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REVIEW ARTICLE

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

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Summary

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 equally serve as standards for both 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 subchapter 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. Standardized recommendations for the organization 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 which 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 also 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, plenty of references are given to facilitate comprehensive studies.

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

Resumen

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

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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.

饲养和选择西方蜜蜂蜂王的标准方法

本章列举了当前蜂王的培育、选择和育种中正在使用或值得推荐的方法。这些方法可做为科学研究和实际养蜂操作的标准方法。我们阐述了培育蜂王的基本条件、不同的饲养管理技术，还推荐了对应的育王设备。 由于蜂王是有选择的同雄蜂进行交配，这一行为会极大的影响育种方案的成功性，因此我们专门设立了分章对交配地点的管理和交配质量控制进行了阐述。由此，蜂王的培育和质量控制组成了蜂王培育部分。评价蜂群的整体性状通常应用蜂群间的对比试验，本章介绍了如何组织、评价蜂群开展常规性状测试的方法。对于大群体的评估，选择了最有效选择法，阐述了评估育种值的统计方法和数据预处理方法，育种值的计算整合了尽可能多的蜂群的家谱和蜂群性状指标数据。简述了针对小群体或某些科学问题而开展的特殊育种方法，包括新的快速发展的分子选择技术。由于蜂王的培育和选择是一很大的研究领域，本文不能完全包含，所以给出了大量文献来表述该领域的综合研究现状。

Keywords: Honey bees, selection characters, performance testing, queen production, mating control, molecular selection, breeding values, *BEEBOOK*, COLOSS

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* 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), which risks lowering the vitality of bee populations.

Responsible breeding activities have to regard 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 bee breeding, and recommend scientific and technical standards. Indeed, internationally approved quality standards for queen rearing, mating and testing are needed for the improvement, comparison and exchange of breeding stock, and to fulfil the demands of the market.

The authors share the vision that these recommendations will help preserve the natural diversity in honey bees and to support the production of high quality queens, both in a physiological and in a 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. Short history of queen rearing

The first queen rearing was practiced in ancient Greece, where bee-keepers put combs with young larvae into queenless colonies in order to raise emergency queen cells. However, at this time very little was known about the biology of honey bee colonies. In 1565 Jacob Nickel was the first in Europe to describe how honey bees can raise queens from worker eggs or very young larvae. In 1861, H Alley, W Carey and E L Pratt, from Massachusetts, USA, began to produce queens for sale. These early producers used narrow strips of comb containing eggs and larvae which they fastened to the top bars of partial combs. Placed in queenless swarms, the bees built queen cells that could be individually distributed to queenless colonies for mating.

The development of modern queen rearing techniques started in the 19th Century. Gilbert Doolittle (1889) in the USA developed a comprehensive system for rearing queen bees which serves as the basis of current production. Essentially, he used wax cups into which he transferred worker bee larvae to start the production of queen cells. His method of queen rearing in queenright colonies with the old queen isolated by a queen excluder (Doolittle, 1915) is still applied. Doolittle emphasized the importance of simulating a swarming or supersedure situation in the cell building colonies and a constant, rich food supply for the production of high quality queens.

Since 1886, queen bees have been delivered by mail with benefits for the beekeepers as well as the breeders (Pellett, 1938). Losses during transit have been reported from time to time, but in general, shipment by mail is satisfactory. Nowadays, about one million queen bees are annually sent by mail, mainly in the USA, Canada, Europe, and Australia (author estimation).

2.1.2. Basic principles of queen rearing

A honey bee colony can produce a new queen without human intervention as long as fertilized eggs 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 and Page, 1997; Ruttner, 1983). Many US beekeepers requeen as often as twice a year.

The key in queen rearing is to take a young (12-24 hours old) larva from a worker cell and place ("graft") it into a queen cell cup suspended vertically in a hive. The larva is fed on a special royal jelly diet by the nurse bees. After 10-11 days, the queen cells, which are ready to emerge, can be transferred to queenless hives or mating nuclei ("nucs") (Woodward, 2007). The success and quality of queen production depends on strong, well fed and healthy nurse colonies and on suitable equipment and colony management.

2.1.3. Equipment for queen rearing

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

2.1.3.1. Cell cups, bars and frames

- Larvae are placed in artificial queen cell cups (grafted). The cups are placed on bars which, in turn, are placed in frames (Fig. 1). Queen cell cups should measure 8-9 mm in diameter at the rim.
- Cell cups can be produced from beeswax as described by Ruttner (1983) or Laidlaw (1979). Cells should always be rinsed, after removal from the dipping sticks ("cell mandrel"), to eliminate traces of soap. Cups made in advance should be kept free of dust by storing in a sealed box. Most queen producers attach their homemade beeswax cell cups directly to a cell bar with hot wax. Queen producers 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.
- Alternatively, plastic cell cups can be purchased from beekeeping suppliers. The most popular are 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/).
- 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). The cups should be left to dry out thoroughly before attaching them to a cell bar. Such cleaning might not prevent an outbreak of black queen cell virus (BQCV), so it is always better to use new ones.

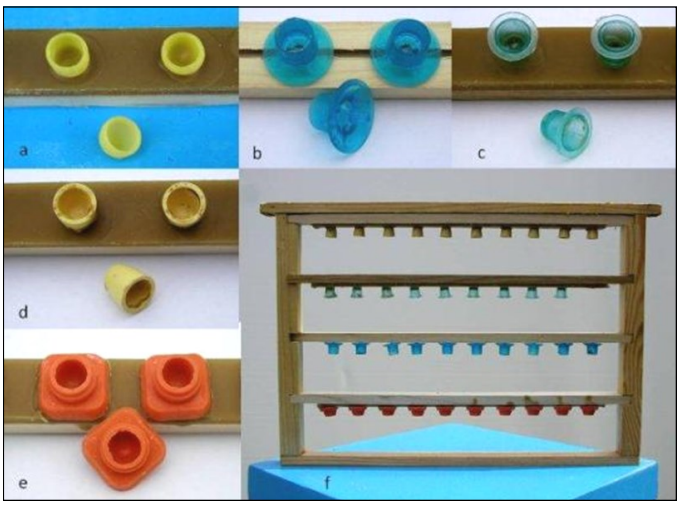


Fig. 1. Different: **a.** wax; and **b-e.** plastic queen cups and ways to attach them to the bars; **f.** frame with bars ready for grafting.

Photos: J Wilde

- Introducing plastic queen cell cups into strong colonies about one day before grafting allows the bees to clean, polish and warm the cells. Plastic cups are attached with molten clean wax as described by Ruttner (1983) or Woodward (2007).
- It is recommended to 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.
- Special push-in queen cell cups make preparing the cell bars simple. 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.
- Usually, 10-20 cells are attached to each bar with 20-60 cell cups per frame.

2.1.3.2. Grafting tools

An assortment of grafting tools can be used effectively:

- Many different versions of metal grafting needles are produced. Some have a magnifying glass fitted to the stem which can help if one's eyesight is insufficient. Usually both ends are designed for grafting; each offers a different configuration.
- A very small (size no. 000 or 00) artist's paint brush is a suitable tool for grafting. The moistened bristles must stick together to easily slide under a larva.
- A "Chinese" grafting tool is a handy and inexpensive grafting tool that looks like a ball point pen. 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 will deposit the larva and any royal jelly that was picked up in the cell to be grafted. A non-slip grip in the middle section gives excellent control. Modern versions of this tool have injection moulded plastic parts, which may help with cleanliness.

In general, grafting is easier from dark wax combs rather than from light wax combs because of the better contrast with the small white larvae. The use of a cool light or an illuminated grafting magnifier will help one see the larvae better. Grafting should be done preferably in a room or in indirect light to ensure the larvae do not dry out or become damaged by UV radiation from direct sunlight.

2.1.3.3. 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 grafting. With a single Karl Jenter kit, about 50 queens can be produced over 50 days. This is suitable for smaller beekeepers producing for their own apiaries. The Ezi-queen system is more effective for a larger production as it uses a cage of 420 cells which can all be transferred in less than 5 minutes. The plastic components used are made of a food grade polycarbonate, which allows for sterilization by autoclaving.

2.1.3.4. 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 the mating colony 1-2 days before emergence (Fig. 2).

Fig. 2. Sealed queen cells, 1-2 days before emergence, ready to be transferred to mating colonies or an incubator. Photo: J Wilde

However, if queen cells are left to emerge in the nurse colonies or in a brood chamber, they have to be protected against attacks of other queens or workers and to prevent the escape of queens. This can be achieved by cell protectors or emergence cages (Figs. 3 and 4).

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 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, which can be used singly or as a block of 10-15 cages, to protect all queen cells on a cell bar.

