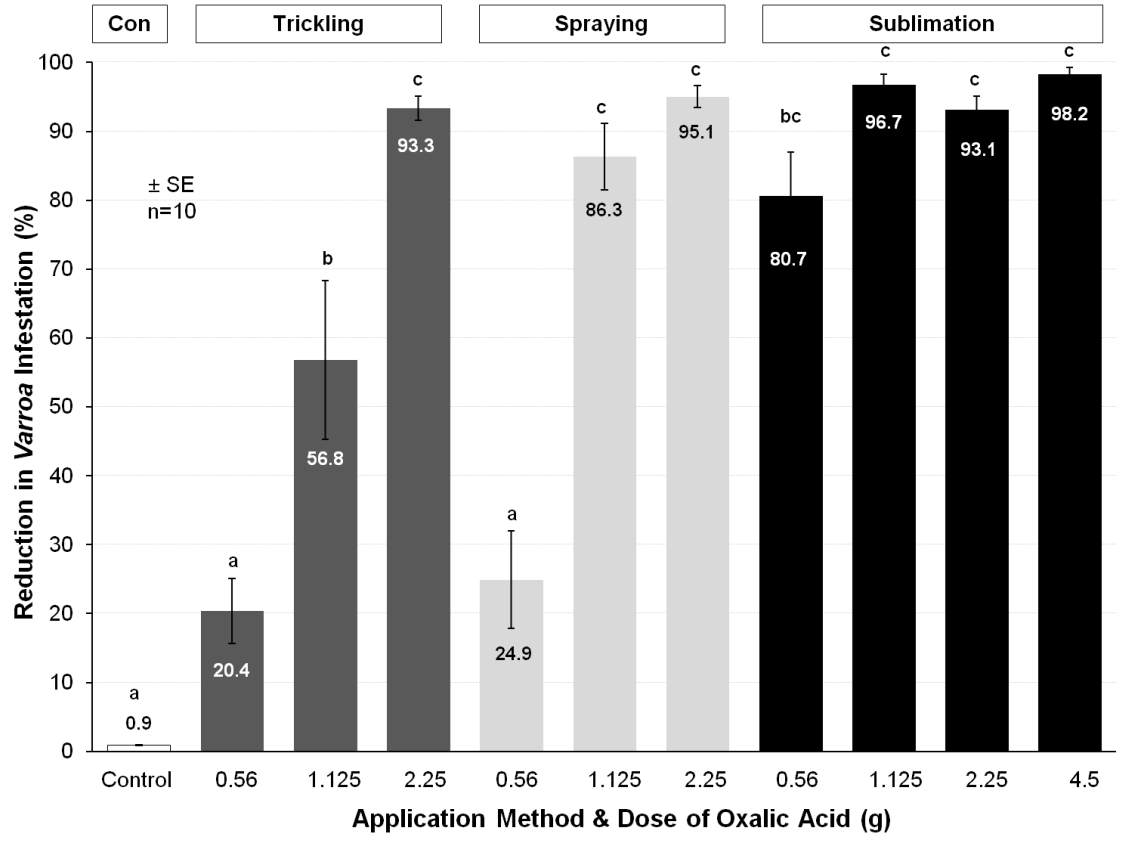

Fig.5.2a. Varroa mortality as determined by the numbers of mites in the samples of worker bees taken immediately before and 10 days after OA treatment. Histogram bars with different letters indicate significant differences, $p < 0.05$. Error bars show the standard error. Numbers on the x-axis refer to the weight in grams of OA applied to each hive, either in 50 ml of sucrose syrup (trickling or spraying methods) or directly via sublimation.

Both trickling and spraying showed clear and statistically significant dose response effects. With sublimation, varroa mortality was high, 81%, even at the lowest dose 0.56 g, and higher still at the three higher doses. However, the post hoc tests showed that these differences among doses were not statistically significant. Overall, the trickling method was the least effective and sublimation the most effective in terms of dose mortality. The sublimation method gave high mite mortality at all doses used. Dose differences were significant when the lowest sublimation dose was compared to the three highest doses combined ($F = 12.89$, $p = 0.001$).

5.4.2.2 Effect of dose and application method on varroa fall onto the hive floor

As above, we found no effect of colony strength, but a significant effect of method ($F = 18.93$, $p < 0.001$), dose ($F = 24.75$, $p < 0.001$) and their interaction ($F = 15.56$, $p < 0.001$), and carried out post hoc analysis with Tukey tests (Table 2).

Table.2.5. P values for pairwise post hoc Tukey tests to determine whether varroa mortality determined from mite fall onto the hive bottom board differed from that determined from numbers of mites extracted from samples of worker bees before and after OA treatment. Bold values are significant, $p < 0.05$. T, Sp, and Su refer to OA application methods trickling, spraying, and sublimation, respectively. Numbers, such as 2.25 g, refer to the amount of OA in the 50 ml of syrup applied or to the weight of OA applied directly via sublimation, per hive.

	T 0.56g	T 1.125g	T 2.25g	Sp 0.56g	Sp 1.125g	Sp 2.25g	Su 0.56g	Su 1.125g	Su 2.25g	Su 4.5g
Control	0.14	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
T 0.56g		**0.007**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
T 1.125g			0.92	0.64	1.00	0.93	0.99	0.93	0.99	0.87
T 2.25g				**0.027**	0.99	1.00	1.00	1.00	1.00	1.00
Sp 0.56g					0.35	**0.031**	**0.15**	**0.029**	**0.15**	**0.018**
Sp 1.125g						0.99	1.00	0.99	1.00	0.98
Sp 2.25g							1.00	1.00	1.00	1.00
Su 0.56g								1.00	1.00	0.99
Su 1.125g									1.00	1.00
Su 2.25g										0.99

The number of varroa falling to the hive floor (Figure 2b) showed a very similar pattern to the proportion of varroa killed (Figure 2a). In particular, trickling and spraying showed a dose response effect, but sublimation did not. In addition, the lowest trickling dose did not differ from the control. The similarity between the two measures is expected, but is also reassuring given that they are independent data sets relevant to the same thing: varroa mortality.

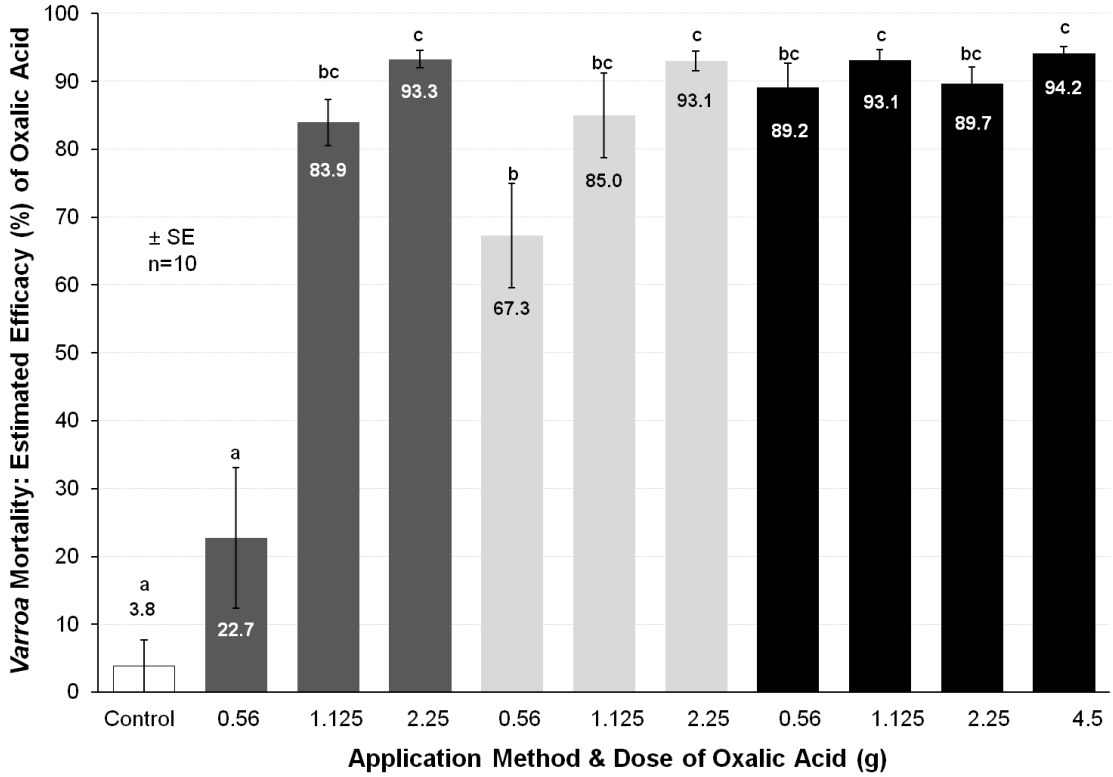

Fig.5.2b. Varroa mortality as determined from the numbers of mites counted on the hive bottom board before and after OA treatment (using formula: (a − b) 100/(a + b) (Calderone and Lin, 2003; Fries et al, 1991; Gregorc and Jelenc, 1996; Ritter, 1981). a = number of mites falling per day after using OA. b = number of mites falling per day before using OA. Mites were counted every 2 days for 8 days before and 10 days after OA treatment, and the numbers averaged. Histogram bars with different letters indicate significant differences, $p < 0.05$. Error bars show the standard error.

5.4.2.3 Effect of application method and dose on bee mortality

We found no significant effect of colony strength on the number of dead bees ($F_{1,109} = 1.495$; $p = 0.106$) so we removed this from the model. We found a significant effect of application method ($F = 4.56$, $p = 0.013$), but no significant effect of dose ($F = 1.35$, $p = 0.262$) or dose/method interaction ($F = 1.33$, $p = 0.265$).

Table.5.3a. P values for pairwise post hoc Tukey tests comparing the number of dead bees from the bee trap and mesh above the bottom board in different OA application methods. Bold values are significant.

	Trickling	Spraying	Sublimation
Control	0.89	0.34	0.99
Trickling		0.53	0.56
Spraying			**0.03**

We then carried out Tukey post hoc tests (Table 3a). Figure 3 shows that sublimation caused lower bee mortality than spraying, and was similar to the control. There was also a trend towards higher mortality with increasing dose in the trickling and spraying methods, but not with sublimation.

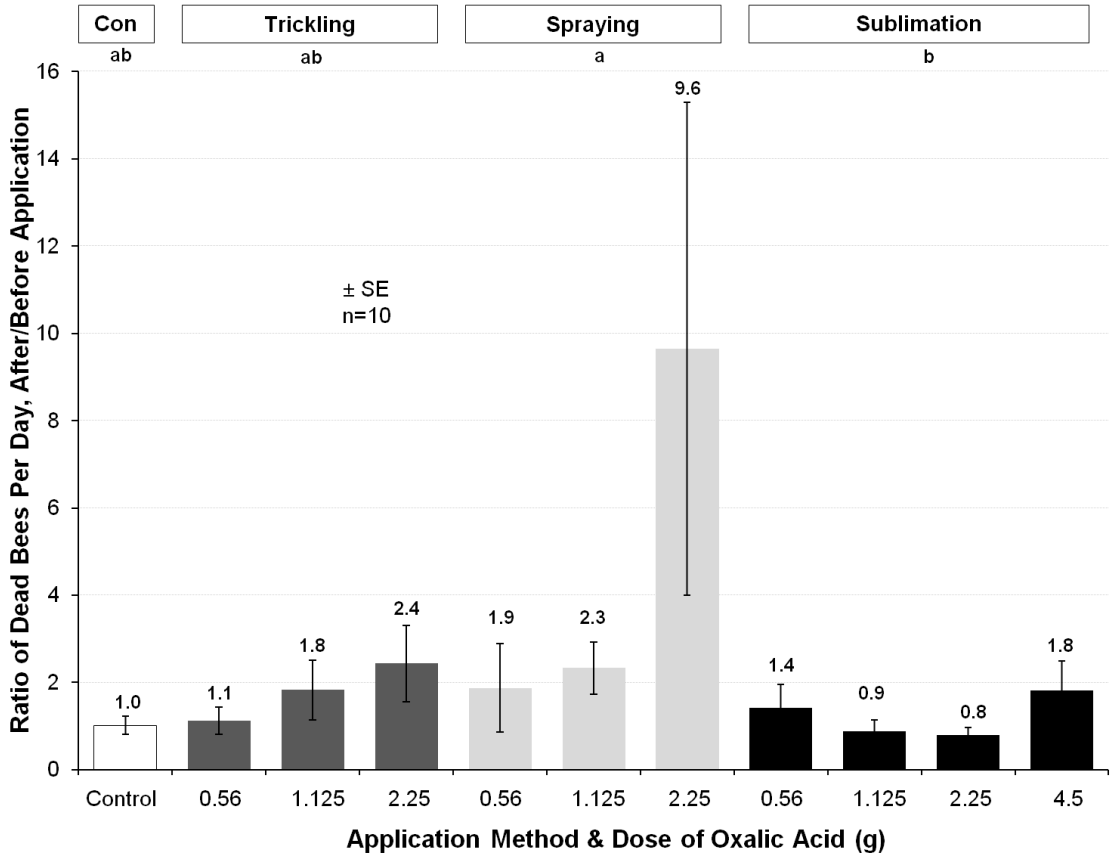

Fig.3.5. Ratio of mean numbers of dead bees per treatment per day in the dead bee traps plus on the meshes above the bottom boards, after treatment divided by before treatment. Bees were counted every 2 days for 8 days before and 10 days after treatment. Histogram bars with different letters indicate significant differences, $p < 0.05$. Error bars show standard errors. Numbers above bars are the mean numbers of dead bees per day per hive.

5.4.3 Effect of OA on colony strength and survival

5.4.3.1 Effect of dose and application method on colony and queen Mortality

Across all doses, more colonies died following spraying (11/30, 37%) than for trickling (5/30, 17%) and sublimation (2/40, 5%), respectively (Figure 4).

Table.5.3b. P values for pairwise post hoc Tukey tests comparing colony survival four months after treatment by different OA application methods. Bold values are significant.

	Trickling	Spraying	Sublimation
Control	0.99	0.41	0.96
Trickling		0.25	0.70
Spraying			**0.01**

Two of the 10 control colonies (20%) also died. We found a significant effect of method ($F = 4.98$, $p = 0.009$) on colony mortality, but no significant effect of dose ($F = 1.49$, $p = 0.22$) and no interaction between these two factors ($F = 1.53$, $p = 0.199$). To determine the effect of method we made post hoc Tukey tests (Table 3b). The difference between sublimation and spraying was significant ($p = 0.01$).

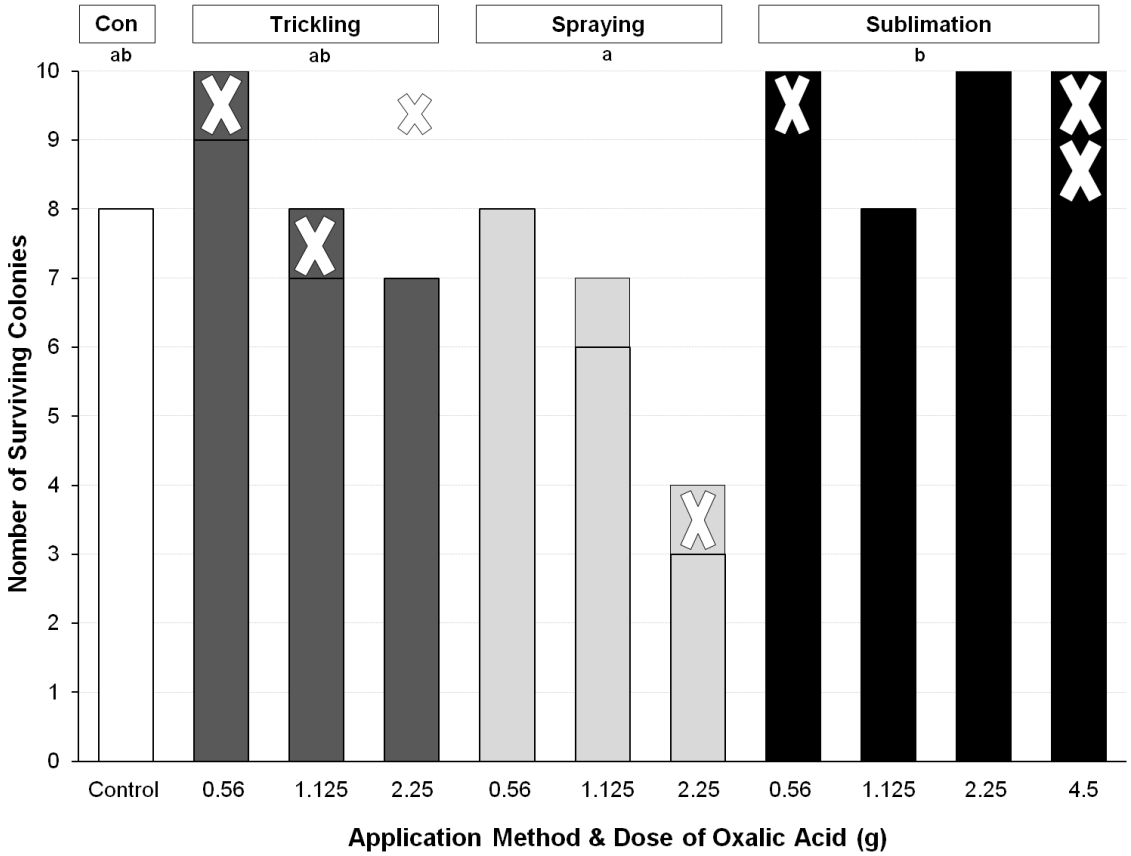

Fig.5.4. Colony survival on 3 May, 111 days after treatment. Crosses represent colonies that were alive but queenless. Treatment groups with different letters indicate significant differences, $p < 0.05$.

5.4.3.2 Effect of dose and application method on colony strength

We found a significant effect of application method ($F = 16.37$, $p < .001$), but no significant effect of dose ($F = .253$, $p = 0.859$) or dose-method interaction ($F = .971$, $p = 0.429$) on colony strength (number of frames of brood) four months after treatment.

Table.5.3c. P values for pairwise post hoc Tukey tests of colony strength in terms of the number of frames of brood in mid-spring four months after the treatment with different OA application methods. Bold values are significant.

	Trickling	Spraying	Sublimation
Control	0.87	0.47	0.44
Trickling		0.76	**<0.001**
Spraying			**<0.001**

Post hoc test analysis using Tukey's test showed that colonies treated by sublimation had significantly more brood than colonies treated using the other methods and also the control colonies (Figure 5; Table 3c).

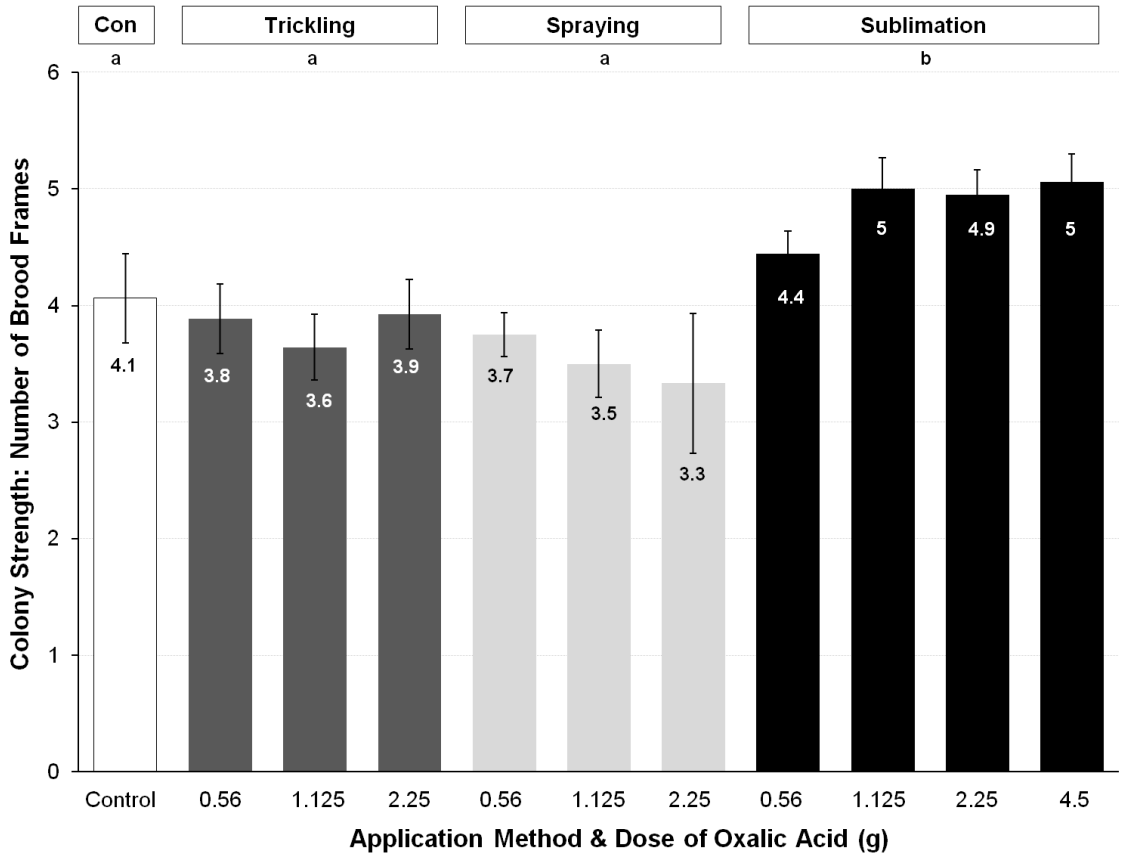

Fig.5.5. Colony strength four months after OA treatment, quantified as the mean number of frames (0.5 per side) with either sealed or open brood cells in the surviving colonies with a queen in each treatment group. Histogram bars with different letters indicate significant differences, $p < 0.05$. Error bars show the standard error.

5.4.3.3 Effect of application method on time taken to treat a colony

Figure 6 shows the amount of time taken for two people working together to treat a colony, or in the case of the control, to open the hive for one minute. As expected, spraying is the most time consuming method, as the frames with bees on them need to be removed from the hive. The sublimation method takes longer with higher doses as it takes longer for the OA to completely sublimate.

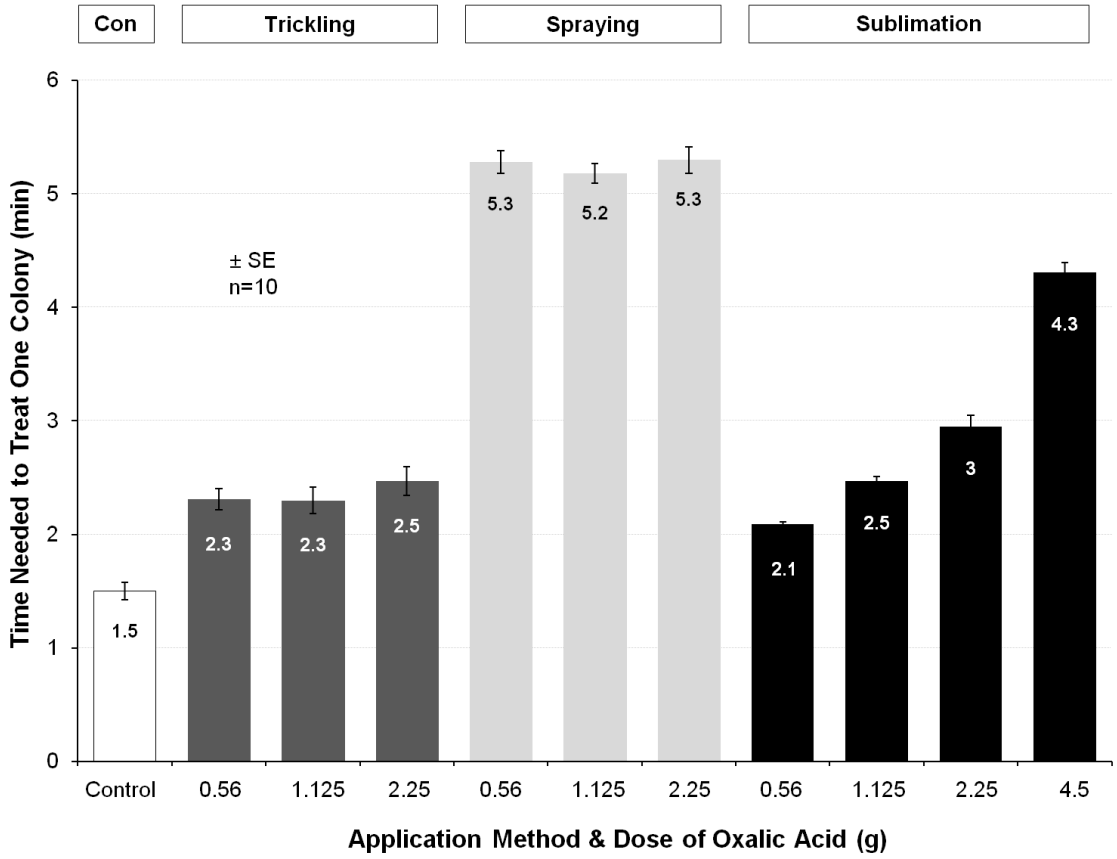

Fig.5.6. Time needed per hive to apply OA. Control hives were opened for 1 min. Error bars show the standard error.

5.4.4 Confirming the effectiveness of 2.25 g sublimation

The initial varroa level was 14.7 (range: 2-33) / 100 bees (89 hives).

5.4.4.1 Determining varroa mortality from samples of worker bees

Sublimation treatment with 2.25 g of OA in December 2013 gave mean varroa mortality of 97.6%. This is significantly higher than the mortality for 2.25 g via sublimation in January 2013 (93.1%, p = 0.006), but not higher than for the three highest doses, (1.125, 2.25, and 4.5 g) combined (96.0%, p = 0.101).

5.4.4.2 Quantifying midterm effects on colony mortality and strength.

The treated colonies had high survival 98% (87/89) after 109 days. All were queenright and had an average of 4.75 frames with brood.

5.5 Discussion

Our results show clearly that OA can be highly effective at killing varroa mites under beekeeping conditions in broodless hives in winter. However, varroa mortality is affected by application method and dose. In addition, bee and colony mortality and colony performance are also affected by application method and dose. The results show that sublimation is the best method, in that in gives greater varroa mortality at lower doses, and results in no harm to the colonies. In fact, colonies treated via sublimation had significantly more brood in spring than controls, and lower winter mortality, although this difference was not significant.

Varroa mortality showed a dose effect in all three application methods, and in all three methods, one or more of the higher OA doses gave mortality of 93% or more (Figures 2a and 2b). Sublimation gave higher varroa mortality at lower equivalent doses (i.e., the same amount of OA per colony) than trickling or spraying. At lower doses, sublimation was the most effective method and spraying was more effective than trickling (e.g. significantly higher mortality at the 1.125 g dose (1.6% OA)). This is probably because spraying results in more of the bees, and hence mites, being contacted by the solution than with trickling. Previous research also indicated that all three methods can give high varroa mortality >90%, although this was based on mite fall (Gregorc and Planinc, 2001; Imdorf et al, 1997; Nanetti et al, 2003; Rademacher and Harz, 2006; Radetzki and Bärmann, 2001a) not on the proportion of mites killed, and that spraying gives greater mite mortality than trickling (Nanetti et al, 1995) although not to a significant degree. In terms of the method used to quantify varroa mortality, with trickling and spraying, the intermediate 1.125 g, dose appeared to be more effective when considering mite fall vs. the proportion of mites killed based on extracting mites from samples of worker bees. This shows the importance of quantifying the actual proportion of varroa killed rather than the number that fall onto the hive floor.

The ratio number of dead bees falling onto the hive floor and collecting in the dead bee trap in the 10 days after OA application was low in all treatments, with a

maximum of 9.6 bees per day at the highest dose (2.25 g) with spraying. Although this was approximately 10 times higher than the control, it is still a low absolute number given that a colony in the winter will contain c. 5–10,000 bees. Thus, 10 days of mortality at 10 bees per day would be only 1–2% of the workers. Sublimation gave significantly lower bee mortality than spraying. Across all doses, sublimation gave mortality rates of 0.8–1.8 bees per day, similar to the control. Trickling at all three doses and spraying at the two lowest doses also gave rates similar to the control (Figure 3). High mortality following spraying has been shown in some previous studies (Higes et al, 1999) but not in others (Rademacher and Harz, 2006). Trickling causes low bee mortality (Aliano and Ellis, 2009). We are unaware of any previous data on the effect of OA sublimation on bee mortality.

Differences among application methods on colony performance were observed in mid-spring, 3 May, 111 days after application (In the study area foraging began in March in 2013). Of the control colonies, 8/10 (80%) survived, and all had a queen. This shows that background colony survival was less than 100%. Of the sublimation colonies, 38/40 (95%) survived and 35/40 (88%) had a queen. Although this difference in colony survival is not significant ($p = 0.43$, chi^2 2 x 2 test) the trend is for the sublimation treated colonies to have higher survival. Survival of the colonies sprayed with OA solution was significantly lower than for sublimation (19/30, 63%; $p = 0.01$) (Figure 4). Survival of colonies dribbled with OA solution (25/30, 83%) was similar and not significantly different to control or sublimation colonies ($p = 0.13$, chi^2 2 x 2 test). Previous studies have also reported colony mortality following OA spraying, but not significantly greater than control colonies (Higes et al, 1999; Toomemaa et al, 2010). We are unaware of any previous research that has quantified the effect of OA treatment via trickling or sublimation on colony mortality.

The amount of brood after four months is also of interest. In particular, sublimation resulted in significantly more brood than controls, at 4.8 (average of the four treatment means) vs. 4.1 frames. The numbers of frames of brood was not different among control, trickling, and spraying colonies. However, there was a trend towards lower amounts of brood with higher doses for both trickling and spraying. Previous research also reported a negative effect on brood rearing following the application of OA via spraying and trickling (Higes et al, 1999; Rademacher and Harz, 2006).

Sublimation is clearly the best method overall, as it is the best in all three criteria studied. Firstly, it requires the least amount of OA to give high varroa mortality and

gives high mortality over the widest range of doses. Our results from the second trial (97.7% varroa mortality with 2.25 g OA) confirmed the high mite kill that can be achieved via sublimation. Secondly, sublimation resulted in no harm to the bees, either at the time of treatment or four months later in mid-spring. In fact, it actually resulted in stronger spring colonies. We do not have any firm explanation for why this may be the case. One possibility is that by killing mites, OA treatment increases colony performance but that this benefit is counteracted in the trickling and spraying methods via harm to the bees, but not with sublimation. In this respect, it is worth noting that the amount of brood in the sublimation colonies was lowest at the lowest dose of OA. If this effect is found in further studies, it would be worthwhile to determine the underlying reason.

The third advantage of sublimation is that it is the simplest method, and quick. In particular, because it does not need the beekeeper to open the hive, it is less work and is well suited for use in winter (Radetzki and Bärmann, 2001a, 2001b), when colonies are broodless but are not normally opened for inspection. It could be applied; for example, on rainy or cool days when opening a hive is not good beekeeping practice (Crane, 1990; Gould and Gould, 1988). The time taken to apply 2.25 g of OA via sublimation, which we consider to be a recommendable dose, is under three minutes per colony. This is slightly more, by about half a minute, than for trickling but less than for spraying.

Sublimation has two small disadvantages. First, it requires the use of a 12 volt car battery, and the special purchase of a mask and heated application tool. But these can be used many times. Second, OA is considered harmful if breathed (Gumpp et al, 2003), although in our experience, the entire vapor was contained within the hive. In part, this was because we sealed the hive entrance with foam immediately after inserting the sublimation tool, and also because the hot tool was inserted into the hive entrance just a few seconds after being loaded with OA. If need be, this could be made certain by only supplying the electricity to the sublimation tool when it has already been loaded with OA and inserted into the hive entrance. However, this would take several minutes extra time per colony to cool down and heat up and would not be practical for a commercial beekeeper treating many colonies. We achieved the same effect by quickly loading the tool with OA at the hive entrance and, within one or two seconds, inserting it before any had sublimated. We did not find it difficult to use the sublimation method.

