STANFREE VALLEY PRESERVATION GROUP

Hygienic Bees

Project Aim and Summary:

Using three local honeybee colonies; Identify the incidence of hygienic behaviour, and study the changes over a 12-month period.

I have a keen personal interest in bees and their role in ecology and have access to beehives from sources both at the Chesterfield Beekeepers Association and from my own colony, which I use to improve my beekeeping skills and practices. Keeping bees enriches garden pollination as well as obtaining honey from a harvest.

Unfortunately, the death of millions of colonies of honeybees over the last 20 years has prompted researchers to investigate the many threats faced by bees.
The outcomes of work so far have led to improved practices and short-term cures. Long-term treatments highlight the potential importance of behavioural mechanisms in bees in response to disease and pests, and this will possibly lead to breeding improved strains of honeybees.

Hygienic behaviour is identified as the tendency of hive bees to locate, uncap and remove diseased, mite infested pupae or dead larvae from the foulbrood, chalkbrood and sacbrood cells. The role of 'hygienic' bees was first observed in the 1930s. A specific response of bees to diseased and parasitised brood is said to be the primary natural resistance against diseases and pests. In honeybees, hygienic behaviour is measured by determining the rate at which experimentally freeze-killed brood is removed. Honeybees can detect and remove varroa-infested brood.

This project focuses on the bees finding, uncapping and removing dead or damaged brood cells. This defines the trait of **hygienic behaviour** in honeybees and can be observed through experimentation. If 50% of the cells are removed – the colony is said to conduct partial hygienic behaviour If 95% of the cells are removed – the colony is said to conduct excellent hygienic behaviour.

The purpose of this project is to research Hygienic behaviour in three local colonies over a period of 12 months. **The hypothesis is that:** If any, or all the hives have the genetic trait of hygienic behaviour, then the differences over time in finding and removing cells will be consistent. Statement: “There will be a consistent pattern in the (%) cells removed over time and thefore a constant level of hygienic behaviour in that colony – proving that the colony is truly hygienic “.

With many thanks to people featured in this project:

Francis Ratnieks is Professor of Apiculture and head of the Laboratory of Apiculture & Social Insects at the University of Sussex. He obtained his PhD at Dyce Laboratory for Honey Bee Studies, Cornell University, and worked for the New York State Apiary Inspection Service and as a commercial beekeeper with 180 hives in California. He has studied honeybees on all continents, taught honeybee biology at 5 universities (Cornell, Berkeley, Sheffield, Sussex, São Paulo) and published 250 articles on honeybees and social insects.
(http://www.sussex.ac.uk/lasi/)

Marla Spivak - Marla's interest in bees began when she worked for a commercial beekeeper from New Mexico in 1975. She later completed her B.A. in Biology from Humboldt State University in northern California, and her PhD from the University of Kansas, under Dr. Orley "Chip" Taylor, in 1989. She spent two years in Costa Rica conducting her thesis research on the identification and ecology of Africanised and European honeybees. From 1989-1992 she was a post-doctoral researcher at the Centre for Insect Science at the University of Arizona. She began as Assistant Professor at the University of Minnesota in 1993. Influenced by Martha Gilliam and Steve Taber from the USDA Bee lab in Tucson, she became interested in hygienic behaviour of honeybees. This interest has expanded into studies of "social immunity", including the benefits of propolis to the immune system of honeybees, and to the health and diversity of native bee pollinators.
(http://www.extension.umn.edu/honeybees/components/meetteam.htm)

Dr. Stephen J Martin Sheffield University, Summary of career and achievements:

- Senior Research Fellow, APS, Univ. of Sheffield, 2009 – present.
- PDRA, Animal and Plant Sciences, Univ. of Sheffield, 2000-2008.
- Higher Scientific Officer, National Bee Unit, York, 1993-2000.
- Scientific Fisheries Officer, Falkland Islands Government, 1988.
- Scientific Officer on British China Expedition, 1982.
- Ph.D., Population dynamics and thermoregulation in hornets, Univ. of Wales.
- M.Sc. Species diversity of ground beetles as environmental indicators in the Japanese Alps, Shinshu University, Japan, 1985-1987.

(http://www.shef.ac.uk/aps/staff/acadstaff/martin-stephen.html)

Introduction.

Keeping bees benefits the owner, the environment and help farmers to achieve healthy crop yields. Many people keep bees as a hobby and some are gardeners. A couple of colonies will improve the pollination in a garden and the surrounding area as well as obtaining around 50lb of honey from a typical harvest.

A bee colony consists of:

- The queen. Her main duty being to lay eggs (up to 2,000 per day at the peak of the colony population in June and July) from which drones, workers and queens are reared. She also influences the activities in the hive and without the queen the hive would not exist.

- Worker bees (females) for building the nests, rearing broods, foraging, hive maintenance and to protect the colony.

- Drones are male bees that mate with the queen and when they are no longer needed at the end of the summer when food becomes scarce – they are expelled from the colony.

Fig 1. The Three Castes of Honey Bees

Drone **Queen** **Worker**

(http://www.i4at.org/lib2/bees.htm)

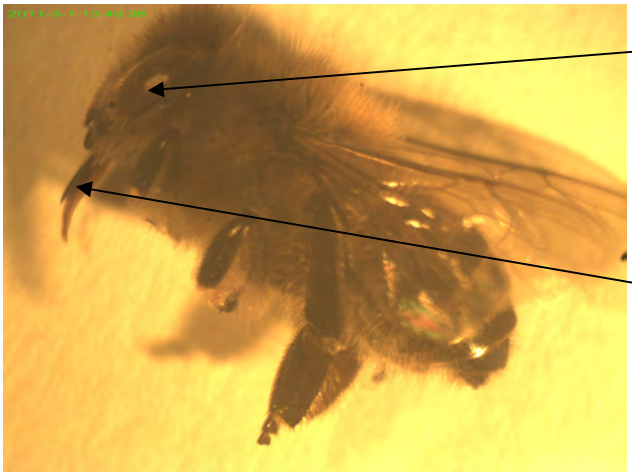
Table 1. Stages Of Growth Of Honey Bees Castes

Stage	Worker (Days)	Queen (Days)	Drone (Days)
Egg hatching	3	3	3
Larva	5	6	7
Cell capped	8	9	10
Pupa	8	12	14
Adult emergence	16	21	24
Life expectancy	6 weeks – 6 months	4 – 5 years	The season

(Chesterfield Beekeepers Association)

Using bees collected during experimentation, these annotated diagrams illustrate the major parts of the Honeybee anatomy.

Creswell sample; Carniolan breed.



Glapwell sample; Buckfast breed.

Sheffield sample; Carniolan breed.

Unfortunately, Honeybees (like most living organisms) face threats from diseases and these may be grouped under brood diseases, adult disease, ineffective human management and pests. A healthy and productive honeybee colony is distinctively free of damaged or dead brood cells, the physical appearance of the adults and their queen is obvious and there are very few dead or dying bees in and around the hive. Infection is not usually apparent until the colony becomes stressed.

The major pests and diseases faced by bees:

- The greater wax moth, causing bald brood. The wax moth's larva chews its way through the comb wax and feeds on larval skins and pollen.
- Nosema - Is a widespread protozoan disease of adult bees. In spring, infected colonies build up very slowly or not at all. Bees appear weak and may crawl around the front of the hive
- Brood diseases (foulbrood, chalkbrood and sacbrood) are usually caused by viruses, bacteria or fungi, which target the brood cells. Chalkbrood often appears in spring and is widespread and is not a notifiable disease. However, American Foul Brood and European foul Brood are caused by a bacterium and its spores can be spread to other colonies. If Foul Brood diseases are confirmed, the regional bee inspector will supervise the burning of bees and combs to eliminate its spread. **Foul Brood diseases are notifiable**.
- Varroasis is an infestation of the varroa mite that lives in the hive and on bees. Varroa mites target the sealed brood cells and can be easily seen on adult bees. The mites transmit a virus causing deformation of wings.

Varroa was first discovered in the UK in April 1992 in Devon. Heavy outbreaks were found in the south where colonies were on the point of collapse. Laboratory examination confirmed that these mites were Varroa jacobsonii. Eventually the whole of Devon was declared to be a Statutory 'Infected' Area (SIA) within which movement of bees was permitted, but bee movement out of the area was prohibited. In 1992 Varroa appeared to be only in the south of England. However, In October 1992 MAFF conducted another nation-wide search, and Varroa was found in 18 apiaries, one in Lincolnshire and 17 in Suffolk. Over the next five years Varroa jacobsonii progressively moved northward as more infestations were discovered. Varroa is now present throughout all of England and Scotland.

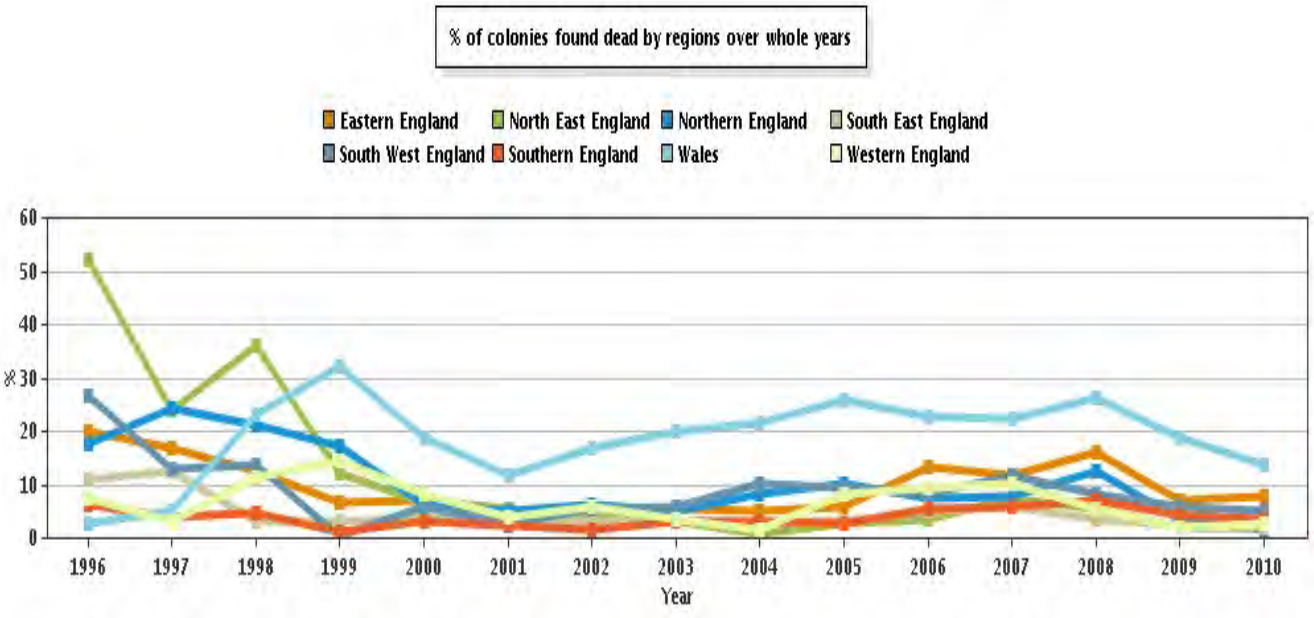
An Economic Evaluation of DEFRA's Bee Health Programme.
(http://www.defra.gov.uk/evidence/economics/foodfarm/evaluation/beehealth/wholereport.pdf) accessed Jan 2011

The Cost of Bee Diseases in England

“As well as producing honey, bees pollinate some £165m worth of crops in the UK annually, as well as wild plants and garden flowers”. (guardian.co.uk/.../apiculture-hygienic-bees-francis-ratnieks).

More than 39 crops grown in the UK for their fruit or seed are insect pollinated therefore the honeybee plays a dominant role in crop production.

The surveys completed by current beekeepers provide evidence on the detrimental effects of bee disease and the costs. Estimated costs of all diseases average £1.87 million per year. This can be divided up and estimated at 4% for AFB, 11% for EFB, and Varroa for 85% of the private costs of bee disease as estimated by current keepers of bees. For every 1% that the bee colony population is reduced by disease there is a loss of £1.2 million in the value of the pollination services of bees.



(https://secure.fera.defra.gov.uk/beebase/public/BeeDiseases/viewGraph.cfm?func=getDead)
This chart shows the number of colonies found dead in regions from 1996 to 2010.

It is very clear why research needs to be undertaken to reduce this deficit, protect our bees from further decline and enhance the knowledge of all beekeepers to help prevent the causes and spread of disease.

