

ORIGINAL RESEARCH ARTICLE



The effect of one generation of controlled mating on the expression of hygienic behaviour in honey bees

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Summary

Honey bee mating cannot be directly controlled in the same way as in many agriculturally important animals. Instrumental insemination is, however, 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 (FKB) bioassay we compared the levels of hygiene in colonies headed by daughter queens reared from hygienic mother colonies that were either instrumentally inseminated with sperm from drones reared from hygienic colonies or allowed to mate naturally with naturally-occurring drones. Hygiene levels were significantly higher in the colonies of the instrumentally inseminated queens than in the colonies of the naturally-mated queens. 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.

El efecto de una generación de apareamiento controlado sobre la expresión del comportamiento higiénico en las abejas melíferas

Resumen

El apareamiento de la abeja de la miel no puede ser controlado directamente de la misma forma que se hace en muchos animales de importancia agrícola. La inseminación instrumental es, sin embargo, posible y puede ser utilizada como ayuda en la cría selectiva. El comportamiento higiénico, en el que las abejas obreras detectan y eliminan la cría muerta o enferma de las celdas operculadas, es un rasgo hereditario que confiere resistencia al nivel de colonia contra enfermedades de la cría. Utilizando el bioensayo de congelar la cría para matarla (BCM) se compararon los niveles de higiene en las colonias gobernadas por hijas de reinas criadas en colonias madre higiénicas que fueron inseminadas instrumentalmente con esperma de zánganos criados de colonias higiénicas o a las que se dejó aparearse naturalmente con zánganos de origen natural. Los niveles de higiene fueron significativamente mayores en las colonias de las reinas inseminadas instrumentalmente que en las colonias de las reinas apareadas naturalmente. Sin embargo, los niveles de higiene en las colonias que se aparearon naturalmente fueron alentadoramente altos e indican que suministrar a los apicultores reinas apareadas naturalmente, o reinas vírgenes para que se apareen localmente, puede dar lugar a colonias con altos niveles de higiene.

Keywords: *Apis mellifera*, hygienic behaviour, instrumental insemination, natural mating, breeding

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; Spivak and Reuter, 1998; Wilson-Rich *et al.*, 2009; Rinderer *et al.*, 2010; Schöning *et al.*, 2012) as hygienic colonies are able to detect, uncap and remove dead or diseased brood (Rothenbuhler,

1964). 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 agricultural animals (Ratnieks, 1998; Pérez-Sato *et al.*, 2009). In particular, controlling mating is difficult. Queens naturally mate in flight with 10–20 males (drones) that gather in drone congregation areas and come from many different hives (Woyke, 1955; Koeniger, 1986; 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, by 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 appear to be hygienic if only a fraction of the workers are hygienic (Arathi *et al.*, 2000), belonging to the a few hygienic patriline among the patrilines present in the colony (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 (Watson, 1927; Nolan, 1932; Mackensen and Roberts 1948; Woyke, 1960; Ball *et al.*, 1983; Laidlaw and Page, 1997; Baer and Schmid-Hempel, 2005). 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, 2007).

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 instrumentally inseminated using 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).

Material and methods

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 (Carreck, 2011). 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, 1998). 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, 1985; 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 an 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 five attendant workers, and fed on honey as needed (Bigio *et al.*, 2012). Virgin queens were inseminated following standard procedures by an experienced queen inseminator 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.

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 plastic frames (Pierco). These hives were kept in two apiaries, one at the laboratory and the other 3km away. Testing for hygiene began six 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 11 NM and 9 II remained alive.

We determined the level of hygienic behaviour using the freeze-killed brood bioassay (Spivak and Reuter, 1998) three times per hive at weekly intervals from 25 August to 10 September 2013. At this time of year, the colonies were actively rearing brood and the hives were 50-75 % full of 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.

Unselected colonies

We also tested 20 randomly selected colonies from our apiaries that did not belong to our breeding programme using the FKB bioassay. The tests were made over the same period but not on exactly the

