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Hygienic Behaviour in Honey Bees

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Summary

This thesis focuses on hygienic behaviour in honey bees. In beekeeping, brood diseases incur heavy economical and biological costs and are no longer effectively treated with chemicals. Previous research has shown how hygienic behaviour, a trait expressed by c. 10% of unselected colonies, can be effective in reducing the impact and presence of such diseases. Hygienic behaviour is experimentally measured using the freeze-killed brood (FKB) bioassay and can be increased by selective breeding, generating lines of hygienic colonies.

Chapter 4 demonstrates that the relative rarity of hygienic behaviour in unselected colonies is not because it incurs a cost via the removal of healthy brood.

Chapters 5 – 6 focus on the impact of external factors on hygienic behaviour. Specifically, we demonstrate that the presence of brood, amount of food, and strength of the colony affect hygienic levels (**Chapter 5**). **Chapter 6** shows that hygienic behaviour does not correlate with aggressiveness or agitated behaviour.

When breeding honey bees, it is possible to exploit instrumental insemination to have complete control over the genetic composition of the resulting progeny. This technique is however laborious and requires particular equipment and training. In **chapter 7** we show that it is possible to obtain acceptable levels of hygienic behaviour without artificial insemination.

Chapter 8 illustrates how we obtained the first breeding line of hygienic honey bees through a selective breeding program that saw its first milestone in autumn 2013 when we detected high levels of hygienic behaviour. The results obtained represent the foundation for future research projects.

Chapter 9 presents a valid, minimal methodology to keep virgin queens. We tested a variety of methods and factors to determine the best, most cost-effective way to maintain queens for the week prior to their introduction into a queenless hive.

The results obtained provide some insights on both basic and applied aspects of honey bee breeding for hygienic behaviour and represent the foundation of what will be an ongoing selection programme towards a disease-resistant honey bee.

Publications arising from this thesis

Bigio, G., Al Toufailia, H., Ratnieks, F. W. L. (2014). Honey bee hygienic behaviour does not incur a cost via removal of healthy brood. *Journal of Evolutionary Biology*, **27**(1):226-230. (**Chapter 4**)

Bigio, G., Schürch, R., Ratnieks, F. W. L. (2013). Hygienic behavior in honey bees (Hymenoptera: Apidae): Effects of brood, food and time of the year. *Journal of Economic Entomology*, 106(6):2280-2285. (**Chapter 5**)

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Bigio, G., Scandian, L., Al Toufailia, H., Ratnieks, F. W. L. (To be submitted). Developing a line of locally-adapted hygienic honey bees at the Laboratory of Apiculture and Social Insects. (**Chapter 8**)

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Part 1 – General Introduction and Methods

Chapter 1: General introduction

1. Historical perspective on beekeeping and basic honey bee biology

Honey bees (*Apis mellifera*) are among the most studied insects due to their importance both from an ecological and economic point of view for honey production and as crop pollinators. From the Palaeolithic period humans interacted with honey bees, first harvesting honey from wild nests, then keeping bees in various types of man-made cavities, traditional hives and finally developing techniques that allowed rational beekeeping (Crane, 1999). In modern rational beekeeping the colonies are kept in hives with movable combs that can be removed for the collection of the honey while the nest containing brood and the bees are left intact. In parallel with the advancements regarding the beekeeping techniques and materials, honey bees were selectively bred in order to enhance some of their most desired characteristics.

Figure 1.1: Honey bee workers and a drone on a comb.

It has been calculated that c. 35% of all human food (Klein et al., 2007) is linked with insect-pollinated crops, and c. 9% of the total value of current agriculture relies on insects for pollination (Gallai et al., 2009). In 2007 the global production of honey was estimated at c. 1 million tonnes, and the number of managed honey bee colonies worldwide was over 72 million (FAO 2009). During the last century the UK has lost c. 75% of its honey bee colonies, since 1985 colonies in mainland Europe declined c. 25% (Potts et al., 2010) and in the United States the number of managed honey bee colonies

has decreased by 61% since 1947 (vanEngelsdorp and Meixner, 2010). Additionally, the number of managed hives has declined in Europe and North America at an average of 1.79% per year (Aizen et al., 2008).

Considering honey bees as a whole unit, the colony at its peak during the summer, consists of two female castes and one male caste. A single queen bee is the mother of up to 60.000 female worker bees and also of 1000 males, called drones. The drones are evicted from the hive at the end of the reproductive season so that they are absent in normal colonies during winter. In addition to the adult bees, the colony also includes the brood, all the pre-imaginal stages of bees: eggs, larvae and pupae. Normally all the eggs are laid by the queen, and she can lay up to 2000 eggs a day in three different types of wax cell. In the smallest cells (5mm diameter) she lays fertilised eggs, which in 21 days become female worker bees. In larger cells (7mm diameter) unfertilized eggs are laid which in 24 days become male drone bees. Drone eggs are not fertilized and the males in the honey bee colony are generated by parthenogenesis. A very special cell that hangs vertically downwards is used to produce new queens 16 days after a fertilized egg is laid, allowing the super-organism hive to reproduce. Honey bee colonies reproduce by swarming, in which the colony splits and each of the resulting colonies is led by a queen. This is achieved when a new queen is produced and the old one leaves the original nest (natural or artificial, like a hive) with c.60% of the worker bees to settle in a sheltered site that is identified by scout bees. The new queen will then mate, lay eggs and start developing a new colony.

2. Challenges facing honey bees

The decline of honey bees and other pollinators has been linked with many reasons. Some of them have debatable importance like mobile phones (Sainudeen Sahib, 2011), while some others have a real impact on honey bee health. Together with pesticides (Gill et al., 2012; Whitehorn et al., 2012) and the lack of food availability due to agricultural intensification and reduction of wild flowers (Couvillon et al., 2014; Westphal et al., 2006), one of the major drivers of this decline is the spread and impact of pests and pathogens.

Brood diseases such as American and European foulbrood (caused respectively by the bacteria *Paenibacillus larvae* and *Melissococcus plutonius*), chalkbrood (caused by

the fungus *Ascosphaera apis*) and the parasitic mite *Varroa destructor* (Le Conte et al., 2010; Guzmán-Novoa et al., 2010; Rosenkranz et al., 2010) represent one of the main threats to managed honey bee colonies. Varroa mites at nymphal stages will feed on the haemolymph of bee pupae, and they are also a very efficient vector for several viruses such as acute bee paralysis virus (ABPV) and deformed wing virus (DWV) (Boecking and Genersch, 2008; Kevan et al., 2006). Varroa eggs are laid by a female mite in a honey bee brood cell just before it is capped, and they will emerge with the adult bee. Their ability of moving from a bee to another allows them to spread from one hive to another and also between apiaries.

Varroa mites were introduced in Europe in 1950s after being recorded on colonies of *Apis cerana* in Eastern Russia (Crane, 1978), and by the 1990s were reported in the south coast of England (BBKA 1992). Varroa populations were controlled very effectively using acaricides such as Amitraz, Coumaphos and Flavulinate but their efficacy faded after mites developed resistance (Floris et al., 2001; Lodesani et al., 2009; Milani, 1995). This has underlined the need for alternative ways to contain Varroa infestations, specifically by breeding bees that show resistance against the mite.

3. Honey bee selective breeding

Both beekeeping and applied honey bee research will benefit from stocks of bees that display particular traits and desirable qualities. Many traits of interest in beekeeping such as honey production (Bienefeld, 1986), wax production, defensive (Bienefeld and Pirchner, 1990) and hygienic behaviour (Boecking et al., 2000; Harbo and Harris, 1999; Lapidge et al., 2002) are heritable and arise from the behaviour of the workers. Controlled breeding and selection will allow for a successful improvement. Bad characteristics can be bred out, and positive traits can be improved, by culling a bad queen and replacing it with a selected queen – possibly mated in a controlled way. The worker bees would be replaced by a new cohort originating from eggs laid by the new queen.

The traits targeted by a breeding program must be detectable and must be possible to rank the individuals based on their different level of expression of a determined trait. Selecting the colonies that would represent the beginning of a breeding line can be complicated as honey bee colonies are influenced by environmental conditions, as most

hives are normally kept under natural, uncontrolled conditions and their behaviours are a result of the impact of available resources and external stresses (Calderone and Page, 1992; Momot and Rothenbuhler, 1971; Pankiw and Page, 2001; Pérez-Sato et al., 2009; Rodrigues et al., 1996; Southwick and Moritz, 1987; Spivak and Reuter, 1998a; Uribe-Rubio et al., 2008). Moreover, honey bee queens are naturally mated with up to 20 drones that travel many kilometres to reach congregation areas where they gather with other males originating from different hives (Tarpy et al., 2004). As a consequence, the workers present in the colony will be half-sisters and the genetic makeup of the colony will be a combination of different patrilines, representing the genotypes of both the queen and the males she mated with.

This aspect of the biology of honey bees makes breeding more challenging than in non-eusocial animals (Ratnieks, 1998). Daughter and mother colonies are genetically less similar than offspring and parents belonging to non-eusocial species. A new colony is headed by a mated queen that is also the full sister of the worker bees in these mother colonies. The workers in the daughter colony have a probability of 0.375 of sharing nuclear genes identical by descent with the workers in the mother colony. This is less than the parent–offspring probability of 0.5 in non-eusocial animals. Furthermore in the honey bees genetic similarity is reduced because the queen in the mother colony is mated to multiple drones (polyandry) and this reduces the probability of sharing alleles between mother and daughter colonies from 0.375 down to 0.15.

A further complication of social life is that many desirable worker phenotypes are not readily detectable in individual queens or workers, so that a whole colony has to be reared before the colony-level trait can be detected. Techniques such as Marker-Assisted Selection (MAS - (Arus and Moreno-González, 1993; Dentine et al., 1999) and intra-colony selection (Pérez-Sato et al., 2009) rely on molecular biology analysis and could help honey bee breeders identify the individuals that possess genetic traits of interest, without the need to wait for the colony to develop fully and to perform assays to detect the actual behaviour.

4 .Hygienic behaviour in honey bees

Known for many years (Park, 1936), hygienic behaviour consists of the detection of dead or diseased brood, the uncapping of the cell and the removal of the content. First

studied as mechanism of resistance to American foulbrood (Park, 1937; Rothenbuhler, 1958), it was then confirmed to be effective also against other brood diseases such chalkbrood (Gilliam et al., 1983; Spivak and Reuter, 1998b) and the parasitic mite *Varroa destructor* (Rinderer et al., 2010; Schöning et al., 2012; Spivak, 1996; Spivak and Reuter, 1998b). Because honey bees reuse brood cells, diseased brood must be removed by the hive, and by doing so with Varroa-infested pupae, it is possible to interrupt the reproductive cycle of the mite (Rath and Drescher, 1990). Recent results show how colonies with high levels of hygienic behaviour have a lower build-up of Varroa population within the hives, and as a consequence, lower levels of deformed wing virus symptoms (Al Toufailia et al. *in prep*).

Hygienic behaviour evolved as a heritable genetic trait of workers that confers social immunity from brood diseases (Wilson-Rich et al., 2009). Initially it was thought to be controlled by two genes (Rothenbuhler, 1964a), one that would control the uncapping phase, and the other for the removal. This hypothesis was recently revised to a multilocus model, where at least six quantitative-trait loci (QTLs) are responsible for the modulation of the propensity of worker bees to engage in hygienic behaviour (Lapidge et al., 2002; Oxley et al., 2010).

Despite being an apparently valuable trait, only a relatively small portion (c. 10%) of colonies in unselected populations normally show high levels of hygiene (Bigio et al., 2013; Pérez-Sato et al., 2009; Waite et al., 2003) and its expression can be increased using selective breeding since hygienic behaviour has a high heritability (Boecking et al., 2000; Harbo and Harris, 1999; Lapidge et al., 2002). It is therefore possible to obtain honey bee colonies with greater disease resistance by selecting the colonies from which to breed among the ones that show higher levels of hygienic behaviour.

Figure 1.2: A visual example of hygienic behaviour. A worker bee is removing a larvae from its cell.

Chapter 2: General materials and methods

1. General beekeeping practices

Figure 2.1: A swarm of bees. A lot of our efforts were dedicated to swarm prevention.

Although often not cited in the scientific literature, basic beekeeping procedures were key throughout my PhD. While experienced beekeepers can assess the status of a colony without inspecting it, a vital skill in winter, most beekeepers, me included, need to open a hive and inspect all of the frames. Modern hives normally hold up to 10 frames in the nest box, and the cells that compose the wax comb are used by the bees to store pollen and nectar, and by the queen to lay eggs. When opening a hive we gently lift the lid and calm the bees using a few puffs of smoke. Next, using a hive tool we proceed to inspect the frames gently lifting them from the hive box. Normally we would look for the presence of the queen, the amount of brood and stores and eventually for the symptoms of the main diseases.

Each winter we prepared our colonies by reducing the number of frames in each hive so that the workers would easily form a cluster around the queen and by doing so, maintain the optimal core temperature (Fahrenholz et al., 1989). Approaching spring the colonies develop, growing in number of worker bees, brood quantities and stores of honey, nectar and pollen. We inspected our colonies, adding frames to the hive so that the bees could expand their nest and brood area. According to the needs of the colonies, we placed additional boxes (supers) containing frames on top of the brood box. A queen excluder grid placed between the boxes was included to prevent the queen from laying eggs in the frames that would only be used to store honey. At the end of the harvest, we would collect the supers and eventually extract the honey, or feed it back to the colonies during winter.

When a colony becomes too crowded, worker bees start to prepare queen cups, in

which a fertilized egg will then develop into a new queen. At this time beekeepers need to intervene to prevent swarming and avoid losing both the old queen and the majority of the workers, which translates into a lower production of honey. Honey production is not the current goal of our breeding program, but we still monitored our colonies to prevent swarming, so we would retain the original queen, and thus the original genetic composition of the colony.

If during our inspections we found queen cups or queen cells we would locate the original queen, move her into a new hive containing a couple of frames of brood, a frame containing stores of pollen and nectar, a couple of empty frames and c. 3000 worker bees. We would move the newly composed colony to a different location, at least 5 km away, so that the worker bees would not go back to their original hive where the queenless colony would carry on, rear a new queen and continue to develop further.

Other operations not directly linked with experiments included checking colonies for food during winter, and feeding them accordingly using either candy, a mixture of honey and icing sugar, sugar syrup, or frames containing honey.

2. Queen rearing

Honey bees have the ability to rear queens voluntarily or in an emergency situation, either in preparation for a swarming event or to replace (supersede) a queen that died or is considered unfit by the colony. For example old queens lay fewer eggs and eventually start laying unfertilized drone eggs as they approach the end of the sperm stored in their spermatheca. Old queens also procude less pheromone and a lack of pheromone is quickly noticed by worker bees. They react by rearing a new queen, starting from a fertilized egg that is placed in a special cell called queen cup. Queen cups are larger than the other cells and protrude from the comb; when larvae hatch from such cells they are fed exclusively on royal jelly. As the larva grows the workers build an acorn-shaped cell known as a queen cell, from which a virgin queen will emerge c. 16 days after the egg was laid.

To artificially produce queens, we used an adaptation of the grafting method (Laidlaw and Page, 1997) as follows. We transferred larvae 1 or 2 days after hatching (ca. 5 days after egg laying) into plastic queen cups mounted on bars using a Swiss metal grafting tool. We then mounted the bars on a dummy frame that was placed

between two frames containing brood in a hive. This hive was made queenless ten days in advance, destroying any rogue queen cells that might have been produced and inspecting the frames for presence of any remaining eggs/young larvae that might lead to other queen cells being reared. This queen rearing hive was strengthened by adding frames of emerging worker brood, frames containing nectar and pollen, and was fed sugar syrup (2M) ad libitum until the queen cells were sealed.

Ten days after grafting, we collected sealed queen cells by carefully detaching the plastic cups from the bars. Timing is very important during this operation, as the first emerging queen normally destroys the other queen cells, and engages in lethal fights with other virgin queens. Depending on the requirement for our experiments, queen cells were either introduced in "Apideas" mating nucs containing worker bees and food, or left to emerge in individually labelled plastic vials kept in an incubator at 34° C. Subsequently the queens were kept in cages (Bigio et al., 2012) until they were introduced into hives using various methods (Graham, 1992; Pérez-Sato et al., 2007), or instrumentally inseminated. Virgin queens that emerged in the apideas mating hives would be accepted by the colony, leave for nuptial flights where they would mate naturally with drones present in the area. After c. 2 weeks we would inspect the nucs looking for the presence of eggs and worker brood, a sign that the queen has successfully mated, and she is ready to be introduced into a full-sized colony.

Figure 2.2: A comb of an Apidea mating nuc, showing freshly-laid eggs, sign of a mated queen

3. Freeze Killed Brood (FKB) Assay

There are two standard methodologies to quantify and experimentally detect hygienic behaviour, by monitoring a colony's ability in removing capped worker brood that has either been pin- (Newton and Ostasiewski, 1986) or freeze-killed (Momot and Rothenbuhler, 1971; Spivak and Reuter, 1998b). When using the pin-killed brood assay, a rhomboid of c. 100 capped worker brood cells is identified on one side of a frame, 50 cells are pierced with an entomological pin and the remaining 50 cells will act as controls. After marking the top bar, the frame is placed back in the original hive where it will remain for c.12 hours then it will be retrieved and the percentage of cleared cells will be calculated.

The other methodology is the freeze killed brood (FKB) assay, where a portion of the comb is cut and frozen or by using liquid nitrogen. Both assays quantify the colony's ability to detect, uncap and remove brood that has been killed by freezing. In the cut-comb method, after identifying a comb containing capped brood, a portion containing approximately 100 cells on each side was cut away from the frame. The comb was placed in a freezer at -20° C for 24 h, and then returned into the original position on the frame in the hive. The frame would be returned to its original colony to test the removal. Hygienic behaviour was quantified by calculating the percentage of cells that were uncapped and cleared by the colony in 24 hours (Spivak and Downey, 1998).

Figure 2.3: A frame being tested using the FKB assay.

For our experiments we adopted the liquid nitrogen FKB assay (Spivak and Reuter, 1998b) since it is perfectly suited to be carried out in the field, and it requires one less visit to the apiary than the cut-comb method. This technique is more convenient because it reduces the number of visits to the apiary site, and hive inspections. As for the previous assays, we identified a suitable frame containing capped brood, we then pressed two metal cylinders (6.5 cm diameter, 8 cm

height) into the comb until they reached the midrib, isolating c.160 capped cells. We then carefully poured approximately 300 mL of liquid nitrogen per cylinder to kill the larvae contained in the selected cells. After c. 10 minutes the nitrogen evaporated, the comb thawed and we removed the cylinders without damaging the comb. We took a picture of the treated frame before returning it to the hive; the same frame was retrieved after 48 hours and after identifying the same treated area, we took another picture. Later using the two pictures we counted the number of intact capped cells that were treated with liquid nitrogen and the cells that were still capped or contained larvae after 48 hours, and from them the percentage of removal. For our purposes colonies that removed more than 95% of the treated brood cells over 48 hours were considered hygienic and of interest for our breeding program.

4. Instrumental insemination

Honey bee virgin queens never mate in the hive. Natural mating happens when the queen leaves the colony, c. 7 days after emergence for up to five nuptial flights (Roberts, 1944). Males from different colonies gather in drone congregating areas and they are attracted to queen pheromones. Honey bee queens mate in free flight and any individual queen mates with 10-20 drones. As a result any naturally mated colony will be composed of groups of worker bees that share the same father, also known as patrilines.