Discussing the ethical implications with this project.

There will be an area of brood cells destroyed in each experiment undertaken.
Some people may argue that killing brood is morally unacceptable or inhumane but unfortunately there are no other methods in which such results should be obtained.

It is necessary to take the beekeeper's feelings into account and sensible to always ask whether or not they wish to attend the experiments. .

If the beekeeper finds out that his/her colony displays bad hygienic characteristics, does the beekeeper decide that they should kill and replace the Queen with another that has been bred to demonstrate Hygienic characteristics?
Again, this raises a moral issue, which can only be answered by the individual in question. They must decide whether replacing a queen in favour of improved colony health is the most suitable direction.

Desk research.

(Summary of key points from papers and articles)

Hygienic behaviour and its link to Bee diseases.

Paper: The resistance to American foulbrood disease by honeybee colonies *Apis mellifera* bred for hygienic behaviour. By: Marla SPIVAK, Gary S. REUTER. 2001. (Accessed March 2011)

American foulbrood (AFB) disease, caused by the bacterium *Paenibacillus larvae* is the most serious of the diseases affecting honeybees *Apis mellifera.* On a colony level, the most important mechanism of resistance to AFB is hygienic behaviour of adult bees toward infected larvae. Worker bees that demonstrate this behaviour rapidly detect, uncap, and remove infected brood from the nest. The spore, or infectious stage of the bacterium appears 10–11 days after egg hatching, when the pre-pupae is developing under its wax capped cell. Hygienic bees remove larvae under capped cells when the bacterium is in the vegetative, non-infectious rod stage. The degree of hygienic behaviour increases in colonies after four years of selection solely on the queens, and that the hygienic colonies have a lower frequency of naturally occurring brood disease than non hygienic colonies.

Paper: The hygienic behaviour of Honeybees in relation to Chalkbrood disease.
Martha Gilliam, Stephen Taber. US dept of agriculture. Agriculture research service. Carl Hayden bee research centre. Gary Richardson. US dept of agriculture. Agriculture research service. Colorado state university. (Accessed Jan 2011)

The severity of Chalkbrood disease in North America has increased dramatically over the last few years. Larvae with the disease become mummified and become pale white because of the mycelium of the fungus (see page 40. Pictures of mummies found at the Creswell hive). No effective chemical reagent has been successfully developed, as the toxicity to bees themselves has to be considered. The project aims to initiate studies on genetically controlled nest cleaning behaviour of bees to determine the possibility of developing resistance or control based on the hygienic behaviour of worker bees. Studies show that resistant strains quickly remove killed larva from the comb and susceptible strains do not. Two recessive genes, one for uncapping and one for removal of larvae control the behaviour.

Journal of Invertebrate Pathology: Socialized medicine: Individual and communal disease barriers in honeybees. November 2009. Jay D. Evans. Marla Spivak. (Accessed Jan 2011)

Bees selected for rapid hygienic behaviour have high olfactory sensitivity to the odour of diseased brood (Masterman et al., 2001; Spivak et al., 2003; Swanson et al., in press). It is possible that the olfactory stimuli used by hygienic bees to detect Varroa-infested pupae are associated with the bee's wound response to mite feeding; also mite's offspring or faeces build up may also be important.

Knowing that the detection of parasitised and diseased brood is based on olfactory stimuli, it would be best to identify the volatile compound(s) specifically associated with mite-infested pupae and present them to the bees in a low concentration such that only the bees in colonies with the highest olfactory sensitivity would respond. Bees had positive electroantennogram response to three specific volatiles collected from infected larvae. Phenethyl acetate, obtained a strong hygienic response in very low concentrations (10^{-9}/ml) by colonies selected for rapid-hygienic behaviour.

Colonies selected for rapid-hygienic behaviour demonstrate resistance to American foulbrood and chalkbrood and also provides an important mechanism of defense against Varroa when bees detect and remove pupae that are infested by mites. Bees that handle diseased brood are between 15–18 days old (older than typical nurse bees).

The removal of mite-infested worker brood through hygienic behaviour is a highly desirable trait because it would interrupt the reproductive cycle of the mite, killing any mite offspring that could have increasing negative effects on the mite population dynamics. It is possible that Varroa populations may largely reflect the seasonal production of drones and thus limit the opportunity for mite reproduction, since mites prefer to lay their eggs in drone cells. (Page 20)

Paper: The relationship between hygienic behaviour and suppression of mite reproduction as honeybee (*Apis mellifera*) mechanisms of resistance to *Varroa destructor.* Abdullah Ibrahim and Marla Spivak. (Accessed March 2011)

Hygienic behaviour is one of various mechanisms of resistance against *V. destructor*. Bees bred for hygienic behaviour detect, uncap, and remove diseased brood from the colony before the disease reaches the infectious stage. Hygienic bees also remove mite-infested pupae from the colony (Spivak, 1996).

Extensive field studies carried out by Spivak and Reuter (1998a, 2001b) showed that the colonies bred for hygienic behaviour produced the same amount of honey as other colonies and had lower levels of mites. However, the degree of removal of infested pupae by hygienic behaviour is still not sufficient to maintain a low mite population.

An article from the American Bee Journal (2009):
The Future of the MN Hygienic Stock of Bees is in Good Hands!
MARLA SPIVAK, GARY S REUTER, KATIE LEE, and BETSY RANUM Department of Entomology University of Minnesota (www.extension.umn.edu/honeybees - Accessed Aug 2010)

Bee breeders from Minnesota have successfully incorporated the hygienic trait into their operations. Their naturally mated colonies are just as hygienic as instrumentally inseminated breeder queens. This means that breeders can select and maintain the (Minnesota breed) MN Hygienic stock on their own and it can be certified that the probability that their queens will produce hygienic colonies is extremely high.

"The daughter queens produced drones that carried the hygienic trait. Now when their new hygienic queens go on mating flights the majority of drones they encounter come from other hygienic colonies".

Reproduction of *Varroa destructor* in worker brood of Africanized honeybees (*Apis mellifera*) 2002. Stephen J Martin, Laura Espinosa and Francis Ratnieks. Africanised honeybees have unique tolerance to Varroa mites (2004). Stephen J. Martin and Luis M. Medina. (Accessed Jan 2011)

Varroa mites feed solely on the hemolymph of honeybees, and their complete reproductive cycle is fulfilled within a sealed brood cell. Varroa kills host colonies indirectly by providing a route for honeybee viruses such as the deformed wing virus.

In Apis cerana (the original host of Varroa), mite reproduction occurs only in the small number of sealed male (drone) honeybee brood cells and mite populations within that colony are low (<800) and no harmful effects are seen. In A. mellifera (European) colonies, V. destructor also reproduces in worker brood cells enabling mite populations to increase 2000-fold annually causing colony death within one year. However, mite populations in similar-sized Africanised colonies stabilize <3000 mites per colony, allowing colonies to survive without a problem.

At low mite populations, mites reproduced in drone brood cells, which have a high reproductive success, contribute to the initial mite population growth. Mites show a tenfold preference to reproduce in drone cells (1–5% of all the honeybee brood) and they soon become overcrowded as the mite population increases. This leads to competition for the limited food and space, causing an increase in mite death rate and resulting in negative reproductive success in these overcrowded drone cells.

In A. cerana, no mite reproduction occurs in worker brood cells per reproductive cycle and the mite population stabilizes at low level, 800 mites per colony. In Africanised Honeybees, limited mite reproduction can occur in worker brood cells and the mite population stabilizes at a higher level 1000–3000 mites. This is linked to the short adult longevity of Africanised Honeybees (21 days versus 25–180 days for European Honeybees) as a result of the tropical or sub-tropical climate. Therefore, In European honeybees mite reproduction in worker brood cells is more than sufficient to compensate for losses as a result of overcrowding in drone brood cells, allowing the mite population to increase until the colony dies.

Genetics and molecular research journal: Sequential hygienic behaviour in Carniolan honeybees (*Apis mellifera carnica*).
Corresponding author: K.P. Gramacho, published June 2009 (Accessed Jan 2011)
Hygienic behaviour can avoid or hinder the development of brood diseases, being considered the primary defence of honeybees against American foulbrood, European foulbrood, chalkbrood, and Varroa infestations. Per Rothenbuhler (1964), HB is genetically controlled by two pairs of recessive genes (u = uncapping and r = remover), which when they are homozygous allow bees to identify sick, killed or infested brood inside capped cells and then to uncap the cell and remove the brood. It is known that the HB is highly variable and is influenced by climatic conditions such as humidity and temperature, as well as the colony conditions.

Methods of Testing and Analysis of Hygienic behaviour.

Genetics and molecular research journal: Sequential hygienic behaviour in Carniolan honeybees (*Apis mellifera carnica*). Corresponding author: K.P. Gramacho, published June 2009. (Accessed Feb 2011)

Experiments done on HB only present the results as of the removal of brood to classify the colony as hygienic or not; they do not examine the steps involved in this behaviour and it was therefore decided to study the steps or sequences of hygienic behaviour. This study was carried out at the Bee Institute (Institute fur Bienenkunde) of the University of Hohenheim, Stuttgart, Germany. Four colonies of Carniolan bees (*Apis mellifera carnica*) were used, with four repetitions at intervals of 15 to 20 days, from June to August 1996. Hygienic behaviour was evaluated by *the pin-killing method*. (See page 27)

Article: The comparison of hygienic behaviour between five honeybee breeding lines. Journal of Apicultural science – volume 54, 2010.
Apiculture dept, University Warmia and marzury in Olsztyn, Poland. Nov 2010. (Accessed May 2011)

An experiment was carried out to compare the HB of five honeybee breeding lines kept in Poland. The five experimental groups included:

- Carniolan “Kortowka” line (carK)
- Carniolan “Dobra” line (CarD)
- Caucasian bees “Woznica line (CauW)
- Mellifera bees “Augustowska” line (MelA)
- A hybrid of the two species *A.m.capensis x A.m.carnica* crossed with Carniolan drones (PCP).

The 40 experimental colonies were observed, eight colonies of each of the five experimental groups. Each year, mating hives were settled with about 1,000 bees and a virgin queen at the beginning of May.

In contrast, the experimental procedure was to *remove a 2-inch square area of brood comb and place in deep freeze (-20C) for 24 hrs*, defrost and the reintroduce to the hive at room temperature. (See page 27 for method)

The American Genetic Association: Journal of Heredity (2009).
Maternal Effects on the Hygienic Behavior of Russian & Ontario Hybrid Honeybees (Apis mellifera L.) PETER UNGER AND ERNESTO GUZMA´N-NOVOA. Published 4th Nov 2009. From the Department of Environmental Biology, University of Guelph, Canada. (Accessed Feb 11)

Strains and hybrids of Russian and Ontario honeybees were evaluated for hygienic behaviour. The objectives were to determine phenotypic and genotypic variability and to study the inheritance of this behaviour using strains and mutual hybrids of Russian and Ontario bees co-fostered in common colonies.

Hygienic behaviour was evaluated at colony level and therefore would incorporate unique interactions within the colony. For the individual level testing, workers of each strain and their hybrids were evaluated in Observation hives to provide a more controlled environment. Newly emerged bees from colonies were collected and tagged prior to release in the observation hive. (A similar approach to Dr. S.J. Martin's stage 3 genotyping method as found on page 21.)

The queens of 15 honeybee colonies were replaced with young Russian queens (RQs), and the same was done for another 15 colonies using Ontario queens (OQs). Colonies of the two bee strains were established after nine weeks, when a new generation of worker bees produced by the new queens had replaced those of the old queen. The colonies of both bee strains that were evaluated for hygienic behavior in full-size hives were ranked based on their performance. The two most hygienic Russian colonies and the two least hygienic Ontario colonies were selected as parental sources for individual testing.

Queens were reared from all four colonies and were instrumentally inseminated (see page 46) with semen collected from drones of each of the other three source colonies. Each possible combination, with the exception of mating queens with their own brothers, was attempted. Therefore, queens from each of the Russian colonies were crossed with drones from each of the Ontario colonies and vice versa. The purpose of the individual observations was to estimate the relative proportion of individuals of each genotype engaging in hygienic tasks as well as the mean number of times that each individual engaged in hygienic behaviour.

Paper: The hygienic behaviour of Honeybees in relation to Chalkbrood disease.
Martha Gilliam, Stephen Taber. US dept of agriculture. Agriculture research service. Carl Hayden bee research centre. Gary Richardson. US dept of agriculture. Agriculture research service. Colorado state university. (Accessed Jan 2011)
Another distinctive method is to use dry ice (See page 27) and this was used in one part of an experiment to kill brood cells in a 2-inch square area. In a second test, a 2-inch square area of brood comb was removed, placed in deep freeze for 24 hours and replaced back on to the comb once thawed at room temperature. Daily observations were made to count the number of cells uncapped or removed.