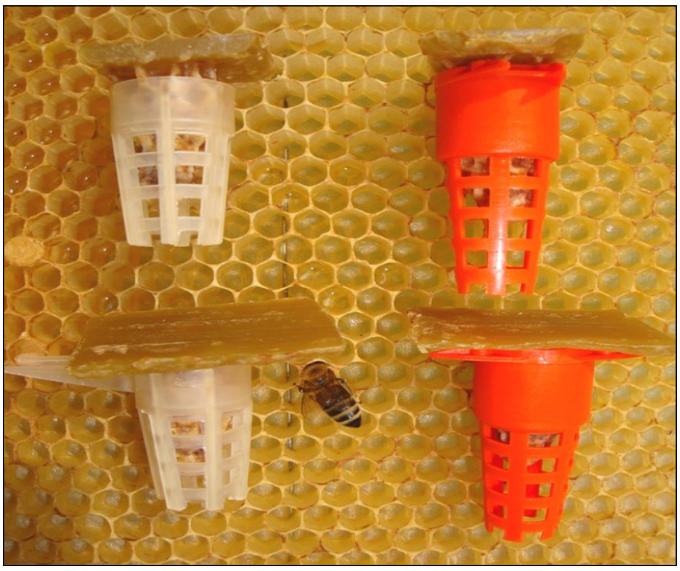


Fig. 3. Two push-in cell protectors (left) and 2 top bar cell protectors (right) from Mann Lake Ltd. Photo: J Wilde

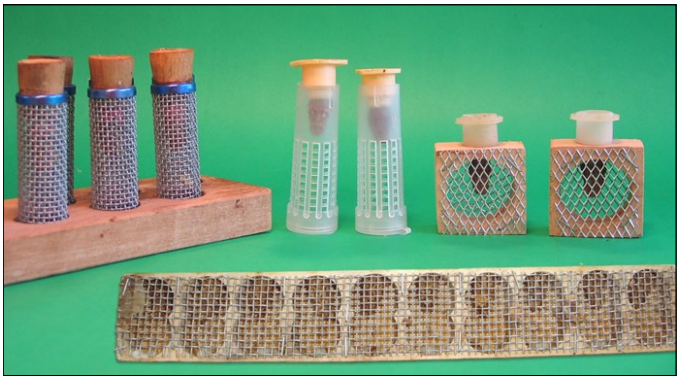


Fig. 4. Queen cells protected by 3 types of cages (from left: iron, plastic and Zander cages) and container for 10 queen cells (below). Photo: B Chuda-Mickiewicz, J Wilde

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

A few queens can be reared very simply by utilizing the natural reproductive impulses of colonies (swarming, supersedure or emergency). For example, in the Alley method (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 surgically separate the cells once they are sealed.

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 or a 9-12 frame hive without a queen or open brood, as described by Laidlaw (1979)	Gives perfect starting results independent of the weather conditions The swarm boxes can easily be transferred and used to transport queen cells	Many manipulations Confined bees in the box are stressed and less active compared to free flying colonies	
Free-flying queenless starter colony	Queenless colony without open brood as described by Laidlaw (1979) or by Morse (1979)	No extra hive equipment (like swarm boxes) needed Achieves necessary number of queen cells at any time of the season	Is necessary to cage the queen Works only with very strong colonies Requires extra colonies for queen cells finishing	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 colony	Several very popular procedures (Mackensen, Ruttner, Sklenar, Mueller) as described by 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	
Queenright starter-finisher	Queenright, two or three story colony as described by Laidlaw & Page (1997)	Achieves optimal cell and queen quality at any time of the season	Needs very strong colony	
Queenless starter-finisher	Queenless two or three story colony, as described by Laidlaw (1979) or one story as described by Morse (1979) or Woodward (2007)	Reliable results widely independent of weather condition and period of season	Needs support of brood and bees from field 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.

However, large scale, systematic production of high quality 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 special colonies and transferred to queenright finisher colonies after about 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 50% sugar syrup 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 are put in 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.

2.1.5. 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, 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 hours 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 (Morse, 1979):

- A simple method is to use a push-in cage made with wire mesh (with 4 mm spaces) or queen excluder. 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. One of the combs should have abundant pollen. The remaining space is filled with one empty comb, sealed and emerging brood and one comb with unsealed honey. Each 24 hours, one comb with eggs is removed and replaced by an empty one. After the 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 (Laidlaw and Page, 1997). 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. Three additional half-combs occupy the other half of the insert, which has open sides (see photo in Laidlaw, 1979). 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. Each day a centre comb with eggs is moved from the queenright partition to the non-excluded half of the insert as described by Laidlaw (1979).

2.1.6. Grafting procedure

Respect of the following conditions when transferring the larva from its original cell to the artificial queen cell (Fig. 5) ensures quality queen production:

- Grafting the larvae from the worker comb to the queen cells should be done rapidly and with suitable environmental conditions (24-26°C and RH > 50%).
- The best place to perform the grafting is in a honey house or a laboratory room, as larvae are sensitive to high temperatures, direct sun light (UV) and low humidity. 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 steps from the breeder colonies and the nurse colonies that receive the grafted cells.
- Cold lighting must be used to avoid generating too much heat which may damage the larvae.
- 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.
- 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.
- With experience and speed, three bars (60 cups) can be completed in 8-10 minutes 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.
- Special carrying boxes for the brood frames and grafted cells exist, which help to protect the larvae from drying from sunshine as well as 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 before the larva are grafted into the cells. If the cells are primed, it is important that the larvae are not immersed in the royal jelly but are floated 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.

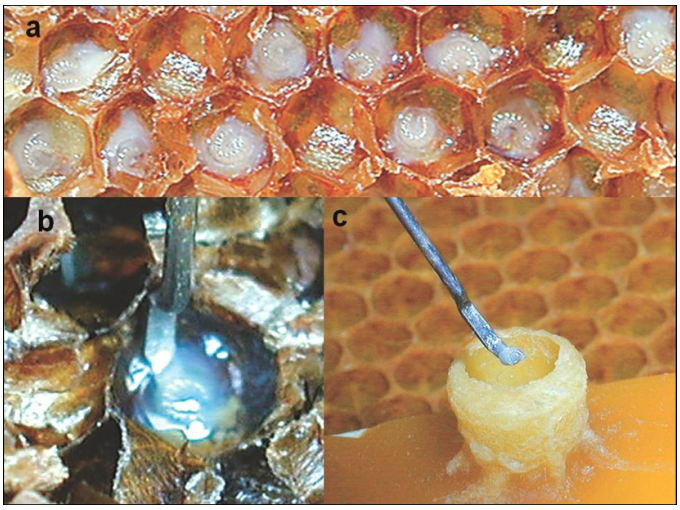


Fig. 5. **a.** Larvae that are a few hours old, floating in royal jelly, and ready for grafting; **b.** a larva taken from dark combs is transferred into wax cups using; **c.** a grafting tool. Photos: L Ruottinen

2.1.7. Acceptance of larvae

The number of accepted larvae depends on different factors, as described in detail by 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

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	-
+ = optimal, 0 = acceptable, - = suboptimal					

the rearing colony and duration of the queenless stage, presence of open brood in the cell-starting colonies, number of grafted cells, rearing sequence 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. Under well managed conditions at least 80% of the larvae should be accepted even in bad weather conditions.

2.2. Mating control

Honey bee breeding programmes and specific research projects depend on controlling the queen's mating process. In addition to the well-developed instrumental insemination technique (see the *BEEBOOK* paper on instrumental insemination (Cobey *et al.*, 2013)) isolated mating stations can serve as an efficient technique for control of honey bee mating for commercial and scientific purposes.

Because drones completely avoid passing over large stretches of water, islands offer an excellent opportunity to establish a fully controlled genetic composition of drones. On the mainland, mating control depends on the isolation of drone colonies by geographic distance (limited flight range of drones and queens) or barriers (high mountains etc.). A comparison of mating apiaries located in both areas is offered in Table 2.

2.2.1. Criteria for establishment of mating stations

- Absence or minimal presence of managed and unmanaged honey bee colonies and airborne drones in a radius of at least 6 km.
- Favourable pollen and nectar resources.
- Weather conditions with long periods of more than 20°C ambient temperature, and wind speed not more than 24 km/h.
- Undulating landscape and sheltered areas for positioning of mating boxes. Obvious markers, such as stones, trees, bushes or specially installed objects help to minimize queen drifting and losses.
- Sufficient drone colonies to ensure a strong drone population for mating. According to Tiesler and Englert (1989), a minimum of 8 to 10 strong drone colonies, or 1 drone colony per 25 queens, are needed.
- Minimal presence of honey bee predator species.

2.2.2. Maintaining mating boxes and mating stations

- For preventing the presence of alien drones in the mating station, only drone-free mating boxes should be used.
- If possible, mating boxes 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 happen about 2 weeks after establishing the mating units. Successful mating should occur within 3 weeks after queen emergence. Later mating 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 colony.
- Regular inspections of the storage and supplementary feeding of mating units is needed if they are used over longer periods.

2.2.3. Drone colonies

The main reason for keeping drone colonies is to provide an adequate number of mature drones of selected origin, in the right period, for mating. A single group of sister queens can be used to control the paternal pedigree, or 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.

- The build up of drone colonies needs to be started in advance of the mating period.
- Drone colonies are managed in standard hives and receive sufficient space to support an optimal population development.
- The drone colonies are established from superior and healthy colonies and special care is taken to provide a continuously rich honey and pollen supply. Regular checks of the health status and the overall development are recommended to achieve a high quality control level.
- Special attention has to be paid to disease treatment. Varroa and other pathogens strongly influence the fitness of drones. Chemical control measures can thus effectively increase the number off fertile drones but at the same time have negative effects on the fertility of drones (De Guzman *et al.*, 1999). On the other hand, reduced treatment can provide a selection pressure that favours colonies with increased varroa resistance. Careful varroa management in drone colonies can thus be an important selection tool within breeding programmes for disease resistance (see Büchler *et al.* (2010) for further details on "tolerance mating stations").
- Up to 2 drone combs are placed within the brood nest of each box to enable a rich production of drones. As the development of drones from egg to maturity takes 40 days and the life expectancy of mature drones last for several weeks, drone production should be started no later than 2 months in advance of the mating period.