In order to have complete control over mating and of the genetic identity of the colony, and to perform specific crosses, bee breeders have two strategies, either by isolating virgins and drones, or relying on instrumental insemination. Islands are an example of locations suited for controlled mating; however they must present particular features (Büchler et al., 2013; Neumann et al., 1999). The method that provides complete control and reduces mating risks, if performed correctly, is artificial insemination.

During the final year of my PhD the laboratory gained an instrumental insemination (II) facility, structured around a Schley device and a Harbo syringe (modified by Peter Schley). Completing the instrumentation is a binocular microscope with its own light source, a carbon dioxide bottle with regulator to anaesthetize the bees and a flight cage for the drones. Additionally we had bottles with ethanol, distilled water and saline

solution (Lens Plus Ocupure, Abbott), plus paper towels, pipettes and a collection of forceps and watchmaker's tweezers in various sizes and shapes.

II being a technique that requires at least ten years of practice before reaching a good level of proficiency (Dalibor Titera pers. com.), for our experiments we relied on the skills and advice of an expert (Mr. Redmond Williams) who very kindly agreed to help us, traveling all the way from Ireland. To successfully perform instrumental insemination, we carefully reared both drones and virgin queens from selected colonies. For the queens, we proceeded as described in the section above, whereas for the drones we followed this protocol. A month before starting to rear queens, we placed frames of empty drone cells into the brood chamber of each selected colony, to account for the difference in developmental time between drones and queens, respectively emerging 24 and 16 days from the laying of the egg. When these frames contained brood they were moved above the queen excluder in each colony. In this way, following emergence, the adult drones (which are too large to pass through the queen excluder) were confined to the upper part of the hive. Periodically, the hive was inspected and these drones were paint-marked on the notum with a colony-specific colour code and placed below the queen excluder so that they could fly at will and mature normally.

On the day we wanted to perform II, we harvested marked drones from the hives and we temporarily kept them in a flight box to minimize the stress. Then in the laboratory we collected sperm (Cobey et al., 2013), by holding them by the wings and crushing their head first, and then thorax. This triggers partial eversion of the endophallus, and by applying pressure to the abdomen the eversion is completed, exposing the semen. Each drone yields 1 µl of semen and we collected it using a microsyringe, looking through the microscope.

Once we collected enough sperm, normally from c.15 drones, we then inseminated the queens. Virgin queens were treated with carbon dioxide twice for c. 3 min, once before the procedure while still in the cages, and a second treatment during the actual insemination. We placed the queen in the plastic holding tube and by using the sting and ventral hooks we exposed the vaginal aperture. Then with the microsyringe tip we delivered the semen into the median oviduct, carefully bypassing the valvefold with a "zig-zag" motion.

While the queen was still unconscious, we applied a paint dot on the thorax and clipped the tip of the left wing, before returning her to her cage where she would be assisted by the attendant bees until we introduced her into a nucleus hive.

Figure 2.4: Collecting semen from the everted endophallus of a drone

Chapter 3: How the thesis evolved

Honey bees have always been a favourite subject for me. I started beekeeping at home in Italy 15 years ago, inheriting the passion from my grandfather Giovanni. I went on studying agricultural and plant biotechnology at the University of Turin (Italy) and during my M.Sc. course I took a module on beekeeping and apiculture, which allowed me to study the subject from a scientific point of view. After working on plant genetics I decided that I wanted to focus on studying honey bees and bee breeding. I contacted Prof. Ratnieks and he offered me a PhD position that I found perfectly suited to me already from the description. I joined the Laboratory of Apiculture and Social Insects (LASI) in April 2010, after suffering the loss of my partner due to a car crash.

The first months were challenging but I was extremely lucky as the field season started right away and I could keep myself busy and entertained with beekeeping and the first experiment, testing the survival of virgin queens in cages (chapter 6). During this project I learnt how to be proficient at obtaining queens and how to manage honey bee colonies more efficiently compared to what I was used to while being a hobbyist beekeeper. This first project also allowed me to learn how to analyse the collected data that we collected and how to produce a manuscript, thanks to the guidance of Prof. Ratnieks and the advice from my colleagues, especially Drs. Grüter and Couvillon.

The following year I started working on hygienic behaviour, trying to assess the impact of external factors on the ability of honey bees to remove freeze-killed brood which resulted in the project described in chapter 2. Later in the year a local beekeeper, Luciano Scandian, joined us after expressing his interest in the work that was carried out at LASI, bringing the number of colonies from 60 to c.150 over 8 apiaries in total. This lead to new options for our research, and in August 2011 we started a long-term hygienic survey of a subset of Mr. Scandian's hives that we carried out until the following year. Thanks to this project, we identified one colony from which we started developing our hygienic line, grafting larvae and obtaining honey bee queens. The results obtained in the main breeding programme are described in chapter 5 and kept us very motivated and committed to carry on and reach our goal.

Towards the latter stages of the breeding program we set up an instrumental insemination facility in the laboratory that allowed us to perform controlled matings. With the help of expert technicians, Mr. Redmond Williams from Ireland and Mr.

Dalibor Titera from the Czech Republic, we learned the basics of this advanced technique and we managed to obtain instrumentally inseminated queens. Some of those queens were used in an experiment compared against naturally mated queens as described in chapter 4.

During my doctoral studies I took part in several outreach events such as talks aimed at sharing our results with the public and workshops to demonstrate and teach techniques to beekeepers. Those events were a great way to discuss ideas with whom I thought were the final users of my research and often from their input we were able to devise projects. Most beekeepers that approached us were hobbyists and expressed their concern about obtaining hygienic bees expressing also undesired traits such as defensive behaviour. Often beekeepers in the United Kingdom keep their bees in allotments, or in gardens that serve recreational purposes, hence bees too inclined to sting are not desirable. In chapters 4 and 6 we to addressed some of those concerns, respectively looking at one of the potential trade-offs limiting the natural occurrence of hygienic behaviour, and correlating the presence of defensive behaviour with hygienic behaviour.

As the end of my doctoral studies approached I was approached by Aspromiele, an Italian beekeepers' association, that offered me a position as a coordinator for their bee breeding program. I plan to start working with them by the beginning of May, continuing the research not far from my home town.

Figure 3.1: The author during a coffee break.

Part 2 – Research Chapters

Chapter 4: Honey bee hygienic behaviour does not incur a cost via removal of healthy brood

Gianluigi Bigio, Hasan Al Toufailia, Francis L.W. Ratnieks

For this chapter I contributed to the design of the project, to the acquisition, analysis and interpretation of the data and to the writing of the manuscript. Hasan Al Toufailia assisted with data collection. Francis Ratnieks assisted with the design of the project and manuscript writing.

Abstract

In the honey bee, hygienic behaviour, the removal of dead or diseased brood from capped cells by workers, is a heritable trait that confers colony-level resistance against brood diseases. This behaviour is quite rare. Only c. 10% of unselected colonies shows high levels of hygiene. Previous studies suggested that hygiene might be rare because it also results in the removal of healthy brood, thereby imposing an ongoing cost even when brood diseases are absent. We tested this hypothesis by quantifying hygienic behaviour in 10 colonies using a standard technique, the freeze-killed brood (FKB) bioassay. At the same time we also quantified the removal of untreated brood. The study colonies showed a wide range in hygienic behaviour, removing 19.7-100% of the FKB. The removal of untreated brood ranged from 2% to 44.4%. However, there was no correlation between the two removal rates for any of the 4 age groups of untreated brood studied (eggs, young larvae, older larvae from uncapped cells and larvae/pupae from capped cells). These results do not support the cost-to-healthy-brood hypothesis for the rarity of hygienic behaviour.

Introduction

Many adaptations involve a trade-off between costs and benefits (Fry, 2003; Kawecki et al., 2012). Examples occur in all types of organisms from bacteria to plants to animals (Feng et al., 2009; Lochmiller et al., 1993; Rose, 2001; Velicer and Lenski, 1999) and in a variety of contexts. For example, in defence the vertebrate immune system uses resources that could be allocated for other purposes (Sheldon and Verhulst, 1996). Eusocial insects also have individual-level defences against pathogens and pests (Schmid-Hempel, 2005; Wilson-Rich et al., 2009) but there are also group-level defences (Evans et al., 2006; Wilson-Rich et al., 2009) subject to similar trade-offs. Some ants have big-headed major workers that aid in colony defence. With their larger body size and low work rate in other tasks, these individuals are more costly to rear and maintain than minor workers (Calabi and Traniello, 1989; Kaspari and Byrne, 1995; Wilson, 1968). Passera et al. (1996) reported that in the ant *Pheidole pallidula*, majors are only reared when a colony is subject to the threat of intraspecific competition.

Hygienic behaviour in the honey bee, *Apis mellifera*, which has been known for many years (Park, 1936), is a heritable genetic trait of workers that confers social immunity (Wilson-Rich et al., 2009) against brood diseases such as American foulbrood (Rothenbuhler, 1964a; Spivak and Reuter, 1998a, 2001), chalkbrood (Gilliam et al., 1983; Spivak and Reuter, 1998b) and the parasitic mite *Varroa destructor* (Rinderer et al., 2010; Schöning et al., 2012; Spivak, 1996; Spivak and Reuter, 1998b). Highly hygienic colonies are able to detect, uncap and remove dead or diseased brood (Rothenbuhler, 1964a). Despite being an apparently valuable trait, only a relatively small portion (c.10%) of colonies in unselected populations normally show high levels of hygiene (Pérez-Sato et al., 2009; Waite et al., 2003).

One possible reason for the rarity of hygienic behaviour is that it results in colony-level costs. Seeley (1985) noted that in Rothenbuhler's (1964) classic behavioural genetics study of hygienic behaviour in the honey bee, hygienic colonies with brood cells experimentally-infected with *Paenibacillus larvae*, the causative agent of American foulbrood, also removed a considerable proportion of healthy brood (c.10%). Given that a honey bee colony will normally have more healthy than diseased brood, and that brood diseases may not always be prevalent, such a cost could outweigh the benefit and lead to natural selection against hygienic behaviour. However, Seeley's

(1985) interpretation of Rothenbuhler's data must be taken with caution. In particular, Rothenbuhler (1964) recorded the removal of healthy brood from cells adjacent to the cells that had been experimentally contaminated with *P. larvae* spores. It is possible that brood from these cells were more likely to be removed due to their proximity.

This experiment tested the cost-to-healthy-brood hypothesis. We quantified levels of hygienic behaviour (removal of dead brood from capped cells using the freeze-killed brood bioassay (Spivak and Reuter, 1998b)) and the removal of untreated brood in 10 study colonies. The results showed no correlation between the removal of dead brood and untreated brood at four age stages, and so do not support the hypothesis.

Materials and Methods

1. Study colonies

We studied 10 colonies located in a single apiary on the University campus. At the time of the experiment, colonies were housed in hives comprising two medium-depth Langstroth hive boxes each with 10 Pierco plastic frames, separated by a queen excluder. Each colony had a laying queen, worker bees, stores of honey and pollen, and four frames of brood at all stages. Colonies were healthy. None had any symptoms of the brood diseases sacbrood, American foulbrood or European foulbrood, which have never seen in the lab's colonies. A small amount of chalk brood is present in the laboratory's colonies, but levels were low in the study colonies (see Figure 4.1).

2. Quantifying hygienic behaviour via the Freeze Killed Brood (FKB) assay

The level of hygienic behaviour in each colony was determined used the freeze-killed brood bioassay (Spivak and Reuter, 1998b), which was carried out three times at weekly intervals in July and August 2012. In each colony, two patches of capped brood, located on the same area on opposite sides of the frame, were located on a single frame that contained brood of all stages (eggs, small and large uncapped larvae, capped cells). A metal cylinder (6.5 cm diameter × 8 cm height) was pressed into the comb until it

reached the mid-rib. Approximately 300 mL of liquid nitrogen was poured into the cylinder to kill the circle of brood inside the cylinder. After 5-10 minutes, the nitrogen had evaporated and the cylinder was removed, and the other side was then treated. Photographs of each patch and the whole frame were made before returning the frame to the hive. After 48 hours the frame was removed from the hive and more photographs were taken. From the photos we determined the proportion of capped cells from which the freeze-killed brood had been removed.

3. Quantifying removal of untreated brood

When photographing both sides of the frame during the FKB assay, additional areas containing untreated brood of different stages and with no evident signs of disease were also photographed and monitored over the same 48 hour period. Up to 100 cells containing each of eggs, young and old larvae in uncapped cells, and capped brood (cells containing fully-fed larvae and pupae) were monitored. Those cells were located at least 2-3 cm away from the patches of capped brood that were treated with the FKB assay, so that there was no effect of the liquid nitrogen (Figure 4.1). Brood stages were visually classified from the photos made at the start of a trial. Identification of the same cells after 48 hours was facilitated by overlaying an empty frame with a wire grid (Figure 4.1a).

4. Statistical analysis

Hygienic behaviour was quantified as the percentage of cells with capped brood that had been cleaned out (cell uncapped and dead brood removed) after 48 hours. We also recorded the developmental stage of the four types of untreated brood (eggs, young larvae, old larvae, capped cells), which were analysed as separate variables. Using R 3.0.0 (R Development Core Team, 2012), we used Spearman correlation coefficient to test the relationship between the median values of hygienic behaviour and the removal of untreated brood at various stages. Subsequently, we performed a power analysis (Champely, 2009) using a power factor of 0.88 in order to correct for the lower efficacy of Spearman compared to Pearson correlation (Siegel and Castellan, 1988).

Results

1. Hygienic behaviour – colony differences and general results

Across all 30 assays (10 colonies × 3 trials), freeze-killed brood (FKB) removal ranged from 19.7 to 100% (mean 64.3%, SD 26%). Per colony, from the range was 22.2 ± 2.6 to 100 ± 0%. Both the study colonies and the individual trials, therefore, spanned a wide range of hygienic behaviour, as required to investigate correlation with the removal of untreated brood.

2. Removal of untreated brood

We monitored 8588 cells containing brood at various stages. Overall, 7559 of these cells still contained brood after 48hrs while 1029 (11.9%) were empty, showing that considerable removal of untreated brood did occur. Of 1071 cells containing eggs, 2136 and 2381 containing young or old larvae in uncapped cells, respectively, and 3000 capped cells, 230 (21.5%), 355 (16.6%), 202 (8.5%), 242 (8.1%) were empty after 48 hrs. Across all stages of untreated brood combined, the removal ranged from 2% (colony 23, trial 1) to 48.4% (colony 54, trial 3).

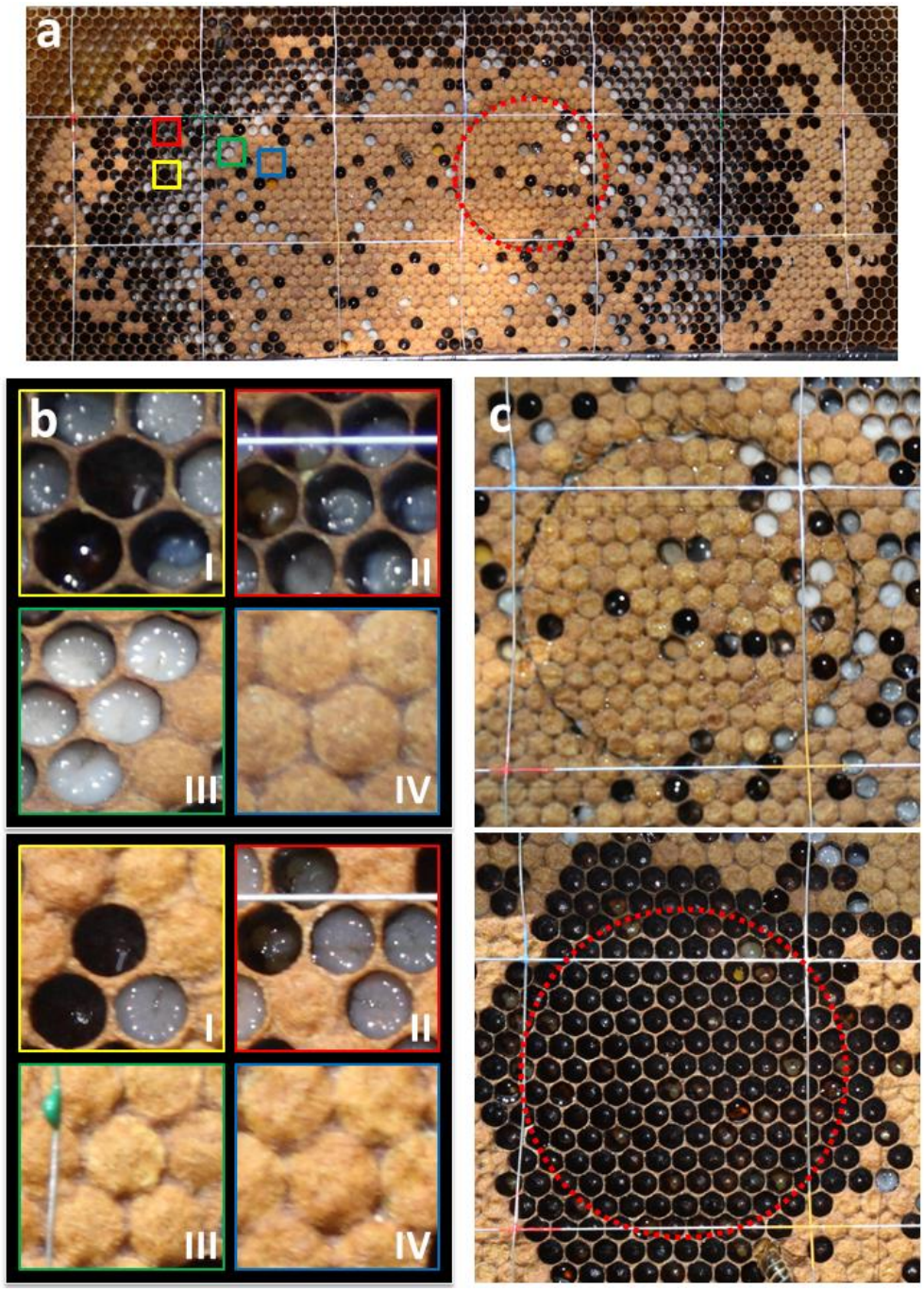

Figure 4.1: Brood treatment, stage and appearance. A) Showing one side of a whole brood comb within a medium-depth Langstroth frame (perimeter of frame not shown) immediately after the area in the red circle had been treated with liquid nitrogen. Also visible is the temporary wire grid that was placed above the tested frame, to facilitate the re-localization of the cells at both time points. B) Showing patches of cells from A). The centre cell in each patch is an example of one of the 4 brood age/stage categories used: I- egg, II- young larva in open cell, III- older larva in open cell, IV- capped cell containing a larva or pupa. The lower photo shows the same patches 48h later; C) Showing the patch of cells treated with liquid nitrogen immediately after (above) treatment and 48h later (below). The circular black line in the upper photo is the mark left by the metal cylinder that was pushed into the brood to contain the liquid nitrogen. It can be seen that the effect of the treatment is concentrated within the circle and extends in a band 1-3 cells wide around the circumference.