Colonies were then classified as to which ones were resistant or susceptible on the basis of good or poor hygienic behaviour. Resistant colonies uncapped > 70% of cells and susceptible colonies removed < 70% of those killed cells. Queens were reared from those colonies, nine in total (Four resistant and five susceptible) were selected and reared. Inoculate containing *Ascosphaera apis* was sprayed 3 times per day on alternating days onto areas of brood of those nine colonies in order to infect those colonies. Mummies soon developed from this application, which were removed and examined microscopically.

Dr. S J Martin and two PHD students of Sheffield University study bees and behaviours. Paper: Multi-level selection for hygienic behaviour in honeybees JA Pe´rez-Sato1, N Chaˆline2, SJ Martin, WOH Hughes and FLW Ratnieks. (Department of Animal and Plant Sciences, University of Sheffield, UK. 2009.)
Research was carried out to investigate whether response to artificial selection for a key resistance mechanism, hygienic behaviour, can be improved using multi-level selection by selecting not only among colonies as normal but also among patrilines (descendant from fathers line) within colonies. This initially involves screening large numbers of colonies to detect the most hygienic colonies, from which queens and/or drones are reared to obtain a hygienic line.

Highly hygienic colonies were identified (between-colony selection), and the specific patrilines within them responsible for most hygienic behaviour were determined using observation hives. Queens reared from these hygienic patrilines (within-colony selection) were identified using DNA microsatellite analysis of a wing-tip tissue sample and then mated to drones from a third highly hygienic colony.

The resulting colonies headed by queens from hygienic patrilines showed approximately double the level of hygienic behaviour of colonies headed by sister queens from non-hygienic patrilines. The results show that multi-level selection can significantly improve the success of honeybee breeding programs.

The aim of this large study was to investigate whether intracolony selection can be used to improve a honeybee-breeding programme. A single generation breeding programme was carried out in four stages: (1) hygienic colonies were identified in an unselected population using a commonly used *freeze-killed brood* method (see page 27) as a bioassay of hygienic behaviour; (2) individual workers in these colonies that performed most hygienic behaviour were identified using observation hives; (3) the workers were genotyped to identify hygienic and non-hygienic patrilines and queens of these patrilines were reared; (4) the queens were mated in a semi-isolated valley with drones from a hygienic colony and allowed to establish colonies. The hygienic behaviour of the colonies headed by queens of hygienic and non-hygienic patrilines and the three original breeder colonies were then compared.

Article: Brood temperature, task division and colony survival in honeybees: A model. Matthias A. Becher, Hanno Hildenbrandt, Charlotte K. Hemelrijk, Robin Moritz.

It appears that there may be an association between brood nest size, brood nest temperature and the duration of the in-hive period in the brood nest in relation to colony dynamics. Data collected in an investigation discovered that the survival of a colony depends on the initial number of “WINTERBEES”. In spring, the colony size will only increase, if enough new workers emerge and hence if the number of available brood cells are high enough. The study found that colony sizes in spring are about 75% of the autumn colony sizes.

This directly depends on the initial number of “WINTERBEES” and found that a minimal number of 3915 initial bees is required for colony survival and is called the “Survival threshold”.

During the annual testing in this report, the Sheffield hive failed to survive the cold winter. It was unfortunately not possible to continue using this hive for data collection

Discussion

Genetics and molecular research journal: Sequential hygienic behaviour in Carniolan honeybees (*Apis mellifera carnica*). Corresponding author: K.P. Gramacho, published June 2009. (Accessed Feb 2011)

After carrying out a Freeze Killed Brood test it was observed that the highest frequency of punctured cell was found at four hours after perforation; it gradually decreased until 24 hours, when it reached 0%. Two hours after perforation all colonies already had some uncapped cells; this reached the highest frequency at four – six hours after perforation; 24 h after perforation most of the cells were already uncapped. The maximum value of brood partially removed was reached at 10 hours; it continued until 48 hours after perforation. This experiment witnessed that normally the workers remove the injured pupae by eating them (cannibalism) instead of simply removing the pupa. However, sometimes they did remove the pupa without eating it.

It was observed that the higher the frequency of brood partially removed, the less hygienic the colony. Brood partially removed occurred more frequently after punctured capping and less frequently after uncapped cell. It may be that the brood partially removed is a sign of cannibalism. This brood condition was observed more frequently in the non-hygienic colony. It was observed that the puncturing of the brood cells had already started two hours after perforation, at a frequency of 42%.

To finally explain the genetic control of hygienic behaviour in *Apis mellifera,* rather than two pairs of genes, the new model involves three pairs of recessive genes (u1, u2 and r). In order to uncap the cell the bee should have both u1 and u2 genes. Only one u1 or u2 gene would determine puncturing while all three genes would be responsible for uncapping and removal (u1/u1, u2/u2, r/r). The sequence of steps would be puncturing and uncapping the capped cells, followed by removal of the brood. The new hypothesis in this study is based on the observations made of individual worker brood cells after pin killing; however it needs to be tested with further observations.

Article: The comparison of hygienic behaviour between five honeybee breeding lines. Journal of Apicultural science – volume 54, 2010.
Apiculture dept, University Warmia and marzury in Olsztyn, Poland. Nov 2010. (Accessed May 2011)
In this research project, it was observed that worker bees of all experimental groups began uncapping cells of dead brood during the first hour after the killed brood combs were introduced. The highest cleaning behaviour after 24 hours were demonstrated by Mellifera (17%) and Carniolan "Dobra" line (13.6%) colonies.

In summary, Mellifera bees and Carniolan breeds may have a higher ability to clean and this suggests that the behavioural trait of these bees is genetically influenced which can be chosen for further breeding programmes to select more Hygienic bees for the future.

There were some setbacks in this study: Based on the observations carried out there were very strong differences in HB within different colonies, but also within the same breeding line. These differences may not only be due to the breeds, but also varied by environmental conditions. The nucleus (nuc) hives used in this study are smaller in comparison to the standard sized hive and this may influence the results obviously having an impact on the colony size.

The American Genetic Association: Journal of Heredity (2009).
Maternal Effects on the Hygienic Behavior of Russian & Ontario Hybrid Honeybees (Apis mellifera L.) PETER UNGER AND ERNESTO GUZMA´N-NOVOA. Published 4th Nov 2009. From the Department of Environmental Biology, University of Guelph, Canada.
More globally it was discovered in one experiment that Russian bees uncapped significantly more cells and removed more brood than Ontario bees, suggesting that Russian bees are above average for the expression of hygienic behavior compared with North American bees, and demonstrates phenotypic variability between bee strains for this behaviour and could be a viable strain for breeding programs aimed at improving the health of colonies. This could mean that breeding hygienic bees would be easier than previously thought, considering that the most difficult part of honeybee selective breeding is controlling the paternal sources. These results demonstrate phenotypic and genotypic variability for hygienic behaviour and the maternal effects in the inheritance of hygienic traits.

It is possible that mitochondrial genes inherited from the mother could be responsible for these effects. It is established that mitochondria are maternally inherited in honeybees (Behura 2006). Therefore, if genes influencing hygienic behavior exist in the mitochondria, it would be expected that workers expressing the behavior inherit it mainly from their mother.

Paper: The resistance to American foulbrood disease by honeybee colonies *Apis mellifera* bred for hygienic behaviour. By: Marla SPIVAK, Gary S. REUTER. 2001.

Colonies that display hygienic behaviour demonstrated resistance to chalkbrood as well as AFB. The hygienic colonies in that study did not have chalkbrood at the beginning of the experiment, but after inoculation with AFB spores, six colonies developed symptoms of chalkbrood. Most likely, chalkbrood spores are present in most colonies, but if larvae become infected in hygienic colonies, the bees removed the diseased larvae from the nest before symptoms of the disease appeared in the larvae. It was presumed that the hygienic colonies were not able to remove all larvae infected with either chalkbrood or AFB, and so disease symptoms appeared temporarily. As the AFB infection recovered, the chalkbrood infection also recovered in both years. The non-hygienic colonies had chalkbrood symptoms prior to inoculation with AFB spores in both years, and after inoculation had symptoms of both diseases. This field study indicated that the presence of chalkbrood in colonies did not inhibit the development of AFB symptoms.

It is important to add that destroying (burning) infected combs is essential in control because AFB spores can successfully germinate in 35-year-old combs. Routine replacement of old combs in thriving colonies is a potentially important component of disease prevention and together with the use of hygienic stocks of bees, could eliminate the routine use of antibiotics to prevent disease outbreak, and reduce the need to treat colonies that become diseased.

Paper: Hygienic behaviour of the honeybee (*Apis mellifera*) is independent of sucrose responsiveness and foraging ontogeny. Katarzyna Goode, Zachary Huber, Karen A. Mesce and Marla Spivak. August 2005.

Honeybees display a range of behaviours that appear to be linked with octopamine. Octopamine (OA) is a key neuroactive substance that modulates a diverse range of behaviours such as nest mate recognition, responsiveness to brood pheromone, the ability to conduct house keeping tasks and foraging duties.

In insects, the effects of this monoamine encompass alterations in olfactory and gustatory information processing, arousal and motor activity and in honeybees it may be linked to Hygienic behaviour. All bees are able to perform the uncapping and removing abnormal brood from the nest, but this rate of initiation and completion is greatly variable among colonies. Honeybees performing hygienic behaviour have been shown to possess a significantly greater level of OA immunoreactivity in the protocerebral neurons (cluster-3 cells) of the brain.

Paper: The hygienic behaviour of Honeybees in relation to Chalkbrood disease.
Martha Gilliam, Stephen Taber. US dept of agriculture. Agriculture research service. Carl Hayden bee research centre. Gary Richardson. US dept of agriculture. Agriculture research service. Colorado state university.
In resistant colonies, the longer the spraying of *Ascosphaera apis* (chalkbrood inoculate), the faster the bees removed the mummies resulting in higher efficiency ratios for resistant bees. The results indicated that bees could detect diseased larva before any human can.

Paper: The relationship between hygienic behaviour and suppression of mite reproduction as honeybee (*Apis mellifera*) mechanisms of resistance to *Varroa destructor.* Abdullah Ibrahim and Marla Spivak.
To establish and maintain the hygienic lines, daughter queens from colonies that displayed the most rapid removal rates were raised. “For each generation, the daughter hygienic queens were instrumentally inseminated with a mixture of semen collected from drones from different hygienic colonies” (previous research: Spivak and Gilliam, 1998). Some bees that maintain low mite levels because the varroa has low reproductive success on the worker brood is another trait to be heritable and is called Suppression of Mite Reproduction (SMR).

An article from the American Bee Journal (2009):
The Future of the MN Hygienic Stock of Bees is in Good Hands!
MARLA SPIVAK, GARY S REUTER, KATIE LEE, and BETSY RANUM Department of Entomology University of Minnesota (www.extension.umn.edu/honeybees)
It has been proven that the time it takes the bees in a colony to remove freeze-killed pupae is correlated with how long it takes them to detect and remove disease and mite-infested brood from the colony. The faster a colony removes this brood; they have increased resistance to American foulbrood, chalkbrood and *Varroa* mites. Minnesota bee breeders tested 100 colonies in this next investigation.

Only those colonies that completely removed ≥ 95% of the freeze-killed brood within 24 hours are chosen as breeders. If a queen is bought from one of these beekeepers, there is 62%-79% chance that the queen will produce a hygienic colony. The colonies with hygienic queens mated with unselected drones were less hygienic than the MN (Minnesota) breeder colonies. This shows that for colonies to be hygienic, the drones in the area where the queen's mate, should be from hygienic colonies. Many hygienic queens sold in the US currently fall in this category: they are mated with unselected drones and on average, are not highly hygienic. However, the colonies with hygienic queens, mated with unselected drones, are more hygienic than the unselected queens mated with unselected drones.

