Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: www.tandfonline.com/journals/tjar20

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To cite this article: Ralph Büchler, Sreten Andonov, Richard Bernstein, Kaspar Bienefeld, Cecilia Costa, Manuel Du, Martin Gabel, Krispn Given, Fani Hatjina, Brock A. Harpur, Andreas Hoppe, Nikola Kezic, Marin Kovačić, Per Kryger, Fanny Mondet, Marla Spivak, Aleksandar Uzunov, Jakob Wegener & Jerzy Wilde (2025) Standard methods for rearing and selection of *Apis mellifera* queens 2.0, Journal of Apicultural Research, 64:2, 555-611, DOI: 10.1080/00218839.2023.2295180

To link to this article: https://doi.org/10.1080/00218839.2023.2295180

Published online: 19 Mar 2024.

JOURNAL OF APICULTURAL RESEARCH
2025, VOL. 64, NO. 2, 555–611
https://doi.org/10.1080/00218839.2023.2295180

REVIEW ARTICLE

Standard methods for rearing and selection of *Apis mellifera* queens 2.0

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ABSTRACT

Here, we cover a wide range of methods currently in use and recommended in modern queen rearing, selection, and breeding. The recommendations are meant to serve as standards for scientific and practical beekeeping purposes. The basic conditions and different management techniques for queen rearing are described, including recommendations for suitable technical equipment. As the success of breeding programmes strongly depends on the selective mating of queens, a subsection is dedicated to the management and quality control of mating stations. Recommendations for the handling and quality control of queens complete the queen rearing section. The improvement of colony traits usually depends on a comparative testing of colonies. Standardised recommendations for the organisation of performance tests and the measurement of the most common selection characters are presented. Statistical methods and data preconditions for the estimation of breeding values that integrate pedigree and performance data from as many colonies as possible are described as the most efficient selection method for large populations. Alternative breeding programmes for small populations or certain scientific questions are briefly mentioned, including an overview of the young and fast developing field of molecular selection tools. Because the subject of queen rearing and selection is too large to be covered within this paper, ample references are given to facilitate comprehensive studies.

Métodos estándar para la cría y selección de reinas de *Apis mellifera 2.0*

Se describe una amplia gama de métodos actualmente en uso y recomendables sobre la cría actual de reinas, su selección y cruzamiento. Las recomendaciones tienen el propósito de servir de igual forma como estándares para fines apícolas tanto científicos como prácticos. Se describen las condiciones básicas y las diferentes técnicas de manejo para la cría de reinas, incluyendo recomendaciones para el equipo técnico adecuado. Dado que el éxito de los programas de mejora depende en gran medida el apareamiento selectivo de reinas, se dedica un subcapítulo a la gestión y control de calidad de las estaciones de apareamiento. Las recomendaciones para el manejo y control de calidad de las reinas completan la sección de cría de reinas. La mejora de las características de colonias por lo general, depende de ensayos comparativos entre colonias. Se presentan recomendaciones normalizadas para la organización de pruebas de rendimiento y la medición de los caracteres de selección más comunes. Aquellos métodos estadísticos y condiciones previas de datos para la estimación de valores de cruzamiento que integren los datos genealógicos y de rendimiento de tantas colonias como sea posible, se describen como los métodos de selección más eficientes para grandes poblaciones. Se mencionan también pero brevemente, otros programas alternativos de cruzamiento para poblaciones pequeñas, o ciertas preguntas científicas, incluyendo una descripción general del reciente campo de rápido desarrollo de las herramientas de selección molecular. Debido a que el tema de la cría de reinas y la selección es demasiado extenso para ser desarrollado en este trabajo, se proporcionan numerosas referencias para facilitar estudios integrales.

ARTICLE HISTORY
Received 5 May 2022
Accepted 12 November 2023

KEYWORDS
Honey bees; selection characters; performance testing; queen production; mating control; molecular selection; breeding values

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饲养和选择 西方蜜蜂蜂王的标准方 法
本文介绍了当前在蜂王培育、选 择和育种中正在使用和 推荐的多种方法。这些
方法旨在为科学研究和实际养蜂操 作提供标准方法。这里介绍了培育蜂王的基本
条件和不同的饲养管理 技术, 并推荐了对应的育 王设备。育种项目成功
与否很大程度上取决于 处女王的选择性交配, 因 此本文有一部分专门阐 述了交尾场的
管理和质量控制。此 外, 蜂王培育部分还包括了关于蜂王的操作和质 量控制的
建议。蜂群性 状的改良通 常依赖于对蜂群的对比 测定。我们提供了组 织性能测定
和常见选择性状测定的 标准化建议。育种值评 估是大种群 的最有效选 育方法, 我
们对育种值评估的统计 方法和数据前提进行了 介绍, 该方法 需要从尽可能多的蜂群
获得性能数 据并与系谱数据相结合 。简要介绍了为小种群 和解决特定科学问
题而开展的育种项目, 包括新 的、发展迅速的分子选 择技术。鉴于蜂王培育 和蜂种选育
涵盖面很广, 本文难以做 到面面俱到, 因此也给出了大量的参 考文献以便于对该领域
的综合研究。

Please refer to this paper as: Büchler, R., Andonov, S., Bernstein, R., Bienefeld, K., Costa, C., Du, M., Gabel, M., Given, K., Hatjina, F., Harpur, B. A., Hoppe, A., Kezic, N., Kovacic, M., Kryger, P., Mondet, F., Spivak, M., Uzunov, A., Wegener, J., Wilde, J. (2024). Standard methods for rearing and selection of *Apis mellifera* queens 2.0. In *V. Dietemann, P. Neumann, N.L. Carreck, & J.D. Ellis (Eds) The COLOSS BEEBOOK 2.0, Volume I: standard methods for Apis mellifera research. Journal of Apicultural Research*, 64(2): https://doi.org/10.1080/00218839.2023.2295180.

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1. Introduction

Adaptation through natural selection is the natural response of bee populations to environmental changes and the challenge of pests and diseases. The richness in biodiversity of races and ecotypes of *Apis mellifera*, the western honey bee, reflects a long lasting, continuous process of adaptation. This diversity represents a highly valuable biological capital that is worth preserving as a basis for future selection and development in response to new ecological and production challenges.

The highly complex reproductive biology of honey bees, including multiple mating of queens, long distance mating flights, male haploidy, excess drone production and drone congregation areas, has

evolved as an effective toolbox for the selection of genetically diverse honey bee populations. However, modern beekeeping and breeding techniques may limit or extinguish these natural selection effects (Bouga et al., 2011), and these risks lowering the vitality of bee populations.

Responsible breeding activities consider the natural reproductive biology of honey bees. Modern techniques of queen rearing, selection and mating control offer very powerful tools to improve the economic, behavioural and adaptive traits of honey bees. Here, we describe the available techniques in honey bee breeding, and recommend scientific and technical standards concerning the most important aspects of breeding programs for genetic improvement and the conservation of honey bees (Uzunov et al., 2017, 2022). This manuscript serves as an update to an earlier BEEBOOK manuscript originally published by some members of the current author team (Büchler et al., 2013). These standards include basic considerations for honey bee breeding, together with recommendations for performance testing, breeding values estimation and selection, mating control, and the multiplication (queen rearing) and propagation of breeding success into the broader population.

It is important to distinguish between queen rearing and bee breeding as many beekeepers confuse the two. Queen rearing simply describes the production of healthy vigorous queens. Most queen producing operations do not breed bees; instead, they produce many queens without a strict selection protocol. In contrast, bee breeding involves the selection of specific heritable traits and controlled mating of selected parents.

Internationally approved quality standards for queen rearing, mating, and performance testing are needed for the improvement, comparison, and exchange of breeding stock, and to fulfil the demands of the market. We share the vision that these recommendations will help preserve natural diversity in honey bees and support the production of high-quality queens in a physiological and genetic sense. The use of standard, high-quality queens is a prerequisite for any research on colony development and behaviour, as well as for economically successful beekeeping.

2. Queen production

2.1. Queen rearing techniques

2.1.1. Basic principles of queen rearing

A honey bee colony can produce a new queen without human intervention as long as fertilised eggs (eggs that result in female honey bees) are present. Beekeepers have developed techniques to rear large numbers of queen bees to requeen colonies regularly (every year or two), to reduce swarming, to increase brood and honey production, to start new colonies, and to change certain genetic characteristics (Laidlaw & Page, 1997; F. Ruttner, 1983). Many US beekeepers requeen as often as twice a year.

The key step in rearing a high-quality queen is beginning with a densely packed colony that has many young nurse bees. Such colonies have a high swarming impulse and want to produce queens. A beekeeper takes a young (12–24 h old) larva from a worker cell and places ("graft") it into a queen cell cup suspended vertically in a hive. The larva is fed a special royal jelly diet by the nurse bees. After 10–11 days, the queen cells, from which queens are ready to emerge, can be transferred to the queenless hives or mating nuclei ("nucs") (Woodward, 2010). These nucs should have all stages of brood present to increase the rate of queen cell acceptance. The success and quality of queen production depends on a strong, well fed, and healthy nurse colony and on suitable equipment and colony management. Over the last century, beekeepers have developed ways to exploit honey bees to produce healthy queens using various techniques.

2.1.2. Equipment for queen rearing

Most systems of queen rearing use standard beekeeping equipment but employ some specialised equipment during the process. Most of the specialised equipment is inexpensive or can be constructed by the beekeeper.

2.1.2.1. Cell cups, bars and frames.

- Larvae are transferred (grafted) to artificial queen cell cups (wax or plastic). The cups are affixed to bars, which are then placed in frames (Figure 1). Queen cell cups should measure 8–9 mm in inside diameter.
- Cell cups can be produced from beeswax as follows:
  1. Select beeswax that is not contaminated with hive medications and pesticides. It's best to get it from newly built beeswax that never had brood or from wax capping after uncapping combs for honey extraction.
  2. Melt beeswax in a double boiler with water at 55–65 °C.
  3. Prepare a „cell mandrel" by dipping it into the soapy water. This will help to take of the cells from the mandrel. Currently, silicone devices are used to make wax cups that do not require soaking in soapy water, as the cups are easily removed after cooling (Figure 2(d)).

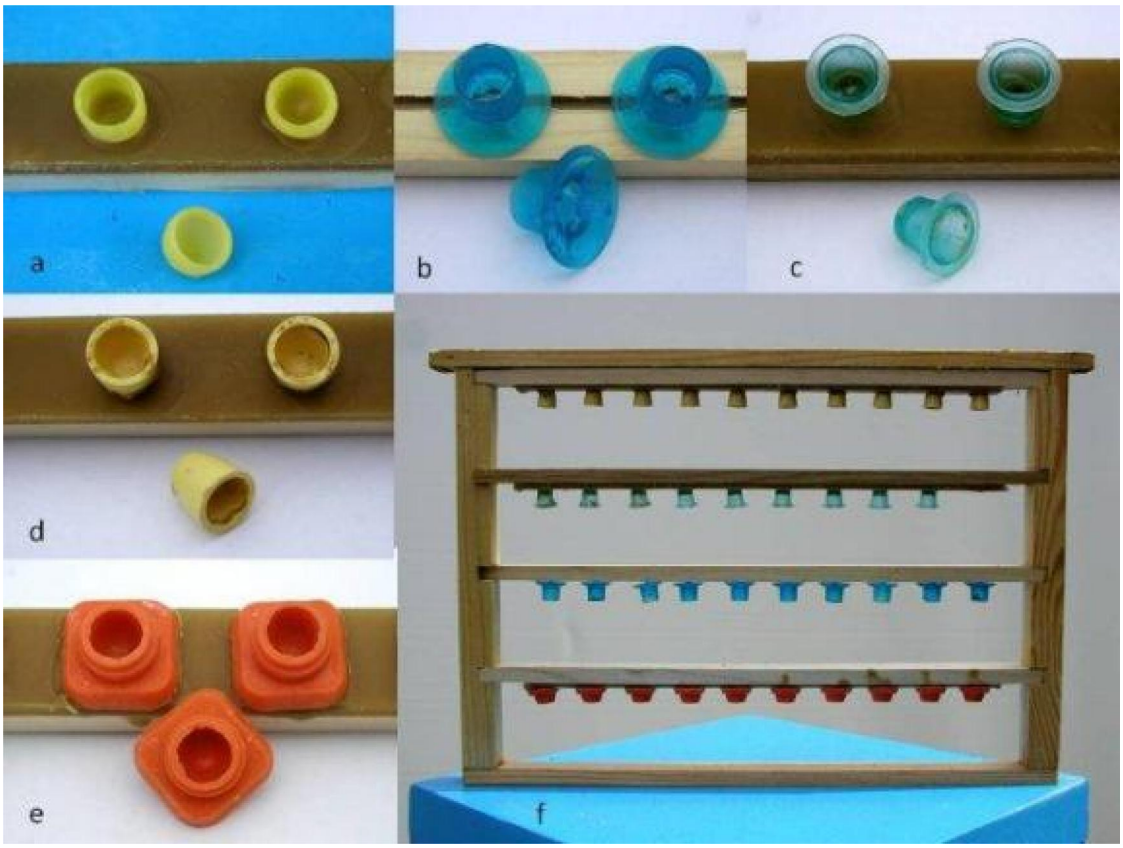


Figure 1. Different: (a) wax; and (b–e) plastic queen cups and ways to attach them to the bars; (f) frame with bars ready for grafting. Photos: J Wilde.

4. Dip the cell mandrel into the melted wax for a few seconds and repeat it 2–3 times (Figure 2(a–c)).
5. After cells are made, wash them in the water to eliminate traces of the soap.
6. Keep prepared cell cups free of dust by storing them in a sealed box.

- Most queen producers attach their homemade beeswax cell cups directly to a cell bar with hot wax. To do this, they dip the base of the cell cups in molten beeswax (beeswax melts at 62.3–65.2 °C) and firmly push the cup base onto the cell bar as the wax cools. Typically, 10–20 cells are attached to each bar with 20–60 cell cups per frame.
- Alternatively, plastic cell cups can be purchased from beekeeping suppliers. Common cell cups include the JZ-BZ Push In and Base Mount Queen Cell Cups from Mann Lake Ltd (http://www.mannlakeltd.com/) in the USA or Nicot in Europe (http://nicot.fr/).
  1. Previously used plastic cell cups can be reused after scraping out royal jelly from the base of the cups and washing the cups in warm water with a little detergent (liquid soap, approx. 2 ml for 1000 ml of water).
  2. The cups should be left to dry thoroughly before attaching them to a cell bar.
  3. Alternatively, you can dip your used plastic cell cups into molten beeswax and reuse them successfully. Such cleaning might not prevent an outbreak of black queen cell virus (BQCV). It is advisable to use new cell cups whenever possible.
  4. Placing plastic queen cell cups into strong colonies about one day before grafting allows the bees to clean, polish and prepare the cell cups (Figure 3). This procedure will increase the acceptance of grafted larvae. Plastic cups are attached with molten clean wax as described above.
  5. Additionally, dip the rim of the outside four cell cups located at each end of the cell bar into wax to increase the acceptance of grafted larvae (4 cell cups in total).
- Special push-in queen cell cups that are available commercially make preparing the cell bars simple (Figure 1(b)). These cells have a raised area on their base that snaps into a groove on the cell bar. The bar then can be inserted into the frame.
- A frame (wooden, plastic or metal) of standard dimensions that will hold 2–4 cell bars can be used (Figures 1(f) and 4).

2.1.2.2. Grafting tools. Grafting tools are used to transfer young female larvae from a cell in a beeswax comb to the cell cups. They are typically composed of a thin shaft and have a scooping surface at the distil end. An assortment of grafting tools are

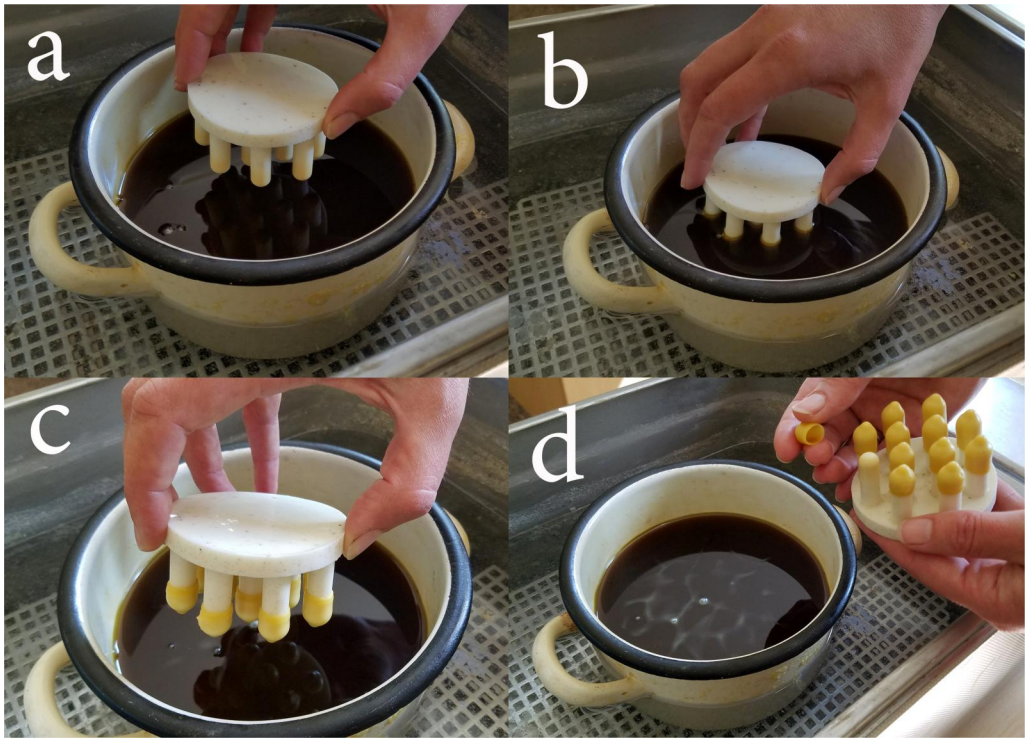


Figure 2. Queen cell cup production from beeswax: a–c. dip the cell mandrel several times in melted wax; d. remove the cups after cooling. Photos: J Wilde.

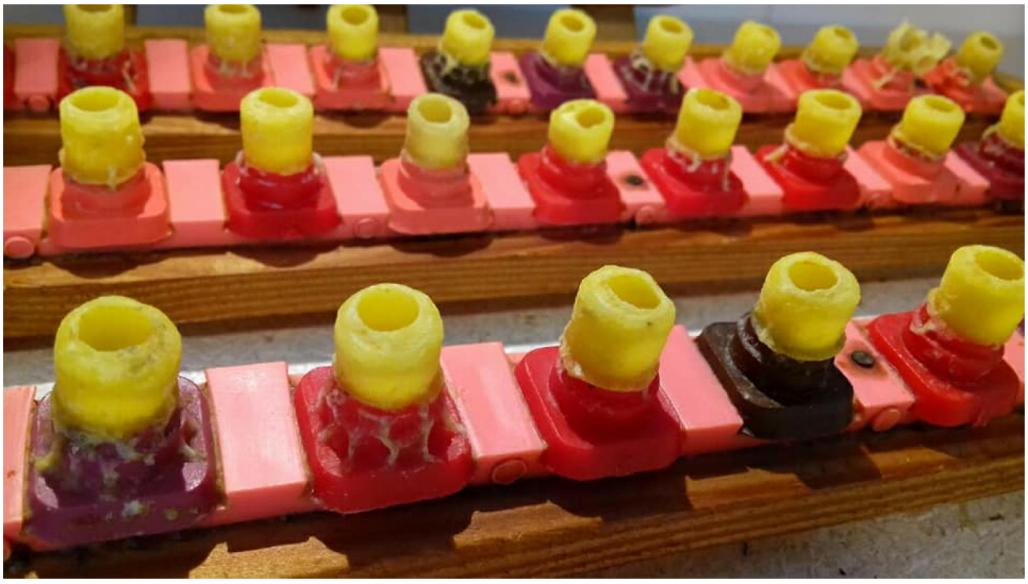


Figure 3. Wax cell cups for grafting. The wax caps have been affixed to a cell base, and the cell base affixed to the cell bar. Photo: M. Leleń.

available for use, representing a variety of preferences by those using the tools (Figure 5).

- Many different versions of metal grafting needles are available commercially. Some have a magnifying glass fitted to the stem. This can help if one's eyesight is insufficient to see the young bee larvae. Sometimes, both ends of the tool are designed for grafting, each with a different scoop configuration.
- A very small (size no. 000 or 00) artist's paintbrush is a suitable tool for grafting (Figure 5(d)). The moistened bristles must stick together to slide under a larva easily.
- A "Chinese" grafting tool is a handy and inexpensive grafting tool that looks like a ballpoint pen (Figure 5(a)). It consists of a spring-loaded bamboo plunger that slides along a thin tongue of flexible plastic. The flexible tongue slips easily under a larva and then a press on the plunger

Figure 4. Sealed queen cells, one to two days after capping, ready to be transferred to an incubator. Photo: M. Kovačić.

Figure 5. Grafting tools: (a) "Chinese"; (b) modern version of "Chinese"; (c) metal grafting spoon; (d) fine brush; (e) flattened match. Photo: J Wilde.

will deposit the larva and any royal jelly into the cell cup. A non-slip grip in the middle section gives excellent control. Modern versions of this tool have injection moulded plastic parts, possibly aiding with cleanliness (Figure 5(b)).

In general, grafting is easier from dark wax combs than from light wax combs because of the better contrast between the dark comb and the small white larvae. Older dark comb also has a more rounded cell base, making it easier to collect larva from the cell. Worker bee cells slant slightly upward when facing the comb. Thus, it helps to have the top of the frame pointing towards you at the grafting station as this will facilitate seeing into the cells easier. The use of a cool light or an illuminated grafting magnifier will help one see the larvae better. Grafting should be done in a lighted room or in indirect light to ensure the larvae do not dry out or become damaged by UV radiation from direct sunlight, as can occur when grafting in the field.

2.1.2.3. Queen rearing kits. There are several queen rearing kits available (Jenter system, Nicot Queen System, Mann Lake Queen Rearing Kit, Ezi-queen queen rearing system) in which the queen is caged on a plastic comb with removable cell bottoms. The kit systems can be used to transfer larvae without

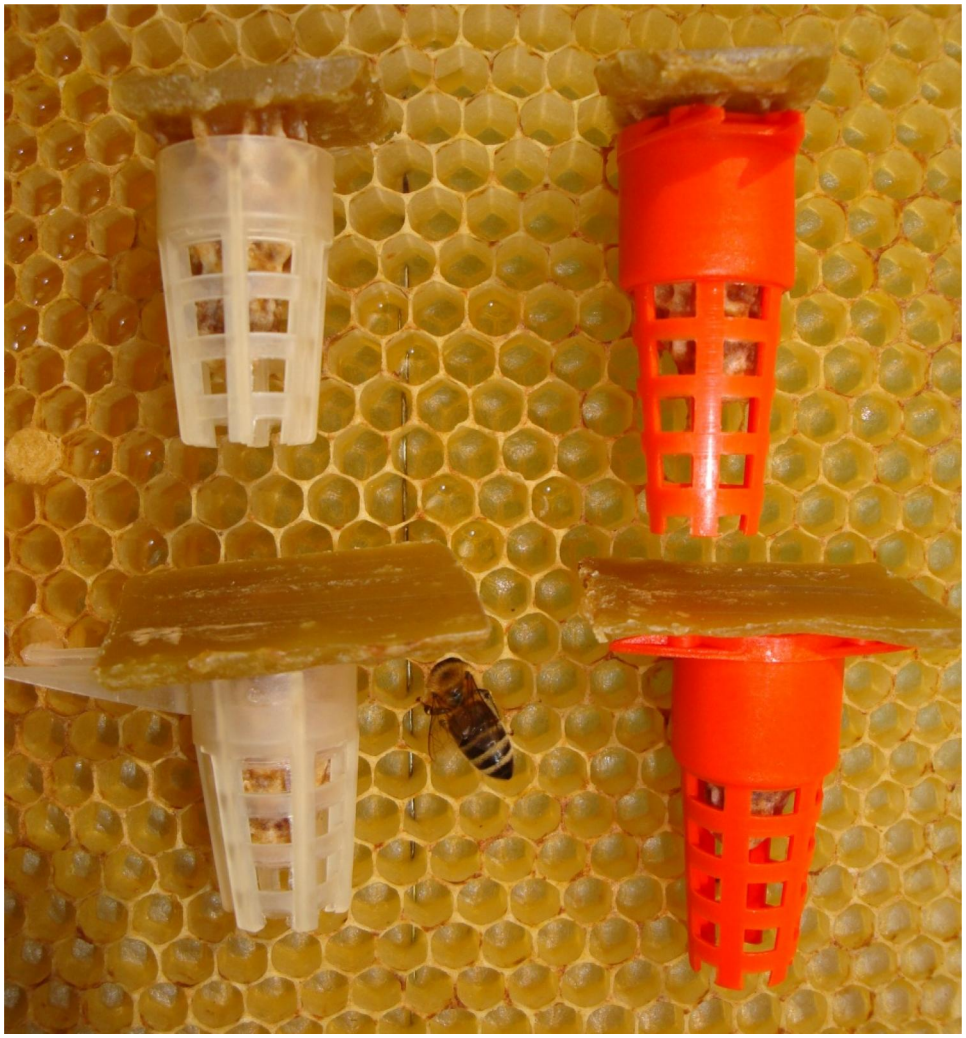


Figure 6. Two push-in cell protectors (left) and two top bar cell protectors (right) from Mann Lake Ltd. Photo: J Wilde.

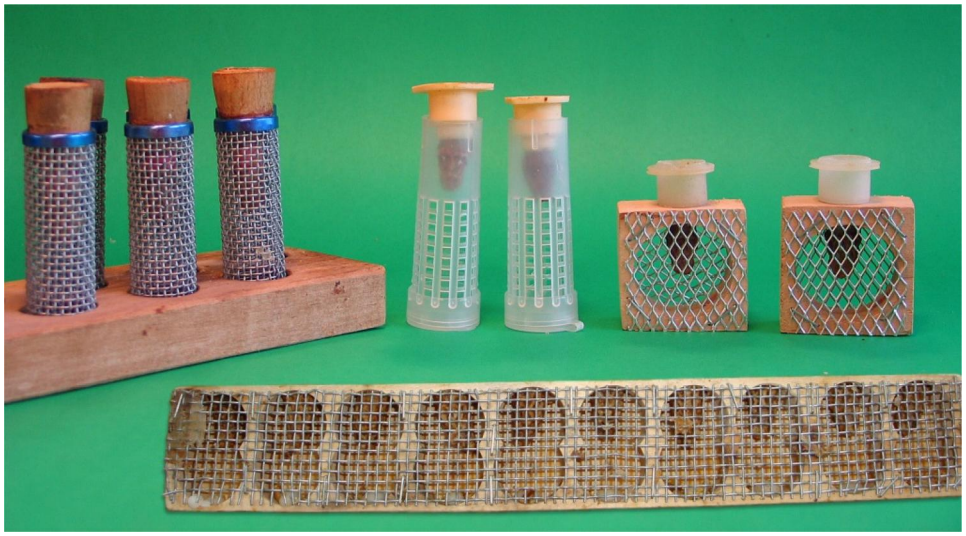


Figure 7. Queen cells protected by three types of cages and container for 10 queen cells (below). Photo: B Chuda-Mickiewicz, J Wilde.

grafting. With a single Karl Jenter kit, about 50 queens can be produced over 50 days. This is suitable for small-scale beekeepers producing queens for their own apiaries. The Ezi-queen system is more effective for larger production of queens as it uses a cage of 420 cells, all of which can be transferred in

less than 5 min. The plastic components used are made of a food grade polycarbonate, which allows for sterilisation by autoclaving.