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

Parameter	Instrument	Unit (Abbreviation)
Temperature	Thermometer	Celsius (°C)
Relative humidity	Hygrometer	Percentage (RH)
Wind speed	Anemometer	Meter 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	meters above sea level (m.a.s)
Position	GPS	Latitude and longitude coordinates
Vegetation	Aerial photography	proportion of different land use, presented as a percentage

- Drone brood combs from selected drone mothers may be removed after capping and placed in nurse colonies, in order to enable production of higher number of drones from the selected queen.
- If the drone colonies are moved to the mating station, queen excluders between the bottom board and the brood box must be used to keep out any other drones. However, those excluders need to be regularly inspected and dead drones removed, which otherwise could block the entrance and ventilation. The queen excluders with all adhering drones should be removed just before moving the drone colonies to the mating station.

2.2.4. Evaluation of a mating station: environmental conditions

In order to better understand and evaluate the requirements and risk factors involved in honey bee mating biology, various research methods have been developed. Consequently, it is useful to characterize mating stations by noting the meteorological phenomena and parameters outlined in Table 3.

2.2.5. 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 and Koeniger, 2007; Zmarlicki and Morse, 1963). Location of DCAs tends 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 be found in Table 4.

2.2.5.1. Traps to estimate worker presence

- Honey traps, consisting of at least 50 ml of liquid honey on 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 boiled in water in order 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 be not less than 3 h. With regard to common flight distance and speed of honey bee workers (Park, 1923; von Frisch, 1967), the continuous control duration on a single trap should not be less than 15 min.

2.2.5.2. Pheromone traps to estimate drone density

Pheromone traps, prepared from synthesized queen pheromone (9-oxo-2-decenoic acid, abb., 9-ODA) or extracted in acetone ($(CH_3)_2CO$) from honey bee queens can be used to lure airborne drones. Additionally, live or model queens, in which the thorax is fixed or tethered, can serve to attract drones. The details of the technique and necessary equipment are given in the *BEEBOOK* paper on behavioural studies (Scheiner *et al.*, 2013).

2.2.6. Assessment of honey bee queen and drone behaviour

Studying honey bee mating behaviour under local environmental conditions and evaluating the reliability of a mating station are complex tasks and should be organized under specifically controlled circumstances.

- Transparent front extensions and queen excluders can be applied to the mating boxes to accurately observe queen activity (Koeniger and Koeniger, 2007). Thus, the time and duration of each flight attempt as well as the presence of any mating sign on the queen can easily be observed. An experienced person is able to simultaneously follow the queen flight activity of up to 10 mating boxes.
- The starting time of oviposition, the sex of the 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).
- For the observation of drone flight activity, the colonies should be equipped with transparent front extensions and entrance reducers to individually follow and count the number of leaving and returning drones in certain intervals as well as to catch and mark individual drones for further observations.

Table 4. A comparison of methods used to determine adult worker and drone honey bee presence in a prospective mating area. + = optimal, 0 = acceptable, - = suboptimal.

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

- Alternatively RFID (Radio Frequency IDentification) technology can be used to individually mark queens and drones and automatically register the exact time of each entrance passage (http://www.microsensys.de).
- Individual drones can be marked with coloured or numbered plates in order to identify them when they return to their colonies or if they are caught again in the field.
- Microsatellite analysis and other molecular methods can be used to identify the individual origin of drones or its semen from certain colonies (see the *BEEBOOK* papers on molecular techniques (Evans *et al.,* 2013), and miscellaneous research methods (Human *et al*., 2013)). This is a very powerful technique to estimate the number of matings per queen, the realized mating distance of queens and drones, the quantitative contribution of certain drones to the female offspring of a queen etc.

2.3. Handling of adult queens

2.3.1. Marking and clipping queens

See the *BEEBOOK* paper 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 each other through the screened parts. The stack of cages can be placed in an envelope with ventilation holes punched in it and labelled "Live bees" and "Protect against sunshine".

Candy for queen cages should contain little water but nevertheless remain soft. A mixture of powder sugar with about 20% honey (weight:weight) gives suitable results. Whilst 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 after shipment. As far as possible, caged queens should be kept in a dark place with a medium and stable temperature.

2.3.3. Storage of queens

Large queen breeding operations often have 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 as described by Morse (1994). It is possible to store up to 60 cages in one frame and up to 120 queens within one colony for 1-2 months with few losses. 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).

Queens lose the ability to fly if the tip of one front wing is clipped (approx. 35 - 40%). 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 alternate years to keep track of the age of queens.

2.3.4. Requeening colonies

There is no perfectly reliable method 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 than mated queens, and egg laying queens are much more easily accepted than queens that have stopped egg laying due to longer transport or other reasons. The best time for requeening is during a good nectar flow. It is important to make the recipient colony queenless for at least 6-8 hours, sometimes for 1 day. Furthermore, it is essential to destroy queen cells being reared by the colony before releasing the queen (even by hand after several days if the workers are not biting the cage). One should use a push-in cage to introduce queens during a low to marginal nectar flow as this allows the queen to begin oviposition, thus increasing the likelihood of her acceptance.

The most popular method is to replace the previous queen directly with the new one in its shipping cage. The candy compartment on the cage is exposed to allow the bees to slowly release the queen after consuming the candy. The success can be improved if the queen to be replaced is caged for about 7 days before requeening.

Under difficult conditions or for the introduction of highly valuable queens, it is recommended to introduce the queen into a nucleus colony (also known as an "artificial swarm", "split" or "nuc"). Those small units usually accept any kind of queen. The queens can then be safely introduced into strong hives by placing the nucleus with the new queen on top of the strong hives separated by an insert with screens on both sides to avoid direct contact of the bees. Heat from the larger parent colony will pass into the upper unit and support the development of the nucleus colony. As soon as the young queen has built a brood nest and is surrounded by her own young bees, it is ready to be combined with the parent colony. The old queen from the strong colony and the double screen are removed and the young queen in its nuc colony is put on top of the brood box of the strong colony, just separated by a sheet of newspaper containing several slits. In this way, a requeening success of 95-100% can be expected.

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 (see the *BEEBOOK* paper on anatomy and dissection (Carreck *et al.*, 2013))
- large size of spermatheca, (see the *BEEBOOK* paper on anatomy and dissection (Carreck *et al.*, 2013))
- high number of spermatozoa (see the *BEEBOOK* paper on miscellaneous research methods (Human *et al.,* 2013).

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

- high brood production (including number of eggs per day) and large bee population (section 2.4.2. and the *BEEBOOK* paper on measuring colony strength parameters (Delaplane *et al.*, 2013))
- brood solidness (section 2.4.3. and the *BEEBOOK* paper on measuring colony strength parameters (Delaplane *et al.*, 2013))
- disease control (Laidlaw, 1979; Cobey, 2007; see the *BEEBOOK* papers on honey bee diseases: De Graaf *et al.*, 2013; De Miranda *et al.*, 2013; Dietemann *et al.*, 2013; Forsgren *et al.*, 2013; 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 fertilized queen can vary considerably due to egg laying intensity, genetic factors (race) and environmental factors that affect egg laying. More uniform conditions can be assured by using very young unfertilised queens and respecting the following conditions:

- Electronic balances with an accuracy of 0.1 mg should be used.
- If unfertilized queens are used, they should be as young as possible. Queens can lose almost 1-2 mg of weight per day after emergence (Skowronek *et al.*, 2004; Kahya *et al.*, 2008).
- Queens can be placed into small cages to facilitate weighing (Fig. 6).
- The genetic origin of the queen influences the weight standards and should thereby be known.
- At least ten queens per line and apiary are collected on the same day when evaluating fertilized queens. Sampling is usually repeated twice during the reproductive season. This parameter can vary considerably due to egg laying intensity and various other factors and mechanisms (genetic, biochemical) that affect egg laying.

2.4.2. Number of eggs per day (fecundity)

- Queen fecundity in a twenty-four-hour 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 hours period, but this can depend on the bee race.
- A simple way of estimating 24 hours fecundity is with the use of a 5 x 5 cm or 2 x 2 cm grid frame (Fig. 7) or by using the Liebefeld method of estimating brood area (see the *BEEBOOK* paper on estimating colony strength parameters (Delaplane *et al.*, 2013)).

Fig. 6. A queen cage for weighing a queen. Photo: F Hatjina

Fig. 7. The 2x2 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

2.4.3. 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%.To determine brood solidness, see the *BEEBOOK* paper on measuring colony strength parameters (Delaplane *et al.*, 2013).

2.4.4. Disease control

- "High quality" of queens means also that they are free from pests and diseases (Laidlaw, 1979; Cobey, 2007).