Beekeepers can successfully incorporate the hygienic trait into their colonies. "Beekeepers can purchase a queen from these beekeepers knowing the probability that the queen's colony will be hygienic, which illustrates how certification of selected stocks or traits of bees might be carried throughout the US". (Marla Spivak)

Paper: Hygienic bee colonies. By Mr. Norman Carreck (researcher), Dr. Karin Alton (researcher), Mr. Gianluigi Bigio (doctoral student), Mr. Mike Kavanagh (volunteer helper). Funding for this project are Mr. Michael Chowen, Rowse Honey Ltd., and The BBKA. 2009 publication. (http://www.sussex.ac.uk/lasi/sussexplan/hygienicbees)

Previous research in the USA has shown that hygienic colonies produce as much honey but are more resistant to brood diseases such as foulbrood and chalkbrood and also disrupts the breeding cycle of *Varroa* mites. Hygiene is a natural genetic trait meaning that it can be bred using normal breeding methods. Previous LASI research by Professor Ratnieks observed that hygiene is found to be rare in British hives with only about 10 per cent hygienic.

Professor Ratnieks has developed an effective method of breeding for hygiene via 'intracolony selection' (*Queens of the same 'patriline' are then reared) w*here a hygienic colony is kept in an observation hive to determine which workers are most hygienic. Daughter queens are reared that have the same father as the hygienic workers. DNA markers are used to identify them. In this way breeding for hygiene is more effective and rapid. This project commenced in autumn 2008. The main aim is to breed and test a stock of hygienic, native British honeybees, *Apis mellifera mellifera.*

Dr. S J Martin and 2 PHD students of Sheffield University study bees and behaviours. Paper: Multi-level selection for hygienic behaviour in honeybees JA Pe´rez-Sato1, N Chaˆline2, SJ Martin, WOH Hughes and FLW Ratnieks. (Department of Animal and Plant Sciences, University of Sheffield, UK. 2009.)

During an investigation at Sheffield University, every colony was screened and the three most hygienic colonies were selected as breeders; two were used to rear virgin queens and one to produce drones. In the 31 colonies tested, the average percentage of Freeze Killed Brood removed ranged from 15 to 98%. Most colonies (65%) removed 50% of FKB within 48 hours. Seven colonies (22%) removed > 70% of FKB and were thus classified as being hygienic. The first two colonies selected for the breeding programme (used for producing queens in the observation hives) removed 72 % and 78 % and one colony (third breeder colony) was very highly hygienic removing 98%.

The next stage was to Identify hygienic workers using one observation hive containing 2000 unmarked bees from the parent colony and a laying marked queen from another colony. Within a few hours of emergence from their cells, young workers were marked with a numbered tag on the thorax and added to their respective observation hive. Over a period of two days, 592 and 634 workers were marked and introduced into two observation hives. The marked workers performing hygienic behaviour were between 15 and 17 days old. Of the marked workers; most were seen uncapping and inspecting cells (68% and 55% in the other) or walking over (23% and 30%). Only a small number (3% and 2%) was ever seen performing full hygienic behaviour (uncapping and removing). There was a similar pattern in the intensity of hygienic behaviour performed, with most bees performing hygienic behaviour less than five times, whereas a very few bees performed frequently at 40–75 times.

Stage 3: Genotyping of hygienic workers and virgin queens for each of the breeder colonies. The 90 marked workers that performed hygienic behaviour most frequently were genotyped. The patrilines (from father's line) of the genotyped workers were then determined based on their paternal alleles, and hygienic and non-hygienic patrilines were identified based on the behavioural data. Virgin queens were reared from colonies (of the observation hives) and individually marked. The wingtips were genotyped to determine which queens belonged to the hygienic and non-hygienic patrilines identified above. The queens were then held for three days whilst genotyping was conducted.

For these colonies 130 and 148 queens were successfully reared and genotyped in this way.

Stage 4: semi-controlled mating and final assessment of the virgin queens reared in Stage three. A total of 60 from each of the two breeder colonies were selected based on belonging either to the three highly hygienic patrilines identified in stage 3.
These queens were introduced into several mating hives with workers in five medium Langstroth frames. The mating hives were then placed during July 2004 at two locations. The third breeder colony (originally to produce drones) was located at the mating apiary to provide drones for the queens to mate with.

The colonies headed by queens of hygienic patrilines removed approximately twice as many FKB as colonies headed by queens of non-hygienic patrilines and there was a clear difference in colonies headed by hygienic and non-hygienic patriline queens.
These results confirm that there is a heritable component to hygienic behaviour. Given that hygienic colonies are a small minority of all colonies, and that within these hygienic colonies, most of the patrilines are most likely to be non-hygienic.
However, this proves that colonies headed by queens from hygienic patrilines removed twice as many FKB (34%) as colonies headed by queens from non-hygienic patrilines (17%). The levels of hygienic behaviour shown by almost all of the colonies headed by queens of hygienic patrilines were greater than of those headed by queens of non-hygienic patrilines. The normal honeybee breeding method in which whole colonies are identified as breeders requires at least four generations of artificial selection to obtain a population of hygienic bees.

The Guardian Newspaper: Wednesday 25 August 2010
Ron Hoskins, a beekeeper from Swindon, has spent the last 18 years looking for a bee that is parasite resistant and yesterday he claimed that his superbee could assure the future of this major pollinating insect. Hoskins, claims to have bred a honeybee that "grooms'" other bees in the hives to remove the blood-sucking varroa mite that spreads viruses and disease. Hoskins calls his strain the "Swindon honeybee". Martin Smith, president of the British Beekeepers' Association, will fund the roll-out of the research to Wiltshire beekeepers as it could hold the answer to halting the number of bee deaths and could help provide the solution to controlling the varroa mite.
This is refered to more on page 46 and 47.

Literature and research review: Conclusion.

From qualitative data collection of the previous studies carried out on bee colonies, it is gathered that the British Beekeepers Association has been calling on the government to donate £8 million from DEFRA over the next few years for research into honey bee decline. Stuart Bailey, chairman of Rowse Honey, is committing £100,000 to help fund the project. 'Clean-up' bees could save endangered hives and there is a plan to use genetically programmed 'hygienic' breeds to combat parasites. (it was reported by The Guardian newspaper: Sunday 9 November 2008).

Professor Francis Ratnieks, the UK's only Professor of Apiculture, at Sussex University, said: "The support for scientific work on honey bees given by organisations such as the BBKA is vital if we are to carry out practical research on honey bee health and wellbeing. By supporting a PhD student, the BBKA is also helping us to train the next generation of bee scientists."
(http://www.britishbee.org.uk/news/current_news/bbka-to-fund-a-phd-project-into-investigating-the-.shtml)

To date, bee research all over the world has vastly contributed to stability in bee populations. More and more conservationists are actively approaching beekeeping as a hobby, attending beekeeping courses (British Beekeepers Association awarded) with their local groups and looking upon research opportunities at universities.

For example: Sheffield University offers PhD opportunities supervised by Stephen Martin, most recently for 2011 applicants "The role of viral pathogens in the over-wintering losses in honeybees".

The Laboratory of Apiculture and Social Insects (LASI) at the University of Sussex offers weekend workshops and short courses in many areas of beekeeping, including: Best plants for bees, decoding the waggle dance and the method behind determination of Hygienic Behaviour. Undergraduate project students, graduate students, postdoctoral researchers, volunteers and visitors under the supervision of Professor Francis Ratnieks carry out LASI research.

Procedures and Methods used in this study.

Access to three beehives from members at Chesterfield Beekeepers Association was permitted to undertake this project. These were:

- One small hive (1 brood box and 1 x honey super) would be from an urban area in a private garden at Sheffield, NetherEdge. Bee breed: Carniolan.

- One small hive (1 brood box and 1 x honey super) – a very rural location on allotments in Creswell, Derbyshire. Bee breed: Carniolan.

- One large hive from commercial beekeeper (at least 2 brood boxes and 3 honey supers). The hive is set in farmland near Whaley. Bee Breed: Buckfast.

Part 1.

Determining hygienic behaviour - Experimental Techniques.

Possible approaches for killing brood cells for this experiment using the information sourced from the literature review:

1. Pin killing method: As used in: Sequential hygienic behaviour in Carniolan honeybees (K.P. Gramacho, June 2009). In this method, the brood is damaged or killed with an insect pin, which is used to pierce the sealed brood cells through the centre of the cell cap, penetrating the body of the pupa. This injury provokes removal of the damaged or dead brood by hygienic worker bees.
2. Section of comb under deep-freeze method. Removal a 2-inch square area of brood comb, placing this in deep freeze (-20C) for 24 hours, defrosting and reintroducing to the hive at room temperature. This method was used in "The comparison of hygienic behaviour between five honeybee breeding lines (Apiculture dept, Poland. Nov 2010)
3. Freeze-killed brood method: A widespread method, particularly used in Francis Ratnieks experiments. Dr. Jerry Bromenshank at the University of Montana was the first to suggest using liquid nitrogen (N2) to freeze a section of sealed brood within the frame. He found that freezing the brood this way was more efficient than cutting, freezing, and replacing comb inserts. (Spivak and Downey, 1998; Spivak and Reuter, 1998).
4. Dry ice could be used to kill brood cells in a 2-inch square area. This method was used in "The hygienic behaviour of Honeybees in relation to Chalkbrood disease". (Martha Gilliam, Stephen Taber. US dept of agriculture. Agriculture research service. Carl Hayden bee research centre. Gary Richardson. US dept of agriculture. Agriculture research service)

Preferred experimental procedure:

It was decided to use the *freeze-killed brood method* (Spivak and Downey, 1998; Spivak and Reuter, 1998) for the determination of hygienic behaviour.

In this procedure, all frames were taken from the same place in each hive and testing carried out where there was most sealed brood area of that frame within a 3-inch area. This method is reproducible.

- A frame with more than a 3-inch diameter circle of sealed brood was selected.
- The frame was laid horizontally across a support. A metal cylinder (soup can cut open at both ends) was twisted carefully into the sealed brood until it reached the mid-rib.
- The number of unsealed cells inside the cylinder (no more than 12 empty cells) was counted and recorded by means of photographic data.
- Liquid nitrogen was poured in stages into the metal cylinder. A recommended 300 ml was required to kill the area of brood.
- It took approximately 5 mins for the frozen cells to thaw before removing the metal cylinder.
- The frame was marked and re-located into the centre of the brood nest.
- 48 hours later, the same frame was removed (containing the FKB). The number of sealed cells remaining within the circle were counted and recorded.

Prior to experimentation, provisional testing was carried out to examine storage of nitrogen in a standard Thermos vessel against the extremes of temperature. A purpose built box (complete with packing material) was made to securely hold a flask containing nitrogen whilst in storage and transit.

Liquid nitrogen can be obtained from several sources such as agricultural supplies, university labs, veterinary surgeons and plumber's merchants or can also be purchased direct from BOC. Storage containers can be purchased or rented on a short-term basis or indefinitely. Liquid nitrogen Dewar containers come in various sizes. The larger sizes will keep nitrogen for a longer period. The container used in this experiment is of 3L capacity, which will store nitrogen for up to two days.

Personal Protective Equipment is essential for the handling, transportation and storage of liquid nitrogen.

- Overalls and thick gloves were worn for Nitrogen handling.
- Personal Protective Equipment, especially safety glasses, were worn to protect against splashes.
- Only containers that have been designed specifically for use with liquids at extreme temperatures were used.

For Health and Safety requirements:

- The extremely low temperature of the liquid can cause burn-like damage to the skin by contact with the fluid.
- Skin can freeze and adhere to liquid nitrogen cooled surfaces causing tearing on removal.
- Thermal stress damage can be caused to containers because of large, rapid changes of temperature.
- The dispensing of liquid nitrogen from pressurised dewers may be carried out only by those trained to do so.
- Only containers that have been designed specifically for use with cryogenic liquids may be used . (http://www.bath.ac.uk/chemistry/safety/cryogen.html)
- Safety training may be obtained in order to handle and use liquid nitrogen safely. The safety Data sheet for Liquid nitrogen is available in the appendix.

Other Personal Protective equipment was purchased as follows:

- Full Bee keeping suit or overalls with a smock
- Bee proof hat and veil
- Bee proof, Leather gloves
- Wellington boots

} All purchased for £70

- Smoker (for calming the bees whilst in the hive) Purchased at £40
- J - shaped tool and Chisel (both for safe hive dismantling and lifting the frames) Purchased at £10
- Bee brush (removes bees gently out of the way) Purchased at £10

Purchased from E. H. Thorne beehives Ltd (Wragby, Market Rasen. Lincs) in August 2010.

The pre-visit Item checklist

	All Personal Protective Equipment: **Bee suite, Veil, Overalls, Gloves, Wellington boots.**
	Smoker + Fuel + light Hive tools
	Liquid N2 in secure box Styrofoam cup for measuring
	3 x Cardboard inserts for hive bottom (14" x 12")
	Labelled sample containers
	Camera
	Tin can with both ends removed
	Notebook and pencil
	Cleaning apparatus and solution

Three hives were visited in one whole day for the initial treatment with nitrogen before being examined two days later (taking a few hours) for counting of cells and the affected area were photographically recorded on both occasions.