Our results are very encouraging for beekeepers. They show that a quick and cheap method, sublimation, can kill approximately 97% of the varroa in a broodless honey bee colony. A broodless period is normal in the honey bee colony's seasonal cycle in many parts of the world, and treatment can be made at this time. Alternatively, colonies with a caged queen could be treated, although this requires considerable additional work by the beekeeper. Swarms and package bees could also be treated within c. eight days of placing into a hive, before any sealed brood is present. Temperature and humidity also need to be taken into account (Aliano and Ellis, 2009; Rademacher and Harz, 2006). One of the goals of varroa control is to develop treatment methods that can be applied at long intervals. Depending on varroa population increase, the level of mite kill from OA sublimation may be sufficient for annual treatment in a winter broodless period without the use of additional control measures in combination with hygienic behavior. This seems to be the case in the study location (Al Toufailia et al, 2014), especially for colonies that are also hygienic. If this is not the case then additional varroa control methods may be necessary.

Chapter 6

Towards integrated control of varroa: Varroa mortality from treating broodless winter colonies twice with oxalic acid via sublimation

6.1 Abstract

We treated 22 broodless colonies twice in winter with 2.25g of oxalic acid (OA) via sublimation. The first OA application was on 23 December 2014 and the second on 6 January 2015. Mean varroa mortality from this double application was 99.6%, which is greater than the 96.8% mortality from a single treatment shown by our previous research. We determined if double OA application was more harmful to colony performance than a single application by comparing 12 colonies that had been double treated with a control group of 12 colonies that had been treated only once. There was no difference in colony performance on 1 May 2015 (100% survival in both groups; 5.5 frames of brood in single-treated colonies versus to 5.3 in double-colonies). Varroa mortality from the first treatment, 96.8%, was significantly higher from the second treatment 87.2%. Double treatment may well be worthwhile to beekeepers. It is not harmful to colonies and reduces varroa populations to such an extent that 8 doublings would be needed to build back to the original level. Ten of the 22 study colonies were killed 14 days after the second OA application in order to precisely quantify varroa levels. The number of surviving varroa averaged only 6 mites (range 2-18). Worker numbers averaged 5644 bees (range 3352-8692).

6.2 Introduction

Research in our laboratory has shown that applying oxalic acid (OA) via the sublimation method to broodless honey bee, *Apis mellifera*, colonies in winter is highly effective at killing *Varroa destructor* mites (97.6% mortality at a dose of 2.25g; Al Toufailia et al, 2016). However, even greater mortality would be advantageous as this would increase the time taken for the mite population to build back up to harmful levels. Our previous research (Al Toufailia et al, 2014) found that in the year following OA treatment varroa populations built up 7.4-65.4 times (mean 40.2), which is approximately 3-6 doublings. If 2.4% of the mites remain alive after OA treatment, it

would take 5 to 6 doublings for the population to build back up. However, if 97.6% could be killed twice, in successive treatments, so that only 0.0576% (=0.024^2) survived, it would take twice as many doublings to build back up.

In this project we treated broodless colonies twice in winter with 2.25g of oxalic acid via sublimation, with 14 days between treatments. The overall mean varroa mortality was 99.6%, which was greater than the mortality caused by the first treatment, 96.8%, showing that double treatment is worthwhile.

6.3 Materials and Methods

6.3.1 Hive set up, treatment with oxalic acid, and quantifying varroa

The basic method followed Al Toufailia et al (2016). The 22 study colonies were each housed in a single commercial hive box and kept in 2 apiaries 0.75km apart on the University of Sussex campus. Colonies were inspected prior to the first treatment with oxalic acid (OA). Most (n=20) had no brood. The small amounts of brood in the other two colonies (c. 200 sealed cells in each) were removed so that all colonies had zero sealed brood during the period of OA treatment.

OA, 2.25g per hive, was applied via sublimation using a commercially-available applicator used in beekeeping (VARROX® M3080, obtained from E. H. Thorne Ltd., a beekeeping equipment supplier from Wragby, UK). The first treatment was made on 23 December 2014 and the second on 6 January 2015. We used an interval of approximately two weeks for two reasons. First, our previous research (Al Toufailia et al, 2016) had shown that mite fall onto the hive bottom board reduced to background levels within one week of OA treatment. As a result, we considered that the two week interval would be more than sufficient for the effect of the first treatment to be complete. Second, many colonies resume brood rearing in January so that a longer interval would have been impractical.

We took samples of worker bees immediately before (mean ± SD: 304 ±26 bees per colony), and 14 days after (1078 ±102) the first OA treatment. 14 days after the second treatment, 10 colonies (C1-10) were sacrificed so that we could make a total count of the bees and surviving mites. We also took a third sample of worker bees (mean 594 ±32) from the other 12 colonies (C11-22). These 12 colonies were not killed so that their performance could be determined. Only c. 600 workers were sampled to reduce the risk of weakening the colonies excessively and harming their performance.

The 10 colonies that were killed were sealed at the apiary, then placed in a cold room at -18C, and then treated with CO_2. We decided to sacrifice the colonies to increase our sample of worker bees because the number of mites per 100 bees, and therefore the power of our statistical tests, was expected to be very low after two OA treatments. For the same reason we increased the sample size from the first to the second sample. After collection, samples of worker bees were frozen. Mites were extracted from each sample using a jet of water and a metal strainer to collect the mites (Dietemann et al, 2013; al Toufailia et al, 2014, 2016). The number of worker bees per sample was counted by hand.

6.3.2 Subsequent colony performance

The 12 hives twice treated with 2.25g oxalic acid via sublimation were inspected on 5 May 2015 to determine if they were alive, whether they had a queen, and to count the number of frames of brood and bees. To determine if a double application of oxalic acid was more harmful to colony performance than a single application, we compared their performance with a control group of 12 colonies that had been treated only once with 2.25g OA via sublimation on 23 December 2014. Previous research had shown that a single application of OA is not harmful to bees or colonies performance (AL Toufailia et al, 2016).

6.3.3 Statistical analysis

Data were analyzed using 'R' software version (Version 3.2) and the lme4 package. We used GLMM (generalised linear mixed-effect model) to test whether the number of bees in the samples between groups had an effect on the number of varroa extracted. We used binomial response (0 or 1) and included "colony" as a random effect to control for non-independence of bees from the same colony (Zuur et al, 2009). We used ANOVA to compare the difference in mortality between the first and second oxalic acid application taking into account the number of varroa extracted per sample of bees. We also tested the effect of single or double application on colony performance in May. If necessary, we log or arcsine transformed the response variable to meet the assumptions of ANOVA (Grafen et al, 2002; Zurr et al, 2010). $P<0.05$ is defined as significant. Descriptive statistics are given as mean ± standard error.

6.4 Results

The first application of OA caused a mean mite mortality of 96.8±0.3%, range 94.5-98.9%, across the 22 study colonies. The second application resulted in a total mortality of 99.6±0.001%, range 98.6-100%, across both applications (Table 1).

Table.6.1. Numbers of worker bees sampled, varroa mites extracted, and varroa mortality. Colonies C1-10 were sacrificed two weeks after the second oxalic acid (OA) treatment. Colonies 11-22 were allowed to survive by taking only a sample of worker bees two weeks after the second oxalic acid (OA) treatment. Numbers of mites in (bold) extracted from samples of worker bees taken from the study colonies at three points in the experiment are given: 1) immediately before the first treatment with 2.25g of OA via sublimation; 2) two weeks after the first OA treatment and immediately before the second OA treatment; 3) two weeks after the second OA treatment. Using colony C1 as an example, varroa mortality, M, was calculated as follows:
Treatment 1. M_1 = ((26/346) – (4/1027))*100/ (26/346) = 94.8%.
Treatment 2. M_2 = ((4/1027) – (4/3369))*100/ (4/1027) = 69.5%.
Treatments 1 & 2 combined. $M_{1\&2}$ = ((26/346) – (4/3369))*100/ (26/346) = 99.1%.

1. Colony Number	2. Initial Number of varroa (bold) in the sample of worker bees	3. Number of varroa after first treatment	4. First treatment efficacy %	5. Number of varroa after second treatment	6. Second treatment efficacy % varroa mortality	7. Double treatments efficacy % varroa mortality
C 1	**26**/346	**4**/1027	94.8	**4**/3369	69.5	99.1
C 2	**37**/302	**5**/993	95.9	**6**/8692	86.3	99.7
C 3	**89**/351	**6**/967	97.6	**7**/7841	85.6	99.3
C 4	**35**/301	**3**/1115	97.7	**3**/3352	66.7	99.5
C 5	**33**/305	**4**/1088	96.6	**2**/5662	90.4	99.7
C 6	**49**/354	**5**/1091	96.7	**4**/7483	88.3	99.5
C 7	**70**/302	**6**/920	97.2	**18**/5784	52.3	98.6
C 8	**51**/284	**4**/1152	98.1	**7**/5293	61.9	99.2
C 9	**39**/279	**4**/956	97.0	**5**/3976	69.9	99.5
C 10	**64**/312	**7**/1129	97.0	**4**/4987	87.1	99.7
Mean	**49.3**/314	**4.8**/1044	96.8	**6**/5644	75.8	99.4
C11	**29**/266	**3**/986	97.2	**0**/634	100	100
C12	**86**/280	**4**/1113	98.8	**0**/594	100	100
C13	**38**/288	**8**/1095	94.5	**0**/613	100	100
C14	**55**/323	**3**/1006	98.2	**0**/566	100	100
C15	**48**/289	**6**/1255	97.1	**0**/642	100	100
C16	**44**/326	**8**/1257	95.3	**0**/543	100	100
C17	**28**/284	**6**/1233	95.1	**0**/601	100	100
C18	**72**/318	**9**/1125	96.5	**1**/571	78.1	98.6
C19	**56**/262	**4**/955	98.1	**0**/611	100	100
C20	**77**/324	**3**/1109	98.9	**0**/552	100	100
C21	**45**/281	**9**/958	94.8	**1**/581	81.7	98.8
C22	**33**/320	**4**/1179	96.7	**0**/622	100	100
Mean	**50.9**/297	**5.6**/1106	96.7	**0.2**/594	96.6	99.8

We tested whether varroa mortality depended on colony strength (number of frames of bees). As this had no significant effect (F= 0.6; P=0.7), we removed strength from the model. We also tested whether the number of bees in the samples from the 12 colonies versus 10 sacrificed colonies (7130 versus 56439 bees in total) after the second treatment had any effect on the number of varroa extracted (2 versus 60 varroa in total,

respectively) relative to the sample size of bees. As there was no significant difference between the two groups (F= 3.5; P=0.08) the data were combined for further analysis.

Of the varroa that survived the first OA treatment, the average mortality from the second OA treatment was 87.2% ± 3.2% (Figure1). This is significantly different from the mortality of the first treatment 96.8% (F= 46.8; P<0.001) taking into account the number of mites extracted from 100 bees as a covariate (F= 44.7; P<0.001).

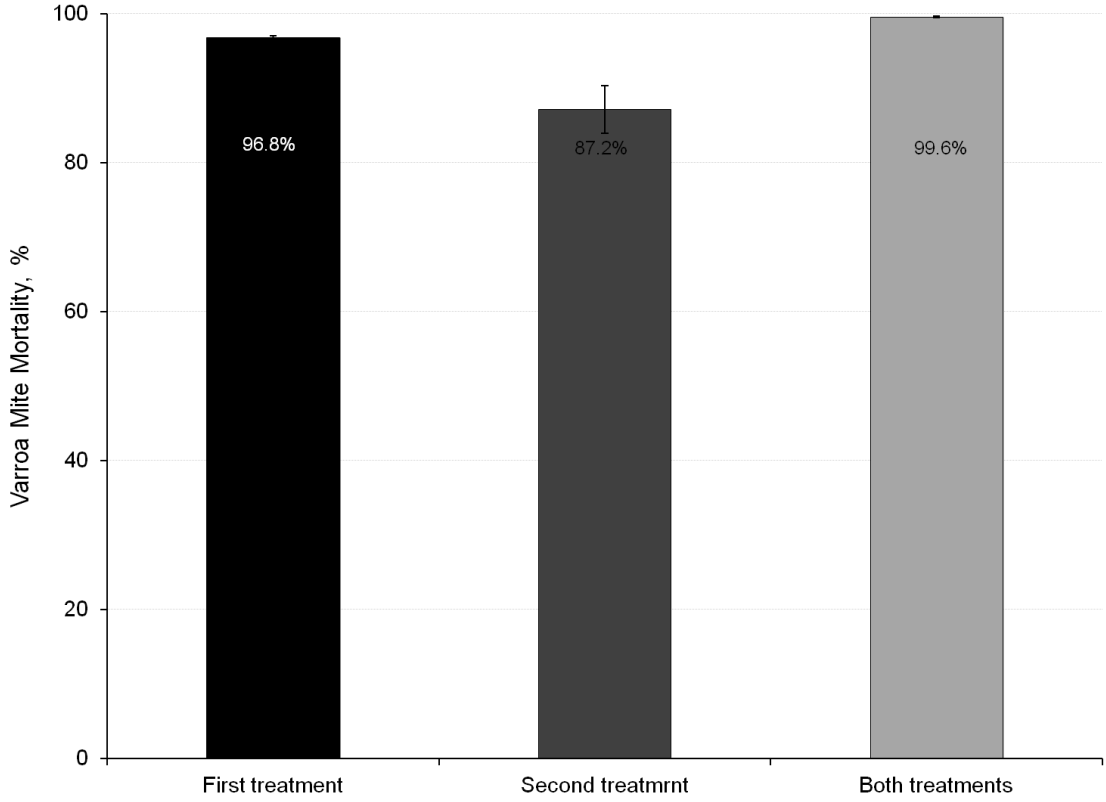

Fig.6.1. Varroa mortality from treatment of broodless colonies in winter with 2.25g oxalic acid via sublimation in the first and second treatments and from both treatments combined. The errors bars show the standard error.

Of the 12 colonies treated twice with OA, all were alive in early spring on 1 May 2015. Of these, 11 had a queen. The queenright colonies had an average of 5.3±0.3 frames of brood. Of the 12 colonies treated only once with OA, all were alive on 1 May 2015, and all had a queen and an average of 5.5±0.2 frames of brood. There is no significant difference in the number of frames of brood between colonies treated twice versus once with OA (F=0.53; P=0.47) or in queen survival (P = 0.5, Fisher's Exact Test, 1-tailed), or colony survival.

6.5 Discussion

Our results show that two treatments of 2.25 g oxalic acid (OA) via sublimation at an interval of 2 weeks in broodless honey bee colonies in winter result in greater varroa mortality than a single treatment, 99.6% v 96.8% (ranges: 98.6-100% v. 94.5-98.9%). Making a second OA treatment was not harmful as the performance (frames of brood, queen and colony survival) of the twice-treated colonies over the next 4 months was not significantly different to the once-treated control colonies.

Varroa mortality from the second OA treatment was lower than in the first, 87.2 v 96.8%. This difference is significant ($P<0.001$). We do not know why the second application was less effective than the first although there are several possibilities. One possible explanation, which would merit further research, is that some varroa are genetically resistant to OA. However, some mites may be less affected due to phenotypic differences that are non-genetic, such as due to age (Kirrane et al, 2012). It is possible that some mites consistently chose more protected phoretic locations on a host bee, or remain on a single host bee that is less contacted by the oxalic acid fumes perhaps because it consistently remains in a location that receives a lower dose of oxalic acid fumes. The final possibility would require that mites remain on the same host bee across the 2 week treatment period. There are currently no data on how frequently phoretic varroa mites change their host bee in broodless colonies, or whether particular bees may consistently be located in parts of the hive more or less contactable by OA fumes from sublimation.

Although the second treatment of OA was less effective than the first the benefits to beekeepers of double OA treatment probably outweigh the costs given that OA is cheap and quick to apply via sublimation and causes no harm to the bees or the colony (this study; Al Toufailia et al, 2016). The main advantage of double treatment is that it will reduce the varroa population to such a low level that it should take at least one year to build up to harmful levels. As a result, annual double treatment with OA in broodless hives may be sufficient to provide full varroa control. The second OA treatment, which gave only 87.2% mortality, will still result in the varroa population requiring approximately 3 additional doublings to reach its original level, and 8 population doublings for both treatments combined. Previous research has shown that a single winter treatment of broodless hives with oxalic acid via sublimation is insufficient to prevent many non-hygienic colonies from showing overt symptoms of deformed wing virus, a pathogen that is vectored by varroa (Al Toufailia et al, 2014). A

further advantage of double OA application is that it could act as insurance in the event that a single application is inadvertently made incorrectly and so kills many fewer varroa than expected.

The ten colonies that we sacrificed in order to count all varroa had an average of just 6 (range 2-18) after double OA treatment. It seems reasonable, therefore, that approximately 4-6 applications of OA could eliminate all varroa from a colony. Provided that such multiple treatments do not harm the colonies it may open up a further avenue of varroa control. Varroa elimination would be most practical for beekeepers with relatively few hives per apiary, with apiaries distant from other apiaries, and who do not move their hives, all of which should reduce recolonization.

Chapter 7

Towards integrated control of varroa: Efficacy of early spring trapping in drone brood

7.1 Abstract

We determined the efficacy of trapping varroa mites, *V. destructor,* in capped drone cells in southern England in spring, when drone rearing was beginning and was at a high level. We introduced two separate test frames of foundation (2/3 drone cells and 1/3 worker cells) into 20 honey bee hives, the first on 2 May, when drone rearing was just starting, and the second on 3 June 2013. Varroa populations were determined per colony by extracting mites from samples of worker bees, the sealed drone and worker cells in the test frame, and from additional samples of sealed worker cells. Samples were collected three times, when the first test frame was introduced, when the first test frame was replaced with the second test frame, and when the second test frame was removed. Colony populations were estimated from photographs of frames of bees and brood. The drone cells in the first and second test frames represented 14.0% and 11.1% of all capped brood cells (drone + worker combined) and trapped 44% and 48% of all the varroa in the colonies, respectively. Sealed drone cells had 13 times as many varroa as sealed worker cells, on average. We conclude that drone trapping is only moderately effective in controlling *V. destructor* within bee hives even when carried out in early spring with a trap frame that allows all the capped drone cells in each hive to be removed. By reducing a colony's varroa population by approximately half, only one population doubling is needed to restore the varroa population to its previous level.

7.2 Introduction

Honey bees, *Apis mellifera,* face many threats (Carreck et al, 2010; Potts et al, 2010; Ratnieks and Carreck, 2010). One of these is the mite *varroa destructor*, which can harm bees both directly (Highfield et al, 2009; van Dooremalen et al, 2012) and indirectly by vectoring virus (Boecking and Genersch, 2008; Brødsgaard et al, 2000; Tentcheva et al, 2004), and can cause colony mortality (Boecking and Genersch, 2008; Guzmán-Novoa et al, 2010).

In recent years varroa has become harder to control because of mite resistance to widely used synthetic acaricides such as fluvalinate, which is the active ingredient in Apistan. Other varroa control methods include natural compounds such as oxalic acid, formic acid, lactic acid and essential oils (Emsen and Dodologlu, 2009; Nanetti et al, 2003; Satta et al, 2005), natural resistance such as hygienic behaviour (Rinderer et al, 2010; Spivak, 1996) and grooming (Andino and Hunt, 2011; Boecking and Spivak, 1999), and biotechnical methods such as trapping mites in sealed drone cells (Calderone, 2005; Charrière et al, 2003).

Varroa mites are particularly attracted to drone brood (Fries et al, 1994; Fuchs 1990; Fuchs and langenbach 1989), in which they are able to produce more offspring (Ifantidis, 1984; Martin, 1994; Sammataro and Avitabile, 1998). A female mite can produce approximately three daughters in an *A. mellifera* drone cell versus one in a worker cell (Fuchs and langenbach, 1989; Martin, 1998; Schulz, 1984). A frame of drone cells can trap 30-70% of the varroa (Calis et al, 1999; Charriere et al, 2003) or even 95% when a frame of drone brood is placed into an otherwise broodless colony with a caged queen (Calis et al, 1999; Boot et al, 1995; Fries and Hansen, 1993).

The aim of our study was to determine the efficacy of trapping varroa in drone cells in early spring. Trapping at this time of year has practical advantages. It is when the first drones of the year are being reared and drone rearing is intense (Page and Metcalf, 1984). Bee hives also have fewer bees in them and so are easier to manipulate. In addition, spring drone trapping does not preclude subsequent drone rearing for mating and bee breeding. Our results show that a test frame of sealed drone comb contained almost half of a colony's varroa. We conclude that drone trapping is only moderately effective in controlling *V. destructor* within bee hives.

7.3 Materials and Methods

7.3.1 Study colonies and data collection

We studied 20 colonies located in one apiary at Steyning, West Sussex, in southern England in spring 2013. Each was housed in a hive consisting of a single "commercial" brood chamber (11 43.8x25.4cm frames, volume 56.4 litres), bottom board with mesh floor with a sticky white plastic sheet underneath, inner cover and telescopic cover. Each hive was given a queen excluder and honey supers as needed. All queens were marked and clipped. All colonies had varroa.

Spring 2013 was unusually late. On 15 April, when nectar was starting to be collected easily, the colonies were equalised so that each had one frame of honey and pollen, and four frames with brood. On 2 May 2013 the first eggs were observed in drone cells. We then introduced the first test frames, one per hive. Each test frame was 2/3 drone cell foundation and 1/3 worker cell foundation, with the worker cells above. We included worker foundation so that each frame would also provide a sample of sealed worker cells.

Hives were inspected every 9 days until the end of the experiment 2 months later. Any drones being reared in small patches of drone cells in other frames were scraped out with a hive tool before they were sealed. Brood frames were neither removed nor added. None of the test colonies swarmed and all remained queenright.

The first test frames were removed on 3 June, before adult drones or workers had started to emerge from the newly-constructed cells. They were replaced with the second test frames, one per hive, placed next to the brood. The second test frames were removed on 28 June. All were frozen at -20C for later analysis. To determine colony populations of worker bees and brood, we took photos of each side of each frame on 2 May, 3 June and 28 June.

7.3.2 Determining varroa numbers on worker bees

To estimate the number of phoretic adult female mites on worker bees, we collected one sample of worker bees per hive on 2 May, 3 June and 28 June. Samples of worker bees were sufficiently large (mean ± SD: 248 ± 11.12), range (218 - 269) to extract many mites. The samples were frozen. Subsequently, the mites were washed off the bees using a jet of water and caught in a fine metal screen (Al Toufailia et al, 2014; Al Toufailia et al, 2016; Dietemann et al, 2013). The number of bees per sample was also counted. The number of mites, all of which are adult females, per 100 worker bees was calculated.

7.3.3 Determining varroa numbers in sealed worker cells

We collected samples of sealed worker cells from each hive on 2 May, 3 June and 28 June. Each colony provided a patch from the middle brood frame plus the patch comprising the top third of the test frame. Samples were sufficiently large (mean = 367.78 ± 27.31 worker cells, range = 301-450) to extract many mites. Samples were frozen. Subsequently, cells were uncapped using a knife. The larva or pupa was

removed by forceps to count the adult mother varroa mites in each cell. A cotton swab was then used to remove and count any adult female varroa in the cell not on the larva or pupa.

The uncapped cells were then washed with a jet of water and any varroa caught in a fine metal screen. The number of worker cells per sample was also counted. The number of adult female mites per 100 worker cells was calculated.

7.3.4 Determining varroa numbers in sealed drone cells

We used the same methods to count mother mites in sealed drone cells. We uncapped a large sample of cells from each test frame (mean = 314.88 ± 14.82 drone cells, range 300-356). The number of drone cells per frame was also counted.

7.3.5 Determining colony strength in terms of sealed brood cells and adult bees

From our photographs we estimated colony strength using the method of Imdorf and Gerig (2001). Photos were taken on cloudy or drizzly days when most bees were inside the colony.

Calculations made after analysing the photos to determine the total number of varroa in each location of the mite. For example, if there were 10 mites per 100 bees in a sample of 300, and the hive has 9000 bees in total, then there are 10 x (9000/100) = 900 phoretic mites.

7.3.6 Statistical analysis

Data were analyzed using IBM SPSS statistical program version 20. If necessary, we log or arcsine transformed the response variable to meet the assumptions of ANOVA (Grafen et al, 2002; Zuur et al, 2010). We then used ANOVA to test the effects of the application time (frame1&2) on varroa population. $P<0.05$ is defined as significant. Descriptive statistics are given as mean ± SE (standard error).

7.4 Results

At the beginning of the experiment on 2 May all varroa were in the sealed worker cells (47.9%±8.7) or phoretic on adult worker bees (52.1%±8.7) as there were no sealed drone cells (Figure 1).

On 3 June colonies had, on average, 857±86 sealed drone cells and 5269 ±149 sealed worker cells (14.0% drone cells). Although sealed worker cells were c. 6 times

more numerous than sealed drone cells, more varroa were in the drone cells (44.4%± 8.6) than in the sealed worker cells (29.6%±8.0); 26.0%±6.4 were phoretic on adult workers (Figure1).

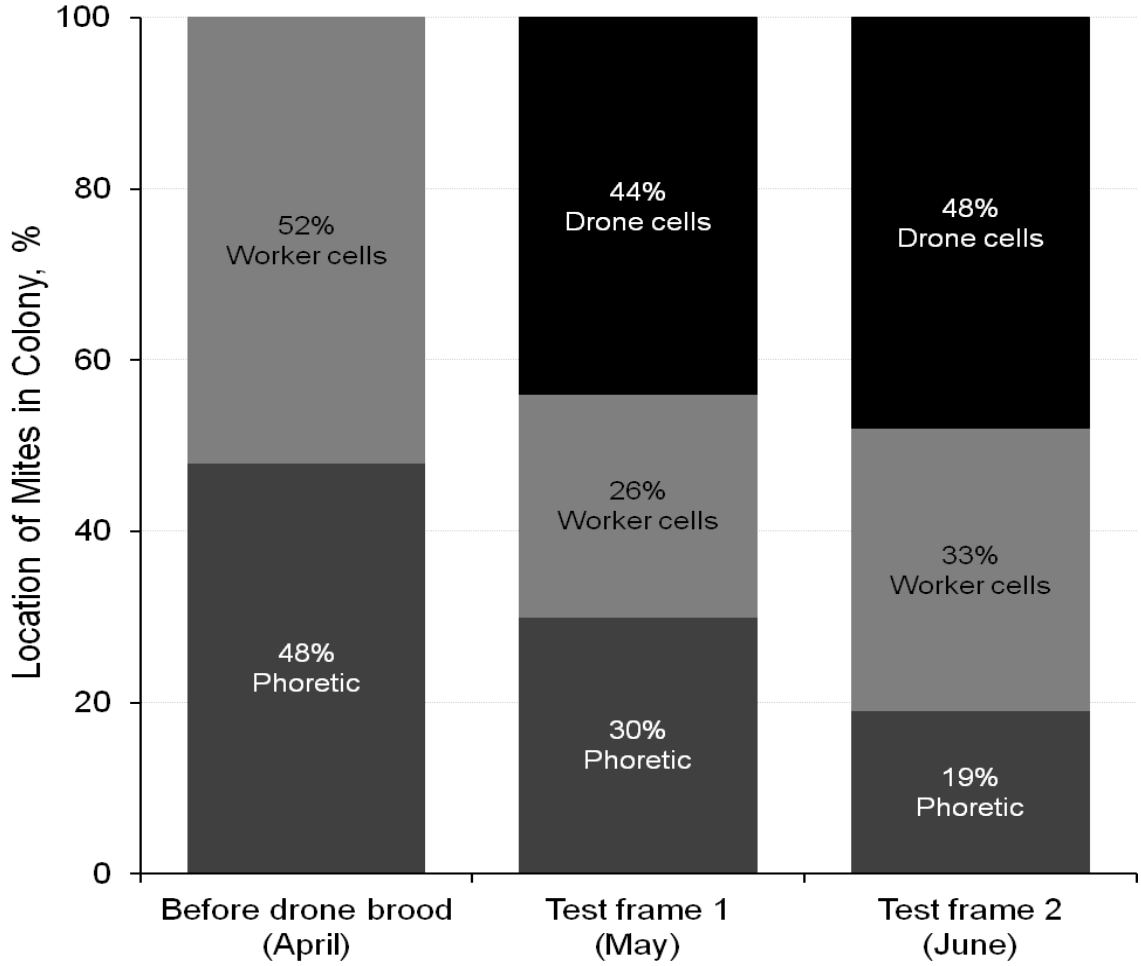

Fig.7.1. Proportion of varroa mites in different colony locations (in sealed drone cells, in sealed worker cells, phoretic on adult workers). In April colonies had no drone brood. The first test frame of drone cells, May, trapped 44% of the varroa mites on average, and the second test frame, June, trapped 48%.

On 28 June colonies had, on average, 807 ±79 sealed drone cells and 6457 ±301 sealed worker cells (11.1% drone cells). As before, although sealed worker cells were approximately 8 times more numerous than sealed drone cells, more varroa were in the drone cells (48.1% ±5.2) than in the worker cells (33.0% ±3.8); 18.9% ±5.5 were phoretic on adult workers (Figure1).

The proportions of varroa in the three locations (in drone cells, in worker cells, phoretic on adult bees) are not significantly different between the May and June tests trials (Anova, F= 0.449; P=0.507).

Discussion

Our results show that trapping varroa in capped drone cells in early spring is reasonably effective at controlling varroa. The first and second test frames of drone foundation removed 44% and 48% of the varroa, respectively (Figure 1).

Our colonies had 7 times more sealed worker cells than drone cells, on average. However, each drone cell had 13 times as many varroa mites, on average, in agreement with previous research which gave values of 5.5-12 (Boot et al, 1995a; Fries et al, 1994; Martin, 1998; Schulz, 1984; Sulimanovic et al, 1982). In our study colonies, more of the varroa were in the relatively few drone cells than in all the worker cells (44% v 26% in May, 48% v 33% in June; Figure 1).

Capped drone cells in test frames 1 and 2 were 14.0% and 11.1% of all capped brood cells in the colonies. This indicates that a single frame with 2/3 drone foundation was sufficient for drone rearing needs in early spring, given that colonies were not of large population. However, beekeepers could use a frame entirely of drone foundation. We used 1/3 worker foundation in part to provide a sample of capped worker cells from which to count varroa.

Overall, our results indicate that trapping in drone brood in spring is probably not sufficiently effective to be able to control varroa populations on its own. It is, for example, far less effective than the 97% kill that can be achieved by applying oxalic acid once via sublimation to broodless hives in winter (Al Toufailia et al, 2016). By killing only approximately half the varroa mites in a colony, the varroa population will build back up to its original level in only one doubling. By contrast, when 97% are killed and 3% survive, it will take approximately 5 doublings for the population to build back to its original level.

Varroa trapping in drone comb may, nevertheless, have a role as part of a program of integrated varroa control. In addition, some beekeepers may value it as a method that does not use chemicals and so may be considered more natural. However, one disadvantage is that it will reduce drone rearing. This would be disadvantageous for beekeepers who are also breeding and selecting bees, such as for hygienic behaviour or other desirable traits. However, one or two rounds of drone brood trapping as early as possible in spring will not prevent subsequent drone rearing and the mating of queens to selected drones one or two months later that would otherwise be possible without drone trapping. Although additional work, it would also be possible not to kill the drone brood following trapping but to place the frames of capped cells in queenless hives for

emergence. The varroa mites in these hives could then be killed using oxalic acid or other methods after the drones have emerged.