Therefore special care has to be taken in order that the productive colonies as well as the mating nuclei show no signs of contaminating diseases such as foulbrood and nosema. Methods for reducing pest/ pathogen loads in colonies can be found in the COLOSS *BEEBOOK* papers on honey bee diseases (De Graaf *et al.*, 2013; De Miranda *et al.*, 2013; Dietemann *et al.*, 2013; Forsgren *et al.*, 2013; Fries *et al.*, 2013; Jensen *et al.*, 2013). One way to ensure that the produced queens are free from nosema 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). According to Rhodes and Somerville (2003), this number should be less than 500,000 spores per queen. However, the queen's attendants in the queen cages can also transmit nosema spores to the queens or to the receiving colony, but the threshold for the accepted limit has still to be evaluated.

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, 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 worst. To do this, individuals must be assessed in a way that allow genetic effects to be distinguished from environmental influences, and according to a uniform method that allows for comparisons across time and space. The basis of performance testing is that colonies in the test station (apiary) should be placed in similar starting conditions and managed according to a standard protocol. The final result obtained from performance testing is a selection index or breeding value for the chosen traits, which is used to select colonies to reproduce (to use as stock for queen and drone production).

The colonies are started from package bees or uniform nucs (see the *BEEBOOK* paper on estimating colony strength parameters (Delaplane *et al.*, 2013)), into which the queens to be tested are placed. The colonies are normally set up at the beginning of the summer, or so that there is sufficient time for the colony to build up before the winter. 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 station. Methods of equalization (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 (overwintering).

3.1.1. Location and organization of testing station

Location of the test apiary should ensure a continuous nectar and pollen flow during the testing period for the number of test colonies. The test colonies may be moved to an apiary for the target (main) honey flow. When planning the location of colonies in the apiary, special care must be taken to reduce drifting. Placing hives in straight, long lines or in rows one in front of another is not allowed. In these conditions, colonies are the strongest at the ends of the lines and in the first and last rows due to drifting of the bees when they fly back to the hive.

The following arrangements of hives in the apiary are recommended to reduce drifting among colonies:

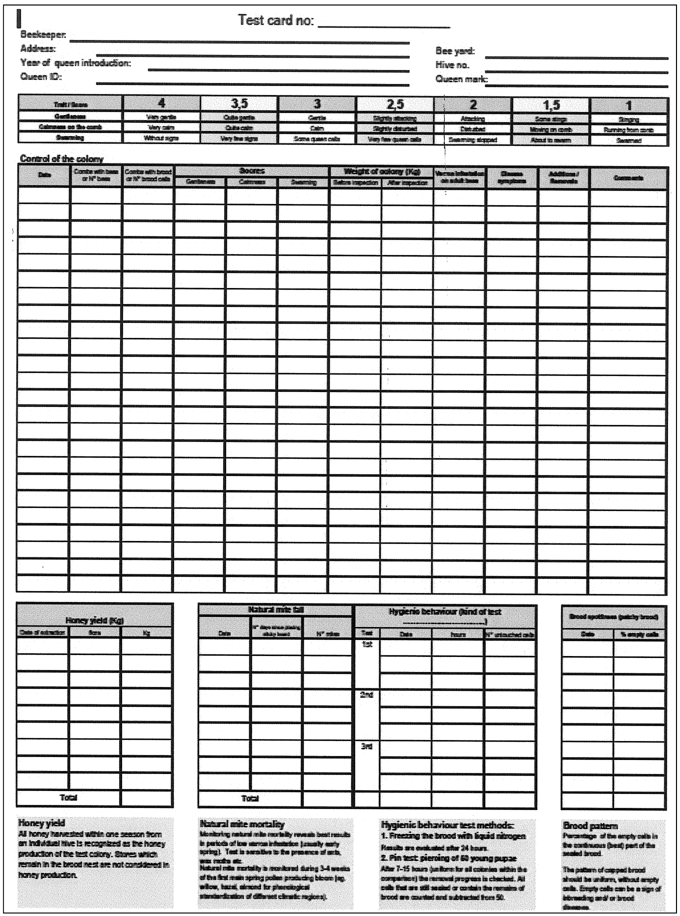
- Hives placed on individual stands – recommended
- Hives placed on small group stand (up to 4 hives) (Fig. 8) - acceptable
- Hives distributed irregularly and in smaller groups with their entrances facing to the four coordinates or somehow different directions (U-shaped or circle groupings) - acceptable
- Groups of hives placed in broken lines - acceptable
- Groups of hives separated by hedge or fence (~2 m high) - recommended in test apiaries with more than 30 colonies.

3.1.2. Size of testing station

The number of colonies in the testing station should be at least 10 (representing different sister groups), to allow for statistical calculations

(see section 4. selection tools), while the maximum number should be obtained by considering the minimal honey flow potential of the area (sufficient nectar for all colonies throughout the season) and the number of beekeepers involved in the testing. Usually, not more than 30 colonies should be placed in one test apiary.

Fig. 8. Hives placed in small groups and with their entrances facing in different directions. Photo: N Kezic



Test card no: ____________

Beekeeper: ____________

Address: ____________ Bee yard: ____________

Year of queen introduction: ____________ Hive no. ____________

Queen ID: ____________ Queen mark: ____________

Trait / Score	4	3,5	3	2,5	2	1,5	1
Gentleness	Very gentle	Quite gentle	Gentle	Slightly attacking	Attacking	Some stings	Stinging
Calmness on the comb	Very calm	Quite calm	Calm	Slightly disturbed	Disturbed	Moving on comb	Running from comb
Swarming	Without signs	Very few signs	Some queen cells	Very few queen cells	Swarming stopped	About to swarm	Swarmed

Control of the colony

Date	Combs with bees or N° bees	Combs with brood or N° brood cells	Scores: Gentleness	Scores: Calmness	Scores: Swarming	Weight of colony (Kg): Before inspection	Weight of colony (Kg): After inspection	Varroa infestation on adult bees	Disease symptoms	Additions / Removals	Comments

Honey yield (Kg)

Date of extraction	Nr. frame	Kg
Total		

Natural mite fall

Date	N° days since placing sticky board	N° mites
Total		

Hygienic behaviour (kind of test ....)

Test	Date	hours	N° uncleaned cells
1st			
2nd			
3rd			

Brood spottiness (patchy brood)

Date	% empty cells

Honey yield
All honey harvested within one season from an individual hive is recognized as the honey production of the test colony. Stores which remain in the brood nest are not considered in honey production.

Natural mite mortality
Monitoring natural mite mortality reveals best results in periods of low varroa infestation (usually early spring). Test is sensitive to the presence of ants, wax moths etc.
Natural mite mortality is monitored during 3-4 weeks of the first main spring pollen producing bloom (eg. willow, hazel, almond for phenological standardization of different climatic regions).

Hygienic behaviour test methods:
1. Freezing the brood with liquid nitrogen
Results are evaluated after 24 hours.
2. Pin test: piercing of 50 young pupae
After 7-15 hours (uniform for all colonies within the comparison) the removal progress is checked. All cells that are still sealed or contain the remains of brood are counted and subtracted from 50.

Brood pattern
Percentage of the empty cells in the continuous (best) part of the sealed brood.
The pattern of capped brood should be uniform, without empty cells. Empty cells can be a sign of inbreeding and/ or brood diseases.

Fig. 9. Recommended protocol to collect all data of repeated performance test controls.

3.1.3. Queens: origin, marking, distribution

Honey bee breeding programmes are based upon evaluation of sister queen groups, in order to estimate the additive genetic component of the chosen traits. At least 12 queens per sister group should be tested, distributed among at least 2 testing apiaries (Ruttner, 1972). Within each test apiary, it is recommended to randomly distribute queens of the same origin. It is not recommended to 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 set up on the basis of the traits chosen for selection. Each control and all specific observations have to be documented in this card. A standard test card that may be used in a testing apiary is shown in Fig. 9.

3.1.4. Timing and duration of test

Performance testing of colonies starts with the last autumn inspection following colony establishment (described in section 3.2.4. establishment of test colonies). Colonies will have been uniformly managed and specific requirements noted. Observations may be made in the first year, taking care that the colony is completely made up of progeny of the test queen (about 40 days after colony establishment). Starting from the spring, qualitative behavioural traits are assessed every time all the hives in the apiary are inspected, with a minimum number of 4 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. Testing continues throughout the season. In the autumn of the queen's second year of life, the test cards are collected and processed for estimation of breeding values.

3.1.5. General recommendations

- The test apiary should be made up of the same kind of material (hives and supers) for uniform management.
- The testing apiary should be run by experienced beekeepers specifically trained to assess production and behavioural traits.
- Assessment of behavioural traits should be performed on all colonies on the same day, preferably by the same tester.
- In migratory beekeeping situations, the apiary should not be split, and the respective colonies should stay together for the whole test period.

3.*2. Colony management*

Colony management is important and has to be planned and prepared in advance, before the beginning of the test. Colony management has to 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, changes in colony management may significantly influence the results.

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

- distribution of queens within the apiary
- type of hives
- kind of wax foundation or comb
- kind of stands for the hives
- water supply
- feeding sources
- nectar and pollen supply / migratory activities.

Large differences exist in different regions regarding colony management. Colony management can significantly influence test results. The main task is to ensure standard conditions for all colonies within each test apiary.