2.1.2.4. Protection of queen cells. In general, the best acceptance and care by nurse bees is achieved when young queens emerge directly into their colony. If possible, ripe queen cells should be transferred from the rearing colony to queenless mating nuclei or colonies one to two days before emergence (Figure 4). However, if queen cells are left to emerge in the nurse colonies or in a brood chamber, they must be protected against attacks from other queens or workers and be prevented from escaping. This can be achieved using cell protectors or emergence cages (Figures 6 and 7).

Queen cell protectors, made from insulation tape, tin foil, or plastic tubing, are placed over the queen cells to prevent the emergence of the queen or to allow the queens emergence but to prevent the workers from chewing down the cells. The most popular cell protectors are push in cell protectors and top bar cell protectors from Mann Lake Ltd. There are many types of wooden or plastic emergence cages available, and these can be used singly or as a block of 10–15 cages to protect all queen cells on a cell bar.

2.1.3. Queen rearing methods and management of nurse (or cell builder) colonies

A few queens can be reared very simply by utilising the natural reproductive impulses of colonies (swarming, supersedure or emergency). For example, in the Alley method (F. Ruttner, 1983), a strip of cells containing one day old larvae is removed from a comb and placed in a frame with the cells pointing downwards. Every 2nd and 3rd larva is destroyed, leaving adequate spacing for queen cells to be started and finished without having to separate the cells surgically once they are sealed.

However, large scale, systematic production of superior queens relies on grafting methods and the application of specific colony management schemes. There are several methods available to stimulate colonies to accept newly grafted queen cells and to rear high quality queens. In starter-finisher systems, the queen cells are started in queenless colonies and transferred to queenright finisher colonies after one or two days. In other systems, the queen cells remain in the same colony for the whole rearing period. The most popular methods are listed in Table 1.

If there is no nectar flow available, all nurse colonies or bees in swarm boxes need to be fed with a 1:1 sugar water syrup-solution or candy (powdered sugar with honey, ratio 4:1 by weight) at least three days before grafting during the whole rearing season. The nurse colonies always need to have a good supply of nectar. If necessary, additional pollen combs can be added from other colonies. In any case, the nurse colony needs plenty of young and well-fed bees to ensure a rich royal jelly supply for the very young larvae.

2.1.4. Obtaining larvae for grafting

Grafting is easier if the larvae can be removed from dark combs (combs from which 8–10 worker generations have emerged). Before use, empty dark combs should be placed close (next) to brood combs so the bees will clean and polish the cells for egg laying.

If many larvae from a single queen are to be grafted on certain dates, it is very useful to confine the queen to single combs for 12–24 h, four days prior to grafting. After this time, the comb with eggs can be transferred to a queenless nurse colony or can be retained in the brood nest of the source colony. There are several commonly used methods of making queen-confining cages (G. D. Morse, 1979):

- A simple method is to use a push-in cage made with wire mesh (with 4 mm spaces) or queen excluder. The cages are pushed into a section of empty comb onto which the laying queen is placed. Push-in cages are usually about 12–15 cm^2. Worker bees move through the holes in mesh as easily as they do in queen excluders. Sometimes the workers bees will chew the comb around the edge of a push-in cage and may release the queen within two days.
- If a breeder colony is to be used for an extended period, the use of 3–5 comb isolators, made from metal queen excluder, is recommended. The isolators are placed in the centre of the hive, between frames. The following number and types of frames should be placed between two isolators: a comb containing an abundant amount of bee bread, one that is empty, one that contains sealed and emerging brood, and a final comb with unsealed honey. Every 24 h, one comb with eggs (the comb that started as "empty") is removed and replaced by another empty comb. After four days, larvae on the first comb will be ready for grafting. The system allows for continuous grafting of large cell numbers every day.

One of the best and most convenient methods of obtaining larvae is to use a special full depth hive body insert (Figure 8). The breeding queen is confined to three small combs, each about half the size of standard combs, in a compartment with sides made of queen excluder that makes up half of the insert (Figure 8(a3, b, d)). Three additional half-combs occupy the other half of the insert, which has

Table 1. Methods to stimulate colonies to accept newly grafted queen cells.

Method	Description	Advantages	Disadvantages	Notes
Swarm box	Artificial swarm with plenty of young bees and feed in a 5–6 frame box that is well ventilated and used only to start cells or a 9–12 frame hive without a queen or open brood, as described by Laidlaw (1979). In a double or triple story hive, the 9–10 frame method uses a "double screen" for starting the cells. After the cells are started, the double screen is removed and the box containing the cells is moved up top and reunited with the box the queen is in and placed below, then a queen an excluder is added in place of the double screen to finish the cells so this unit becomes a queenright finisher.	Gives great starting results independent of weather conditions. The swarm boxes can easily be transferred and used to transport queen cells.	Many manipulations are needed. Confined bees in the box are stressed and less active compared to those in free flying colonies. The five-frame swarm box needs to be moved to a cool location temporally. It can only start with a small number (max. 20) of cells at a time. Need specialised, modified nuc box.	
Free-flying queenless starter colony	Queenless colony without open brood as described by Laidlaw (1979) or by G. D. Morse (1979)	No extra hive equipment (like swarm boxes) needed. Achieves necessary number of queen cells at any time of the breeding season.	Is necessary to cage the queen. Works only with very strong colonies. Requires extra colonies for queen cell finishing. If a honey flow starts, more space is needed (i.e., add additional supers). Otherwise, the bees will connect the queen cells with wax bridges.	Need to be supported by the addition of sealed or emerging brood at 7–10 day intervals. Bees should be collected in the morning from open brood of support colonies in other apiaries. The bees should be fed sugar syrup and left caged in a cool dark place until late afternoon before they are added to the starter colonies.
Free-flying, queenright finishing colony	Several very popular procedures (Mackensen, Ruttner, Sklenar, Mueller) as described by F. Ruttner (1983)	Excellent queen quality (Cengiz et al., 2009). Used for starting and finishing the queen cells. Possible to graft every day.	Swarm prevention necessary. Additional space needed to place nectar, especially during a flow.	
Queenright starter-finisher	Queenright, two- or three-story colony as described by Laidlaw and Page (1997)	Achieves optimal cell and queen quality at any time of the season	Needs very strong colony. Need to watch for swarming impulse and cut out any queen cells the colony tries to produce.	
Queenless starter-finisher	Queenless two- or three-story colony, as described by Laidlaw (1979) or one story as described by G. D. Morse (1979) or Woodward (2010)	Reliable results independent of weather condition and period of season.	Needs support of brood and bees from field colonies. Need to watch for swarming impulse and cut out any queen cells the colony tries to produce from missed larva you may have introduced with the brood frames from the foster colony. Need to place a queen excluder between the bottom board and hive body to keep any virgin queen from flying in from other colonies.	Maintained by the addition of about 300–400 g of bees in the evening before each new graft. A frequent addition of this amount of bees is preferable to adding more bees at less frequent intervals. If almost all brood is gone, emerging brood combs are given as well.

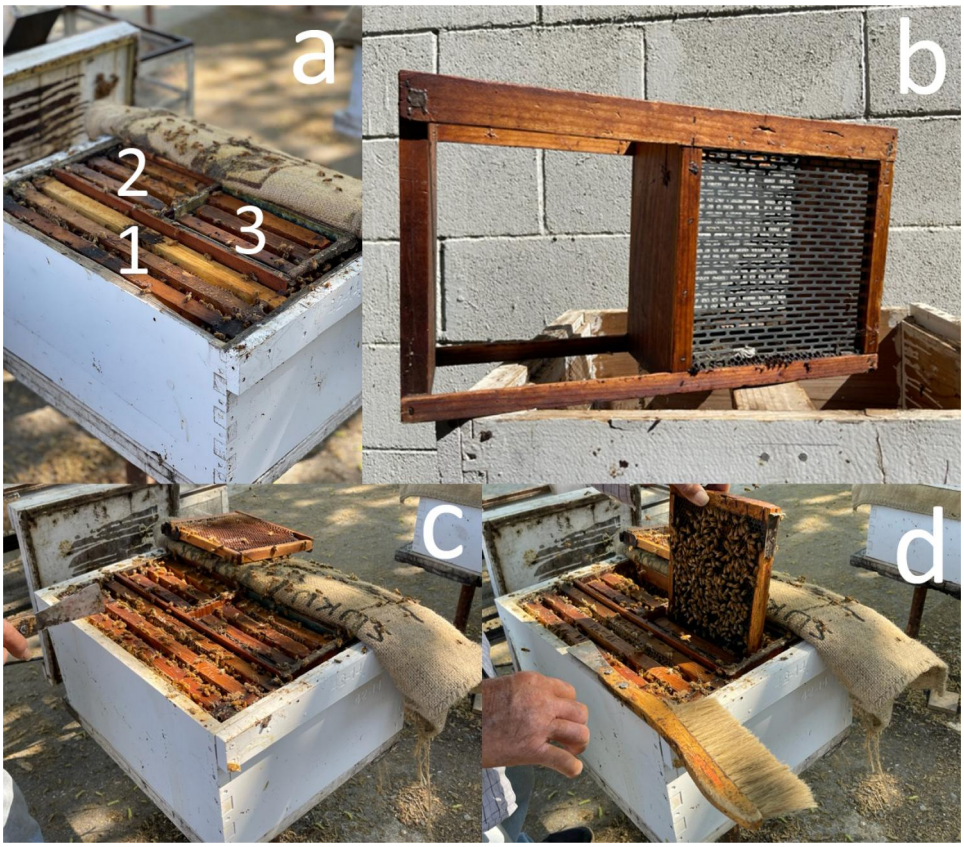


Figure 8. Hive body insert to obtain larvae for grafting: (a) inside the hive, a1. with open brood combs aside, a2. an open and a3. a closed half with queen excluders; (b) empty insert with open (left) and closed by queen excluders (right) part; (c,d) each day a half comb with eggs is moved from the queen right (separated by queen excluders) to the open part. Photo: J Wilde.

open sides (Figure 8(b)). A standard comb well filled with pollen is placed next to one side of the insert, such as to the left, and combs with sealed or emerging brood are put in the remaining spaces of the body (Figure 8(a1)). Each day, a centre comb with eggs is moved from the queenright partition to the non-excluded half of the insert (Figure 8(c)).

When fewer than hundred queens are produced, the "hunt method" is sufficient. Simply select the young larvae from a comb taken out from the brood nest. For this method, previous experience in selecting larvae of proper age is recommended.

2.1.5. Grafting procedure

The following conditions should be observed when transferring a larva from its original cell to an artificial queen cell (Figure 9). This will ensure quality queen production.

- Grafting the larvae from the worker comb to the queen cells should be done rapidly (no longer than 2–3 min for 10–15 larvae) under suitable environmental conditions (24–26 °C and RH $>$ 50%). Under these conditions, combs could be 1–2 h outside the hive. If the comb is not used for grafting for a few minutes (e.g., due to moving grafted cells to nurse colonies), it should be covered with a moist towel.
- Avoid trying to graft larvae that are less than 12 h old. They do not have sufficient royal jelly and are easily damaged when grafted.
- It is best to graft larvae inside a climate-controlled room (a honey house, laboratory, etc.) as larvae are sensitive to high temperatures, direct sun light (UV) and low humidity. Furthermore, grafting in a room is comfortable for the operator and protects against robbing bees. The location of the grafting room should be just a few metres from the breeder colonies and the nurse colonies that receive the grafted cells. However, if one ensures proper humidity of the grafted larvae (covering with a wet cloth), then the combs can stay outside the hive for 2–3 h, sometimes even being transported to other apiaries.
- The grafting frames should not be illuminated with a hot light source. The generated heat may desiccate the larvae, resulting in high mortality. A headlamp works well, especially when coupled with a pair of reading glasses.
- Attention must be placed in selecting larvae which are sitting in a pool of royal jelly, as "hungry larvae" will not be readily accepted by the nurse bees nor develop into strong queens.

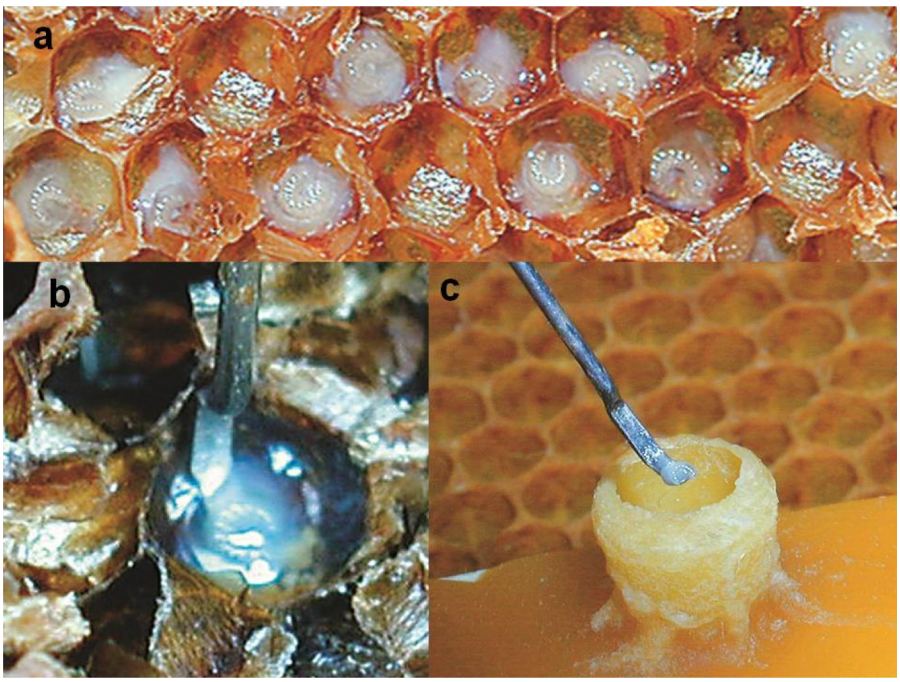


Figure 9. (a) Larvae that are a few hours too old for grafting, floating in royal jelly; (b) a larva taken from dark combs is transferred into wax cups using; (c) a grafting tool. Photos: L Ruottinen.

- The cells and the brood comb should be kept out of the bright sunlight as much as possible. When the weather is hot and dry, a damp cloth may be spread over the cells to prevent them from drying out. A damp cloth also protects the larvae from light and dust. Care shood be taken to avoid chilling the larva as well.
- With experience and speed, three bars (60 cups) can be completed in 8–10 min or less. As soon as one bar is finished, it should be covered with the damp cloth. The grafted cells should be placed into the starters as soon as possible with a maximum of 30–45 min after grafting (at 24–26 °C and RH $>$ 50%).
- Special carrying boxes for the brood frames and grafted cells exist. These help to protect the larvae from desiccating in the sun and from chilling on cold days.
- Queen cells can be "primed" by placing a small drop (about twice the size of a pinhead) of a mixture of half royal jelly and a half warm water into the cell before the larva is grafted into the cell. If the cells are primed, it is important that the larvae are not immersed in the royal jelly but float off the grafting tool on top of the centre of the drop. Usually, it is necessary to prime the queen cells if a standard grafting tool is used while there is no need if a Chinese grafting tool or automatic needle is employed, which tend to transfer royal jelly along with the larva. It is also a good idea to have a small well of warm water to use for washing the grafting tool periodically. Using a Chinese tool will keep it clean, moisturised and running smoothly. It is best to leave it in warm water before the first graft to soften the tongue.

2.1.6. Acceptance of larvae

The number of accepted larvae depends on different factors, as described in detail by F. Ruttner (1983). The most important factors are: quality, strength and developmental stage of the nurse colonies, age of the workers, age of the grafted larvae, presence or absence of queen in the rearing colony and duration of the queenless stage, presence of open brood in the cell-starting colonies, number of grafted cells, rearing sequence, nectar intake and method of rearing.

Environmental conditions are of major importance for final queen rearing success. Essential factors are regulation of humidity and temperature by the rearing colony or in the incubator, and vitality of queen cells and the feed supply (nectar flow, supplemental feeding) of the nurse colony. There is also some indirect influence of the weather conditions and of the season. It is more difficult to produce queens when the night-time temperature drops below freezing. Under well managed conditions, at least 80% of the larvae should be accepted, even in bad weather conditions.

2.1.7. Use of incubators in queen rearing

After queen cells are sealed by bees, they can be placed in the incubator (Figure 10). Currently, it is beneficial to candle (view from above a light source) the cells to ensure they have a viable pupa in them

and cull any small inferior cells. This will save time later by ensuring a higher take and longevity of queens heading future colonies. The temperature in the incubator should be maintained at 34.5 °C and the humidity at 70%. There are several benefits when using incubators. First, breeder colonies could be used to produce a new set of queen cells. Second, the queen cells are not in danger of being destroyed by a young queen. Third, you can mark the virgin queens on the day they emerge. Finally, you can provide honey directly to the caged virgin queens. On the other hand, when using incubators, one must take precaution to protect against power outages. Queen cells can be introduced into colonies or mating boxes before the queens emerge, or queens can emerge in an incubator. Queen cells should be placed in a special cage if allowed to emerge in an incubator (Figure 10). After emergence, young queens are very hungry, and a supply of quality honey should be available to them.

2.1.8. Introduction of queen cells

Queen cells should be introduced into a hive or mating box at least one day before the queen emerges. To ensure queen quality, the queen cell should be checked against a source of light (candling – section 2.1.8) to confirm the queen inside is fully developed. This way, the introduction of undeveloped queens can be avoided. To achieve the highest acceptance of queen cells, a receiving colony should have all development stages of young brood. If there is no young brood in the colony or mating box, one frame should be introduced prior to queen cell introduction. It is best to introduce two queen cells into a hive. That way, you can avoid the situation in which a queen does not hatch or a queen cell is destroyed by bees (Szabo, 1982). However, it may introduce other problems, if the two queens emerge the two maybe fight to the death. If one queen cell is introduced, it is necessary to confirm the queen emerged from the cell. This can be done about three days after cell introduction into the hive.

2.1.9. Introduction of virgin queens

If a queen hatches in an incubator or breeder colony, it is necessary to introduce her into a swarm or mating box. The acceptance of virgin queens is not as high as when working with queen cells, but following some basic rules, acceptance could be quite satisfactory (even more than 85%). The main advantage of working with virgin queens in comparison to queen cells is that the queens can be examined for any deformations and small body sized queens could be excluded. Virgin queens can be introduced in a broodless or broodright mating box.

The most common method is to introduce a virgin queen at the same time when stocking the mating box with bees. Bees for stocking mating boxes are “shocked” by spraying them with water (10% lactic acid can be added to the water for spraying) and shacking. With this procedure, bees are disoriented and easier to handle. When bees are introduced to a mating box, the virgin queen is introduced on top of the worker bees. Another good method is to spray them with a light sugar syrup (20%) upon introduction.

To successfully introduce a virgin queen in to a broodright mating box (where laying queen was present) it is necessary to follow three basic rules: (1) The virgin queen should be introduced in a natural

Figure 10. Incubator with queen cells. Photo: M. Leleń.

or artificial queen cell, (2) The young queen should be not older than 12 h and receiving mating box should be queenless for two to three days (Perez-Sato et al., 2007; Pérez-Sato & Ratnieks, 2006).

2.2. Mating control and its importance

Honey bee breeding programmes and specific research projects depend on controlling the queen's mating process. Commonly, bee breeders have full control over the origin of honey bee queens, i.e., the maternal side. Drones, on the other hand, are often the "neglected gender;" yet, it is well recognised that we enhance selection success by selecting the paternal side. Plate et al. (2019) demonstrated that mating control is a crucial prerequisite for long-term selective breeding. Without applying it, the progress made in a breeding program is limited to a few generations (Uzunov et al., 2022).

In addition to the well-developed instrumental insemination technique (see the *BEEBOOK* paper on instrumental insemination (Cobey et al., 2013)) mating stations can serve as an efficient technique for controlling honey bee mating for commercial and research purposes. On the mainland, mating control depends either on isolation by geographic distance (limited flight range of drones and queens), geographic barriers (high mountains, water surfaces etc.), or the saturation of mating places with drones of the desired genotype. Great distances (minimum 6 km) from known apiaries or mating stations established in high altitudes (high mountains above 1200 m) can ensure control of mating given the absence of unwanted drones. Similarly, valleys also act as isolating areas, keeping unwanted drones away from mating activities in the valley. Furthermore, the saturation of an area with desired drones increases the probability of queens mating with selected males, given the selected males outnumber unselected ones. In any case, the conditions and the cost for the establishment will determine the method to be used.

In contrast to mainland areas, islands offer an excellent opportunity to establish a fully controlled abundance of selected drones because they avoid flying over large stretches of water. A comparison of mating apiaries located in both areas is offered in Table 2.

2.2.1. Criteria needed for the establishment of mating stations

- Absence or minimal presence of managed and unmanaged colonies and airborne drones in a radius of at least 6 km.
- Diverse nectar and pollen resources.
- Weather conditions with long periods of $> 20\,^{\circ}C$ ambient temperature, and wind speed ≤ 24 km/h.
- Sheltered areas for positioning of mating boxes with obvious markers, such as stones, trees, bushes, or specially installed objects help to minimise queen drifting and losses.
- Sufficient drone colonies to ensure a strong drone population for mating. According to Tiesler et al. (2016), the required number of drone colonies ranges from 8 (for 50 virgin queens) to a minimum of 20 for 500 virgin queens.
- Minimal presence of honey bee predator species, such as lizards, frogs, bee-eaters, and hornets.
- Proper logistical (roads, water and electricity supply) and communication infrastructure (mobile network).

2.2.2. Evaluation of a mating station: environmental conditions

In order to understand and evaluate the requirements and risk factors involved in honey bee mating biology better, various parameters to be taken into consideration have been suggested. Consequently, it is useful to characterise mating stations by noting the meteorological phenomena and parameters outlined in Table 3.

2.2.3. Evaluation of a mating station: biological conditions

Mating between the virgin honey bee queen and numerous mature drones occurs in the air, at a certain distance from the hives, in rendezvous sites called "Drone Congregation Areas" (DCA) (Koeniger & Koeniger, 2007; Zmarlicki & Morse, 1963). The locations of DCAs tend to remain constant over time. When establishing a mating station, it can be useful to assess the presence of surrounding colonies and DCAs. This can be achieved in several ways, as described in the sections below. A comparison of the methods described below can also be found in Table 4.

2.2.3.1. Traps to estimate worker presence.

- The presence of worker bees implies the possible presence of drones in an area.
- Honey traps, consisting of at least 50 ml of liquid honey of known or controlled origin on a small plate, are positioned in the area surrounding the mating station (see the *BEEBOOK* paper on miscellaneous methods (Human et al., 2013) for more information on using honey traps to estimate worker presence and colony density).
- Alternatively, dark brood combs can be melted in boiling water (wax melting trap) to attract bees by the intensive and specific smell.

- The traps are regularly checked for the presence of worker honey bees. The total testing time should not be < 3 h. A single trap should not be viewed <15 mins, given common flight distance and speed of honey bee workers (Park, 1923; Von Frisch, 1967). However, one should keep in mind that the attractiveness of any trap significantly depends on the presence of natural food source availability.
- Alternatively, in isolation areas, a pre-testing mating can be performed, with a few mating nuclei. The frequent failure of queen mating, a high percentage of lost queens (presumably due to frequent unsuccessful mating flight attempts) and/or drone laying queens indicate unsuccessful mating. These are a good sign of the absence/scarcity of drones in the selected area. In some areas, dragonflies or birds can be a problem for queens mating on wing. Queen producers are sometimes impacted with significant numbers of queens lost.

2.2.3.2. Estimation of drone density and mating behaviour. The *BEEBOOK* manuscript on behavioural studies (Scheiner et al., 2013) describes in the section entitled "Equipment and devices for experiments on mating behaviour" suitable methods to investigate the mating parameters useful to characterise potential mating stations. These parameters include the number and diversity of drones in an area. Drones can be attracted to living/dead queens, queen extracts, or synthetic blends of queen pheromones.

- Pheromone traps made by small pieces of sponge or cigarette filter for instance, prepared from synthesised queen pheromone (9-oxo-2-decenoic acid, abb., 9-ODA) or extracted in acetone ((CH3)2CO) from honey bee queens, can be used to lure airborne drones. Additionally, live or model queens, the latter in which the thorax is fixed or tethered, can serve to attract drones.
- The number of observed queen mating flights and their duration indicate the distance to DCAs and the density of the drone population.
- The starting time of queen's oviposition, the sex of the resulting larvae, and the rate of brood mortality can be used as indicators of successful mating.
- The spermathecae of mated queens can be dissected (see the *BEEBOOK* paper on anatomy and dissection of the honey bee (Carreck et al., 2013)); to estimate the number of stored spermatozoa see the *BEEBOOK* paper on miscellaneous research methods (Human et al., 2013).

2.2.3.3. Molecular verification of drone presence. Microsatellite analysis and other molecular methods can be used to identify the individual origin of captured airborne drones or their semen from certain

Table 2. Parameters associated with locating mating apiaries on islands or the mainland.

Mating station type	Accessibility & applicability	Mating control	Mating risks	Weather conditions	Costs per queen
Mainland	+	o	+	o	+
Island	–	+	o	o	–

Note: + = optimal, 0 = acceptable, – = suboptimal.

Table 3. Meteorological parameters, instruments used to measure the parameters, and units of measure that can be used to characterise mating stations.

Parameter	Instrument	Unit (abbreviation)
Temperature	Thermometer	Celsius (°C)
Relative humidity	Hygrometer	Percentage (RH)
Wind speed	Anemometer	Metre in second (m/s)
Wind direction	Anemometer	Wind rose (NESW)
Precipitation	Rain gauge	Millimetres on hour (mm/h)
Cloud cover	Campbell–Stokes recorder	Campbell–Stokes recorder card / Subjective cloud coverage in %
Altitude	GPS	Metres above sea level (m.a.s)
Position	GPS	Latitude and longitude coordinates
Vegetation	Aerial photography	proportion of different land use, presented as a percentage, and pollen availability

Table 4. A comparison of methods used to determine adult worker and drone honey bee presence in a prospective mating area.

Method	Accessibility	Efficacy	Price	Notes
Honey traps	+	o	+	Attracts worker bees
Wax melting traps	+	+	+	Attracts worker bees
Synthesised 9-ODA	–	+	–	Attracts drones
Extracted queen pheromone	o	+	o	Attracts drones
Fixed live queen	+	+	o	Attracts drones
Fixed model queen + pheromone	–	+	o	Attracts drones

Note: + = optimal, 0 = acceptable, – = suboptimal.

colonies (see the *BEEBOOK* papers on molecular techniques (Evans et al., 2013), and miscellaneous research methods (Human et al., 2013). This is a powerful technique to estimate the paternity on the worker bees, the number of matings per queen, the realised mating distance of queens and drones, the quantitative contribution of certain drones to the female offspring of a queen, etc.

2.2.4. Management and administration of mating stations

The mating station usually consists of the mating boxes/hives (small hives into which queen cells are introduced) and drone producing colonies that need to be in the best health condition to avoid spreading pathogens and pests. Sometimes, queen cell building colonies (colonies used for rearing grafted female larvae into queens) and colonies selected for grafting (colonies from which female larvae are grafted) may be present. However, all colonies that are not used as drone source colonies should be managed to prohibit their drones from participating in the mating process (e.g., by removing drone brood in colonies that are sources for grafting).

There are various options for administering the mating station. These options depend on the capacity, location, and the responsible partner(s) for administering the mating station. The small-scale mating stations usually are under the supervision of a single beekeeper/breeder who takes responsibility for the following tasks:

- Maintenance of mating station property and facilities.
- Managing Drone Producing Colonies (DPC).
- Communication and coordination of arrangement with arriving and departure of the mating boxes and subsequent control of drone presence.
- Checking the mating boxes at the end of the mating period (usually two to three weeks) by assessing parameters such queen survivability, mating success, laying performance, health status etc.
- Recordkeeping and editing various books/cards/software that enable full traceability of the executed activities.
- Taking care of other responsibilities and duties.

Mating stations with larger capacities (hundreds or thousands of queens) demand more intensive coordination between multiple responsible persons.