Chapter 8

Towards integrated control of varroa*:* Monitoring honey bee brood rearing in winter and the proportion of varroa in small patches of sealed cells

8.1 Abstract

Oxalic acid is used by beekeepers to control varroa mites, *Varroa destructor*. Oxalic acid is effective against varroa that are phoretic on adult bees but not against varroa in sealed brood cells. In Britain and other temperate areas, brood rearing diminishes in winter and may even fall to zero providing a natural window of opportunity for varroa control with oxalic acid. We monitored brood rearing from September to March for 3 years in hives located in Sussex, southern Britain. We also sampled varroa phoretic on adult bees and in brood cells to determine the proportion of varroa protected from oxalic acid in brood cells. Our results showed that December is the month with the least brood. However, winter reduction in brood rearing varied among years and even in December some colonies still had sealed brood (range 9 - 52 %, n = 4 winters). Although the amounts of sealed brood were low, even a small patch of c. 500-600 sealed cells (300 cm^2) could contain 14% of the varroa in a colony. This will halve the duration of control provided by an oxalic acid treatment. Our results indicate that beekeepers should check their hives in winter if they plan to apply oxalic acid. This will show which month(s) have the least capped brood, and which hives have patches of capped brood to remove.

8.2 Introduction

Oxalic acid has been used by beekeepers for several decades to control varroa mites, *Varroa destructor* (Aliano and Ellis, 2008; Marinelli et al, 2006; Nanetti et al, 2003; Rademacher and Imdorf, 2004; Popov et al, 1989). Oxalic acid kills varroa phoretic on the bodies of adult bees (Charriére and Imdorf, 2002; Gregorc and Planinc, 2002; Nanetti et al, 1995) but not those in sealed brood cells (Gregorce and Poklukar, 2003). As a result, it is most effective when applied to colonies without sealed brood (Marinelli et al, 2000; Bacandritsos et al, 2007; Nanetti and Stradi, 1997). In a broodless colony, one application of 2.25g of oxalic acid via the sublimation method kills 97% of the

varroa (Al Toufailia et al, 2016) and two applications at an interval of 10 days kill 99.6% (Chapter 6).

Colonies without capped brood can occur in various ways. For example, the queen can be caged to prevent egg laying (Jabde, 2005; Schmidt-Bailey et al, 1996; Van Dung et al, 1997). However, as the brood period is 21 days for workers and 24 days for drones (Seeley, 2009; Winston, 1991), the queen needs to be caged for approximately 3 to 4 weeks before treating with oxalic acid. In the biology of the honey bee, colonies without capped brood occur during swarming (Winston, 1991), after emergency queen replacement, and, in temperate areas, in winter when there is a natural reduction in brood rearing (Jeffree, 1956; Nolan, 1925).

The aim of this study was two fold. First, to determine the winter month when sealed brood is lowest in Sussex, southern Britain. Second, to determine the proportions of varroa mites in sealed brood in winter, and especially the proportion in small patches of sealed brood that beekeepers might think were too small to have much effect.

8.3 Materials and Methods

8.3.1 Study site

Colonies were located in apiaries in or within 20 km of the University of Sussex in southern England, UK. Sussex has a temperate climate with a mild winter. Winter foraging varies across years, but is generally zero or close to zero in December and January and low in November and February.

8.3.2 Investigation 1: Sealed brood amount, September to March, 2012-2015

This part was carried out in the winters of 2012-3 (n = 28 colonies), 2013-4 (n = 30), and 2014-5 (n = 34). Colonies were housed in hives consisting of a single "commercial" brood chamber (11 frames each 43.8 x 25.4 cm, volume 56.4 l), bottom board, inner cover and telescopic cover, were queenright and with adequate honey stores for overwintering, and were kept in two apiaries with an equal number in each. Data were collected from September to March, which is from early autumn to early spring. Colonies had previously been equalised to four frames of brood in late August.

Hives were inspected in the middle of each calendar month, day 15-19, to check for queen presence and colony strength. Photos were taken of each frame with bees or sealed brood. Photos were taken on cloudy or drizzly days when all the bees were in the hive. From the photographs we estimated the numbers of bees and sealed brood cells

(Imdorf and Gerig, 2001). When the amount of sealed brood was low the numbers of sealed cells were counted directly.

8.3.3 Investigation 2: Sealed brood amount in December and January, 2012-2016

From our beekeeping experience in the study area we knew that brood rearing was lowest in mid winter, December and January. Therefore, we gathered additional data for these months using other full-sized colonies belonging to our laboratory in 10 apiaries. Sample sizes were: 2012-3, n = 110 (December), 28 (January) colonies; 2013-4, n = 140, 30; 2015, n = 132, 34; 2015-6, n = 64, 20.

8.3.4 Investigation 3: Proportions of varroa in sealed brood

The aim of this part was to estimate the proportion of varroa in sealed worker brood cells versus phoretic on adult workers in order to determine the degree to which even small patches of brood can reduce the proportion of phoretic varroa, and so make treatment with oxalic acid less effective.

In 2012 twelve colonies were monitored once per calendar month from September to December, as brood rearing diminished going into winter. Colonies were set up as in Investigation 1. However, all were strong colonies as used in commercial beekeeping with 8 frames of bees and 5 with brood in September. To estimate the proportion of phoretic adult female varroa we collected and froze one sample of worker bees (mean 861.16 ± 75.43 SD; range: 799-1265 adult worker bees) per month. We also took and froze one frame with a patch of sealed brood. The number of sealed cells varied considerably (544.27 ± 370.46; 18-1120) as at some inspections, especially in December, there was little brood in some hives. As in Investigation 1, we took photos to estimate the numbers of sealed brood and adult bees. No sealed drone cells or adult drones were observed in the study colonies during the data collection period.

Data collection was repeated in 2013 using 12 different colonies kept in the same apiary as 2012. The sample sizes were 839.14 ± 43.54 (765-933) adult bees and 826.43 ± 244.55 (365-1463) sealed cells. Because bee and brood samples were taken from these study colonies, the brood levels seen in November and December may have been higher than in unmanipulated colonies, to compensate for this loss. However, this is not a problem for the investigation as its aim was not to monitor brood levels, but to determine the effect of brood amount on the proportion of phoretic varroa.

8.3.5 Counting varroa on worker bee samples

Following established methods, the frozen bees were washed with a jet of warm water for 5 minutes to dislodge the varroa, which were caught in a fine metal screen with 20 wires per cm (Al Toufailia et al, 2014; Al Toufailia et al, 2016; Dietemann et al, 2013).

8.3.6 Determining varroa numbers in sealed worker cells

Following previous methods (Chapter 7) worker cells were uncapped with a knife after thawing out. The larva or pupa was removed from each cell and any adult female varroa mites were counted. The cell was then washed with a jet of water and any varroa caught in a fine metal screen to count any additional adult females.

8.3.7 Determining the proportion of phoretic varroa per colony

The total number of varroa in a particular colony on a given sampling day was calculated using the capped brood and adult worker bee populations estimated from the photos, combined with the numbers of adult female varroa extracted from samples of adult workers and capped cells. For example, if there were 10 varroa per 100 cells or bees, and the hive had 5000 sealed cells or bees in total, then the estimate is 10 x (5000/100) = 500 mites in the sealed brood or phoretic.

8.3.8 Statistical analysis

Data were analyzed using the IBM SPSS statistical program version 20. If necessary, we log or arcsine transformed the response variable to meet the assumptions of ANOVA (Grafen and Hails, 2002; Zuur et al, 2010). In investigations that used multiple apiaries or years, we then used ANOVA to test the effect of apiary or year. Colony strength, in terms of bees and brood, was initially included in the analyses as a covariate and removed where it was not significant. Finally, we used Tukey's post-hoc test to compare months of different years (Investigation 2). The level of significance was $a = 0.05$. Descriptive statistics are given as mean ± standard error.

8.4 Results

8.4.1 Investigation 1: Sealed brood amount, September to March, 2012-2015

We found no significant effect of apiary (F=0.12; P=0.73) or colony strength (F=0.96; P=0.33) on brood amount (2012-3: F=0.22, P=0.64; 2013-4: F=0.49, P=0.48; 2014-5: F=0.004; P=0.95). This was as expected as the colonies had been equalized and the

apiaries were close. We then pooled the data across apiaries to compare brood rearing across the three study years. There was a significant effect of year on brood amount (F=3.02; P=0.049). From the *post hoc* Tukey's test the only two-year comparison that was significant was 2012-3 versus 2013-4 (P =0.04) (2012-3 to 2014-5: P=0.22; 2013-4 to 2014-5: P=0.66). The number of bees in the colony also had a significant effect on the amount of capped brood (F= 591.82; P<0.001).

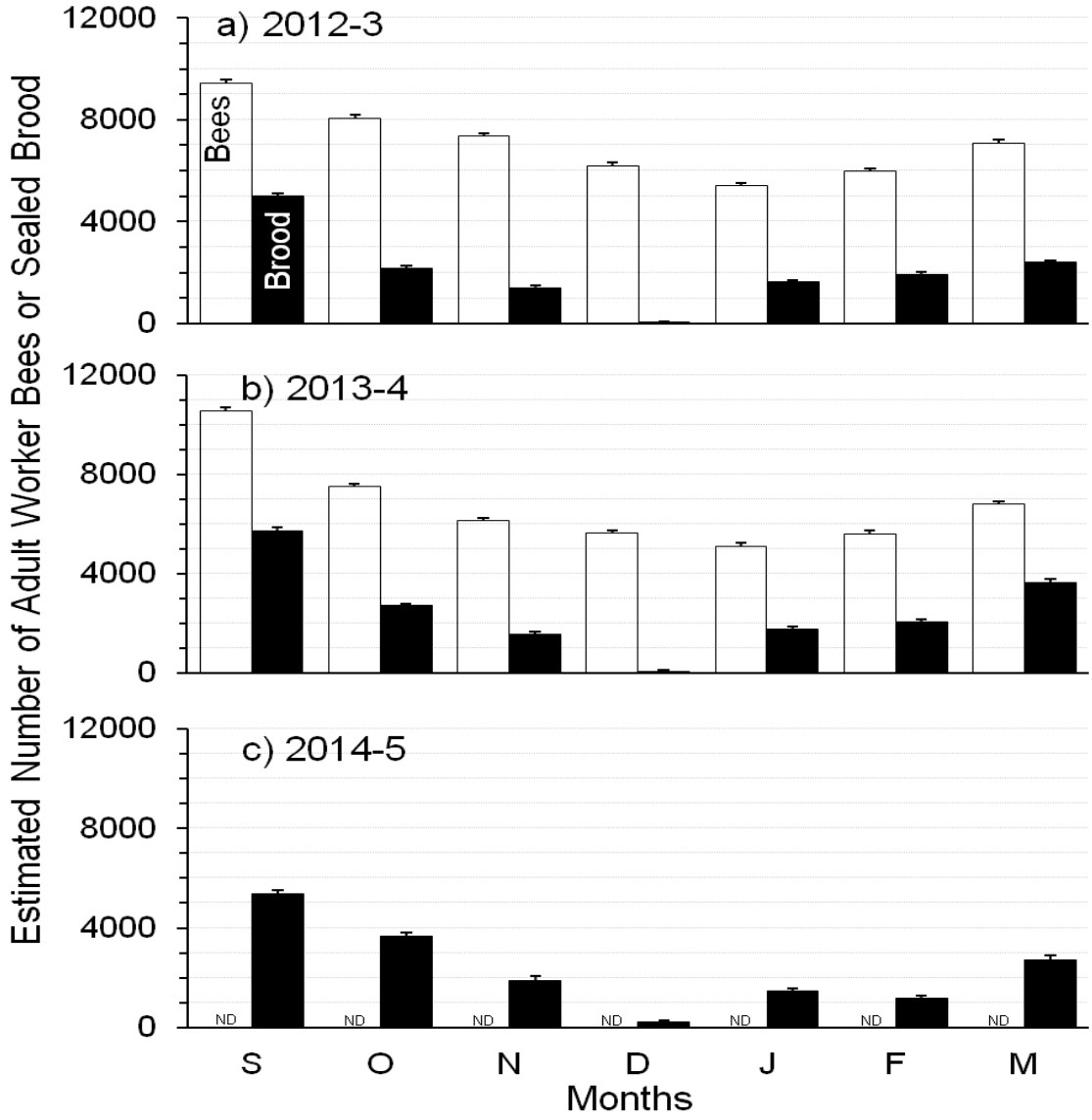

Fig.8.1. Estimated numbers of sealed brood cells (back bars) and adult worker bees (white bars) from September to March in the three study years: a) 2012-3, n = 28 colonies; b) 2013-4, n = 30; c) 2014-5, n = 34). No data on the number of adult bees were collected in 2014-5. Bars show the standard error.

Figure 1 shows that in each of the three study years the number of sealed brood cells was least in December, with the numbers of capped cells across the study colonies

averaging 43, 29, and 211 (mean of the 3 year means = 94) cells in the winters of 2012-3, 2013-4 and 2014-5, respectively. This is 17.2 times less than in November and 17.3 times less than in January (November: 1400, 1552 and 1906 (mean of 3 years = 1619); January: 1631, 1782 and 1473 (mean of 3 years = 1629).

8.4.2 Investigation 2: Sealed brood amount in December and January, 2012-2016

Figure 2a shows that in January 100% of the colonies had sealed brood in each of the 4 study winters. However, in December the proportion with capped brood was less than 100%, ranging from 9% to 52%. This difference between January and December is significant ($F=452.27$; $P<0.001$). There is no significant effect of year on the proportion of colonies with brood in January ($P=1.00$). However, there is great variation among years in the proportion of colonies with brood in December ($F=22.28$; $P<0.001$). The *post hoc* Tukey's test showed a significantly greater proportion in 2015 (versus 2012: $P < 0.001$; 2013: $P < 0.001$; 2014: $P < 0.001$), and in 2014 versus 2013 ($P = 0.01$). Differences were not significant in 2012 versus 2013 ($P = 0.96$) or 2014 ($P=0.066$).

Figure 2b shows that in the colonies that had sealed brood, the mean number of sealed brood cells is much lower in December (578, range 452-788) than in January (1405, range 1208-1582). This difference is significant ($F=171.58$; $P<0.001$). In addition, there is a significant effect of year on brood amount in January ($F=2.87$; $P=0.04$). From the *post hoc* Tukey's test the only two-year comparison that was significant was 2014 versus 2016 ($P =0.02$) (Jan-13 to 14: $P=0.58$; Jan-13 to 15: $P=0.99$; Jan-13 to 16: $P=0.34$; Jan-14 to 15: $P=0.36$; Jan-15 to 16: $P=0.43$).

There is also great variation among years in the number of sealed brood in December ($F= 4.17$; $P=0.008$) with significantly more in 2015 (than in 2012: $P =0.03$; 2013: $P=0.04$). Differences were not significant in 2012 (versus 2013: $P = 0.99$; and versus 2014: $P=0.59$) and in 2013 (than in 2014: $P = 0.70$) and in 2014 (than in 2015: $P =0.15$).

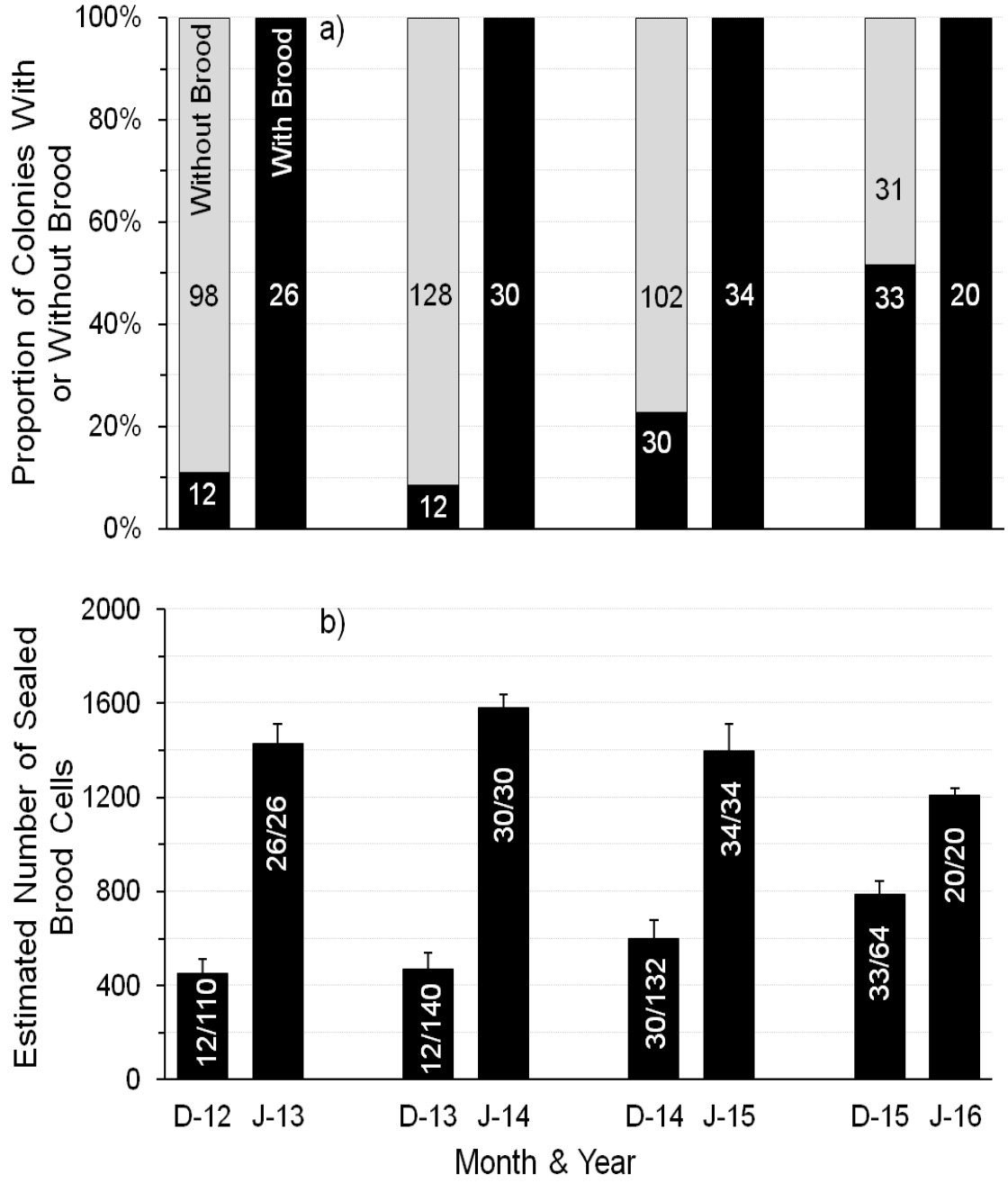

Fig.8.2. Sealed brood in December and January in four study winters, 2012-3 to 2015-6. a) Proportion of colonies with sealed brood; numbers in bars indicate the number of colonies with or without brood. b) Estimated mean number of sealed brood cells only in the colonies with non-zero sealed brood; numbers in bars indicate the number of colonies with brood divided by the total number of colonies. Bars show the standard error.

8.4.3 Investigation 3: Proportion of varroa in sealed brood from September to December

The study colonies had medium to high levels of varroa. At the start of each year's data collection, the number of adult female varroa per 100 sealed cells was 28.8 ± 11.9

(range: 12-53) in September 2012 and 18.3 ± 5.1 (range: 10-27) in September 2013 (n = 12 hives per year). The number of phoretic varroa per 100 adult worker bees was 6.7 ± 2.4 (range: 3-11) in September 2012 and 12.9 ± 2.1 (range: 9-16) in September 2013.

Figure 3 shows that the proportion of varroa in sealed cells reduced from September to December in both 2012, from 56.1 to 14.3%, and in 2013, from 64.3% to 13.9%. As there was no significant effect of year (F= 2.82; P=0.097) we pooled the data across both years. As expected from Investigation 1, the amount of sealed brood differed among months. We found a significant effect of the amount of sealed brood (F= 20.59; P<0.001) and month (F= 9.16; P<0.001) on the proportion of varroa in sealed cells versus phoretic.

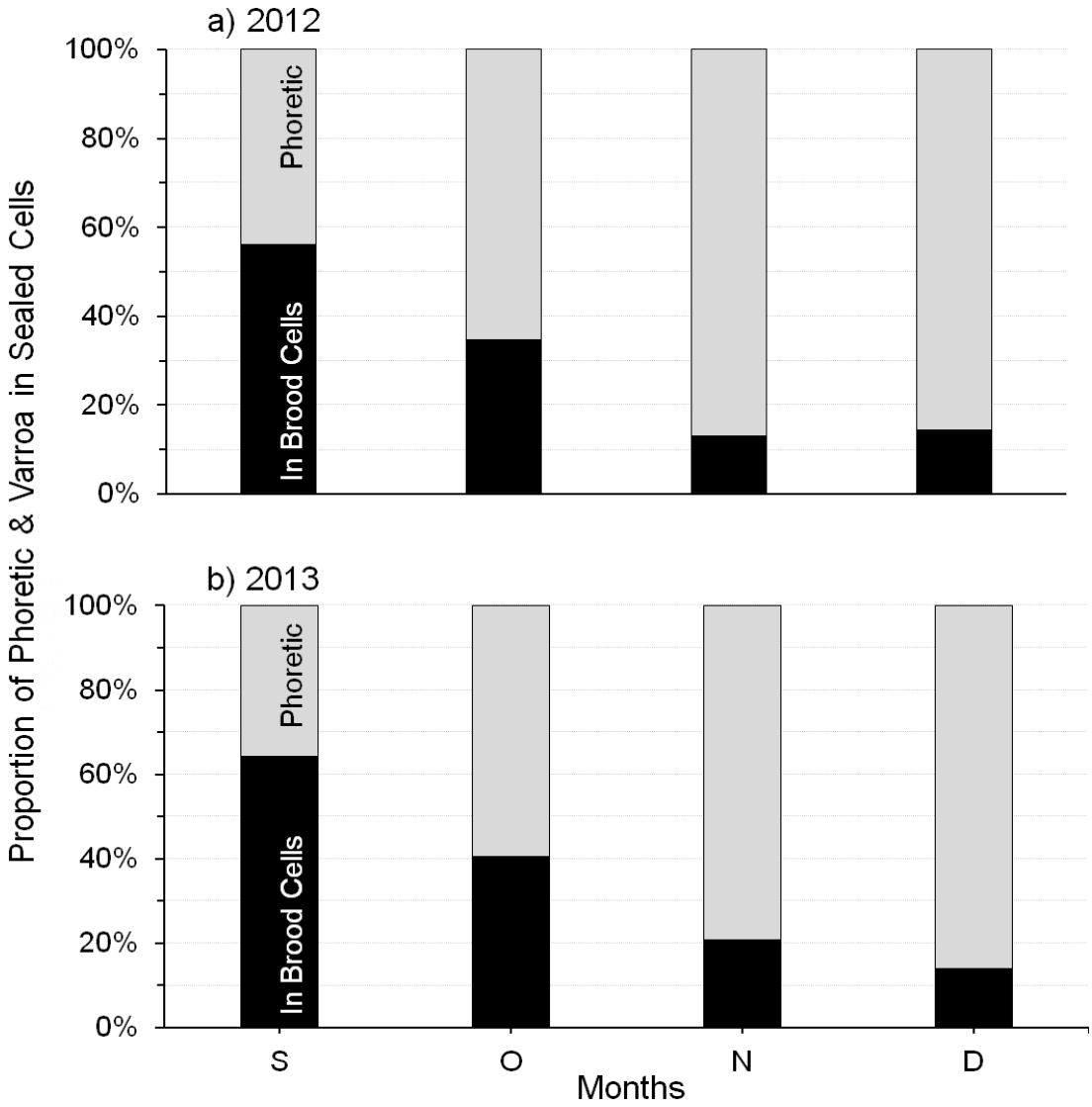

Fig.8.3. Estimated proportions of varroa phoretic on the bodies of adult worker bees versus in sealed brood cells (black bars) from September to December in 2012 and 2013, n = 12 colonies in each year.

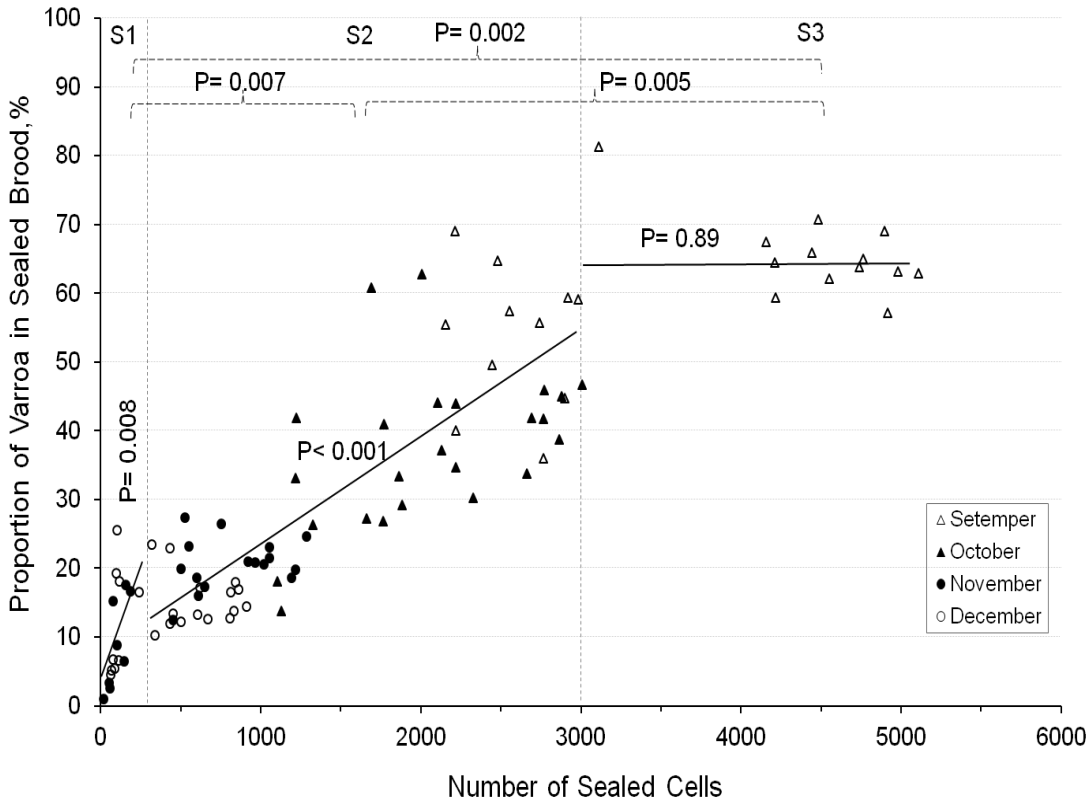

Fig.8.4. Relationship between the number of sealed brood cells in a colony and the proportion of varroa in the sealed cells (versus phoretic on adult bees). The relationship appears non-linear with the proportion in sealed cells reaching a maximum of c. 60-70%. Therefore, we divided the data points into three groups, S1, S2 and S3, corresponding to higher gradient (1-250 sealed cells), lower gradient (251-3000), and horizontal (>3000). The plotted lines are the least squares linear regressions for these 3 data sets. The gradients of S1 and S2 are both significantly greater than 0, but S3 is not. The three regressions are also significantly different from each other. The data were collected from 12 hives in 2012 and 12 in 2013, one estimate in the middle of each month September to December.

Figure 4 uses the same data as Figure 3 but plots the data to show the relationship between the amount of sealed brood and the proportion of adult female varroa in sealed brood cells versus phoretic on adult bees. The relationship appears non linear, with a higher gradient near the origin, suggesting more adult female varroa per sealed brood cell, and appearing to level off at high numbers of sealed brood to approximately 64%, on average. We tested this by determining the gradients of the linear regression lines for subsets of the data corresponding to these three parts of the relationship: <250 sealed cells (Subset 1), 251-3000 sealed cells (Subset 2), and >3000 sealed cells (Subset 4). The gradients are significantly greater than zero for S1 (P = 0.008) and S2 (P< 0.001), showing an increasing total proportion of adult varroa in sealed brood cells as the number of cells increases to 3000. The gradient for S3 is not

significantly different to zero (P= 0.89), showing that the relationship levelled off after approximately 3000 cells. The differences between subsets are highly significant (S1 v. S2, P = 0.007; S2 v S3, P = 0.005; S1 v. S3, P = 0.002; all P values include Tukey's corrections). The comparison between the gradients for S1 and S2 confirm the visual impression from the figure that there are significantly more varroa mites per sealed cell when the number of sealed cells is lower.

8.5 Discussion

The results of investigations 1 and 2 (Figures 1, 2) show that December is the best month in the study area to treat honey bee colonies with oxalic acid as this month has the least amount of sealed brood, both in terms of the proportion of colonies with zero sealed brood and in the number of sealed brood cells in the colonies with brood. Figure 2 show that in mid January all colonies had sealed brood, showing that the gap in brood rearing in the study area is in early winter (December). However, in none of the 4 study years did all colonies have zero sealed brood in December.

The amount of sealed brood differs greatly between years. The variation in December, the month with the least sealed brood and so the best month for oxalic acid treatment, is of particular importance in terms of oxalic acid treatment and ranged from 9 to 52% with sealed brood.

The amount of sealed brood in December 2015 was particularly high, both in the proportion of colonies with sealed brood (52%) and in the number of sealed brood cells (mean = 788 ± 336) in the colonies with brood. This was almost certainly due to the warm conditions in autumn and early winter. Temperatures in December 2015 (mean maximum 13.5C, mean minimum 9.9C) were higher than in the other years (2014: 9.5C; 3.9C; 2013: 10.8C, 4.8C; 2012: 9.6C, 4.3C). We noted that ivy, *Hedera* spp., the main source of autumn pollen and nectar for honey bees in the study area (Garbuzov and Ratnieks, 2014), flowered for an unusually long period in 2015. Honey bees were still collecting ivy pollen in early December (FR personal observations).

Figure 4 shows that the proportion of varroa in sealed cells reaches a maximum of c.70%, and that when colonies have little sealed brood, there are more varroa per cell. This means that even the small numbers of sealed brood cells seen in December (means of 452, 472, 601, 788 for 2012-5, respectively [Figure 2b]) could protect approximately 11-18% of the varroa from oxalic acid (Figure 4).

The proportion of varroa surviving a treatment of oxalic acid, P_{surv}, is

$$P_{surv} = P_cS_c + P_pS_p \qquad \text{(equation 1)}$$

Where P_c and P_p are the proportions of varroa in sealed brood cells and phoretic, and S_c and S_p are the proportions that survive at each location. S_c is close to 1, as oxalic acid is ineffective at killing varroa in sealed brood cells (Gregorce and Poklukar, 2003) and S_p is approximately 0.03 for the most effective oxalic acid application methods (Al Toufailia et al, 2016). From equation 1, the proportions of varroa surviving an oxalic acid treatment in a colony with 5, 10, or 14% of the varroa in sealed cells are (0.05 + 0.95*0.03) = 8%, (0.1 + 0.9*0.03) = 13%, and (0.14 + 0.86*0.03) = 17%.

These increases in varroa survival will considerably reduce the time that it will take for the varroa population in a colony to increase back to its original level after oxalic acid treatment. In a colony without sealed brood only 3% of the varroa will survive oxalic acid treatment (Al Toufailia et al, 2016), and it will take just over 5 doublings for the population to build back up to 100% (i.e., 3, 6, 12, 24, 48, 96%). But if 5% of the varroa are in sealed cells, so that survival increases to 8%, it will now take only 3 to 4 doublings to build back up (8, 16, 32, 64, 128%). The figures for 10% and 14% varroa in sealed cells are close to 3 doublings and 2.5 doublings, respectively (13, 26, 52, 104%; 17, 34, 68, 136%). This means that if only 14% of the varroa are in sealed cells (Figure 3a and b), this will reduce the duration of control (number of doublings need to build back up) from an oxalic acid treatment by 50%. Figure 4 shows that in our study colonies it requires only approximately 500-600 sealed cells, equivalent to a small patch of c. 300 cm^2, to allow 14% of a colony's varroa to survive.