3. Correlation between removal rates

We tested the correlation between the removal of freeze-killed brood and untreated brood separately for the four different untreated brood stages (table 4.1). None were significant. In addition, there was no consistent trend. The slope was negative for young and old larvae in open cells and positive for eggs and capped brood.

Power analysis showed that the minimum sample sizes (N_2) that would have been required for the trends we observed between the removal of untreated and freeze-killed brood was large. These (table 4.1) ranged from 53 colonies for old larvae in uncapped cells and for eggs. That is, if the trends that we have observed were real, these numbers of colonies would have been needed for them to have been significant.

Correlation	**ρ**	**S**	**p-value**	**N_2**
FKB and untreated eggs	0.1463415	140.8537	0.6866	455.062
FKB and untreated young larvae	-0.1945298	197.0974	0.5902	255.8575
FKB and untreated old larvae	-0.4146341	233.4146	0.2335	53.25538
FKB and untreated capped brood	0.2477076	124.1282	0.4902	156.3089

Table 4.1: Showing correlation coefficients between the removal of FKB and the removal of brood monitored at 4 stages and the results of the power analysis (ρ: Spearman's rank correlation coefficient, S: Test statistic for ρ, N_2: number of colonies that would have been needed for the correlations being significant).

Discussion

The wide range of hygienic behaviour (FKB removal) that we observed in our study colonies shows that they were appropriate for testing the cost-to-healthy-brood hypothesis. Our results show that the ability of honey bee colonies to perform hygienic behaviour, measured as the removal of freeze-killed brood (FKB), is statistically uncorrelated with the removal of untreated, presumably healthy, brood. Although there

was a slightly positive, but non-significant, correlation between FKB removal and the removal of both eggs and capped brood the trend was negative for the removal of both young and old larvae from open cells. Power analysis results confirmed that all trends were very weak, and so were not likely to be biologically important. Overall, the results do not support the cost-to-healthy-brood hypothesis.

In the context of honey bee breeding, resistance towards brood diseases via hygienic behaviour is a desirable heritable trait (Boecking et al., 2000; Harbo and Harris, 1999). Our results are a further reason to promote the use of hygienic bees in commercial beekeeping. Previous research has also shown that hygienic colonies are as or more productive in making honey as non-hygienic colonies (Spivak and Reuter, 1998a).

Based on our results we propose an alternative explanation to the one put forth by Seeley (1985) for the high removal rate of control brood from capped cells in hygienic colonies by Rothenbuhler (1964). In his experiment, Rothenbuhler (Rothenbuhler, 1964a) measured the removal of control "check" larvae in adjacent rows to the young larvae that he infected with *P. larvae* spores. It is possible that this uninfected brood may have been sufficiently close to the infected brood for any odour indicating infection to be strong in adjacent cells. In addition, it is possible that the worker bees adopt a strategy of removing adjacent brood as a precautionary measure, analogous to a surgeon who prophylactically removes healthy tissue surrounding a tumour (T. Seeley, personal communication to F. R.). Further studies would be necessary to test these hypotheses.

Chapter 5: Hygienic behavior in honey bees (Hymenoptera: Apidae): effects of brood, food and time of the year

Gianluigi Bigio, Roger Schürch, Francis L.W. Ratnieks

For this chapter I contributed to the design of the project, to the acquisition, analysis and interpretation of the data and to the writing of the manuscript. Roger Schürch assisted with data analysis. Francis Ratnieks assisted with the design of the project and manuscript writing.

Abstract

Hygienic behavior in honey bees is a heritable trait of individual workers that confers colony-level resistance against various brood diseases. Hygienic workers detect and remove dead or diseased brood from sealed cells. However this behavior is quite rare, with only c.10% of unselected colonies showing high levels of hygiene. Beekeepers can potentially increase this by screening colonies for hygiene and breeding from the best. However, the level of hygiene expressed by a colony is variable, which poses a challenge to colony selection. In this study we systematically varied two factors thought to be of importance in influencing hygiene levels, "nectar" availability, by feeding or not feeding sucrose syrup, and brood amount, by adding or removing brood, to determine what effect they had on hygienic behavior. We tested 19 colonies repeatedly over a 4 month period using the freeze-killed brood assay (FKB), a standard technique to quantify hygienic behavior. Two days after FKB treatment, our colonies showed a wide range of brood removal levels, with colony means ranging from 31.7 ± 22.5 to 93 ± 6.9 (mean % ± SD). Neither the food nor the brood manipulation had an effect on hygiene levels. Colony size and time of year were also non-significant. The only significant effect was a three-way interaction between syrup availability, amount of brood and time of the year, resulting in reduced hygienic behavior early in the season (spring), in colonies with added brood that were not fed sucrose syrup. Overall these results suggest that hygienic behavior is not greatly affected by environmental

conditions typical of real-life beekeeping, and that screening of colonies can be done any time without special regard to nectar conditions or brood levels.

Introduction

For the last half century the number of managed honey bee colonies, *Apis mellifera*, has declined in both N. America and Europe (Neumann and Carreck, 2010; Oldroyd, 2007; vanEngelsdorp and Meixner, 2010). There are several reasons for this. One, which also affects other bee and wildlife species (Goulson et al., 2005; Potts et al., 2010), is the loss of forage due to agricultural intensification. Another, which affects only honey bees, is the increasing importance of honey-bee-specific pests and diseases. Challenges include newly-introduced pest and pathogen species (Danka et al., 1995; Higes et al., 2006) and resistance to chemical treatments, such as against varroa mites (*Varroa destructor*) and the bacterium *Paenibacillus larvae*, that causes American foulbrood (Milani, 1999; Miyagi et al., 2000; Pettis, 2004). However, honey bees have natural methods of resistance to pests and pathogens (Evans et al., 2006). A practical and desirable strategy, therefore, is to breed bees that express high levels of natural resistance, such as hygienic behavior.

Known to science since the 1930s (Park, 1936), hygienic behavior comprises several behavioral traits expressed by individual worker honey bees and controlled by multiple loci (Lapidge et al., 2002; Moritz, 1988; Oxley et al., 2010; Rothenbuhler, 1964a; Woodrow and Holst, 1942) which result in dead or diseased brood being removed from their cells and discarded, thereby reducing infection of healthy brood. When sufficient workers in a colony perform hygienic behavior it confers colony-level resistance (Wilson-Rich et al., 2009) against diseases that affect honey bee brood such as American foulbrood (Spivak and Reuter, 1998a, 2001), chalkbrood (Gilliam et al., 1983; Spivak and Reuter, 1998b) and varroa mites (Rinderer et al., 2010; Schöning et al., 2012; Spivak, 1996; Spivak and Reuter, 1998b) which breed in brood cells although the adult female mites are also phoretic on adult honey bees.

In addition to genetic control, and as with many other honey bee behaviors (Calderone and Page, 1992; Pankiw and Page, 2001; Uribe-Rubio et al., 2008) the removal of dead brood from sealed cells is also affected by environmental factors (Momot and Rothenbuhler, 1971; Pérez-Sato et al., 2009; Rodrigues et al., 1996; Spivak and Reuter, 1998a). Early research indicated that a colony's level of hygienic behavior, measured as the removal of diseased or dead brood, was affected by nectar conditions, with lower hygienic behavior in a period of nectar scarcity (Momot and Rothenbuhler,

1971; Thompson, 1964) or when there was a high ratio between the amount of brood to care for and the population of worker bees that nurse the brood (Thompson, 1964).

However, these experiments were primarily designed to investigate the effects of genotype and age on hygienic behavior, with any evidence for reduced hygienic behavior during a nectar dearth being incidental. Variation in brood amount and nectar availability are normal in honey bee ecology and beekeeping, and could lead to increased variability in measured levels of hygienic behavior, thereby causing difficulties when evaluating honey bee colonies for this trait as part of a breeding program. In this study we experimentally varied these two factors. Contrary to the earlier findings, our results showed no significant effect of either syrup feeding or brood addition/removal, or of time of year or colony population. The only significant effect was a three-way interaction of reduced hygienic behavior in early spring in colonies with added brood that were not fed sucrose syrup.

Materials and Methods

1. Study colonies and hive set up

We used 19 colonies kept in a single apiary on the University campus. In order that our work would be of relevance to beekeepers involved in the early stages of stock improvement, the study colonies had not been previously selected for hygienic behavior and were expected to show a wide and natural range in hygiene levels. In early spring 2011, brood and workers were removed from the strongest colonies to equalize initial populations to c. 4 frames of brood and c. 6 frames of bees. Sixteen colonies were used initially with three colonies kept as replacements for colonies in which the queen was superseded during the experiment. Each colony was at first kept in a hive consisting of a single medium-depth Langstroth hive box with 10 Pierco plastic frames with foundation. As colony population increased an additional medium box was given above a queen excluder. Each hive had a standard bottom board, inner cover with integrated syrup feeder capable of holding 4 liters, and an outer telescopic cover.

2. Experimental design

We assigned the hives to create four blocks each of four hives, one per treatment in a 2 × 2 design. For each experimental week we tested two factors, namely brood and syrup. Half of the colonies were fed sucrose syrup (F+ treatment) while the other half of the colonies were not fed syrup (F- treatment). To create Brood Plus (B+) and Brood Minus (B-) treatments, we exchanged one frame containing young brood with an empty frame between a pair of hives at the time the hives were opened to carry out the freeze-killed brood assay. Both treatments were tested simultaneously in the 2 × 2 design (B+F+, B+F-, B-F+, B-F-) replicated in four blocks (n = 16 hives per trial) across 10 repeated trials. We changed treatments across trials so that every colony would experience each treatment combination at least twice.

3. Colony strength estimation

At the beginning of each experimental week, the strength of each colony was estimated counting the number of side of frames containing more than 200 worker brood cells, at any stage of development.

4. Quantifying levels of hygienic behavior via Freeze Killed Brood (FKB) assay

Hygienic behavior was quantified at the colony level using the freeze-killed brood assay (FKB) (Spivak and Reuter, 1998b). To do this, in each colony we located a suitable patch of sealed brood on one frame. Two metal cylinders (6.5 cm diameter × 8 cm height) were pressed into this patch to reach the plastic foundation, to temporarily isolate 160 cells in each cylinder (Spivak and Reuter, 1998b). Approximately 300 ml of liquid nitrogen were poured into each cylinder to kill the brood in the selected area. After 5-10 minutes, the nitrogen had evaporated and the cylinders were removed. Photographs of each patch were then taken and the frame was replaced in its hive. Forty-eight hours later, the frame was removed and the patches were photographed again. The photographs were then used to determine the percentage of capped brood cells that had been removed by each colony.

5. Manipulating syrup levels

F+ treatment colonies were given 2L of 2M sucrose (molar mass 342.3 g/mol) solution two days before performing the FKB assay. Additional solution was given *ad libitum* to any colony that had emptied its feeder, when checked daily. Sucrose feeding was terminated after four days, at the end of the FKB assay. This treatment mimicked a strong nectar flow in progress at the time of the bioassay. F- treatment colonies were not given any syrup but could forage naturally.

6. Manipulating brood levels

B- treatment colonies acted as brood donors. When hives were open for the freeze-killed brood assay one frame containing mostly uncapped brood on both sides, was removed from each B- colony, marked and transferred to a B+ treatment colony. This brood frame was returned to its own B- colony 48h later, in order to prevent worker bees from emerging in the B+ colony, and altering the genetic profile of that colony.

7. Statistical analysis

Raw data on the proportions of freeze-killed brood that had been removed were arcsine transformed to normalize errors. All calculations and statistics were done on the transformed values, and the data were then back transformed for presentation (Sokal and Rohlf, 1981). In R (R Development Core Team, 2012) we used a linear mixed-effect model (Bates et al., 2013; Zuur et al., 2009) to investigate the relationship between treatments, brood removal (i.e. level of hygienic behavior), and season. Colony was included in the model as a grouping factor with a random intercept per colony. We started with a model containing all covariates and all interactions, and then performed a backward model selection process (Faraway, 2006) dropping single terms and testing (loglikelihood ratio test LRT) for the significance of each removed term or interaction between terms (Zuur et al., 2009). We dropped factors and interactions that did not lead to significant increases in deviance ($p > 0.05$) and we re-fitted the resulting model. In

order to determine the proportion of the variance in FKB removal that was explained by colonies, we calculated the intraclass correlation coefficient (ICC) according to Zuur et al. (Zuur et al., 2009).

Results

1. General results and colony differences

Across 160 trials on 19 colonies over 10 trial dates from June 2011 to September 2011, freeze-killed brood (FKB) removal at 48 h ranged from 9 to 100%, with mean of 62.6% and standard deviation (SD) of 24.5%. Per colony, the mean and SD varied from 93% ± 6.9% to 31.7% ± 22.5% (see also Fig. 5.1). Across all 160 measurements 16 (10%) were above 95%, and 9 of 19 (47%) colonies had at least one trial with > 95% FKB removal, which is considered an appropriate cut off point between hygienic and non-hygienic colonies (Spivak and Reuter, 1998b). No colony had a mean FKB removal rate above 95%, and only one colony scored above 90% (colony 55, 93 ± 6.9%). Despite having a mean removal rate of only 79.5 ± 19.1%, a second colony, number 100, had high FKB removal (> 90%) in 6 of 10 trials.

As expected, colonies differed significantly in FKB removal (LRT: $\chi 2 = 45.21$, $df = 1$, $p < 0.001$). This also justifies inclusion of colony as a random effect in the mixed-effect model. The intraclass correlation coefficient (ICC) was 45.5%, meaning that almost half of the variance in FKB removal was explained by colony.

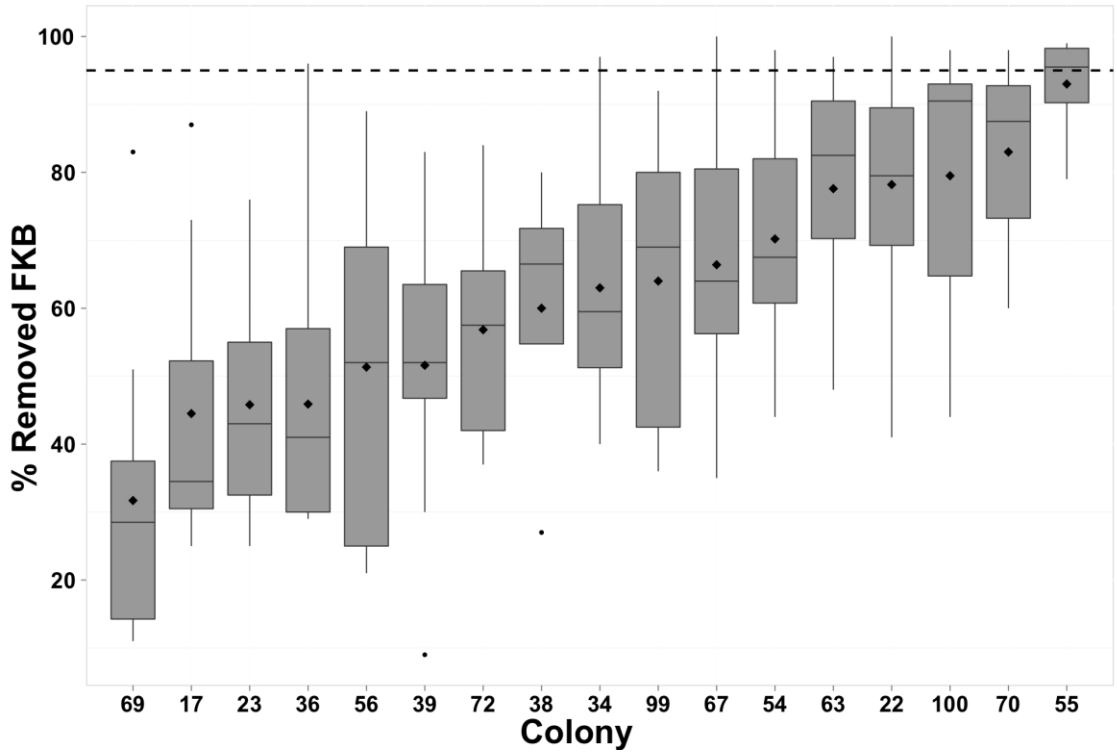

Figure 5.1: Percentage of freeze-killed brood removed within 48 hours by unselected colonies that were each screened in 10 trials from June 2011 to September 2011. The boxes represent the interquartile range and the bar indicates the median. Whiskers extend to 1.5 times the interquartile range, with black dots representing outliers. The dotted line represents the 95% threshold. Black dots on each boxplot represent mean FKB removal.

2. Effect of brood, syrup and season

To analyze the impact of the experimental treatments (brood, syrup), other variables (colony strength, season) and their interaction, we started with a model containing all the effects and their interactions. Despite equalizing colonies in the spring (20 April), colony strength became non-equal as the season and the experiment progressed, for example from 4-13 sides containing brood in late spring (14 June) to 7-16 sides in mid-summer (15 August). In addition mean colony size varied from a minimum of 8.9 frame sides containing brood in late spring to a maximum of 11.7 in summer. However, colony strength did not have an effect on FKB removal ($\chi 2 = 0.006$, $df = 1$, p= 0.9385) and was removed from the final model (Fig. 5.2, see also table 5.1).

As shown in Fig. 5.2, FKB removal was approximately constant across the season. Of the three factors remaining in the model (syrup, brood, day) and their interactions, after backward selection only one was significant and was kept as part of the best fitting

model. This was the three way interaction syrup × brood × day ($\chi 2 = 9.9375$, $df = 1$, $p = 0.0016$). The distance between the dotted and the solid line in Fig. 5.2b graphically shows that FKB removal was lower in spring, in the colonies that received a frame of brood (B+) without being fed with syrup (F-).

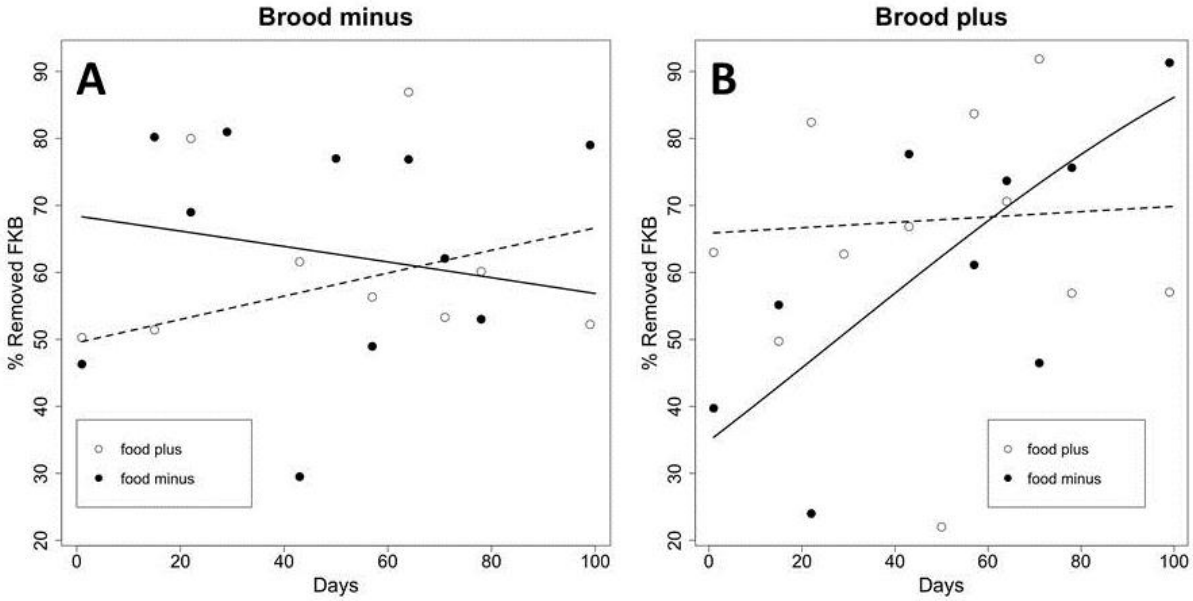

Figure 5.2: Variation in removal of freeze-killed brood under different experimental conditions: with a frame of young, uncapped brood removed (A), or added (B). In both graphs the dotted line and empty dots represent colonies that were given additional syrup and the solid line and dots represent colonies that had only natural forage.