2.2.4.1. Maintaining mating boxes.

- Mating boxes (sometimes called matting nuclei or nucs) house small colonies, making them prone to robbing and other stressors. Care should be taken to prevent robbing behaviour by feeding mating boxes with a solid food. Make sure that the established colonies have partial shade in the afternoon and avoid siting them in full sun all day The bees may leave their hives when temperatures exceed 32 °C.
- For preventing the presence of alien drones in the mating station, only drone-free mating boxes should be used. Using queen excluder, bees can be "filtered" and all drones separated from the worker bees.
- Mating boxes with virgin queens should not be disturbed during the queen flight period (between 11:00 and 16:00 h).
- Depending on weather conditions, a first inspection of the queens' mating success should occur about two weeks after establishing the mating boxes. Any inspection before the queen has conducted her orientation flights may result in her flying off the comb, thus losing her from the hive altogether. Successful mating should occur within three weeks after queen emergence. Mating after week 3 will result in a reduced fecundity and life expectancy of queens.
- A final evaluation of successful mating should occur upon the appearance of sealed brood in the mating box.
- Regular inspections of the storage and supplementary feeding of mating boxes (preferably with solid food) is needed if they are used over longer periods.
- Each mating box should have a record sheet to record the queen origin. If all mating boxes are prepared on the same day with sister queens (e.g., in large operations), then the records could be kept in the recordkeeping card or book.

2.2.4.2. Drone producing colonies (DPCs) and their maintenance. The main reason for keeping DPCs is to provide an adequate number of mature drones of selected origin, during the right period, for mating. A single group of sister queens can be used to control the paternal pedigree. Alternatively, several groups of sister queens, each of them derived from a selected breeder colony, can be used for drone production within one mating station, depending on the breeding programme/design.

- The build-up of a DPC needs to be started in advance of the mating period. As the development of drones from egg to sexual maturity takes 40 days and the life expectancy of mature drones last several weeks, drone production should be started at least two months prior to the mating period. If drones are required early in spring, drawn drone combs (also available as plastic

combs) can be placed within the hives before overwintering.
- DPCs are managed in standard hives and receive sufficient hive space to support an optimal population development.

2.2.4.2.1. Producing drones.

- Up to two drone combs are placed within the brood nest of each hive box to enable a rich production of drones. This will ensure you get the highest quality drones that are adequately fed and capable of producing the maximum number healthy sperm cells. Extra care and attention to details are important if you are expecting a colony to produce many drones. If the weather conditions become unfavourable, many drone pupae or larvae may be cannibalised.
- Drone combs could be wired frames in which bees will produce drone comb. You can also use drone wax foundation. Drone brood combs from selected drone mothers may be removed after capping and placed in nurse colonies to enable the production of a greater number of drones from the selected queen.
- The DPCs are established from superior and healthy colonies and particular care is taken to provide a continuously rich honey (alternatively other sugar supplements) and pollen supply.
- Regular checks of colony health and overall drone development are recommended to achieve a high-quality control level. If there is a honey flow occurring when you place the drone combs in the hives, the bees will fill the combs with nectar. Regularly inspect the colonies to enable adequate conditions for drone rearing.
- If the drone colonies are moved to the mating station from other apiaries, queen excluders must be placed between the bottom board and the brood box of the hives. This prohibits drones from other colonies from entering the DPC. Dead drones should be removed from these excluders at regular intervals to prevent blockage of the entrance and lack of ventilation for the colony. The queen excluders and any dead drones should be removed from the hive just before moving the drone colonies to the mating station. Execute such tasks early morning or late evenings.

Drone colonies are also sensitive to heat, as they are highly populated. In hot areas, particular care for the ventilation of those colonies or even extra space should be provided when they are going to be moved to the mating stations. Alternatively, the DPCs could be moved to other locations when the drones are still in the pupal stage.

2.2.4.2.2. Controlling pests and pathogens in DPCs.

- Special attention must be given for *Varroa destructor* control (see Dietemann et al., 2013) in a DPC as it is well documented that *V. destructor* and other pathogens strongly influence the fitness of drones (Peng et al., 2015; Rinderer et al., 1999).
- The semen can also be negatively impacted by exposure to agrichemicals (Ben Abdelkader et al., 2015; Straub et al., 2016). One of the reasons for frequent and repeated queen replacement is the low viability of sperm due to agrochemicals (Ben Abdelkader et al., 2015). However, the same might be caused by miticides intended to protect the colonies from major disease vectors (Rinderer et al., 1999). That is why special attention must be paid to disease prevention and miticide treatments should be avoided while drones are being reared. That said, reduced chemical treatment can provide a selection pressure that favours colonies with increased resistance to *V. destructor*. Consequently, allowing *V. destructor* presence in drone colonies can be an important selection tool within breeding programmes for disease resistance (see Büchler et al. (2010) for further details on "tolerance mating stations").
- *Nosema* spp. infection imposes energetic costs and reduced flight ability for drones. Care should be taken to avoid producing drones from colonies either with evident *Nosema* spp. infection or with counts $>$ 1.000.000 *Nosema* spp. spores/bee (Emsen et al., 2020; Punko et al., 2021).

2.2.5. Alternative approaches for mating control

The Delayed Flight-Time Method (DFM) is a manipulation of the mating hours of queens and drones to achieve natural mating with a controlled drone population (Freudenstein, 1938; Oxley et al., 2010). According to this system, queens are released approximately half an hour after the end of natural drone flight in the area (which usually takes place at early afternoon, but this should be determined *a priori* using drone luring baits) and drones slightly later. In addition, determining the time the last drones are returning to their colonies from orientation flights, one could close the entrance of the colonies in the early afternoon and monitor the number of the drones returning. The time of the day when no more drones are found in front of the closed entrances is the time for starting point for the DFM method. The queens/drones are allowed to go on mating flights for 2–3 h, depending on the latitude. Following this period, the hive entrances of the drone colonies and mating boxes are closed with queen excluders, or else closed completely. Alternatively, a secondary flight entrance can be opened, an entrance that can only be used by worker bees. This process is repeated for several days to ensure adequate time for the queens to mate.

For regulating the flight times of queens, two slightly different approaches are currently in use. They include the labyrinth mating boxes and the Joe Horner system.

2.2.5.1. Use of "labyrinth" mating boxes. DPCs used for this form of DFM have two flight entrances. One is only accessible through a queen excluder and is intended for daytime flights of worker bees. The entrance should be sufficiently big (fully opened to prevent congestion by dead drones on the excluder) and oriented or arranged in such a way that no daylight can enter the hive. The second flight hole is intended for afternoon flights of drones and workers. The two flight holes are equipped with a mechanism that allows the user to control if they are open or closed. They can be operated manually or automatically (Figure 11). Ideally, the placement of the hives should ensure that they are shaded until late afternoon. They must receive direct daylight during the drone flight time.

Like the DPCs, the mating boxes or nuclei have two separate flight holes that are opened and closed alternatively. The one intended for diurnal flights of workers is equipped with a piece of queen excluder material and leads into a dark tunnel with at least one U-turn (the "labyrinth"), which shields the interior of the box from light. The flight hole for queen flights is a simple hole in the front wall, allowing light to enter freely to attract the queen to it quickly once it is opened. A semi-automatic version of this approach is described in Musin et al. (2021). In Germany, the optimum time of queen release has been found to be around 3h 15 min. before sunset under fair weather conditions. Until now, this method has only been validated under the environmental conditions of Central and Southeast Europe (Macedonia).

2.2.5.2. The Joe Horner system. The Joe Horner system (Oxley et al., 2010) uses a structure that looks like a miniature train, with rails and wagons, where the wagons are the mating boxes or nuclei containing the virgin queens. They are connected to one another with 2 m chains, and slide on metallic rails (hence their nickname: the train of virgin queens) (Hatjina & Charistos, 2018). The mating boxes are pushed close to one another and stored in a dark cooling chamber (at about 14–15 °C) for most of the day. They can be pulled out of the cooling chamber daily, to allow a controlled flight period for queens and drones.

The rails and chains ensure that the mating boxes remain in the same position every time they are moved outside the cooling chamber (Figure 12). This facilitates virgin queen orientation to the mating nucs. The low temperature in the cooling chamber helps the queens remain calm during confinement, and the temperature differences between the cold chamber and the atmosphere act as stimuli for the queens to begin their mating flights outside of their normal mating flight window. For the queens to warm and get ready for flight, the mating boxes should be released about 30 min before the drones.

The DPCs are kept outside but their entrances are restricted during the day with a queen excluder. Given the DPCs are large colonies with high numbers of bees and mature drones, care should be taken to ensure adequate shade or ventilation. This can be accomplished using a ventilated bottom board and/or an extra super. Drone overheating can be a serious problem, especially in warm climates.

2.3. Handling of adult queens

2.3.1. Marking and clipping queens

Queens lose the ability to fly if the tip of one front wing is clipped (approx. 35–40% of the wing). Wing clipping has no negative effects on the vitality or longevity of the queens and is therefore a common technique to delay, but not prevent, swarming of the colony. Beekeepers may clip alternate wings in

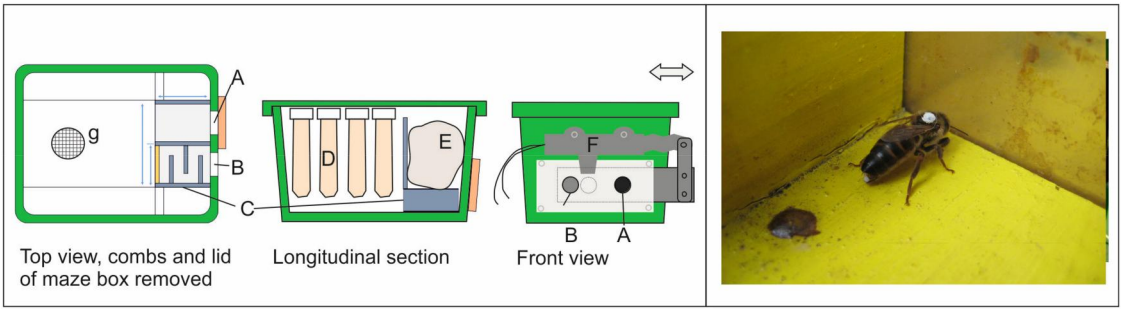


Figure 11. An implementation of the "labyrinthum"-approach to controlled mating *via* delayed flight times (from Musin et al., 2021) Left: Schematic drawing of mechanism to shield the queen from light during periods of nonflight and to switch between flight holes; A: flight hole for queen and workers; B: flight hole with "labyrinth"; for workers only; C: plywood box containing the "labyrinth" system; D: combs; E: sugar dough; F: step motor; G: bottom hole for ventilation; Right: a queen returning with mating sign in front of the mating box. Photo: A. Uzunov.

Figure 12. The Joe Horner mating system or "Train of Virgin Queens" during application in Greece. Photo: F. Hatjina.

alternate years to keep track of the age of queens. See the *BEEBOOK* chapter on miscellaneous research methods (Human et al., 2013) for techniques of clipping or marking queens.

2.3.2. Shipment of queens

Queen cages for shipment by mail are usually made from plastic and are offered in a variety of sizes and shapes. The most popular cage has two compartments; the larger one is used to house the queen and 6–12 attendant worker bees, while the smaller one is filled with queen candy to provide food during shipping. If the shipping cages are used to introduce the queen into a colony, a small hole can be created in the end of the candy compartment through which the workers from the hive can slowly reach and free the queen. Several cages can be packed together if care is taken that the queens cannot reach one another through the screen mesh. Multiple cages can be placed in an envelope containing ventilation holes. The envelope should be labelled with the phrases "Live bees" and "Protect against sunshine". If the shipments take more than two days during low or high outside temperature, it is recommended to add loose attendant bees inside the shipment box so they can help to regulate and keep temperature of 26 °C, as low or high temperatures may decrease sperm viability in queen's spermatheca (Rousseau et al., 2020)

Candy for queen cages should contain little water but remain soft. A mixture of powdered sugar with about 20% honey (weight:weight) gives suitable results. To avoid the possible spread of pathogens found in honey, glucose syrup is increasingly used to make candy. While is not necessary to give water to queens during transport, it is a good idea to place a drop of water on the screen of a queen cage as soon as it is received. Queens should be introduced to colonies as soon as possible (within two to four days) after shipment, no later than the next day. When possible, caged queens should be kept in a dark place with a medium and stable temperature (18–24 °C).

2.3.3. Storage of queens

Large queen rearing operations often produce more queens than they can use or ship immediately. They may need to remove mated queens from mating nucs to make space for new emerging queen cells. Mated queens can be caged in regular cages without worker bees or candy and placed together with other similarly caged queens in a "queen bank" colony. A strong queenright colony is needed to prepare a queen bank.

1. Put queens in regular queen cages for shipping but without attendant bees.
2. Close the cages so that bees from the colony cannot enter the cage.
3. Arrange cages on a special "cage carrier" (see Figure 13).
4. In the honey chamber of a strong queenright colony (at least two boxes of bees), place 2–3 combs with open brood from the brood chamber and 3–4 combs of honey. Open brood attracts young bees from brood chamber to take care of brood and the banked queens.
5. Arrange the combs with the queens between brood combs.
6. After 15–20 days, replace emerging brood combs with new open brood combs from the brood chamber.

7. It is possible to store up to 60 cages in one frame and up to 120 queens within one colony for one to two months with few losses (Figure 13).
8. Make sure you place a queen excluder between the hive bottom board and lowermost brood box. This limits the accidental movement of a queen from another hive into the queen bank. For example, sometimes, a virgin queen can fly into the queen bank from another colony and be accepted. When these queens begin laying, the resident workers will lose their impulse to take care of the banked queens.

While queen banking is very popular in the USA, European breeders avoid storing mated queens this way because the queens may become damaged by the workers who may injure the queens' feet, legs, wings and antennae (Woyke, 1988).

If queens are banked for periods of one week or longer, it will take some time (usually a few days) for her to regain her full egg laying capacity after being banked. Unfortunately, queens that were banked are often subject to supersedure impulse from the workers the first two weeks after their introduction. If supersedure cells are removed by the beekeeper during this period, the queen usually develops a normal egg laying profile. The supersedure impulse will stop and these queens will be allowed to lead a productive colony. Some queens may also die from starvation if banked for long periods (over two weeks). It is a good idea to supply each queen cage with candy to avoid this.

2.3.4. Requeening colonies

There is no perfectly reliable method one can use to introduce new queens to a colony. The success of queen introduction depends on the attractiveness of the new queen and the previous queen status of the colony. Unmated queens are less attractive to queenless colonies than are mated queens. Furthermore, egg laying queens are more readily accepted than queens that have stopped laying due to transport or other reasons. The best time for requeening is during a good nectar flow. If there is no nectar flow, it is recommended to feed colonies with syrup (50:50 water-sugar). It is important to make the recipient colony queenless for at least 6–8 h, sometimes for up to one day.

There are two general methods one can use when requeening with mated queens: requeening with a caged queen and requeening with a nuc.

2.3.4.1. Requeening with a caged queen.

1. Find the queen in a colony and remove her.
2. Put the new queen in its shipping cage on the top of the bars or between the combs. Either way, it is important that candy compartment on the cage be exposed to allow the bees to release the queen slowly after consuming the candy.

Figure 13. In total, 128 queens were banked in the upper box of queenright colony. Three open brood combs are placed between the frames containing the queens to attract nurse bees. Photo: M. Kovačić.

3. Introduction success can be improved if the queen to be replaced is caged for about seven days before requeening.

2.3.4.2. Requeening using a nuc.

1. Under difficult conditions, or for the introduction of highly valuable queens, we recommend introducing the queen into a nucleus colony containing mainly young bees (also known as an "artificial swarm", "split" or "nuc"). Those small units usually accept any kind of queen. Once ample bees and brood are present, the nuc can be used to requeen another colony.
2. Dequeen the colony to be requeened.
3. The nuc containing a young, mated queen, bees and brood is placed on the top of the brood box and sheltered by an empty super or half-size super (Figures 14 and 15).
4. The bottom of the nuc could be removed, if possible, or the mating nuc can be turned upside down on the hive to be requeened.
5. A sheet of paper with some slits or a cover with a hole (Figure 15) can be used as a separator between the mating nuc and the queenless colony (brood box). This helps the bees from both colonies mix slowly.
6. Following this method, a requeening success of 95–100% can be expected.

Figure 14. Introduction of a queen into a queenless colony using a mating nuc. The nuc containing the young mated queen, bees and brood is placed on the top of the brood box and sheltered with half-size super. Photo: N. Kezić.

2.4. Queen quality control

"Quality" is a subjective term used in relation to queens and drones to describe certain quantitative physical and performance characteristics. It is generally believed that a queen of "high quality" should have the following physical characteristics:

- high body weight (described in section 2.4.1),
- *large number of ovarioles (depending on subspecies, the number of ovarioles per ovary ranges

Figure 15. Introduction of a queen into a queenless colony using a Mini Plus mating nuc. The Mini Plus containing a young mated queen, bees and brood is placed on the top of the brood box (right) and is covered with a lid that contains a hole (left). Photos: M. Leleń.

from 130 to 175, for details see Carreck et al., 2013, section 3.5.2.4; Hatjina, 2012)
- *large size of spermatheca with a diameter > 1.2 mm (see Carreck et al. 2014, section 3.5.2.5; Hatjina et al., 2014)
- *high number of spermatozoa (see Human et al., 2013).
- Visually, the best queens tend to have a large thorax with nice branching legs. The tibia is typically wider on superior queens.

A* indicates that the measurement of this parameter results in the death of the queen.

Once active as the queen of a hive, some colony performance traits can be used as criteria for accessing queen quality:

- high brood production (including number of eggs per day) and large bee population (section 2.4.2 in Delaplane et al., 2013)
- brood solidness (section 2.4.3 in Delaplane et al., 2013)
- disease control (Cobey, 2007; Laidlaw, 1979; see de Graaf et al., 2012; De Miranda et al., 2013; Dietemann et al., 2012; Forsgren et al., 2012; Fries et al., 2013; Jensen et al., 2013).
- increased honey yield (see section 3.3.1)
- low defensive behaviour (see section 3.3.2)
- low swarming tendency (see section 3.3.3)
- intensive hygienic behaviour (see section 3.3.4)

2.4.1. Body weight

The weight of a fertilised queen can vary considerably due to egg laying intensity, genetic factors (subspecies) and environmental factors that affect egg laying. More uniform conditions can be assured by weighing very young unfertilised queens and respecting the following conditions:

- Electronic balances with an accuracy of 0.1mg should be used. Calibrate the balance before use. Also, ensure the balance is level.
- If unfertilised queens are used, they should be as young as possible. Queens can lose almost 1–2 mg of weight per day after emergence (Kahya et al., 2008; Skowronek et al., 2004).
- Queens can be placed into small cages to facilitate weighing (Figure 16). The cage can be weighed with and without the queen. The difference in weights is the weight of the queen. Alternatively, the empty cage can be placed on the balance, and the balanced tared prior to adding the queen.
- The genetic origin of the queen influences the weight standards and should be known.
- At least 10 queens per line and apiary should be collected on the same day when evaluating fertilised queens. Sampling is usually repeated twice during the reproductive season.

Figure 16. A queen cage for weighing a queen. Photo: F Hatjina.

Figure 17. The 2 × 2 cm grid frame is placed over the surface of the comb and used to estimate the amount of brood (or eggs) in the comb. Photo: F Hatjina.

2.4.2. Number of eggs per day (fecundity)

- Queen fecundity in a 24-h period is estimated either once, when the laying of eggs is at its maximum, or several times during the productive period.
- The queen should lay more than 2000 eggs in 24 h period, but this can depend on the bee race and time of year.
- A simple way of estimating 24 h fecundity is with the use of a 5 × 5 cm or 2 × 2 cm grid frame (Figure 17) or by using the Liebefeld method of estimating brood area (see section 5.1 in Delaplane et al., 2013).

2.4.3. Brood solidness

Brood solidness is expressed by the percentage of empty worker cells in a brood patch of a given area. An acceptable level of empty cells is usually less than 10%. Higher levels can indicate an increased brood mortality due to inbreeding or genetic

deficiencies. To determine brood solidness, see section 4.1 in Delaplane et al., 2013).

2.4.4. Health status of the queens

"High" quality queens should also be free of pests and diseases (Alaux et al., 2011; Amiri et al., 2017; Cobey, 2007; Laidlaw, 1979). Although *V. destructor* does not affect queens directly (the mite does not parasitise queen larvae), it affects queen reproductive potential through effects on drones (e.g., drone flight activity and sperm production). Undoubtedly, virus prevalence in queens is associated with egg-laying deficiencies and reduced survival (Gauthier et al., 2011).

It is important to note that Deformed wing virus (DWV) prevalence has been found to be higher in mated than in virgin queens, and given that sperm can transmit viruses to newly mated queens, mating is probably one main route of transmission. Relative to its mass, the spermatheca also bears more virus titres than other tissues in queens (Francis et al., 2013; see also section 2.2.5). Virus titres were higher in queens showing discolouration on their ovaries and had reduced egg laying capacity. Keeping the reproductive colonies with maintaining low *V. destructor* numbers, in colonies used for rearing queens might also limit virus prevalence.

One way to ensure that the produced queens are free from *Nosema* spp. spores is to count the number of spores in the alimentary canal on the same sample of queens sacrificed for the other characteristics mentioned above (number of ovarioles, diameter of spermatheca and number of spermatozoa). This can be done following Fries et al. (2013). The same applies to the queen's attendants.

Exposure to miticides during development (through direct treatment or residues in wax) severely compromises queen's reproductive health (Degrandi-Hoffman et al., 2013; Rangel & Tarpy, 2015; Ricke et al., 2021). Thus, it is necessary to use pure uncontaminated wax from a proven source for grafting and queen cell production, as well as uncontaminated food sources for the nurse bees. Furthermore, *V. destructor* treatments should be performed with great care, as they can affect the survival of virgin queens.

3. Performance testing of bee colonies

Performance tests refer to the testing parameters of queen performance across the season, including brood and population production, honey and pollen yield, score of hygienic behaviour, general disease-resistance, swarming tendency, calmness, overwintering, food consumption etc.

3.1. Preconditions and general recommendations

A breeding programme entails selection of the best individuals for specific traits, and elimination of the individuals displaying the worst manifestations of those traits. To do this, individuals must be assessed in a way that allows genetic effects to be distinguished from environmental influences, according to a uniform method that allows for comparisons across time and space. The basis of performance testing is that colonies in the test apiary should be placed in similar starting conditions (i.e., hive type, comb supply, colony strength, etc.) and managed according to a standard protocol. The result obtained from performance testing is a standardised set of observations for phenotypes of interest. These values can then be synthesised into a selection index or breeding value for the chosen traits.

The colonies are started from package bees or uniform nucs (see section 3, Delaplane et al., 2013) into which the queens to be tested are placed. The colonies are normally established in the spring, or when there is sufficient time for the colony to grow sufficiently. The size of the starting package of bees or nuc and the establishment of the test colonies depends on the climatic conditions of the testing apiary. Methods of equalisation (food, space, diseases) of the test colonies are allowed until the last autumn observation, when the first assessment data are taken. This represents the starting point of the test.

3.1.1. Location and organisation of the testing apiary

The apiary location should have a sufficient nectar and pollen flow during the test period to support the number of test colonies used. The test colonies can be moved during the testing period as long as all comparative colonies are kept together. When planning the location of colonies in the apiary, special care must be taken to reduce drifting.

The following arrangements of hives in the apiary are recommended to reduce drifting among colonies and make inspections more efficient:

- Hives placed on individual stands separated by at least 1 m
- Hives placed on small group stand (up to 4 hives) with their entrances facing in different directions (Figure 18)
- Groups of hives placed in broken lines
- Groups of hives separated by hedge or fence (~2 m high)

Figure 18. Hives placed in small groups and with their entrances facing in different directions. Photo: R Büchler.

3.1.2. Size of the testing apiary

Testing apiaries should include at least ten colonies, representing different sister groups (see 3.1.3). Having multiple replicate colonies in a single test apiary is critical to account for apiary-level environmental variation (see section 4 selection tools). We recommend a minimum of eight colonies. Similarly, the maximum number of colonies in an apiary will depend on the local conditions and experience of the beekeeper(s) involved. Usually, not more than 30 colonies should be placed in a single test apiary. Ideally, many testing apiaries are established to have hundreds to thousands of colonies to phenotype and from which to select.

3.1.3. Queens: origin, marking, distribution

Honey bee breeding programmes are usually based upon evaluation of sister queen groups in order to estimate the additive genetic components of the chosen traits. Sister queens may originate from controlled or open mating (in the first case, the whole pedigree is known, in the latter, only the mother line). At least 12 queens per sister group should be tested and distributed among at least two testing apiaries (H. Ruttner, 1972; Uzunov et al., 2015). This number can be reduced if BLUP breeding value estimation is applied (Plate et al., 2020; see sections 4.1 and 5.1). Within each test apiary, queens of the same origin should be randomly distributed: do not group and/or isolate sister queens in separate positions within the testing apiary.

The sister queens submitted to performance testing should belong to the same rearing series and be mated at the same mating station (i.e., with the same array of drones). To increase the accuracy of the breeding value calculation, it is important that pedigree information of the queens is known. Each test queen should have an individual code and be unambiguously marked (see section 2.3.1 for details). Hives in the apiary should also be individually numbered and equipped with a test card, on which the performance of the colony is noted. The test card is developed on the basis of the traits of interest chosen for selection. Each control and all specific observations have to be documented on this card. An example is shown in Figure 19.

3.1.4. Timing and duration of tests

Performance testing of colonies can begin anytime. Timing will depend on the traits of interest and local climatic factors. When possible, multiple tests should be performed for each phenotype of interest, for example, for hygienic behaviour in test apiaries to identify the highest expressing colonies (Harpur et al., 2019). Colonies will have been uniformly managed and specific requirements noted. Testing should start when it is sure that the queen present in the colony is the mother of the progeny being tested. It takes at least two brood generations (42 days) after queen introduction to replace most of the previous queen's worker population. Qualitative behavioural traits (such as gentleness, calmness, swarming tendency, see sections 3.3.2 and 3.3.3) are

Beekeeper's name			Apiary			Origin of stock				
Starting date			Autumn 2019 inspection date			Spring 2020 inspection date			Summer 2020 inspection date	Final Varroa check date
Colony	Queen	No. combs with bees			Gentleness [+ / 0 / -]	Swarming [+ / 0 / -]	Honey harvest [kg]	Mites per 10 g/bees			Observation and comments
		Autumn	Spring	Summer				Autumn	Summer	Final	
!	Column "Observation and comments": Here you should provide information related to disease incidents, applied treatments, production of swarms or nucleuses, number of added or taken frames and suppers, colony and/or queen losses etc.										

Figure 19. Recommended data sheet to collect all relevant data of repeated colony controls.

assessed every time all the hives in the apiary are inspected, with a minimum number of four evaluations per trait. Behavioural traits should be evaluated under the same environmental conditions; in other words, tests should be performed on the same day for all colonies present in the testing apiary.

3.1.5. General recommendations

- The testing apiary should be run by experienced beekeepers specifically trained to assess desired traits.
- Assessment of traits should be performed on all colonies on the same day, preferably by the same individual or team.

3.2. Colony management

Colony management is essential and must be planned/prepared in advance, before beginning the test. The management schedule should also be agreed upon by all parties involved in testing and managing test apiaries. Colony management must fulfil specific requirements of the test. Standard procedures should be adopted for all colonies in the test to enable comparative results. After the test has started, any differences in colony management may significantly influence the results.

During the planning process, decisions should be made on the following issues:

- colony design (Langstroth, top bar, etc)
- type of wax foundation or comb
- type of stands for the hives
- distribution of queens within the apiary
- water supply
- supplementary feeding sources
- nectar and pollen supply/migratory activities
- health management (disease control) plan

Large differences in colony management exist in different regions. Colony management can significantly influence test results. The main task is to ensure standard conditions for all colonies within each test apiary and, if feasible, between the apiaries.