3.2.1. Hives (types, painting, hive components, identification)

3.2.1.1. Type of hive

The type of the hives used must be included in a research report. Common standard hives such as Langstroth or Dadant, are recommended for use, whilst modifications of traditional hives are not recommended.

Use of stands is recommended for the following reasons:

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

3.2.1.2. Painting and colouring

Hives should be protected with paint that does not harm bees. If oil dyes are used for hive protection, the overlaying paint has to dry and the polymerisation process has to be finished prior to hive use. Special care should be placed in choice of dyes in order to ascertain that they do not contain insecticides or other components that are long retained in the wood and gradually released. The hive entrances can be painted different colours to help bees in orientation 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). Super(s) should be removed when bees occupy less than two thirds of the combs in lower super.

Fig. 10. Screened bottom boards ensure good hive ventilation and allow for easy control of mite mortality. Photo: B Binder-Köllhofer

It is recommended that hives in the testing apiary be equipped with screened bottom boards (Fig. 10).They guarantee good ventilation and allow for easy varroa mortality control. The size of the hive entrance has to be adjustable according to colony strength, and time of the year. During winter, a metal mesh / comb should be placed across the entrance as a protection against rodents. The size of the landing board is not important. It is recommended that landing boards should be the same size, but in 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 recognized (e.g. to prevent robbing). The use of a queen excluder is not recommended, but if used, it should be placed / removed on all test colonies at the same stage of development. Feeders do not have to be in the hives all the time. If feeding is needed, feeders should be placed in all colonies at the same time and of the same capacity.

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 that combines the colony number, hive position in the apiary and number of the queen. Hive identification is complex and can cause problems if the test is long lasting. Clearly identified colonies 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 recognized. 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.

3.2.2. Water supply

Colonies need to have a sufficient and continuous source of clean water (Figs 11 and 12). Bees can have difficulties in 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. If there is an interruption of water supply from the designated source, bees may find an alternative water source, and then it is much more difficult to return them to desired water source again. So the water source must be suited to the apiary requirements. Most importantly, the water source has to be protected in such a manner that bees' faeces or dead and dying bees do not end up in the water (Hegić and Bubalo, 2006). It is not recommended to add salt or any other substance in the water. A lack of water may cause problems in digestive tract, especially to young bees intensively feeding on pollen. Also water is needed during hot weather to maintain temperature and humidity in the brood nest.

Fig. 11. Water source in test apiary. Photo: N Kezic

Fig. 12. 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

3.2.3. Wax source

It is recommended that colonies be established on high quality wax foundation, free from pesticides (confirmed with a residue analysis). 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 have to be disinfected (acetic acid fumes, gamma rays) (de Ruijter and van der Steen, 1989; Baggio *et al.*, 2005). Frames and supers treated with acetic acid fumes need to be well ventilated prior to use.

3.2.4. Establishment of test colonies

We recommend the use of package bees ("artificial swarms"; Fig. 13) as the healthiest and most uniform start of test colonies. The artificial swarm has to contain at least 2 kg of young and healthy bees. The bees are placed on wax foundation in a disinfected hive. The queen is introduced at the same time as the bees. Bees should have access to sugar solution in feeder. Newly formed colonies are fed for the first few days with small amounts of sugar solution (1:1).

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 has to 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* paper on measuring colony strength parameters (Delaplane *et al.*, 2013)). The source of the bees and combs with brood and honey must be from healthy colonies.

Fig. 13. 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 in order 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 10 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 test apiaries.

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 (Ruttner, 1972) which still 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, first of all due to challenges posed by varroa, but also because of rapid environmental and climatic changes (Neumann and Carreck, 2010). Reviews of recent developments in breeding for resistance to *Varroa destructor* in Europe and the USA have been published by Büchler *et al.* (2010) and Rinderer *et al.* (2010) respectively.

The recommendations in the sections below were largely revised and approved by the members of COLOSS Working Group 4 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.).

3.3.1. Honey productivity and feed consumption

- All honey harvested within one season from an individual hive is recognized as the honey production of the test colony. A potential crop of swarms or permanent splits, coming from the test colony, is not regarded.
- Honey stored in the brood nest is not considered toward honey production.
- The supers filled with honey combs are weighed before and after extracting and the difference is noted as the honey harvest. If the extraction procedure does not allow following individual combs, 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.
- 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 in order 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 has to be checked in regular intervals. The net weight of all added or replaced equipment has to be noted to calculate the net weight development in defined control intervals, for example during overwintering. See the *BEEBOOK* paper on miscellaneous research methods for techniques associated with weighing full colonies (Human *et al.*, 2013).
- Programmable hive scales are on the market. Some models store the total hive weight in 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

- As a standard protocol in performance testing, defensive behaviour and response of the bees during handling are subjectively classified by an experienced tester (Table 5).
- In accordance with the Apimondia guidelines, 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 better describe slight differences within the population.
- To ensure the comparability of test results colonies should be scored according to the following descriptions. Use intermediate scores (3.5, 2.5, 1.5) if the observed behaviour is somewhere between the given descriptions.
- The evaluation of the behaviour has to be repeated 3-6 times during the season without regard to specific conditions (like weather, honey flow etc.). The arithmetic mean of all evaluations is calculated at the end of season and used as 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 varied among successive evaluations.

Table 5. Standard scoring criteria for colony defensiveness.

Points	Gentleness	Calmness
4	No use of smoke and no protective clothes are necessary to avoid stings during normal working procedure.	Bees stick to their combs "like fur" without any notable reaction to 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	In spite of the use of smoke the colony shows a strong defence reaction on being handled, or 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 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 with 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.).

Table 7. Methods for determining the level of hygienic behaviour expressed by a colony. *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 hours, will show very high consistency in results between assays, irrespective of strength of colony and nectar flow.

Method	Repeatability	Costs & efforts	Remarks
Freeze killed brood*	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
Pin test	Medium	Low	Piercing of 50 young pupae
VSH	Unclear	High	Tests for varroa specific hygiene

- For quantitative research results, black leather balls about the size of tennis balls, marked with alarm pheromone (isopentyl acetate) can be moved in front of the hive entrance to provoke
- stinging by guard bees (Collins and Kubasek, 1982; Free, 1961; Guzman-Novoa *et al.*, 2003; Stort, 1974). The number of stings remaining in the leather after 1 or 5 minutes of exposure can serve to measure differences in defence behaviour.

3.3.3. Swarming behaviour

- As with other behavioural traits (see section 3.3.2.), a 4 point scale is used to classify the swarming behaviour of test colonies (Table 6).
- Note that typical supersedure queen cells are not considered as swarm cells.
- All symptoms 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 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 be intermediate scores (3.5, 2.5, 1.5).

3.3.4. Hygienic behaviour

Hygienic behaviour is recognized as a natural antiseptic defence against the brood diseases, American foulbrood and chalkbrood, and against varroa (Boecking and Spivak, 1999; Evans and Spivak, 2010; Spivak and Reuter, 2001; Wilson-Rich *et al.*, 2009) and thus may be relevant in breeding programmes for resistance to these pathogens and parasite. Standardized methods for testing hygienic behaviour are based on the removal of freeze killed (Momot and Rothenbuhler, 1971; Spivak and Reuter, 1998) or pin killed brood (Newton and 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 purple-eyed pupae containing approximately 100 cells on each side (5 x 6 cm) from a frame and freeze it for 24 hours at -20°C.
2. Insert the frozen comb section into a frame of sealed brood in the colony being tested (Fig. 14). 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 and Downey, 1998).
3. Remove the frames no more than 24 hrs later.
4. Record the 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.
5. The tests should be repeated on the same colony at least twice
6. A hygienic colony will have uncapped and completely removed over 95% of the frozen brood within 24 hours on both tests. This is the most conservative (strict) assay for hygienic behaviour that should be used for breeding purposes.
7. 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 hours.

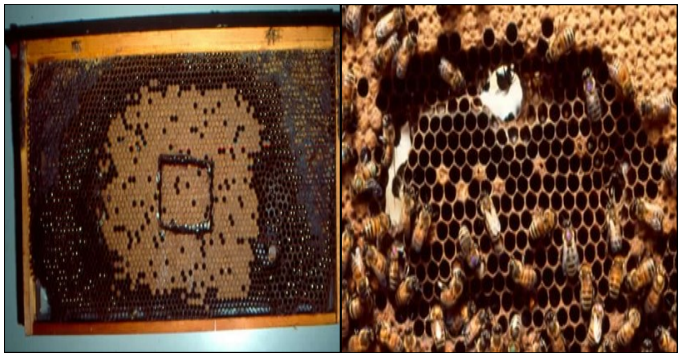


Fig. 14. Freeze-killed brood assay: cutting brood out of comb to freeze. Left: Frozen section of sealed brood is carefully placed into hole cut through comb. Right: Twenty-four hours after being returned to a colony, the amount of freeze-killed brood uncapped and removed is recorded. Photos: M Spivak

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 (Fig. 15)

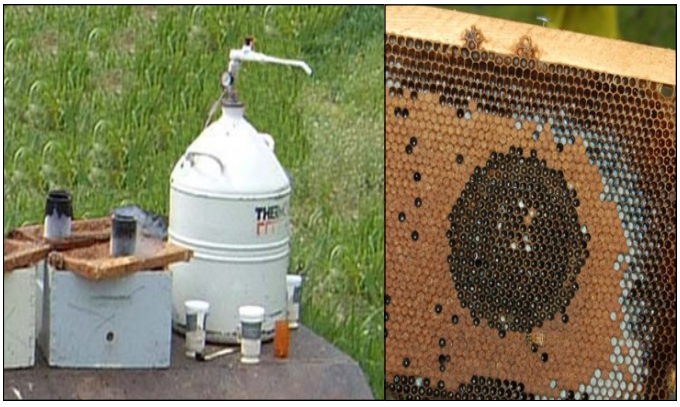


Fig. 15. Freeze-killed brood assay: freezing brood within comb using liquid N_2. Left: Dewar tank with valve to dispense liquid nitrogen, polystyrene foam cups for pouring liquid N_2 into PVC pipes (black pipes in combs). Right: After 24 hours, this hygienic colony uncapped and removed > 95% of the freeze-killed brood. Photos: M Spivak

2. Make a 75 mm diameter tube to pour the liquid nitrogen directly on the comb. A metal vent pipe or PVC plumbing pipe can be used. A wider 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 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 hours.
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 hours 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 calculates the number of frozen pupae completely removed plus those that are in the process of being removed after 24 hours.