Discussion

None of the 19 study colonies had a mean FKB removal levels over 95%, which is a convenient threshold level above which colonies are considered fully hygienic. However, one colony had a mean of 93% over 10 trials. These results are in general agreement with previous studies that reported variable hygienic behavior levels in unselected honey bee colonies, and confirms that hygienic behavior at a level high enough for breeding under ideal circumstances is normally rare (> 95% FKB removal: 10% (JA Pérez-Sato, 2007; Waite et al., 2003). We found 1/19 (5%), 3/19 (16%) and 6/19 (31%) colonies with mean hygiene levels respectively greater than 90%, 80% and 70% which is similar to the 3%, 3% and 23% found earlier also in England (JA Pérez-Sato, 2007).

Our results show that the level of hygienic behavior in honey bee colonies, as

measured by FKB removal, is not affected in any systematic way by the time of the active season (spring-summer) that the trial was conducted, colony population (frames of brood), the manipulation of brood levels (by adding or removing a frame of brood during the 2-day FKB bioassay period) or food availability (by providing or not providing several liters of sucrose syrup during and for a few days before the FKB bioassay). The one significant interaction was between season, whether colonies were given additional food, and whether they were given additional brood: hygienic behavior decreased in colonies when in spring they received additional brood without being given supplementary syrup at the same time.

Our experimental results are in disagreement with earlier observational studies (Momot and Rothenbuhler, 1971; Thompson, 1964). Thompson's (Thompson, 1964) data indicate that honey bee colonies show a lower rate of hygienic behavior during a period of relative lack of nectar, but no statistics are presented. In regard to the brood ratio, Thompson (Thompson, 1964) only refers to unpublished data. Similarly, Momot and Rothenbuhler (Momot and Rothenbuhler, 1971) report data that suggest that more foragers switch back to hygienic behavior if enough food is available. These results were based on observation of just one colony, and no statistics were presented. Given the limitations of these earlier studies in this respect, it is hard to compare their results to those of this study.

Colony explained 45.5% of the total variation of FKB removal. This measure of repeatability confirms, as expected, that hygienic behavior has a strong heritable component given that each colony had the same genetic structure across the season (i.e., the same queen) even though the workers who carried out the hygienic behavior would have been different in each trial given that hygienic behavior is mainly carried out by workers aged between 15 and 17 days (Arathi et al., 2000). Our 45.5% also agrees with a previous estimate of 50.4% carried out in England, in which three FKB removal bioassays were made per colony (JA Pérez-Sato, 2007).

Our results are encouraging in their implications for honey bee breeding. The screening of colonies for hygienic behavior using the FKB or other bioassays is time consuming and needs to be carried out at least several times per colony given the variation within a colony across trials (Pérez-Sato et al., 2009; Spivak and Reuter, 1998a). However, our findings suggest that there is no need to worry unduly about variation caused by differences between spring and summer, variation in nectar availability, or brood or adult bee populations as long as colonies are reasonably strong

as would be the case for colonies used in this experiment, housed in standard equipment that is very similar to what is used by beekeepers. The only combination of factors we found that affected hygiene was the 3-way interaction, with lower hygiene in spring for colonies given extra brood but not given syrup. When applied to a real-life scenario, this is unlikely to be a problem for any program of screening for hygiene carried out by beekeepers. In particular, it would not be advisable to add frames of brood to test colonies as this would result in the genetic profile of the colony being altered, and weakening the validity of any results from c. 2-5 weeks later when worker bees from the added frame were of any age to carry out hygiene. Although beekeepers sometimes feed syrup, our results show that this did not have a significant effect on hygiene levels.

In both this study and that of Pérez-Sato (JA Pérez-Sato, 2007), colony only explained about half of the observed variation on FKB removal. Our study indicates that brood and food levels have little effect on their own, which will make it more practical to screen bees for hygienic behavior. Further research will be necessary to determine what other factors are responsible for the additional variation, but for now the information available is sufficient for bee breeding purposes.

			Model parameters					Likelihood ratio tests			
Model	Model Description	Effect tested	Fixed effects	Number of parameters	ln Lik	AIC	ΔAIC	X^2	d.f.	Reference model	p
1	Full model			15	6.7013	22.598			1		
2		F × B × S × D	Model 1 - F × B × S × D	14	6.15	21.7	0.898	1.1026	1	1	`0.2937
3		F × B × S	Model 2 - F × B × S	13	6.1293	19.741	1.959	0.0413	1	2	0.8389
4		B × S × D	Model 2 - B × S × D	13	5.6874	20.625	1.075	0.9252	1	2	0.3361
5		F × S × D	Model 2 - F × S × D	13	6.1455	19.709	1.991	0.009	1	2	0.9246
6		F × B × D	Model 2 - F × B × D	13	0.9529	30.094	-8.394	10.394	1	2	0.0012**
7		F × B × S	Model 5 - F × B × S	12	6.1277	17.745	1.964	0.0355	1	5	0.8505
8		B × S × D	Model 5 - B × S × D	12	5.6578	18.684	1.025	0.9753	1	5	0.3234
9		F × B × D	Model 5 - F × B × D	12	0.9265	28.147	-8.438	10.438	1	5	0.0012**
10		B × S × D	Model 7 - B × S × D	11	5.6501	16.7	1.045	0.9551	1	7	0.3284
11		F × B × D	Model 7 - F × B × D	11	0.7925	26.415	-8.67	10.670	1	7	0.0010**
12		F × S	Model 10 - F × S	10	5.3374	15.325	1.375	0.6254	1	10	0.429
13		B × S	Model 10 - B × S	10	5.5359	14.928	1.772	0.2285	1	10	0.6327
14		S × D	Model 10 - S × D	10	5.6446	14.711	1.989	0.011	1	10	0.9166
15		F × S	Model 14 - F × S	9	5.3366	13.327	1.384	0.616	1	14	0.4325
16		B × S	Model 14- B × S	9	5.5142	12.972	1.739	0.2609	1	14	0.6095
17		F × S	Model 16 - F × S	8	5.0894	11.821	1.151	0.8497	1	16	0.3566
18		F × B × D	Model 17 - F × B × D	7	0.1190	19.762	-7.941	9.9407	1	17	0.0016**
19	Final	S	Model 17 – S	7	5.0864	9.8272	1.99	0.006	1	17	0.9385
20		F × B × D	Model 19 - F × B × D	6	0.1176	17.7648	-7.938	9.9375	1	19	0.0016**

Table 5.1: Eleven linear mixed effect models are compared with LTR to find the best fitting model to predict hygienic behaviour. The full model (Model 1) contained the fixed effects Food (F), Brood (B), Day (D) and Strength (S) plus the following interactions: F × B × S × D, F × B × S, F × B × D, F × S × D, B × S × D, F × B, F × S, F × D, B × S, B × D, S × D. All the factors and interactions were tested, and the non-significant (p > 0.05) ones were removed from the model to improve its fit. In the final model (Model 19) we retained the following factors F + B + D + F × B + F × D + B × D + F × B × D. See table 5.2 for the parameter estimates.

Fixed effect	**Estimate ± SE**	**t**
	0.974027±0.076467	12.738
Food	-0.193731± 0.099992	-1.937
Brood	-0.342626± 0.100733	-3.401
Day	-0.001199± 0.001191	-1.007
Food × brood	0.509148± 0.145612	3.497
Food × day	0.002946± 0.001759	1.675
Brood × day	0.006781± 0.001785	3.789
Food × brood × day	-0.008100± 0.002561	-3.163

Table 5.2: Parameter estimates obtained for the final model (see Table 5.1) predicting hygienic behaviour. Reference categories for food and brood were minus.

Chapter 6: Evaluation of hygienic, defensive and running behaviour in commercially managed honey bee colonies: a UK case study

Gianluigi Bigio, Luciano Scandian, Francis L.W. Ratnieks

For this chapter I contributed to the design of the project, to the acquisition, analysis and interpretation of the data and to the writing of the manuscript. Luciano Scandian assisted with hive management and data collection. Francis Ratnieks assisted with the design of the project and manuscript writing.

Abstract

Honey bee colonies display many traits that are the result of collective behaviour. Some of these are desirable in beekeeping while others are undesirable. In this experiment we monitor the variation of freeze-killed brood (FKB) removal in 36 honey bee colonies owned by a local beekeeper over a total of 14 months. Our goal was to assess how the seasonal development of commercially-managed colonies can affect the expression of hygienic behaviour (from the FKB bioassay), and to determine if there is any correlation between this desirable trait and two undesirable traits: defensivity/stinging and running (rapid movement of worker bees on combs during hive inspections). The results show that honey bees can express valued traits, such as hygienic behaviour, without showing detrimental traits, such as defensive or running behaviour. In addition, we also demonstrate that hygienic behaviour is not influenced by time of the year in which the colonies are tested with the FKB assay. However, colonies that are either too weak or too strong, close to swarming, should not be tested as they do not represent a viable colony. Additionally, we suggest that selecting against the presence of chalkbrood, a fungal disease of capped brood, also selects for hygienic behaviour. This is because the average rate of FKB removal observed, 75%, was much higher than expected for a population of colonies previously unselected for hygiene. However, the beekeeper had been selecting for low levels of chalkbrood in his colonies, and it is likely that this was indirectly selecting for hygienic behaviour.

Introduction

Honey bees have a range of heritable traits that affect the colony as a whole and that are relevant to beekeeping. Some of these can be detected directly, such as honey production, the presence or absence of diseased brood, defensivity (stinging) and running behaviour of the bees during hive inspection. Some others, such as hygienic behaviour, are not obvious and require special methods to measure the colony phenotype.

Known for over half a century (Park, 1936; Woodrow and Holst, 1942) hygienic behaviour is a genetic trait controlled by six or seven loci (Lapidge et al., 2002; Oxley et al., 2010) and is a form of social immunity (Wilson-Rich et al., 2009) against several diseases that can affect honey bee brood. Hygienic behaviour is expressed at the colony level when the colony contains enough workers that have the ability of detecting, uncapping and removing dead or parasitized brood from sealed cells, and has been proven to be an effective control measure against American foulbrood (Rothenbuhler, 1964a; Spivak, 1996; Spivak and Reuter, 2001), chalkbrood (Gilliam et al., 1983; Trump et al., 1967) and Varroa (Boecking and Drescher, 1992; Rinderer et al., 2010; Spivak, 1996; Spivak and Reuter, 1998a). Hygienic behaviour is a naturally occurring trait that generally occurs at high levels only in c.10% of unselected colonies (Bigio et al., 2013; Pérez-Sato et al., 2009; Waite et al., 2003). It can be experimentally detected using the Freeze Killed Brood (FKB) assay (Spivak and Reuter, 1998b), an assay that allows for practical testing under field conditions. Honey bee colonies can be selectively bred for the expression of high levels of hygienic behaviour, resulting in disease resistant colonies.

The life cycle of a honey bee colony is affected by the season of the year and environmental factors. For example, swarming occurs mainly in spring and colony population occurs from spring to summer (Graham, 1992). Behaviour can also vary, such as nestmate recognition which is less permissive during periods of nectar dearth (Downs and Ratnieks, 2000). When breeding for a specific trait, it is useful, therefore, to know the effect of seasonal or environmental factors (Bigio et al., 2013) on the trait in order to contextualize the results and to allow for any externally-caused variation. In addition, when breeding for a desirable trait such as hygienic behaviour it is important to avoid co-selection for undesirable traits. In particular, when selecting honey bees for

hygienic behaviour it is important to avoid also selecting for defensive behaviour/stinging or for bees that run over the combs when making a hive inspection. Both traits make beekeeping more difficult, and are commonly seen in honey bees in Britain. However, what is not known is if there is a correlation between hygienic, defensive and running behaviour.

Here we monitored the variation of FKB removal in 36 honey bee colonies managed using standard beekeeping procedures over 14 months. Our goal was to assess how seasonal development and honey bee colonies management affects the expression of hygienic behaviour, and to correlate hygiene with defensive and running behaviour.

Materials and Methods

1. Study colonies

We began by identifying 36 suitable colonies belonging to a semi-commercial beekeeper with c.150 hives, located in West Sussex. For more than 20 years he had focused his efforts on improving his bees so that they would show low levels of defensive and running behaviour, together with low levels of chalkbrood. Chalkbrood is a very common brood disease that has no method of chemical control and it is easily detected by a beekeeper. It kills larvae in capped cells, which turn into white or black-coloured "mummies" that can easily be seen on the bottom board of the hive, near the hive entrance, and in brood cells after the cell has been uncapped but before the mummy has been removed.

The experiment ran from August 2011 to September 2012. During this period the colonies were managed by the beekeeper to prevent swarming, hence retaining the same queen and genetic structure of each colony. We dropped from the experiment any colonies that, upon inspection, did not possess the original paint-marked queen. Each colony was housed in one "national deep boxes" containing up to 11 deep frames. According to the need and the time of the year, we gave each colony a queen excluder grid and one or two honey boxes ("supers") as needed depending on the flow of nectar and the honey crop.

2. Strength estimation

At the beginning of each experimental week, we estimated the strength of each colony as the number of side of frames containing worker brood of any stage with the cluster size of the worker bees (Nasr et al., 1990). Due to our need to retain the genetic identity of each experimental colony, colonies would be dramatically weakened (frames containing bees and brood removed, and brought to another apiary) when we found queen cells. Empty frames were added to replace those removed and to give the colony space for brood. As a consequence, colony strength became non-equal depending on the laying rate of the queens and any brood removal to prevent swarming.

3. Quantifying levels of hygienic behaviour via Freeze Killed Brood (FKB) assay

We indirectly measured the ability of each colony to uncap and remove diseased or parasitized brood using the freeze-killed brood (FKB) assay described by Spivak and Reuter (Spivak and Reuter, 1998b). On experimental day 0, after locating suitable patches of sealed brood, we inserted two metal cylinders (6.5 cm diameter and 8 cm height) into the sealed brood up to the comb mid-rib. We poured approximately 300 mL of liquid nitrogen into each cylinder to kill the brood present in the enclosed circular area. After 5-10 minutes, the nitrogen had evaporated and we removed the cylinders. Before returning the frame to its original hive, we took photographs of each patch. Forty-eight hours later, at experimental day 2, we retrieved the same frame and took photographs of the treated patches to determine the number of freeze-killed brood that had been removed, and the number uncapped.

4. Assessing defensive behaviour

We assessed the defensivity of each colony on experimental day 0 before proceeding with the inspection of the hive and the FKB assay. By doing this we allowed enough time for the colonies to recover from the disruption caused by the previous visit. Each hive was opened by the same operator, using a hive tool but no smoke. We recorded the colony response on a 5 point scale as follows. 1 – very docile: bees not responsive,

slight buzzing. 2 – docile: bees react at being handled, some might leave the hive and fly around the operator without stinging. 3 – medium: bees leave the hive and fly around the operator, with some stinging, some smoke calms them down. 4 – defensive: bees show propensity to sting the operator on the gloves and fly around and a lot of smoke is required. 5 – very defensive: honey bee workers instantly fly against the operator to attack and tend to follow him away from the hive, stinging gloves and bee suit; repeated use of smoke is required to calm the bees. Colonies that scored 4 or 5 in the aggressive scale also released alarm pheromone, which could be smelt.

5. Assessing running behaviour on the comb

We assessed the running behaviour on the comb of the workers in each colony on experimental day 0 before proceeding with the inspection of the hive and the FKB assay. By doing this we allowed enough time for each colony to recover from the disruption caused by the previous visit. Each hive was opened by the same operator, using a hive tool but no smoke and the behaviour was observed on a frame containing brood. We assigned a score on a scale from 1 to 3 as follows. 1 – calm: bees remain undisturbed, move on the comb quietly and don't leave the comb. 2 – medium: bees react to being handled, move on the comb without running and cluster on the edges. 3 – agitated: bees nervously run on the comb, leaving the hive and the frames flying towards the operator.

6. Statistical analysis

Hygienic behaviour was quantified as the percentage of cells removed. We arcsine transformed the results to normalize errors. In R (R Development Core Team, 2012) we used a linear mixed effect model (Bates et al., 2013) to investigate the relationship between levels of hygienic behaviour and season. Colony was included in the model as a grouping factor with a random intercept per colony. To analyse the impact of the two variables (colony strength and season) and their interaction on the removal of FKB, we started with a model containing all the effects and their interactions. We then performed a backward model selection process (Faraway, 2006) dropping single terms and testing (loglikelihood ratio test LRT) for the significance of each removed term or interaction

between terms (Zuur et al., 2009). We dropped factors and interactions that did not lead to significant increases in deviance ($p > 0.05$) and we re-fitted the resulting model.

Results

1. General results

In total we performed 255 trials on 36 colonies over 9 occasions, collecting data for hygienic, defensive and running behaviour. Freeze-killed brood removal (FKB) ranged from 19 to 100%, with mean of 74.81% and standard deviation of 20.88%. Per colony, the mean and SD varied from 45.62 ± 18.36 to 96.23 ± 3.64%. Only one colony had a mean FKB removal rate above 95% (colony A9, 96.23 ± 3.64%) and 3 colonies in total had a mean FKB removal rate above 90% (colonies ENR1, 09E3N1 and W3, respectively 93.75 ± 3.4%, 92.4 ± 12.66% and 90.75 ± 5.85%. See also figure 6.1).

Over all the trials, defensive behaviour scores varied from 1 to 5, with a mean of 1.57. Across each colony, the mean varied from 1 to 2.33. Running behaviour scores varied from 1 to 3, with a mean of 1.43. Across each colony, the mean varied from 1 to 1.8.

Over the whole duration of the study, from August 2011 to September 2012, the strength of the colonies showed a great degree of variation, ranging from a minimum of 1 frame side containing brood, to a maximum of 20 sides.

2. Correlations between behaviours and effects of colony strength and time of year on hygienic behaviour

We first tested the correlation between defensive and running behaviour. As the two values were highly correlated (Spearman's correlation coefficient: S = 4203.523, p-value = 0.004865, rho = 0.4590061) for the rest of the analysis we discarded running behaviour and focused on defensiveness as a factor because of the more detailed score. We then proceeded in assessing the correlation between the average values (average calculated colony-wise) of hygienic behaviour and defensiveness, and the results showed no correlation (Spearman's correlation coefficient: S = 6579.546, p-value =

0.3723, rho = 0.1532116).

Colony strength varied greatly over the whole duration of the experiment. We recorded colonies with as little as one side of one frame with brood (colony A1 on 1 May 2012 and on 20 June 2012) or as much as 20 sides (colony A2W on 17 August 2011 and on 20 June 2012). In addition, across experimental days mean colony size varied from a minimum of 4.9 frame sides containing brood on 20 June 2012 to a maximum of 12.6 when the experiment started on the 17 August 2011.