3.2.1. Hives

3.2.1.1. Type of hive. All hives in the apiary must be of the same type. The type of hives used must be included in the report. Common hive types, such as Langstroth or Dadant, are recommended for use if possible because standardised equipment can be purchased or made for them with relative ease. Hive type will, of course, vary by geographic region and desire.

The use of stands is recommended for the following reasons:

- The hive can be placed on a horizontal level regardless of the terrain configuration.
- Stands facilitate a comfortable working position for the tester.
- Stands provide protection for the hive from ground moisture.

3.2.1.2. Painting and colouring. Hives should be protected with paint that does not harm bees (water-based products for instance). If oil dyes are used for hive protection, the overlaying paint must dry and the polymerisation process must conclude prior to hive use. Special care should be placed in the choice of dyes to ascertain that they do not contain components that are toxic to bees or those that

are retained in the wood and gradually released. Hive entrances can be painted in different colours (bees learn colours very well, as reviewed by Hempel de Ibarra et al., 2014) or decorated with different shapes to help bees orient and to reduce drifting between hives.

3.2.1.3. Hive components. Sufficient space for colony development must be provided. Super(s) are added when bees occupy most combs in the brood box (at least ¾ of the combs). Super(s) should be removed when bees occupy <2/3 of the combs in lower super.

It is recommended that hives in the testing apiary be equipped with screened bottom boards (Figure 20). They guarantee good ventilation and facilitate inspections of *V. destructor* mortality (natural, or after a treatment). They can also allow one to estimate grooming and mite biting behaviour of the bees. The size of the hive entrance should be adjusted according to colony strength, and time of the year. During winter, a metal mesh or other device should be placed across the entrance as a protection against rodents, but allowing bees to pass. The size of the landing board is not important. It is recommended that landing boards should be the same size across all hives, but of different colours within the apiary. Regular maintenance of the landing board is important, since it is the place where disturbances to the colony can be noticed and recognised (e.g., to prevent robbing). The use of a queen excluder is not recommended as it may impair colony development. If used, it should be placed/removed on all test colonies at the same stage of colony development. Feeders do not have to be in the hives continuously. If feeding is needed, feeders should be placed in all colonies at the same time and be of the same capacity.

Figure 20. Screened bottom boards ensure good hive ventilation and allow for easy determination of mite mortality. Photo: B Binder-Köllhofer.

3.2.1.4. Hive and colony identification. Multiple types of hive/colony identification are recommended. It is recommended to use an identification number on the bottom board. The identification should include the colony number, hive position in the apiary and number of the queen within the hive. Hive identification is complex and can cause problems if the test is long lasting. Clearly identified hives are the basis for successful test processing. Identification of the queen is not reliable, since queen tags can be removed and an unmarked queen is not easily recognised. Queen identification is, however, useful as an additional ID system.

Frequently used hive identifications:

- An accompanying card under the roof of the hive is good but harsh weather conditions can damage it. Furthermore, during regular work with colonies, cards can be mixed up between neighbour hives.
- Marks on the roof of the hive are good, but roofs are easily switched between hives during regular work.
- Marks regarding hive position within apiary (number on the stand) are a reliable system of identification in the test.
- The best position for hive identification is on the hive bottom board. Usually, these hive parts are constant and they need to be changed only in case of damage or for cleaning purposes. Therefore, it is recommended to have clean and disinfected bottom boards at the beginning of an experiment.
- The identification tag can be with standard numeration/description or with barcode or QR codes in case a digitalised data collection is used.

3.2.2. Water supply

Colonies need to have a sufficient and continuous source of clean water (Figures 21 and 22). Bees can have difficulties accepting the water source provided by the beekeeper. Therefore, it is important to provide water early in the spring, just after night temperatures are above freezing, or when first establishing the apiary. It is recommended to place the water source at or close to the test station. If there is an interruption of water supply from the designated source, bees may find an alternative water source,

Figure 21. Water source in test apiary. Photo: N Kezic.

Figure 22. A useful water dispenser which can be connected to a water butt in order to provide continuous supply over longer periods. Note that the access to water is covered to reduce the risk of contamination by faeces. Photo: N Kezic.

and then it is much more difficult to return them to desired water source. Most importantly, the water source must be protected in such a manner that bees' faeces or dead and dying bees do not end up in the water (Hegić & Bubalo, 2006). It is not recommended to add salt or any other substance in the water.

3.2.3. Wax source

It is recommended that colonies be established on high quality wax foundation, free from pesticides, veterinary drugs (confirmed with a residue analysis) and adulteration. Residues in wax can significantly influence test results, especially if the wax comes from different suppliers. A part of, or entire supers can contain frames with drawn (built) combs. However, these combs should be disinfected (acetic acid fumes, gamma radiation) (Baggio et al., 2005; Ruijter et al., 1989). Frames and supers treated with acetic acid fumes need to ventilated for at least 24 h prior to use.

3.2.4. Establishment of test colonies

We recommend the use of package bees ("artificial swarms"; Figure 23) as the healthiest and most uniform beginning for test colonies. The artificial swarm should contain at least 2 kg of young and healthy bees. The bees are placed on wax foundation in a new or clean and disinfected hive box. The hive box can be disinfected either by use of heat (jet flame lighter), gamma radiation, or soda wash (check this fact sheet for detailed instructions: https://www.nationalbeeunit.com/assets/PDFs/3_Resources_for_beekeepers/articles_reports/BBKA_news/BBKA_07_Hive_Cleaning_and_Sterilisation.pdf). The queen is introduced at the same time as the bees. Bees should have access to sugar solution in a feeder. Newly formed colonies are fed for the first few days with small amounts of 1:1 sugar solution (500 to 1000 ml).

Starting test colonies by requeening existing hives or as nucs with brood is less recommended as it bears a higher risk of contamination with diseases that are not always clearly visible (Varroa, *Nosema*, chalkbrood, viruses). However, if this method must be used for practical reasons, we recommend establishing nucs with at least two frames with brood, two frames with pollen and honey and the rest of the frames with wax foundation. At least 1 kg of bees should be in each nuc (see the COLOSS *BEEBOOK* chapter on measuring colony strength parameters, Delaplane et al., 2013). The bees and combs with brood and honey must be sourced from healthy colonies.

Figure 23. A uniform and hygienic establishment of test colonies can be achieved by placing artificial swarms placed on wax foundation. Photo: D Krakar.

3.2.5. Feeding

It is not recommended to feed bees with honey to avoid the spread of any diseases. During build-up, all colonies in the test apiary should receive the same quantity of sugar solution. Test colonies should always contain of minimum of about 8 kg stored honey to support optimal and healthy development. Rescue of weak colonies by adding brood frames or by combining weak colonies is not allowed in the test apiaries. If colonies are too weak to survive, they should be removed from testing.

3.2.6. Health management plan

The test colonies are also subject to tests and observations of colony health parameters. Thus, careful and well-planned control measures should be taken in case of occurrence of any pathogen, pest or disease. A record should be taken and the condition described when any health problem is noted. Then, if the situation represents a risk for overall testing, the tester/breeder should decide between the following possibilities:

- Do not apply any treatment if there is no risk for the remaining colonies. Treat and leave the compromised colony on site if the incident does not affect the ongoing testing and does not represent a serious risk for the remaining colonies.
- Remove the compromised colony if there is a risk for the remaining colonies.
- Apply treatment to all colonies if the performance testing would not otherwise be affected (for instance, treatment of condition/disease/pathogen/pest that is not a breeding criteria).

Any health management decision must take into account the legal aspects regarding honey bee diseases in the country where the testing is occurring. Otherwise, a special permission for experimental studies needs to be provided. Precautions should be taken in the regions affected by some honey bee predators and pest species such as *Vespa orientalis*, *Vespa velutina*, *Aethina tumida*.

3.3. Testing criteria

At the Apimondia symposium "Controlled mating and selection of the honey bee" held in Lunz in 1972, technical recommendations for methods to evaluate the performance of bee colonies were developed (H. Ruttner, 1972). These continue to serve as an international standard for testing and selecting honey bees. However, much technical progress has been achieved since then, and today the beekeeping community is facing new challenges, posed by Varroa, but also by rapid environmental and climatic changes (Neumann & Carreck, 2010).

Several reviews of recent developments in breeding for resistance to *V. destructor* in Europe and the USA have been published (Büchler et al., 2010; Guichard et al., 2020; Le Conte et al., 2020; Rinderer et al., 2010)

The recommendations in the sections below were largely revised and approved by the members of COLOSS working group on breeding and selection (Research Network on Sustainable Bee Breeding – https://www.beebreeding.net/) who cooperated in a European-wide experiment with more than 600 test colonies for assessing the impact of genotype-environment interactions on the vitality of honey bee colonies (Costa et al., 2012), the EU FP7-SMARTBEES project and the EurBeSt study (European Commission, Directorate-General for Agriculture and Rural Development, 2021). Within the EurBeST study, a detailed and visualised method guideline was developed for performance testing (Uzunov et al., 2021b). Furthermore, progress has been made for standardising the testing under commercial beekeeping conditions (Uzunov et al., 2021a). Both handbooks are available for download in different languages (www.eurbest.eu/downloads <http://www.eurbest.eu/downloads>).

3.3.1. Honey productivity and feed consumption

- All honey harvested within one season from an individual hive is recognised as the honey production of the test colony. A potential crop of swarms or permanent splits, coming from the test colony, is not regarded. To the opposite, brood removal is strictly limited, as this would jeopardise the uniformity of testing conditions for evaluation of honey production. Honey stored in the brood nest is not considered towards honey production, unless the type of hive does not include separate brood chamber and supers (e.g., Layens format).
- The supers of each colony filled with honey combs are weighed before and after extracting and the difference is noted as the honey harvest of each colony. If the extraction procedure does not allow following individual supers, an average net weight of extracted supers can be used instead of weighing individual supers after extraction.
- The result is noted in kg.
- The balance should ensure an accuracy of 100 g, be calibrated, and levelled.
- Repeated honey harvests during one season are totalled to calculate the total honey production.
- The honey harvest of different periods, however, should be reported separately to document the colony's development and adaptability to different crops.
- For more accurate investigations of colony development and food consumption, the total weight of the hives can be checked at regular intervals. The net weight of all added or replaced equipment (foundations, supers, queen excluders, etc) must be noted to calculate the net weight development in defined control intervals, for example during overwintering. See the BEEBOOK chapter on miscellaneous research methods for techniques associated with weighing full colonies, part 4.1 (Human et al., 2013).
- Programmable hive scales are on the market. Some models store the total hive weight at short intervals and can transfer the data via cell phone to central computers. This allows a continuous real-time monitoring of the honey production and food consumption of test colonies.

3.3.2. Gentleness and behaviour on combs

- The honey bees' defensive behaviour (the trait is termed from the beekeepers' point of view: "gentleness") and the bees' response during handling are classified by an experienced tester based on a standard classification (Table 5).
- In accordance with the Apimondia guidelines (H. Ruttner, 1972), the classification of gentleness and calmness are scored on a scale from 1 to 4, where 1 represents the most negative and 4 the most positive phenotype. Intermediate scores (0.5) can be used to describe slight differences within the population better.
- The behaviour must be evaluated 4–6 times during the season without regard to specific conditions (i.e., weather, honey flow, etc.). The arithmetic mean of all evaluations is calculated at the end of season and used as a test result.
- All colonies within one test yard need to be evaluated on the same date. As defensive colonies can influence the reaction of neighbouring hives, the order of management should be randomised among successive evaluations.

3.3.3. Swarming behaviour

- As with other behavioural traits (see section 3.3.2), a four-point scale is used to classify the swarming behaviour of test colonies. See Table 6 for detailed scoring criteria.
- All indications of swarming behaviour (score 1–4) are noted on each inspection.
- At the end of the testing season, the lowest registered score, representing the most extreme expression of swarming behaviour, will be assigned as a test result.
- All observed (and usually destroyed) queen cells can be counted throughout the season to quantify slight differences between colonies within the same score. Those differences can be expressed as intermediate scores (3.5, 2.5, 1.5).

3.3.4. Hygienic behaviour

Hygienic behaviour is recognised as a natural antiseptic defence against the brood diseases, American foulbrood and chalkbrood, and against Varroa (Boecking & Spivak, 1999; Evans & Spivak, 2010; Spivak & Danka, 2021; Spivak & Reuter, 2001; Wilson-Rich et al., 2009) and thus may be relevant in breeding programmes for resistance to these pathogens and parasite. Standardised methods for testing hygienic behaviour are based on the removal of freeze killed (Facchini et al., 2019; Momot & Rothenbuhler, 1971; Spivak & Reuter, 1998) or pin killed brood (Newton & Ostasiewski, 1986). Furthermore, Harbo and Harris (2005) described a method to check for a specific hygiene behaviour induced by reproducing mites in brood cells, called Varroa Sensitive Hygiene (VSH). See Table 7 for more information.

Freezing the brood with liquid nitrogen is more efficient and less destructive to the combs than cutting, freezing, and replacing comb inserts.

3.3.4.1. Freeze-killed brood assay: cutting brood out of comb to freeze.

1. Cut a comb section of sealed brood with pink to purple-eyed pupae containing approximately 100 cells on each side (5–6 cm rhombus or 6–8 cm radius) from a frame and freeze it for 24 h at −20 °C. Alternatively, the section can immediately be frozen through immersion in liquid nitrogen for about 2 min.
2. Count the number of capped cells.
3. Insert the frozen comb section into a frame of sealed brood in the colony being tested (Figure 24). Tests have shown that it does not matter if the frozen section comes from the same colony from which it was removed or from a different colony (Spivak & Downey, 1998).
4. Remove the frames 24 h after introduction into the colony.
5. Record the remaining number of sealed cells. In addition, the number of cells that have been partially or fully uncapped and the dead pupae that have not yet been completely removed from the cells can be recorded.
6. The tests should be repeated on the same colony at least twice.
7. A hygienic colony will have uncapped and completely removed over 95% of the frozen brood within 24 h on both tests. This is the most conservative (strict) assay for hygienic behaviour that should be used for breeding purposes.
8. A less conservative measure of hygienic behaviour calculates the number of frozen pupae completely removed plus those that are in the process of being removed after 24 h.
9. Formula 3.3.4.1:

$$removal\ [\%] = \frac{(number\ of\ sealed\ cells\ at\ the\ beginning - number\ of\ remaining\ sealed\ cells)}{number\ of\ sealed\ cells\ at\ the\ beginning} \times 100$$

Table 5. Standard scoring criteria for honey bee colony defensiveness.

Points	Gentleness	Calmness
4	No use of smoke and no protective clothes are necessary to avoid stings during normal working procedure.	Bees adhere to their combs "like fur" without any notable reaction when being handled.
3	Colony can easily be worked without stings, if using some smoke.	Bees are moving, but do not leave their combs during treatment.
2	Single bees attack and sting during working procedure, even if smoke is used intensively.	Bees partly leave their combs and cluster in the edges of frames and supers.
1	Despite the use of smoke, the colony exhibits a strong defence reaction when being handled, or the bees attack without being disturbed.	Bees nervously leave the combs, run out of the supers and cluster inside or outside the hive.

Table 6. Standard scoring criteria for honey bee colony propensity to swarm.

Points	Symptoms of swarming behaviour
4	The colony does not show any swarming tendency. There are no swarm cells containing eggs, larvae or pupae.
3	Low swarming tendency: some queen cells containing brood are present, but the overall colony condition does not indicate immediate swarming activities. The preparations for swarming may be stopped by destroying the swarm cells and offering additional comb space.
2	Strong swarming tendency as indicated by repeated queen cell construction and advanced symptoms of preparation for swarming (reduction of open brood, emaciated queen, limited comb construction).
1	Active swarming: the test colony swarmed or swarming could be prevented only by extensive intervention (interim nucleus etc.).

3.3.4.2. Freeze-killed brood assay: freezing brood within comb using liquid N_2.

1. Liquid nitrogen must be kept in an appropriate tank (e g. a Dewar tank) and gloves should be used when handling liquid N_2 (Figure 25)
2. Make a 75 mm diameter tube through which to pour the liquid nitrogen directly onto the comb. A metal vent pipe or PVC plumbing pipe can be used. A thicker walled tube will reduce leakage of the nitrogen through empty cells along the perimeter. The tube should be at least 100 mm long.
3. Find a section of sealed brood with pink to purple eyed pupae to freeze.
4. Put the frame horizontally across a support (i.e., an empty super). Press the tube down to the midrib of the comb with a twisting motion until it seals.
5. Record the number of unsealed cells inside the cylinder.
6. Pour 300–400 ml of liquid nitrogen into the tube. Less liquid N_2 may not freeze-kill the brood. Use a 300 ml or larger polystyrene foam (coffee) cup for measuring and pouring. First pour about 5 mm of the liquid nitrogen in the tube. When it evaporates, pour the rest.
7. Wait for the liquid nitrogen to evaporate and the tube to thaw before trying to remove it (may take 10 min or more).
8. Return the frames to the colony for 24 h.
9. The tests should be repeated on the same colony at least twice.
10. A hygienic colony will have uncapped and completely removed over 95% of the frozen brood within 24 h on both tests. This is the most conservative (strict) assay for hygienic behaviour that should be used for breeding purposes.
11. A less conservative measure of hygienic behaviour involves calculating the number of frozen pupae completely removed plus those that are in the process of being removed after 24 h
12. Formula 3.3.4.2.

$$removal\ [\%] = \frac{(100 - number\ of\ unsealed\ cells\ at\ the\ beginning - number\ of\ remaining\ sealed\ cells\ after\ 24\ hours)}{(100 - number\ of\ sealed\ cells\ at\ the\ beginning} \times 100$$

Historically, colonies that removed freeze-killed brood within 48 h were considered hygienic, and if they took more than a week, they were considered non-hygienic (Gilliam et al., 1983). There is, however, a better correlation between the removal of freeze-killed brood and disease resistance when only the

Table 7. Methods for determining the level of hygienic behaviour expressed by a honey bee colony.

Method	Repeatability	Costs & efforts	Remarks
Freeze killed brood[a]	High in colonies that remove >95% of the freeze-killed brood in 24h; variable, in colonies that do not	Moderate	Introduction of freeze killed brood pieces or use of liquid nitrogen to kill a section of brood in the comb
Pin test	Medium	Low	Piercing of 50 young pupae through the cell capping
Varroa sensitive hygiene	Unclear	High	Tests for removal of Varroa infested brood cells

[a]Colonies that are considered hygienic based on the freeze-killed brood assay, i.e., colonies that remove >95% of the freeze-killed brood within 24 h, will show very high consistency in results between assays, regardless of the strength of the colony or a given nectar flow.

Figure 24. Freeze-killed brood assay: cutting brood out of comb to freeze. Left: Frozen section of sealed brood is carefully placed into hole cut through comb. Right: Twenty-four hours after being returned to a colony, the amount of freeze-killed brood uncapped and removed is recorded. Photos: M Spivak.

Figure 25. Freeze-killed brood assay: freezing brood within comb using liquid N_2. Left: Dewar tank with valve to dispense liquid nitrogen, polystyrene foam cups for pouring liquid N_2 into PVC pipes (black pipes in combs). Right: After 24 h, this hygienic colony uncapped and removed >95% of the freeze-killed brood. Photos: M Spivak.

removal of freeze-killed brood within 24 h is considered (Spivak, unpublished data).

3.3.4.3. Pin-killed brood assay. The pin test method is at least in Europe widely used as a standard in field selection programmes. It can be performed easily by beekeepers. A statistical tool has been established to include pin test data in the estimation of breeding values for Varroa resistance (see 4.1). For the pin-killed brood assay protocol, see Figure 26 while following the numbered protocol below. Additionally, Figure 27 shows images of the protocol being applied in the field.

1. A rhomboid frame of a 10×10 cell wide template (Figure 26, number 2) is placed on a brood comb containing young pupae (Figure 26, number 1).
2. The upper left and lower right cells are marked with a colour felt-tip pen (Figure 26, number 3).
3. 50 capped brood cells are pierced (Figure 26, number 4) row by row from left to right with a fine insect pin (entomological pin size No 2).
4. Cell 51 is marked with a colour felt-tip pen to identify the treated brood area (Figure 26, number 3).
5. The comb is marked on the top bar and placed back to the brood nest in its former position.
6. After 6 h, the removal progress is checked. All cells that are still sealed are counted and subtracted from 50.
7. The highest discriminatory power of the test is reached when the total average removal of all test colonies is about 50%. Therefore, the time interval between piercing the cells and checking should be adapted to the average removal response of the test population. If the average removal rate is much lower than 50%, the time interval should be prolonged to 7 or 8 h and if the average removal is much higher than 50%, the control interval should be shortened to 5 or 4 h in further test repetitions to yield higher differences between colonies with high and low hygienic behaviour.
8. The test should be repeated 2–3 times per colony during the main brood season.
9. Formula 3.3.4.3.

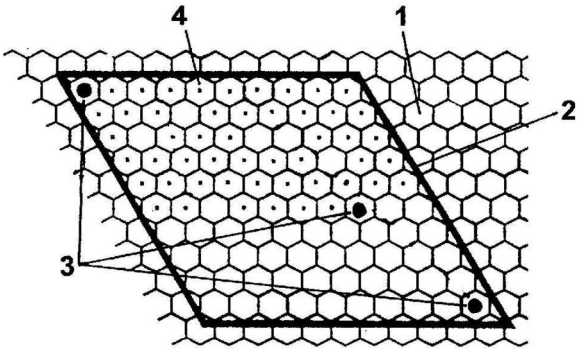


Figure 26. Pin-killed test for hygienic behaviour. The numbers correspond to text references in section 3.3.4.3.

$$removal\ [\%] = (50 - number\ of\ sealed\ cells\ after\ 6\ hours) * 2$$

3.3.5. Varroa infestation

Regular monitoring of *V. destructor* populations is not only a precondition for integrated mite control, but also an important basis for the selection of mite resistant stock. Several different methods have been developed and tested regarding systematic field evaluation of *V. destructor* densities (Lee, Moon, et al., 2010; Lee, Reuter, et al., 2010). Please also refer to the *BEEBOOK* chapter on *V. destructor*, part 4.2 (Dietemann et al., 2012). We outline in Table 8 the methods commonly used to determine *V. destructor*

populations in colonies and include information pertinent to the methods' uses in stock selection.

As a standard for performance testing, repeated checks of the mite infestation level are recommended. In periods of low infestation (usually early spring), monitoring natural mite mortality with sticky boards at the bottom of the hive reveals best results. Counting the mites on samples of adult bees is more effective with higher infestation levels that occur later in the season (Büchler et al., 2020). The estimation of breeding values (see 4.1) for *V. destructor* resistance is based on mite population growth during the season. For these calculations, natural mite mortality during three to four weeks of the first main spring pollen producing bloom (e.g., willow, hazel, almond for phenological standardisation of different climatic regions) is combined with the mite infestation of bee samples estimated during summer (Büchler et al., 2020). Repeated measurements of the bee infestation in intervals of three to four weeks improves the accuracy of the test and allows prolongation of the test period without treatment against *V. destructor* until defined threshold values (usually 5–10 mites/10 g bees, depending on environmental and beekeeping conditions) are reached.

3.3.6. Other diseases

In general, any signs of pathogen infection within performance test colonies should be carefully registered and documented. Special care should be taken with diseases that can be influenced by the genetics of the bees. These include American foulbrood, chalkbrood, nosemosis and chronic bee paralysis (CBPV or hairless black syndrome). Usually, no prophylactic or acute treatments against those diseases are recommended on test colonies to facilitate observation of potential susceptibility or resistance of the test stock to the disease. However, for a more systematic selection, a uniform initial infection of all colonies should be provided.

A simple, qualitative documentation (signs of infection observed: yes/no) may be sufficient for identification and removal of infected colonies from the breeding programme if the disease prevalence is low among colonies. Furthermore, such data can be used to identify differences among genotypes if results of related colonies in different test environments and seasons are available. Chalkbrood resistance is a regular breeding trait in www.beebreed.eu, and CBPV and nosemosis resistance have recently been added (Hoppe et al., 2022). Quantitative protocols may be used for highly prevalent diseases or for more intense selection for resistance to certain diseases. See the respective pest and pathogen BEEBOOK chapters (de Graaf et al., 2012; De Miranda et al., 2013; Dietemann et al., 2012; Forsgren et al., 2012; Fries et al., 2013; Jensen et al., 2013).

3.3.7. Colony development and wintering

The seasonal development of the bee population and brood activity are important parameters to describe local adaptation, wintering ability and productive potential of test colonies. Therefore, regular notes on the bee and brood status are essential components of each performance test.

The strength of the colony (bee population and brood extension) should at least be evaluated before and after wintering (i.e., during the first pollen flow but before plenty of young bees emerge), at the beginning of the honey flow and at the peak of

Figure 27. Pin test: (a) piercing 50 cells containing young pupae; (b) control of brood removal after about 8 h, many cells are opened but not removed; (c) nearly all cells are completely cleaned. Photos: R Büchler.

Table 8. Methods for estimating Varroa populations in honey bee colonies (see the *BEEBOOK* chapter on Varroa part 4.2 for more information on each method, including how to perform the method, Dietemann et al., 2012).

Method	Repeatability	Effort	Remarks
Natural mite mortality (i.e., mite fall or mite drop)	low	low	Results depend on the amount of emerging brood and colony size; sensitive to the presence of ants, wax moths et. al.
Bee samples – washing technique	medium	medium	Does not work with very low infestation rates; independent from colony size; bees are killed
Bee samples – powdered sugar	medium	low	Similar to washing technique, but bees are kept alive; evaluation directly at the bee yard possible; depends on dry weather
Brood samples	low	high	Time consuming; can be combined with investigations on mite reproduction

development. An overwintering index, calculated as: bee population at the end of the winter/bee population before winter, yields important information on the health of wintered colonies and the wintering ability of the colony. This index can be combined with the amount of honey consumed during winter (see section 3.3.1) to select for winter hardiness. A high overwintering index and low food consumption indicate healthy colonies that clearly stop rearing brood and have a stable winter cluster. The relation of bees and brood in spring and the overwintering index can be used to classify the spring development of colonies. Colonies with high brood activity and a quick increase in population are more suitable to exploit a good spring honey flow.

Population estimates measured with high accuracy, as may be needed for scientific investigations, can be achieved by the methods described in the *BEEBOOK* chapter on measuring colony strength parameters (Delaplane et al., 2013). When field testing of large numbers of colonies (as in most honey bee selection programmes), satisfactory results can be achieved using the methods outlined in sections 3.3.7.1 and 3.3.7.2).

3.3.7.1. Bee population.

1. Open the colony without or with limited use of smoke.
2. Check each hive box (or super) from the top and from the bottom (you do not need to take out individual combs) immediately after opening the hive to estimate the bee population.
3. Count fully covered beeways (spaces between combs) as 1, partially covered ones proportionately in quarters of a full beeway (0.25, 0.5, 0.75).
4. Calculate the average number of fully covered beeways from top and from bottom for each hive box.
5. For colonies with several boxes, sum each box's averages.
6. For performance testing, all colonies within one apiary need to be inspected on the same day.
7. Seasonal differences in the average density of bees in the cluster do not need to be recorded as the data are mainly used to compare colonies to one another. They are not meant to be an absolute measure of the number of bees.

3.3.7.2. Brood area.

1. Inspect the whole brood nest and count the number of combs containing open or sealed brood.
2. Even combs with small areas of brood of at least about 7,5 cm diameter (= 100 cells) are counted as brood combs.
3. Count the brood as 0.5 if the brood is just on one side of the comb.
4. In addition, the brood area on a central brood comb gives useful information on the brood activity of the hive. A four-point scoring is recommended for the protocol according to following the scheme:
   - 4 points: brood present on more than 75% of the comb,
   - 3 points: brood present on 50–75% of the comb,
   - 2 points: brood present on 25–50% of the comb,
   - 1 point: less than 25% of the whole comb area is covered with brood.
5. For performance testing, all colonies within one apiary need to be inspected on the same day.