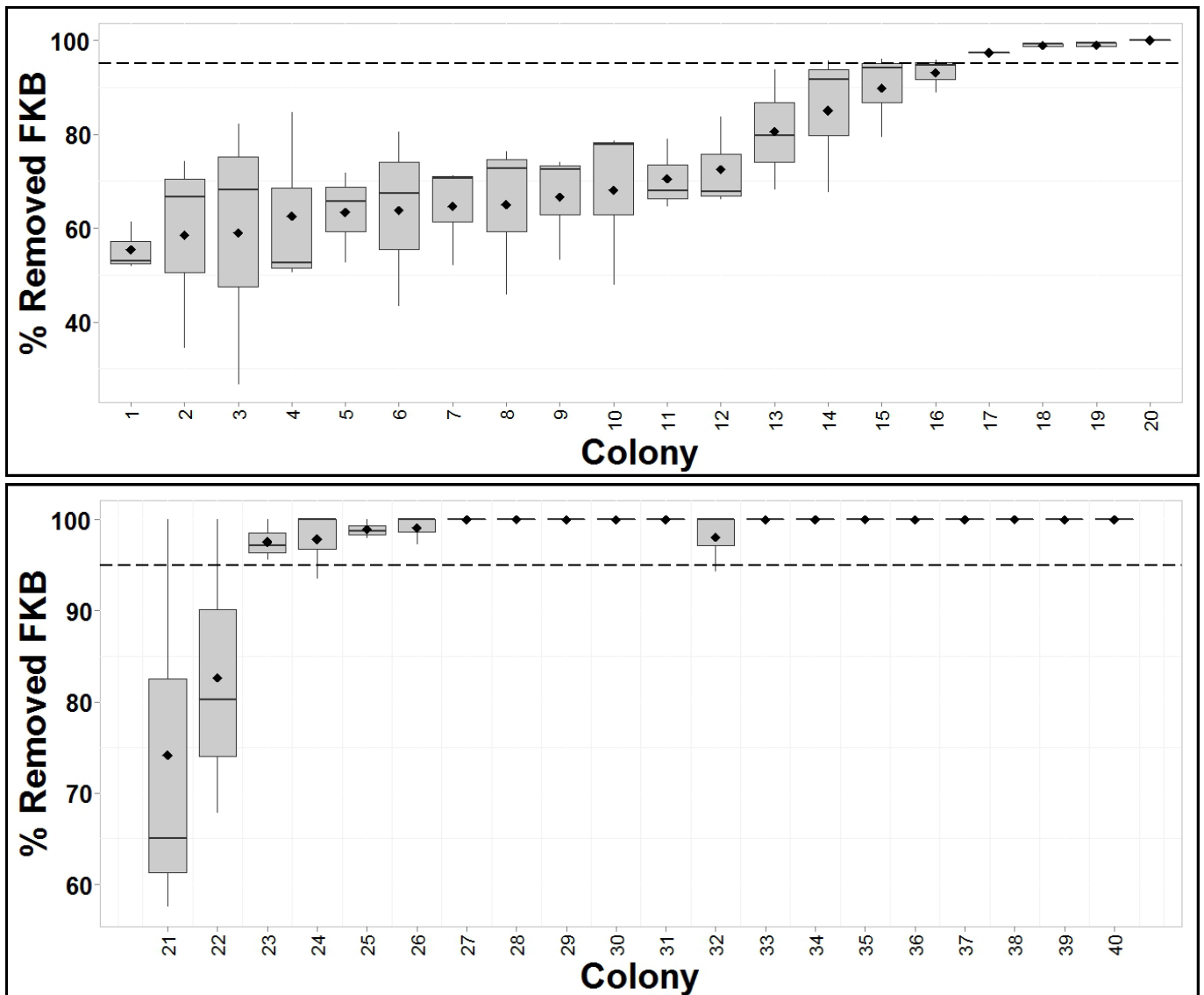


Fig. 1. Percentage of freeze-killed brood removed within 48 hours by unselected (**1a, top**) and selected (**1b, bottom**) colonies that were each screened in four trials. The boxes represent the interquartile range and the bar indicates the median. Whiskers extend to 1.5 times the interquartile range, with black dots representing outliers. The dotted line represents the 95 % threshold. Black dots on each boxplot represent mean FKB removal. In **Fig. 1b**, colonies 21 to 31 were naturally mated, colonies 32 to 40 were instrumentally inseminated.

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.

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 (IBM SPSS, 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 \times 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.

There was a significant (Wald $\chi^2 = 14.6$, $df = 1$, $P < 0.001$) difference in the removal of FKB between selected and unselected colonies. FKB removal in the unselected colonies ranged from 26.6 to 100 % (mean 75.7 %, SD 18.9 %) (Fig. 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 (Fig. 1b). All colonies headed by an instrumentally inseminated ($n = 9$) queen had 100 % hygiene in all trials apart from one (Colony 32, 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 %. 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 (Wald $\chi^2 = 32.4$, $df = 3$, $P < 0.001$) effect of the mother colony from which the queens were reared, as expected given that the four mother colonies varied in their own levels of hygienic behaviour.

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, 1964) a breeding programme 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 (Waite *et al.*, 2003; Pérez-Sato *et al.*, 2009; Bigio *et al.*, 2013).

Our results have encouraging implications for beekeeping because they show that a breeding programme for hygiene without the use of II can still 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 programme 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 programmes 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 two 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 have been 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.

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