While the time of the year in which we performed the FKB assay did not have an effect on hygienic behaviour, and was then removed from the final model ($\chi 2 = 2.5388$, df = 4, p= 0.1111), colony strength ($\chi 2 = 0.0413$, df = 4, p= 0.0032) had a significant effect on FKB removal (see also Table 6.1 and 6.2).

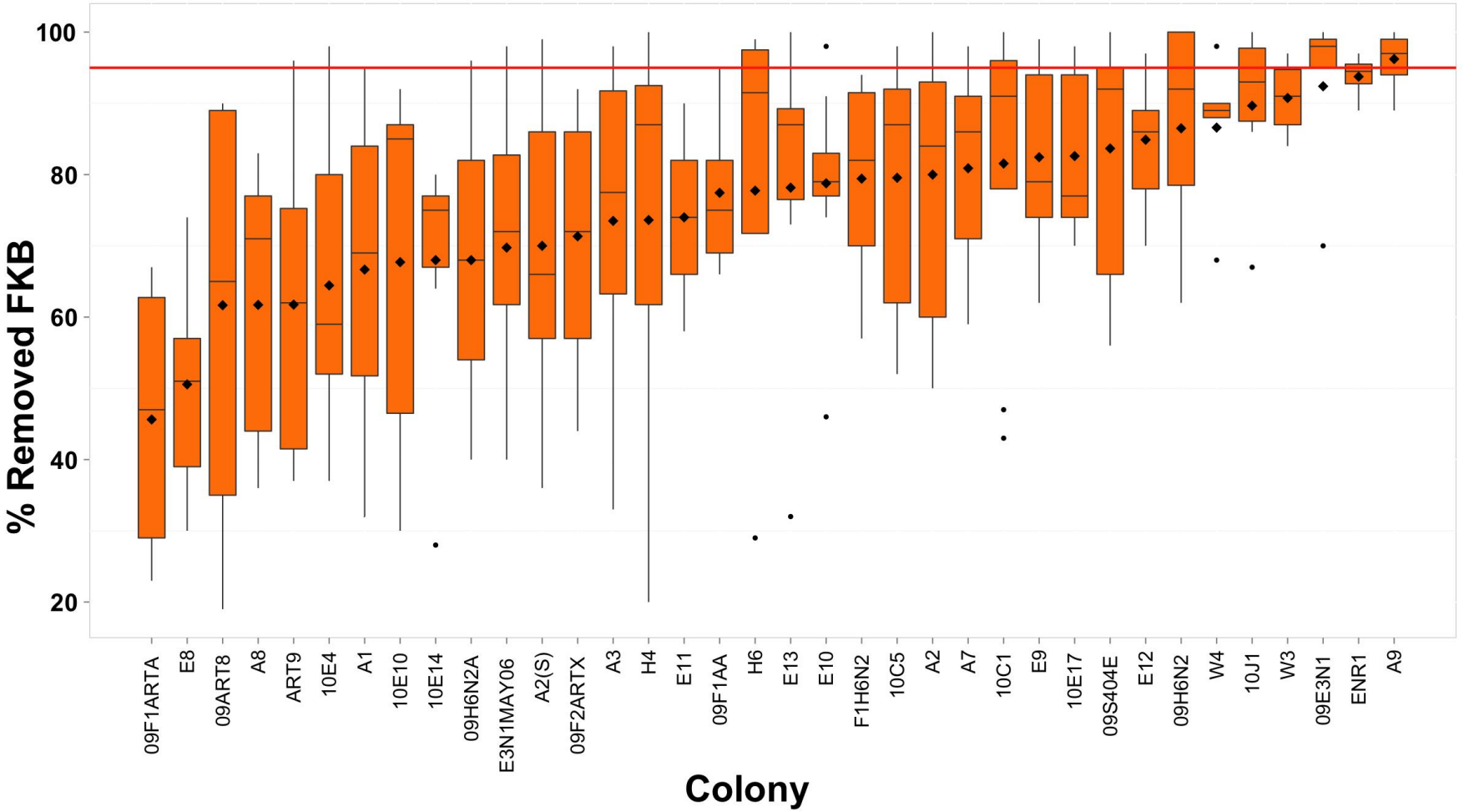

Figure 6.1: Percentage of freeze-killed brood removed within 48 hours by 36 unselected colonies that were each screened in 9 trials from August 2011 to September 2012. The boxes represent the interquartile range and the bar indicates the median. Whiskers extend to 1.5 times the interquartile range, with black dots representing outliers. The red line represents the 95% threshold. Black dots on each boxplot represent mean FKB removal.

			Model parameters					**Likelihood ratio tests**			
Model	Model Description	Effect tested	Fixed effects	Number of parameters	ln Lik	AIC	ΔAIC	X^2	d.f.	Reference model	p
1	Full model		S + D + S × D	3	2.226	7.549			6		
2		S × D	S + D	2	1.316	7.369	0.18	1.8201	5	1	0.1773
3		S	D	1	-3.03	14.06	6.691	0.0413	4	2	0.003198**
4	Final	D	S	1	0.04626	7.907	0.538	2.5388	4	2	0.1111
4		D	S	1	0.04626	7.907	6.153	6.1522	4	3	< 2.2e-16 ***

Table 6.1: Four linear mixed effect models are compared with LTR to find the best fitting model to predict hygienic behaviour. The full model (Model 1) contained the fixed effects Strength (S) and Day (D) plus the interaction S × D. In the final model (Model 4) we retained Strength (S) as a factor. Both factors and their interaction were tested, and the significant ($p < 0.05$) ones were kept in the model to improve its fit. See table 6.2 for the parameter estimates.

Fixed effect	**Estimate ± SE**	**t**
	0.958747 ± 0.040899	23.442
Strength	0.014587 ± 0.003535	-1.937

Table 6.2: Parameter estimates obtained for the final model (see Table 6.1) predicting hygienic behaviour

Discussion

Over all 9 trials only one of the 36 study colonies had a mean FKB removal level of over 95%, which is the conventional threshold level above which colonies are considered fully hygienic (Büchler et al., 2013). Additionally, we found 5/36 (13.9%), 15/36 (41.7%) and 26/36 (72.2%) colonies with mean hygiene levels greater than 90%, 80% and 70%, respectively, which are considerably higher when compared to the 3%, 3% and 23% found in an earlier survey of colonies, also in England (JA Pérez-Sato, 2007). While results from previous experiments (Bigio et al., 2013) are in general agreement with previous studies that reported variable hygienic behaviour levels in unselected honey bee colonies, and confirms that hygienic behaviour at a level high enough for breeding under ideal circumstances is normally rare (> 95% FKB removal: 10% - (Waite et al., 2003), 3% - (JA Pérez-Sato, 2007)), the average level of FKB removal in the study colonies was very high at 75%.

One possible reason for this difference is that the colonies had actually been selected for hygienic behaviour. Although the beekeeper had not directly selected for hygienic behaviour, he had selected against the occurrence of chalkbrood. As hygienic behaviour is known to reduce chalkbrood (Gilliam et al., 1983; Trump et al., 1967), he may have been inadvertently selecting for hygiene. This parallels the situation in the USA with the hygienic Brown line studied by Rothenbuhler in the 1960s (Rothenbuhler, 1964a; Thompson, 1964). This line had been bred by a beekeeper from Iowa, Brown, who operated a wax rendering plant. He kept an apiary at the plant to rob out residual honey from dead hives kept beside the plant before rendering. Any colonies showing symptoms of American foulbrood (a contagious and virulent brood disease of honey bees that would be expected to proliferate under these conditions) were allowed to requeen themselves using brood taken from colonies without AFB at the apiary. (Information given to F. Ratnieks by beekeeper Harold Merrill of New York State who had visited Brown). This process led to selection for highly hygienic colonies, as shown by Rothenbuhler. The evidence that the high rates of FKB removal in our study colonies was due to the beekeeper selecting for low chalk brood is circumstantial but highly plausible. If so, it indicates that beekeepers can improve disease resistance simply by requeening colonies with high levels of chalkbrood with queens reared from their own colonies that show low levels of chalkbrood. Chalkbrood is a common disease and so

provides a ready target for evaluation and selection.

The results obtained also show that more defensive bees tend also to run around the hive and on the frames during inspection. Both characteristics are undesirable in beekeeping. However, neither correlates with hygienic behaviour. This shows that it is possible to have hygienic colonies that are calm and not over defensive. These results are in agreement with previous studies that managed to obtain bees that were both hygienic and did not display defensive behaviour, concluding that those two behaviours were not genetically co-inherited (Rothenbuhler, 1964a; Spivak and Reuter, 1998b).

During our previous experiments (Bigio et al., 2013) the time of year in which we performed the FKB bioassay explained part of the variation in hygienic behaviour that was detected. However in this experiment, which monitored hives over a much greater time period, time of year did not have an effect on the removal of freeze-killed brood. Conversely, in this experiment the number of frames containing brood, a proxy for colony strength, did explain some of the variation of FKB removal, with stronger colonies showing higher levels of FKB removal but this was not a factor in our previous research (Bigio et al., 2013). This is likely due, at least in part, because in the previous study the colonies were monitored for a shorter period of time and were manipulated so that their strength was kept as similar as possible. In contrast, this experiment lasted for over a year and the amount of brood showed a much greater variation. These results suggest that colonies that are either too weak or too strong should not be tested for hygienic behaviour, but this is unlikely to be problematic in breeding program as colonies would be evaluated when at normal sizes and not when excessively weak or strong.

In this experiment we have demonstrated that honey bee colonies can express hygienic behaviour without necessarily showing detrimental traits such as defensive or lack of calmness during hive inspections. Our results also show how hygienic behaviour is not influenced by time of the year in which the colonies are tested with the FKB assay. However colonies that are either too weak or too strong should not be tested. Additionally, the study provides strong circumstantial evidence that by selecting against the presence of chalkbrood, a beekeeper can select for hygienic behaviour.

Chapter 7: Comparing levels of hygienic behaviour in honey bee colonies with naturally mated vs. instrumentally inseminated queens

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For this chapter I contributed to the design of the project, to the acquisition, analysis and interpretation of the data and to the writing of the manuscript. Hasan Al Toufailia assisted with data collection. William Hughes assisted with data analysis and manuscript writing. Francis Ratnieks assisted with the design of the project and manuscript writing.

Abstract

Honey bee mating cannot be directly controlled in the same way as in many agriculturally-important animals. Instrumental insemination, however, is possible and can be used as an aid in selective breeding. Hygienic behaviour, in which worker bees detect and remove dead or diseased brood from capped cells, is a heritable trait that confers colony-level resistance against brood diseases. Using the freeze-killed brood bioassay we compared the levels of hygiene in colonies headed by daughter queens reared from hygienic mother colonies (mean FKB removal = 90.4%) that were either instrumentally inseminated with sperm from drones reared from hygienic colonies (n = 9) or allowed to mate naturally with naturally-occurring drones (n = 11). Hygiene levels were significantly higher ($p < 0.005$) in the colonies of the instrumentally inseminated queens (FKB removal 99.8%, n =3 trials per colony) than in the colonies of the naturally-mated queens (95.5%). However, the hygiene levels in the naturally-mated colonies were encouragingly high and indicate that supplying beekeepers with naturally-mated queens, or virgin queens to mate locally, can result in colonies with high levels of hygiene.

Introduction

Hygienic behaviour in honey bees (*Apis mellifera*) is a naturally occurring, heritable trait known for many years (Park, 1936). Hygiene confers social immunity against various brood diseases (Gilliam et al., 1983; Rinderer et al., 2010; Schöning et al., 2012; Spivak and Reuter, 1998b; Wilson-Rich et al., 2009) as hygienic colonies are able to detect, uncap and remove dead or diseased brood (Rothenbuhler, 1964a). These characteristics make it a trait of potential benefit to beekeeping.

Due to their biology and mating behaviour, honey bee breeding is more complicated than in many other animals used in agriculture (Pérez-Sato et al., 2009; Ratnieks, 1998). In particular, controlling matings is difficult. Queens naturally mate in flight with 10-20 males (drones) that gather in drone congregation areas and come from many different hives (Tarpy et al., 2004). Queens and males from hives many kilometres apart can mate. Some control over natural mating can be achieved by providing the queens with selected drones to mate with, using areas isolated from other hives such as islands (Neumann et al., 1999), mountain valleys (Jensen et al., 2005) or areas where honey bees do not normally live (Szabo, 1986).

From the perspective of breeding for high levels of hygienic behaviour, multiple matings by queens creates additional challenges. A colony can be hygienic even if only a fraction of the workers are hygienic (Arathi et al., 2000), belonging to the few hygienic patrilines (Pérez-Sato et al., 2009). As a result daughter queens reared from a hygienic colony may belong to non-hygienic patrilines.

To precisely control honey bee mating, researchers and breeders can exploit instrumental insemination (II). Honey bees are among the few insects for which this technique is available (Baer and Schmid-Hempel, 2005; Ball et al., 1983; Laidlaw and Page, 1997). Instrumental insemination has many potential applications in research and breeding because it enables complete control over mating and the genetic composition of the daughter colony, allowing specific crosses to be made. However, its technical nature has meant that it has never been widely adopted by the beekeeping industry, despite the fact that instrumentally inseminated queens can have the same performance as naturally mated queens (Cobey, 2007a).

The aim of this experiment was to compare the levels of hygienic behaviour in colonies headed by daughter queens reared from colonies with high levels of hygienic

behaviour and mated in two different ways. One group of queens were fertilised using instrumental insemination with semen from drones reared in colonies with high levels of hygienic behaviour, and which presumably carried hygienic genes. The other group were allowed to mate naturally in a local area with whatever drones were naturally available (i.e., without using an isolated area).

Materials and Methods

1. Obtaining and mating hygienic-stock queens

In our laboratory we have been quantifying and breeding for hygienic behaviour for several years using open mating without instrumental insemination. From the colonies available, we chose four "mother" colonies (A, B, C, D) that showed high levels of hygienic behaviour as shown by the freeze-killed brood (FKB) bioassay (Spivak and Reuter, 1998b). Average FKB removal in these colonies, based on four trials per colony, was 86, 88, 92 and 96% respectively. Queen cells were reared by grafting one-day old larvae, a standard queen rearing method (Laidlaw and Page, 1997). These queen cells were used to produce fertilised queens via natural mating (NM) or instrumental insemination (II).

For NM, ripe queen cells were placed individually in queenless Apidea mating nucleus hives in a nearby apiary, ca. 20km away, in Shoreham (West Sussex – Grid Ref. TQ 21460 06338). For II, queens emerged from their cells in an incubator and were then placed individually into wooden queen-mailing cages with 5 attendant workers, and fed on honey as needed (Bigio et al., 2012). Virgin queens were inseminated following standard procedures by an experienced queen inseminator (Mr. Redmond Williams) using a Schley device with semen extracted from mature drones from colonies A-D. Queens were inseminated with semen from several drones from each of the other colonies. For example, daughter queens from colony A were inseminated with drones from colonies B, C and D, etc. This was to avoid inbreeding via brother-sister mating. To inseminate each queen we used a capillary tube and Harbo syringe (modified by Peter Schley) to collect and inject semen from 10-15 males, 3-4 per drone-mother colony. This was to ensure genetic diversity in the resulting workers. Natural mating to

many males leads to colonies that are characterized by high levels of genetic diversity in the workers, which has been shown to have a beneficial impact on colony productivity (Mattila and Seeley, 2007), exploitation of food sources (Mattila et al., 2008) and disease infections (Tarpy and Seeley, 2006). All inseminated queens were paint marked and had their wings clipped. Clipping ensured that they were unable to mate naturally.

To ensure that the drones used for II were from the correct breeder colonies, and had not drifted among colonies in the apiary (Pfeiffer and Crailsheim, 1998), we adopted the following procedure. First we placed frames of empty drone cells into the brood chamber of each colony. When these frames contained brood they were moved above the queen excluder in each colony. In this way, following emergence, the adult drones were confined to the upper part of the hive. Periodically, the hive was inspected and these drones were paint-marked on the notum with a colony-specific colour code and placed below the queen excluder so that they could fly at will and mature normally. Marked drones were harvested when needed for insemination.

2. Testing for hygienic behaviour

The resulting naturally mated (n =15) and instrumentally inseminated (n = 11) queens that were observed laying eggs were removed from the mating nucleus hives and introduced into queenless hives, consisting of 1 medium depth Langstroth hive box with 10 frames (Pierco). These hives were kept in two apiaries, one at the laboratory and the other 3km away. Testing for hygiene began 6 weeks later, at which time the workers that were old enough to carry out hygiene (Arathi et al., 2000) were the offspring of the new queens, of which 12 NM and 9 II remained alive.

We determined the level of hygienic behaviour using the freeze-killed brood bioassay (Spivak and Reuter, 1998b) three times per hive at weekly intervals from August 25 to September 10. At this time of year the colonies were actively rearing brood and the hives were 50-75% full with bees. Previous research has shown that colonies of this strength show levels of hygienic behaviour that are not significantly different to stronger colonies (Bigio et al., 2013). For each colony, two suitable patches of capped worker brood were tested on the same side of the same frame. Two metal cylinders (6.5 cm diameter × 8 cm height) were pressed into the comb until they reached the mid-rib. Approximately 300 mL of liquid nitrogen was poured into each

cylinder to kill the circle of brood inside. After 5-10 min the nitrogen had evaporated, the cylinders were removed, and photographs of each patch and the whole frame were taken before returning the frame to the hive. After 48 h we removed the frame from the hive to photograph the treated areas. From the photos we determined the proportion of capped cells from which the freeze-killed brood had been removed.

3. Unselected colonies

We also tested 20 randomly selected colonies from our apiaries that did not belong to our breeding program using the FKB bioassay. The tests were made over the same period but not on the exact same days due to practical constraints. This was to provide a general comparison to the colonies headed by the experimental queens above, and to verify that the high levels of hygiene seen in the selected colonies (see Results) were not found in all colonies at this time of year and in this region. Colonies were housed in commercial hives and kept in two apiaries within 15 km of the laboratory.

4. Statistical analysis

Hygienic behaviour was quantified as the proportion of capped cells killed with liquid nitrogen from which the dead brood had been removed after 48 h. We used generalized estimating equations with binomial distributions and log link functions to investigate the levels of hygienic behaviour shown, with the three FKB trials being included as a repeated measure. We first compared the selected and unselected colonies. We then carried out a second analysis comparing the II and NM selected colonies, and including the mother colony of each daughter queen as a factor. All analyses were carried out in IBM SPSS 21.0 (2012).

Results

A total of 60 FKB bioassays were made using the colonies with hygienic queens and another 60 with the unselected colonies (20 colonies × 3 trials per group). A total of 20,248 capped cells (mean: 169 cells per colony per trial) were treated with liquid

nitrogen, of which 17,234 (85%) were removed after 48h.