3.3.8. Varroa resistance traits

Several characters may be included to improve *V. destructor* resistance, in addition to looking for colonies with limited mite population growth (MPG, see 3.3.5, Mondet, Beaurepaire, et al., 2020). Brood related *V. destructor* resistance traits appear as the most promising traits for selection and are described below.

3.3.8.1. Measuring mite non-reproduction (MNR).

The reproductive success of *V. destructor* within the brood cells is a key factor for mite population development in the colony. Mite reproduction can fail for various reasons (Büchler et al., 2020; Mondet, Beaurepaire, et al., 2020; Mondet, Parejo, et al., 2020). Due to the tight synchronisation between honey bee

pupal development and the ontogenesis of mite offspring, a modulated development or composition of mite offspring in relation to the stage of brood development characterises disturbed mite reproduction (Büchler et al., 2017). A failure of mite reproduction occurs when the mother mite lays no eggs at all (i.e., infertile), starts to oviposit too late (i.e., delayed reproduction) or male offspring is missing (i.e., no son and only daughters, see Figure 28). The investigated mother mites can be described as reproductive (i.e., at least one mature mated Varroa daughter could potentially hatch from the cell), or non-reproductive (i.e., infertile, delayed reproduction or male missing). Based on this, MNR is usually expressed as the proportion of all single infested cells investigated in which mites fail to reproduce.

The test colonies should have an undisturbed brood development for at least three brood cycles (approx. 60 days) prior to sampling (no brood interruption, no queen exchange, Gabel et al., 2023). The sampled brood should mainly contain older pupae (purple eyes stage to moult completed, corresponding to 7–12 days post capping). Depending on the brood pattern, infestation level and investigation approach, whole combs in frames or excised pieces of the brood comb might be sampled. Depending on the work capacities, the brood samples can be evaluated immediately or stored in freezer (−18 °C) until examination. An immediate examination is recommended, since it easily reveals if mother mites were already dead at the time of sampling. However, large sample sizes and scarce working capacities during the sampling season often make a postponed brood examination necessary. In this case, the absence of Varroa faeces (Figure 29) as well as a shrivelled and dried up appearance of the mother indicate that the mite was already dead before it was frozen. When working with frozen samples, the brood combs need to be thawed to room temperature for approximately 30 min prior to examination.

To gain a sufficient accuracy of the measurement, a minimum number of 35 single Varroa infested brood cells is recommended for selection purposes (Büchler et al., 2017; Eynard et al., 2020) and should be extended whenever possible.

Material needed for brood investigation:

- binocular stereo microscope or similar optics with 5× to 10× magnification
- light source (preferably LED) for sample investigation
- fine forceps (e.g., size 7)
- fine paintbrush (e.g., size 1 or smaller)
- two hand counters
- prepared form sheets for record keeping

Investigation of mite reproduction:

1. Gently open the cell capping by cutting its rim with the sharp forceps until it can be unfolded.
2. Flip the cell capping over to open the cell, the remaining connection between capping and cell wall can work like a hinge (Figure 30).
3. Gently remove the pupa and determine its age; details for age determination of honey bee brood are given in the section "Obtaining brood and adults of known age" in the BEEBOOK chapter on miscellaneous methods (Human et al., 2013).
4. Count the cell with a hand counter if the bee within the cell is older than seven days post capping (discard the cell and do not count it if it is younger); the end-count of this hand counter gives the total number of cells investigated.
5. Check for Varroa in the cell and on the pupa.
6. Move to the next cell if no signs of infestation (mites, mite faeces) are visible.
7. If the cell is infested, note the age of the host pupae, the number of mother mites, the presence of male mites, and the development stadium of the oldest female offspring in the record keeping sheet; determination of mite offspring is presented in the BEEBOOK chapter on standard methods for Varroa research (Dietemann et al., 2012). See also Figure 31.

Figure 28. Types of failed reproduction at 10 days post capping: male missing (left), delayed reproduction (centre) and infertility (right). Photos: M. Gabel.

Figure 29. Varroa faeces (white spots at the lower sidewall) inside a broodcell. Photo: F. Mondet.

Figure 30. Cell capping opened for investigation. Photo: M. Gabel.

8. Evaluate the reproductive success of the single infested cells using the scheme given in Figure 32.
9. Calculate the proportion of single infested non-reproductive cells on all single infested cells according to formula 338.1.

Formula 3.3.8.1 – *MNR* :

$$non\ reproductive\ cells\ [\%] = \frac{number\ of\ non\ reproductive\ single\ mite\ infested\ cells}{number\ of\ all\ single\ mite\ infested\ cells\ sampled} \times 100$$

3.3.8.2. Recapping of brood cells. The recapping behaviour (REC) consists of the uncapping and subsequent recapping of already sealed brood cells by worker bees. Although described since the 1990s, REC has recently regained attention in the light of both research and breeding (Aumeier et al., 2000; Boecking & Spivak, 1999; Corrêa-Marques & De Jong, 1998; Danka et al., 2013, 2016; Harris et al., 2010, 2012; Martin et al., 2020; Oddie et al., 2018). This trait seems to be associated with Varroa resistance in honey bees (Mondet, Beaurepaire, et al., 2020), though it is also expressed in Varroa-naïve populations (Martin et al., 2020). Indeed, several surviving populations of honey bees display enriched levels of recapping targeted towards infested brood. High levels of recapping could be linked to high levels of mite reproduction failure. It has also been shown that artificial uncapping of cells and subsequent recapping by the worker bees can trigger a decrease in mite reproductive success (Oddie et al., 2018). Thus, brood cell recapping stands as a candidate trait to explain the ability of honey bee colonies to survive Varroa infestation.

To assess whether a cell was recapped or not, the underside of the cell capping has to be investigated. Usually, this part of the inner cell walls is covered by the silk fibres of the pupal cocoon. If the cocoon is damaged so that the waxen cell capping is visible from underneath, the cell was opened after pupation of the brood and later resealed with wax (Figure 33). The size of the holes in the cocoon varies in accordance to the size of the prior cell openings. Recapping is usually investigated alongside the reproductive success of the mites. Measurements of recapping can be performed between Step 2 and 3 of the investigation protocol described in *3.3.8.1*.

Note that recapping is typically recorded on cells older than seven days post capping. If younger brood is examined, the results might not be comparable to reference values. Likewise to MNR measurements, at least 35 single mite infested cells should be investigated for a sufficient accuracy of recapping of brood cells infested with Varroa mites values (RECinf). For values of recapping of all cells (RECall), a minimum of 300 brood cells of the right age should be investigated.

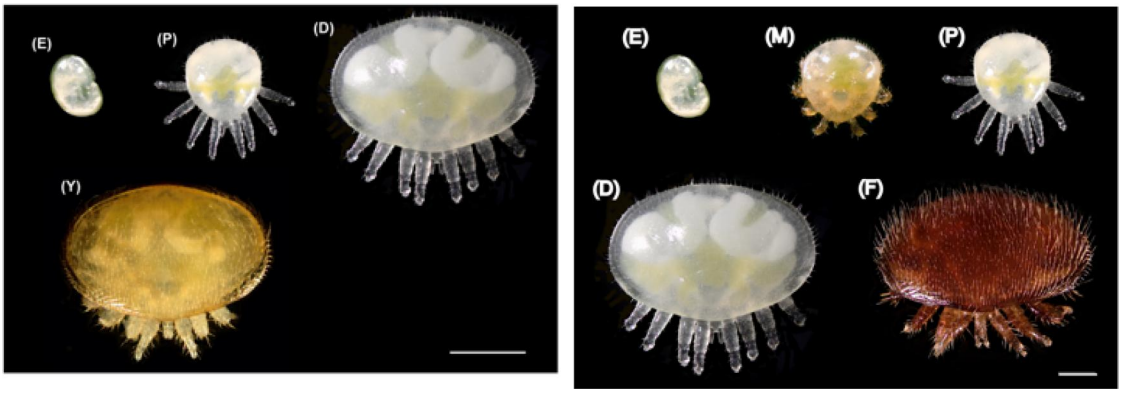


Figure 31. Developmental stages of *Varroa destructor*: (E), egg; (P), male protonymph; (M) adult male; (D) deutonymph; (Y) young adult female; (F) adult female. Scale-bar left: 5 mm; Scale-bar right: 2 mm. Photos: F. Mondet.

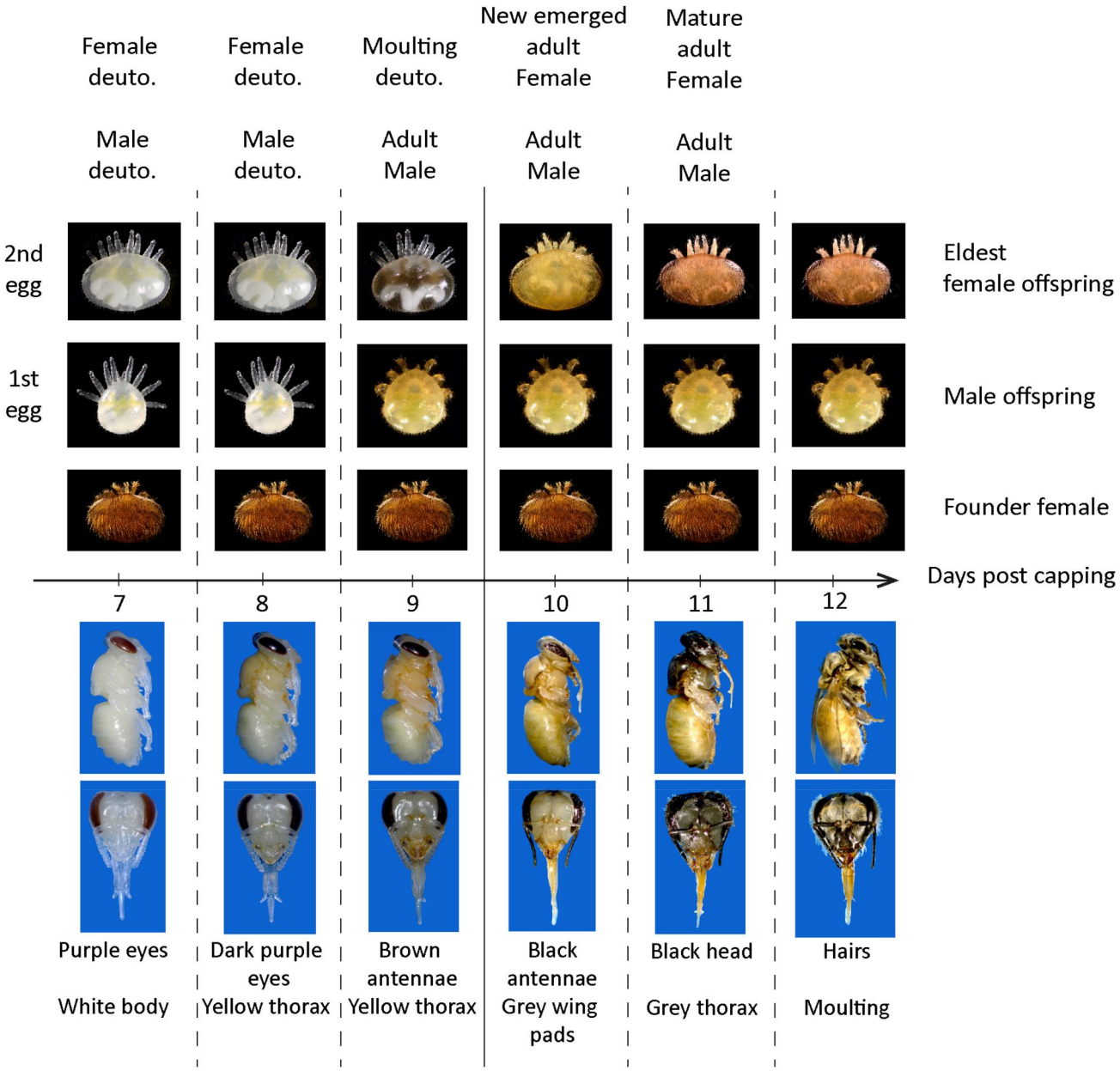


Figure 32. Comparative development of A.m.Honey bees (bottom) and *Varroa destructor* mites (first two eggs)(Top), 7 to 12 days post-capping of brood cells. Photos: F. Mondet.

For the investigation of recapping

1. Gently open the cell capping by cutting its rims with sharp forceps until it can be unfolded.
2. Flip the cell capping over to open the cell. The remaining connection between capping and cell wall can work like a hinge.

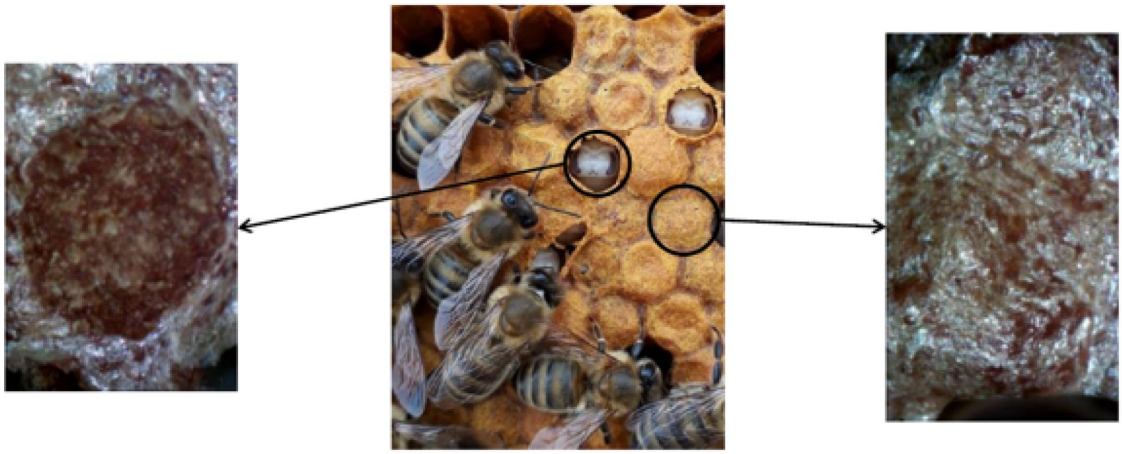


Figure 33. Recapping behaviour of worker bees (center), underside of a recapped (left), and untouched (right) cell capping with damaged and intact pupal cocoon respectively. Photos: M. Buchegger (left and right) and M. Gabel (centre).

3. Gently remove the pupa by grabbing it on the thorax with forceps. Check if its age fits your research question; details for age determination of honey bee brood are given in the chapter "Obtaining brood and adults of known age" in the BEEBOOK chapter on miscellaneous methods (Human et al., 2013).
4. Move to a new cell if the brood is too young (prepupa) or does not fit your research question for other reasons.
5. Check for holes in the silken pupal cocoon through which the waxen cell capping below is visible (Figure 33).
6. Count the investigated cell with one hand counter (for all cells investigated).
7. Note whether the cell was recapped or not. This is easily done with another hand counter for all recapped cells.
8. Continue with the steps described in *3.3.8.1.* if reproductive success of mites shall be investigated or note at least if the cell is infested or not.
9. Calculate the proportion of recapped cells (RECall-value) on all investigated cells in the right age stadium according to formula 3.3.8.2.
10. Calculate the proportion of recapped cells (RECinf-value) on all infested cells investigated according to formula 3.3.8.3.

Formula 3.3.8.2 – *RECall* :

$$recapped\ cells\ [\%] = \frac{number\ of\ all\ recapped\ cells}{number\ of\ all\ investigated\ cells} \times 100$$

Formula 3.3.8.3 – *RECinf* :

$$recapped\ infested\ cells\ [\%] = \frac{number\ of\ all\ recapped\ infested\ cells}{number\ of\ all\ infested\ cells} \times 100$$

3.3.8.3. Varroa sensitive hygiene. Varroa sensitive hygiene (VSH) describes a brood hygiene behaviour specifically targeting *V. destructor* infested brood cells. Infested brood cells are cleaned by bees that chew the infested pupa and remove the parasitising mites (Harbo & Harris, 2005; Ibrahim & Spivak, 2006). By performing this behaviour, bees can decrease the brood infestation rate (Harris, 2007) and lower the reproductive success of mites in the long term (reviewed in Mondet, Beaurepaire, et al., 2020). VSH can be evaluated at colony scale through two different methods:

- Evaluation of brood infestation (see section 4.2.3.2 in the Beebook Standard methods for Varroa research, Dietemann et al., 2013) before and after transferring naturally *V. destructor* infested brood combs to test colonies (Harris, 2007).
- Examination based on individual artificial cell infestations. To do this, freshly capped brood cells (within 6 h post capping) are artificially infested with a single mother mite harvested by powdered sugar shakes from mite donor colonies (see section 4.2.3.1.2.1 in the Beebook Standard methods for Varroa research, Dietemann et al., 2012, which also provide details on both rearing of mites in donor colonies (section 4.6), as well as artificial infestation of brood cells (section 4.5.2.3.1). A minimum of 30 cells should be infested per colony and compared to a similar number of control cells (i.e., manipulated but no mite inserted) to account for possible effects of the cell opening on brood removal.

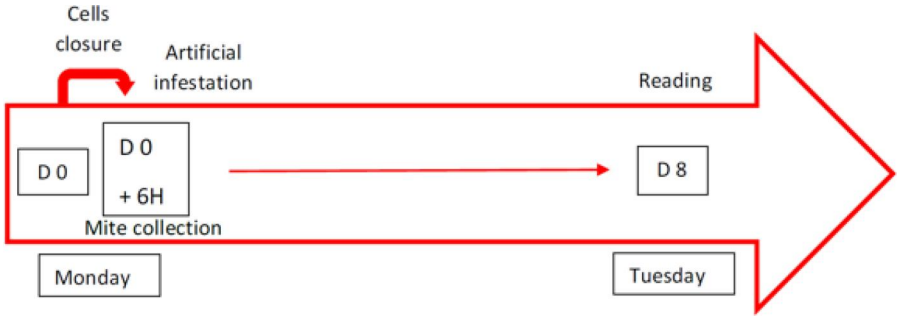


Figure 34. Workflow of Varroa sensitive hygiene examinations based on artificial cell infestations. Weekdays are given as examples for application. Abbreviations: D = day, H = hour. Copyright: F. Mondet.

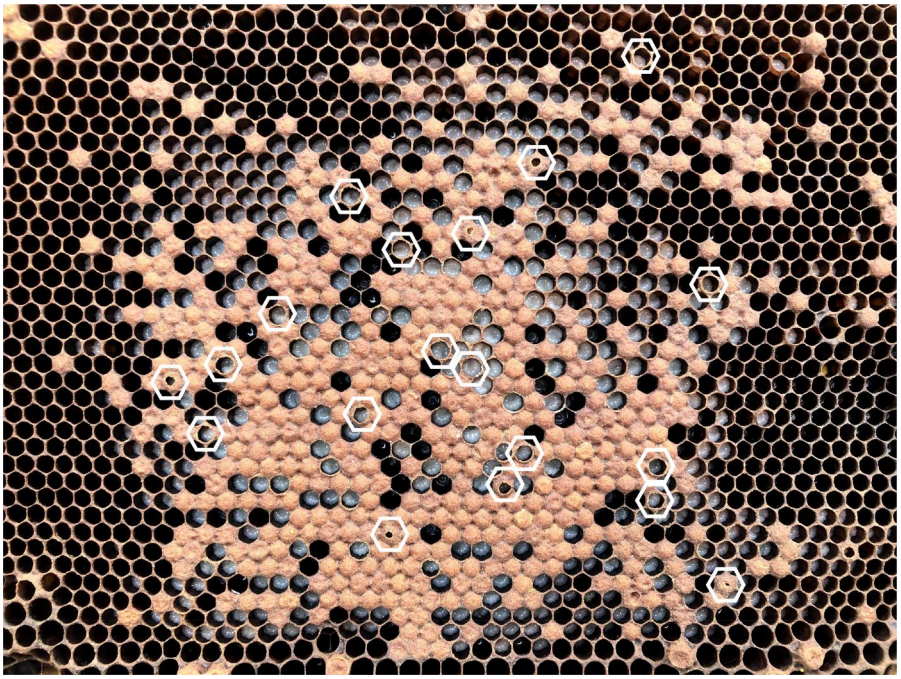


Figure 35. Ideal brood cells close to being capped (white outlines). Photo: F. Mondet.

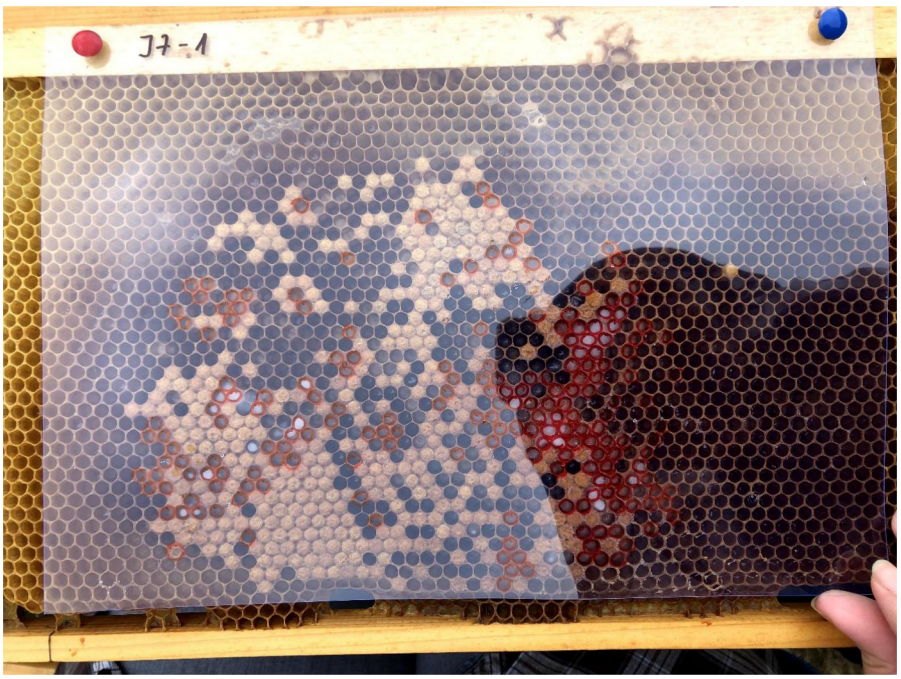


Figure 36. Cells close to capping are marked using a transparent sheet. Photo: M. Kovačić.

Figure 37. Collection of Varroa for artificial infestation after powdered sugar shakes. Photo: M. Gabel.

Figure 38. Opening the edge of a capped brood cell with a scalpel. Photo: M. Kovačić.

Investigation of VSH through artificial cell infestation

Good preparation is necessary for performing the VSH testing more easily. Varroa donor colonies should be identified prior to testing. This could be done by checking for natural mite fall a few weeks before study initiation or by checking adult bee infestation (see chapter 3.3.5.).

Equipment

- Transparent acetate sheets
- Thumbtacks to attach sheets to the frames
- Markers (two different colours)
- Scalpel
- fine paintbrush (e.g., size 1 or smaller)
- Petri dishes (tight lid) with moist filter paper (to reduce *V. destructor* movement)

Day 0 morning (see workflow chart in Figure 34)

1. Find a comb containing many brood cells that are partially capped or close to being capped (L5 stage larvae; Figure 35).
2. Attach the transparent sheet with thumbtacks to the top bar of the frame. Mark its position (i.e., the sheet margins) with two lines (left and right) on the top bar.
3. Mark brood cells that are partially capped or close to being capped on the transparent sheet (approx. 150–200 cells are usually sufficient; Figure 36).

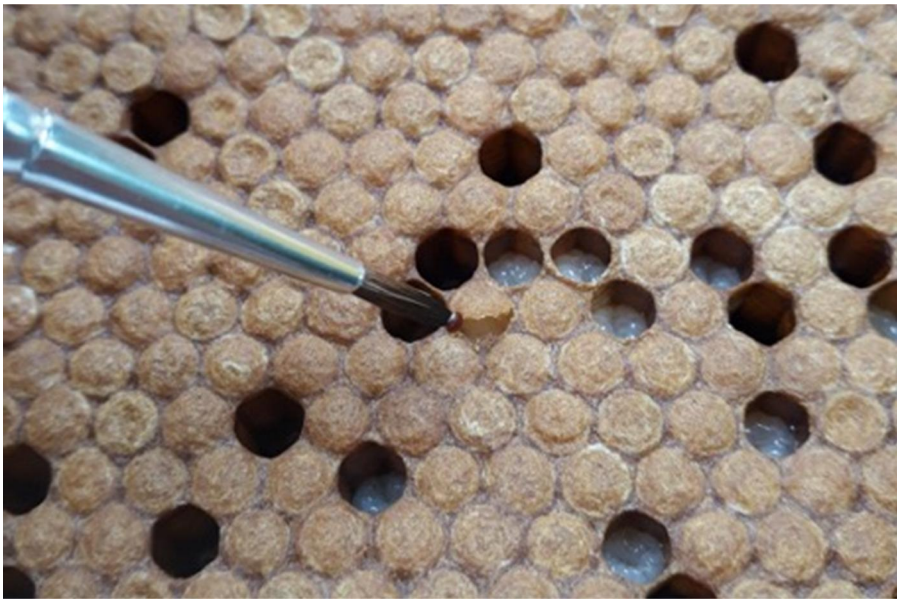


Figure 39. Transfer of a living mother mite into the opened brood cell. Photo: M. Gabel.

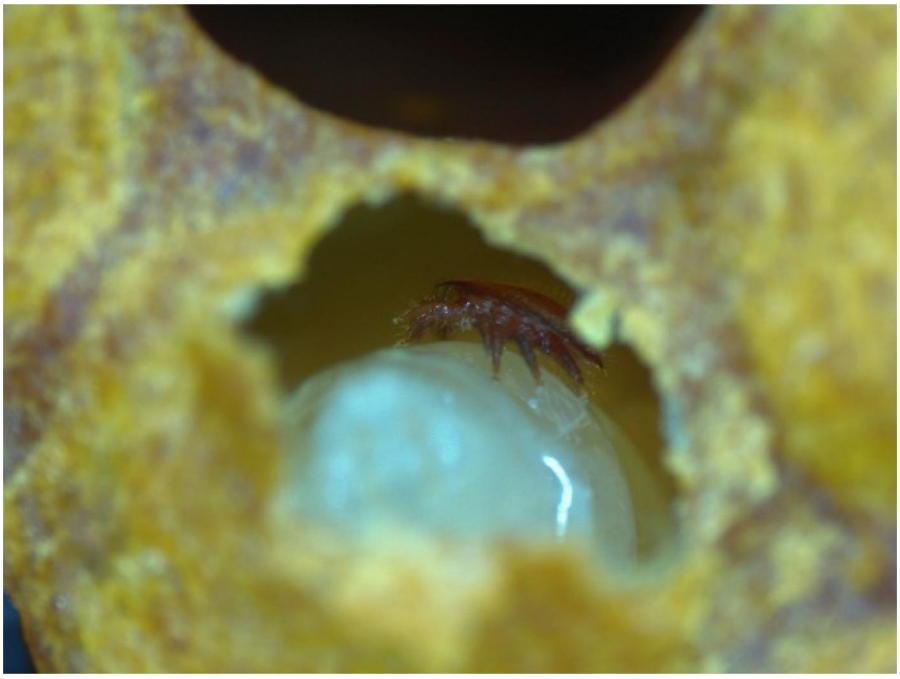


Figure 40. Mother mite in artificially infested cell. Photo: M. Gabel.

4. Label frame and sheet with the same code and remove the sheet (but return the thumbtacks to the frame to facilitate repositioning of the sheet later).
5. Return the comb to its former position in the hive.

Day 0 afternoon (collecting Varroa*)*

6. Collect Varroa using powdered sugar shake method from the donor colonies (see section 4.2.3.1.2.1 in Dietemann et al., 2013).
7. Gently rinse the mites with clear tap water of room temperature, keep them in petri dishes with moist filter paper, and use them for infestation as soon as possible (Figure 37).