In the study area, the varroa population in a colony builds up approximately 40 fold, on average, in the year following oxalic acid treatment (Al Toufailia et al, 2014). This is equivalent to just over 5 doublings. This means that oxalic acid treatment to a broodless colony that kills 97% of the varroa provides approximately one year's control. However, this would drop to half a year if the treated colonies had 300 cm^2 of sealed brood, and will be less than one year for even any amount of sealed brood at the time of treatment.

The aim of this project was to provide practical information to beekeepers. The results show clearly that even small amounts of sealed brood will lower the effectiveness of oxalic acid in killing and controlling varroa. This means that

beekeepers should check their colonies before applying oxalic acid and remove any small patches of capped brood. It is not enough to assume that because it is winter the colony is not rearing brood. The results show that in the study area there is a consistent month, December, in which the amount of brood rearing is lowest. However, capped brood never fell to zero in all colonies in December, and also varied year to year, with a mild autumn in 2015 leading to greatly increased amounts of sealed brood in December 2015. This shows the importance of understanding local conditions and how these vary year to year.

In Aberdeen, Scotland, Jeffree (1956) inspected 367 colonies over 9 years and found that brood rearing was least in October and November, with only 14% and 25% of the colonies having brood, respectively, and with an average area of 2 square inches (= 12.5 cm^2). He also found that in December and January more brood was present (10 and 14 square inches) with 58% and 50% of the colonies having brood, respectively. Butler (1954) noted that honeybee colonies in Britain contain very little brood between the middle of November and February and commented "sometime after Christmas, usually early in February, brood rearing begins again". Our results show somewhat different patterns even though all three sets of observations are from Britain. This suggests that winter brood rearing conditions vary in different locations in Britain, or have changed in the past 80 years. In Wisconsin, a northern US state with a severe winter, Farrar (1934) noted that brood rearing was least in January. These differences among locations in winter brood rearing make clear the importance of determining winter brood rearing locally.

Beekeepers are often recommended not to open their hives in winter (Morse, 1975). However, in our experience hives can be opened in winter on suitable days (no rain, low wind mild temperatures, c. 6-12C) without harming the colony. For example, 87 of 89 hives that we inspected in December to check for brood and then treated with oxalic acid survived to spring (Al Toufailia et al, 2016). Winter inspection is needed to maximise the effectiveness of oxalic acid application in winter, both to determine the month(s) when the amount of sealed brood is least and, if there is no month in which it is zero in all colonies, to remove any small patches of capped brood prior to application. This extra effort will result in more effective varroa control.

PART 2: HYGIENIC BEHAVIOUR

Chapter 9

Towards integrated control of varroa: Effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence

9.1 Abstract

Hygienic behaviour in the honey bee, *Apis mellifera*, is the uncapping and removal of dead, diseased or infected brood from sealed cells by worker bees. We determined the effect of hygienic behaviour on varroa population growth and incidence of deformed wing virus (DWV), which can be transmitted by varroa. We treated 42 broodless honey bee colonies with oxalic acid in early January 2013 to reduce varroa populations to low levels, which we quantified by extracting mites from a sample of worker bees. We quantified varroa levels, again when the colonies were broodless, 48 weeks later. During the summer the hygienic behaviour in each colony was quantified four times using the Freeze Killed Brood (FKB) removal assay, and ranged from 27.5 % to 100 %. Varroa population increased greatly over the season, and there was a significant negative correlation between varroa increase and FKB removal. This was entirely due to fully hygienic colonies with >95 % FKB having only 43 % of the varroa build up of the less hygienic colonies. None of the 14 colonies with >80 % FKB removal had overt symptoms of DWV, whilst 36 % of the less hygienic colonies did. Higher levels of FKB removal also correlated significantly with lower numbers of DWV RNA copies in worker bees, but not in varroa mites. On average, fully hygienic colonies had c. 10,000 times less viral RNA than less hygienic colonies.

9.2 Introduction

Honey bees, *Apis mellifera,* face many threats (Carreck et al, 2010; Potts et al, 2010; Ratnieks and Carreck, 2010). Probably the most serious is the parasitic mite *Varroa destructor*, which can harm colonies both directly, by damaging individual worker pupae so that the resulting adult's lifespan and body weight are reduced (van Dooremalen et al, 2012), and indirectly by transmitting virus diseases (Ball and Allen, 1988; Boecking and Genersch, 2008; Guzmán-Novoa et al, 2010; Highfield et al, 2009).

There has been considerable research on varroa control, including synthetic chemicals (Alonso de Vega et al, 1990), natural chemicals such as oxalic acid (Nanetti et al, 2003), biotechnical methods such as drone brood trapping (Calderone, 2005; Charriére et al, 2003), and natural resistance such as hygienic behaviour (Rinderer et al, 2010; Spivak, 1996) and grooming behaviour (Andino and Hunt, 2011; Boecking and Spivak, 1999).

Hygienic behaviour is a natural defence against brood diseases in which hygienic worker honey bees uncap cells containing brood that is dead or infected and remove the contents (Rinderer et al, 2010; Rothenbuhler, 1964; Spivak, 1996). In this way, diseases such as chalk brood, American foulbrood and varroa infestation can be fully or partly controlled (Boecking and Spivak, 1999; Spivak and Gilliam, 1998). Despite a number of recent reviews on varroa resistance (Büchler et al, 2010; Carreck, 2011; Rinderer et al, 2010), there has been relatively little research on the role of hygienic behaviour in varroa control.

Colonies selected for hygienic behaviour using the Freeze Killed Brood (FKB) removal assay had fewer varroa mites than unselected commercial colonies (Delaplane et al, 2005; Harbo and Harris, 2001; Ibrahim and Spivak, 2006; Ibrahim et al, 2007). Colonies selected for Varroa Sensitive Hygiene (VSH), which have greater mite removal than hygienic colonies selected with the FKB bioassay (Delaplane et al, 2005; Ibrahim and Spivak, 2006), show reduced varroa population growth (Ibrahim and Spivak, 2006; Peng et al, 1987). Schöning et al (2012) showed that hygienic colonies uncap cells containing a female varroa mite seven days after capping. However, only cells containing a mother mite infected with deformed wing virus (DWV) were uncapped and cleaned out. Cells with uninfected mites were not uncapped.

In this study we quantified hygienic behaviour in honey bee colonies using the FKB assay to determine the effect of intercolony variation in FKB removal on varroa population increase and incidence of DWV over one year.

9.3 Material and Methods

9.3.1 Study colonies and data collection

We studied 42 honey bee colonies in four apiaries within 20 km of the University of Sussex. The colonies were managed using normal beekeeping methods. Each was housed in a hive consisting of a single “Commercial” brood chamber (11 frames 43.8

cm x 25.4 cm; total volume 56.4 l), bottom board with mesh floor, inner cover and telescopic cover. Each hive was given a queen excluder and honey supers as needed, and the honey crop was removed in early August.

All colonies were treated with oxalic acid when broodless on 2 January 2013. In broodless colonies all the varroa are phoretic on adult bees so can be killed by oxalic acid (Gregorc and Planinc, 2001). Ten days later, which is sufficient time for the complete mortality effect of the oxalic acid (Al Toufailia et al, 2015), a sample of c. 300 worker bees was taken from each colony, which were all still without sealed brood for varroa to enter, and frozen. The varroa mites were then washed from the sampled bees using a jet of water (Dietemann et al, 2013; Al Toufailia et al, 2016) to determine the initial number of mites per 100 bees. As the colonies were broodless when the sample was taken, this gave an estimate of the whole varroa population in each colony. No other treatments against varroa were used.

Starting on 19 August 2013, each colony was tested four times at weekly intervals using the Freeze Killed Brood removal assay (Bigio et al, 2014a; Spivak and Downey, 1998; Spivak and Reuter, 1998a,b) to quantify hygienic behaviour. In September 2013 each hive was inspected for the presence or absence of worker bees showing overt symptoms (shrivelled wings: see Figure 1) of DWV. To do this, each frame of bees was viewed on both sides during a hive inspection. On 12 December 2013 a second sample of c. 300 worker bees was taken from each colony, frozen and used to estimate the final varroa population. The colonies were all broodless at this time.

9.3.2 Virus quantification

Analysis of DWV in worker bee and varroa samples followed previously used methods (Francis et al, 2013). Briefly, c. 50 bees and 10 mites from each of the December samples were placed into a 15 ml bottle together with 7 – 10 steel ball bearings. For the mites, 10 individuals per colony were placed in a 1.5 ml Eppendorf tube with 2 steel ball bearings and freeze-dried for three days at 0.009 hPa and - 93 °C. After homogenisation of the samples, total RNA was extracted using NucleoMag 96 RNA kit (Machery- Nagel; Düren, Germany) on a Kingfisher Magnetic Extractor according to the manufacturer's guidelines.

A two-step real-time RT-PCR assay was used to quantify virus levels in the samples. Quantitative PCR amplifications were carried out on a vii7 apparatus (Applied

Biosystems) in duplicate for each sample using SYBR® Green DNA binding dye (Applied Biosystems).

Table.9.1. DNA primer sequences used for quantitative PCR assays and for establishing standard curves.

Source	Primer name	Primer sequence	Product size(bp)	Reference
DWV	F-DWV	5'-GGATGTTATCTCCTGCGTGGAA	69bp	Gauthier *et al*., 2007
	R-DWV	5'-CTTCATTAACTGTGTCGTTGATAATTG		
Varroa.β.Actin	FV-β-Actin	5'- GTTCATCGGAATGGAGTCATGCGGT	108bp	Francis *et al*., 2013
	RV-β-Actin	5'- CCAGAGAGAACGGTGTTAGCGTACA		
Bee.β.Actin	F-β-Actin	5'-TGCCAACACTGTCCTTTCTGGAGGT	96bp	Francis *et al*., 2013
	R-β-Actin	5'- TTCATGGTGGATGGTGCTAGGGCAG		

Viral loads in each sample were quantified using methods for absolute quantification based on standard curves obtained through serial dilutions of known amounts of PCR amplicon (Francis et al, 2013). Species specific β-Actin primers were included in the analysis, as an internal control for either honey bee or varroa samples. The primer sequences are shown in Table 1.

9.3.3 Statistical analysis

Data were analysed using the IBM SPSS statistical program version 20. If necessary, the response variable was log or arcsine transformed to meet the assumptions of ANOVA (Grafen et al, 2002; Zuur et al, 2010). Linear regression was then used to test for the effects of hygienic behaviour on varroa population increase and t tests and Fisher's exact tests for the effects of varroa population build up in the presence or absence of DWV symptoms. Descriptive statistics are given as mean ± standard deviation.

9.4 Results

9.4.1 Varroa population increase in one year and DWV incidence

Mean freeze-killed brood (FKB) removal, a measure of hygienic behaviour, ranged widely among the colonies from 27.5 % to 100 % with an overall mean of 72.6 %. Eight

colonies had FKB removal of >95 %, a threshold commonly used to signify “fully hygienic” (Spivak and Downey, 1998).

Initial levels of varroa mites per 100 worker bees were low following oxalic acid treatment (mean & SD: 0.55 ± 0.34). Nearly one year later, this had increased 36.25 fold to 17.38 ± 7.25, on average (Figure 1). Population increase in the number of varroa mites per colony was determined using this increase in the number of mites per 100 workers, combined with change in colony population, measured as the number of frames covered with bees at the time that worker samples were taken. For example, if the number of mites had increased from 0.5 to 20 per 100 workers, but the colony population had increased from four to five frames of bees, then the total varroa population increase was (20/0.5) x (5/4) = 50.

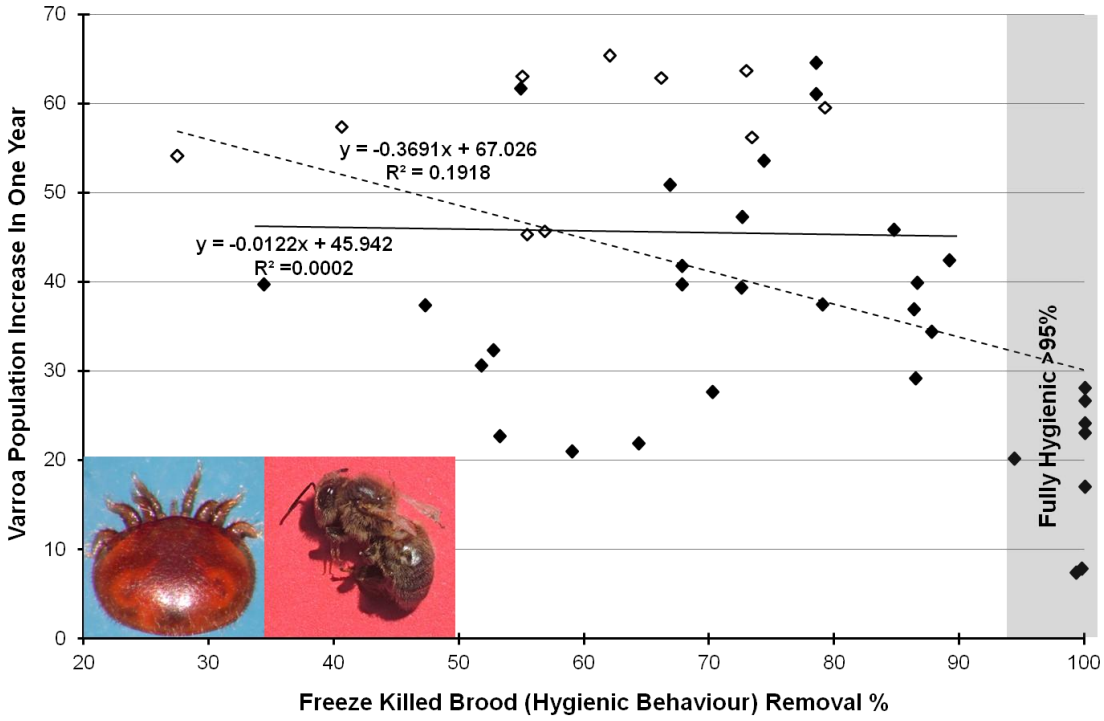

Fig.9.1. Proportional increase of varroa population from 12 January to 12 December 2013 in the 42 study colonies as a function of Freeze Killed Brood removal. Colonies with workers showing symptoms of deformed wing virus are shown as open symbols. The photos show (left) an adult female varroa mite and (right) an adult worker bee with shrivelled wings, an overt symptom of DWV.

Overall, the mean increase in varroa population was 40.23 fold (range 7.4 – 65.4) (Figure 1). The mean increase in the fully hygienic colonies was 19.37 (range 7.4-28.2) versus 45.14 (range 21.1 – 65.4) in the non-hygienic colonies (<95 % FKB).

Across all 42 study colonies, there was a significant negative relationship (F=17.068, P<0.001; R^2= 0.19) between FKB removal and varroa population increase (Figure 1 dashed regression line). However, this effect was entirely due to the influence of the fully hygienic colonies. In the 34 colonies with <95 % FKB removal (range 27.5 – 90 %) there was no trend to lower varroa increase with higher FKB removal (F=0.006, P=0.937; R^2= 0.0002; Figure 1 solid regression line).

None of the 14 colonies with >80 % FKB removal had workers with shrivelled wings, an overt symptom of DWV, whereas 10 of the 28 (36 %) colonies with <80 % FKB removal did. This difference is significant (P = 0.017, Fisher's Exact Test, two tailed). The mean final number of varroa per 100 worker bees was greater in colonies with overt DWV symptoms (28.23±2.27) than without (24.80±5.56) (F=8.68; P=0.005). Colonies with DWV symptoms also had significantly greater varroa build up (57.36±7.19) than those without symptoms (34.87±14.36) (F=4.36; P=0.043).

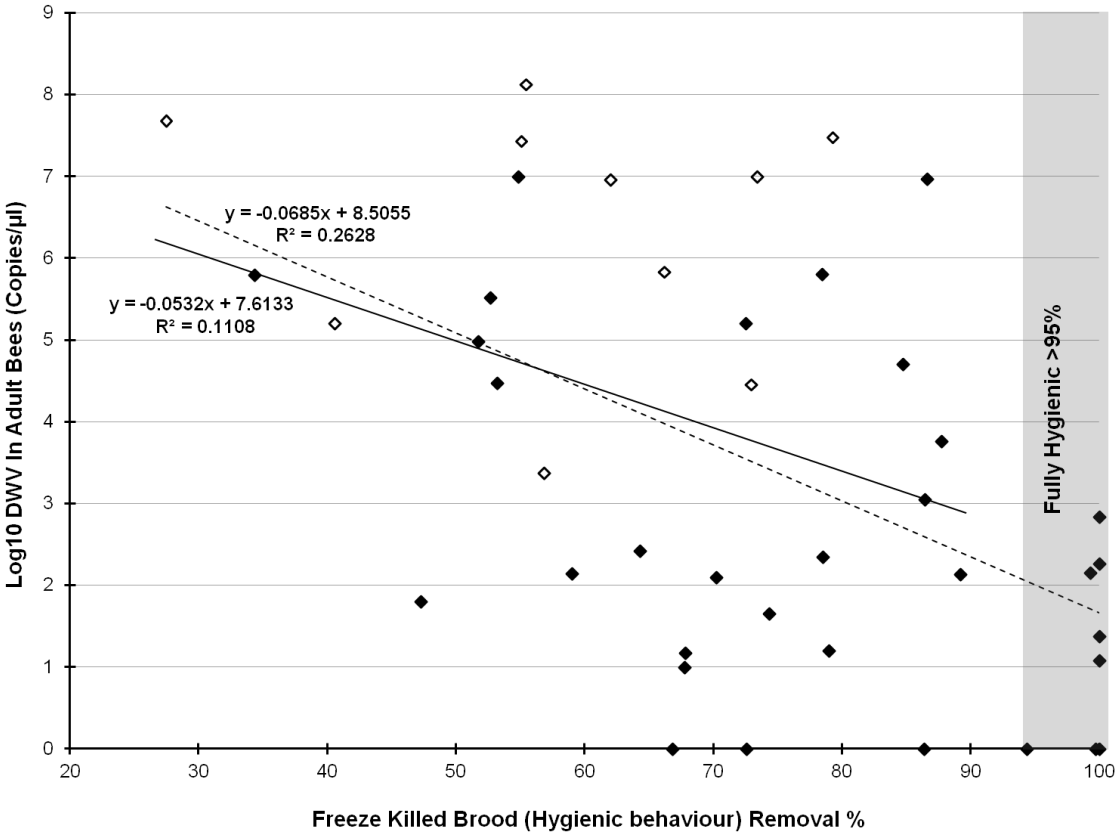

Fig.9.2. Number of deformed wing virus RNA copies in adult bee samples collected on 12 December 2013, 11 months after treating with oxalic acid, in the 42 study colonies. Colonies that had some workers with overt symptoms of DWV (shrivelled wings) are shown as open symbols.

9.4.2 Virus quantification

Overall, the range of viral RNA levels in the pooled worker bee samples in December 2013 was 0 - $1.3x10^8$ copies/µl (Figure 2). The average level in the 8 fully hygienic colonies (>95 % FKB removal) was 8.6 x 10^2 copies/µl vs. $8.2x10^6$ copies/µl in the 34 non-hygienic colonies (<95%). Across all 42 study colonies, there was a significant negative relationship (F=14.258, P<0.001; R^2= 0.26) between FKB removal and viral RNA levels in worker bees (Figure 2. Dashed regression line). However, this effect was entirely due to the influence of the fully hygienic colonies. In the 34 colonies with <95% FKB removal (range 27.5 – 90 %) there was a trend to lower viral level increase with higher FKB removal but the relation was non-significant (F= 3.989, P=0.06; R^2= 0.1108; Figure 2. solid regression line).

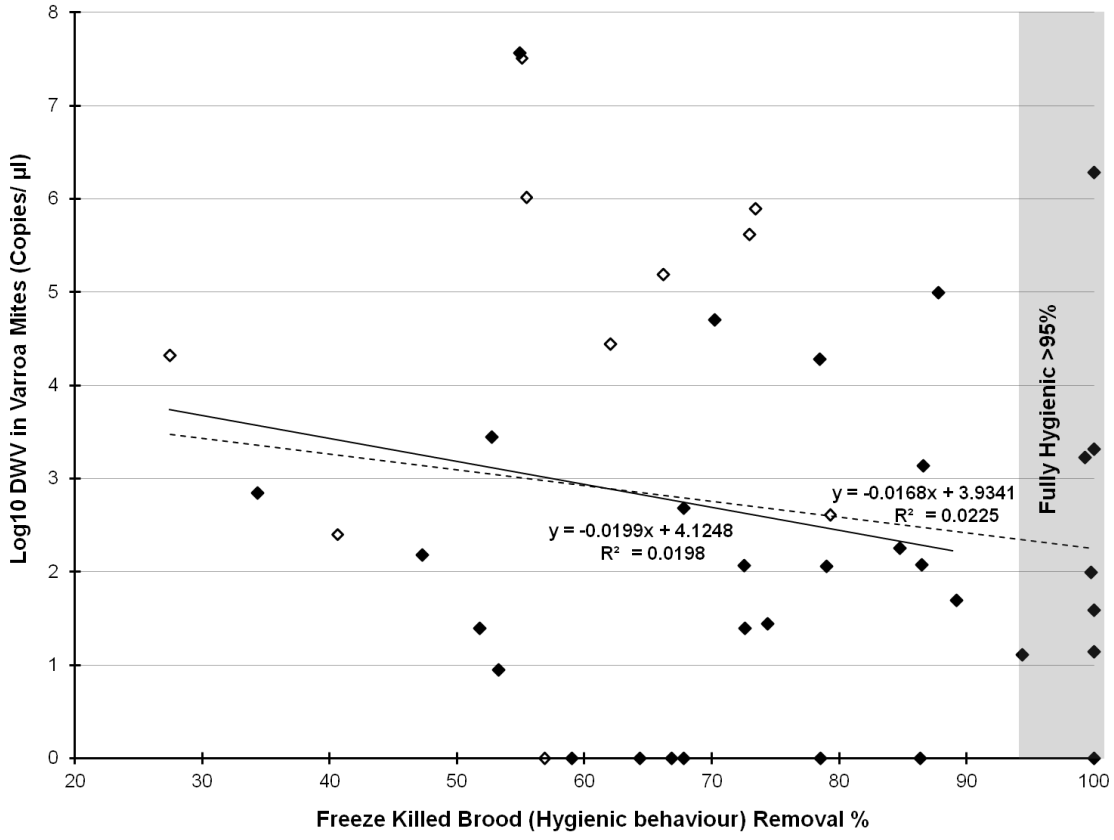

Fig.9.3. Number of deformed wing virus RNA copies in varroa mite samples collected on 12 December 2013, 11 months after treating with oxalic acid, in the 42 study colonies. Colonies with workers showing overt symptoms of deformed wing virus are shown as open symbols.

The mean viral level in the varroa mites collected from the 8 fully hygienic colonies (> 95 % FKB removal) was $2.5x10^5$ copies/µl versus $21x10^5$ copies/µl in the 34 non-hygienic colonies (<95 %). Across all 42 study colonies, there was a non-significant relationship (F=0.918, P= 0.34; R^2= 0.022) between FKB removal and viral RNA levels in varroa mites (Figure 3. dashed regression line). In the non-hygienic colonies with <95 % FKB removal, there was a non-significant relationship (F= 0.647; P= 0.43; R^2= 0.019; Figure 3. solid regression line).

9.5 Discussion

Our results show clearly that hygienic behaviour can be effective at reducing the one-year population growth of varroa in honey bee colonies. In particular, the fully hygienic colonies (>95 % FKB removal) showed, on average, only 43 % of the varroa population growth of the non-fully hygienic colonies (<95 % FKB removal) (mean 19.37-fold, range 7.4 to 28.2, v 45.14-fold, range 20.1 to 65.4, respectively). The significant negative correlation between varroa build up and FKB removal was entirely due to the fully hygienic colonies (n = 8, >95 % FKB removal) having lower varroa build up than the colonies with FKB <95 % removal. There was no trend to lower varroa build up among the 34 colonies with FKB <95 % removal.

The annual varroa increase we quantified is greater than the 12-fold estimate based on simulation modelling (Martin, 1998) but is within the wide range, 10-300 fold, found in previous empirical research (De Guzman et al, 2007; Fries et al, 1991; Kraus and Page, 1995). These earlier studies were carried out in different locations and conditions, and estimated varroa increase indirectly by counting mite fall onto the hive bottom board rather than directly, as in our study.

Our results also show that hygienic behaviour reduces the occurrence of DWV in that none of the 14 colonies with >80 % FKB removal showed overt symptoms of DWV. That is, the presence of workers with shrivelled wings. The fact that lower levels of FKB removal (80 v 95%) seemed to be effective in controlling DWV but were not effective in slowing varroa population growth may be because hygienic behaviour particularly targets capped cells containing a mother mite infected with DWV (Ibrahim and Spivak, 2006; Schöning et al, 2012). It seems that DWV transmission to the immature bee results in odours that trigger cell uncapping.

Intercolony variation in the number of viral RNA copies show that the highly hygienic colonies (FKB removal > 95 %) had much lower levels of DWV in worker

bees, some 10,000 times less than in the non-hygienic colonies (FKB removal < 95 %; $8.6x10^2$ versus $8.2x10^6$ copies/1 µl extract; Figure 2.). However, varroa mites from hygienic colonies had only 8 times less viral RNA than those from non-hygienic colonies ($2.5x10^5$ versus $21x10^5$; Figure 3.). It appears, therefore, that hygienic behaviour is more effective at reducing levels of DWV in worker bees than in the mites.

Our study does not reveal why this is the case. Previous research has shown that hygienic bees remove worker pupae infected with DWV by varroa (Schöning et al, 2012). This would tend to reduce virus levels more in worker bees than in mites, as infected pupae are killed while the female mite is probably not killed. However, this process could also reduce virus levels in mites, as infected mites would not be as successful in breeding as uninfected mites.

Overall, our results are encouraging to beekeepers, as they demonstrate that hygienic behaviour, which is a heritable and natural form of disease resistance, can reduce the build up of varroa mites and the incidence of DWV. There is now evidence that hygienic behaviour is beneficial against four honey bee pests and diseases (varroa, DWV, chalkbrood, American foulbrood (Boecking and Spivak, 1999; Boecking et al, 2000; Spivak and Gilliam, 1998a,b). Our results also support the advice given by Spivak and Reuter (2001) that hygienic colonies should require fewer additional treatments against varroa, possibly only annual treatment.

Under the conditions of southern England, where our colonies were studied, it seems that hygiene combined with winter treatment of broodless hives with oxalic acid (Al Toufailia et al, 2016) is sufficient for one year. Our results show that breeding hygienic bees is worthwhile for beekeepers, and support the recommendation of Spivak and Downey (1998) that an FKB removal of 95 % is a suitable criterion for “fully hygienic” colonies. Our results suggest, however, that it is possible that slightly lower levels of FKB removal (>80 %) may also provide protection against DWV, even if these intermediate levels (80-95% FKB removal) do not reduce varroa population build up compared to even lower levels of hygiene.

Chapter 10

Hygienic behaviour saves the lives of honey bee colonies

10.1 Abstract

Hygienic behaviour is a naturally occurring heritable mechanism of disease resistance in the honey bee, *Apis mellifera*. Hygienic workers uncap sealed brood cells containing dead or diseased larva and pupae and remove the contents, thereby reducing levels of several brood diseases. Deformed wing virus is a serious disease that shortens worker life span and kills colonies due to the collapse of the worker population including causing the winter collapse of colony populations. Here we show that requeening colonies with DWV symptoms using hygienic queens greatly reduces colony mortality. Our results indicate that breeding and using hygienic honey bees should be encouraged as a way of reducing disease and resulting colony losses.

10.2 Introduction

Hygienic behaviour is a naturally occurring heritable mechanism of disease resistance in the honey bee, *Apis mellifera* (Rinderer et al, 2010; Rothenbuhler, 1964a,b; Spivak, 1996; Wilson-Rich et al, 2009). Hygienic workers uncap sealed brood cells containing dead or diseased larvae and pupae and remove the contents, thereby reducing levels of several brood diseases (Al Toufailia et al, 2014; Gilliam et al, 1983; Rothenbuhler, 1964a,b; Schoning et al, 2012; Spivak and Reuter, 1998a,b). Deformed wing virus is a serious disease that shortens worker life span (Dainat et al, 2012; Francis et al, 2013) and kills colonies due to the collapse of the worker population (Berthoud et al, 2010; Martin et al, 2010) including winter collapse (Berthoud et al, 2010; Genersch et al, 2010; Highfield et al, 2009).

Here we show that colonies with overt DWV symptoms that were requeened using hygienic queens have significantly greater survival, 73% v 24% over the first year of our 20 month trial, than those requeened with non-hygienic queens. We also show significant positive correlations between colony hygiene level and survival duration, including significant effects across intermediate levels of hygienic behaviour. Our results indicate that beekeepers should be encouraged to use hygienic bees as a way of reducing disease and resulting colony losses. What is needed to do this is already

available. Hygienic honey bees occur naturally (Oldroyd, 1996; Perez-Sato et al, 2009; Spivak and Gilliam, 1991) at low levels and can be increased by selective breeding (Bigio et al, 2014a; Spivak and Reuter, 1998b). Queen breeding, rearing and hive requeening are also standard beekeeping practices (Laidlaw and Page, 1997).

10.3 Materials and Methods

Workers with shrivelled wings (Figure 1a) are an overt symptom of DWV (Dainat and Neumann, 2013; Yue and Genersch, 2005) that predicts colony death (Dainat and Neumann, 2013). In May 2014 we inspected 120 hives in our apiaries in Sussex, UK, and found 15 with workers showing this symptom, which is easily seen during a standard beekeeping hive inspection in which frames of brood covered in worker bees are briefly removed from the hive and scanned by eye.

Our previous research had already shown that DWV is present in almost all our colonies and is at high levels in colonies with deformed workers (Al Toufailia et al, 2014). On 4 or 5 August 2014 we divided each of these 15 colonies into 3 hives, each receiving equal amounts of bees and brood (Figure 1a). One division retained the original queen. The other two were given either a hygienic or a non-hygienic queen. The newly-divided hives were then moved to a single experimental apiary and fed with sucrose syrup. By moving the hives at the time of colony division we ensured that the worker bees remained equally divided.

Because our laboratory has been breeding and researching hygienic behaviour, we had both highly-hygienic and non-hygienic colonies available in our apiaries. We have bred hygienic bees using the the freeze-killed brood (FKB) bioassay (Al Toufalia et al, 2014; Perez-Sato et al, 2009; Spivak and Reuter, 1998a,b) in which patches of sealed brood cells are killed with liquid nitrogen. To obtain hygienic and non-hygienic queens, we tested 75 of our colonies using FKB bioassay. Each colony was tested 4 times at weekly intervals in July 2014. Hygienic queens were taken from colonies showing 100% FKB removal within 48 ± 2 hours. Non-hygienic queens were taken from colonies showing low to medium FKB removal (mean 34%, range 23-55%). We also made FKB tests on the original queens in the 15 colonies.

If a colony became queenless during the experiment it was given a tested queen of the appropriate treatment. The worker bees in a colony are daughters of the single queen and the males she mated with when 1-2 weeks of age and whose sperm is stored in her body and used for the rest of the queen's life. Thus, requeening results in a

colony's worker population being replaced with a new worker population comprised of the daughters of the replacement queen and her mates (stored sperm) that is genetically similar to that of the colony from which the queen was taken, and which had been tested for FKB removal.