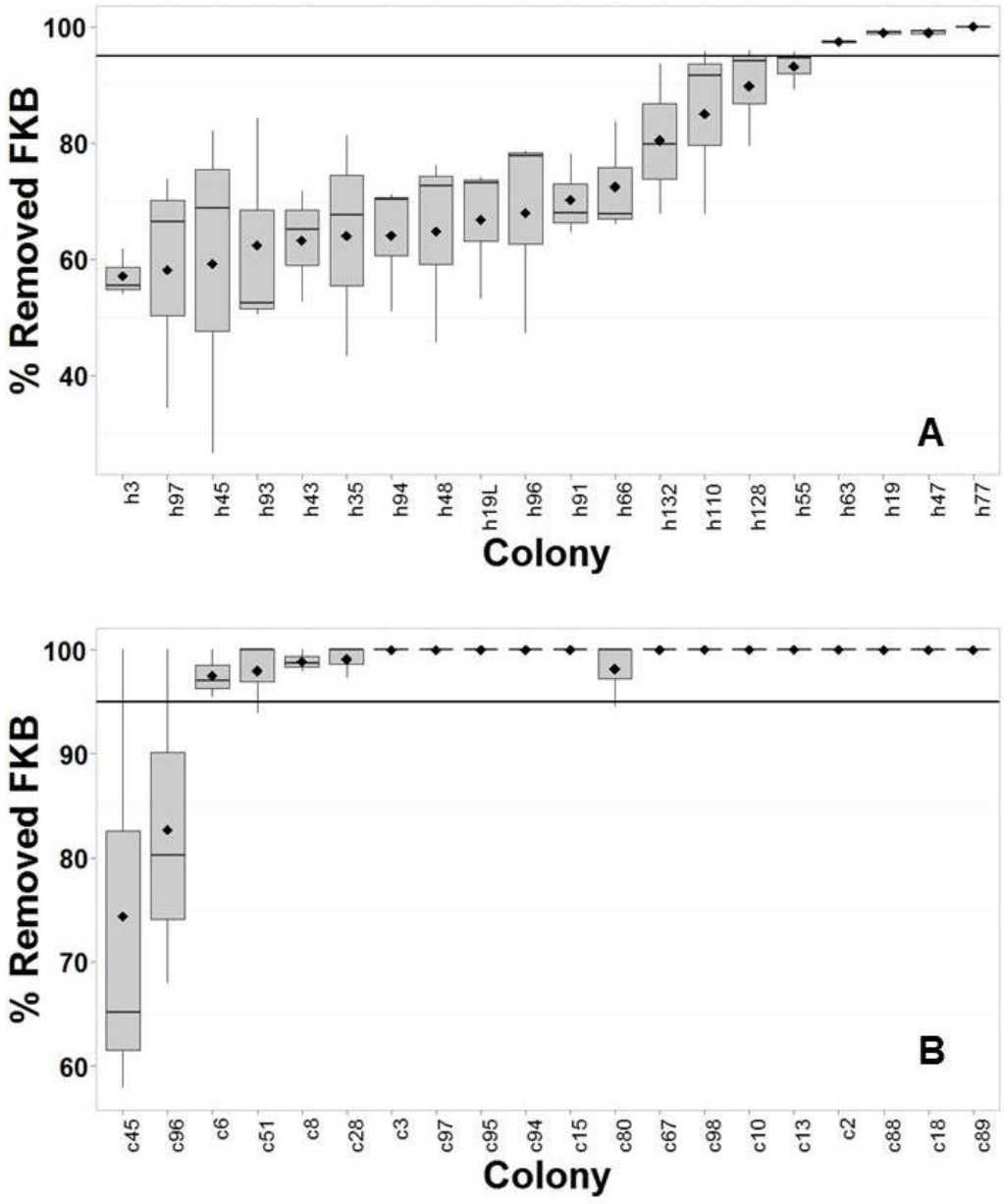

Figure 7.1 a/b: Percentage of freeze-killed brood removed within 48 hours by unselected (1a) and selected (1b) colonies that were each screened in 4 trials. The boxes represent the interquartile range and the bar indicates the median. Whiskers extend to 1.5 times the interquartile range, with black dots representing outliers. The dotted line represents the 95% threshold. Black dots on each boxplot represent mean FKB removal.

There was a significant difference in the removal of FKB between selected and unselected colonies (Wald χ^2 = 14.6, *df*= 1, P < 0.001) (Figure 7.2a). FKB removal in the unselected colonies ranged from 26.6 to 100% (mean 75.7%, SD 18.9%) (Figure 7.1a). FKB removal in colonies with hygienic queens ranged from 94.5 – 100% (mean

99.8%, SD 1.1%) for the instrumentally inseminated queens and 57.8 – 100% (mean 95.5%, SD 11%) for the naturally mated queens (Figure 7.1b). All colonies headed by an instrumentally inseminated (n = 9) queen had 100% hygiene in all trials apart from one (Colony 80, Trial 3, 94.5%), showing that they were all extremely hygienic. Colonies headed by naturally-mated queens (n = 11) also had high levels of hygiene. Of these 11 colonies, 9 had FKB average levels above 97% with 5 at 100% (Figure 7.2b). The difference in FKB removal between colonies of the II and NM queens was significant (Wald $\chi^2 = 10.1$, $df = 1$, P = 0.002). There was also a significant effect of the mother colony from which the queens were reared, (Wald $\chi^2 = 32.4$, $df = 3$, P < 0.001), as expected given that the four mother colonies varied in their own levels of hygienic behaviour.

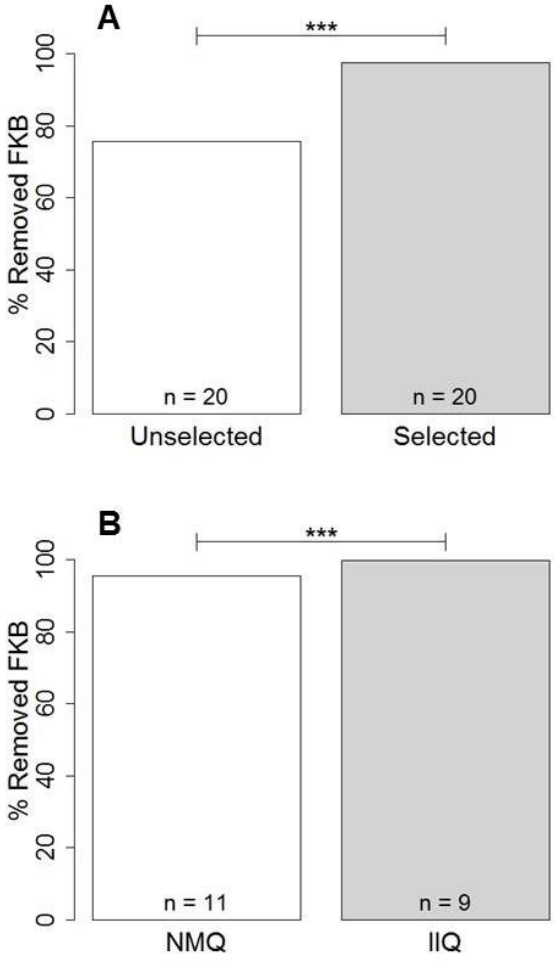

Figure 7.2 a/b: Difference in average removal of freeze-killed brood, when comparing selected/unselected colonies (2a) and colonies led by naturally mated(NMQ)/instrumentally inseminated (IIQ) queens (2b). Both graphs show the number of colonies tested.

Discussion

Both groups of experimental colonies had high levels of hygienic behaviour, greater than that of the unselected colonies. This shows that the high levels of hygiene observed when using the FKB bioassay were not simply due to common environmental conditions, something which was unlikely but which now is clearly excluded (Bigio et al., 2013).

The levels of hygiene in the 9 colonies headed by instrumentally inseminated (II) queens were almost 100% in all three FKB trials per colony. This shows that, as expected (Rothenbuhler, 1964a) a breeding program can result in very high levels of hygiene especially when mating control is exercised over both the males and females (Spivak, 1996).

Although the mean level of hygiene (n = 3 FKB trials per colony) in the 11 colonies with naturally mated (NM) queens was lower on average, 95.5%, than in the colonies with instrumentally inseminated queens, 99.8%, this is still high in absolute terms. Nine out of the 11 colonies had FKB removal of 97% or more, which is above the 95% threshold recommended for considering a colony to be hygienic. The two that were below this threshold had hygiene levels of 74%% and 83%, which is still high when compared to background levels of hygiene in unselected populations detected in previous studies (Bigio et al., 2013; JA Pérez-Sato, 2007; Waite et al., 2003).

Our results have encouraging implications for beekeeping because they show that a breeding program for hygiene without the use of II can be successful in breeding hygienic bees. This is well within the capability of any individual beekeeper or association who can rear their own queens and learn how to make a FKB bioassay. These results confirm that II is a valuable tool for selective breeding, but also show that several (3 or 4) generations of colonies headed by naturally-mated, selected queens can also provide colonies with high levels of hygiene. Indeed, the breeding program in our laboratory has been based on natural mating, with the II used in this experiment being the first time that we used this technique. Other breeding programs have also obtained good results without using II (Guzman-Novoa and Page, 1999; Pérez-Sato et al., 2009).

Our results are also encouraging in terms of overcoming the major challenge of supplying other beekeepers with hygienic queens. In particular, it is much harder to supply instrumentally inseminated (II) than naturally mated (NM) queens, and also

harder to supply mated than virgin queens. In commercial queen rearing, it is possible to rear c. 40 queen cells in a single finisher colony in c. 5 days. Each of these cells can give rise to one virgin queen. To mate these queens, they each have to be placed into a hive of their own (usually a small nucleus colony). The mating process from the time a queen cell is placed into a nucleus hive to the time in which a queen is confirmed to be laying worker eggs, hence ready to be harvested, is c. one month (Graham, 1992). This shows that the bulk of the resources in the queen mating process are to convert queens from virgin to mated status. For example, a beekeeper operating just 2 finisher colonies could easily rear 300-400 virgins per month. But to mate these would require 300-400 nucleus hives.

The high levels of hygiene we have shown for the naturally-mated daughter queens reared from hygienic mother colonies suggest that a queen rearer could supply virgin queens of hygienic stocks to other beekeepers, who would introduce them into their own hives to mate locally. Although virgin queens are considered to be harder to introduce into hives, the success rate can be almost 100% if the correct and simple methods are used (Pérez-Sato et al., 2007). One advantage of supplying virgin queens is that, by mating locally, the resultant colonies are combining hygienic traits with any locally-adapted or selected traits. A second advantage is the greater ease by which virgins can be supplied, compared to naturally-mated queens.

In recent years, honey bees are much in the news due to the challenges they face. It is encouraging, therefore, to report on something that may be used to improve the health of colonies and which is practical in terms of beekeeping.

Chapter 8: Developing a line of locally-adapted hygienic honey bees at the Laboratory of Apiculture and Social Insects

Gianluigi Bigio, Luciano Scandian, Hasan Al Toufailia, Francis L.W. Ratnieks

For this chapter I contributed to the design of the project, to the acquisition, analysis and interpretation of the data and to the writing of the manuscript. Hasan Al Toufailia and Luciano Scandian assisted with data collection and hive management. Francis Ratnieks assisted with the design of the project and manuscript writing.

Abstract

In order to develop a stock of honey bees that show high levels of hygienic behaviour, we performed freeze-killed brood (FKB) assays and selective breeding. Starting from colonies with queens that were naturally mated to locally-available drones, we systematically screened the colonies using the FKB assay, and reared honey bee queens using larvae from the best performing hives. Selective breeding was carried on for 4 generations of honey bee colonies over 3 years, and resulted in colonies that showed high levels of hygienic behaviour that are suitable both for research and to provide to beekeepers for further evaluation. Starting with an average FKB removal of 74.9% and one colony out of 36 with a mean FKB removal rate above 95%, after four generations the average FKB removal was 99.5% and all eight colonies had a mean FKB removal rate above 95%.

Introduction

Studied since the late 30s (Park, 1936), hygienic behaviour in honey bees (*Apis mellifera*) is a heritable trait believed to be controlled by multiple quantitative trait loci (Lapidge et al., 2002; Oxley et al., 2010) that confers social immunity to the colony (Wilson-Rich et al., 2009) against diseases that affect brood. Even a fraction of worker bees expressing this phenotype is enough to make a colony hygienic, resulting in dead or parasitized brood in capped cells being detected and removed (Arathi et al., 2000). Hygienic behaviour has been proven to be an effective measure against various brood diseases such as American foulbrood (Rothenbuhler, 1964b; Spivak, 1996; Spivak and Reuter, 2001), chalkbrood (Gilliam et al., 1983; Trump et al., 1967), against the parasitic mite *Varroa destructor* (Boecking and Drescher, 1992; Spivak, 1996; Spivak and Reuter, 1998b, Al Toufailia et al. *in prep*) and viruses that are transmitted by Varroa such as DWV (Al Toufailia et al. *in prep*).

The spread of fluvalinate-resistant strains of *Varroa destructor* (Milani, 1999) increases the value of natural resistance such as hygienic behaviour. Colonies left untreated for Varroa may sustain damage not only by the direct feeding action of the mite on the larvae and adult bee, but also from viral infections vectored by the mite (Bowen-Walker et al., 1999; Shen et al., 2005). Previous studies (Harbo and Harris, 1999; Rothenbuhler, 1964a; Spivak and Reuter, 1998a) have shown that hygienic behaviour is heritable and can successfully be bred for. In the United Kingdom a few beekeepers each year produce small quantities of honey bee queens (Lodesani and Costa), but to our knowledge nobody in the UK has an ongoing breeding program aimed at selecting honey bees that display high levels of hygienic behaviour. In recent years some beekeepers in the USA have started commercial rearing of hygienic queens (Spivak et al., 2009).

We carried out a breeding program to select for hygienic honey bees and obtain stocks that would repeatedly display high levels of freeze-killed brood removal in which brood is killed with liquid nitrogen (Spivak and Reuter, 1998b). It had two major goals which were both accomplished. First, to provide colonies suitable for further research on hygienic behaviour (Bigio et al., 2013, Al Toufailia et al. *in prep*) and second, to provide British beekeepers with a source of locally adapted, hygienic bees thereby limiting the importation of non-indigenous ones. Hygienic behaviour occurs in bee

populations wherever it has been looked for but generally at low levels (Bigio et al., 2013; JA Pérez-Sato, 2007; Waite et al., 2003) and local bees provide an advantage as they may be better adapted to the local conditions (Büchler et al. In Press.), hence there is a value in breeding hygienic bees in particular locations, such as in England.

Materials and Methods

1. Study colonies

For the duration of the breeding program (from August 2011 to September 2013) colonies were managed to minimize swarming, thereby retaining their queens and genetic structure. Any colony that replaced its original paint-marked queen was dropped from the program. Colonies were either housed in National deep brood boxes with up to ten deep frames, or in the case of the last trial, in medium-depth Langstroth hives. Colonies were given additional hive boxes above a queen excluder as needed, depending on colony population, time of year, and honey stores.

The project started by repeatedly screening colonies belonging to a semi commercial beekeeper for hygienic behaviour. The beekeeper had been keeping hives in the local area for more than 25 years. During this time he reared most of his own queens by providing queenless nucleus colonies with a comb containing young brood taken from a colony that satisfied his requirements, which included being non-defensive and having low amounts of chalk brood visible in the combs.

In August 2011 we identified 36 suitable colonies housed in National deep brood boxes, located in two of the beekeeper's apiaries in the Shoreham area in Sussex. We carried out nine freeze-killed brood (FKB) assays during the first season, and we reared queens from the hive that showed the highest levels of hygienic behaviour. The second generation was composed by 13 colonies housed in National deep brood boxes, located in two of the beekeeper's apiaries. Those colonies were FKB tested eight times and we reared queens from the five most hygienic colonies. The third generation was composed by 19 colonies housed in both National deep and Langstroth medium brood boxes. Those colonies were screened seven times and we reared queens from the two most hygienic colonies. Second and third generation colonies were kept in apiaries within 10

miles of Brighton. The fourth generation was composed by eight colonies housed in Langstroth medium brood boxes and were located in apiaries located on the University campus. Those colonies were FKB screened 3 times and will be screened again in 2014 to identify the best performing colonies and continue this breeding program.

2. Quantifying levels of hygienic behaviour via Freeze Killed Brood (FKB) assay

The ability of each colony to uncap and remove diseased or parasitized brood was measured via the FKB assay described by Spivak and Reuter (Spivak and Reuter, 1998b). After locating suitable patches of sealed brood, two metal cylinders (6.5 cm diameter and 8 cm height) were pushed into the sealed brood until reaching the mid-rib of the comb. Approximately 300 mL of liquid nitrogen was poured slowly into the cylinders to kill the brood in the selected area. After 5-10 minutes, the nitrogen had evaporated and the cylinders were removed. Photographs of each patch were then taken and the frame was returned to its hive. Forty-eight hours later, the frame was removed and the same patches were photographed again to determine the number of cells from which the brood in the capped cells had been removed.

3. Artificial selection and production of daughter colonies

The colonies that showed the highest levels of average FKB removal after several trials were considered suitable to act as sources of young queens for the following generation. Virgin queens were obtained adapting the methodology described in (Büchler et al., 2013) in which 1-2 day old larvae are collected from the hives previously identified, and transferred to plastic queen cups, placed in a dummy frame that is then placed in the middle of a very strong, queenless hive. The resulting queencells are then introduced into queenless Apidea mating nucleus hives. The resulting queens then mated naturally with locally available drones. In some colonies that were in the more advanced stages of the breeding program, we also used instrumental insemination to preform controlled crosses (Bigio et al., 2014). Successfully mated queens that were laying eggs were transferred from the mating nucleus hive into a normal-sized hive, which would develop into a full sized colony that

could itself be evaluated for hygienic behaviour.

4. Statistical analysis

Hygienic behaviour was quantified as the proportion of capped cells killed with liquid nitrogen from which the dead brood had been removed after 48 h. We used generalized estimating equations with binomial distributions and log link functions to investigate the levels of hygienic behaviour shown, with the FKB trials performed on the same colony over time included as a repeated measure. We compared the effect of breeding among colonies belonging to different generations and then we performed pairwise comparisons between generations 1 to 4, using a sequential Bonferroni procedure to correct for multiple comparisons. All analyses were carried out in IBM SPSS 21.0 (2012).

Results

1. Hygienic behaviour – general results, differences between generations and effects of selection and breeding

The study started in August 2011 and the last set of data was collected in September 2013. In total 77 colonies were screened over 20 FKB trials, for a total of 396 individual freeze-killed brood (FKB) assays performed. Overall FKB removal ranged from 19 to 100%, with mean of 79.26 % and standard deviation of 21.64 %. Considering each generation separately, the 1st, 2nd, 3rd and 4th generations had mean FKB removal of 74.92%, 87.28%, 82.72%, 99.46% respectively (SDs were 20.81%, 18.06%, 24.77% and 1.53%, see figure 8.1).