To reduce the impact between colonies, a key factor in the prevention of spread of infection and disease (such as the microbes that cause American or European foul brood (AFB and EFB) (*Paenibacillus larvae* and *Melissococcus plutonius)* is good hygiene. It is of the up most importance that the hive tools and equipment are cleaned between hives. If these items are not cleaned after each visit, an accumulation of hive debris on the equipment will possibly carry spores.

A washing solution was made with bleach and water, which effectively dissolved propolis and removed germs and potential contamination. A "brillo" pad was also used to scrub off any propolis or hive debris on equipment. Gloves were additionally wiped between hives.

Times of Year for Hygienic Behaviour experimentation:

- August, **Autumn 2010**. Date of experiment 25th August (Wednesday), follow up 48 hours later for results analysis on the 27th (Friday).

To minimise colony losses, over the winter months' apiary visits are made to make sure that hive roofs have not been blown off; that hives are still upright and to check for stray animal interference or vandalism and to monitor varroa levels. Treatments can be carried out typically in December or January using oxalic acid.

Hefting (lifting) hives will indicate whether to carry out emergency feeding. Lifting the crown board also enables assessment of stores, which can be done in conjunction with winter Varroa treatment.

The first part of the year is a critical time for honeybee colonies as the overwintering population rears brood to replace itself and develop the colony for spring and summer. Bee disease can impact seriously on colony development at this crucial time causing the over winter bees to die before replacement brood hatches. This causes colonies to diminish but often they recover through the summer.

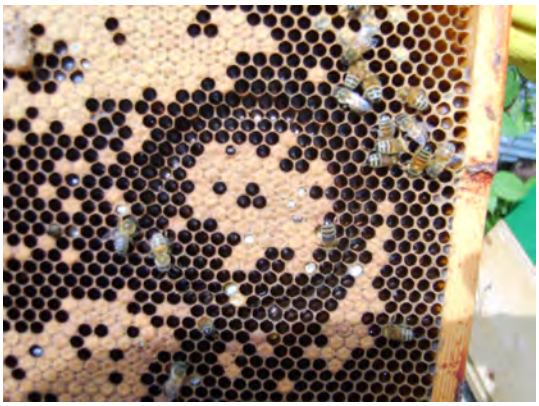
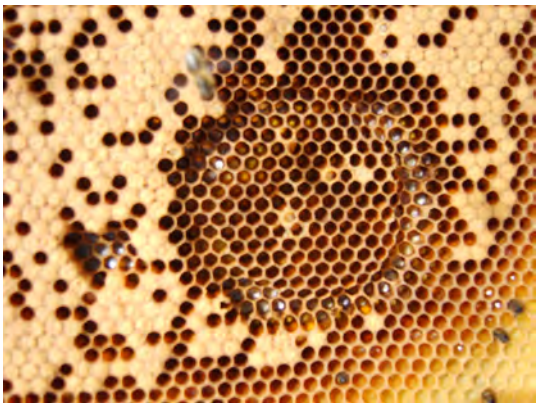
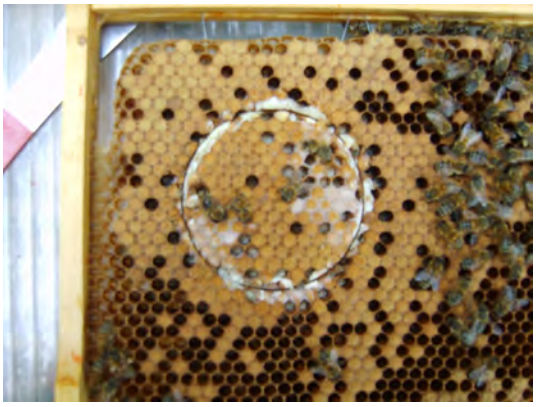
If varroa had previously been poorly controlled in autumn, the winter bees could have suffered serious damage and been infected with viral diseases.

Spring checks should be completed by late February or early March and must not wait until April. This is the period of the year when many colonies are starving.
(The National Bee Unit. Best Practice Guideline No. 6. Spring checks)
NB: If there is sufficient brood in the colony, it is OK to test the colony so early into the spring.

- March. **Early spring 2011**. Date of experiment 23rd and 25th March.
- May. **Late spring 2011**. Date of experiment 18th and 20th May.
- June. **Early Summer 2011**. Date of experiment 20th and 22nd June.
- July – **August, Late summer 2011**. Date of experiment 18th and 20th July.

Hygienic Behaviour Results:

August, Autumn 2010. Date of experiment 25th August (Wednesday), follow up 48 hours later for results analysis on the 27th (Friday)

	
Creswell Hive	**58 – 60 % HB**
	
Glapwell Hive	**94 – 96 % HB**
	
Sheffield Hive	**90 – 92 % HB**

Weather affecting the progress of the project. Winter 2010 and Spring 2011.

The Guardian - Wednesday 6 January 2010: The Met Office issued an alert warning that nearly half a metre of snow was due to fall in some areas, while freezing conditions spread after having brought chaos to the North of England and Scotland today. Forecasters say that the cold snap, which began in mid-December, is the longest since 1981.

It was a very mixed Winter, December was very cold with long lasting and frequent snow and ice, but the weather became milder with January seeing temperatures very close to the average and February warmer than average.

After the harsh winter, hive inspections are carried out to ensure the health and survival of the colonies and the beekeeper to provide food in the form of sugar solution, which gives the bees a kick-start to the season. At the point, it has been observed that the Sheffield colony has not pulled through the winter very well. The population seems very week and there are no signs of brood laid by the queen. This is not a good situation for the colony and unfortunately for this study as well. There could be several explanations for the collapse to a colony during the winter months. On a positive note; the Creswell and Glapwell hives look very strong indeed and so the study will go ahead using those hives.

The honeybee colony's ability to survive the winter depends on their food stores.
The workers form a cluster around the queen and brood and as temperatures fall, the bees form a tight group within their hive to stay warm.
(Keeping Bees by Pam Gregory, Claire Waring and Paul Peacock - May 2011)

Two potential winter problems resulting in loss of bees or colony collapse:

- **Dysentery**

Workers routinely defecate outside as they fly however; dysentery is a result from a combination of long periods without cleansing flights in cold winter. Warm days during winter are critical for honeybee survival. However, when the workers cannot fly because of extreme cold or stormy weather, they retain their faeces in the rectum and wait. Honeybee dysentery is not caused by a pathogen; it is caused by an excess amount of faecal material in the honeybee stomach. When the time between cleansing flights is too long, they will void inside the hive or just outside of it.

Sever dysentery leads to death because of stress, disease resulting from unsanitary conditions, or a breakdown in the internal communication system due to the overpowering odour inside the hive.

Colonies that are found dead in spring from dysentery will show signs of defecated material smeared over the frames and other hive parts.
(http://www.edinburghbeekeepers.org.uk/diseases/Dysentery.pdf)

- **Starved brood**

Under the absence of nectar and pollen, larvae and pupae are frequently removed or eaten by adult worker bees. If there is little or no brood, honey or pollen present, dead adult bees may be present inside the hive facing headfirst into cells.

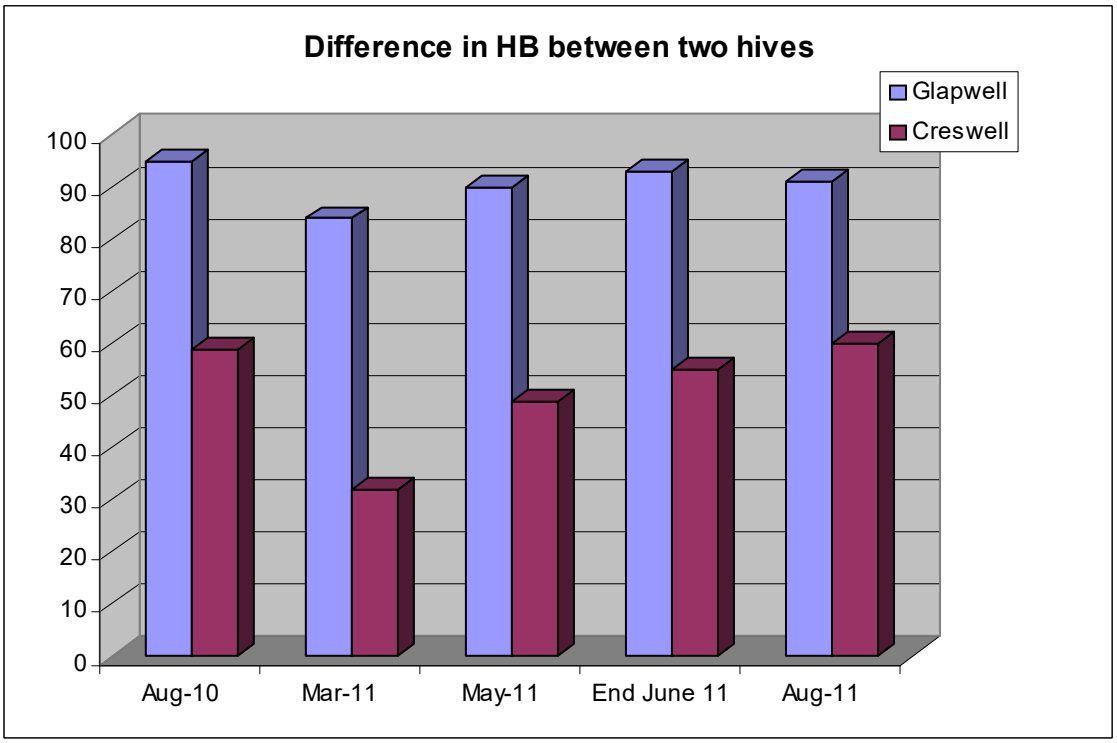
When a sudden shortage of adult bees occurs and there are insufficient adult bees to feed the brood, the brood starves to death. Bee larvae also crawl out of their cells or move to abnormal positions in their cells.
(http://www.dpi.nsw.gov.au/data/assets/pdf_file/0020/77123/non.infectious_disorders_of_honeybees_-_primefact_40-final.pdf)

After careful examination, no evidence was found to suggest that any of these factors were the specific cause for the Sheffield hive colony failure.

Hygienic Behaviour Results:

Time of Year % HB	Creswell Hive	Glapwell Hive	Sheffield Hive
Aug - 2010	59	95	91
March - 2011	32	84	-
May - 11	49	90	-
End June - 11	55	93	-
Aug - 11	60	91	-

Statistical data:



Statistical analysis for Glapwell Hive: Mean = 90.6 median = 91 StDev = 3.72

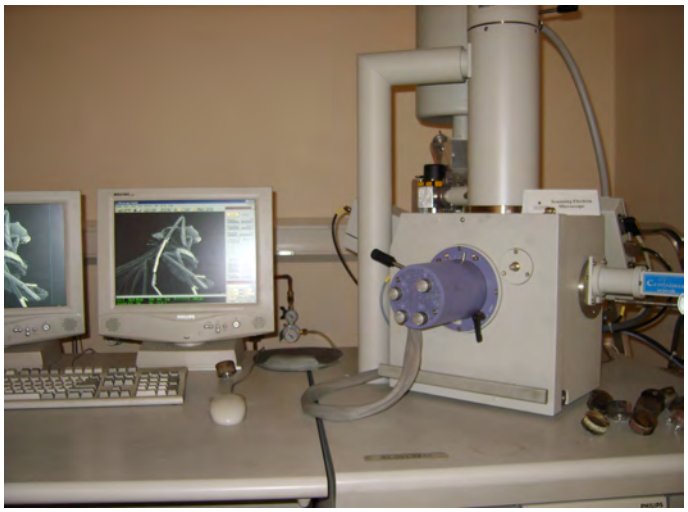
Statistical analysis for Creswell Hive: Mean = 51 median = 55 StDev = 10.26

[Standard deviation is recorded but is of little significance due to the small number of observations]

Part 2.

Examination of hive debris using the Scanning Electron Microscope.

As well as determining the results for HB, all hive bottom debris over the 48-hour period of testing was collected into sterilised receptacles and taken back to the laboratory for microscope analysis. Observing the hive debris from the 3 study hives produced examples of specimens of dead bees, organisms, possibly dead Varroa. Images of pollen grains were also discovered.



SEM. Author: Melissa Rose (July 2010)

Philips 30XL Scanning Electron Microscope
Work and safety Instructions

Carbon Film Sputter Coater

- To examine samples by the SEM a conductive coating earthed to the base of the sample holder must first be applied.