In September 2014 all colonies were fed sucrose syrup to ensure they had sufficient honey stores for winter survival. In October 2014 the 45 divided colonies all had 3-5 frames of brood, which is more than sufficient worker bees and brood for successful overwintering in the study area provided that the worker population does not collapse. Colonies were inspected monthly for survival until April 2016, and any workers with shrivelled wings were counted. No frames of brood were introduced into any study hive during the experiment. In spring and summer 2015, following common beekeeping practice, a few frames of brood were removed from some hives to reduce overcrowding and so prevent swarming. This was necessary as the queen departs with the swarm. Other than the hygienic queens, colonies were given no other treatment against pests and diseases.

10.4 Results

Figure 1 shows colony mortality at three key points. Across the first winter until April 2015, 13 of the 15 colonies given a hygienic queen survived versus only 6 of the 15 given a non-hygienic queen ($P = 0.0209$. Fisher's Exact Test, 2-tailed). At the end of the next summer, September 2015, and after the second winter, April 2016, survival remained significantly higher for the colonies with hygienic queens (September 2015: 11/15 v 2/15, $P = 0.0025$; April 2016: 6/15 v 0/15, $P = 0.0169$).

In the first winter, colony mortality began between the January and February inspections (Figure 1b). That is, the populations in some colonies with many bees in autumn had already collapsed by mid winter to leave no bees in the hive. Survival analysis showed that colonies given a hygienic queen remained alive significantly longer than colonies with original queens or given non-hygienic queens (Figure 1b).

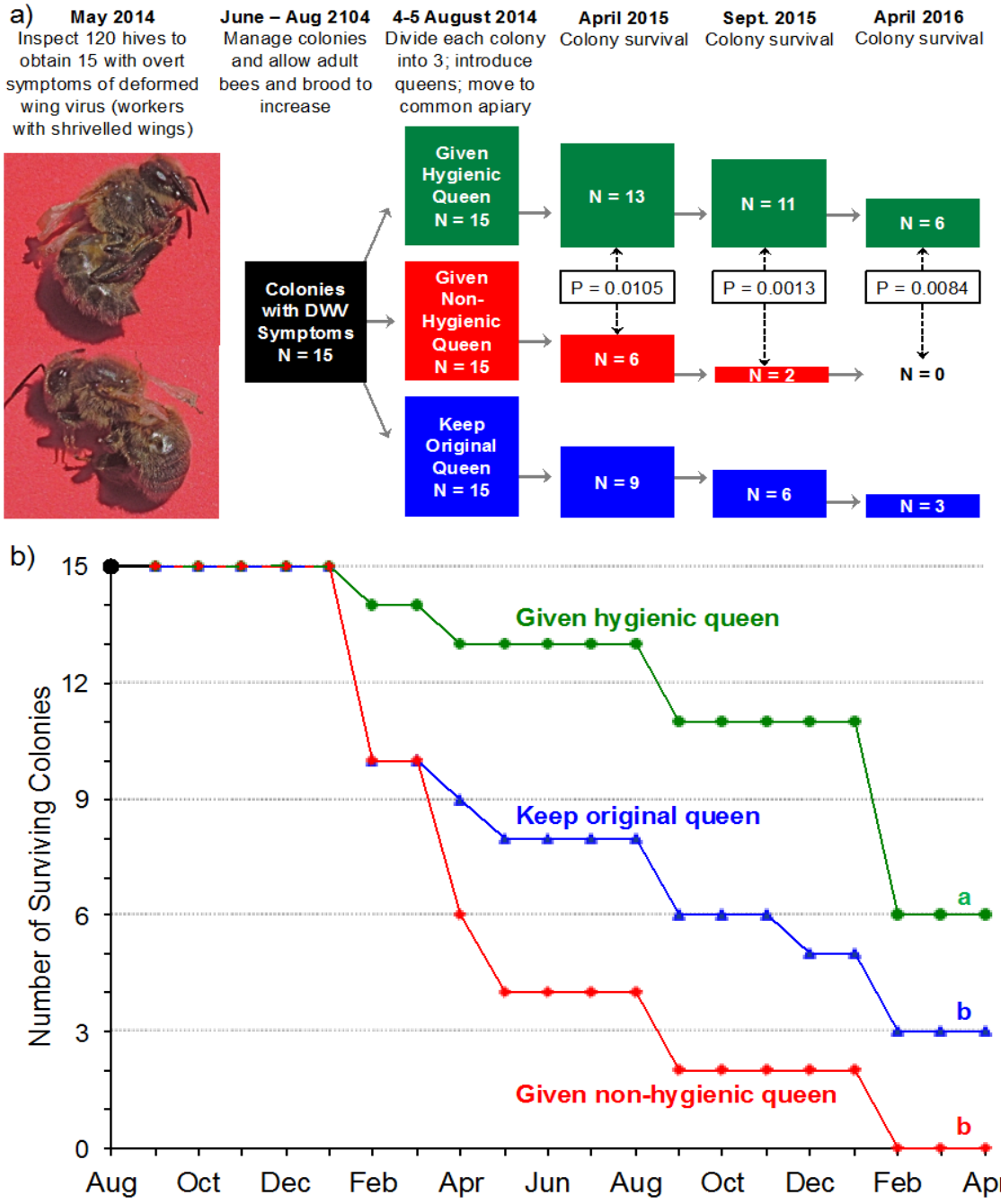

Fig.10.1. a) Schematic of experimental design and survival of colonies given hygienic queens (n =15, all had 100% Freeze-Killed Brood removal within 48 ± 2 hours) versus non-hygienic queens (n = 15, 23-55% FKB removal, mean = 34%) and original queens (n = 15, 32-95% FKB removal, mean = 57%). P values give results of Fisher's Exact tests, 2-tailed, comparing the number of surviving colonies at 3 key points. b) Monthly survival of colonies, August 2014 to April 2016. Colonies given a hygienic queen survived for significantly longer than colonies given a non-hygienic queen or with their original queen combined (P = 0.01, Cox Regression Survival Analysis, Wald = 9.23). Breslow pairwise comparisons showed that the survival of colonies given hygienic queens (Kaplan-Meier Survival Test, using SPSS [Kalbfleisch and Prentice, 1980]) was significantly longer than for colonies given non-hygienic queens (P < 0.001) or with their original queens (P = 0.041).

Across all 45 colonies there are highly significant positive correlations, using both parametric and non-parametric statistical tests, between hygienic behaviour (FKB removal) and colony survival duration (Figure 2). There are also significant positive correlations in the 15 colonies with original queens and the 15 given non-hygienic queens, showing benefits of intermediate levels hygienic behaviour. The three original-queen colonies that survived the full 20 months of the experiment had the three highest levels of FKB removal (82, 86, 95%).

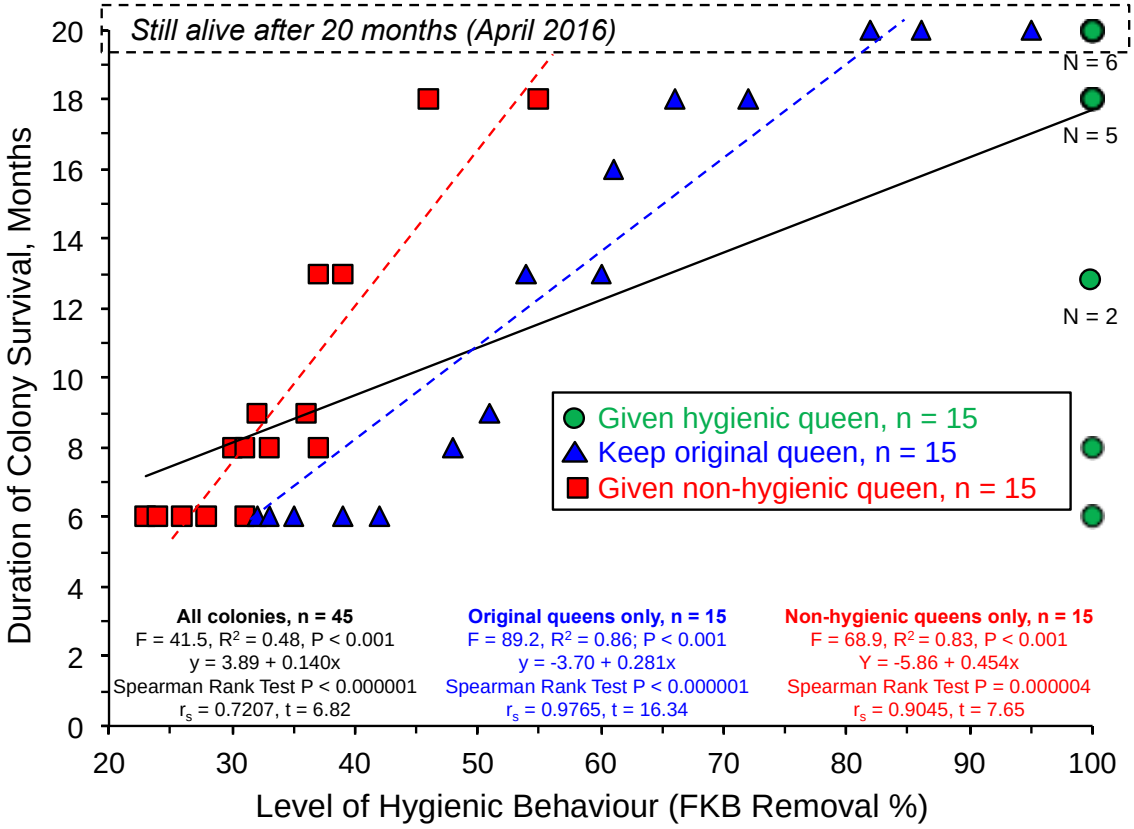

Fig.10.2. Relationship between colony survival duration and level of hygienic behaviour (FKB Removal: % freeze-killed brood removed in 48 ± 2 hours). Colonies were checked for survival once per month. Across all 45 colonies there is a highly significant positive relationship (solid regression line). The relationship is also positive and highly significant for the 15 colonies with mother queens (dashed blue line) or given non-hygienic queens (dashed red line). The same relationships are also highly significant when tested using the Spearman Rank Test, which is non-parametric. Analyses used IBM SPSS v. 20. Adjusted R^2 values are given for the regression analyses. Colonies given a hygienic queen all had 100% FKB removal. As a result, some data points lie on top of each other as indicated (e.g., n = 6).

In all of the 12 months for which counts were made there was a significant negative correlation between hygienic behaviour (FKB removal) and the number of worker bees with shrivelled wings seen in that month's hive inspection (Figure 3). This

suggests, as expected (Al Toufailai et al, 2014), that hygienic behaviour reduced DWV within colonies.

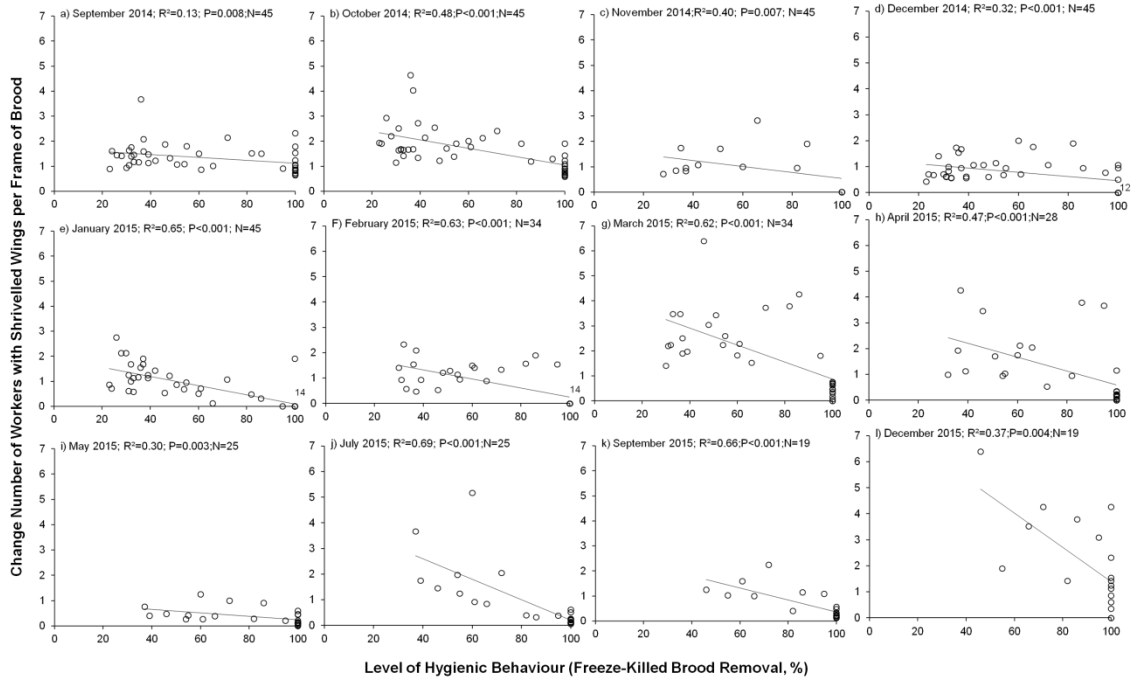

Fig.10.3. Numbers of worker bees with shrivelled wings seen in monthly hive inspections, n = 12, of all surviving colonies with sealed brood present at that time. The y-axis gives the number seen relative to the number seen in the final inspection (August 2014) of the 15 study hives before colony division, and the amount of sealed brood. Workers with shrivelled wings are young workers which have recently emerged from their cells, and which soon leave the colony and die (De Miranda and Genersch, 2010). The number seen, therefore, should depend on the amount of sealed brood (i.e., cells containing pupae). In all cases there is a significant negative correlation between colony levels of hygienic behaviour and relative numbers of workers with shrivelled wings. Linear regression analysis used IBM SPSS v. 20. Adjusted R^2 values are given (Grafen et al, 2002; Zurr et al, 2010).

10.5 Discussion

Our results show that hygienic behaviour saves the lives of honey bee colonies with shrivelled wings, a visible symptom of DWV that is considered a predictor of colony death (Dainat and Neumann, 2013). Although hygienic behaviour gave increased survival it did not give full survival. The most likely reason for this is that we did not provide any additional disease or pest management. In particular, beekeepers, including ourselves, normally control varroa mites, which are also a vector of DWV (Bowen-Walker et al, 1999; Martin et al, 2010; Santillan-Galicia et al, 2010). Although hygienic behaviour does not fully control varroa, it does reduce the annual growth of a colony's varroa population (Al Toufailia et al, 2014). To further increase colony survival,

hygienic behaviour could be combined with other measures in an integrated varroa control program.

Higher than expected winter colony losses due to collapse of the worker bee population have occurred in recent years (Berthoud et al, 2010; Genersch et al, 2010; Highfield et al, 2009; vanEngelsdorp et al, 2009). In areas with a temperate climate and a winter break in brood rearing, we suggest that it would be advantageous not to delay requeening colonies with DWV symptoms with a hygienic queen later than August because requeening is expected to take time to exercise a beneficial effect. It takes several days for the new queen to be released from her cage and start laying, then 21 days for the eggs to develop into adult workers, and then another 15-17 days before these genetically hygienic workers actually carry out hygienic behaviour within their age-based polyethism schedule (Arathi et al, 2000).

Thus, our colonies would not have benefitted from increased levels of hygienic behaviour until mid-September, by which time brood rearing is already starting its autumn decline. In our 45 study colonies the mean number of frames of brood declined from 3.4 (September) to 2.1 (October) to 0.1 (November) and 0.3 (December) in 2014.

Two of the 15 colonies requeened with a hygienic queen died in the first winter. This suggests that our requeening date in early August may have been later than ideal. It is likely that requeening in spring or early summer would likely be more effective in helping colonies with DWV symptoms (workers with shrevilled wings). However, requeening at any time would be beneficial as a preventive measure.

Honey bees are important to humans. They provide half of all the value of bee pollination to crops (Kleijn et al, 2015) plus an annual honey harvest of c. 1.6 billion kg (Moritz and Erler, 2016). At a time when the most news about honey bees is mainly bad we are pleased to report something optimistic. Natural populations of honey bees generally have low average levels of hygienic behaviour, for example 46% (range: 15-98%), FKB removal across 31 colonies in a study in the UK (Perez-Sato et al, 2009), but have some highly hygienic colonies, 15-98% (Perez-Sato et al, 2009) from which hygienic stocks can be bred within a few generations using standard queen rearing methods (Bigio et al, 2014a). It would be helpful if the bees themselves controlled their own diseases. Our study shows that this is possible.

Chapter 11

Hygienic behaviour by non-hygienic honey bee colonies: all colonies remove dead brood from open cells

11.1 Abstract

Eusocial insects have both individual and group-level defences against disease. Hygienic behaviour is a group defence against brood diseases in which workers remove dead and dying brood from sealed cells. In the honey bee, *Apis mellifera*, hygienic behaviour is a rare heritable trait. In this experiment we compared the removal of dead and diseased larvae from open and sealed cells in 20 colonies. Larvae in open cells were either killed by freezing with liquid nitrogen, or exposed to the chalkbrood fungal parasite, which killed all small larvae (0-1 days old) within a few days but did not kill all medium (2-3 days old) or large (4-5 days old) larvae. The study colonies varied greatly in the rate at which freeze-killed brood were removed from sealed cells, with 52-100% removal within two days. However, all colonies removed 100% of the freeze-killed larvae from open cells within 1 day. In addition, all colonies removed 100% of the young larvae treated with chalkbrood spores within a few days and before cell capping. The results show that all honey bee colonies are highly hygienic in response to dead or diseased brood in open cells. This suggests that there may be alternative adaptive responses, high and low levels of hygienic behaviour, to diseased brood in sealed cells in honey bees, in comparison to the consistently high levels of hygienic behaviour expressed towards dead or diseased brood in open cells in honey bees and to brood in sealed cells in stingless bees.

11.2 Introduction

Diseases are a challenge to all organisms, but often more so for group living versus solitary species due to increased opportunities for pathogen transmission (e.g., birds: Møller et al, 2001; humans: Anderson et al, 1989; Lepidoptera: Hochberg, 1991; lizards: Godfrey et al, 2005). However, group living organisms also have group level defences against disease (birds: Møller et al, 2001; humans: Curtis and Cairncross, 2003; primates: Freeland, 1979). Often referred to as the pinnacle of social evolution, the eusocial insects display a wide variety of different group defences (Wilson-Rich et

al, 2007). These include allogrooming behaviour (Hughes et al, 2002; Nunn and Altizer, 2006; Schmid-Hempel, 1998), the use of antimicrobial resins in nests (Christe et al, 2003; Simone et al, 2009; Stow et al, 2007), social fever (Starks et al, 2000), antimicrobial secretions (Brown, 1968; Tranter and Hughes, 2015), and hygienic behaviour (Wilson-Rich et al, 2007).

Hygienic behaviour is a group defence against diseases in which workers remove dead, dying and infected individuals from the nest (Wilson-Rich et al, 2007). In the honey bee, *Apis mellifera*, the term hygienic behaviour has a well-established and specific meaning which refers to the removal of dead, dying and infected brood (larvae, pupae) from sealed cells (Park, 1937; Rinderer et al., 2010; Robinson and Page, 1988; Rothenbuhler, 1964ab; Spivak, 1996). This helps defend the colony against diseases such as American foulbrood (Spivak and Reuter, 2001), chalkbrood (Oldroyd, 1996), varroa mites (Al Toufailia et al, 2014; Locke et al, 2014; Spivak, 1996) and deformed wing virus (Al Toufailia et al, 2014; Schöning et al, 2012). It is a heritable trait and not a learned behaviour (Momot and Rothenbuhler, 1971; Rothenbuhler, 1964a,b), and seems to have no negative effect on colony performance as it does not result in increased removal of healthy brood (Bigio et al, 2014b) or reduced honey production (Spivak and Reuter, 1998b).

Hygienic behaviour also occurs in stingless bees, Meliponini, which, like the honey bee, rear brood in individual cells. In stingless bees, however, cell sealing takes place immediately after an egg is laid on a food provision mass previously placed there by worker bees (Michener, 1974), whereas in *A. mellifera* cell sealing does not occur until 5-6 days after the egg hatches due to the progressive food provisioning in this species (Gould and Gould, 1988; Winston, 1991). Most colonies of honey bees are not hygienic (Perez-Sato et al, 2009), defined as removing 95% of dead and diseased brood within two days (Spivak and Downey, 1998), while most colonies of stingless bees, in the six species that have been studied so far, are consistently defined as hygienic (Al Toufailia et al, accepted; Medina et al, 2009; Nunes-Silva et al, 2009).

Although there has been much investigation of hygienic behaviour in honey bees, we still have a limited understanding of how the defence varies across colonies in response to parasite infections and how its expression changes depending on the age of brood. The aim of this study was to compare levels of hygienic behaviour against dead or parasite-infected larvae in open versus sealed cells in the honey bee. The 20 study colonies varied greatly in the rate at which freeze-killed brood were removed from

sealed cells, with 52-100% removal within two days. However, all colonies removed 100% dead larvae, killed either by freezing or by chalkbrood spores, before cell sealing. This shows that all honey bee colonies are highly hygienic to dead and diseased brood in open cells, and suggests that the lower levels of hygienic behaviour observed in response to dead and diseased brood in sealed cells is a specific trait or adaptation in its own right.

11.3 Materials and Methods

11.3.1 Study colonies

We studied 20 colonies of the honey bee, *Apis mellifera*, located in a single apiary on the University of Sussex campus in summer 2013, July-September. Each colony was housed in a hive consisting of a single 'commercial', 56 litre brood chamber, containing 11 frames (43.8 x 25.4 cm), bottom board, inner cover and telescopic cover. From May 2013 no frames of brood were transferred into any study colony from any other colony, so the young worker bees in each colony were the progeny of the colony's queen at the time of the experiment. As part of normal hive management, all colonies were inspected regularly for visible symptoms of brood diseases. Dead brood showing symptoms of sac brood, American foulbrood, European foulbrood were never seen. Chalkbrood mummies were observed in brood cells in 8 of the 20 colonies, but always at low levels (<12 cells per colony per inspection).

11.3.2 Quantifying hygienic behaviour in sealed cells using the Freeze Killed Brood (FKB) bioassay

We determined the level of hygienic behaviour towards dead larvae in sealed cells in the 20 study colonies using the freeze-killed brood (FKB) bioassay (Al Toufalia et al, 2014; Bigio et al, 2014a; Spivak and Downey, 1998; Spivak and Reuter, 1998a,b). Each colony was tested four times from late July to late August at 7-10 day intervals. At this time the colonies were all strong and with approximately equal population (8-9 frames of brood and 10-12 frames of bees), and all had several frames of honey, a marked egg-laying queen, and empty frames for egg laying. For each FKB bioassay we followed standard methods (Al Toufailia et al, 2014; Bigio et al, 2014a; Perez-Sato et al, 2009).

We removed a test frame with a large area of sealed brood from each colony. We pressed two metal cylinders (6.5 cm diameter × 8 cm height) into the sealed brood. Approximately 300 ml of liquid nitrogen was poured into each cylinder to kill the circle

of brood inside. After a few minutes, when the liquid nitrogen had evaporated, we removed the cylinders. The frame was then photographed and returned to the hive, and photographed again 48 ± 2 h later. From these photographs we determined the proportion of FKB cells removed by the hygienic behaviour of the workers.

11.3.3 Preparation of chalkbrood (Ascosphaera apis) spore suspension

Black chalkbrood mummies (larvae with visible symptoms of chalkbrood disease, in which the fungus is sporulating [Spiltoir, 1955]), n=20, were collected from University of Sussex honey bee colonies. The mummies were placed in pairs onto Sabouraud dextrose agar (SDA) media plates and incubated at 28ºC. Hyphae grew from each mummy. Spores formed where the two hyphal mats of different mating types met (Spiltoir, 1955; Jensen et al, 2009b; Jensen et al, 2013). Spores were allowed to mature before being harvested from above the SDA media. A spore suspension was made by gently grinding a small amount of spore material in a glass tissue homogenizer with 200 µl of sterile deionized water. The supernatant was made up to 1 ml with sterile deionized water and left to stand for 20 min to ensure any spore clumps had settled to the bottom. A 0.5 ml aliquot was taken from the central column of the spore suspension and placed in an Eppendorf tube. The concentration of the spores in the aliquot was determined using a FastRead disposable hemocytometer (Immune Systems, UK) and the viability determined as described by Vojvodic et al (2011). A final dilution gave a concentration of $1x10^6$ spores per ml, which is enough to infect and kill honey bee larvae (Aronstein et al, 2010).

11.3.4 Quantifying removal of larvae treated with chalkbrood spores

In September, after the FKB tests were completed, two frames of brood with larvae of different ages, including both capped and uncapped cells, were chosen from each colony and taken into a warm room (25ºC) in the laboratory. A clear acetate sheet was attached to each of these two test frames with drawing pins. On the first frame, nine groups of 30 cells, each containing a worker larva in an uncapped cell, were traced onto the acetate sheet so that the position of each cell was precisely recorded. Three groups contained small larvae (< 1 day old; Figure 1), three groups contained older larvae that half-filled the base of the cell (c. 2-3 days old; Figure 1), and three groups contained large larvae that filled the base of the cell and would normally be capped within a day (c. 4-5 days old; Figure 1). In each of these three larval size categories, one group of cells acted as a

control with no treatment, one acted as a control treated with 5 µl sterile deionized water, and the third was treated with 5 µl of chalkbrood spore suspension, corresponding to c. 5,000 spores.

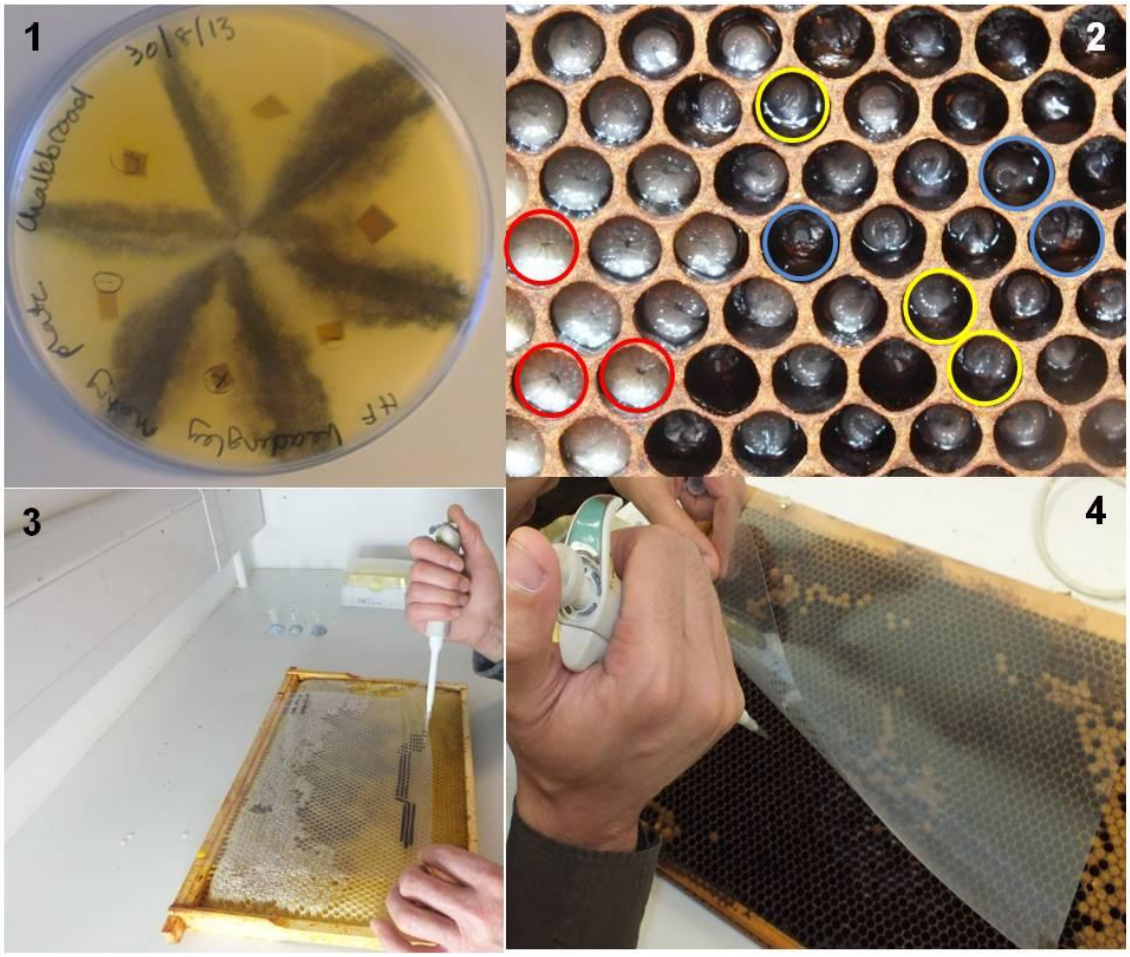

Fig.11.1. Treating brood cells with chalkbrood spores. 1) Agar plate with chalkbrood culture showing black spore formation areas from which spores were obtained (Photo, S.Evison); 2) Examples of small and medium and old larvae in open cells. Blue (small larvae, aged <1 day), yellow (medium, aged c. 2-3 days old) and red (large, aged c. 4-5 days old and within 1 day of being capped [solid line]; 3, 4) placing spore suspension into cells with pipette.

The water or spore suspension was placed in the larval food at the base of the cell using a Gilson pipette. Care was taken not to touch the larva with the pipette to avoid physical damage. On the second frame, a further 90 cells in three patches, small, medium and large, were traced following the same procedure. The whole patch of larvae were then killed using liquid nitrogen in the same way as for the FKB bioassay (see above), except that the larvae were in uncapped cells and no photographs were taken because the content of each individual cell was already recorded. The acetate

sheets allowed the status (i.e. presence/absence of a larva) of each study cell to be precisely recorded before and after treatment.

Whether or not a larva had been removed from each cell was determined 1, 2, 3, 4, 5, 6, 7, 8, 10, and 12 days after treatment. Any cells in which larvae had not been removed after 12 days were uncapped to check the contents, and in particular to determine if this was a healthy pupa or a chalkbrood mummy within the cell.

11.3.5 Statistical analysis

Data were analysed using IBM SPSS version 20. We used generalised linear mixed models (GLMM) (Zuur et al, 2010) fitted with a Poisson distribution and a log link function. The number of larvae removed was the response variable, and one or more of the following variables included as the predictor, depending on the aim of the test: 1. Treatment type (control [CON_0]; water control [CON_w]; freeze killed brood removal (of open cells only) [FKB]; chalkbrood spore suspension [CHB]); 2. Days since treatment; 3. Larval size. In each model the rate of FKB removal (as a %) from sealed cells was included as a covariate to account for the hygienic level of each colony, and colony was fitted as a random effect to account for the repeated measures made within each colony. $P < 0.05$ is defined as significant and descriptive statistics are given as mean ± standard error.

11.4 Results

Our 20 study colonies showed considerable variation in the removal of freeze-killed brood by hygienic behaviour from sealed cells in the two days after freezing (53-100%). Each of the fixed effects (treatment type, larval size, days since treatment, and each of their 2-way interactions, as well as the FKB removal rate from sealed cells) had a highly significant effect on the removal of larvae treated in open cells (Table 1).

Table.11.1 Significance levels in the statistical analysis of fixed effects on the removal of larvae treated in open cells.