Day 0 afternoon (artificial infestation 6 h after cell marking)

8. Place the transparent sheet marked in the morning back on its respective frame.
9. Choose 60 brood cells that have been capped.
10. Infest half of the brood cells (30) with one living Varroa per cell.
11. Carefully open one side of brood capping (not to injure the bee larvae) by holding the

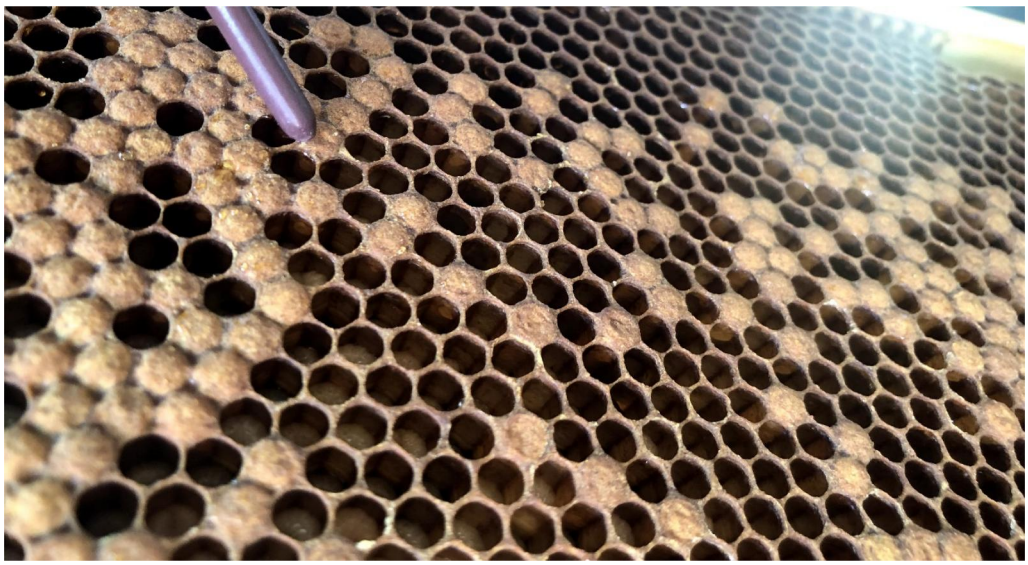


Figure 41. Closing of the infested brood cell using the brush handle. Photo: M. Kovačić.

Figure 42. Cell marks on transparent sheet (red = infested, blue = control, unfilled = not manipulated and/or not sealed in time). Photo: M. Gabel.

scalpel flat above the comb surface (Figure 38).

12. Transfer the mite with a fine brush into the opened cell (Figures 39 and 40).
13. Gently close the cell using the handle of the brush (Figure 41).
14. Mark on the transparent sheet the position of these infested cells (e.g., red cross in the previously marked cell margins).
15. Open and close 30 control cells. Follow the same procedure from the previous steps (steps 11, 13 and 14) without introducing the mite. Mark the cells on the sheet as controls (use a different colour, Figure 42).
16. Return the comb to its former position in the hive.

Day 8 (reading the results)

17. Place the transparent sheet (with marked cells) back on the frame.
18. Record if originally marked cells are capped, opened, or emptied for both control and infested cells.
19. Remaining capped cells should be additionally investigated according to the protocols given in 3.3.8.1. and 3.3.8.2. to check for recapping, number of mother mites and presence of offspring.
20. Calculate the proportion of removed cells (VSH-value) according to formula 3.3.8.4.

Formula 3.3.8.4 – *VSH* :

$$removed\ infested\ cells\ [\%] = \left(\frac{number\ of\ removed\ infested\ cells}{number\ of\ initially\ infested\ cells} - \frac{number\ of\ removed\ control\ cells}{number\ of\ initial\ control\ cells}\right) \times 100$$

3.3.9. Additional test characters

Bees can be tested and selected for additional traits of interest. Such traits include pollen gathering behaviour, longevity, and breeding for morphological characters (Rinderer, 1986).

Additional characters may be selectable when attempting to improve disease resistance of bees. For *V. destructor* resistance, various traits such as grooming behaviour of bees, post-capping period of cells, etc. have been discussed as potential selection criteria but have not yet been effectively involved in field selection programs.

Testing and selection may be more effective if focused on fewer characters. Usually, each additional test parameter needs additional effort and results in additional stress for the colonies. Furthermore, simultaneous selection for several independent characters reduces the selection power for each single trait. Thus, the breeding success depends very much on a clearly defined selection goal and a focused testing scheme.

4. Selection tools

The goal of beekeeping is to manage bees to produce many quality hive products and provide pollination services with maximum efficiency. An important factor in achieving this goal is genetic improvement in terms of economic, behavioural and adaptive traits of honey bees. Genetic improvement is achieved with selection (Falconer & Mackay, 1996). The rate of improvement is directly linked to accuracy with which queens are ranked based on their breeding value, the intensity with which they are selected, the amount of genetic variation available in the traits and generation interval. These issues are components of a breeding programme.

The standardisation of performance testing as described in section 3.3 is a prerequisite for successful breeding. The results will indicate differences between individual colonies that can be utilised for improvement, but these data alone are insufficient. The environment varies greatly between and within apiaries and test stations, and the traits measured are strongly affected by these environmental effects. Only the hereditary disposition (the genes conferred) is significant in breeding, as only the hereditary disposition of the animals influences the quality of the offspring. The environmental conditions under which the colonies live, unfortunately, mask or influence their hereditary properties (breeding value). Thus, a breeding programme requires a breeding value or selection index for one to choose which queens to reproduce, according to the aims of the breeding programme.

There are several instruments available for separating the environmental effects of colony performance from those of genetic disposition. The most sophisticated and accurate method for calculating a selection index is a statistical model called the "BLUP (Best Linear Unbiased Prediction) Animal Model" (Henderson, 1988), which was modified for use in honey bee breeding programmes (Bienefeld et al., 2007; Brascamp & Bijma, 2014; described in section 4.1). However, for small scale breeding programmes, simpler indicators may be used (section 4.2).

4.1. Genetic evaluation with BLUP

The use of the BLUP Animal Model is referred to as "Genetic evaluation" and its outcome, the "breeding value", refers to the expectation value of the progeny of the selected individual in relation to the population for a considered trait.

In genetic evaluation, a genetic value is assigned to each animal to rank animals and, thus, guide selection. Compared to other livestock which undergo genetic improvement, honey bees have peculiar genetic and reproductive characteristics (haplo-diploid sex determination, arrhenotoky, polyandry) which require a sophisticated calculus to estimate the numerator relationship matrix (Bernstein et al., 2018; Brascamp & Bijma, 2014) and reduce the impact of selection. The colony's performance and behaviour result from the combined activities of the queen (maternal effect) and workers (direct effect). As queen and worker effects are negatively correlated (Bienefeld & Pirchner, 1990), selection response is further limited (Willham, 1963). Models for genetic evaluation must take this into account to be precise (Bienefeld et al., 2007).

Genetic evaluation needs the individual result of each colony's performance test, information regarding the apiary and season it was tested, the ancestry of each queen, and how/where the queen was mated. All this information can be collected using a web service where the person who gathers the information (the tester, mating station head, inseminator) can enter the data directly.

The requirements for such a web service:

- Controlled (i.e., password-protected) access for data input.

- Software-assisted checking for coherence with existing information, outliers, and logical inconsistencies.
- Clear definition of access rights if several people have writing access (e.g., breeder and administrator of a breeding association).
- Data format consistent with the genetic evaluation model.
- Open access of the results of the genetic evaluation.

At the moment, just one international database for the honey bee fulfils these requirements (www.beebreed.eu, see also Figure 43), and so its specifications have been chosen as a standard.

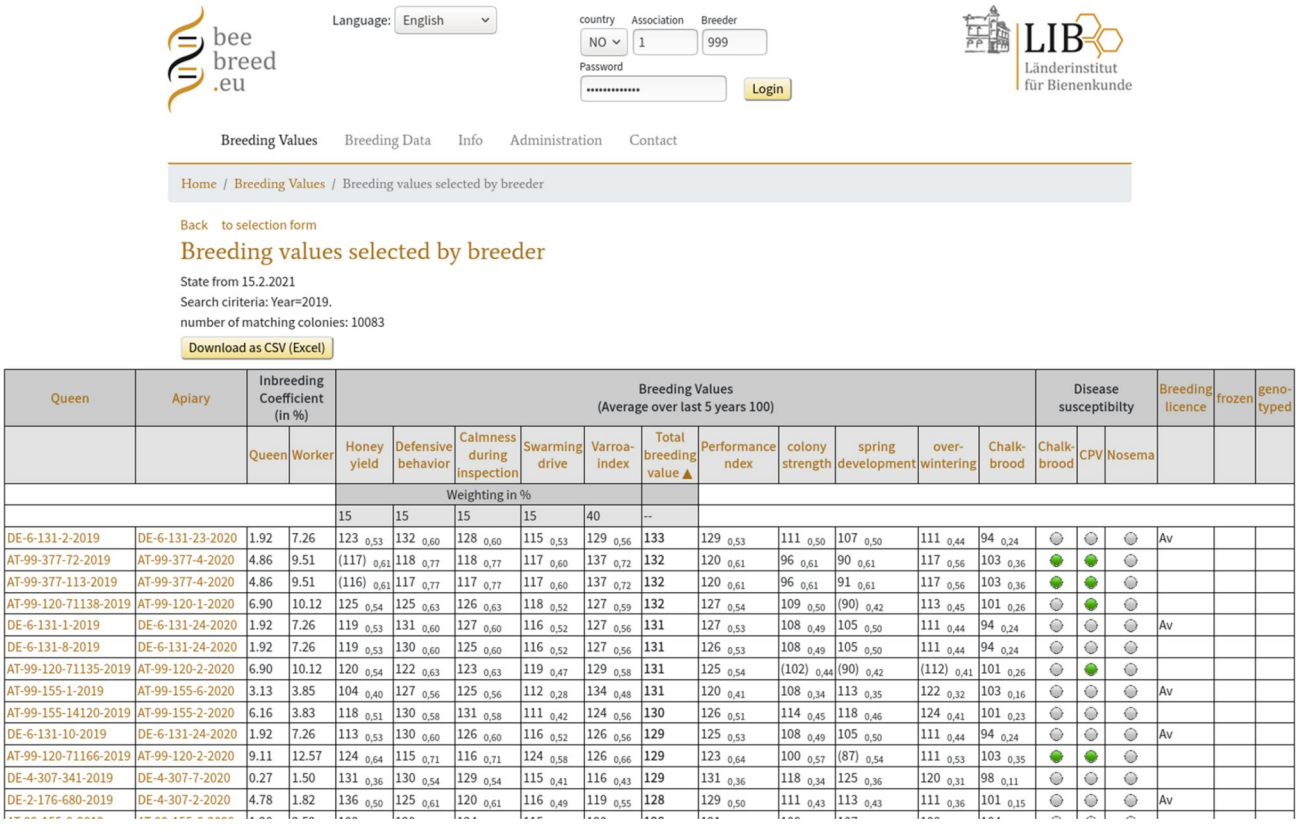
Genetic evaluation highly benefits from links within the population and is promoted by the simultaneous testing of the different genetic origins of the same breeding population at each apiary, and vice-versa from the distribution of sister groups among different testing apiaries.

At least 10 colonies per apiary allow to account for the environmental effect of an apiary reliably; fewer colonies per apiary reduce the accuracy of the estimation.

4.1.1. Nomenclature and pedigree data

The cornerstone of a breeding programme is the unambiguous identification of queens, breeders, test apiaries and associations. In www.beebreed.eu, a hierarchical system is chosen which directly embeds an association in a country, links a breeder to an association, a breeding queen to its breeder, and a test apiary to the breeder who executes the performance test, by nomenclature. For example, a queen code DE-4-1-4-2021 shows that it was born in 2021, in Germany (DE), under the supervision of LV Brandenburgischer Imker (DE-4), raised by Bee Institute Hohen Neuendorf (DE-4-1), where it is queen number four. A structured code helps to minimise errors, eases recognition, and improves the identification with the system.

Because of polyandry, the pedigree specification of honey bees differs to that of other species in that the concept of *father* is not practically applicable. The basic pedigree element consists of the actual queen, her mother, and the mating partner. The latter is either *a mating station* (represented by the drones present in the area) or a particular *drone colony* (in case of single-colony insemination). A mating station (which can be a controlled mating place for free mating or an insemination station with provided drone colonies) is specified by the season and the ancestry of the drone colonies in the area. In many cases, the drone colonies are daughters of a single mother colony, also called father colony (F. Ruttner, 1988). The paternal descendent of each queen needed for genetic evaluation is (software-assisted) generated using pedigree information of her mother. For each drone producing sister group, a special item is inserted into the pedigree.



Queen	Apiary	Inbreeding Coefficient (in %)		Breeding Values (Average over last 5 years 100)											Disease susceptibilty			Breeding licence	frozen	geno-typed
		Queen	Worker	Honey yield	Defensive behavior	Calmness during inspection	Swarming drive	Varroa-index	Total breeding value ▲	Performance ndex	colony strength	spring development	over-wintering	Chalk-brood	Chalk-brood	CPV	Nosema			
				Weighting in %																
				15	15	15	15	40	--											
DE-6-131-2-2019	DE-6-131-23-2020	1.92	7.26	123 0,53	132 0,60	128 0,60	115 0,53	129 0,56	133	129 0,53	111 0,50	107 0,50	111 0,44	94 0,24				Av		
AT-99-377-72-2019	AT-99-377-4-2020	4.86	9.51	(117) 0,61	118 0,77	118 0,77	117 0,60	137 0,72	132	120 0,61	96 0,61	90 0,61	117 0,56	103 0,36						
AT-99-377-113-2019	AT-99-377-4-2020	4.86	9.51	(116) 0,61	117 0,77	117 0,77	117 0,60	137 0,72	132	120 0,61	96 0,61	91 0,61	117 0,56	103 0,36						
AT-99-120-71138-2019	AT-99-120-1-2020	6.90	10.12	125 0,54	125 0,63	126 0,63	118 0,52	127 0,59	132	127 0,54	109 0,50	(90) 0,42	113 0,45	101 0,26						
DE-6-131-1-2019	DE-6-131-24-2020	1.92	7.26	119 0,53	131 0,60	127 0,60	116 0,52	127 0,56	131	127 0,53	108 0,49	105 0,50	111 0,44	94 0,24				Av		
DE-6-131-8-2019	DE-6-131-24-2020	1.92	7.26	119 0,53	130 0,60	125 0,60	116 0,52	127 0,56	131	126 0,53	108 0,49	105 0,50	111 0,44	94 0,24						
AT-99-120-71135-2019	AT-99-120-2-2020	6.90	10.12	120 0,54	122 0,63	123 0,63	119 0,47	129 0,58	131	125 0,54	(102) 0,44	(90) 0,42	(112) 0,41	101 0,26						
AT-99-155-1-2019	AT-99-155-6-2020	3.13	3.85	104 0,40	127 0,56	125 0,56	112 0,28	134 0,48	131	120 0,41	108 0,34	113 0,35	122 0,32	103 0,16				Av		
AT-99-155-14120-2019	AT-99-155-2-2020	6.16	3.83	118 0,51	130 0,58	131 0,58	111 0,42	124 0,56	130	126 0,51	114 0,45	118 0,46	124 0,41	101 0,23						
DE-6-131-10-2019	DE-6-131-24-2020	1.92	7.26	113 0,53	130 0,60	126 0,60	116 0,52	126 0,56	129	125 0,53	108 0,49	105 0,50	111 0,44	94 0,24				Av		
AT-99-120-71166-2019	AT-99-120-2-2020	9.11	12.57	124 0,64	115 0,71	116 0,71	124 0,58	126 0,66	129	123 0,64	100 0,57	(87) 0,54	111 0,53	103 0,35						
DE-4-307-341-2019	DE-4-307-7-2020	0.27	1.50	131 0,36	130 0,54	129 0,54	115 0,41	116 0,43	129	131 0,36	118 0,34	125 0,36	120 0,31	98 0,11						
DE-2-176-680-2019	DE-4-307-2-2020	4.78	1.82	136 0,50	125 0,61	120 0,61	116 0,49	119 0,55	128	129 0,50	111 0,43	113 0,43	111 0,36	101 0,15				Av		

Figure 43. Screen shot from the breeding value database at www.beebreed.eu.

4.1.2. BLUP Animal Model

The Statistical model used in the modified BLUP Animal Model is the following:

$\mathbf{y}=\mathbf{Xb}+\mathbf{Z_1u_1}+\mathbf{Z_2u_2}+\mathbf{e}$, where: = a vector of records/traits of the colonies (e.g., honey production, defence behaviour); $\mathbf{b}$ = a vector of fixed year/beekeeper/location effects; $\mathbf{u_1}$ = a vector of random worker (direct) effects; $\mathbf{u_2}$ = a vector of random queen (maternal) effects; $\mathbf{c}$ = a vector of random residual effects; $\mathbf{x}$ = incidence matrix relating the observations to the corresponding environment (apiary within tester and year effect);

$\mathbf{Z_1}$ = incidence matrix relating the observations to corresponding worker effects; $\mathbf{Z_2}$ = incidence matrix relating the observations to the corresponding queen effects.

Solutions are obtained from the following mixed model equations:

$$\begin{pmatrix} \mathbf{X'X} & \mathbf{X'Z_1} & \mathbf{X'Z_2} \\ \mathbf{Z'_1X} & \mathbf{Z'_1Z_1}+\mathbf{A^{-1}}\alpha_1 & \mathbf{Z'_1Z_2}+\mathbf{A^{-1}}\alpha_2 \\ \mathbf{Z'_2X} & \mathbf{Z'_2X_1}+\mathbf{A^{-1}}\alpha_2 & \mathbf{Z'_2X_2}+\mathbf{A^{-1}}\alpha_3 \end{pmatrix} \cdot \begin{pmatrix} \mathbf{b} \\ \mathbf{u_1} \\ \mathbf{u_2} \end{pmatrix} = \begin{pmatrix} \mathbf{X'y} \\ \mathbf{Z'_1y} \\ \mathbf{Z'_2y} \end{pmatrix}$$

where

$$\begin{pmatrix} \alpha_1 & \alpha_2 \\ \alpha_2 & \alpha_3 \end{pmatrix} = \begin{pmatrix} \alpha_{12} & \alpha_{12} \\ \alpha_{12} & \alpha_{22} \end{pmatrix}^{-1} \cdot \alpha_e^2$$

with: σ^2_1 = additive genetic variance for worker effects; σ^2_2 = additive genetic variance for queen effects; σ_{12} = additive genetic covariance between worker and queen effects; σ^2_e = residual error variance; $\mathbf{A}^{-1}$ = inverse of the additive genetic relationship matrix.

The genetic parameters can be estimated by solving an optimisation problem (Bienefeld & Pirchner, 1990), where several algorithms such as REML and AIREML are established. A first hint on the adequacy of a breeding model is whether the parameter estimation converges robustly to a consistent solution.

Many production and behavioural traits are correlated genetically (are influenced by some of the same genes). The more traits that are targeted with the breeding programme, the less progress can be made for any single trait. A multi-trait approach, which considers the genetic correlation between traits, is applied so that predicted breeding values for individual traits in the breeding goal are combined according to the demands of the breeders (Hoppe et al., 2020).

Within the framework of a BLUP animal model, there is a variety of modelling decisions related to questions such as:

- Are the data sufficient to support worker and queen effects (Bienefeld & Pirchner, 1991)?
- Which fixed effect(s) (such as apiary and test year combination) is/are implemented?
- Which traits are combined to a multi-trait model?
- Is any data pre-transformation helpful?
- Which components form the bee pedigree (queen with or without semen, mating station, drone colonies, drone semen, drone, worker community)?
- Which variance model is applied to the community of worker bees (i.e., do all workers contribute equally or is the trait determined by a fraction of workers)?

For answers to these questions, exemplary breeding values estimations (BVE) can be viewed from two different perspectives:

- the plausibility of results, scientifically not clearly defined, but highly relevant for the breeders to accept them as a selection tool.
- on validation, where the data are split into learning data, for which BVE is applied, and test data, on which the breeding values are evaluated. Validation comes in many flavours: cross-validation vs. breeding year validation, evaluation of correlations, mean deviation and bias, relation of breeding values with data or reference breeding values, filters of test data etc. A breeding model should be preferred when it shows acceptable results in different validations because robustness of the model is of major importance.

Both perspectives are needed to target modelling artefacts which can lead to wrong breeding decisions, finally hampering breeding progress. Artefacts are generally caused by overfitting, i.e., there are not enough data or the quality of the data is not sufficient to get meaningful predictions (genetic quality of the progeny) from the model. In case of overfitting, a simpler model is often the solution.

The accuracy of genetic evaluation depends on the quality of the relationship information, the size of testing apiaries, the share of exchanged queens for testing, the quality of performance testing and the adequacy of the breeding model including its parameters. In the beginning of a breeding program, the estimation of genetic parameters is often inaccurate and may even lead to extremely wrong parameters. In this situation, generic parameters can be used with only little negative impact to the breeding progress (Du et al., 2022). In the stage when own genetic parameters are used, they may change in the progress of breeding and need to be re-estimated from time to time.

4.1.3. Outcome of genetic evaluation: breeding values

The breeding value of a queen is the estimated expectation value of the progeny for a particular characteristic (honey production, Varroa tolerance,

etc.). Breeding values are expressed in relation to the distribution of genetic values of the population. To ensure the comparability between traits, breeding values are transformed to an average of 100 and a standard deviation of 10. Under the assumption of a Gaussian distribution of breeding values, a queen with a breeding value of, e.g., 110 is better than 84.1% of the population as a basic distribution property. The population is represented by all tested queens of the last five birth years. Consequently, breeding values usually depreciate if genetic response is achieved.

To enable the breeder to make an informed decision, additional information and tools supplement the list of breeding values:

- Inbreeding coefficients
- Customisable total breeding value (weighted combination of traits)
- Filtered list of queens for thresholds of breeding values and other parameters
- Prediction of breeding values and inbreeding coefficients for potential queens, either for public mating stations or freely selected mating colonies.

Breeding values and inbreeding coefficients are published yearly after data collection and curation. Only consistent and thorough testing, precise recording, and rational selection leads to sustained breeding progress at low increase rate in inbreeding (Hoppe et al., 2020).

4.2. Selection indexes and scores

Due to various reasons, there are cases where an organised data collection as described in section 4.1 is not possible or there is an incomplete data structure. In such cases, a direct comparison of the queens based on their performance can be used. However, one should be aware that this ranking is based on phenotypic value only and does not reflect the genetic potential of the queens. In addition, a lack of pedigree information can lead to inbreeding, and it is not reliable in producing the next generation of queens. However, the following approaches can be useful if a breeding programme is not yet established or is in its infancy:

- Regression analyses: In most breeding programmes, several traits are of interest (morphological, behavioural, and production level). Evaluation of the colonies is only based on their own performance of these traits. Additional information gained from ancestors and progeny cannot be linked to them. In most cases, regression analyses can be applied, e.g., linear, logistic or even ordinal, depending on the quantity of information complementing the performance data. The adequate choice is subject to understanding the data structure and statistical methods. Nevertheless, in traits that are described quantitatively (such as honey yield, food consumption, brood development, hygienic behaviour, Varroa infestation), linear regression can be sufficient, with or without previous data transformation for obtaining normality. If the traits are described in categorical values (such as gentleness, calmness, swarming behaviour), logistic regression can be used. The estimations will be a compromise between the potential for corrections in environmental factors and the observed individual performance leading to lower accuracy. In some cases, survival analyses are appropriate (Rhodes et al., 2004), particularly in disease tolerance.
- Z-score: a simple way for comparing colonies across apiaries. It assumes that differences between apiary average scores are entirely due to location differences (this is not completely true due to interactions between the genetic origin and the location). Each testing apiary is described in terms of its own mean and standard deviation, then the individual colony performances are transformed into standard deviation units and compared (Rinderer, 1986). The resulting individual score is called z-score: $z = X - M/s$ where: $X =$ colony score; $M =$ apiary average score; $s =$ apiary standard deviation.
- Selection index according to Rinderer (1986): the aim of a selection index is to express the breeding value from the point of view of several traits in a single number. The selection index proposed by Rinderer (1986) considers the colony's individual phenotypic scores, the heritability (h^2) of the traits and the genetic correlations between them, as well as the economic value of the characteristics (based on breeding programme and beekeeper preference). A simple version of the index considers only the z-scores and the relative economic value of the chosen traits: $I = z_a\ V + z_b$ where: $z_a =$ z-score for trait A; $z_b =$ z-score for trait B; $V =$ relative importance of trait A compared to trait B (e.g., if trait A is half as important as trait B, then $V = 0.5$).
- The above equation can further incorporate the heritability and genetic correlations between traits: $I = z_a\ V\ (h^2_a/h^2_b) + z_b\ (1 - r_g)$ Where: $h^2_a =$ heritability of trait A; $h^2_b =$ heritability of trait B; $r_g =$ genetic correlation between traits (correlation between breeding values).
- Selection index according to Cornuet and Moritz (1987): When groups of sister queens are considered in the testing programme, a selection index J, which considers the relationships inside the family (mother-daughter covariance, between

sisters covariance and aunt-niece covariance), can be used. Plausible values for covariances result in the following formula, which considers a single trait: $J_{ij} = 0.163\ (m_{ij} - m_i) + 0.348\ m_i$ Where: m_{ij}= colony value; m_i = average family value.

4.3. Molecular selection tools

Note: The BEEBOOK paper on molecular research techniques (Evans et al., 2013) outlined how molecular data can be obtained from *Apis mellifera*. We focus on the data evaluation for queen selection.

The completion of the honey bee genome project held the promise for fast selection of colonies with desirable traits (Weinstock & Robinson, 2006). Knowing the genes coding for any particular trait would, in theory, allow for the selection of queens and drones with desired genotypes for further breeding without evaluation of colony traits. However, compared to other livestock species, complications arise from the complexity of honey bee genetics. For example, colonies that perform best, do so due to a high level of genetic diversity amongst the workers (Seeley & Tarpy, 2007). The colony harbours two generations, namely the queen and her worker offspring, which are subject to the combinational effects of mostly more than ten chromosome sets due to the multiple matings of the queen. This makes the role that selection for a single trait at individual level can play questionable, especially when transferred into colony performance (but see Cook et al., 2020). In more advanced and complex breeding programmes, genome-wide marker assisted selection may boost accuracy of genetic improvement in honey bees (Meuwissen et al., 2001). Recently, it has been shown that genomic breeding values can be significantly more accurate than pedigree-based breeding values for honey bees (Bernstein et al., 2023)

A variety of markers with accurate linkage maps today exist for the preliminary screening for quantitative trait loci (QTL):

- At first, the DNA microsatellites carefully mapped by Solignac et al. (2004) became the marker of choice.
- Since the genomic information became available (Weinstock & Robinson, 2006), single nucleotide polymorphisms (SNPs) also allow cheap and accurate targeting of QTL. A marker set of 44,000 is commercially available (Spötter et al., 2012), providing a robust coverage of the honey bee genome. Using this set of markers in a study of "Varroa-specific defence behaviour", it has been shown that it is important to examine several control populations to avoid randomly significant SNPs. In the study at hand, 122 cases were compared to 122 controls, and 6 SNPs showed highly significant associations to the trait investigated. Inspection of the surrounding genomic regions led to the discovery of candidate genes (Spötter et al., 2016).