Historically, colonies that removed freeze-killed brood within 48 hours 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 removal of freeze-killed brood within 24 hours is considered (Spivak, unpublished data).

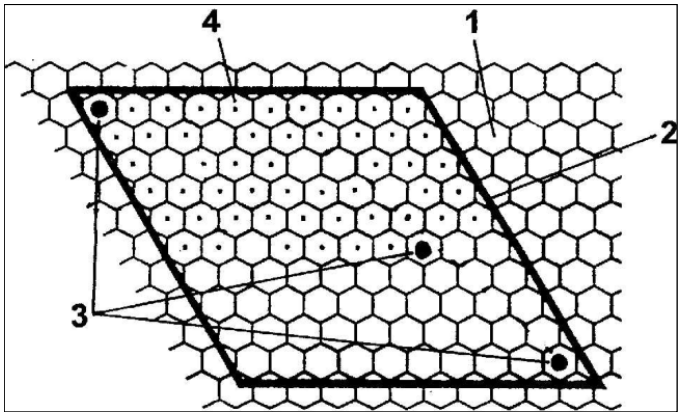


Fig. 16. Pin-killed test for hygienic behaviour. The numbers correspond to text references in Section 3.3.4.3.

Fig. 17. Pin test: **a.** Piercing 50 cells containing young pupae; **b.** Control of brood removal after about 8 hours, many cells are opened but not removed; **c.** Nearly all cells are completely cleaned. Photos: R Büchler

3.3.4.3. Pin-killed brood assay

The pin test method is recommended in Europe as a standard in field selection programmes, because it shows a significant correlation with the removal of varroa infested brood, can be standardized and is easily handled by beekeepers. A statistical tool has been established to include pin test data in the estimation of breeding values for varroa tolerance (see 4.1). For the pin-killed brood assay protocol, see Fig. 16 while following the numbered protocol below. Additionally, Fig. 17 shows images of the protocol being applied in the field.

1. A rhomboid frame of a 10×10 cell wide template (Fig. 16, number 2) is placed on a brood comb containing young pupae (Fig. 16, number 1)
2. The upper left and lower right cells are marked with a colour felt-tip pen (Fig. 16, number 3)
3. 50 capped brood cells are pierced (Fig. 16, number 4) row by row from left to right with a fine insect pin (entomological pin size No 2).
4. Cell 51 is marked to identify the treated brood area (Fig. 16, number 3).
5. The comb is marked on the top bar and placed back to the brood nest in its former position.
6. After 7-15 hours (uniform for all colonies within the comparison) the removal progress is checked. All cells that are still sealed or contain the remains of brood are counted and subtracted from 50. The percentage of completely cleaned cells is noted in the protocol.
7. The highest discriminatory power of the test is reached when all of the test colonies remove an average of 50% of the pupae within the time interval. 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 yield higher differences between colonies with high and low hygienic behaviour. If the average removal is much higher than 50%, a shorter time interval should be realized in further test repetitions.
8. The test should be repeated 2-3 times during the main brood season.

3.3.5. Varroa infestation

Regular monitoring of varroa populations is not only a precondition for integrated varroa control, but also an important basis for the selection of mite resistant stock. Several different methods have been developed and tested with regard to systematic field evaluation of varroa densities (Lee *et al.*, 2010). Please also refer to the *BEEBOOK* paper on varroa (Dietemann *et al.*, 2013). We outline in Table 8 the methods commonly used to determine varroa populations in colonies and include information pertinent to the method's 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 reveals best results. Sampling bees is more effective with higher infestation levels that occur later in the season (Büchler, unpublished data). The estimation of breeding values (see 4.1) for varroa resistance is based on mite population growth during the season. For these calculations, natural mite mortality during 3-4 weeks of the first main spring pollen producing bloom (e.g. willow, hazel, almond for phenological standardization of different climatic regions) is combined with the mite infestation of bee samples estimated during summer. Repeated measurements of the bee infestation in intervals of 3-4 weeks improves the accuracy of the test and allows prolongation of the test period without treatment against varroa 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 disease symptoms of performance test colonies should be carefully registered and documented. Special care should be taken with diseases which can be influenced by the genetics of the bees. These include American foulbrood, chalkbrood and chronic bee paralysis

Table 8. Methods for estimating varroa populations in honey bee colonies (see the *BEEBOOK* paper on varroa 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	Doesn´t 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

virus (CBPV or hairless black syndrome). Usually, no prophylactic or acute treatments against those diseases are recommended on test colonies so as to observe potential susceptibility or resistance. However, for a more systematic selection, a uniform initial infection of all colonies should be provided.

A simple, qualitative documentation (symptoms observed: yes/no) may be sufficient for identification and removal of infested 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. An estimation of breeding values for chalkbrood resistance has recently been developed at the institute in Hohen Neuendorf, Germany (Ehrhardt, pers. communication), based on such a simple data structure. 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* papers (De Graaf *et al.*, 2013; De Miranda *et al.*, 2013; Dietemann *et al.*, 2013; Forsgren *et al.*, 2013; 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 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. It can be combined with amount of honey consumed during winter (see 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* paper 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 3.3.7.1. and 3.3.7.2.).

3.3.7.1. Bee population

- Check each hive box (or super) from the top and bottom (you do not need to take out individual combs) immediately after opening the hive to estimate how many spaces between combs are populated with bees.
- Add up the total number of combs covered with bees. Fully covered spaces between combs count as 1. Partially covered ones are counted proportionately in quarters of a comb (0.25, 0.5, 0.75).
- 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

- Count the number of combs containing brood. Count the brood as 0.5 if the brood is just on one side of the comb.
- In addition, the brood area on a central brood comb gives useful information on the brood activity of the hive. A 4 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.

3.3.8. Additional test characters

With regard to specific needs, bees can be tested and selected for further traits. Pollen gathering, length of life and breeding for morphological characters are some examples for successful selection activities (Rinderer, 1986).

Further characters may be included to improve the disease resistance of bees. With regard to varroa resistance, various traits such as the grooming behaviour of bees, the post-capping period duration and others, have been discussed as potential selection criteria but have not been demonstrated to be effective.

However, 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 consequent testing scheme.

4. Selection tools

The goal of beekeeping is to produce many quality products and 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 and 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. All of these issues are part of the breeding programme.

The standardization of performance testing as described in Section 3.3. is a necessary prerequisite for successful breeding. The results will indicate differences between individual colonies that can be utilized 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 is significant in breeding, as only the hereditary disposition (genes) of the animals influence the quality of the offspring. The environmental conditions under which the colonies live unfortunately mask or influence their hereditary properties (breeding value). A breeding programme therefore requires a breeding value or selection index in order 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 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 by Bienefeld *et al.* (2007) (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 values", refers to the probability that the progeny of the selected individuals will be above or below the population average for a certain considered trait.

Genetic evaluation aims at assigning a genetic value to each animal with the goal of ranking animals and selecting animals with the best genetic values. Compared to other livestock which undergo genetic improvement, honey bees have peculiar genetic and reproductive characteristics (haplo-diploid sex determination, arrhenotoky, polyandry) which make simple appliance of the BLUP Animal Model not appropriate (difficulties in calculating the numerator relation matrix, which links information from related colonies (Bienefeld *et al.*, 1989; Fu-Hua and Sandy, 2000)). However, the main methodological problem is that the colony's performance and behaviour result from the interaction between the queen and worker bees. Thus, a trait measured in the honey bee colony is the result of the combined activities of the queen (maternal effect) and workers (direct effect). Bienefeld and Pirchner (1990) found queen and worker effects to be negatively correlated, which strongly hinders selection response (Willham, 1963). Therefore, the BLUP animal model approach was modified to consider worker and queen effects and the negative correlation between them (Bienefeld *et al.*, 2007).

Genetic evaluation via BLUP combines the phenotypic data of the animal itself with data of related animals to rank them according to their (environmentally adjusted) genetic merit. Therefore, this approach needs the individual results of performance tests of all animals and the genetic relationship (pedigree information) between them. All this information must be combined in an appropriate database.