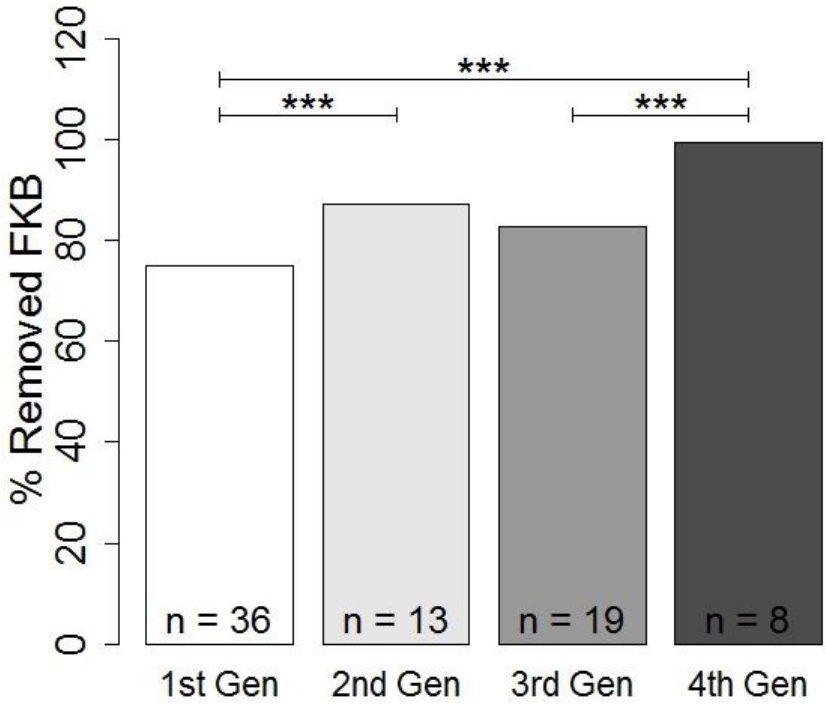

*Figure 8.1: Average removal of freeze-killed brood, between colonies belonging to subsequent generations. In each bar is noted the number of colonies tested. *** mark the statistically significant differences.*

2. Hygienic behaviour –within generation results and choice of mother colonies

Within the 36 colonies belonging to the 1st generation across all 255 trials, over which FKB removal ranged from 18.75% to 100%, only one colony had a mean FKB removal rate above 95% and four colonies had a mean FKB removal rate above 90%. One colony (A9 see Figure 6.1) was selected as "mother colony" and acted as a source of young larvae to produce the next generation of queens.

Those daughter queens resulted in 13 second generation colonies. Across a total of 53 trials, FKB removal ranged from 32.21% to 100%. Five colonies had a mean FKB removal rate above 95% and seven colonies in total had a mean FKB removal rate above 90%. Five colonies (F1A9A, H1, F109F1AA, F1E9 and H3 see Figure 8.2), therefore, were highly hygienic (>95% FKB removal) and were selected to act as "mother colonies".

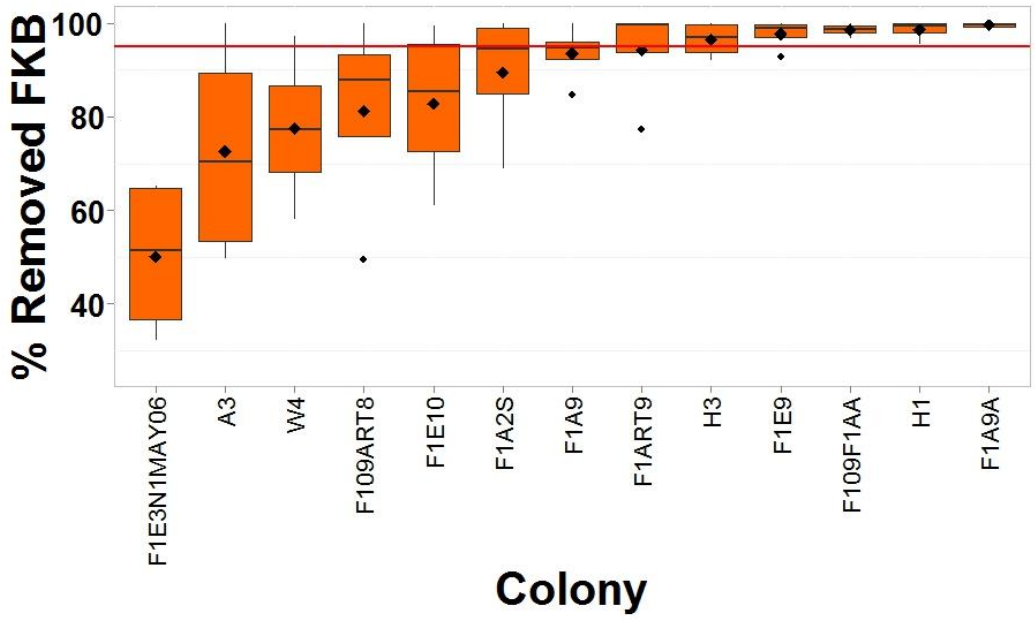

Figure 8.2: Percentage of freeze-killed brood removed within 48 hours by 13 honey bee colonies of second generation each screened in 8 trials. The boxes represent the interquartile range and the bar indicates the median. Whiskers extend to 1.5 times the interquartile range, with black dots representing outliers. The red line represents the 95% threshold. Black dots on each boxplot represent mean FKB removal.

Daughter queens from these colonies gave 19 third generation colonies. They were tested for a total of 64 trials and FKB removal ranged from 20.1 % to 100 %; eleven colonies had a mean FKB removal rate above 95% and the remaining eight colonies had a mean FKB removal rate below 90%. Two colonies met our criteria (89 and 80 see Figure 8.3) and the daughter queens bred from them led to eight colonies 4th generation colonies. These were tested over three occasions. Across 24 trials FKB removal ranged from 94.27% to 100 %; and all of the eight colonies had a mean FKB removal rate above 95%.

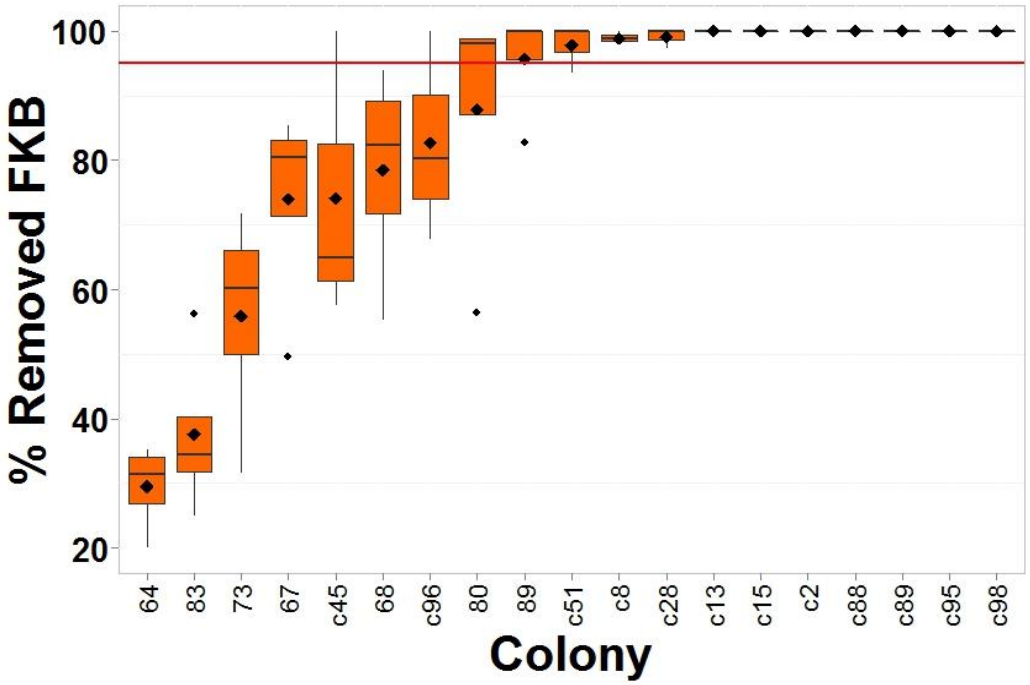

Figure 8.3: Percentage of freeze-killed brood removed within 48 hours by 19 honey bee colonies of third generation each screened in 8 trials. The boxes represent the interquartile range and the bar indicates the median. Whiskers extend to 1.5 times the interquartile range, with black dots representing outliers. The red line represents the 95% threshold. Black dots on each boxplot represent mean FKB removal.

When we applied the model, generation was a significant predictor for the removal of FKB (Wald χ^2 = 126.82, *df* = 3, P < 0.001) and there were significant differences in FKB removal between colonies belonging to different generations (Table 8.1, Figure 8.1). In particular average removal increased significantly from 74.92% to 87.28% between the 1st and the 2nd generation, decreased to 82.72% between the 2nd and the 3rd although in a non-significant way and significantly increased to 99.46% from the 3rd to the 4th generation (Sequential Bonferroni test).

Discussion

Our results confirm previous experiments, and show that is possible to breed honey bee colonies that express high levels of hygienic behaviour using standard beekeeping techniques in combination with the freeze-killed brood (FKB) bioassay. These results are extremely encouraging for the beekeeping community and for existing breeding

programs being carried out by beekeepers. They show that in a few generations it is possible to obtain colonies that display high levels of hygienic behaviour with two methods that beekeepers can use: 1) queen rearing, which is a standard beekeeping technique; 2) The FKB bioassay, which can be carried out in an apiary by a beekeeper and does not require specialized equipment. Other studies being carried out in our laboratory link high levels of hygienic behaviour (90% and greater) with lower build-up rates of Varroa mites and less symptoms of deformed wing virus (DMV), (Al Toufailia et al. *in prep*) underlining even further the advantages of hygienic behaviour in beekeeping.

Our breeding programme relied exclusively on locally obtained honey bee genetic material. Studies have shown that locally-adapted honey bees perform better than imported ones (Büchler et al. *in prep*) and one of the main goals was to provide beekeepers with bees that would be better suited to the environmental conditions found in Britain.

Our results underline the importance of constant screening and selective choice of the colonies that would act as mother colonies, that is as donors of female larvae be used to rear into next generation virgin queens. The results we obtained until the 3rd generation relied on commonly used beekeeping techniques paired with the FKB assay, and overall the reiterated selection significantly accounted for the increase of hygienic levels. The slight decrease in average FBK removal observed from the 2nd to the 3rd generation is not statistically significant and the trend is inverted when we consider the 3rd and 4th generation. After only 4 generations of selection, we achieved very high (near 100%) levels of hygienic behaviour with a variance in FKB removal also close to zero.

Most of the queens heading the colonies used in this experiment were naturally mated, reducing the risk of inbreeding. In another study (Bigio et al 2014) we showed how instrumental insemination can give even better results than open mating. This is a technique that can be combined with open mating. However, it requires specific training and practice to be carried out proficiently. In addition, because instrumental insemination allows the control of the source of both the young queens and the drones, care needs to be taken to avoid inbreeding.

The hygienic line that we obtained represents the foundation of a breeding program that can be continued and expanded both by selecting for other traits of interest, such as low levels of defensiveness, and by increasing the levels of hygiene expressed. One way

to do this, if FKB removal were to reach 100% after 48 h would be to reduce the time to 24 h. Another way of enhancing the breeding program is to provide hygienic queens to experienced beekeepers in order to evaluate their performance in commercial beekeeping.

As the breeding programme described here is only a part of the overall breeding plan carried out at our laboratory, future plans also include screening of previously unselected colonies looking for high levels of hygienic behaviour to include in the program. Focusing on a single line using strong truncation selection (i.e., choosing few colonies from which to rear queens) is not ideal in the long term, but if several such programs are being followed, starting from a different "mother colony" they would add genetic variability and we would limit the chances of accidental inbreeding.

Chapter 9: Comparing alternative methods for holding virgin honey bee queens for one week in mailing cages before mating

Gianluigi Bigio, Christoph Grüter, Francis L.W. Ratnieks

For this chapter I contributed to the design of the project, to the acquisition, analysis and interpretation of the data and to the writing of the manuscript. Christoph Grüter assisted with data analysis and manuscript writing. Francis Ratnieks assisted with the design of the project and manuscript writing.

Abstract

In beekeeping, queen honey bees are often temporarily kept alive in cages. We determined the survival of newly-emerged virgin honey bee queens every day for seven days in an experiment that simultaneously investigated three factors: queen cage type (wooden three-hole or plastic), attendant workers (present or absent) and food type (sugar candy, honey, or both). Ten queens were tested in each of the 12 combinations. Queens were reared using standard beekeeping methods (Doolittle/grafting) and emerged from their cells into vials held in an incubator at 34° C. All 12 combinations gave high survival (90 or 100%) for three days but only one method (wooden cage, with attendants, honey) gave 100% survival to day seven. Factors affecting queen survival were analysed. Across all combinations, attendant bees significantly increased survival (18% vs. 53%, $p < 0.001$). In addition, there was an interaction between food type and cage type ($p < 0.001$) with the honey and plastic cage combination giving reduced survival. An additional group of queens was reared and held for seven days using the best method, and then directly introduced using smoke into queenless nucleus colonies that had been dequeened five days previously. Acceptance was high (80%, 8/10) showing that this combination is also suitable for preparing queens for introduction into colonies. Having a simple method for keeping newly-emerged virgin queens alive in cages for one week and acceptable for introduction into queenless colonies will be useful in honey bee breeding. In particular, it facilitates the screening of many queens for genetic or phenotypic characteristics when only a small proportion meets the desired

criteria. These can then be introduced into queenless hives for natural mating or insemination, both of which take place when queens are one week old.

Introduction

Beekeepers and researchers often keep honey bee queens alive outside a colony for short periods of time. For example, queens are frequently sent through the mail from a queen breeder to another beekeeper. In this situation the queen is generally mated and spends a few days in the cage and is then introduced into a queenless hive. A recent paper from Gençer (2003) highlighted the need for mated queens in times of the year when queen rearing is not possible, and devised a successful methodology to overwinter them in reservoir colonies in order to have queens available in early spring. Virgin queens may also be held outside a colony as part of the queen rearing process, allowing a greater flexibility in the schedule. Commercial queen rearing typically produces a sequence of mated queens from each mating nucleus hive and if the mating flights of the current batch of queens are delayed due to poor weather, the queen cells can be emerged in an incubator and the resulting virgin queens would be introduced into the mating hives instead of ripe queen cells. This methodology can provide beekeepers with additional time, up to approximately one week (Pérez-Sato and Ratnieks, 2006).

Another advantage of emerging and keeping virgin queens out of a colony is that it provides an opportunity for selection and testing. Selection can be very simple and quick, such as when a queen is visually inspected for wing deformities or appropriate body colour, or more technical, such as when wing morphometry or genetic tests are performed (Pérez-Sato and Ratnieks, 2006). For example, when selecting for certain behavioural traits like hygienic behaviour, many virgin queens from a hygienic colony can be reared, then genotyped using a small piece of wing tissue (Châline et al., 2004) in order to identify queens that have the same father as the workers that are most hygienic (Pérez-Sato et al., 2009). With the progress being made in honey bee genetics, including genome sequencing (Munoz-Torres et al., 2011) and the identification of genes (Ben-Shahar et al., 2002) and quantitative trait loci (Hunt et al., 1995, 1998, 2007; Oxley et al., 2010; Rüppell et al., 2004) linked with behavioural or other phenotypical traits, it is likely that in the future molecular markers that denote desirable characteristics will be available as tools for marker-assisted selection.

One challenge in using intra-colony selection with molecular markers is that the majority of queens will often be discarded. If, for example, behavioural tests on the workers in a hygienic colony show that only one or two patrilines are hygienic (Pérez-

Sato et al., 2009), then only approximately 10% of any queens reared will belong to these patrilines given that honey bee queens mate with multiple males (Estoup et al., 1994; Page Jr, 1986; Tarpy et al., 2004). If every daughter queen has to be held in a colony while testing is being carried out, rather than held in a cage outside a colony, this will require much greater resources, effort and cost.

Therefore, there is an incentive to streamline the process to find the most economical method to keep a majority of queens alive for the greatest number of days.

Nelson and Roberts (1967) report that of 12 virgin queens stored in bespoke wooden cages, one died after three days, one after five days and five after 17 days. The five surviving queens were artificially inseminated and introduced into colonies on day 17. The purpose of our study was to investigate the effects of three factors (cage type, food type, presence or absence of attendant workers) on the survival of newly-emerged virgin queens stored in commercially available mailing cages. We measured survival for the first week of life because this is a biologically relevant duration given that natural mating (Eigil Holm, 2009; Gerula et al., 2011; Laidlaw and Page, 1997; Oertel, 1940) occurs approximately one week into adult life. Additionally, one week is also the optimum time for instrumental insemination (Cobey, 2007a; Woyke and Jasinski, 1976). Lastly, one week is sufficient time to carry out genetic tests, which may involve a few days delay to deliver samples to a testing lab. Our results show that the different combinations of factors gave very different survival rates, ranging from 0% to 100% after one week. Only one method (wooden cage, honey, attendants present) gave 100% survival at day seven.

Methods

1. Rearing and preparation of virgin queens

Queen cells were reared using standard beekeeping methods in which one-day-old larvae from worker cells were transferred (“grafted”) into queen cups and reared in queenless colonies (Laidlaw and Page, 1997). The hives used were in the apiary adjacent and belonging to the laboratory. The bees were of mixed European races, predominantly *Apis mellifera mellifera*. Ten or eleven days after grafting, sealed queen

cells were placed individually into glass vials in an incubator (34° C) and were kept there until emergence, after which they were placed in cages.

2. Conditions under which queens were held

Queens were held individually in cages in a temperature controlled room, 22° C and given water *ad libitum* by placing droplets on the mesh covering each cage. Survival was determined at 09.00 and 18.00 each day. Three factors (cage type, food type, attendant workers) were tested in a complete three-way design, with 12 combinations and 10 queens per combination.

Cage type. (2 treatments: wooden cage, plastic cage). We used two commercially-available and commonly-used queen mailing cages, one made of wood and one of plastic: "Three-hole" wooden cages with a metal mesh top (manufacturer W. T. Kelley, USA) and plastic "Puzzle" cages (manufacturer Swienty A/S, Denmark) (Figure 9.1).

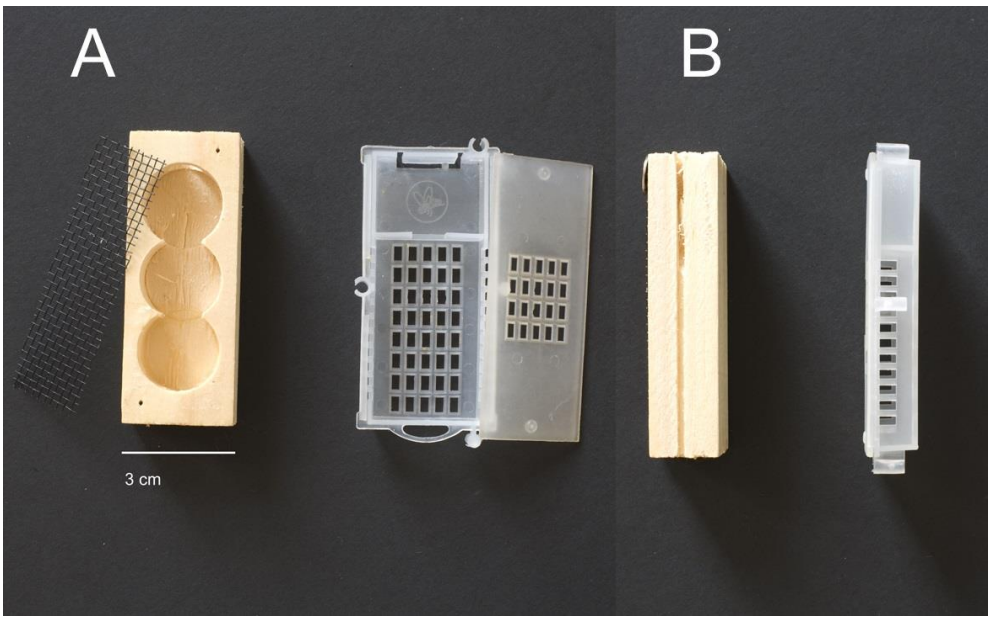

Figure 9.1: Cage types used in the experiment, top (A) and side (B) view: "Three-hole" wooden cage (left) and "Puzzle" plastic cage (right).