QTL methods are particularly applicable to honey bees, due to the rather small genome with a high rate of recombination. Furthermore, the haploid stage of the drone allows for direct testing of traits linked to the individual level (e.g., Cook et al., 2019), but it might remain more complex for colony level traits. If workers can be observed to harbour a significant fraction of a colony's traits, like those engaging in hygienic behaviour, these too can be employed for these types of studies. Due to multiple matings of the queen with haploid drones, a colony will typically consist of more than 10 subfamilies. Each subfamily, often referred to as a "patriline", effectively acts as linkage group sharing the paternal fraction of the genome. Bees with a particular patriline are variable for the remaining queen contributions. This allows for the testing of genotype interactions, both at the individual worker level and at the colony level. Finding QTLs or genes affecting complex colony traits, like swarming behaviour, honey production or gentleness will demand thorough testing and considerable skills both at the molecular and computational level.

Since the late 1980s, animal breeders designed models to incorporate QTL information into BLUP-breeding value estimation (Fernando & Grossman, 1989). However, most economically important traits are polygenic, i.e., their phenotypes are controlled by many thousands of QTL, each with small effect. For these traits, individual QTL play a minor role, but genome-wide dense marker maps can be used to achieve significantly more genetic gain than with pedigree-based selection (Meuwissen et al., 2001). The initial approaches assumed that all animals were genotyped. In practical applications, not all animals were genotyped, and multiple step genomic BLUP (GBLUP) was used, where multiple analyses were done to obtain genomic breeding values (Kachman et al., 2013). The disadvantages of multistep genomic BLUP (GBLUP) are the complexity of evaluation, approximation of the information, and limitation in different models application. They also may include double counting in related individuals.

Misztal et al. (2009) proposed single step GBLUP (ssGBLUP), where phenotypes, pedigree, and genotypes are combined in a single evaluation. The method alters the relationship between individuals based on the similarity of their genotypes. In diploid species, the pedigree-based relationship between full-siblings is 0.5 (meaning 50% of their DNA in common), while it varies from 0.2 to 0.7 (Lourenco

et al., 2015) in reality. ssGBLUP is easier to implement, with high flexibility in modelling, provides proper weighting of all available information and can be used in small and large populations with any number of genotyped individuals. For honey bees, Bernstein et al. (2021) found in simulation studies that with ssGBLUP, the accuracies were over 100% higher compared to pedigree-based BLUP in different scenarios for the size of the reference population and the trait considered (Figure 44). The authors also recommend that in the beginning of breeding programs, at least 1000 queens should be genotyped per year.

Recently, an array for 100,000 SNPs has been developed and used to genotype about 3000 queens to initiate genome-wide marker assisted selection in the honey bee (Jones et al., 2020). ssGBLUP was used to estimate genomic breeding values. Cross-validations showed that the genomic breeding values were significantly more accurate than pedigree-based breeding values for honey yield, swarm drive, and gentleness (Bernstein et al., 2023).

Genomic breeding value estimation with ssGBLUP and its preparation include the following tasks:

- Selection of markers based on the known QTL for traits of interest
- Producing a SNP chip for rapid, cost-efficient genotyping
- Genotyping queens with phenotyped colonies to calibrate and validate the genomic breeding value estimation
- Quality control, e.g., by the number of successfully called SNPs per sample, and the comparison of pedigree and genotype data which must account for the reproduction biology of honey bees (Bernstein et al., 2022)
- Calculation of a relationship matrix combining pedigree and genotype information
- Calculation of breeding values as with pedigree-based BLUP
- Additional steps or variations from pedigree-based BLUP are possible. For example, Bernstein et al. (2023) applied weighted ssGBLUP, where weights for each marker are used to calculate the relationship matrix.

While the arrival of molecular markers allows for rapid selection, simulation studies in honey bees showed that new breeding schemes are required for optimal genetic improvement from genomic selection (Bernstein et al., 2021). Breeders should focus on genotyping unphenotyped queens and consider only the ones of high genomic breeding value for reproduction (Figure 45). However, an ongoing program of genomic selection will require that genotyped queens are also phenotyped to maintain the accuracy of selection over several generations.

5. Breeding designs

The tools described in section 4 provide an indication on which colonies to use in breeding, i.e., which colonies to use for the production of queens and drones. However, how many colonies should be chosen and how these breeder colonies should be combined depends on the aims, size, and resources of the breeding programme.

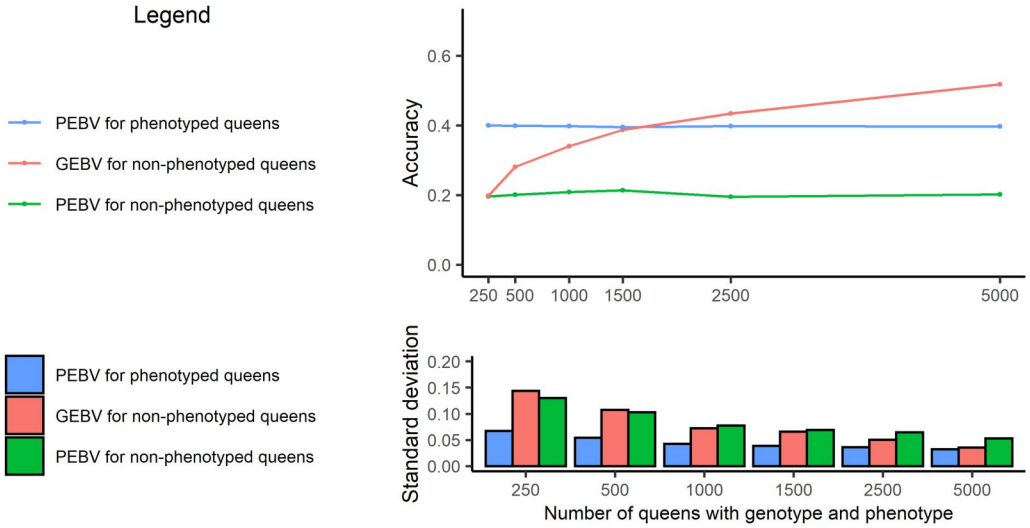


Figure 44. Accuracies of estimated breeding values and the standard deviations of these accuracies in 100 replicates of a computer simulation (Bernstein et al., 2021). The population comprised 1000 phenotyped colonies per year, distributed over 10 years with pedigree records. The genotyped queens were randomly drawn from the last year without phenotypes and from the preceding five years with phenotypes. The breeding values were calculated either based on pedigree information alone (PEBV) or with genomic data in addition to the pedigree information (GEBV).
Adapted from: Figure 3 in Bernstein et al. (2021).

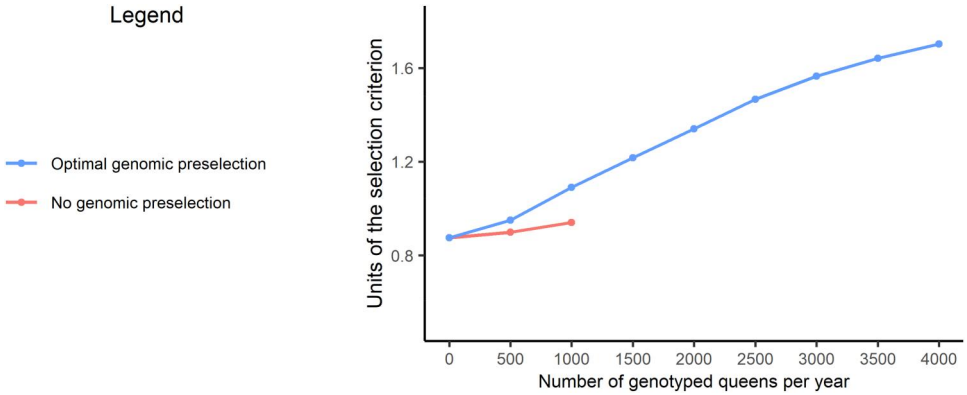


Figure 45. Predicted genetic gain according to a deterministic model (Bernstein et al., 2021). The population comprised 1000 phenotyped colonies per year. Under genomic preselection, however, a higher number of queens is produced, and the candidates of low genomic breeding value are not used for breeding. Without genomic preselection, queens were selected based on their genomic breeding value after they were phenotyped. Genetic gain was calculated in 14 million scenarios. The numbers of genotyped queens per year for which breeding schemes were compared are highlighted. Among all breeding schemes employing genomic preselection, only the one of highest genetic gain is shown for each number of genotyped queens per year. Adapted from: Figure 4 in Bernstein et al. (2021).

5.1. General recommendations

Hoppe et al. (2020) showed that the introduction of BLUP methodology boosts the improvement rates in honey bee breeding programs. Furthermore, it has been demonstrated mathematically (Du et al., 2021) and in simulation studies (Plate et al., 2019) that breeding success is much higher with controlled mating than under free mating conditions. However, breeding systems with BLUP breeding value estimation and controlled mating also come with an increased risk of high inbreeding rates and reduced genetic variance. To implement sustainable breeding strategies, it is paramount to limit the exploitation of genetic resources. In a large-scale simulation study, Plate et al. (2020) derived concrete recommendations for honey bee breeding programs:

- Breeding strategies that select the best queens of each generation for reproduction are feasible for population sizes from $\geq$200 breeding colonies per year.
- In small populations (200 colonies per year), the top 25% to 35% of tested queens should be selected for queen production. In larger populations, selection can be sharper (20% to 25% for 500 colonies per year, 15% to 20% for 1000 colonies per year, 10% to 15% for larger populations).
- Conversely, this means that in a population of 200 colonies per year, each selected queen should produce three to four daughter queens (four to five, five to six, and six to ten daughters per selected queen for populations of 500, 1000, and $>$1000 colonies per year, respectively).
- To maintain genetic variance, the variety of controlled mating opportunities should be as great as possible. If mating is controlled solely via isolated mating stations, the minimum number of such stations should be 12 for 200 breeding colonies per year (20 mating stations for 500 colonies, 40 mating stations for 1000 colonies).

These results were derived with extensive Monte Carlo simulations assuming standardised conditions. The outcomes may vary. For example, if the best queens are not selected, but instead the best daughter of each queen is chosen for reproduction, inbreeding rates will generally be lower. This strategy is called *within-family selection* by Moritz (1986) and *queen supersedure* by Laidlaw and Page (1997). In practice, it is highly recommended to monitor the actual development of inbreeding rates closely. Their increase should not exceed 0.5% to 1% per generation (Food and Agricultural Organization of the United Nations, 2013).

5.2. Possible adjustments

Only few breeding programs will be able to match all the assumptions that led to the recommendations of section 5.1. Here, we specify adjustments that can be made for several common scenarios.

5.2.1. Shortage of mating stations

The recommended number of 12 mating stations for a population of 200 colonies will generally be difficult to achieve, because isolated mating stations have high demands on the geography of their area. In case of a shortage of suitable mating stations, we recommend artificial insemination (see BEEBOOK chapter on instrumental insemination, Cobey et al., 2013). Here, a wide range of drone producing

colonies can be used. If all drones that are used for the insemination of a single queen come from the same colony, the pedigree information and thus the accuracy of breeding value estimation will be improved. Note that this strategy may have a higher risk of producing scattered brood due to homozygosity at the *csd* locus (Laidlaw & Page, 1997). Thus, we recommend close monitoring of the genetic variance, particularly in small populations.

5.2.2. Phenotypical selection

If queens are selected based on phenotype or selection indices (see section 4.2), inbreeding rates will be lower than when selected under BLUP selection. Particularly, when the heritability of the selection trait is low, simulations have shown that inbreeding rates under phenotypical selection may be reduced by up to a factor five (Du et al., 2022). Accordingly, sharper selection regimes can be applied. It is necessary to select fewer queens for reproduction since larger sister group sizes of tested queens are required to obtain reliable information on the colonies' genetic qualities. H. Ruttner (1972) recommended to test sister groups of at least 12 queens; this corresponds to selecting only 8.3% of queens for reproduction (see also section 3.1.3).

5.2.3. Non-closed populations

Many real breeding populations of honey bees are not closed and allow introgression of foreign genetic material. This can happen by planned crossings with other subspecies (as for example in many Buckfast breeding programs) or unplanned if mating is not controlled. In such breeding schemes, there is virtually no risk of inbreeding depression and selection can follow a very sharp regime. On the contrary, large sister groups of tested queens are necessary to make up for the missing pedigree information. Note, however, that uncontrolled mating not only bears the risk of unwanted hybridisations but also creates a huge reduction in genetic progress that cannot be overcome by other measures (Du et al., 2021; Plate et al., 2019).

5.3. Special designs for scientific purposes

Sometimes, honey bee breeding is performed not to achieve a general improvement of a stock, but to answer specific scientific questions. For such breeding experiments, other considerations may apply. Since they are often planned for a limited number of generations, requirements on sustainability are lower. In some cases, high inbreeding rates may even be the desired outcome. Here, we present two strategies that are of use in scientific settings.

5.3.1. Single drone mating

In some experiments, it is useful to minimise genetic differences among colonies to establish the extent of an external factor. For this aim, instrumental insemination (see the *BEEBOOK* chapter on instrumental insemination; Cobey et al., 2013) of one or more queens with semen from a single drone can be used (spermatozoa of a single drone are genetically identical). According to the number of individuals needed for the experiment, the scientist may decide whether to inseminate up to three queens with semen from a single drone. However, success in single drone insemination is more likely when a single queen is inseminated. Daughter queens from the single mated queen may then be raised (they will be closely related with degree of relationship = 0.75, i.e., "super-sisters") and according to the level of homozygosity required in the experiment, may then be inseminated with pooled homogeneous semen, or naturally mated in an isolated mating station with selected drones. To a limited degree, single drone inseminations can also be integrated in commercial breeding endeavours, since they provide better relationship information and thus more accurate BLUP breeding values. Note, however, that Kistler et al. (2021) found single drone inseminations not to be beneficial under phenotypical selection.

5.3.2. Bi-directional selection

To understand the physiological or genetic mechanism underlying a specific trait, it can be useful to obtain individuals that manifest extreme values for this trait. A breeding design in which the best and worst individuals are chosen and reproduced is referred to as "bi-directional selection". An example of a bi-directional design is described in detail in Page and Fondrk (1995). The basic steps are the following:

- The 10 best (contain the trait at highest expression in the population) and 10 worst (contain the trait at lowest expression in the population) colonies are selected.
- Five sublines within the best and worst groups are created by inseminating virgin queens with semen from a different colony of the same group, preferably from a single drone to ensure uniformity (see section 5.3.1 and the BEEBOOK paper chapter on instrumental insemination (Cobey et al., 2013).
- At each generation, the best colony of the "best" group and the worst colony of the "worst" group are used to produce virgin queens and drones.
- The colonies from the third generation queens are used for the experimental observations.

6. Conclusions

- Queen and drone reproduction techniques are widely used in modern beekeeping. Although there are plenty of technical and regional variations existing, the methodology is based on general biological preconditions and well standardised.
- Mating control is a major issue for honey bee breeding as queens naturally mate in the open with many drones and over long distances. Strategies to control the flying drone population by spatial or temporal isolation are challenging and strongly depend on local environmental conditions. The technique of artificial insemination is well established. Research is needed to better understand the biological implication of polyandry and improve the stocking of mating populations to produce quality colonies.
- The identification of genetic traits depends on standardised testing of colonies under comparable conditions. Testing protocols need to be adapted to the individual breeding programs and evolve with breeding progress and changing challenges. Objective evaluation methods are missing for some relevant selection criteria. Therefore, the quality of data collection strongly depends on clear trait descriptions and the training and experience of the testers.
- The statistical analysis of performance test data is complex as it must regard interactions of individual bees on colony level, pedigree and environmental data simultaneously.
- Genes linked to traits associated with disease resistance have been identified, but are not coherent across studies, and practical validation of the results of genetic studies are still missing. To obtain progress in this field, phenotypic observations need to be paired with genetic analyses, and more data across different environments and subspecies need to be collected.
- Breeding designs must be optimised along the genetic parameters of the selection criteria, the available resources and dimension of the breeding programs. This can be approached by simulation programs.
- Queen and drone rearing, controlled mating, and colony selection are of major relevance for the genetic adaptation of bees to various and changing environments, pest and diseases, and the success of commercial beekeeping.
- The large reproduction potential of selected individuals, the haploidy of drones and multiple mating of queens in combination with controlled mating techniques – especially by artificial insemination, opens unique opportunities for genetic research in honey bees.

Acknowledgments

The COLOSS (Prevention of honey bee COlony LOSSes) Association aims to explain and prevent massive honey bee colony losses. It was originally funded through the COST (European Cooperation in Science and Technology) Action FA0803. The COLOSS Association is now supported by the Ricola Foundation – Nature & Culture.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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References

Alaux, C., Folschweiller, M., Mcdonnell, C., Beslay, D., Cousin, M., Dussaubat, C., Brunet, J. L., & Le Conte, Y. (2011). Pathological effects of the microsporidium *Nosema ceranae* on honey bee queen physiology (*Apis mellifera*). *Journal of Invertebrate Pathology*, *106*(3), 380–385. https://doi.org/10.1016/j.jip.2010.12.005

Amiri, E., Strand, M. K., Rueppell, O., & Tarpy, D. R. (2017). Queen quality and the impact of honey bee diseases on queen health: Potential for interactions between two major threats to colony health. *Insects*, *8*(2), 48. https://doi.org/10.3390/insects8020048

Aumeier, P., Rosenkranz, P., & Gonçalves, L. S. (2000). A comparison of the hygienic response of Africanized and European (Apis mellifera carnica) honey bees to Varroa-infested brood in tropical Brazil. *Genetics and Molecular Biology*, *23*(4), 787–791. https://doi.org/10.1590/S1415-47572000000400013

Baggio, A., Gallina, A., Dainese, N., Manzi, Nello, C., Mutinelli, F., Serra, G., Colombo, R., Carpana, E., Sabatini, A. G., Wallner, K., Piro, R., & Sangiorgi, E. (2005). Gamma radiation: A sanitizing treatment of AFB contaminated beekeeping equipment. *Apiacta*, *40*, 22–27.

Ben Abdelkader, F., Barbouche, N., Belzunces, L., & Brunet, J.-L. (2015). Effects of some insecticides on the viability and the ATP synthesis of honeybee drone's

spermatozoid *in vitro* exposed. *Tunisian Journal of Plant Protection*, *10*(1), 79–93.

Bernstein, R., Du, M., Hoppe, A., & Bienefeld, K. (2022). *New approach to identify Mendelian inconsistencies between SNP and pedigree information in the honey bee* [Paper presentation]. Proceedings of 12th World Congress on Genetics Applied to Livestock Production, Rotterdam, The Netherlands. https://doi.org/10.3920/978-90-8686-940-4_622

Bernstein, R., Du, M., Du, Z. G., Strauss, A. S., Hoppe, A., & Bienefeld, K. (2023). First large-scale genomic prediction in the honey bee. *Heredity*, *130*(5), 320–328. https://doi.org/10.1038/s41437-023-00606-9

Bernstein, R., Du, M., Hoppe, A., & Bienefeld, K. (2021). Simulation studies to optimize genomic selection in honey bees. *Genetics Selection Evolution*, *53*(1), 64. https://doi.org/10.1186/s12711-021-00654-x

Bernstein, R., Plate, M., Hoppe, A., & Bienefeld, K. (2018). Computing inbreeding coefficients and the inverse numerator relationship matrix in large populations of honey bees. *Journal of Animal Breeding and Genetics*, *135*(4), 323–332. https://doi.org/10.1111/jbg.12347

Bienefeld, K., & Pirchner, F. (1990). Heritabilities for several colony traits in the honey bee (*Apis mellifera carnica*). *Apidologie*, *21*(3), 175–183. https://doi.org/10.1051/apido:19900302

Bienefeld, K., & Pirchner, F. (1991). Genetic correlations among several colony characters in the honey bee (Hymenoptera: Apidae) taking queen and worker effects into account. *Annals of the Entomological Society of America*, *84*(3), 324–331. https://doi.org/10.1093/aesa/84.3.324

Bienefeld, K., Ehrhardt, K., & Reinhardt, F. (2007). Genetic evaluation in the honey bee considering queen and worker effects – A BLUP-animal model approach. *Apidologie*, *38*(1), 77–85. https://doi.org/10.1051/apido:2006050

Boecking, O., & Spivak, M. (1999). Behavioural defences of honey bees against *Varroa jacobsoni* Oud. *Apidologie*, *30*(2–3), 141–158. https://doi.org/10.1051/apido:19990205

Bouga, M., Alaux, C., Bienkowska, M., Büchler, R., Carreck, N. L., Cauia, E., Chlebo, R., Dahle, B., Dall'olio, R., DE LA Rúa, P., Gregorc, A., Ivanova, E., Kence, A., Kence, M., Kezic, N., Kiprijanovska, H., Kozmus, P., Kryger, P. L., Conte, Y., … Wilde, J. (2011). A review of methods for discrimination of honey bee populations as applied to European beekeeping. *Journal of Apicultural Research*, *50*(1), 51–84. https://doi.org/10.3896/IBRA.1.50.1.06

Brascamp, E. W., & Bijma, P. (2014). Methods to estimate breeding values in honey bees. *Genetics Selection Evolution*, *46*(1), 53. https://doi.org/10.1186/s12711-014-0053-9

Büchler, R., Andonov, S., Bienefeld, K., Costa, C., Hatjina, F., Kezic, N., Kryger, P., Spivak, M., Uzunov, A., & Wilde, J. (2013). Standard methods for rearing and selection of Apis mellifera queens. *Journal of Apicultural Research*, *52*(1), 1–30. https://doi.org/10.3896/IBRA.1.52.1.07

Büchler, R., Berg, S., & Le Conte, Y. (2010). Breeding for resistance to *Varroa destructor* in Europe. *Apidologie*, *41*(3), 393–408. https://doi.org/10.1051/apido/2010011

Büchler, R., Costa, C., Mondet, F., Kezic, N., & Kovačić, M. (2017). RNSBB SMR recapping protocol. www.beebreeding.net/wp-content/uploads/2017/11/RNSBB_SMR-recapping_protocol_2017_09_11.pdf

Büchler, R., Kovačić, M., Buchegger, M., Puškadija, Z., Hoppe, A., & Brascamp, E. W. (2020). Evaluation of traits for the selection of *Apis mellifera* for resistance against *Varroa destructor*. *Insects*, *11*(9), 618. https://doi.org/10.3390/insects11090618

Carreck, N. L., Andree, M., Brent, C. S., Cox-Foster, D., Dade, H. A., Ellis, J. D., Hatjina, F., & van Englesdorp, D. (2013). Standard methods for *Apis mellifera* anatomy and dissection. *Journal of Apicultural Research*, *52*(4), 1–40. https://doi.org/10.3896/IBRA.1.52.4.03

Cengiz, M., Emsen, B., & Dodologlu, A. (2009). Some characteristics of queen bees (*Apis mellifera* L.) rearing in queenright and queenless colonies. *Journal of Animal and Veterinary Advances*, *8*(6), 1083–1085.

Cobey, S. W. (2007). Comparison studies of instrumental inseminated and naturally mated honey bee queens and factors affecting their performance. *Apidologie*, *38*(4), 390–410. https://doi.org/10.1051/apido:2007029

Cobey, S. W., Tarpy, D. R., & Woyke, J. (2013). Standard methods for instrumental insemination of *Apis mellifera* queens. *Research Journal of Apicultural Research*, *52*(4), 1–18. https://doi.org/10.3896/IBRA.1.52.4.09

Cook, C. N., Lemanski, N. J., Mosqueiro, T., Ozturk, C., Gadau, J., Pinter-Wollman, N., & Smith, B. H. (2020). Individual learning phenotypes drive collective behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(30), 17949–17956. https://doi.org/10.1073/pnas.1920554117

Cook, C. N., Mosqueiro, T., Brent, C. S., Ozturk, C., Gadau, J., Pinter-Wollman, N., & Smith, B. H. (2019). Individual differences in learning and biogenic amine levels influence the behavioural division between foraging honeybee scouts and recruits. *Journal of Animal Ecology*, *88*(2), 236–246. https://doi.org/10.1111/1365-2656.12911

Cornuet, J.-M., & Moritz, R. F. A. (1987). Selection theory and selection programmes. In Moritz, R (Ed.), *The instrumental insemination of the queen bee* (pp 125–141). Apimondia Publishing House.

Corrêa-Marques, M. H., & De Jong, D. (1998). Uncapping of worker bee brood, a component of the hygienic behavior of Africanized honey bees against the mite *Varroa jacobsoni Oudemans*. *Apidologie*, *29*(3), 283–289. https://doi.org/10.1051/apido:19980307

Costa, C., Berg, S., Bienkowska, M., Bouga, M., Bubalo, D., Büchler, R., Charistos, L., Le Conte, Y., Drazic, M., Dyrba, W., Fillipi, J., Hatjina, F., Ivanova, E., Kezic, N., Kiprijanovska, H., Kokinis, M., Korpela, S., Kryger, P., Lodesani, M., … Wilde, J. (2012). A Europe-wide experiment for assessing the impact of genotype-environment interactions on the vitality of honey bee colonies: Methodology. *Journal of Apicultural Science*, *56*(1), 147–158. https://doi.org/10.2478/v10289-012-0015-9

Danka, R. G., Harris, J. W., & Dodds, G. E. (2016). Selection of VSH-derived "Pol-line" honey bees and evaluation of their Varroa-resistance characteristics. *Apidologie*, *47*(3), 483–490. https://doi.org/10.1007/s13592-015-0413-7

Danka, R. G., Harris, J. W., Villa, J. D., & Dodds, G. E. (2013). Varying congruence of hygienic responses to *Varroa destructor* and freeze-killed brood among different types of honeybees. *Apidologie*, *44*(4), 447–457. https://doi.org/10.1007/s13592-013-0195-8

de Graaf, D. C., Alippi, A. M., Antúnez, K., Aronstein, K. A., Budge, G., De Koker, D., De Smet, L., Dingman, D. W., Evans, J. D., Foster, L. J., Fünfhaus, A., Garcia-Gonzalez, E., Gregore, A., Human, H., Murray, K. D., Nguyen, B. K., Poppinga, L., Spivak, M., van Engelsdorp, D., Wilkins, S.,

& Genersch, E. (2012). Standard methods for American foulbrood research. *Journal of Apicultural Research*, *52*(1), 1–28. https://doi.org/10.3896/IBRA.1.52.1.11

De Miranda, J. R., Bailey, L., Ball, B. V., Blanchard, P., Budge, G., Chejanovsky, N., Chen, Y.-P., Van, Dooremalen, C., Gauthier, L., Genersch, E., De Graaf, D., Kramer, M., Ribière, M., Ryabov, E., De Smet, L., & Van Der Steen, J. J. M. (2013). Standard methods for virus research in *Apis mellifera*. *Journal of Apicultural Research*, *52*(4), 1–56. https://doi.org/10.3896/IBRA.1.52.4.22

Degrandi-Hoffman, G., Chen, Y., & Simonds, R. (2013). The effects of pesticides on queen rearing and virus titers in honey bees (*Apis mellifera* L.). *Insects*, *4*(1), 71–89. https://doi.org/10.3390/insects4010071

Delaplane, K. S., Van Der Steen, J., & Guzman, E. (2013). Standard methods for estimating strength parameters of *Apis mellifera* colonies. *Journal of Apicultural Research*, *52*(1), 1–12. https://doi.org/10.3896/IBRA/1.52.1.03

Dietemann, V., Nazzi, F., Martin, S. J., Anderson, D. L., Locke, B., Delaplane, K. S., Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B., Rosenkranz, P., & Ellis, J. D. (2012). Standard methods for varroa research. *Journal of Apicultural Research*, *52*(1), 1–54. https://doi.org/10.3896/IBRA.1.52.1.09

Du, M., Bernstein, R., Hoppe, A., & Bienefeld, K. (2021). A theoretical derivation of response to selection with and without controlled mating in honeybees. *Genetics Selection Evolution*, *53*(1), 17. https://doi.org/10.1186/s12711-021-00606-5

Du, M., Bernstein, R., Hoppe, A., & Bienefeld, K. (2022). Consequences of incorrect genetic parameter estimates for single-trait and multi-trait genetic evaluations in honeybees. *Journal of Animal Breeding and Genetics = Zeitschrift Fur Tierzuchtung Und Zuchtungsbiologie*, *139*(6), 666–678. https://doi.org/10.1111/jbg.12728

Emsen, B., De la Mora, A., Lacey, B., Eccles, L., Kelly, P. G., Medina-Flores, C. A., Petukhova, T., Morfin, N., & Guzman-Novoa, E. (2020). Seasonality of Nosema ceranae infections and their relationship with honey bee populations, food stores, and survivorship in a North American region. *Veterinary Sciences*, *7*(3), 131. https://doi.org/10.3390/vetsci7030131

European Commission, Directorate-General for Agriculture and Rural Development. (2021). EurBeST Pilot Project: Restructuring of the Honey Bee Chain and Varroa Resistance Breeding & Selection Programme, Final Study Report AGRI-2017-0346. BRUSSELS. https://doi.org/10.2762/470707

Evans, J. D., & Spivak, M. (2010). Socialized medicine: Individual and communal disease barriers in honey bees. *Journal of Invertebrate Pathology*, *103 Suppl 1*, S62–S72. https://doi.org/10.1016/j.jip.2009.06.019

Evans, J. D., Schwarz, R. S., Chen, Y. P., Budge, G., Cornman, R. S., De la Rua, P., de Miranda, J. R., Foret, S., Foster, L., Gauthier, L., Genersch, E., Gisder, S., Jarosch, A., Kucharski, R., Lopez, D., Lun, C. M., Moritz, R. F. A., Maleszka, R., Muñoz, I., & Pinto, M. A. (2013). Standard methods for molecular research in *Apis mellifera*. *Journal of Apicultural Research*, *52*(4), 1–54. https://doi.org/10.3896/IBRA.1.52.4.11

Eynard, S. E., Sann, C., Basso, B., Guirao, A. L., Le Conte, Y., Servin, B., Tison, L., Vignal, A., & Mondet, F. (2020). Descriptive analysis of the Varroa non-reproduction trait in honey bee colonies and association with other traits related to Varroa resistance. *Insects*, *11*(8), 492. https://doi.org/10.3390/insects11080492

Facchini, E., Bijma, P., Pagnacco, G., Rizzi, R., & Brascamp, E. W. (2019). Hygienic behaviour in honeybees: A comparison of two recording methods and estimation of genetic parameters. *Apidologie*, *50*(2), 163–172. https://doi.org/10.1007/s13592-018-0627-6

Falconer, D. S., & Mackay, T. F. C. (1996). *Introduction to quantitative genetics* (4th ed.), Longmans Green, Harlow, Essex, UK.