- A comprehensive set of instructions is available for the safe operation of this device. Please refer to Operators Manual CC7650 Carbon Coater, located in SEM Room at Vesuvius, Barlborough.

Experimental Procedure for using the Scanning Electron Microscope.

All specimens must be dried at 110C overnight to avoid any water, volatile and internal fluids escaping into the equipment and causing serious damage.

By removing the water from the specimen, chemical changes will have occurred and many of the elements relocated as the water is removed. Unfortunately, this process could cause the specimen to be delicate and could cause shrinkage. The alternative preferred option to above will be improved by placing the specimen in a desiccator for the drying medium, and dried for a longer period – producing only minimum damage to the specimen. After drying, the sample is ready and is positioned onto a "stub" (sticky holder) for placement into the microscope. To avoid any bad surface imagery, the non-conductive specimen must be coated. This prevents defects in the image in the form of brightly coloured centres and streaking. These cannot be overcome without coating, as a very high beam is required to achieve resolution at high magnification. Coating can be metallic or carbon. Two coats of carbon coat will be administered in this case.

Please note:

- Ideal working distance is around 10mm from sample surface to base of Centaurus Detector. A height gauge is provided for this purpose to ensure the sample is not too close. This must be used to check sample clearance before the ESEM door is closed.
- Major height adjustments should only be carried out by rotating the sample holder table.
- Please be aware that sample surface to be examined should be as closely parallel to the base plate as practicably possible.

For method of microscope operation please refer to 30XL ESEM TMP Operating Instructions manual kept in the SEM Room at Vesuvius, Barlborough.

Nikon Photo Microscope
Work Instructions

The Nikon photomicroscope has the following features.
Microscope feature - reflected light
Microscope feature - transmitted light
Instrument connected to server, Image capture facility, Camera Download procedure & Scanner attached.

Lens capacities

M2.5	=	4.5mm field width
M10	=	0.8mm
M20	=	0.5mm
M40	=	0.25mm
M60	=	0.15mm

Image Capture Procedure

Capturing an image from the microscope is done using the PC programme “Aquis”.
In Aquis, click on LHS red camera icon, then the adjacent green icon, then go “File”, “Save As”, and navigate to where the image is to be stored. Before saving, change the file type to “jpg” type.
To return to the live camera image, click again on the LHS green camera icon, which will turn red again.

An example of the Repair, maintenance, inspection and comments form.
OPERATIVE MUST SIGN AND GIVE A BRIEF DESCRIPTION OF ENTRY.

Date	Signed	Repair	Maintenance	Inspection	Comments

Examination of bees and hive debris using Stereomicroscope Imagery.

The stereoscope is used for low magnification analysis and photographs of samples.

Stereomicroscopes (or dissecting microscopes) are two compound microscopes, which focus on the same point from slightly different angles. This allows the specimen to be viewed in three dimensions. Stereomicroscopes are low power compared with compound microscopes. They can have a single fixed magnification, several discrete magnifications, or a zoom magnification system. Working distance is much longer than with a typical compound microscope allowing work to be done on the specimen while it is being observed through the microscope.

(http://www.martinmicroscope.com/MicroscopePages/Stereomicroscopes.htm)

Hypothesis: "There will be a strong correlation in (%) HB and the amount of debris found (per mg) on the hive bottom board. Therefore: The less debris found on the bottom board, the higher the (%) HB will be in the colony thus confirming, "The HB is linked to the cleanliness of the hive"

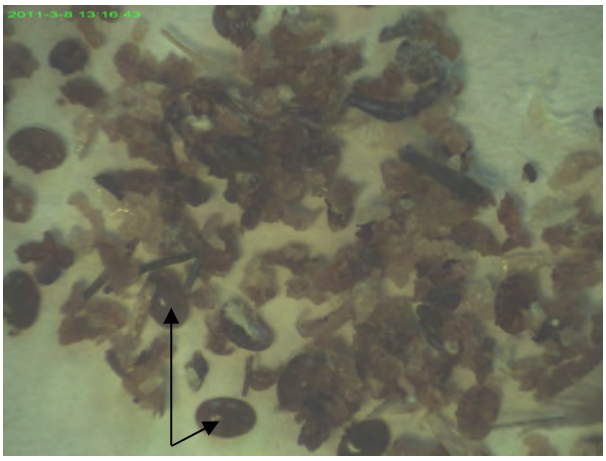
Hygienic behaviour VS Hive debris on bottom boards.

Often it is asked if hygienic colonies tend to have clean bottom boards, and if they tend to remove debris from the colony more quickly than other colonies. Removing debris and rotting material from the hive will to an extent eliminate disease and bacteria. Mayer (1996) suggested that "Removing debris from the hive is a form of cleanliness, but it is not necessarily a sign that the bees carry the hygienic trait. Although the common usage of the word hygienic denotes cleanliness, hygienic behaviour is a specific response by the bees to diseased and parasitised brood. There is a possible fact that "A colony that keeps its hive clean does not imply that it will be resistant to diseases" (Marla Spivak). Colonies must be screened for hygienic behaviour using an assay such as the one being carried out in Part 1 of this assignment.

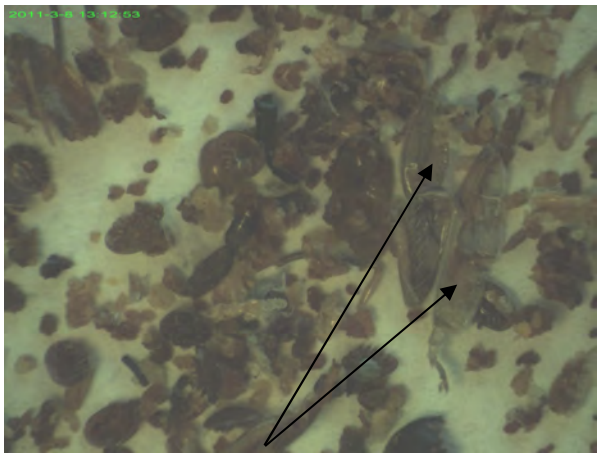
Examination and analysis of Hive debris.

The hive debris consists of wax, particularly the flake like structures and large lumps of propolis. The rest is likely to be: bits of bees, "muck" from cleaning out cells (Larval skins, faecal matter, spoilt brood food), and often chalk brood fruiting bodies. For each hive, the bottom board debris is weighed (mg).

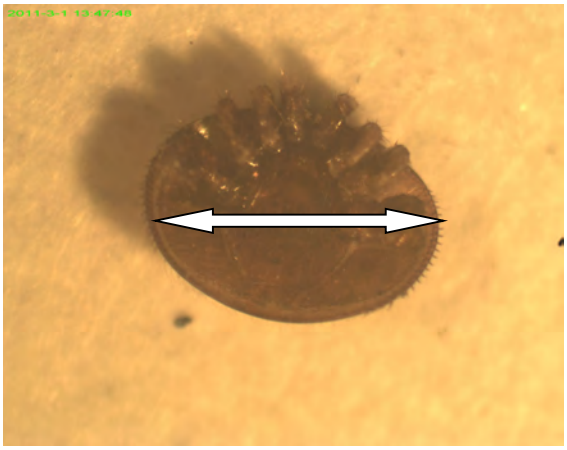
Light microscope and Stereomicroscope Images.



Hive debris (Arrows to Varroa mites)

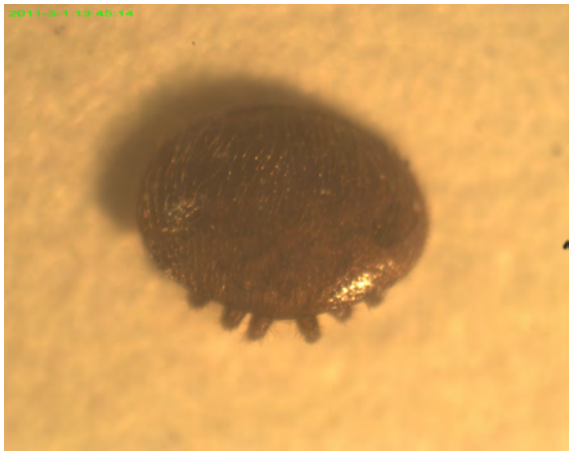


Hive debris (Arrows to bee body parts)

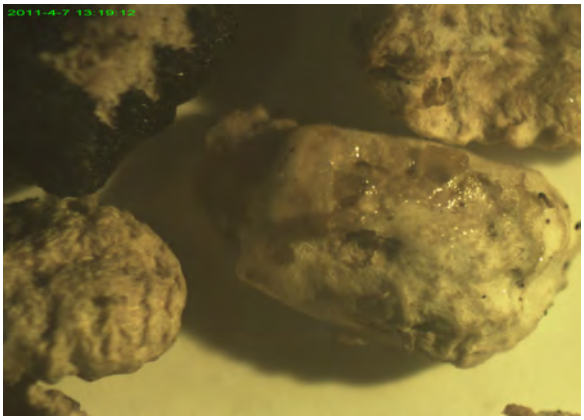


Up-turned Varroa mite

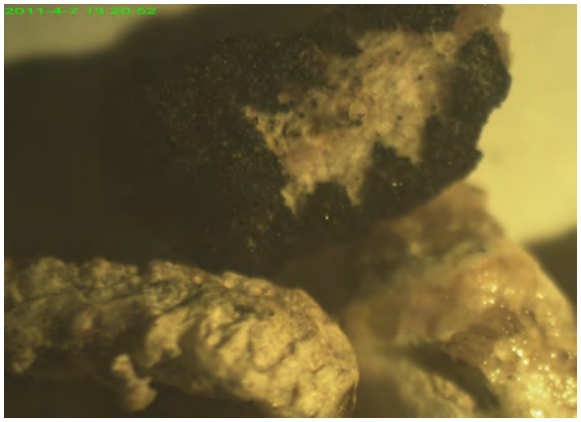
(Approx. 1.2 mm long x 1.6 mm wide)



Top view, Varroa mite.

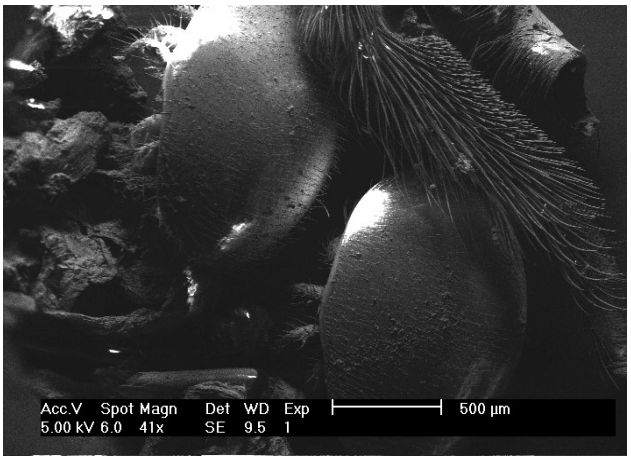


Chalkbrood mummies found in the Creswell hive (Size of bee larvae).

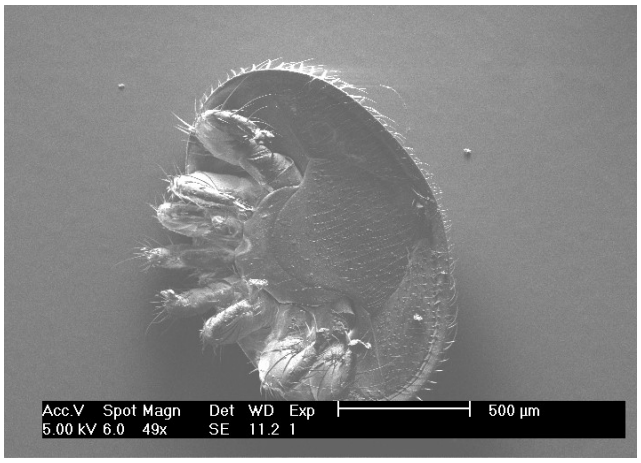


Mummy covered by ascocysts (black) and mycelium (white).