Food. (3 treatments: candy, honey, candy+honey). Beekeepers have various preferences for feeding queens in cages. Queen cage candy is widely used, especially when sending queens by mail, because it is solid. Honey is natural and although it is not suitable for use when mailing queens, it is suitable for keeping queens alive if mailing is

not needed. Candy was prepared by mixing together semi-crystallised honey and powdered sucrose in an approximate 1:4 weight ratio. Each cage was given 0.5-0.8g honey in one of the end holes of the cage, or 8-10g of candy by filling the food compartment in a plastic cage or one of the three circular cavities in a wooden cage, or both. Whenever honey was used, either alone or in combination with candy, more was given when daily inspections showed that existing supplies were low. The honey used was from our own apiary and so was known to be free of American foulbrood spores, as this disease is extremely rare in Britain and has never occurred in our apiary. We recommend that where AFB is common, honey should either not be used or, if practical, first be sterilized with γ-rays.

Attendant workers. (2 treatments: 5 or 0 workers) A frame containing sealed brood was placed in the incubator. Five newly-emerged workers were collected and introduced in each cage.

3. Survival of queens and introduction in nucleus hives

Following the experiment that measured cage survival, we reared ten additional queens and held them for one week using the method that gave the greatest survival (wooden cage, honey, five attendants) to test their acceptance into queenless nucleus hives. These were five-frame medium depth Langstroth with two to three frames of bees including one to two with brood, and were fed twice with 300 ml of 2M sucrose solution prior to and during queen introduction. Queens were introduced using the direct method with smoke (Perez-Sato et al., 2008) five days after queen removal, which maximises acceptance rate. We determined the acceptance of each introduced queen 24h later by inspecting the hive. Any hive in which the queen was not seen was closed and checked again after one hour to verify it was absent.

4. Statistical analysis

Data were analysed in R 2.10.1 (R Development Core Team, 2012) by fitting a linear generalised mixed-effects model using the LMER function of the LME4 package (Bates et al., 2013). We included colonies from which the larvae used to rear queens were collected as a random effect to control for the non-independence of the data (Zuur

et al., 2009). For model selection, we used the protocol proposed by Zuur et al. (2009 chapter 5). To explore the best random effects structure, we compared random intercept models with random intercept and slope models (Zuur et al., 2009).

We tested the significance of fixed effects (food, cage, attendants) on queen survival at day seven by treating queen survival as a binomial response: 0 for queens that died and 1 for queens that survived. Additionally, queen weight at emergence was entered as a covariate. We used the Wald test to determine the significance of each fixed effect and the likelihood ratio test was used to test for significant interactions (Zuur et al., 2009). Since food treatments had three levels we performed pairwise comparisons using the multicomp package (Hothorn et al., 2008) and corrected significance levels with sequential Bonferroni (Sokal and Rohlf, 1981).

Results

1. Queens obtained

In total 120 virgin queens were obtained from larvae that were transferred from six "mother colonies" (14 queens obtained from colony A, 17 from B, 53 from C, 6 from D, 19 from E and 11 from F). We randomly allocated 10 queens to each of the 12 combinations.

2. Queens survival in cages

All 12 combinations initially performed well with 90% or 100% queen survival up to day three (Table 9.1). However, by day 7 survival varied from 0 to 100% with only one combination (wooden cage, honey, with attendants) achieving 100% survival (10/10 queens alive; Table 9.1).

To analyse queen survival we started with a model containing all the effects and interactions and then removed the non-significant interactions (Crawley, 2007). The presence of attending bees was an important factor, increasing mean survival probability by 35% across all combinations (Figure 9.2; survival without attendants = 18%, with attendants = 53%, z = 3.743, $p < 0.001$).

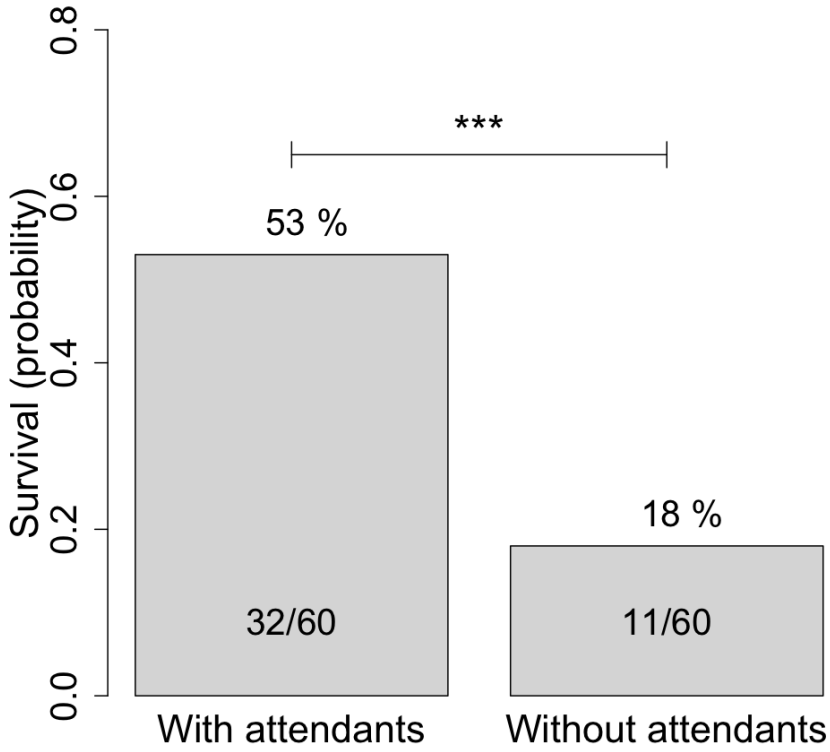

Figure 9.2: Probability of surviving until day 7 of virgin queens in cages with and without attendants, across the other treatments.

We found a significant interaction between food type and cage type (Figure 9.3; LRT = 25.352, *df* = 2, $p < 0.001$). Because of this interaction, we analysed the effect of food separately in plastic and wooden cages. In plastic cages, honey alone had a negative impact on survival compared to candy alone, reducing survival by 35% (z = -2.588, $p = 0.0206$). Conversely, using honey with candy improved the survival duration by 35% compared to honey alone (z = 2.704, $p = 0.0206$). The difference between candy alone or candy with honey was not significant (z = 0.215, $p = 0.8314$).

In wooden cages the differences in survival duration among food types, (proportion alive after 7 days: candy 40%, honey 65%, both 25%), were not significant when comparing honey and candy (z = 1.224, $p = 0.4418$) and honey and candy with candy alone (z = -1.208 , $p = 0.4418$). However using honey with candy instead of honey alone showed a tendency to reduce survival (z = -2.305 , $p = 0.0636$).

There was no effect of queen weight on survival (z = -0.484, $p = 0.6282$).

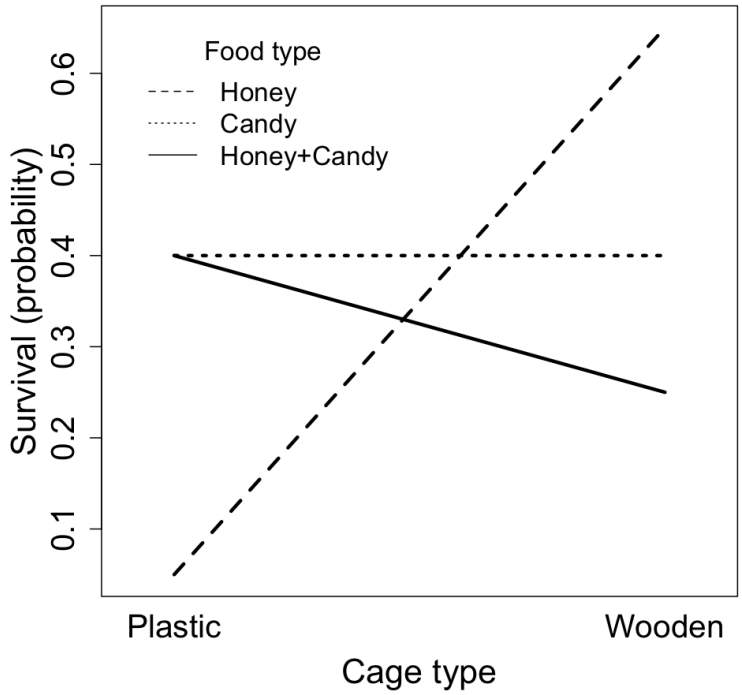

Figure 9.3: The interaction between cage and food type shows that honey alone negatively affects queens' survival in plastic cages.

3. Acceptance of queens into queenless nucleus hives

Eight of ten (80%) queens were successfully accepted into the queenless nucleus hive. This proportion is not significantly different from the 100% acceptance rate previously reported by Pérez-Sato et al. (Perez-Sato et al., 2008), (p = 0.1082, chi-square test).

Discussion

Our results show clearly that there is significant variation in the survival of virgin queens under different combinations of cage conditions during their first week of adult life. The best combination (wooden cage, honey, with attendant workers) gave 100% (10/10) survival in experiment 1 and also 100% in experiment 2 (10/10). The worst combination (plastic cage, honey, without attendant workers) gave 0% (0/10) survival.

We suggest the following reasons why certain combinations resulted in low survival.

Plastic cages are designed to use candy not liquid honey. Even the small amount of honey we placed in the cage resulted in the queen becoming covered, which presumably hastened death. However, in the wooden cages the queens did not get covered presumably because the honey was partly absorbed into the wood. Attendant workers were the most important factor increasing queen survival by 35%. Therefore we highly recommend that they should be provided. Although an important goal of our project is to find a method that minimises the workload needed to maintain queens alive, the additional work needed to provide attendants is not great. It takes just a few minutes per cage, and workers are easily obtained either from a colony or by emerging brood in an incubator.

The only method to give 100% 7-day survival is simple to use and also gave high acceptance, 80%, of queens introduced into queenless colonies. This was not significantly lower than the 100% acceptance measured in a previous study using the same introduction method (Perez-Sato et al., 2008). As a result, we are confident in recommending this method to beekeepers and scientists who are rearing and breeding queens as it provides similar survival rate to previous studies (Gencer, 2003) but uses commercially available materials.

With the progress being made in honey bee genetics it is likely that in the future, there will be greater use of marker-assisted selection (MAS) on queens to decide which to retain in a breeding program or provide to beekeepers. Genetic tests can be made within a few days, and individual queens kept or discarded according to the results. A honey bee queen's one week pre-mating (or pre-insemination) period is an ideal time to perform tests as each queen can be kept in a cage, rather than in a colony, thereby saving time, effort and hive resources when compared to the alternatives, such as keeping queens in hives or in queen "banks". Queen banking, in which caged queens are held in a populous and well-fed queenless colony, is often used by commercial queen rearers to keep mated queens alive for a few days or weeks prior to being sold. Virgin queens can also be kept alive in queen banks prior to insemination (Cobey, 2007a, 2007b). However, based on our own experience (Ratnieks, personal observation), virgin queens have poor survival when banked compared to mated queens.

Queen rearing has been practised for over 100 years using the same basic method developed by Doolittle in the USA, involving the transfer one–day-old larvae. Queen breeding has benefited from the development of instrumental insemination, and will likely soon see benefits coming from the use of molecular markers (MAS) for desirable

characteristics. But to use these methods effectively they will need to be combined with modifications of traditional procedures, such as keeping queens alive in cages.

Conditions:			Number of virgin queens alive after experimental day:						
Attendants	Cage	Food	1	2	3	4	5	6	7
0	Plastic	Candy	10	10	10	10	5	1	1
5	Plastic	Honey	10	10	10	10	6	2	1
0	Plastic	Honey	10	9	9	4	1	1	0
0	Wood	Candy	10	10	9	9	7	6	4
5	Wood	Candy	10	10	10	10	9	9	4
0	Wood	Honey	10	9	9	9	8	7	3
0	Plastic	Honey+Candy	10	10	9	6	3	2	2
5	Plastic	Candy	10	10	10	10	9	8	7
5	Plastic	Honey+Candy	10	10	10	10	10	10	6
5	Wood	Honey+Candy	10	10	10	9	9	9	4
0	Wood	Honey+Candy	10	10	9	8	6	3	1
5	Wood	Honey	10	10	10	10	10	10	10

Table 9.1: Survival of virgin queens during the first seven days of adult life under specified conditions

Part 3 – Final Discussion and References

Chapter 10: Final discussion and future directions

Hygienic behaviour is a complex and fascinating subject. In this thesis I focused on some aspects that are of particular relevance for bee breeders and bee researchers to gain more insight on this subject. The aim was to help streamline the process of breeding better honey bees, with increased disease resistance and other traits of interest. While we managed to get some answers, often these topics led to further and wider questions. In this last section will try and summarize what this thesis adds to the field and what future ideas and plans have arisen from it.

1. Variation in levels of hygienic behaviour

A large part of this thesis focuses at understanding what factors could cause variation in the detected levels of hygienic behaviour in unselected colonies. Screening colonies using the freeze-killed brood (FKB) assay is time consuming and variation in the results can complicate the choice of the best performing hive. In chapters 2 and 3 we show that of all the monitored factors the ones causing significant variation in FKB removal rates were also not likely to occur in a real-life scenario.

When testing honey bee colonies a breeder would normally choose strong, productive colonies and would not perform assays on colonies that are not in optimal conditions. During our experiments we monitored food availability, brood amount, strength of the colony and the time of the year in which we performed the FKB assay. Based on our results we can conclude that environmental conditions have a limited impact on the ability of colonies of removing freeze-killed brood, and the colony genetic identity explained over 40% of the total variation of hygienic behaviour.

2. Assessing the potential costs of hygienic behaviour

One of the laboratory main goals was to obtain a genetic strain of hygienic bees that could be distributed to local beekeepers. To achieve this, we had to ensure that the bees we were breeding would not present negative traits, limiting their acceptance and use.

Previous studies (Rothenbuhler, 1964a) have hypothesised that hygienic bees would

also display defensive behaviour. Defensive behaviour is an undesirable trait that represents a cost in managed honey bee colonies because it slows beekeeping operations and spoils what can be an enjoyable activity. Results presented in chapter 3 show how FKB removal does not correlate with defensive behaviour and indeed the hygienic colonies we obtained show very little defensive attitude.

In chapter 4 we investigated one of the possible reasons for which hygienic behaviour is quite rare, with only c. 10% of unselected colonies showing high levels of hygiene. Previously (Seeley, 1985) it has been hypothesised that hygienic bees would have a colony-level cost by removing also more healthy brood and this would have limited the diffusion of hygienic behaviour. The results we obtained do not support the hypothesis and further investigations are required to find out if hygienic behaviour has a trade-off. We can conclude that we did not find any detrimental aspect to hygienic behaviour.

Figure 10.1: Because beekeeping is a fun activity, we wanted to obtain bees that were also docile.

3. Technical advancements in beekeeping techniques

Bee researchers borrow a lot of techniques from beekeepers and during my studies we investigated two aspects related to selective breeding: the survival of virgin queens in cages and the importance of controlled mating when breeding for hygienic bees.

Chapter 6 presents a valid methodology to keep virgin queens using a minimal set-up prior to their mating and introduction in a queenless hive. With the progress being made in molecular biology, it is likely that in the future, there will be greater use of marker-assisted selection (MAS) to decide which queen to retain and which to discard for a breeding program. And our proposed method would allow introducing queens only after they have been tested with molecular markers, optimizing the resources.

Instrumental insemination is the most reliable technique for complete control over honey bee queen mating and obtaining specific crosses. However due to the experience needed to achieve proficiency, it has been slowly adopted by the commercial industry. In chapter 4 we show that it is possible to obtain acceptable levels of hygienic behaviour without artificial insemination.

4. Breeding hygienic bees

In parallel with all the experimental projects, we set out to develop a genetic line of hygienic honey bees. We started screening locally obtained honey bee colonies, and we obtained queens from the best performing ones. After 4 generations over 3 years, the resulting colonies had a mean FKB removal rate of almost 100%.

The results we obtained are extremely encouraging for the beekeeping community, as they show that selective breeding for hygienic behaviour can be carried out in an apiary by a proficient beekeeper. Using two techniques, queen rearing and the FKB bioassay, and repeated events of screening and selective breeding, it is possible to considerably increase the levels of hygienic behaviour.

Additionally, as described in chapter 3, we can suggest that breeding bees that display low levels of chalkbrood will result in obtaining bees with high levels of FKB removal.

5. Final thoughts and future directions

Luckily for me, hygienic behaviour still generates interesting questions, and I plan to continue my investigations on this topic in my future career.

The fundamental steps to obtain bees that express high levels of hygienic behaviour are constant screening and selective breeding and both are methods well suited as an

activity carried out by beekeeping clubs. During my doctoral studies, I took part in various outreach activities, such as talks and workshops aimed at sharing our results with the public and to train beekeepers. Given our results as described in this thesis, I hope that either single beekeepers or beekeeping clubs take up selective breeding and obtain their own hygienic colonies, or they get involved with the laboratory helping to further improve the line that we developed.

Figure 10.2: Beekeepers were very keen in knowing what we studied. Pictured here, a queen rearing workshop with Worthing BKA members. (Photo courtesy of J. Scrace)

The hives resulting from the project described in chapter 5 will be screened by my colleagues Luciano Scandian and Hasan Al Toufailia even further, both for FKB removal and also for effective disease resistance, by assessing the levels of Varroa and chalkbrood infestation. We will obtain queens from the colonies that display high hygienic behaviour and low infestation. In our quest towards a better bee we will continue to select the least defensive colonies, and we will as well include honey productivity as a trait to monitor.

In parallel, we will screen other unrelated colonies, in order to obtain hygienic colonies starting from a different "mother colony" and add genetic variability and limit the chances of accidental inbreeding. Genetic diversity impacts several traits and behaviours, not only in honey bees (Mattila and Seeley, 2007; Mattila et al., 2008) but also in other social insects (Oldroyd and Fewell, 2007).

An interesting experimental idea comes from the one described in chapter 4, on the importance of instrumental insemination when breeding for hygienic behaviour. It

would be interesting to perform controlled mating between queens and drones obtained by previously unselected colonies that showed high levels of hygienic behaviour, and compare the FKB removal ability with colonies headed by “sister queens” that were openly mated. The hypothesis is that when dealing with queens representing the first selected generation, the use of instrumental insemination to obtained controlled matings will be of greater importance.

Lastly, by developing a line of bees that constantly display low levels of FKB removal, we would be able to further investigate where and if hygienic behaviour has a cost. By having colonies managed under the same conditions, the only difference being the different FKB removal ability, we could compare other traits of interest, such as honey production and colony development. Moreover, following on from previous studies (Lapidge et al., 2002; Oxley et al., 2010), the DNA extracted from bees belonging to both lines could be analysed using next-generation sequencing techniques, hopefully providing further knowledge on what portions of the honey bee genome control the expression of hygienic behaviour.

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