Fixed Effects	F value	P value
1. Larval-size when treated in open cell (small, medium, large)	303.10	<0.001
2. Days since larval treatment	182.84	<0.001
3. Treatment (control, water control, freezing, chalk brood spores)	1999.54	<0.001
4. Freeze-killed brood removal (%) from sealed cells in the same colony	29.17	<0.001
1 x 2 interaction	22.05	<0.001
1 x 3 interaction	486.55	<0.001
2 x 3 interaction	173.86	<0.001

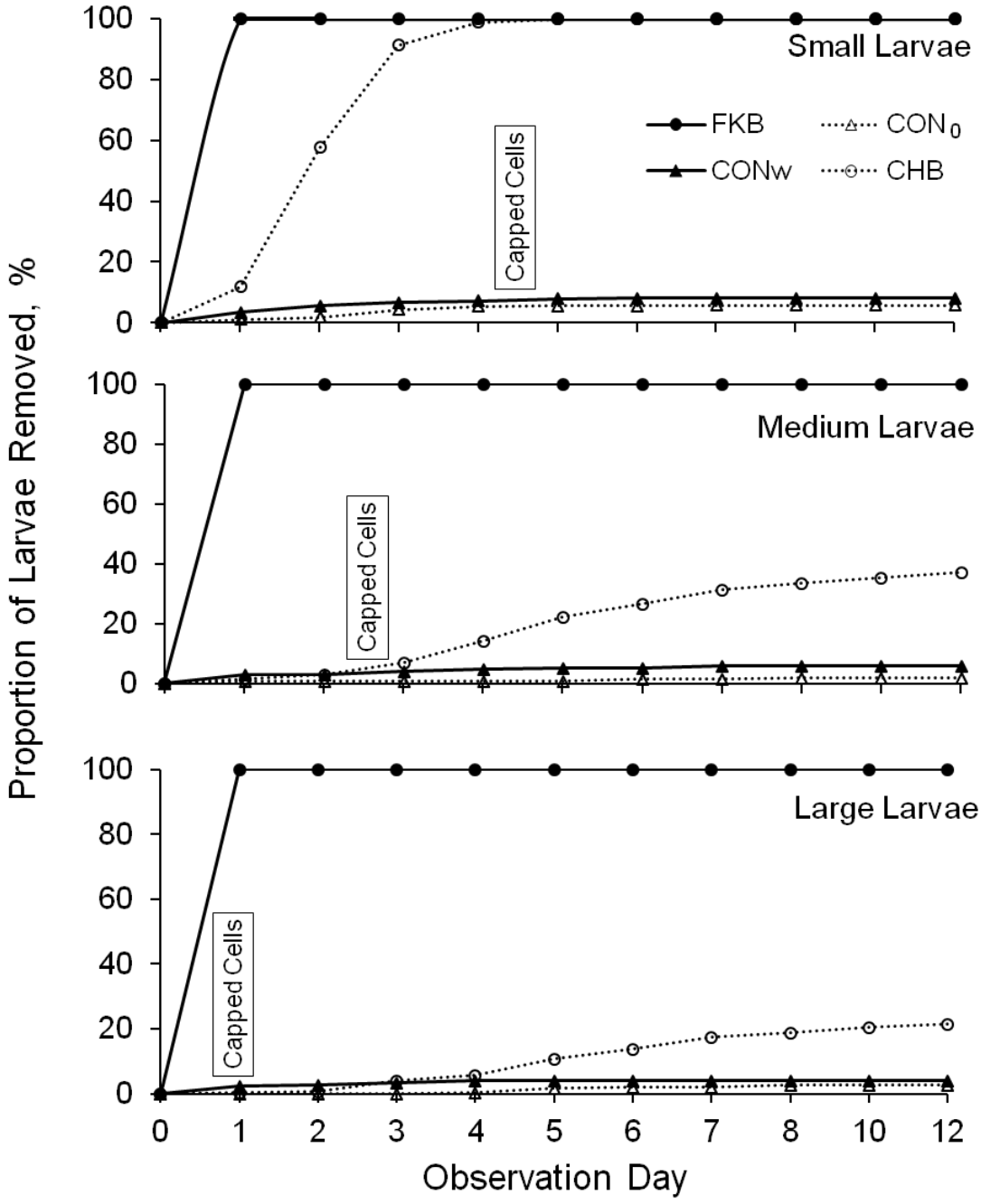

Fig.11.2. Mean proportions of small (0-1 day old), medium (2-3 days) and large (4-5 days) larvae removed from cells in 20 *A. mellifera* colonies. All larvae were in unsealed cells when treated. Cell sealing occurs when a larva is c. 6 days old, which was c. 5, 3 and 1 day after treatment for small, medium and large larvae. Treatments were: CON_0, cell monitored but not treated; CON_w, 5µl distilled water placed in the base of a cell beside the larva at day 0; CHB, 5µl water suspension of chalkbrood spores placed in base of cell beside larva at day 0; FKB, larva killed with liquid nitrogen at day 0.

Figure 2 shows the mean proportion of small, medium and large larvae removed from all colonies. The removal rate of all three sizes of larvae that had been freeze killed with liquid nitrogen was 100% within one day (Figure 2, solid line and circles).

As there was no variation, larval-size, FKB removal from sealed cells, and days since larval treatment were all non-significant (F= 0.00, P = 1.00). Removal was low for both control groups (CON_W [water control; solid line and triangles]; CON_0 [monitored but not treated; dashed line and open triangles]; Figure 2).

The percentage of CON_0 larvae removed were 5.7, 2.0 and 2.8% for small, medium and large larvae, respectively, in the 12 days after treatment, and CON_w larvae showed similar but slightly higher removal rates (8, 6 and 4%). All, 100%, of the small larvae treated with chalkbrood spores were removed before cell sealing in all 20 colonies. Removal rate was much lower for medium (mean 37%, range 23 - 60%) and large (21.5, 13 - 40%) larvae (Figure 2). FKB removal from sealed cells had a non-significant effect (P = 1.00) on the proportion of small larvae removed from open cells because all colonies removed all small infected larvae.

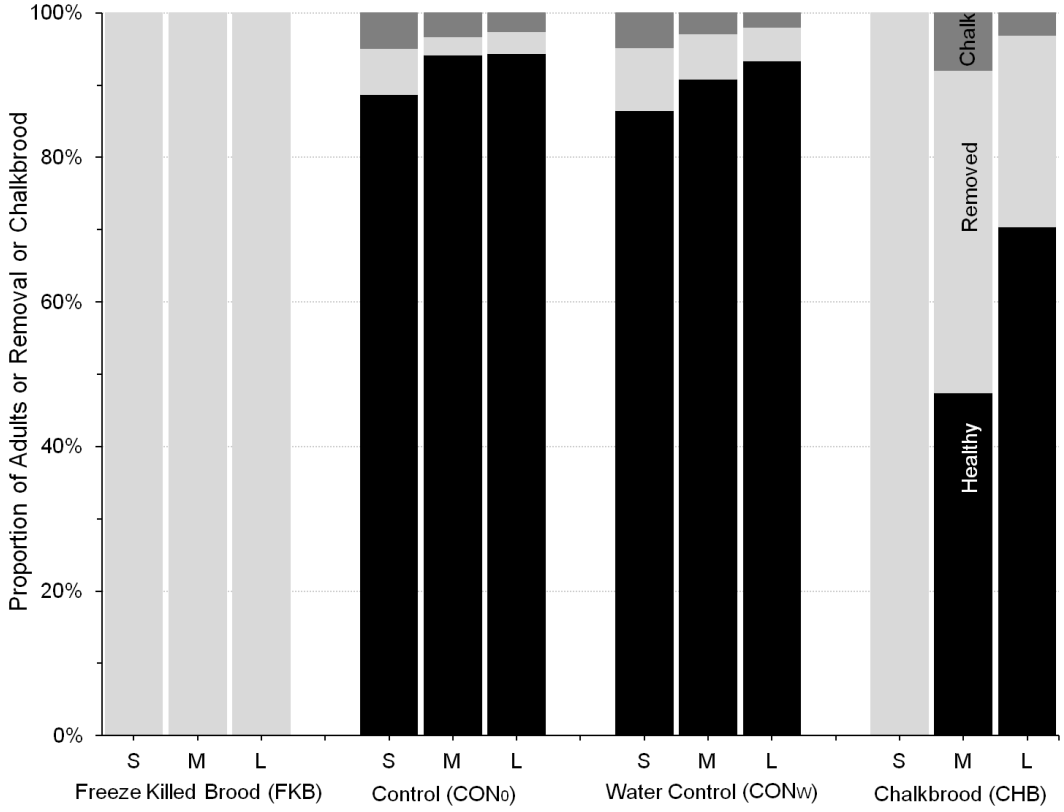

Fig.11.3. Mean % of cells 12 days after treatment that contained either a healthy pupa (black stack), were empty (light grey stack), or contained a chalkbrood mummy (dark grey stack). Each stacked bar shows the mean of 20 colonies x 30 cells per colony. Larvae were all treated in open cells when small (0-1 day old), medium (2-3 days), or large (4-5 days).

Figure 3 shows the proportion of cells, for each initial larval size, that were empty or contained a healthy pupa or a chalkbrood mummy 12 days after treatment.

Chalkbrood mummies occurred in a small proportion of all cells from all treatments, at levels of 1.9-7.8% (Figure 3). Even the CON_0 control resulted in 5, 3.3 and 2.6% chalkbrood mummies for small, medium and large larvae, respectively, probably because our study colonies had mild chalkbrood infections. Large proportions of the medium and large-sized larvae treated with chalkbrood spores also survived to form healthy pupae (medium larvae: mean 49%, range 37-63%; large larvae: mean 73%, range 60-80%; Figure 3).

11.5 Discussion

The results clearly show that all honey bee colonies remove dead brood from open cells, because larvae killed by freezing with liquid nitrogen and larvae with chalkbrood disease were both removed at a rate of 100%. This effect was true even for colonies with low levels of removal of dead brood from sealed cells, which would traditionally be considered as non-hygienic colonies. In the case of chalkbrood, a large proportion of the medium and large sized larvae were not killed and so were not removed, or died and were removed after cell sealing. Previous research has shown that older honey bee larvae are less likely to die from infection by disease spores (Aronstein et al, 2010; Bambrick and Rothenbuhler, 1961; Brødsgaard et al, 1998) and that it takes several days or more for the larva to die. This is also seen in our small larvae treated with chalkbrood, which took up to 4 days to die and be removed. As the larvae were 0-1 days old when treated with the chalkbrood spore suspension, and sealing does not takes place until 5-6 days after the egg hatches (Gould and Gould 1988; Winston, 1991), there was enough time for death and removal before capping.

Previous research on hygienic behaviour has shown that it is a rare trait. For example, of 31 colonies screened using freeze-killed brood only 1 was fully hygienic (defined as removing >95% of the dead brood within 2 days [Spivak and Downey, 1998]), with an average of 46% of the dead brood being removed (Perez-Sato et al, 2009). This contrasts to stingless bees, Meliponinae, in which studies have shown much higher levels of hygienic behaviour. A total of 5 species have been studied using brood freeze killed with liquid nitrogen. Two days after freezing removal was 65% in *Melipona beecheii* and 98% in *Scaptotrigona pectoralis* (Medina et al, 2009; n = 8 colonies per species, Mexico), and 99% in *M. scutellaris*, 80% in *S. depilis* and 62% in *Tetragonisca angustula* (Al Toufailia et al, Chapter 13; n = 8 colonies per species,

Brazil). Across the 5 stinglesss bee species the average was 81% and all colonies removed all cell contents within a maximum of 3-7 days.

The comparison with stingless bees and the fact that all honey bee colonies remove dead and diseased larvae from open cells gives an additional perspective to hygienic behaviour in the honey bee. In particular, it strongly suggests that hygienic behaviour against dead, dying and diseased brood in sealed cells has been reduced by natural selection. In other words, that low hygienic behaviour is actually a trait in its own right, and not simply the absence of a trait.

Hygienic behaviour in the honey bee is known to be a heritable trait (Bigio et al, 2014a; Spivak and Downey, 1998) under the control of multiple genetic loci (Lapidge et al, 2002; Oxley et al, 2010; Rothenbuhler, 1964;). However, it is puzzling that so much variation exists, with both hygienic and non-hygienic colonies (and hence genetic variation) in the same population (Perez-Sato et al, 2009), and with enough genetic variation available to carry out a program of selective breeding which in a few generations results in colonies with >95% removal of freeze-killed brood from sealed cells (Bigio et al, 2014a).

One obvious general hypothesis for the rarity of hygienic behaviour is that it is costly to the colony. However, hygienic colonies have similar or better performance (honey production) in comparison to non-hygienic colonies (Spivak and Reuter, 1998b). In addition, there is no correlation between the removal of healthy brood with the removal of freeze-killed brood indicating that hygienic colonies do not mistakenly remove healthy brood (Bigio et al, 2014b).

We propose another hypothesis for the rarity of hygienic behaviour to explain why genetic and phenotypic variation for this trait is high. This hypothesis builds on an idea previously suggested by Marla Spivak (personal communication). We consider hygienic behaviour to be a defence against brood diseases. This is shown by its effectiveness against a wide variety of diseases (American foulbrood (Spivak and Reuter, 2001), Chalk brood (Oldroyd, 1996), varroa and deformed wing virus (Al Toufailia et al, 2014; Locke et al, 2014; Spivak, 1996).

However, there may be two alternative adaptive responses to diseased brood in sealed cells. First, to remove the diseased brood quickly, thereby eliminating the source of infection from the colony and thereby reducing pathogen transmission (i.e., removal before the pathogen forms spores, as in American foulbrood [Ratnieks, 1992]). Second, leave the diseased brood sealed in its cell so that the source of infection is isolated,

which would also reduce transmission. Each of these twin adaptive peaks might be favoured under distinct conditions. High levels of dead brood removal (i.e., hygienic behaviour) would most likely be selected when disease is abundant, and low levels (i.e., non-hygienic behaviour) when disease is rare. This could lead to periods when selection is changing, resulting in an intermediate situation.

This hypothesis may also explain why stingless bees have high levels of hygienic behaviour. Stingless bees never reuse brood cells (Nogueira Neto et al, 1997). Brood cells are always torn down after being used, and new cells are constructed. As a result, leaving cells containing dead brood is not an option, and as a result there is only one adaptive peak. Another difference between honey bees and stingless bees is colony lifespan. Wild honey bee colonies probably survive only a few years (Seeley, 1978) whereas stingless bee colonies may live for decades (Nogueira Neto et al, 1997). If a honey bee colony leaves cells with disease sealed, they cannot be used for food storage or brood rearing. If a colony is very long lived, but not if the colony is short lived, the number of such cells might increase to a level where much of the nest space was wasted.

Chapter 12

Removal of honey bee larvae infected by different fungal diseases in hygienic and non-hygienic colonies

12.1 Abstract

Hygienic behaviour is a defence against brood diseases in which workers remove dead and diseased brood from sealed cells. This study investigated the fate of larvae treated with two different strains of *Ascosphaera apis*, the causative agent of chalkbrood, and two species of *Aspergillus*, the causative agent of stonebrood, in both hygienic and non-hygienic colonies. The results show that hygienic colonies removed all brood infected with *Aspergillus* from capped cells, and also removed all infected brood with different strains of *Ascosphaera apis*. Non-hygienic colonies did not remove all dead brood from capped cells, as uncapping of cells showed the presence of dead larvae, "mummies", killed by the fungi. The results provide further evidence that hygienic behaviour is a broad-spectrum defence against honey bee brood diseases. The results also show that the freeze-killed brood bioassay indentifies colonies that are hygienic against disease-killed brood in sealed cells.

12.2 Introduction

Disease is one of the challenges faced by honey bees, *Apis mellifera* (Ratnieks and Carreck, 2010; Potts et al, 2010). One widespread brood disease is chalkbrood, which is caused by the fungus *Ascosphaera apis* (Maassen, 1913) and is found worldwide (Aronstein and Murray, 2010; Reynaldi et al, 2003). Chalkbrood kills infected larvae in approximately 2-3 days (Aronstein et al, 2010). Although it rarely kills colonies (Carreck et al, 2010; Highfield et al, 2009) it weakens colonies by killing larvae and can reduce honey production by 5-37% (Bailey, 1963; Heath, 1982a; Wood, 1998; Zaghloul et al, 2005). Stonebrood, causative agent *Aspergillus*, is another fungal brood disease that kills honey bee brood (Gilliam and Vandenberg, 1997; Foley et al, 2014). Although not as common as chalkbrood it is reported to be able to kill colonies (Burnside, 1930).

Hygienic worker honey bees remove dead, dying and infected individuals from their cells thereby reducing disease levels (Park et al, 1937; Rothenbuhler, 1964a,b; Wilson-Rich et al, 2007). Larvae infected by a wide variety of pests and pathogens,

including varroa mites, chalkbrood, American foulbrood, and deformed wing virus are removed (Al Toufailia et al, 2014; Gilliam et al, 1983; Oldroyd, 1996; Spivak and Reuter, 2001) showing that it is a generalized defence against brood diseases. However, not all potential brood diseases and strains have been investigated. The aim of this study was to investigate the fate of larvae treated with different strains of *A. apis* and *Aspergillus* in both hygienic and non-hygienic colonies. Our results show that hygienic bees remove brood infected with both pathogens.

12.3 Materials and Methods

12.3.1 Study colonies and data collection

We studied ten colonies of the honey bee, *Apis mellifera*, located in a single apiary on the University of Sussex campus in August and September 2014. Each colony was housed in a hive consisting of a single "commercial" brood chamber, volume 56 litres, containing 11 frames, 43.8 x 25.4 cm, bottom board, inner cover and telescopic cover. All ten colonies had several frames of honey, an egg-laying queen, empty frames for egg laying, and were of approximately equal population (five frames of brood and nine frames of bees).

During the 4 months leading up to data collection, no frames of brood were introduced into any study colony from another colony. As a result, the worker bees in each colony were the progeny of the colony's queen, other than those that may have drifted between hives. Drifting was minimized by placing hives at least 2 m apart in the apiary, and with the entrances of neighbouring hives pointing in different directions. Colonies were inspected for visible symptoms of brood diseases. Sac brood, American foulbrood and European foulbrood were not seen. Chalkbrood mummies were seen in the 5 non-hygienic study colonies at a level of 30 or less per colony (number seen in brood combs during a hive inspection) but not in the 5 hygienic study colonies.

12.3.2 Quantifying hygienic behaviour using the Freeze Killed Brood (FKB) bioassay

We determined the level of hygienic behaviour in each study colony using the freeze-killed brood (FKB) bioassay (Bigio et al, 2014a, Spivak and Downey 1998; Spivak and Reuter, 1998a,b). Each colony was tested four times at weekly intervals starting in early July. For each FKB bioassay we took a test frame with a large area of capped brood from each colony. We pressed two metal cylinders (6.5 cm diameter × 8 cm height) into the capped brood. Approximately 300 ml of liquid nitrogen was then poured into each

cylinder to kill the circle of capped brood inside. After a few minutes, when the liquid nitrogen had evaporated, we removed the cylinders. The test frame was then photographed and returned to the hive, then removed again and photographed 48 ± 2 hours later. From these photographs the proportion of frozen capped-brood cells which had been cleaned out by hygienic behaviour was determined (Bigio et al. 2014a; Perez-Sato et al. 2009).

Fig. 12.1. Examples of small and medium larvae in open cells: Blue (small, aged <1 day) and yellow (medium, aged c. 2-3 days old) [solid line]). Also shown but not used are: White (large larvae aged c. 4-5 days old and within 1 day of being capped [solid line], or already being capped (dashed circle); Red (eggs); Unmarked: larvae of intermediate sizes, sealed cells with brown wax cappings, and empty cells.

12.3.3 Fungal treatments:

We used four different types of fungi with either low or high levels of virulence. Two strains of the causative agent of chalkbrood, *Ascospharea apis* Strain F (low virulence)

[CH_L] and Strain I (high virulence)[CH_H], and two species of the causative agent of stonebrood, *Aspergillus niger* (low virulence) [ST_L] and *A. flavus* (high virulence) [ST_H]. Each was grown on Sabouraud Dextrose Agar media plates (Jensen et al, 2013). As *Ascosphaera apis* is a heterothallic fungus, spores were produced by the mating of two different mating type isolates where their mycelium growth touched on the media plate (Strain F by isolates KVL 06123 and KVL 06132; Strain I by isolates ARSEF 7405 and ARSEF 7406).

12.3.4 Preparation of spore suspensions

Spores were allowed to mature before being harvested from the SDA media plates. From each fungal strain or species, spore suspensions were made by grinding a small amount of spore-rich material (ca. 0.01g) in a glass tissue homogenizer with 200 µl sterile deionized water. The spores released by this process were made up to a volume of 1 ml with sterile deionized water and left to stand for 20 min to ensure any spore clumps settled to the bottom. A 0.5 mL aliquot was taken from the central column of the resulting medium-density spore solution and stored in a separate Eppendorf tube. The concentration of the medium density spore solution was determined using a FastRead disposable hemocytometer (Immune Systems, UK) and diluted to a concentration of 2 x 10^6 spores per ml, which is enough to kill a honey bee larva. Spore viability was determined as detailed in Vojvodic *et al*. (2011), and the suspension adjusted accordingly. 5 µl of each spore suspension was applied directly to the area around a larva at the bottom of a test cell with a pipette, where it would mix with the larval food, being careful not to cause any physical damage to the larva.

12.3.5 Quantifying removal of larvae treated with chalkbrood spores

One frame of brood with larvae of different ages was chosen from each colony and taken into a warm room, 25°C, in the laboratory. A clear acetate sheet was attached to the test frame with drawing pins. On each frame, 12 groups of 50 cells, each containing a worker larva in an uncapped cell, were marked on the acetate sheet so that the position of each cell was precisely recorded. Six groups of very small larvae (< 1 day old; Figure 1), and six groups of medium larvae (c. 2-3 days old; Figure 1) were located. In each of these two larval size categories, one group was a control, CON_0, and was not manipulated in any way. One, CON_w, was a control treated with 5µl sterile deionized

water. The other four groups were each treated with one of the four spore treatments. Whether or not a larva had been removed from each cell was determined 1, 2, 3, 4, 5, 6, 7, 8, 10, and 12 days after treatment. Any cells that were not removed after 12 days were uncapped to check the contents, and in particular to check if it contained a healthy pupa or adult bee, or a fungal mummy.

12.3.6 Statistical analysis

Data were analysed using IBM SPSS version 20. We used Generalised Linear Mixed Models (GLMM) (Zuur et al. 2010) fitted with a Poisson distribution and a log-link function. The number of larvae removed was the response variable, and one or more of the following variables included as the predictor, depending on the aim of the test: 1. treatment type (control [CON_0]; water control [CON_w]; four different fungal spore suspensions: chalkbrood low [CB_L] and high [CB_H] virulence strains; stonebrood low [ST_L] and high [ST_H] virulence strains); 2. days since treatment; 3. larval size; 4. hygienic type (hygienic or non-hygienic) colony, and colony was fitted as a random effect to account for the repeated measures made within each colony. $P < 0.05$ is defined as significant, and descriptive statistics are given as mean ± standard error.

12.4 Results

Table 1 shows the results as P values for the different, effects, treatments, and interactions.

Table.12.1. F and P values of the statistical analyses of the removal of young versus medium larvae in open cells treated with 4 types of fungal spore suspensions and 2 control treatments from their cells for 12 days after treatment.

Fixed Effects	F value	P
Larvae-size	1130.96	<0.001
Observation day	629.54	<0.001
Treatment type	80.59	<0.001
Hygienic type	52.96	<0.001
Larvae-size* Observation	438.02	<0.001
Larvae-size* Treatment	24.02	<0.001
Larvae-size* Hygienic type	63.51	<0.001
Observation day*	16.37	<0.001
Observation day* Hygienic	4.98	0.026
Treatment type*Hygienic	4.12	0.001

Figure 2 shows the proportion of small and medium sized larvae removed. Only small proportions of the two controls (CON_W, CON_0) were removed. Even though the removal of control larvae that were completely untreated, CON_0, was low there was a significant effect of larval size: ($F=22.30$, $P<0.001$) However, hygienic type (low or high) had no effect ($F=0.24.6$, $P=0.62$).

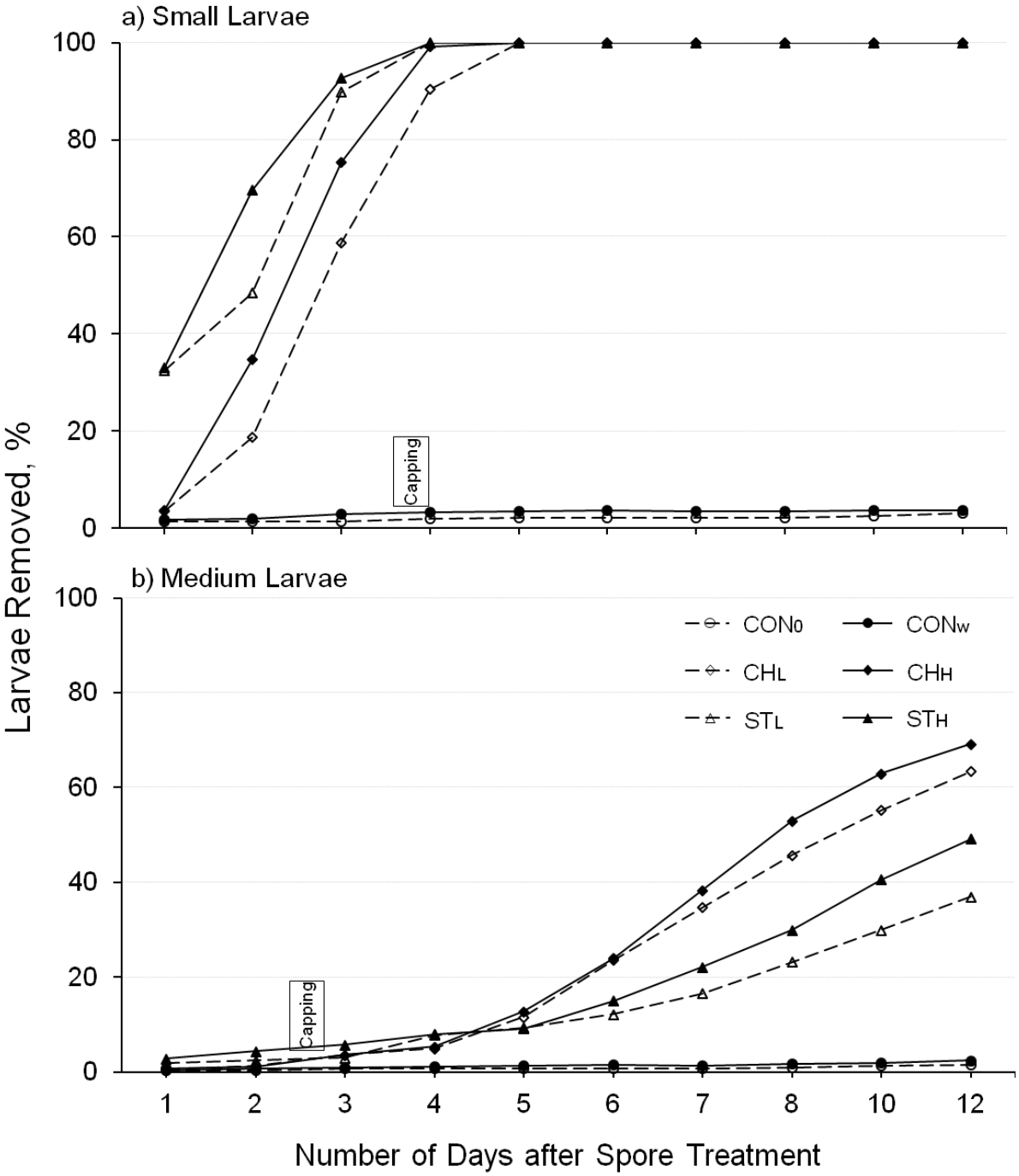

Fig. 12.2. Proportion of small (above) and medium (below) sized larvae removed. Data points show means for the 10 study colonies. Cells were sealed 4-5 and 2-3 days after treatment for small and medium larvae, respectively.

In the control cells treated with water only, CON_w, there was slightly higher removal of larvae than in CON_0 (3.08 vs 2.06% small larvae; 1.4% vs 0.88% medium). Larval size ($F=29.92$, $P<0.001$) and hygienic type ($F=15.93$, $P<0.001$) both had a significant effect on the proportion removed. Bonferroni post-hoc comparison test showed that the removal of small and medium larvae in CON_0 is not significantly different from CON_w (small: $P=0.055$; medium: $P=0.457$).

Small larvae treated with all 4 spore suspensions were 100% removed before the larvae reach the capping stage, 4-5 days after treatment with spore suspension (Figure 2). There was a significant effect of Treatment type ($F=4.85$, $P=0.003$) but not of hygienic type ($F=1.16$, $P=0.29$). Bonferroni post-hoc tests showed that the removal of small larvae was only significantly different between stonebrood both high and low virulence strains from chalkbrood low virulence (ST_H v. CH_L: $P=0.029$; ST_L v. CH_L: $P=0.004$).

The removal of medium larvae varied among colonies in all 4 treatments (mean in medium $ST_H=18.7\%$, range: 3-49; $ST_L=14.36$, range: 2-37; $CH_H=27.1$, range: 1-69; $CH_L=24.5$, range: 1-63). There was a significant effect of treatment type ($F=32.47$, $P<0.001$) and hygienic behaviour also has a significant effect ($F=87.62$, $P<0.001$). Figure 3 shows that the proportion of medium larvae removed in hygienic colonies was higher in $CH_H > CH_L > ST_H > ST_L$ but Bonferroni post-hoc comparison test showed this is only significantly different in ST_H v. ST_L: $P=0.031$ and ST_L v. CH_H: $P=0.002$ (Figure 3). In the non-hygienic colonies, the proportion of medium larvae removed was higher in $CH_H > CH_L > ST_H > ST_L$ but Bonferroni post-hoc comparison test showed that is only significantly different in ST_H v. CH_H: $P<0.001$; ST_H v. CH_L: $P<0.001$; ST_L v. CH_H: $P<0.001$; ST_L v. CH_L: $P<0.001$.

On day 12 after treatment any capped cells were uncapped. We found cells containing healthy pupae and adults or mummies. In hygienic colonies, there were zero mummies in all treatments. In the non-hygienic colonies, there were 3.7 and 2.8% mummies in small and medium CON_0 control cells, and 3.7 and 3.6% in small and medium CON_w control cells, respectively. In fungal treatment cells mummies were found only in medium-sized larva cells, (11.6, 16.4, 1.2 and 1.9% in CH_L, CH_H, ST_L and ST_H, respectively).