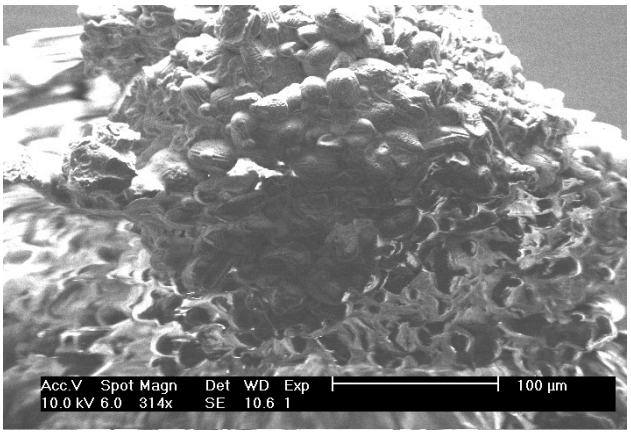
SEM Images.



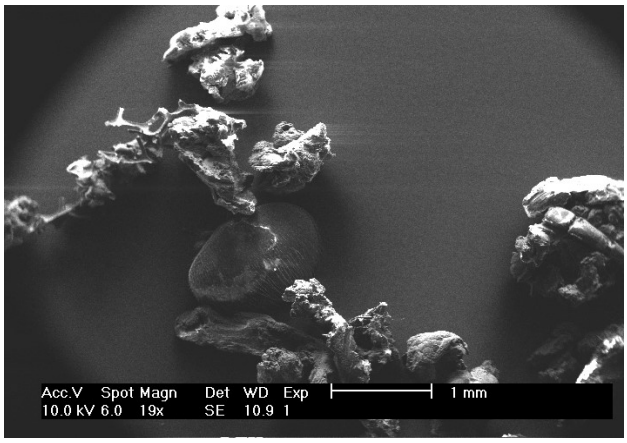
Varroa mites and bee body parts



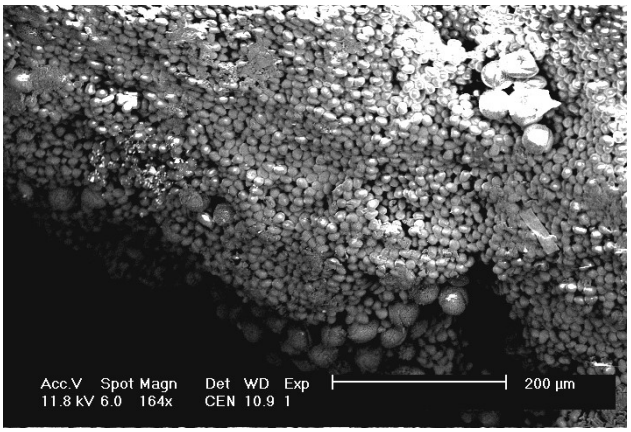
Up-turned varroa mite



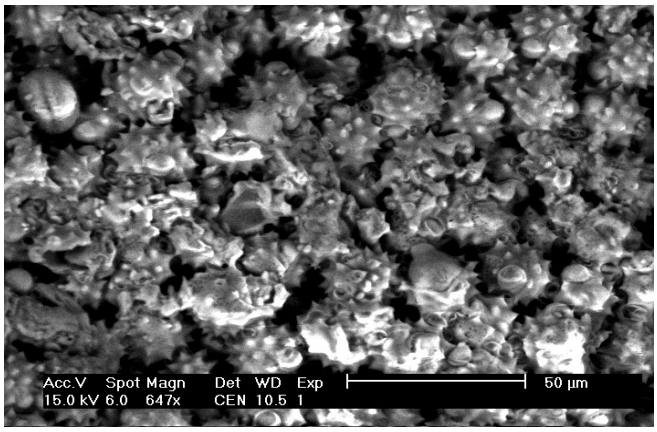
Pollen grains



Sticky bee propolis and Varroa



Pollen grains (various)



Pollen grains

(Possible identity: Rosebay willowherb)

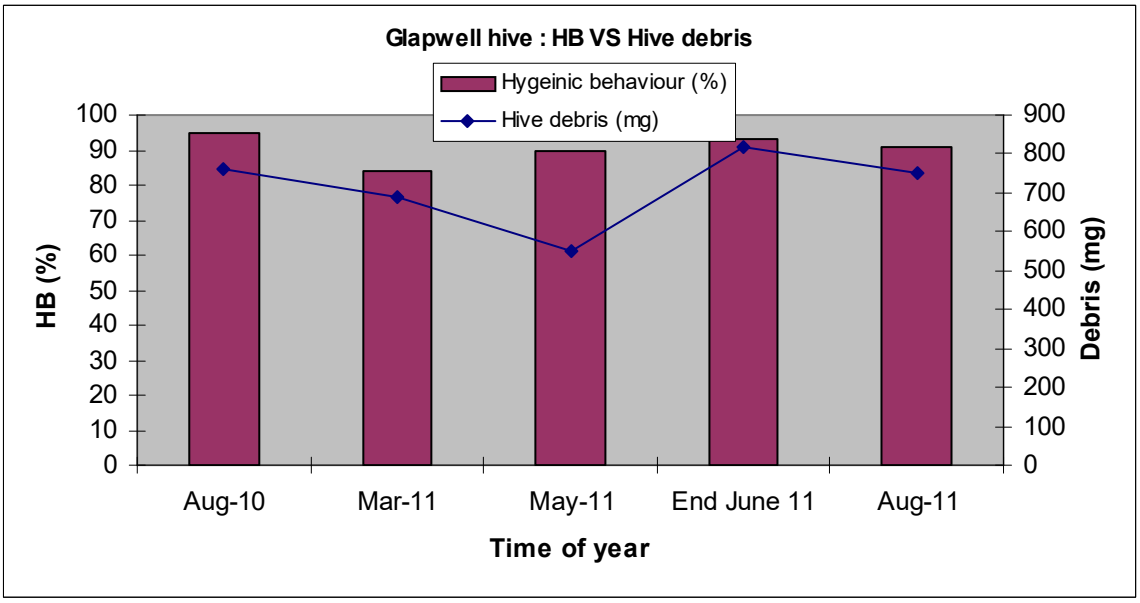
For more Hive debris pictures see appendix.

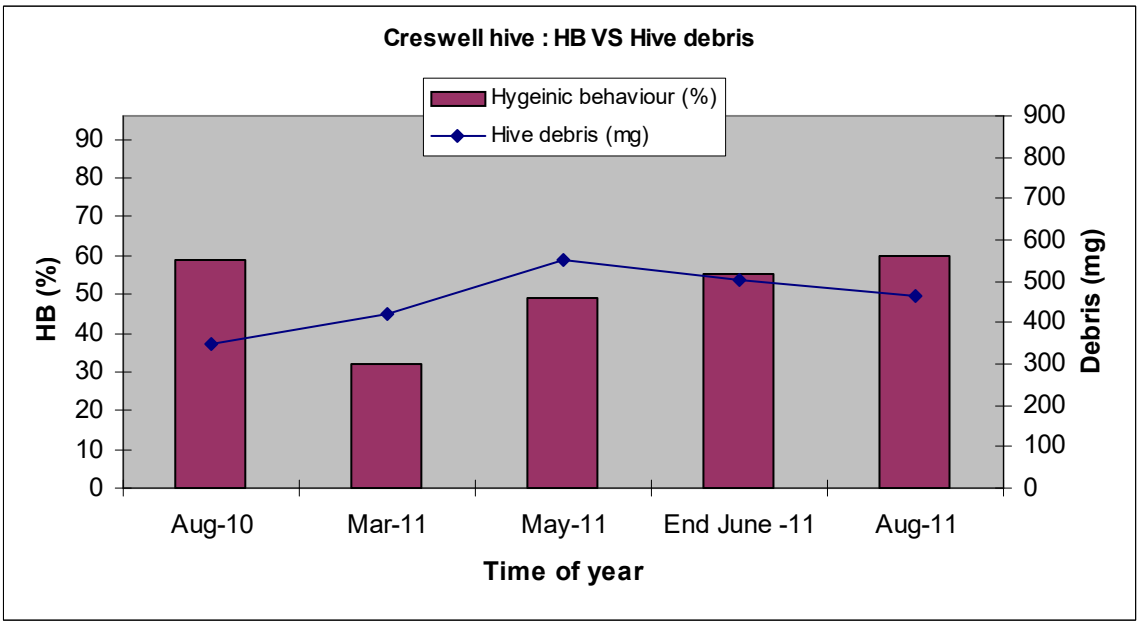
Hive Debris Results:

Time of Year Hive Debris (mg)	Creswell Hive	Glapwell Hive	Sheffield Hive
Aug - 2010	350	762	300
March - 2011	421	690	-
May - 11	550	550	-
End June - 11	502	820	-
Aug - 11	465	750	-

Statistical analysis for Glapwell Hive: Mean = 714.4 median = 750 StDev = 91.99

Statistical analysis for Creswell Hive: Mean = 457.6 median = 465 StDev = 68.53





Discussion.

From the research undertaken in this project, it appears that the three colonies were not hygienic, although the Glapwell colony showed superior hygienic behaviour to the Creswell colony. The year long monitoring proves that levels of HB change throughout the year; unless the colony is truly hygienic, although this was not found to be the case. When there is a nectar flow, all colonies will clean out the brood much faster than when there is no flow. It is a relative test and results among colonies are relative to the time of year they were tested. But colonies that are truly hygienic will clean out >95% in less than 24 hours no matter what time of year whether there is a nectar flow or not, however results from this project have not shown this.

Many senior beekeepers say that the type of breed has no impact on traits such as HB and that it is the change of queen that could improve the characteristics of the colony. If a colony suffers continually from brood diseases or a viral disease that is linked to the laying queen, then it is always best to replace her. A new queen would mate with a variety of drones on her first mating flight and a combination of her eggs and new sperm should change the colony.

Hygienic behaviour is a natural defence mechanism against brood diseases in which dead and diseased cells are removed before the disease reaches the infectious 'sporulating' stage, which has been shown in many studies, as illustrated in the literature review of this project. It also may help to reduce, or hopefully eradicate, varroa mite levels in the colony, although this is debatable. Although bees have been shown to be able to detect varroa, the main gain appears to be the interruption of the mite's reproductive cycle so it is unable to reproduce successfully.

All bees remove dead cells, but some more than others. This must mean that they are all hygienic – but at different levels. Colonies remove dead/ diseased brood more speedily when there is a nectar flow. Most likely, they need space for more brood and honey. It looks from the data that the HB might be related to the growth of the population of the colony – i.e. more bees are available to do the cleaning and remove dead cells. Truly hygienic colonies (95%+) remove dead brood quickly at any time of year and irrespective of colony strength and nectar flow. This has been proven as a genetic trait, and these colonies are the ones that should be selected to raise daughter queens.

Even though they are removing diseased, dead and mite-infested brood, they are performing all other tasks well. Highly hygienic colonies still produce as much, if not more honey compared to less hygienic colonies.

HB vs. Hive Debris

Visually, there is no link between HB and the amount of Hive debris collected at the hives during testing. The amount of hive debris is more likely to be correlated with house cleaning abilities and the amount of house bees in the hive, which is in turn linked to population size.

The microscope work (Light and SEM) on hive debris collected has been useful in this report to visually illustrate hive debris/contents and varroa mites to the reader.
All images have also been forwarded to members at LASI (professor Ratnieks), Chesterfield Beekeepers Association and other researchers, who have also found them useful in their studies and for educational purposes.

Marla Spivak and Stephen Martin (researchers) have been working on HB for over ten years. They have allowed their personal comments to be used in this report and are extremely useful and give a detailed insight into two sets of beliefs with regards to Hygienic Behaviour.

Hygienic Behaviour: An alternative view.

Article from Beekeeping journal of BBIBA. 2000. By Stephen Martin

With respect to the Varroa mite there are two main aspects to hygienic behaviour. The first is the ability of bees to remove mites from their bodies (auto-grooming) or their nestmates (allo-grooming). The second is the detection and removal of the mite and pupae from infested sealed brood cells. Two distinct behaviours, which are not believed to be genetically linked, so selecting colonies, which show high occurrences of both aspects, is by far not easy. If this were the case, in theory, Varroa would no longer be a problem and would probably become extinct. However, Varroa has existed in *Apis cerana* colonies for many thousands of years and is now widespread.

The mite has only could survive in this very hostile environment by becoming highly adapted to avoid detection and being killed by the bees. These include a flattened crab shape, chemical camouflage, stiff ventral hairs, retractable lobed suckers and hooks on their feet.

For any real effect on the mite population the bees must capture and kill the mites, not just force the mite to move to another bee. Once the mite is fixed in the feeding position between the abdominal plates it is impossible for the bee to remove the mites. Selecting strains of *A. mellifera*, which can detect and remove infested sealed brood, has been the goal of several research programmes. The basic assumption is that the fast removal of killed sealed brood is a good trait and can be selected for. This is true in the case of AFB since the dead brood needs to be detected, uncapped and removed before the disease reaches the infectious 'sporulating' stage. The problem now arises that it is not possible to reuse that cell. It is known that when a disease starts to overwhelm an *A. cerana* colony it reacts by absconding from the hive thus allowing predators such as wax moth to destroy the combs and in doing so remove the diseased brood entombed in the cells.