Fernando, R. L., & Grossman, M. (1989). Marker-assisted selection using best linear unbiased prediction. *Genetics Selection Evolution*, *21*(4), 467–477. https://doi.org/10.1186/1297-9686-21-4-467

Food and Agricultural Organization of the United Nations. (2013). Breeding strategies for sustainable management of animal genetic resources. *FAO Animal Production and Health Guidelines,* vol. 3. Rome, Italy.

Forsgren, E., Budge, G. E., Charrière, J.-D., & Hornitzky, M. A. Z. (2012). Standard methods for European foulbrood research. *Journal of Apicultural Research*, *52*(1), 1–14. https://doi.org/10.3896/IBRA.1.52.1.12

Francis, R. M., Nielsen, S. L., & Kryger, P. (2013). Patterns of viral infection in honey bee queens. *Journal of General Virology*, *94*(3), 668–676. https://doi.org/10.1099/vir.0.047019-0

Fries, I., Chauzat, M.-P., Chen, Y.-P., Doublet, V., Genersch, E., Gisder, S., Higes, M., McMahon, D.P., Martín-Hernández, R., Natsopoulou, M., Paxton, R. J., Tanner, G., Webster, T. C. & Williams, G. R. (2013). Standard methods for nosema research. *Journal of Apicultural Research*, *52*(1), 1–28. https://doi.org/10.3896/IBRA.1.52.1.14

Gabel, M., Scheiner, R. & Büchler, R. (2023). Immediate and long-term effects of induced brood interruptions on the reproductive success of Varroa destructor. *Apidologie* *54*(2), 20. https://doi.org/10.1007/s13592-023-00998-x

Gauthier, L., Ravallec, M., Tournaire, M., Cousserans, F., Bergoin, M., Dainat, B., & De Miranda, J. R. (2011). Viruses associated with ovarian degeneration in *Apis mellifera* L. queens. *PLoS One*, *6*(1), e16217. https://doi.org/10.1371/journal.pone.0016217

Gilliam, M., Taber Iii, S., & Richardson, G. V. (1983). Hygienic behavior of honey bees in relation to chalkbrood disease. *Apidologie*, *14*(1), 29–39. https://doi.org/10.1051/apido:19830103

Guichard, M., Dietemann, V., Neuditschko, M., & Dainat, B. (2020). Advances and perspectives in selecting resistance traits against the parasitic mite *Varroa destructor* in honey bees. *Genetics Selection Evolution*, *52*(1), 2020. https://doi.org/10.1186/s12711-020-00591-1

Harbo, J. R., & Harris, J. W. (2005). Suppressed mite reproduction explained by the behaviour of adult bees. *Journal of Apicultural Research*, *44*(1), 21–23. https://doi.org/10.1080/00218839.2005.11101141

Harpur, B. A., Guarna, M. M., Huxter, E., Higo, H., Moon, K. M., Hoover, S. E., Ibrahim, A., Melathopoulos, A. P., Desai, S., Currie, R. W., Pernal, S. F., Foster, L. J., & Zayed, A. (2019). Integrative genomics reveals the genetics and evolution of the honey bee's social immune system. *Genome Biology and Evolution*, *11*(3), 937–948. https://doi.org/10.1093/gbe/evz018

Harris, J. W. (2007). Bees with Varroa sensitive hygiene preferentially remove mite infested pupae aged ≤ five days post capping. *Journal of Apicultural Research*, *46*(3), 134–139. https://doi.org/10.1080/00218839.2007.11101383

Harris, J. W., Danka, R. G., & Villa, J. D. (2010). Honey bees (Hymenoptera: Apidae) with the trait of Varroa sensitive hygiene remove brood with all reproductive stages of Varroa mites (Mesostigmata: Varroidae). *Annals of the Entomological Society of America*, *103*(2), 146–152. https://doi.org/10.1603/AN09138

Harris, J. W., Danka, R. G., & Villa, J. D. (2012). Changes in infestation, cell cap condition, and reproductive status of *Varroa destructor* (Mesostigmata: Varroidae) in brood exposed to honey bees with Varroa sensitive hygiene. *Annals of the Entomological Society of America*, *105*(3), 512–518. https://doi.org/10.1603/AN11188

Hatjina, F. (2012). Greek honey bee queen quality certification. *Bee World*, *89*(1), 18–20. https://doi.org/10.1080/0005772X.2012.11417453

Hatjina, F., & Charistos, L. (2018). Observations of the mating behaviour of *Apis melifera macedonica* and *Apis melifera cecropia* under natural conditions and under conditions of a control mating system (the Train of Virgin Queens). *EurBee 2018 – 8th International Congress of Apidology*, September 18–20, 2018, Belgium.

Hatjina, F., Bieńkowska, M., Charistos, L., Chlebo, R., Costa, C., Dražić, M. M., Filipi, J., Gregorc, A., Ivanova, E. N., Kezić, N., Kopernicky, J., Kryger, P., Lodesani, M., Lokar, V., Mladenovic, M., Panasiuk, B., Petrov, P. P., Rašic, S., Skerl, M. I. S., Vejsnæs, F., & Wilde, J. (2014). A review of methods used in some European countries for assessing the quality of honey bee queens through their physical characters and the performance of their colonies. *Journal of Apicultural Research*, *53*(3), 337–363. https://doi.org/10.3896/IBRA.1.53.3.02

Hegić, G., & Bubalo, D. (2006). Hygienic water supply for the bees. *Journal of Central European Agriculture*, *7*(4), 743–752.

Hempel de Ibarra, N., Vorobyev, M., & Menzel, R. (2014). Mechanisms, functions and ecology of colour vision in the honeybee. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, *200*(6), 411–433. https://doi.org/10.1007/s00359-014-0915-1

Henderson, C. R. (1988). Theoretical basis and computational methods for a number of different animal models. *Journal of Dairy Science*, *71*, 1–16. https://doi.org/10.1016/S0022-0302(88)79974-9

Hoppe, A., Bernstein, R., D. U., M., & Bienefeld, K. (2022). *Heritability of disease resistance to chronic bee paralysis, chalkbrood and nosemosis in the honeybee (A.m.carnica)* [Paper presentation]. Proceedings of 12th World Congress on Genetics Applied to Livestock Production, Rotterdam, The Netherlands. https://doi.org/10.3920/978-90-8686-940-4_618

Hoppe, A., Du, M., Bernstein, R., Tiesler, F.-K., Kärcher, M., & Bienefeld, K. (2020). Substantial genetic progress in the international *Apis mellifera carnica* population since the implementation of genetic evaluation. *Insects*, *11*(11), 768. https://doi.org/10.3390/insects11110768

Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J. D., Forsgren, E., Fries, I., Hatjina, F., Hu, F.-L., Jaffé, R., Jensen, A. B., Köhler, A., Magyar, J. P., Özkýrým, A., Pirk, C. W. W., Rose, R., Strauss, U., Tanner, G., Tarpy, D. R., … Zheng, H.-Q. (2013). Miscellaneous standard methods for *Apis mellifera* research. *Journal of Apicultural Research*, *52*(4), 1–53. https://doi.org/10.3896/IBRA.1.52.4.10

Ibrahim, A., & Spivak, M. (2006). The relationship between hygienic behavior and suppression of mite reproduction as honey bee (Apis mellifera) mechanisms of resistance to Varroa destructor. *Apidologie*, *37*(1), 31–40. https://doi.org/10.1051/apido:2005052

Jensen, A. B., Aronstein, K., Flores, J. M., Vojvodic, S., Palacio, M. A., & Spivak, M. (2013). Standard methods for fungal brood disease research. *Journal of Apicultural Research*, *52*(1), 1–20. https://doi.org/10.3896/IBRA.1.52.1.13

Jones, J. C., Du, Z. G., Bernstein, R., Meyer, M., Hoppe, A., Schilling, E., Ableitner, M., Juling, K., Dick, R., Strauss, A. S., & Bienefeld, K. (2020). Tool for genomic selection and breeding to evolutionary adaptation: Development of a 100K single nucleotide polymorphism array for the honey bee. *Ecology and Evolution*, *10*(13), 6246–6256. https://doi.org/10.1002/ece3.6357

Kachman, S. D., Spangler, M. L., Bennett, G. L., Hanford, K. J., Kuehn, L. A., Snelling, W. M., Thallman, R. M., Saatchi, M., Garrick, D. J., Schnabel, R. D., Taylor, J. F., & Pollak, E. J. (2013). Comparison of molecular breeding values based on within- and across-breed training in beef cattle. *Genetics Selection Evolution*, *45*(1), 30. https://doi.org/10.1186/1297-9686-45-30

Kahya, Y., Gençer, H. V., & Woyke, J. (2008). Weight at emergence of honey bee (*Apis mellifera caucasica*) queens and its effect on live weights at the pre and post mating periods. *Journal of Apicultural Research*, *47*(2), 118–125. https://doi.org/10.1080/00218839.2008.11101437

Kistler, T., Basso, B., & Phocas, F. (2021). A simulation study of a honeybee breeding scheme accounting for polyandry, direct and maternal effects on colony performance. *Genetics Selection Evolution*, *53*(1), 71. https://doi.org/10.1186/s12711-021-00665-8

Koeniger, N., & Koeniger, G. (2007). Mating flight duration of *Apis mellifera* queens: As short as possible, as long as necessary. *Apidologie*, *38*(6), 606–611. https://doi.org/10.1051/apido:2007060

Laidlaw, H. H. (1979). *Contemporary queen rearing* (pp. 199). Dadant & Sons.

Laidlaw, H. H., & Page, R. E. (1997). *Queen rearing and bee breeding* (pp. 224). Wicwas Press.

Le Conte, Y., Meixner, M. D., Brandt, A., Carreck, N. L., Costa, C., Mondet, F., & Büchler, R. (2020). Geographical distribution and selection of European honey bees resistant to *Varroa destructor*. *Insects*, *11*(12), 873. https://doi.org/10.3390/insects11120873

Lee, K. V., Moon, R. D., Burkness, E. C., Hutchison, W. D., & Spivak, M. (2010b). Practical sampling plans for *Varroa destructor* (Acari: Varroidae) in *Apis mellifera* (Hymenoptera: Apidae) colonies and apiaries. *Journal of Economic Entomology*, *103*(4), 1039–1050. https://doi.org/10.1603/ec10037

Lee, K. V., Reuter, G. S., & Spivak, M. (2010a). Standardized sampling plan to detect varroa densities in colonies and apiaries. *American Bee Journal*, *149*(12), 1151–1155.

Lourenco, D. A. L., Fragomeni, B. O., Tsuruta, S., Aguilar, I., Zumbach, B., Hawken, R. J., Legarra, A., & Misztal, I. (2015). Accuracy of estimated breeding values with genomic information on males, females, or both: An example in broiler chicken. *Genetics, Selection, Evolution: GSE*, *47*(1), 56. https://doi.org/10.1186/s12711-015-0137-1

Martin, S. J., Hawkins, G. P., Brettell, L. E., Reece, N., Correia-Oliveira, M. E., & Allsopp, M. H. (2020). Varroa destructor reproduction and cell re-capping in mite-resistant *Apis mellifera* populations. *Apidologie*, *51*(3), 369–381. https://doi.org/10.1007/s13592-019-00721-9

Meuwissen, T. H., Hayes, B. J., & Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics*, *157*(4), 1819–1829. https://doi.org/10.1093/genetics/157.4.1819

Misztal, I., Legarra, A., & Aguilar, I. (2009). Computing procedures for genetic evaluation including phenotypic, full pedigree, and genomic information. *Journal of Dairy Science*, *92*(9), 4648–4655. https://doi.org/10.3168/jds.2009-2064

Momot, J. P., & Rothenbuhler, W C. (1971). Behaviour genetics of nest cleaning in honey bees. VI. Interactions of age and genotype of bees, and nectar flow. *Journal of Apicultural Research*, *10,* 11–21. https://doi.org/10.1080/00218839.1971.11099665

Mondet, F., Beaurepaire, A., Mcafee, A., Locke, B., Alaux, C., Blanchard, S., Danka, B., & Le Conte, Y. (2020a). Honey bee survival mechanisms against the parasite *Varroa destructor*: A systematic review of phenotypic and genomic research efforts. *International Journal for Parasitology*, *50*(6–7), 433–447. https://doi.org/10.1016/j.ijpara.2020.03.005

Mondet, F., Parejo, M., Meixner, M. D., Costa, C., Kryger, P., Andonov, S., Servin, B., Basso, B., Bieńkowska, M., Bigio, G., Căuia, E., Cebotari, V., Dahle, B., Dražić, M. M., Hatjina, F., Kovačić, M., Kretavicius, J., Lima, A. S., Panasiuk, B., … Büchler, R. (2020b). Evaluation of suppressed mite reproduction (SMR) reveals potential for varroa resistance in European honey bees (*Apis mellifera* l.). *Insects*, *11*(9), 595. https://doi.org/10.3390/insects11090595

Moritz, R. F. A. (1986). Comparison of within-family and mass selection in honeybee populations. *Journal of Apicultural Research*, *25*(3), 146–153. https://doi.org/10.1080/00218839.1986.11100708

Morse, G. D. (1979). Queens and queen introduction (*Apis mellifera*) gleanings. *Bee Culture*, *107*(7), 342–344.

Morse, R. A. (1994). *Rearing queen honey bees* (pp. 128). Wicwas Press.

Musin, E., Bienefeld, K., Skerka, H., & Wegener, J. (2021). Delayed flight time of drones and queens as a method for mating control in small-scale honey bee breeding. *Journal of Apicultural Research*, *62*(3), 450–458. https://doi.org/10.1080/00218839.2021.2006983

Neumann, P., & Carreck, N. L. (2010). Honey bee colony losses. *Journal of Apicultural Research*, *49*(1), 1–6. https://doi.org/10.3896/IBRA.1.49.1.01

Newton, D. C., & Ostasiewski, N. J. (1986). A simplified bioassay for behavioural resistance to American Foulbrood in honey bees (*Apis mellifera* L.). *American Bee Journal*, *126*, 278–281.

Oddie, M., Büchler, R., Dahle, B., Kovacic, M., Le Conte, Y., Locke, B., DE Miranda, J. R., Mondet, F., & Neumann, P. (2018). Rapid parallel evolution overcomes global honey bee parasite. *Scientific Reports*, *8*(1), 7704. https://doi.org/10.1038/s41598-018-26001-7

Oxley, P. R., Hinhumpatch, P., Gloag, R., & Oldroyd, B. P. (2010). Genetic evaluation of a novel system for controlled mating of the honeybee, *Apis mellifera*. *Journal of Heredity*, *101*(3), 334–338. https://doi.org/10.1093/jhered/esp112

Page, R. E., & Fondrk, M. K. (1995). The effects of colony-level selection on the social organization of honey bee (*Apis mellifera* L.) colonies: Colony-level components of pollen hoarding. *Behavioral Ecology and Sociobiology*, *36*(2), 135–144. https://doi.org/10.1007/BF00170718

Park, O. W. (1923). Flight studies of the honey bee. *American Bee Journal*, *63*, 71.

Peng, Y., Baer-Imhoof, B., Millar, H. A., & Baer, B. (2015). Consequences of *Nosema apis* infection for male honeybees and their fertility. *Scientific Reports*, *5*, 10565. https://doi.org/10.1038/srep10565.

Pérez-Sato, J. A., & Ratnieks, F. L. W. (2006). Comparing alternative methods of introducing virgin queens (*Apis mellifera*) into mating nucleus hives. *Apidologie*, *37*(5), 571–576. https://doi.org/10.1051/apido:2006036

Perez-Sato, J. A., Hughes, W. O. H., Couvillon, M. J., & Ratnieks, F. L. W. (2007). Improved technique for introducing four-day old virgin queens to mating hives that uses artificial and natural queen cells for introduction. *Journal of Apicultural Research*, *46*(1), 28–33. https://doi.org/10.1080/00218839.2007.11101363

Plate, M., Bernstein, R., Hoppe, A., & Bienefeld, K. (2019). The importance of controlled mating in honeybee breeding. *Genetics Selection Evolution*, *51*(1), 74. https://doi.org/10.1186/s12711-019-0518-y

Plate, M., Bernstein, R., Hoppe, A., & Bienefeld, K. (2020). Long-term evaluation of breeding scheme alternatives for endangered honeybee subspecies. *Insects*, *11*(7), 404. https://doi.org/10.3390/insects11070404

Punko, R. N., Currie, R., Nasr, M. E., & Hoover, S. H. (2021). Epidemiology of Nosema spp. and the effect of indoor and outdoor wintering on honey bee colony population and survival in the Canadian Prairies. *PloS One*, *16*(10), e0258801. https://doi.org/10.1371/journal.pone.0258801

Rangel, J., & Tarpy, D. R. (2015). The combined effects of miticides on the mating health of honey bee (*Apis mellifera* L.) queens. *Journal of Apicultural Research*, *54*(3), 275–283. https://doi.org/10.1080/00218839.2016.1147218

Rhodes, J. W., Somerville, D. C., & Harden, S. (2004). Queen honey bee introduction and early survival – effects of queen age at introduction. *Apidologie*, *35*(4), 383–388. https://doi.org/10.1051/apido:2004028

Ricke, D. F., Lin, C. H., Johnson, R. M. (2021). Pollen treated with a combination of agrochemicals commonly applied during almond bloom reduces the emergence rate and longevity of honey bee (Hymenoptera: Apidae) queens. *Journal of Insect Science (Online)*, *21*(6), 1–8. https://doi.org/10.1093/jisesa/ieab074

Rinderer, T. E. (1986). *Bee genetics and breeding* (pp. 426). Academic Press. https://doi.org/10.1093/jisesa/ieab074

Rinderer, T. E., Guzman, L. I., Lancaster, V. A., Delatte, G. T., & Stelzer, J. A. (1999). *Varroa* in the mating yard. I. The effects of *Varroa jacobsoni* and Apistan on drone honey bees. *American Bee Journal*, *139*(2), 134–139.

Rinderer, T. E., Harris, J. W., Hunt, G. J., & de Guzman, L. I. (2010). Breeding for resistance to *Varroa destructor* in North America. *Apidologie*, *41*(3), 409–424. https://doi.org/10.1051/apido/2010015

Rousseau, A., Houle, É., & Giovenazzo, P. (2020). Effect of shipping boxes, attendant bees, and temperature on honey bee queen sperm quality (*Apis mellifera*). *Apidologie*, *51*(5), 724–735. https://doi.org/10.1007/s13592-020-00756-3

Ruijter, D. E., Van Der, A., & Steen, J. J. (1989). Disinfection of combs by means of acetic acid (96%) against nosema. *Apidologie*, *21*, 503–506.

Ruttner, F. (1988). *Biogeography and taxonomy of honey bees* (pp. 284). Springer-Verlag.

Ruttner, F. (Ed.). (1983). *Queen rearing: Biological basis and technical instruction* (pp. 358). Apimondia Publishing House.

Ruttner, H. (1972). Technical recommendations for methods of evaluating performance of bee colonies. In F Ruttner (Ed.), *Controlled mating and selection of the honey bee* (pp. 87–92). Apimondia Publishing House.

Scheiner, R., Abramson, C. I., Brodschneider, R., Crailsheim, K., Farina, W. M., Fuchs, S., Grünewald, B., Hahshold, S., Karrer, M., Koeniger, G., Koeniger, N., Menzel, R., Mujagic, S., Radspieler, G., Schmickl, T., Schneider, C., Siegel, A. J., Szopek, M., & Thenius, R. (2013). Standard methods for behavioural studies of *Apis mellifera*. *Research Journal of Apicultural Research*, *52*(4), 1–58. https://doi.org/10.3896/IBRA.1.52.4.04

Seeley, T. D., & Tarpy, D. R. (2007). Queen promiscuity lowers disease within honey bee colonies. *Proceedings. Biological Sciences*, *274*(1606), 67–72. https://doi.org/10.1098/rspb.2006.3702

Skowronek, W., Bieńkowska, M., & Kruk, C. (2004). Changes in body weight of honey bee queens during their maturation. *Journal of Apicultural Science*, *48*(2), 61–68.

Solignac, M., Vautrin, D., Baudry, E., Mougel, F., Loiseau, A., & Cornuet, J.-M. (2004). A microsatellite-based linkage map of the honey bee, Apis mellifera L. *Genetics*, *167*(1), 253–262. https://doi.org/10.1534/genetics.167.1.253

Spivak, M., & Danka, R. G. (2021). Perspectives on hygienic behavior in *Apis mellifera* and other social insects. *Apidologie*, *52*(1), 1–16. https://doi.org/10.1007/s13592-020-00784-z

Spivak, M., & Downey, D. L. (1998). Field assays for hygienic behaviour in honey bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, *91*(1), 64–70. https://doi.org/10.1093/jee/91.1.64

Spivak, M., & Reuter, G. S. (1998). Honey bee hygienic behaviour. *American Bee Journal*, *138*, 283–286.

Spivak, M., & Reuter, G. S. (2001). Resistance to American foulbrood disease by honey bee colonies, *Apis mellifera*, bred for hygienic behaviour. *Apidologie*, *32*(6), 555–565. https://doi.org/10.1051/apido:2001103

Spötter, A., Gupta, P., Mayer, M., Reinsch, N., & Bienefeld, K. (2016). Genome-wide association study of a varroa-specific defense behavior in honeybees (*Apis mellifera carnica*). *Journal of Heredity*, *107*(3), 220–227. https://doi.org/10.1093/jhered/esw005

Spötter, A., Gupta, P., Nürnberg, G., Reinsch, N., & Bienefeld, K. (2012). Development of a 44K SNP assay focussing on the analysis of a varroa-specific defence behaviour in honey bees (*Apis mellifera carnica*). *Molecular Ecology Resources*, *12*(2), 323–332. https://doi.org/10.1111/j.1755-0998.2011.03106.x

Straub, L., Villamar-Bouza, L., Bruckner, S., Chantawannakul, P., Gauthier, L., Khongphinitbunjong, K., Retschnig, G., Troxler, A., Vidondo, B., Neumann, P., & Williams, G. R. (2016). Neonicotinoid insecticides can serve as inadvertent insect contraceptives. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1835), 20160506. https://doi.org/10.1098/rspb.2016.0506

Szabo, T. I. (1982). Requeening honeybee colonies with queen cells. *Journal of Apicultural Research*, *21*(4), 208–211. https://doi.org/10.1080/00218839.1982.11100543

Tiesler, F.-K., Bienefeld, K., & Büchler, R. (2016). *Selektion bei der Honigbiene*. Buschhausen Druck und Verlaghaus.

Uzunov, A., Brascamp, E. W., & Büchler, R. (2017). The basic concept of honey bee breeding programs. *Bee World*, *94*(3), 84–87. https://doi.org/10.1080/0005772X.2017.1345427

Uzunov, A., Brascamp, E. W., Du, M., & Büchler, R. (2022). Initiation and implementation of honey bee breeding programs. *Bee World*, *99*(2), 50–55. https://doi.org/10.1080/0005772X.2022.2031545

Uzunov, A., Büchler, R., & Bienefeld, K. (2015). Performance testing protocol. A guide for European honey bee breeders. In *Landesbetrieb Landwirtschaft Hessen* (1st ed.). Kirchhain. https://doi.org/10.13140/RG.2.2.21829.86244

Uzunov, A., Büchler, R., Costa, C., Mondet, F., Bienkowska, M., Hatjina, F., Meixner, M., Andonov, S., Kovačić, M., Dall'olio, R., Basso, B., Wilde, J., Panasiuk, B., Charistos, L., Brechensbauer, D. (2021a). Book of methods for performance testers, EURBEST project (AGRI-2017-0346), Bee Institute in Kirchhain, Germany. https://www.eurbest.eu/resources/EN/EurBeST_Book_of_methods_PT_EN_20_July_2021_Uzunov_et_al.pdf (accessed on 7 February 2022)

Uzunov, A., Büchler, R., Costa, C., Mondet, F., Bienkowska, M., Hatjina, F., Meixner, M., Andonov, S., Kovačić, M., Dall'olio, R., Basso, B., Wilde, J., Panasiuk, B., Charistos, L., Brechensbauer, D. (2021b). Book of methods for commercial beekeepers, EURBEST project (AGRI-2017-0346), Bee Institute in Kirchhain, Germany. https://www.eurbest.eu/resources/EN/EurBeST_Book_of_methods_CB_EN_20_July_2021_Uzunov_et_al.pdf (accessed on 7 February 2022)

Von Frisch, K. (1967). *The dance language and orientation of bees* (pp. 566). Harvard University Press.

Weinstock, G. M., & Robinson, G. E. (2006). Insight into the social insects from the genome of the honey bee *Apis mellifera*. *Nature*, *443*, 931–949. https://doi.org/10.1038/nature05260

Willham, R. L. (1963). The covariance between relatives for characters composed of components contributed by related individuals. *Biometrics*, *19*(1), 18–27. https://doi.org/10.2307/2527570

Wilson-Rich, N., Spivak, M., Fefferman, N. H., & Starks, P. T. (2009). Genetic, individual, and group facilitation of disease resistance in insect societies. *Annual Review of Entomology*, *54*(1), 405–423. https://doi.org/10.1146/annurev.ento.53.103106.093301

Woodward, D. (2010). *Queen bee: Biology, reading and breeding* (pp. 137), Northern Bee Books.

Woyke, J. (1988). Problems with queen banks. *American Bee Journal*, *124*(4), 276–278.

Zmarlicki, C., & Morse, R. A. (1963). Drone congregation areas. *Journal of Apicultural Research*, *2*(1), 64–66. https://doi.org/10.1080/00218839.1963.11100059