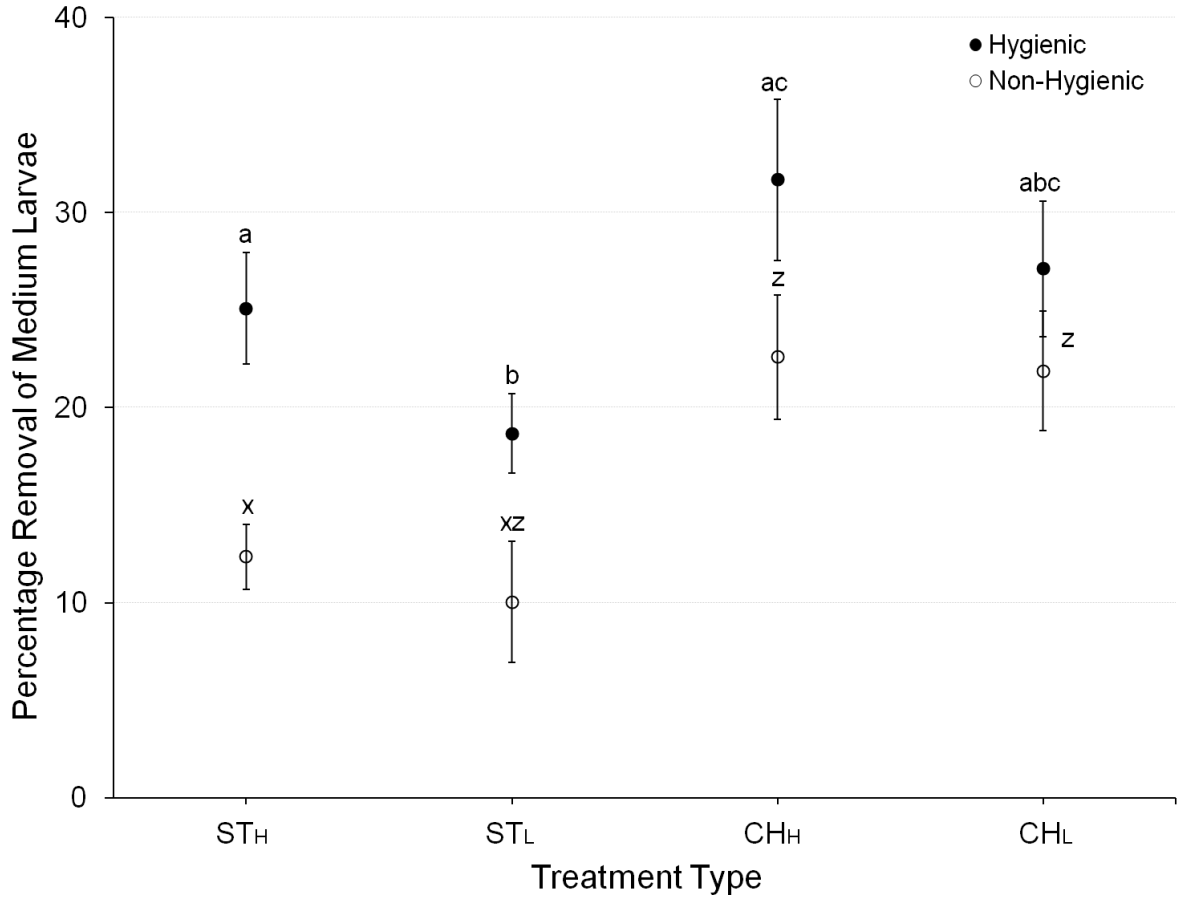

Fig. 12.3. Mean proportions, ± standard error, of medium-sized larvae treated with the 4 fungal spore suspensions removed from cells in hygienic (n = 5) and non-hygienic (n = 5) colonies. ST (stonebrood), treated with *Aspergillus* of low, L, and high, H, virulence. CH (chalkbrood), treated with *Ascosphaera apis*.

12.5 Discussion

Our results show that hygienic workers bees remove larvae from sealed cells that are infected with two species of *Aspergillus*, the causative agent of stonebrood, and two strains of *Ascosphaera*, the causative agent of chalkbrood. This shows that hygienic behaviour is likely to be effective against natural occurences of stonebrood and is further evidence that hygienic behaviour is effective against all brood diseases, including different strains of the same disease. It is also further evidence that the freeze-killed brood bioassay (Spivak, 1996; Spivak and Reuter, 2001) allows for the selection of disease resistance, even though it is based on the removal of freeze-killed brood rather than disease-killed or infected brood.

The results also support the conclusion from Chapter 11, that the level of hygienic behaviour had no effect on the removal of larvae that die in open cells, all of which are removed. In both this chapter and the previous chapter, 100% of the small

larvae infected with disease spores were removed from their cells before capping (Figure 2). However, removal was one day faster for stonebrood than chalkbrood, and faster with the more virulent strains.

Hygienic behaviour had a significant effect on the removal of dead medium larvae ($P< 0.001$). Hygienic bees removed all 4 groups of infected dead medium larvae and responded with higher removal in chalkbrood than stonebrood and more in the virulent strains in either of the two types of fungi (Figure 3). When we uncapped all remaining cells after 12 days, there were no mummies in the cells in hygienic colonies. This shows that hygienic behaviour was effective against larvae killed by all 4 strains.

In the non-hygienic colonies we had less variation in removal between strains of either of the two fungi but there was higher removal of dead medium larvae infected with chalkbrood than stonebrood (Figure 3). In fungal treatment cells, mummies were found only in medium sized larva cells in the non-hygienic colonies (11.6, 16.4, 1.2 and 1.9% in CH_L, CH_H, ST_L and ST_H respectively). The low level of stonebrood mummies in the non-hygienic colonies after 12 day could be because the dose was insufficient to cause high mortality, unlike with chalkbrood.

Our results provide further evidence to support the view that hygienic behaviour is a useful defence against diseases that affect honey bee brood. Not all honey bees are hygienic, but it is possible to breed hygienic bees by selecting queens and males from hygienic colonies detected by using the freeze-killed brood (FKB) removal bioassay (Ibrahim et al, 2007; Perez-Sato et al, 2009; Spivak, 1996).

Chapter 13

Hygienic Behaviour in Brazilian Stingless Bees

13.1 Abstract

Social insects have many defence mechanisms against pests and pathogens. One of these is hygienic behaviour, which has been studied in detail in the honey bee, *Apis mellifera*. Hygienic honey bee workers remove dead and diseased larvae and pupae from sealed brood cells, thereby reducing disease transfer within the colony. Stingless bees, Meliponini, also rear brood in sealed cells. We investigated hygienic behaviour in three species of Brazilian stingless bees (*Melipona scutellaris, Scaptotrigona depilis, Tetragonisca angustula*) in response to freeze-killed brood. All three species had high mean levels of freeze-killed brood removal after 48 hours c. 99% in *M. scutellaris*, 80% in *S. depilis* and 62% in *T. angustula* (N = 8 colonies per species; 3 trials per colony). These levels are greater than in unselected honey bee populations 46%. In *S. depilis* there was also considerable intercolony variation, ranging from 27% to 100% removal after 2 days. Interestingly, in the *S. depilis* colony with the slowest removal of freeze-killed brood, 15% of the adult bees emerging from their cells had shrivelled wings indicating a disease or disorder, which is as yet unidentified. Although the gross symptoms resembled the effects of deformed wing virus in the honey bee, this virus was not detected in the samples. When brood comb from the diseased colony was introduced to the other *S. depilis* colonies, there was a significant negative correlation between freeze-killed brood removal and the emergence of deformed worker bees (*P= 0.001*), and a positive correlation with the cleaning out of brood cells (P= 0.0008). This shows that the more hygienic colonies were detecting and removing unhealthy brood prior to adult emergence. Our results indicate that hygienic behaviour may play an important role in colony health in stingless bees. The low levels of disease normally seen in stingless bees may be because they have effective mechanisms of disease management, not because they lack diseases.

13.2 Introduction

Hygienic behaviour is one of a large number of defence mechanisms that honey bees, *Apis mellifera*, have against pests and diseases (Park, 1937; Rothenbuhler, 1964a,b; Spivak and Gilliam, 1998; Wilson-Rich et al, 2009). In particular, it is a social defence

against diseases of brood (larvae and pupae) in sealed cells. Worker honey bees showing hygienic behaviour detect and uncap cells containing dead and infected brood, and remove the contents from the colony (Rothenbuhler, 1964a,b). Hygienic behaviour has been shown to help control varroa mites, deformed wing virus (DMV), chalkbrood, and American foulbrood (Al Toufailia et al, 2014; Spivak and Gilliam, 1998a,b; Wilson-Rich et al, 2009). Hygienic behaviour is not learned. Rather it is an instinctive heritable trait controlled by multiple genetic loci (Jones and Rothenbuhler, 1964; Momot and Rothenbuhler, 1971; Rothenbuhler, 1964a,b; Wilson-Rich et al, 2009). Hygienic behaviour does not result in the excess removal of healthy brood (Bigio et al, 2014b) or reduce honey production (Spivak and Reuter, 1998b).

Historically, honey bee hygienic behaviour was investigated by introducing the spores of *Paenibacillus larvae*, the causative agent of American foulbrood, into cells with young larvae, by killing whole frames of brood using hydrogen cyanide gas, by wounding pupae in capped cells with a pin inserted through the wax capping, by freezing whole brood frames or by cutting out patches of brood from combs to be frozen and then placed back into the hive (Rothenbuhler, 1964a,b; Spivak and Downey, 1998). Nowadays, the level of hygienic behaviour in a colony is commonly determined by killing an area of capped cells by freezing with liquid nitrogen *in situ* (Spivak and Downey, 1998). Two days later, the cells with dead brood are checked to determine the proportion cleaned out. Unselected populations of honey bees show wide variation among colonies in the level of hygienic behaviour, but with a low mean and with few colonies that have high levels of hygienic behaviour (Perez-Sato et al, 2009). By selective breeding it is possible to obtain colonies that are fully hygienic, defined as >95% removal of freeze-killed brood within 2 days (Bigio et al, 2014a; Ibrahim et al, 2007; Spivak and Reuter, 1998a,b).

Stingless bees, Meliponini, are a large group of eusocial bees found in the tropics worldwide and in the southern subtropics (Michener, 2000). They are important pollinators of wild plants and are increasingly being studied and used in Brazil and other countries for crop pollination (Heard, 1999; Slaa et al, 2006) and honey production (Cortopassi-Laurino et al, 2006). Like honey bees, stingless bees live in colonies with a queen and many workers and rear brood individually in cells. In contrast to honey bees, in which food is given progressively to larvae in open cells, in stingless bees the brood cells are mass provisioned. Cells are filled with larval food regurgitated by worker bees just before oviposition by the queen, and sealed immediately after

(Michener, 1974). Stingless bees are known for their varied nest defence against predators (Kerr and de Lello, 1962; Shackleton et al, 2015; van Zweden et al, 2011), but compared to honey bees, far less is known about their diseases and disease resistance. An important Brazilian book on beekeeping with stingless bees (Nogueira-Neto, 1997) has only the barest mention of diseases, in contrast to the considerable attention given to diseases of honey bees in beekeeping books. Indeed, there are numerous books just on honey bee pests and diseases or pathology (Bailey and Ball, 2013; Morse and Flottum, 1997; Morse and Richard, 1990). This may be because stingless bees have fewer pathogens and diseases, or it may be because they have highly effective ways of controlling diseases so that disease problems are rarely seen.

Hygienic behaviour has been studied in two species of stingless bees from Mexico, *Melipona beecheii* and *Scaptotrigona pectoralis* (Medina et al, 2009), using the freeze-killed brood bioassay. On average *M. beecheii* colonies took 4.4 days to remove all the frozen brood whereas *S. pectoralis* took significantly less, 2.2 days (n = 8 colonies per species). Compared to unselected honey bees, these are high levels. Indeed, all 8 colonies of *S. pectoralis* would have been considered highly hygienic, >95% removal within 2 days, by honey bee standards. In Brazil, *Plebeia remota*, has also been studied for hygienic behaviour using pin-killed brood, which resulted in 96.4% removal after 48 hours (Nunes-Silva et al, 2009).

The aim of the current project was to gather further information on hygienic behaviour in stingless bees. We chose one species each of the two genera studied in Mexico (*M. scutellaris, S. depilis*) and one additional species, *Tetragonisca angustula* to test using the freeze-killed brood. In addition, we also studied the effect of hygienic behaviour on the removal of *S. depilis* brood suffering from a novel and naturally occurring disease or disorder that we fortuitously observed.

13.3 Materials and Methods

13.3.1 Study site and species

The study was carried out in the Laboratory of Useful Insects (Laboratório de Insetos Úteis) at the University of São Paulo "ESALQ", Piracicaba, São Paulo State, Brazil, between 21 February and 22 March 2015. The study colonies of *Scaptotrigona depilis* and *Tetragonisca angustula* were kept in wooden box hives (inside measurements 26 x 26 x 35 (high) cm and 25 x 13 x 13 (high) cm, respectively), in an outdoor meliponary shelter beside the laboratory. The wooden hives (inside measurements 43 x 24 x 18

(high) cm) of *Melipona scutellaris* were kept in a brick laboratory building with tubes leading from the hive entrances through holes in the walls to the outside to allow foraging. All colonies had a queen, brood of all ages, and pollen and honey stores, and were typical of their species in regard to colony size. Colonies were of similar population within a species, and the combs and food pots filled most but not all of each hive (mean: *M. scutellaris* 70% of hive filled, *S. depilis* 63%, *T. angustula* 80%).

The three study species were chosen as they are among the species of stingless bees most used in Brazil for honey production and pollination. For example, controlled queen-rearing methods are currently being developed for *S. depilis* in order to provide a supply of colonies for crop pollination (Menezes et al. 2013). As a result, information on disease resistance is of value, and could also be incorporated into a breeding program.

13.3.2 Experiment 1. Removal rates of freeze-killed brood (FKB)

At the start of the study, each colony was inspected and a brood comb containing larvae and pupae was removed and divided into several pieces. Each piece was put into a plastic petri dish with lid and frozen at -20°C. These pieces were then used in a series of 3 trials of freeze-killed brood removal, one per week, starting 2 days later. Each trial used an average of 319.5±10.4 (Mean ± SE) sealed brood cells.

To carry out a trial, one or two pieces of the previously-frozen brood comb were photographed to count the number of capped cells. These comb pieces were then placed into the top of each study hive (n = 8 per species). The hive was then inspected every 24±1 hour for the next 6 days or until all the previously-frozen brood had been removed. Daily photographs were taken to determine the number of brood cells that still remained, and whether these had been fully cleaned out or just uncapped.

13.3.3 Experiment 2. Removal of live brood in S. depilis, in which some cells produced workers with shrivelled wings

At the start of the study we noticed that below one of our *S. depilis* study colonies (Colony 6) there were many young worker bees on the concrete floor of the meliponary shelter. On inspection, we saw that these had shrivelled wings (Figure 5, photo b). Experiment 1 showed that Colony 6 had the lowest level of freeze-killed brood removal of the 8 study colonies of *S. depilis*. We, therefore, hypothesised that more hygienic *S. depilis* colonies would be able to detect and clean out cells with these diseased or

disordered brood leading to lower production of adult worker bees with shrivelled wings.

To test this hypothesis we introduced patches of live brood (cells containing older larvae and pupae; mean 185±8.0 sealed cells), taken from colony 6 into all 8 colonies and quantified the cleaning out of cells for 5 days. We also introduced a patch of live brood taken from a healthy colony (colony 24) into all 8 colonies. This was a non-nestmate control, to allow for the possibility that removal was not due to disease but because the comb had come from another colony. We monitored the combs as before by taking daily photographs. In addition, at the end of the test period each piece of live comb was placed into an incubator (28°C), to allow the worker bees to emerge from their cells. We then determined the proportion emerging with shrivelled wings. We carried out two trials during the final 2 weeks of the study period.

The cause of the shrivelled-wing workers remains to be determined. Their overall appearance was similar to that of honey bee workers with overt symptoms of deformed wing virus (DWV) (Al Toufailia et al, 2014). However, cDNA generated from RNA extracted from pooled samples of workers from both the diseased colony and healthy *S. depilis* colonies did not amplify for primers of DWV-F2 (strain A) and DWV-R2a (strain B) (McMahon et al, 2015) (Robert Paxton and Kristin Gößel helped analysing samples of *S. depilis* for deformed wing virus). Nevertheless, the unidentified cause was naturally occurring, and resulted in a serendipitous opportunity to gather important additional information of the role of hygienic behaviour in removing unhealthy brood from sealed cells.

13.3.4 Statistical analysis

Data were analysed using the IBM SPSS statistical program version 20. If necessary, we log or arcsine transformed the response variable to meet the assumptions of ANOVA (Grafen et al, 2002; Zuur et al, 2010). We then used ANOVA to test the effect of colony, trial and species on the time taken to remove all freeze-killed brood. Linear regression was used to test for the effects of hygienic behaviour on the removal of live brood from colony 6. $P<0.05$ is defined as significant. Descriptive statistics are given as mean ± standard error.

13.4 Results

13.4.1 Experiment 1. Removal rates of freeze-killed brood

There was no significant difference between the three trials (F= 1.08; P= 0.35). All three study species showed high levels of hygienic behaviour, in that freeze-killed brood were removed rapidly. After 2 days, the mean ± SE removal of freeze-killed brood was 99.3 ±0.5% in *M. scutellaris*, 79.5±9.6% in *S. depilis* and 62±12.4% in *T. angustula* (Figure 1). The time taken to remove all freeze-killed brood ranged from 1 to 3 days (1.3±0.2) in *M. scutellaris*, 2 to 4 days (3.0±0.3) in *T. angustula*, and 1 to 6 days (2.7±0.6) in *S. depilis* (Figure 1). *M. scutellaris* was significantly faster than *S. depilis* and *T. angustula* (P<0.001) which were not significantly different from each other (P= 0.58).

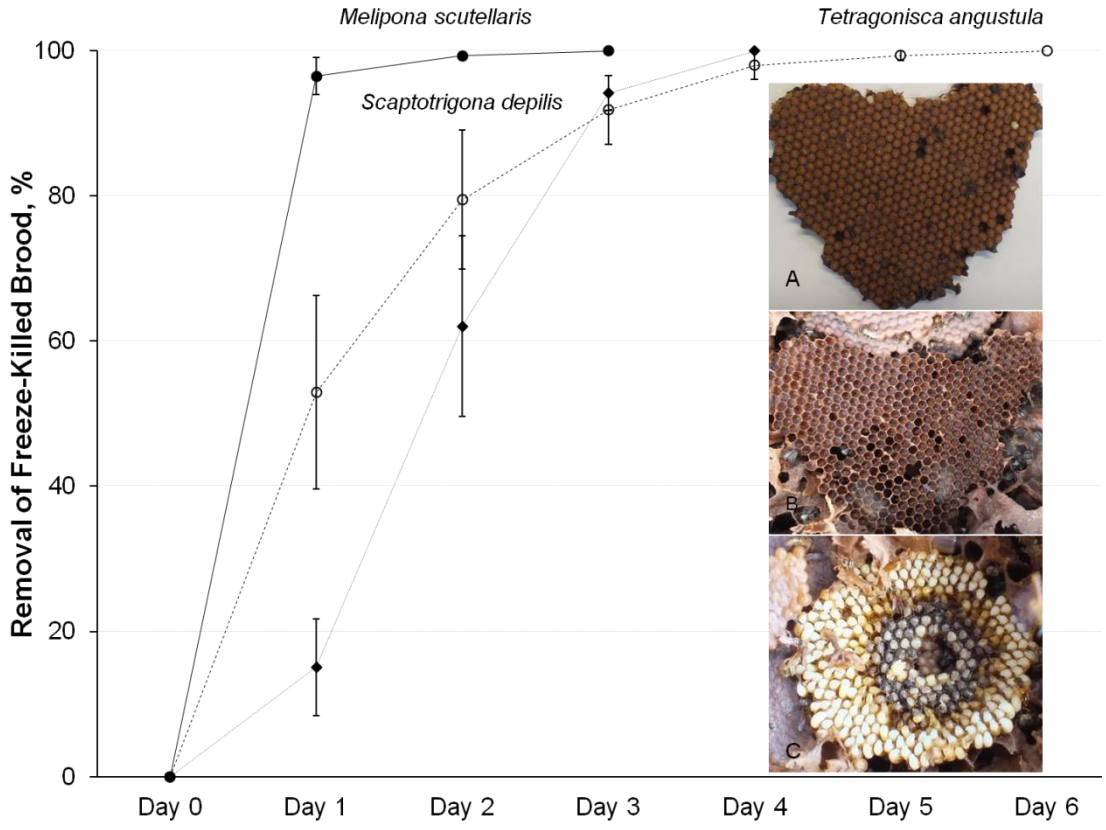

Fig.13.1. Experiment 1. Removal of freeze-killed brood in the 3 study species of stingless bees. Each data point shows the mean and standard error of 24 trials (8 colonies per species x 3 trials). The photos show: a) a piece of previously-frozen comb from *S. depilis* with c. 420 cells as placed into a test colony on day 0 of a trial; b) the same piece of comb after 3 days with 100% of the dead brood removed; c) a piece of previously-frozen comb after 1 day in a colony of *T. angustula* in which all cells have been uncapped but few dead brood have so far been removed.

Figure 2 shows intraspecific variation in FKB removal, which was significantly different between colonies within species (F= 17.96; P< 0.001). Variation is minimal in

M. scutellaris but noticeable in the other two species and especially in *S. depilis*, which ranged between 27 and 100% removal after 2 days.

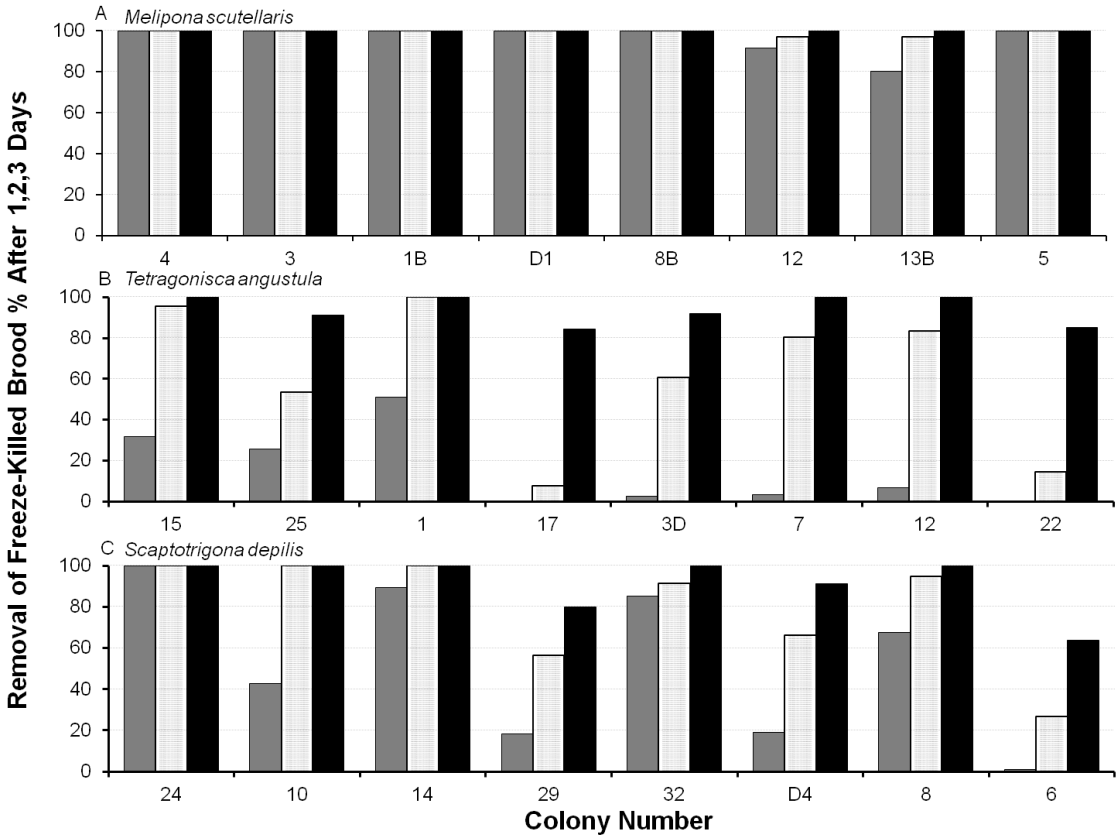

Fig.13.2. Intercolony variation in hygienic behaviour, the removal of freeze-killed brood after 1, 2, 3 days (dark grey, light grey, black, respectively), in *M. scutellaris, T. angustula* and *S. depilis*. a) in *M. scutellaris* there is very little intercolony variation; b, c) in the other 2 species intercolony variation is much greater. Each histogram bar is the mean of 3 trials per colony.

In *T. angustula* the removal of FKB in the first day was lower than in the other two species. In this species most colonies had a delay of approximately one day between uncapping a cell and then removing its contents (Figure 3). 60±15.1% of the freeze-killed brood cells were uncapped in the first day (range: 28 to 100%) but only 15±10.4 % were removed (range: 0-51%). By the end of the second day, 97±3.1% had been uncapped (range: 89-100%) and the contents had been removed from 62±13.9% (range: 8-100%). After 48 hours colonies did not differ greatly in the proportion of uncapped cells, but differed more in the proportion from which the contents had been removed. For example, colonies 15 and 1 were able to uncap and remove the FKB quicker than colonies 17 and 22.

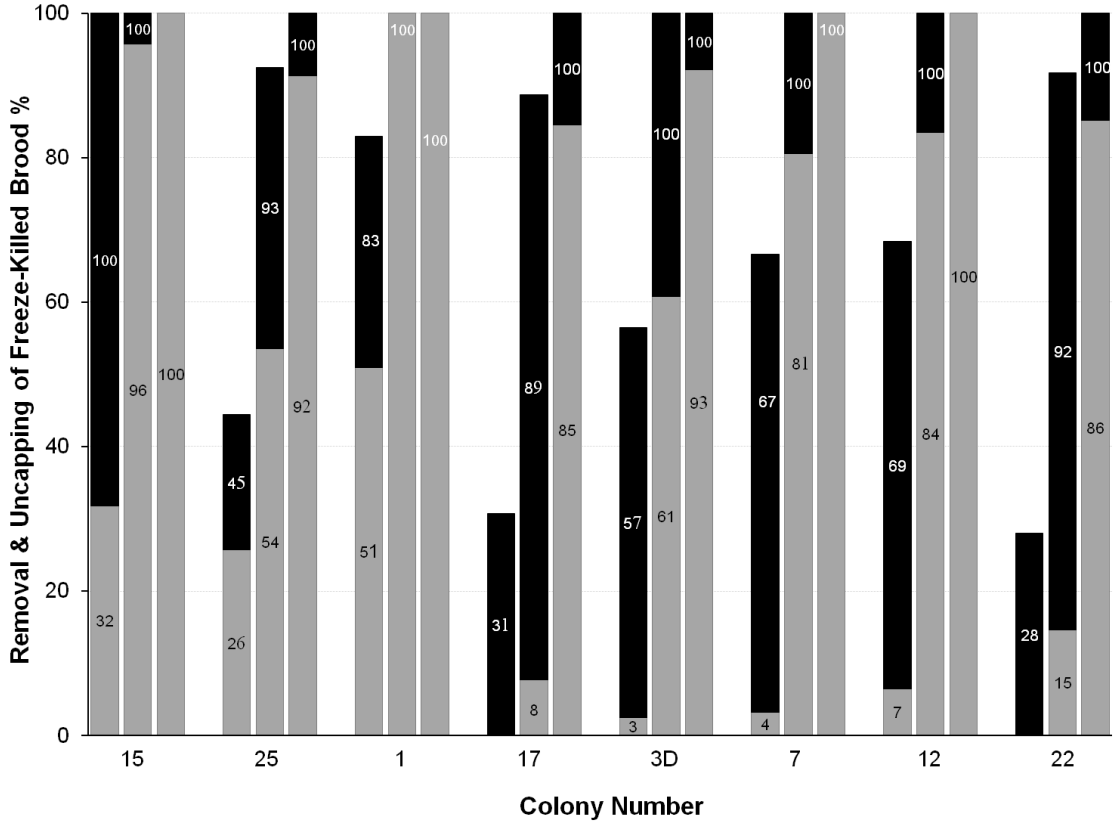

Fig.13.3. Variation in uncapping and removing of freeze-killed brood between colonies of *T. angustula* 1, 2 and 3 days after introduction to the test colony. Most of the variation between uncapping and removing was in the first day. Black: cells uncapped but dead brood not yet removed. Grey: cell contents removed.

13.4.2 Experiment 2. Removal of live brood in S. depilis, in which some cells produced workers with shrivelled wings

Figure 4 shows that all 8 *S. depilis* study colonies removed very few live healthy brood (1 ± 0.2%) taken from colony 24, showing a zero or negligible tendency to remove healthy non-nestmate brood (F= 3.84; P= 0.10). Removal of this healthy brood was not different between the 2 trials (F=2.31; P=0.15). However, colonies varied greatly in the removal of live unhealthy brood, taken from colony 6 (0.5-12.5%). The proportion removed was positively and significantly correlated with the removal of freeze-killed brood in Experiment 1 (F= 38.62; P=0.0008). Removal of live brood was not significantly different between the 2 trials (F= 0.013; P= 0.91) (Figure 4).

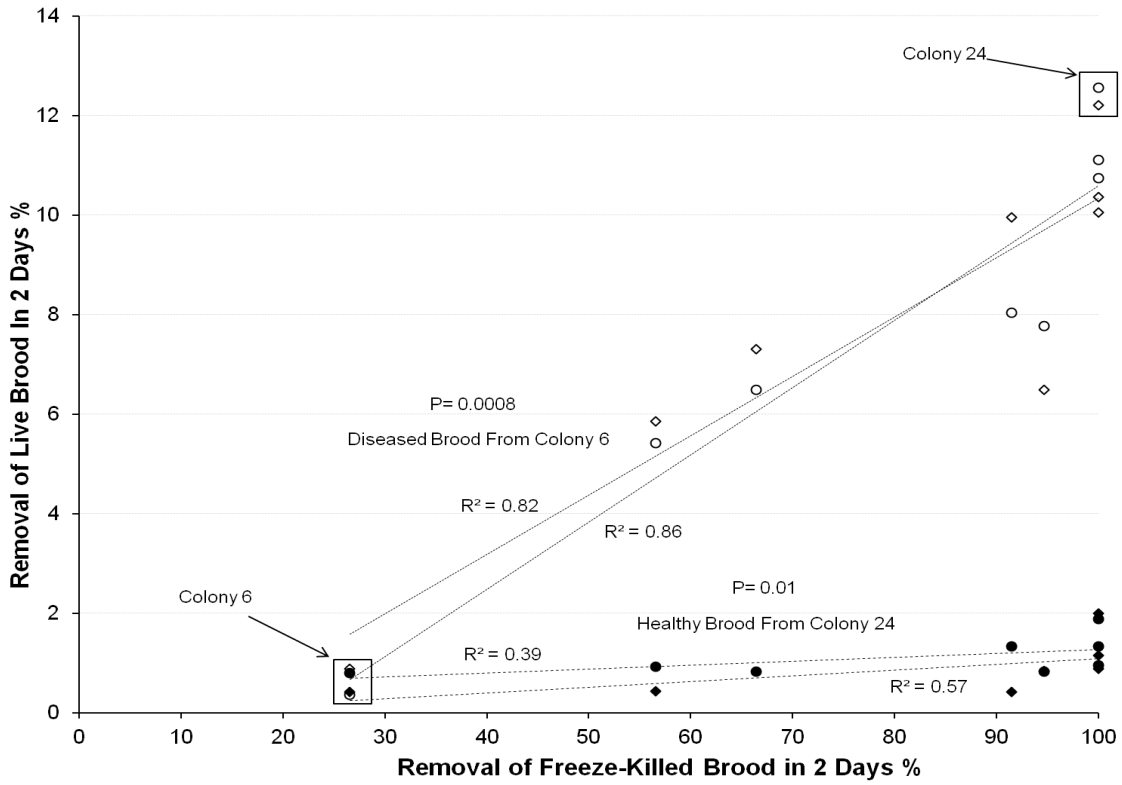

Fig. 13.4. Removal of live brood versus freeze-killed brood in 8 colonies of *S. depilis*. Colonies showing greater levels of hygienic behaviour against freeze-killed brood (Experiment 1) removed significantly more live brood taken from colony 6, the colony with unhealthy brood, but did not remove more healthy brood. The unhealthy brood all came from colony 6 and the healthy brood from colony 24. Open and full circles refer to the first trial and diamonds refer to second trial.

When the remaining live brood from colony 6 were placed in an incubator to allow the adult bees to emerge from their cells, the opposite correlation was seen. That is, fewer workers with shrivelled wings emerged from brood combs taken from colony 6 that had been placed in the colonies with greater removal of freeze-killed brood ($F= 32.4$; $P=0.001$) (Figure 5). This indicates that unhealthy brood had been removed via hygienic behaviour. The results of the two trials were very similar ($F= 0.03$; $P= 0.86$) (Figure 4). The proportion of brood cells in colony 6 giving rise to workers with shrivelled wings was c. 15%.