Varroa can transmit various bee viruses and it is these, which can eventually lead to the collapse of the colony. Breaking the viral transmission cycle becomes a key factor in colony survival. Varroa is an extremely effective vector of these viruses once they become established within the colony.

In a highly hygienic colony where 95%+ of killed brood is removed, then any brood killed by a virus will be quickly uncapped and removed periodically, allowing the mite to escape and infect more bees with the virus. Mites can live several months and it appears that the bee viruses do not affect the survivorship of the mite, and a single mite could do a significant amount of damage during its life.

The twist is that in the non-hygienic colony the dead infested bee brood may remain left for several days. The longer it is left the more chance of the entombed mites perishing within the cell along with the already doomed pupae. If this occurs, then the mite-bee viral transmission cycle is broken. So, the speedy removal of killed pupae may not always be a beneficial to the colony, which is counter initiative.

It has long been known but often overlooked that in *A. cerana* infested drone cells which die are not uncapped which results in the death of all mites trapped within the cell. It has also been observed that the adult workers sometimes block the pore in the capping of an infested *A. cerana* drone cell, so killing both the drone and any mites.

“Researchers have done some great work on HB especially on the mechanisms that underpin it and in many cases HB is linked to colony health and well-being. There are many other problems bees face eg AFB. It’s the link with Varroa control that is troubling. Over the past 20 years people have been breeding/selecting bees on traits linked to HB and so far all have failed. The Varroa problem in A. cerana and possibly in Africanised Honeybees may be down to non-HB, since the key behaviour is that brood killed by Varroa are not removed so die within the cells, HB would let them out to do more damage epically since it’s the viruses that are the problem”. (S.J.Martin)

Currently, Marla Spivak is working one-to-one with bee breeders from Minnesota to help them select for hygienic behaviour and is also working one-to-one with the bee breeders in California to help them begin to select for hygienic behaviour.

There are about 20 queen-breeding operations with almost half of the queens sold across the U.S. The goals are to increase the level of hygienic behaviour from diverse stocks and to look at levels of Nosema and Varroa mites to select for colonies with the lowest levels.

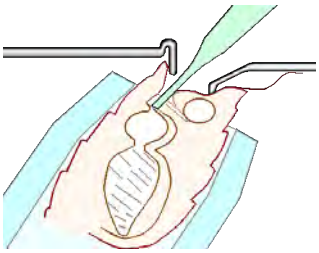
In the U.S., hygienic bees represent a small proportion of the total population because bee breeders sell queens that do not express the hygienic trait. The effects of man assisted selection override natural selection here. As for feral populations, such as AHB in South America, the low proportion suggests that bees use other traits in addition to hygienic behaviour to defend against mites.
The freeze killed brood assay is correlated with the bee’s ability to remove diseased and mite-infested brood. As far as releasing the mite: Even if the female mite escapes during the removal process, her reproductive cycle is interrupted (none of her offspring survive), and if she is continually interrupted, she will run out of sperm, or does not lay male eggs in a subsequent reproductive cycle.

“Hygienic colonies do lower mite loads. The Hygienic line is thriving, and mite loads are lower in colonies from this line. But the colonies still require treatment when mite levels increase, so they are not "resistant". HB is a good start in selection, but there are other traits that will increase mite resistance for example grooming.
Since we know how to select for HB and it helps the bees naturally fight chalkbrood, AFB and mites. Why not try to select for grooming?” (Marla Spivak).

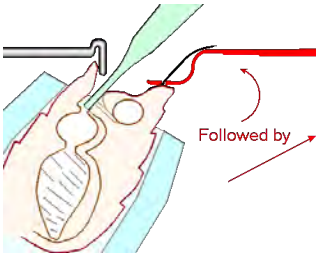
To successfully breed Hygienic bees on a large scale, there are moral issues to consider. Instrumental or Artificial insemination is a modern technique and the queen bank system looks to be a real business for any beekeeper, as it assures producing and commercialisation of a higher number of artificial inseminated and tested queens at the highest profit rate. “Instrumental Insemination is a great tool in bee breeding. It is a research or breeding tool and not for everyone”. (Marla Spivak)

Ron Hoskins has been leading a local bee breeding group for years, selecting for native, and most recently hygeinic bees and using artificial insemination.
The conventional insemination method used is illustrated in the sketches and shows the difficulty and inherent risk of injury to the queen.

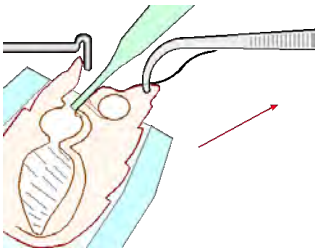
Step 1.



Step 2.



Step 3.



To carry this out, the queen must be aneasthetised with Carbon dioxide.

Using tweezers to apply tension to the sting limits damage to the abdomen.
This method (steps 1, 2 and 3) gives the easiest entry into the queen’s sex organ.

The maximum volume of semen is around 85 µl and is injected by the Harbo device which is a micrometer driven syringe.

Semen can be stored for six to eight weeks, with a gradual reduction in viability. After 9 months, this viability is reduced to 50%. For best results honey bee semen, should be stored at 21°C

(www.dave-cushman.net/bee/instrumental.html)

The cost of undertaking this task is commercially too expensive, “unless you took the trouble to set up your own tooling and learned the technique yourself”. The method as located on the Internet (www.dave-cushman.net/bee/instrumental.html) He suggests that amateurs and unskilled people could carry out this procedure.

Preparation for procedure: Anaesthetising the selected queen: A very difficult and precise process. How would you know the dose of CO2 to give the queen? How many times has this been tried to acquire the correct dosage? And how many queens killed to fulfill this? Does the dose of CO2 affect the abilities of the queen after?

The process of insemination (or AI): The moral implications are huge. Some might consider it as detaining and raping the queen, taking away her biological mating process and freedom to mate naturally. This is a very difficult and complicated procedure to carry out. How many queens killed to fulfill this? It only takes a small jolt of the administrator to damage or even kill the queen. As most know, once a queen is damaged, the colony will reject her. As well as the effect on queens, the drones (sperm donors) must be taken into consideration when they are mutilated. First, the head and thorax of the drone are crushed. This causes the abdominal muscles to contract and turn part of the drone inside out. The rest of the body is then crushed to turn the endophallus fully inside out. The semen from many drones of the same blood line is collected and combined

Finally, it is required to obstuct the freshly inseminated queen from making any additional mating flights that could obscure results and so she is confined and never allowed to undertake her natural mating flight. Normally the queen mates once in her life, in mid-air with 7-17 drones. The AI queen has no choice of this.

The moral issues of this artificial insemination method are huge, too disturbing, some might say. “It implies that humans can come up with better solutions than nature, that humans have the right to manipulate other creatures for our own gain, and it is the use of technology to try to solve a problem brought about by technology in the first place”. (http://www.vegetus.org/honey/art.htm).

Genotyping for Hygienic behaviour (as used by SJ Martin, WOH Hughes and FLW Ratnieks. Multi-level selection for hygienic behaviour in honeybees) and then Selected mating in a semi-controlled environment appears to be a more natural method and still very reproducible and effective. To control matings of queens, select from queen lines, let them naturally mate, but make sure the area is saturated with drones from other hygienic colonies. Some naturally mated colonies are just as hygienic as the instrumentally inseminated breeder queens (The Future of the MN Hygienic Stock of Bees is in Good Hands! Page 18)

Conclusions:

1) The work carried out in this set of Hygiene experiments shows that individual colonies have different tendencies to remove Freeze Killed brood, and thus a different intensity of % Hygienic behaviour.

2) The task also indicates that Hygienic colonies demonstrate very good/excellent hygienic behaviour throughout the course of a year.

3) The work illustrates that non-hygienic colonies are more likely to remove freeze killed brood during spring and summer to make room for new brood and food stores, but may not remove dead cells at other times of the year.

4) It appears that chalkbrood disease, found at the Creswell hive is most certainly linked to low HB. No chalkbrood mummies were detected at the Glapwell colony which showed “resistance” to chalkbrood, removing >70% (average) of FKB cells.

5) It has been shown that the total weight of debris on the hive floor is a poor indicator of the bee’s hygienic behaviour, since an indefinite amount of this debris has always been taken outside the hive by the bees.

Evaluation and Recommendations.

This study has proved that honeybee colonies, which show signs of hygienic behaviour throughout the year, are truly hygienic. It was fortunate that the Glapwell colony performed good HB throughout this study. However, the study hive at Sheffield couldn't be examined after spring due to collapse. It too might also have performed well. It would have been valuable to this study if the Sheffield colony had been healthy to examine, as this one appeared to show a pleasing first result at above 90% HB.

To gather more data, a bigger sample size should be undertaken for investigation. If only a small percentage of colonies are truly hygienic, an initial sample size of no less than 20 – 30 colonies could be tested. The colonies that show positive results for HB should then be monitored over a course of the year.

It would potentially be an advantage for the researcher to carry out this experiment on their personal hives. This would eradicate communication problems with the hive keeper. The researcher will always know when a hive was last treated for Varroa, whether of not the queen has been superseded by another (thus changing the rate of HB) and the overall state and history of the colony.

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Wilson, E.O., 1971. The Insect Societies. Oxford University Press, London.

Spring Watch “Un-sprung” 8th June. Feature of Ron Hoskins and his “Swindon bees” Queen Breeding.

Useful websites

http://www.bibba.com/index.php

http://www.honeybeeworld.com

http://www.britishbee.org.uk/ (accessed June 2010)

http://www.beefarmers.co.uk

http://www.devonbeekeepers.org.uk

http://www.defra.gov.uk

http://www.honeybeeworld.com/misc/hygienic.htm (accessed June 2010)

http://www.honeybeekeeping.co.uk/bee-breeding.php (accessed June 2010)

http://www.ibra.org.uk

http://www.nationalbeeunit.com

http://www.culturaapicola.com.ar/apuntes/revistaselectronicas/apidologie/27-4/07.pdf (accessed June 2010)

Books:

Beekeeping For Dummies by Kim Flottum and Howland Blackiston (Mar 2009)

Self-sufficiency Beekeeping by Joanna Ryde (Apr 2009)

Keeping Bees (Green Guides Series) by Pam Gregory, Claire Waring and Paul Peacock (May 2011)

Teach Yourself Beekeeping (Teach Yourself General) by Adrian Waring (Apr 2006)

Bee Keeping: Inspiration and Practical Advice for Would-be Smallholders by Andrew Davies (May 2007)

Keeping Bees: A Complete Practical Guide by Paul Peacock (Jun 2008)

The Bad Beekeeper's Club: How I Stumbled into the Curious World of Bees - and Became (perhaps) a Better Person by Bill Turnbull (May 2010)

Keeping Bees and Making Honey by Alison Benjamin and Brian McCallum (May 2008)

Guide to Bees and Honey by Ted Hooper (Mar 2008)

Chesterfield Beekeepers meetings, seminars and talks.

Attended:

4th May 2010: Regular meetings and getting to know people.

7th June:

2nd August:

6th September:

18th February 2011: Presentation delivered by S. J. Martin on Varroa.

4th March 2011. AGM Chesterfield Beekeepers.

9th May 2011: Regular meeting and presentation delivered by Robin Bagnall about the management of bee swarming.

6th June: Honey extraction and colony health

1st April 2011: Presentation and demonstration: Ben Jones (National Bee Unit's Head of Laboratory Practice)

May 2011: Basic beekeeping Certificate (Chesterfield Beekeepers)

19th May 2011: Professor Francis Ratnieks talk at Sheffield University.

BBKA Membership commenced May 2011.

Telephone conversations:

Stephen Martin at Sheffield University

14th May 2010

7th and 8th June

6th August

Regular telephone conversations and email communication throughout this project and beyond:

Members (Chesterfield Beekeepers)

Marla Spivak (Department of Entomology University of Minnesota)

Francis Ratnieks (Department of Entomology, University of Essex)

Local beekeeper (allotment bees)

Local beekeeper (Chesterfield Beekeepers)

Sheffield beekeeper (Sheffield Bees)

Local commercial beekeeper (commercial hive)

Appendices

Safety Data Sheets for Liquid Nitrogen.

Scanning Electron Microscope images of Hive Debris.