The requirements for the database are the following:

- 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 written access (e.g. breeder and administrator of a breeding association).
- Data format should fit the requirements of the of the genetic evaluation software.
- Open access for all users regarding the results of the genetic evaluation.

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

Most breeders use the database not only for efficiently making data of their colonies available for genetic evaluation, but also for running their private studbook. Not all entries of the studbook (e.g. day of birth, tag colour of queen, etc.) are needed for genetic evaluation. To adjust for the environmental effect, information concerning the contemporary group is of central importance. A contemporary group comprises all colonies tested at the same location and management conditions at the same point in time. For genetic evaluation, the

contemporary group is formed by combining the following variables: year of birth, login ID of the tester (who is not necessarily the breeder), and a code for the apiary belonging to the tester (one tester may run several apiaries). Ten to 15 colonies per apiary are needed to be able to correctly adjust for the environmental effect of an apiary. However, fewer colonies per apiary are accepted for genetic correlation, but then the colony information at these apiaries is downgraded. Genetic evaluation requires genetic links within the population and is promoted by the simultaneous testing of the different genetic origins (of the same race) at each apiary.

For the reasons explained above (reproductive peculiarities of honey bees) and in contrast to other species, the full pedigree specification in the database used for genetic evaluation consists of the identification number of the (actual) queen, of her mother, of her mating partner, and NOT her father. This model is adapted to the breeding scheme according to which a single drone line is used: a mother queen is selected from whom a group of queen daughters is reared, which will be used for drone production (Ruttner, 1988). The paternal descendent of each queen needed for genetic evaluation is (software-assisted) generated by using pedigree information of her mother. For each drone producing sister group, a dummy father is inserted into the pedigree. The identification number of the mother is a mandatory field in the database, but not for the mating partner, because controlled single-line mating is not adopted by all associations. Pedigree data is combined with performance data for genetic evaluation.

4.1.1. Access to the data input feature

Two options are available:

1. The administrator of a breeding association receives the pedigree and test data from the breeders via lists, copies of their studbooks, etc. to input these data under the breeders login ID.
2. The administrator of a breeding association activates password-protected access to the data input module of database for each breeder: in this case, each breeder inputs all his data alone. However, this stand-alone data input by a breeder requires additional data checking and confirmation by the administrator of the responsible breeding association before these data can be released for genetic evaluation.

4.1.2. Pedigree data

A unique queen identification number is a central requirement for genetic evaluation. The international unique queen identification number (QID) (see www.beebreed.eu for coding) consists of:

- Country code: 2 digits
- Breeder ID (within country) 3 digits
- Queen no. within the studbook of the breeder 5 digits
- Year of birth of the queen 4 digits

The international QID is automatically linked with an alphabetic race code (**C** for *A. m. carnica*, **L** for *A. m. ligustica* and **M** for *A. m. mellifera)* if the authorized breeder enters the corresponding database with his password.

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

$$y = Xb + Z_1u_1 + Z_2u_2 + e,$$

where: y = a vector of records/traits of the colonies (e.g., honey production, defence behaviour); b = a vector of fixed year/beekeeper/location effects; u_1 = a vector of random worker (direct) effects; u_2 = a vector of random queen (maternal) effects; e = a vector of random residual effects; X = incidence matrix relating the observations to the corresponding environment (apiary within tester and year effect); Z_1 = incidence matrix relating the observations to corresponding worker effects; Z_2 = incidence matrix relating the observations to the corresponding queen effects.

Solutions are obtained from the following mixed model equations:

$$\begin{pmatrix} \mathbf{X'X} & \mathbf{X'Z_1} & \mathbf{X'Z_2} \\ \mathbf{Z_1'X} & \mathbf{Z_1'Z_1} + \mathbf{A}^{-1}\alpha_1 & \mathbf{Z_1'Z_2} + \mathbf{A}^{-1}\alpha_2 \\ \mathbf{Z_2'X} & \mathbf{Z_2'Z_1} + \mathbf{A}^{-1}\alpha_2 & \mathbf{Z_2'Z_2} + \mathbf{A}^{-1}\alpha_3 \end{pmatrix} \cdot \begin{pmatrix} \mathbf{b} \\ \mathbf{u_1} \\ \mathbf{u_2} \end{pmatrix} = \begin{pmatrix} \mathbf{X'y} \\ \mathbf{Z_1'y} \\ \mathbf{Z_2'y} \end{pmatrix}$$

where

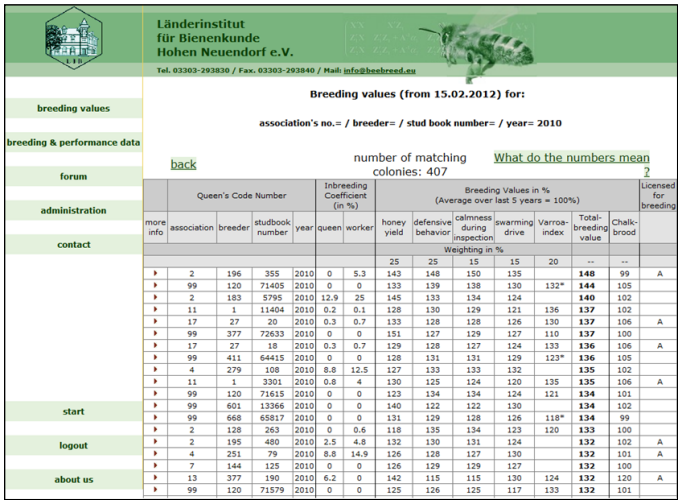
$$\begin{pmatrix} \alpha_1 & \alpha_2 \\ \alpha_2 & \alpha_3 \end{pmatrix} = \begin{pmatrix} \sigma_1^2 & \sigma_{12} \\ \sigma_{12} & \sigma_2^2 \end{pmatrix}^{-1} \cdot \sigma_e^2$$

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; A^{-1}= inverse of the additive genetic relationship matrix.

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 (Ehrhardt and Bienefeld, unpublished).

Phenotypic and genetic parameters (Bienefeld and Pirchner, 1990; Bienefeld and Pirchner, 1991) are re-estimated from time to time. All aspects of estimation procedures for the estimation of variance components (data structure, method and model of estimation, effects included in the model, and so on) should be as similar as possible to the estimation procedures for breeding values.

The accuracy of genetic evaluation depends on the quality of the relationship information and the possibility of the statistical procedures to distinguish the genetic component from the total phenotypic variance. The estimations may even lead to misinterpretation if they are not statistically adequate. Breeding values, inbreeding coefficients, and tools for breeding plans should be published. Breeding values are estimated once a year and are published mid-February of each year.



more info	Queen's Code Number: association	breeder	studbook number	year	Inbreeding Coefficient (in %): queen	worker	Breeding Values in % (Average over last 5 years = 100%): honey yield	defensive behavior	calmness during inspection	swarming drive	Varroa-index	Total-breeding value	Chalk-brood	Licensed for breeding
Weighting in %							25	25	15	15	20	--	--	
▸	2	196	355	2010	0	5.3	143	148	150	135		**148**	99	A
▸	99	120	71405	2010	0	0	133	139	138	130	132*	**144**	105	
▸	2	183	5795	2010	12.9	25	145	133	134	124		**140**	102	
▸	11	1	11404	2010	0.2	0.1	128	130	129	121	136	**137**	102	
▸	17	27	20	2010	0.3	0.7	133	128	128	126	130	**137**	106	A
▸	99	377	72633	2010	0	0	151	127	129	127	110	**137**	100	
▸	17	27	18	2010	0.3	0.7	129	128	127	124	133	**136**	106	A
▸	99	411	64415	2010	0	0	128	131	131	129	123*	**136**	105	
▸	4	279	108	2010	8.8	12.5	127	133	133	132		**135**	102	
▸	11	1	3301	2010	0.8	4	130	125	124	120	135	**135**	106	A
▸	99	120	71615	2010	0	0	123	134	134	124	121	**134**	101	
▸	99	601	13366	2010	0	0	140	122	122	130		**134**	102	
▸	99	668	65817	2010	0	0	131	129	128	126	118*	**134**	99	
▸	2	128	263	2010	0	0.6	118	135	134	123	120	**133**	100	
▸	2	195	480	2010	2.5	4.8	132	130	131	124		**132**	102	A
▸	4	251	79	2010	8.8	14.9	126	128	127	130		**132**	101	A
▸	7	144	125	2010	0	0	126	129	129	127		**132**	100	
▸	13	377	190	2010	6.2	0	142	115	115	130	124	**132**	120	A
▸	99	120	71579	2010	0	0	125	126	125	117	133	**132**	101	

Fig. 18. Screen shot from the breeding value database at www.beebreed.eu.

4.1.3. Outcome of genetic evaluation: breeding values

The breeding value states for a particular characteristic (honey production, varroa tolerance, etc.) the extent to which an animal is genetically different from the average of the population. Breeding values can be expressed as the percentage of a moving genetic average of the population. The moving basis is the last-five-year-genetic-average for each trait. Consequently, breeding values usually depreciate, if genetic response is achieved. Because the traits used for honey bee breeding strongly differ with respect to phenotypic variation (honey 0-150 kg, gentleness 1-4), their breeding values also differ. To ensure their comparability, breeding values of all traits are transformed by fitting to an identical standard deviation of 10.