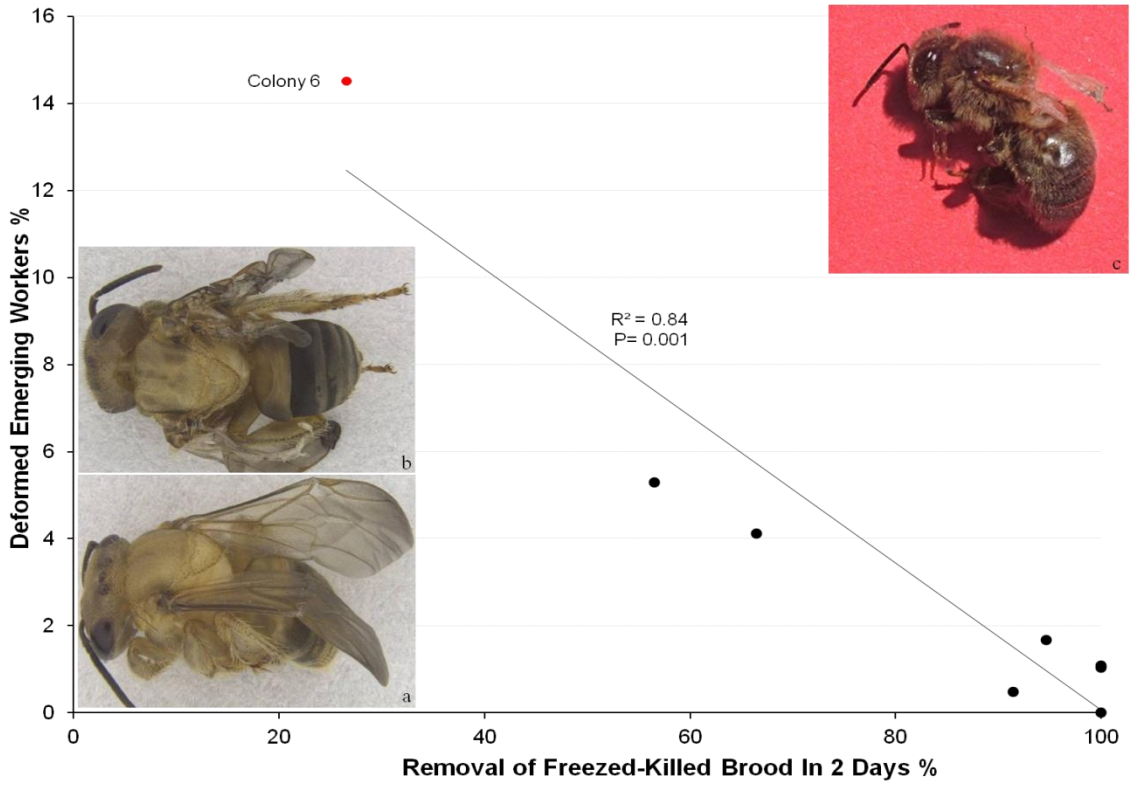

Fig.13.5. Proportions of workers with shrivelled wings emerging from the combs taken from the *S. depilis* colony with unhealthy brood (colony 6) in Experiment 2 versus freeze-killed brood removal (Experiment 1). Significantly more unhealthy bees emerged from combs that had been kept for 5 days in less hygienic colonies. Photos: a) a newly emerged worker *S. depilis* with normal wings; b) a newly emerged worker *S. depilis* with shrivelled wings taken from colony 6; c) honey bee with shrivelled wings, as a result of deformed wing virus, for comparison.

13.5 Discussion

The results of Experiment 1 show that the three stingless bee species studied all have high levels of hygienic behaviour, quantified as the removal of freeze-killed brood. Removal after 2 days was 99% in *Melipona scutellaris*, 80% in *Scaptotrigona depilis* and 62% in *Tetragonisca angustula* (N = 8 colonies per species; 3 trials per colony). This is much greater than in unselected populations of the honey bee (46%; Perez-Sato et al, 2009) and similar to previous research on the stingless bees *M. beecheii* and *S. pectoralis* in Mexico (Medina et al, 2009).

In *T. angustula* there was a delay of approximately one day between uncapping cells and removing the contents. In the honey bee, *Apis mellifera*, Rothenbuhler's (1964b) classic experiment on behavioural genetics showed that the uncapping of cells containing diseased larvae and the removal of the diseased larvae from uncapped cells are distinct behaviours which are, at least partly, under the control of different genes.

Further study will be needed to determine if this is the case in *T. angustula*. However, our results are also compatible with an alternative mechanism: that there is a greater delay between uncapping and removing in this species than in the other study species.

Our results also showed considerable intercolony variation in the time taken to remove freeze-killed brood in *T. angustula* and, most noticeably (Figure 2), in *S. depilis*. This is more similar to the situation in the honey bee, in which there is great variation among colonies in the proportion of freeze-killed brood removed within 2 days (Perez-Sato et al, 2009). In *S. depilis*, intercolony variation in the removal of freeze-killed brood (Experiment 1, Figure 1) was positively correlated with the removal of brood that was naturally producing workers with shrivelled wings (Figure 4) (P= 0.0008) (Figure 5). This is important as it shows that the freeze-killed brood bioassay, in which brood are experimentally killed, was relevant to a naturally-occurring brood disease.

The colony producing adult workers with shrivelled wings, Colony 6, removed only c. 27% of freeze-killed brood in Experiment 1, and only cleaned out c. 1% of live brood cells taken from colony 6 when c. 15% of these contained a larva or pupa that would produce an adult with shrivelled wings if not removed (Figure 5). By contrast, *S. depilis* colonies that had removed a greater proportion of freeze-killed brood in Experiment 1 cleaned out more cells from comb taken from Colony 6. This resulted in a smaller proportion of adults with shrivelled wings emerging from these cells. These data show clearly that the more hygienic colonies were removing brood that would otherwise have gone on to produce crippled adults, but that colony 6 was removing none or almost none of these.

Experiment 2 also shows that more hygienic colonies do not remove more healthy brood that non-hygienic colonies (Figure 4). This has a parallel to recent research on hygienic behaviour in honey bees, which also found that colonies that removed higher proportions of freeze-killed brood did not remove higher proportions of healthy brood (Bigio et al, 2014b).

Overall, the results of this study and those of previous research (Nunes-Silva et al, 2009; Medina et al, 2009) suggest that most stingless bee species and colonies exhibit high levels of hygienic behaviour, removing brood that has been killed by freezing or, in the case of *S. depilis*, by an as yet unidentified disease. The presence of one *S. depilis* colony which was producing many workers with shrivelled wings and had a low level of hygienic behaviour leads to the hypothesis that hygienic behaviour is

playing an important but previously unrecognized role in combating brood diseases in stingless bees. If this is the case, then the low levels of disease normally seen in stingless bees may be because they have effective mechanisms of disease management, and not simply because they do not have diseases. Our discovery of worker bees with shrivelled wings in *S. depilis* should be followed up in order to determine the cause, and in particular if is caused by a pathogen. Furthermore, our discovery of great intercolony variation in hygienic behaviour in *S. depilis* has implications for beekeeping with this species, and also in the further development of queen rearing and breeding methods now underway (Menezes et al, 2013). Selection could take place during queen rearing to produce colonies that show high levels of hygienic behaviour for commercial use. In addition, selection could take place to produce colonies with low levels of hygienic behaviour, for use in further research on the underlying mechanisms of hygienic behaviour and its importance in colony health and performance.

PART 3: DISCOVERY OF THE SMALL HIVE BEETLE

Chapter 14

First Record of Small Hive Beetle, *Aethina tumida* Murray, in South America

14.1 Abstract

We report the discovery of adult small hive beetles, *Aethina tumida*, in a honey bee, *Apis mellifera*, apiary in Piracicaba, São Paulo State, Brazil, in March 2015. This is the first record for South America of this honey bee pest.

14.2 Introduction

The small hive beetle (Coleoptera: Nitidulidae, *Aethina tumida* Murray) is a pest and scavenger in honey bee, *Apis mellifera*, nests where it feeds on brood, adult bees and stored food (Neumann and Ellis, 2008; Pirk and Neumann, 2013). The species is native to sub-Saharan Africa. In the past two decades, it has colonized countries outside its native range including Italy, Philippines, Australia, Canada, USA, Mexico, El Salvador, Nicaragua, Cuba, and Jamaica (Genaro, 2008; Lounsberry et al, 2010; Mutinelli et al, 2014; Neumann et el, 2016). Here we report the discovery of adult small hive beetles in an apiary of *A. mellifera* in Piracicaba, São Paulo State, Brazil. This is the first record for this pest in South America.

The presence of small hive beetles in Piracicaba, Brazil, was officially reported to the OIE, the *World Organisation for Animal Health,* (Report reference: 35387090008 REF OIE 19529, Report Date: 23/02/2016, Country: Brazil) by Dr Henrique Figueiredo, the Director of the Department of Animal Health of the Brazilian Ministry of Agriculture. This report is largely based on our data and report materials collected in March 2015.

14.3 Discovery

14.3.1 Presence of small hive beetle (A. tumida) adults in A. mellifera hives and identification

Adult small hive beetles were detected in the honey bee apiary of the Laboratory of Useful Insects, Department of Entomology and Acarology, University of São Paulo

(ESALQ), Piracicaba, São Paulo State, Brazil. The apiary had been established for many decades, it measures 20 × 30m and is surrounded by trees.

On 4 March 2015, we inspected the nine honey bee colonies managed in Langstroth hives in the apiary and observed what appeared to be adult small hive beetles. We collected samples and prepared photographs to help with preliminary identification by HAT and FLWR.

Fig.14.1. Photographs of specimens collected at ESALQ, March 2016. (left) Dorsal view of habitus; (right) Dorsal view of habitus. Scale bar 2 mm.

Identification was further confirmed by JDE and ARC using these photographs (Figure 1). Subsequently, DCB (Museum of Zoology, University of São Paulo, São Paulo) was sent dead adult female beetles. Beetles were examined by DCB using Wild SM-Lux and Wild M5A stereomicroscopes. DCB made drawings (Figure 2) using a camera lucida attached to the Wild SM-Lux. Mouth parts, wing and genitalia were studied using dissected specimens and by relaxing body parts in hot water. The dissected structures were mounted on slides in 100% glycerin medium. The dissected structures were stored in capped microvials with a few drops of glycerin and pinned beneath the same specimen from which they were removed. DCB confirmed that they were small hive beetles (*A. tumida)* by making comparisons to morphological descriptions (Ellis et al, 2001; Habeck, 2002).

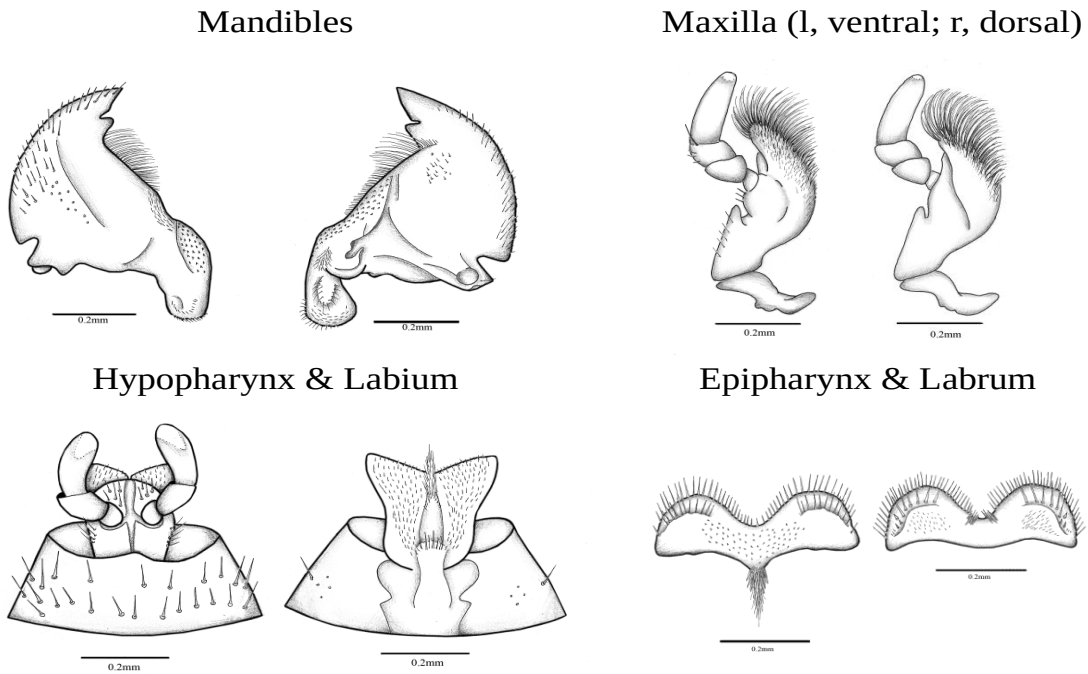

Fig.14.2. Drawings of selected morphological characters of the collected beetle specimens. Dorsal and ventral view of mandibles; dorsal and ventral view of maxillae; ventral and dorsal view of labium; dorsal and ventral view of labrum; dorsal view of left antenna; dorsal view of left metathoracic wing; dorsal view of female genitalia (composite view). Scale bar = 0.2mm.

Although we visually inspected every frame in each hive (Neumann et al, 2013), we did not detect any beetle larvae. The adult beetles were mainly in groups on the hive bottom boards, hiding in corners and cracks. Some (c. 10%) were observed walking on frames or hiding inside wax cells. We did not see any beetle damage to combs or brood. The hives were in either one or two deep hive boxes, were of low to mid strength with brood (1-5 frames), approximately 3-10 frames of bees, and 1-2 frames of honey.

In August 2015, six of the eleven hives (two colonies were brought into the apiary after March 2015) were dead with no sign of small hive beetle damage. We think that this high mortality was largely as a result of insecticide spraying to control ticks or crop pests.

One year later, 10 March 2016, only one colony remained alive. The other ten were present but dead. The wax combs in these dead hives were either fully consumed (n = 10) or in the process of being consumed (n = 1) by both greater (*Galleria mellonella*) and lesser (*Achroia grisella*) wax moth larvae, as evidenced by the presence of cocoons, adult moths, and the characteristic damage caused by wax moth infestation (large amounts of silk and frass [Ellis et al, 2013]).

The one living colony was extremely weak, with low forager traffic of 1-2 bees per minute, one frame of bees, and approximately one quarter of one frame with emerging capped brood. The hive appeared to be queenless as no larvae were present. No honey stores were apparent and the combs not covered by worker bees had wax moth larvae tunnelling through them. A few adults of the small hive beetle were observed in this one surviving colony and we estimated the colony would have survived no more than one additional week. This last inspection on 10 March was made by workers from the São Paulo State Government (Coordenadoria de Defesa Agropecuária) and the living colony was burned. On 29 March 2016, workers destroyed the other 10 dead hives, with 66 frames, and 4 wooden stands.

14.3.2 Absence of small hive beetle in nearby hives of stingless bees (Meliponini)

The honey bee apiary contained no hives of stingless bees. However, six species of stingless bees (*Melipona scutellaris* [n = 8 colonies], *Melipona quadrifasciata* [n = 6], *Tetragonisca angustula* [n = 9], *Nannotrigona testaceicornis* [n = 3], *Scaptotrigona depilis* [n = 12], *Frieseomelitta varia* [n = 10]) were being kept in hives in two locations on the ESALQ campus. The first location, where the *M. scutellaris* colonies were kept, was 40 m from the nearest honey bee hive, inside a 7 × 10 m brick building in which foraging bees could enter and leave hives via tubes through holes in the building walls. The second location, a meliponary shelter 7 × 6 × 3 m tall with a roof but no walls, held the other colonies and was 50 m from the nearest honey bee hive.

These stingless bee colonies were inspected every two weeks as part of routine hive maintenance by DAA, and more frequently during ongoing experiments. For example, in the period mid-February to mid-March 2015, eight hives each of *M. scutellaris*, *T. angustula* and *S. depilis* were inspected on a nearly daily basis as part of a research project on hygienic behaviour by HAT, DAA and FLWR. No small hive beetles were ever detected in any of these stingless bee colonies, despite regular inspections by DAA from the time the hives were established, from March 2014 for the *M. scutellaris* hives and from February 2015 for the hives of the other species.

No survey of wild stingless bees on the ESALQ campus has been made. However, with c. 300 m of the Laboratory of Useful Insects we are aware of approximately 10 wild colonies of *T. angustula* and 2 of *Partamona helleri*.

14.4 Discussion

Due to our samples originating from a single small apiary, it is not possible to know the extent to which small hive beetle (*A. tumida)* may have spread in Brazil, or where it was initially introduced. Brazil possesses a significant beekeeping industry that involves hive movement via truck. Therefore, the potential for rapid and extensive geographical spread is strong and may already have occurred, although this is not known currently. However, the February 2016 OIE (the World Organisation for Animal Health) reports a Brazilian notification that small hive beetles have been found within an apiary in a nearby location.

Why small hive beetles were found first in a university laboratory where honey bees and beekeeping are studied remains unclear. Honey bees, hives, and honey bee products (wax, pollen) were never imported from other countries to the study apiary, laboratory or beekeeping workshop by LCM, the head of the Laboratory of Useful Insects. However, he notes that wild colonies of honey bees were collected, on campus or within a few kilometres, to repopulate dead hives. Further investigations by the Brazilian authorities are needed to determine the geographic spread of the small hive beetle at local, state and national levels. We believe it is quite likely that the small hive beetle will be found to be widespread, with the discovery at the ESALQ apiary being mainly a consequence of personnel trained to recognise the small hive beetle, rather than the beetles only being present at this location.

The honey bees in Brazil are mainly of African origin. The small hive beetle is native to sub-Saharan Africa and honey bee subspecies there seem tolerant of it (Neumann et al, 2001; Neumann and Ellis, 2008). In this respect, the Brazilian situation is different to that of most other countries where the small hive beetle has been introduced and where European and Mediterranean subspecies of honey bees without natural defences against that the small hive beetle are managed. Within tropical America, some countries (Cuba and Jamaica) in which small hive beetles have been reported have European honey bees while others (Mexico, El Salvador, Nicaragua) have African honey bees.

In South Africa, from where the African bees now present in tropical and sub-tropical America were introduced, Neumann and Ellis (2008) considered the small hive beetle to be only a minor pest of local African honey bee colonies. In contrast, beekeepers completing a questionnaire on colony losses in South Africa (Pirk et al.

2014) noted small hive beetles as one of the 4 main causes of colony losses (*Varroa destructor*, chalkbrood, and absconding were the other three).

Compared to other countries where that the small hive beetle has been introduced, Mexico is more similar to Brazil in terms of climate and in possessing mainly African-derived honey bees and a well-developed beekeeping industry. Thus, Mexico could be a useful source of advice. However, the Mexican experience with this pest is limited. The small hive beetle was first detected in Coahuila State, which borders Texas and from where small hive beetles were presumably moved (Loza et al, 2014). In 2014, it was found in eight Mexican States, including two states, Yucatan and Quintana Roo, far from the US border (Loza et al, 2014). At present, it seems there are no reports of major problems caused by small hive beetles to honey bee colonies and beekeeping in Mexico.

Reports exist of the small hive beetle being found in colonies of stingless bees, Meliponini. For example, in Cuba, adult beetles were detected in *Melipona beecheii* (wild colonies: in 1 of 13 colonies, 8.3%; managed hives: in 7 of 258, 2.71%, with larvae in 2) (Peña et al, 2014). As noted above, we did not observe small hive beetle adults in stingless bee hives on the ESALQ campus despite making over 1000 hive inspections of c. 50 colonies of 6 species over the past two years, February 2014 to March 2016. Stingless bees are not present in N. America, Europe, or North Africa. In Australia, workers of the native stingless bee *Tetragonula carbonaria* kill introduced small hive beetles by coating them with resin, wax and mud (Greco et al, 2009). This defensive behaviour shares some similarities to the use by native South African honey bees of resins (propolis) at small hive beetle prison sites (Ellis, 2005; Neumann et al, 2001). Another Australian stingless bee, *Austroplebeia australis*, also showed effective defences against small hive beetles (Halcroft et al, 2011). Therefore, it is quite possible that that the small hive beetle may not become a problem to Brazilian stingless bees. In temperate parts of South America, such as much of Argentina, honey bees are predominantly of European origin and are likely to be affected in a similar way to that of the honey bees in the USA and Australia if the small hive beetle spreads to that region. Given that honey bees in Brazil originate from Africa, and that African honey bees have defensive adaptations against that the small hive beetle, we are hopeful that it will not be a problem for Brazilian beekeeping. However, beekeepers need to become aware of the potential problems associated with small hive beetles, and practice simple

precautions similar to those that are already used against wax moth larvae, which may also destroy weak colonies and unattended wax combs.

Chapter 15

Final Discussion

This chapter considers some of the main implications arising from the results of this thesis for honey bee colony health and notes some key questions for further research.

15.1 Varroa control

The research in this thesis shows that applying oxalic acid via sublimation is better than spraying or trickling (Chapter 5; Figure 1). Sublimation is more effective at killing varroa at lower doses, causes no harm to the colonies, and results in stronger colonies in spring. One treatment of 2.25 grams to a broodless hive in December kills 97%. Double treatment, at an interval of 10 days, kills 99.6% (Chapter 6). Double treatment is not harmful to colonies and reduces varroa populations to such an extent that 8 doublings would be needed to build back to the original level, versus 5 doublings for a single oxalic acid treatment. This will take more than one year.

Another important result concerning the use of oxalic acid to control varroa is to show the importance of treating broodless colonies. Even a small patch of sealed brood, 500-600 sealed worker cells, could contain 10-14% of the varroa in a colony and would greatly reduce the duration of varroa control provided by a single oxalic acid application to 2.5 doublings versus 5 doublings in a broodless colony (Chapter 8). This is because oxalic acid does not kill varroa in sealed brood cells. The research also shows that beekeepers cannot assume that a hive will be broodless in winter. There was no winter month in which all colonies were broodless. Although December was the month with least brood, some colonies had brood and the amount of brood in December varied across years, with high levels following the warm autumn of 2015.

15.2 Hygienic behaviour

The research in the thesis clearly shows that hygienic behaviour can benefit colony health. In particular, the results show its effectiveness against varroa mites and deformed wing virus. In the case of deformed wing virus, hygienic behaviour was able to save the lives of colonies containing workers with shrivelled wings, which is considered to be a predictor of colony death (Dainat and Neumann, 2013).

It is worth noting that we did not directly select for resistance to specific diseases. Rather, we tested and selected for the removal of dead brood using the FKB-bioassay. Our results, therefore, also show that this simple test, which does not necessitate that the hives have pests or pathogens present in the brood to cause the death of larvae and pupae, is highly effective in identifying colonies that have higher levels of disease resistance.

Fig. 15.1. 1) The author checking a colony selected for high levels of hygienic behaviour in a hive at the LASI home apiary. LASI's hygienic bees are not highly defensive as shown by the open veil. 2) Worker bee removing a dead larva killed in a freeze-killed brood (FKB) removal bioassay.

Chapter 11 shows that all honey bee colonies, whether with high or low levels of hygienic behaviour, remove dead larvae from open cells. This poses a question about hygienic behaviour in the honey bee. Why is rare? It is suggested that both low and high levels of hygienic behaviour are alternative adaptations to diseased brood in sealed cells. It may be the case that for much of their evolutionary history honey bees have been selected to leave dead brood in sealed cells, rather than to uncap these cells and remove the contents. Chapter 13 shows that hygienic behaviour may well be important in stingless bees to reduce levels of diseases.

15.3 Future directions

15.3.1 Outreach activities

Many of the results of this thesis are of practical importance to beekeepers and beekeeping. For example, the research on oxalic acid shows the best application method

and dose to use to control varroa. Therefore, one important future direction is to make the results of this thesis more widely known to beekeepers.

This is already underway. I have given numerous talks to more than 15 beekeeper groups, including to an audience of hundreds at the 2015 BBKA (British Beekeepers Association) Spring Convention. I will be giving a talk at the 2016 National Honey Show which is attended by over one thousand beekeepers. I have also demonstrated at several outreach workshops on varroa control that we organized for beekeepers at the Laboratory of Apiculture and Social Insects, with more planned for 2017 including one on integrated varroa control and a new workshop on hygienic behaviour.

I also helped to write popular articles for beekeeping magazines that give the results of the research on controlling varroa with oxalic acid in a way that beekeepers will find easy to follow, and have helped write a “how to” pamphlet on applying oxalic acid via sublimation.

Fig.15.2. Outreach activities in LASI. 1) Demonstrating to a group of beekeepers at a LASI open day; 2) Demonstrating to a group of beekeepers at a LASI workshop on varroa control (Photos F. Ratnieks).

Other activities and approaches are also useful to disseminate the research results to beekeepers and teach them the practical side, such as how to breed hygienic bees or apply oxalic acid. One additional way that we plan to do this is via the LASI You Tube Channel, which already has many videos on bee foraging, but so far does not have any videos on disease control.

Beekeepers are already showing great interest in the results on varroa control using the sublimation of oxalic acid (Chapter 5). This paper has been downloaded more than three thousand times from the Journal of Apiculture Research in the five months since it was published, and rapidly became the JAR’s most downloaded paper. We also

know that numerous beekeepers have purchased the tool needed to apply oxalic acid via sublimation. I am expecting more to consider this method in the next few years as it is currently the best method for controlling varroa mites. There are also other reasons why this method will be attractive to beekeepers. Most importantly, it does not harm the bees or colonies. It is also low cost, as low as a few pence per treatment. In addition, the UK regulatory authorities have recently approved the use of oxalic acid to control varroa in bee hives. As with all the applied research in this thesis, it is not aimed only at the UK as the disease challenges facing honey bees are international. However, it is good that UK beekeepers can now take advantage of oxalic acid to control varroa without breaking the law.

The results on hygienic behaviour clearly show that it can play a very important role in colony health. One clear message from this research that needs to reach beekeepers is that hygienic behaviour combined with a single or double oxalic acid treatment in broodless colonies can provide more than one year's control of varroa (Chapters 5, 6 and 8). Research in the USA has shown that hygienic colonies can fully control AFB (Spivak and Reuter, 2001), chalkbrood (Oldroyd, 1996), and partly reduce the varroa population in the colony. The research in this thesis shows that hygienic behaviour can reduce varroa population build up over one year by more than 50% (Chapter 9), can reduce levels of deformed wing virus by 10,000 times (Chapter 9), and can increase the lifespan of colonies with overt symptoms of deformed wing virus for approximately one year without additional treatments against varroa, the vector of DWV (Chapter 10).

One novel direction that LASI is also taking in terms of hygienic behaviour is to provide queens bred from hygienic stocks to beekeepers. During my PhD, we would sometimes supply a few hygienic queens to beekeepers. However, in spring 2016 LASI set up a spin-off business, LASI Queen Bees, through which we hope to supply thousands of hygienic queens to beekeepers over the coming years. This project has been supported by the University of Sussex's Enterprise scheme and will employ me as a research technician, in day to day charge of things. LASI Queen Bees would not have gone ahead without the results of my PhD thesis, and also the results of the earlier PhD thesis by Gianluigi Bigio. Together, and in combination with the research carried out in the USA, these studies established and tested the methods for rearing and breeding hygienic bees and also showed their value in disease control.

One important recommendation arising from the research is that beekeepers need to check their hives in winter to determine both the month with the least amount of sealed brood and whether individual hives have sealed brood. If sealed brood is present it should be removed (Chapter 8), as it will greatly reduce the effectiveness of oxalic acid. During the PhD research I contacted beekeepers in the search for volunteers to monitor hives in other parts of the UK. However, it seems that most beekeepers have been told not to open their hives in winter as this will harm the bees. My research shows that it is not harmful if done with care, and is essential if oxalic acid is to be applied effectively to control varroa.

15.3.2 Further research

The results of the thesis also indicate future directions for further research. Chapter 5 used oxalic acid to control varroa. With double treatment of oxalic acid, the proportion of varroa killed in the second application was lower than in the first application. Could this be because some varroa have resistance? If so, and if heritable, this could lead to the evolution of resistant varroa as has happened with synthetic acaricides such as fluvalinate. However, it is possible that the lower mortality in the second application was due to non-heritable differences among varroa mites.

On a more positive side, after the second application of oxalic acid an average of only 6 mites per hive survived. This suggests that it may be possible to kill all the varroa in a hive by applying oxalic acid several more times, perhaps 5 or 6 times in total, per hive. This might allow beekeepers to eliminate varroa totally from their hives. Whether or not this was worthwhile would depend very much on the rate at which hives are recolonized by varroa, but it might possible to provide several years of control if recolonization was slow. This method might be of particular value to beekeepers who have small numbers of hives and who do not move them.

One obvious future research direction is to investigate the reason why hygienic behaviour is rare. Only c. 10% of unselected colonies is showing high levels of hygiene in the UK (Perez-Sato et al, 2009). Seeley (1985) hypothesised that hygienic bees cause a continuous colony-level cost by removing healthy brood. However, the results of Bigio et al (2014) did not support this hypothesis and showed that the removal of freeze-killed brood (i.e., hygienic behaviour) had no effect on the removal of healthy brood across colonies. Hygienic behaviour also has no effect on honey production (Spivak and Reuter, 1998b). The rarity of this behaviour is still not understood. Chapter

11 proposes the hypothesis of two adaptive peaks in honey bees (Chapter 11). That is, hygienic behaviour (i.e., the removal of diseased brood from sealed cells) is only one possible adaptation to brood diseases. Another adaptation is to leave dead brood in their cells. One possible way of testing this hypothesis is by comparing with other species. Chapter 11 indicates that in stingless bees there can only be one adaptive peak, because brood cells are torn down after use. Thus, the option of leaving dead brood sealed in their cells is not available to stingless bees, and may be why they have higher levels of hygienic behaviour than honey bees. In addition, in the Asian hive bee *Apis cerana*, drone brood that have died of European foulbrood and Thai sac brood virus are not removed from their sealed cells (Boecking 1999). The question of why hygienic behaviour is rare is challenging and needs more work.

There are many other questions about hygienic behaviour, both in the honey bee and in stingless bees. Chapters 11 and 12 showed that all honey bee colonies remove dead brood from open cells. European foulbrood (EFB) is a notifiable disease in the UK caused by the pathogenic bacterium *Mellissococcus pluton*. Honey bee larvae killed by EFB are young and most of the affected larvae die before their cells are capped (Shimanuki and Knox, 2000; Delaplane, 1998). Would hygienic behaviour also be effective against EFB? This has never been investigated. EFB is a common honey bee disease in England. This is just one of many possible follow on questions in honey bee hygienic behaviour that are of practical significance to beekeeping, and in this case of particular significance in the UK.

There are many opportunities for research in stingless bees on hygienic behaviour and its role in controlling diseases. In Chapter 13, *Tetragonisca angustula* showed a delay of approximately one day between uncapping cells and removing the contents (Figure 3). In the honey bee, *Apis mellifera*, Rothenbuhler's (1964b) classic experiment on behavioural genetics showed that the uncapping of cells containing diseased larvae and the removal of the diseased larvae from uncapped cells are distinct behaviours which are, at least partly, under the control of different genes. Further study will be needed to determine if this is the case in *T. angustula*. However, our results are also compatible with an alternative mechanism: that there is a greater delay between uncapping and removing in this species than in the other study species.

In Chapter 13, the discovery of worker bees with shrivelled wings in the stingless bee *Scaptotrigona depilis*, should be followed up to determine the cause. In particular, is it caused by a pathogen? The discovery of great intercolony variation in

hygienic behaviour in *Scaptotrigona depilis* has implications for beekeeping with this species, and also in the further development of queen rearing and breeding methods now underway with this species (Menezes et al, 2013). In addition, the results of this study show that hygienic behaviour in stingless bees is effective at removing diseased brood, even if the cause of the disease is not known. The stingless bees comprise hundreds of species, and further research is needed to investigate interspecies and intercolony variation in levels of hygienic behaviour, and on diseases. The whole area of stingless bee diseases is essentially virgin territory.

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