At www.beebreed.eu, several features are available to select queens meeting the specific demands of breeder or buyers of queens:

- Breeding and inbreeding values of specific queens
- List of queens that meet specific requirements (e.g. breeding value for varroa tolerance > 125% and for other traits ≥ 100%). An example is given in Fig. 18.
- List of queens, including a total breeding value (combination of all traits used for selection) that meets the specific weighting of the traits in which the breeder or buyer is interested.

A breeding plan program is also available at www.beebreed.eu. Entry of the QID of potential parents makes available an estimation of the inbreeding and breeding values of the expected offspring. This allows breeders to visualize the potential results that a specific cross will produce to avoid inbreeding. Inbreeding has been found to be of crucial importance for honey bee breeding programmes. Additionally, a tool is available to search for the mating station that best suits the individual breeding goal.

4.2. Selection indexes and scores

Due to various reasons, there are cases where an organized 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 and 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 of 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, linear regression can be sufficient, with or without previous data transformation for obtaining normality. If the traits are described in categorical values, 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 bee-keeper 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 half as important as trait B then V = 0.5).
- The above equation can further incorporate the heritabilities 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: many of the methods mentioned below are outlined in the *BEEBOOK* paper on molecular research techniques (Evans *et al.,* 2013).

The completion of the honey bee genome project held the promise for fast selection of colonies with desirable traits (Weinstock *et al*., 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, at present much knowledge is still needed before delivery on this promise can come through. Complications further arise from the complexity of honey bee genetics. It seems that those colonies that perform best, do so due to a high level of genetic diversity amongst the workers (Seeley and Tarpy, 2007). The colony composition of two generations in form of the queen and her worker offspring and 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. 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). The recent developments in sequencing single nucleotide polymorphisms (Harismendy *et al*., 2009) and bioinformatics' approaches in data evaluation (Pérez-Sato *et al*., 2010) can make breeding programmes for honey bees more reliable. However, such an approach needs considerable resources and expensive laboratory work.

Even before completion of the honey bee genome, scientists started the search for quantitative trait loci (QTL) in honey bees using different kinds of markers:

- Hunt *et al.* (1995) used bees preselected for variation in their pollen hoarding behaviour to search for the underlying genetic traits. Using genetic markers derived from a technique called random amplification of polymorphic DNA (RAPD), they identified first two and later a third marker (Page *et al*., 2000). Each marker held predictive power, concerning the preference of a given forager for the collection of either nectar or pollen. The RAPD loci observed are not thought to be directly responsible for the variance in the traits, they are merely closely linked to a genetic region that primes the bees' behaviour in the direction of pollen or nectar collection.
- Using similar RAPD markers with the addition of DNA microsatellites and a sequence tagged site, Lapidge *et al*. (2002) detected seven loci linked to hygienic behaviour in honey bees. This finding conflicts with the only two loci described by Rothenbuhler (1964; see however Moritz, 1988); still it may result from the usage of strains less extremely selected as compared to the earlier studies.
- Today RAPD are all but forgotten, as is their cousin methodology of amplified fragment length polymorphism (AFLP) used by Rüppelt *et al.* (2004) to search for additional markers linked to pollen hoarding behaviour.

  A variety of markers with accurate linkage maps today exist for the preliminary screening for 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 *et al*., 2006), single nucleotide polymorphism (SNPs) also allow cheap and accurate targeting of QTL. Recently a marker set of 44000 has become commercially available (Spötter *et al*., 2011), 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, more than 151 SNP differed between the reference sample of "varroa-defence bees" and a set of bees from completely unhygienic colonies, against 7 SNPs differing between varroa-defence bees and related workers not engaging in defensive behaviour, taken at the highest level of significance. Comparing all three groups, merely a single SNP remained. This result demonstrates the value of having appropriate samples available.

The current rapid developments in availability and pricing of DNA sequencing may eventually replace all these linkage bound methods with a direct sequence based search for the underlying genetic variance for each trait.

- A separate methodology to identify marker genes has emerged from the use of microarray techniques. Microarrays consist of a set of known honey bee genes. Using the microarray allows for the detection of mRNA levels in specific workers. The microarrays are built based on expressed sequence tags (EST) results from mRNA of bees, which after cDNA transformation are cloned and can be analysed rather swiftly (Whitfield, 2002). Based on genetic information from *Drosophila melanogaster* many of the gene functions are well known. An example of the application of this technique is the study of honey bee brood reaction to parasitism by varroa mites (Navajas, 2008). The strength of this technique lies in the immediate detection of differential gene activity in bees

with variable traits. It is thus feasible to directly identify the action of genes related to specific traits. The currently available microarrays allow for the screening of more than 8000 genes identified from the honey bee brain. Any gene unidentified or not included in the microarray however, will go undetected. This is particularly important for those promoter regions that act as switches for coding genes, as these are likely to go unnoticed from such studies.

- While interactions between coding genes and their regulator genes may go unnoticed by microarray techniques, the use of SNP markers might be particular suitable for the detection of promoter regions. In humans two independent SNPs have been shown to generate lactose tolerance in adults (Tishkoff, 2007).

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, but it remains 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 type of studies. Due to the 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 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. The main problem remains, i.e. to demonstrate, in a considerable set of colonies, that heritable variance exists for the trait of choice. Only once a large sample size is available, representing both variation and similarity between the screened colonies, would it seem worthwhile to conduct a molecular genetic screening.

A caveat in the interpretation of genetic marker data results from the vast number of genes screened, either genetically mapped markers or from microarray studies. Chance differences in marker diversity between tested bees or in the activity of genes unrelated to the trait under study are rather likely given the vast number of comparisons. Hence it is advisable to demand particular strict statistical testing, before accepting a particular marker as involved. One way to reduce this problem is to repeat the study in several independent populations.

While the arrival of molecular markers will allow for rapid selection, some words of caution are needed. It may seem straightforward to select for the identified genotype in a separate population, if this has been found to be associated with particular valuable traits. As a shortcut, it may be equally tempting to inter-cross a set of genes into an unrelated population, and based on marker assisted selection follow their fate in following generations. Organisms resulting from this technique have been termed cis genetically modified organisms, in contrast to trans genetically modified organisms, as the genetic exchange happens via traditional interbreeding, and genes are not introduced from other completely unrelated species. In theory it could be possible to incorporate a single gene into an unrelated population, however, unless considerable care is taken this will go hand in hand with a significant genetic bottleneck. Whether consumers, be it bee-keepers or honey buyers, will accept such cis techniques as being less problematic than standard trans GM techniques remains an open question. Furthermore, searching for identical genotype variations in unrelated populations hold no warranty for success, as our knowledge of the complex underlying mechanisms are still rather rudimentary. While the future of honey bee breeding may benefit from more advanced molecular methods, it is still an emerging field.

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.

5.1. Closed-population breeding

In a closed population, there is no introduction of unknown genetic material: this can be achieved by use of completely isolated mating stations (section 2.2.2.) or instrumental insemination (see the *BEEBOOK* paper on instrumental insemination, Cobey *et al.*, 2013). The aim of this kind of design is to rapidly achieve improvement while limiting loss of genetic variability (which would lead to inbreeding depression). Laidlaw and Page (1986) list 3 basic strategies:

- Daughters from all of the breeding queens are each mated (instrumentally inseminated) to 10 drones selected at random from the entire population; replacement breeder queens are selected at random from all the daughters of all the breeder queens, without considering their parentage. To operate this design as a long term plan, about 50 breeder colonies must be selected at each generation, in order to reduce inbreeding.
- Each breeder queen is replaced by one of her daughters, reared as above.
- All queen daughters are inseminated with the same aliquot of mixed semen originating from drones of all breeder queens.

5.2. Open population breeding

In this kind of design, the introduction of foreign genetic material into the population is allowed, thereby reducing the risk of inbreeding. Performance testing with sister queen groups placed in different testing apiaries is particularly useful for the calculation of breeding values.

More simply, significant differences among families, distributed across different apiaries, reveal a heritable effect of the performance. An example of an open breeding scheme is the following (from Cornuet and Chevalet, 1987):

- First generation: selection based on individual values.
- Second generation: colonies ranked according to selection index or breeding value (combination of performance and pedigree data) – the best 10 colonies are used for queen and drone production.
- Mating occurs in mating station where selected and unselected drones are present.

5.3. Special designs for scientific purposes

5.3.1. 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, 1994. The basic steps are the following:

- The 10 best and 10 worst colonies are selected.
- Five sublines within the best and worst groups are created by inseminating virgin queens with semen from a single drone (from a different colony of the same group). See the *BEEBOOK* paper 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 for the production of virgin queens and drones.
- The colonies from the 3rd generation queens are used for the experimental observations.

5.3.2. Single drone mating

In some experiments, it is useful to minimize genetic differences among colonies in order to establish the extent of an external factor. For this aim, instrumental insemination (see the *BEEBOOK* paper 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 3 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 required level of homozigosity required in the experiment, may then be inseminated with pooled homogonous semen, or naturally mated in an isolated mating station with selected drones.

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