



# BREEDING DISEASE RESISTANT HONEYBEES

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## INTRODUCTION

### Scope of the manual

This document attempts to integrate the results from the EU financed project BEE SHOP (STREP, Contract no.: PL 022568) with current methods for breeding disease resistant bees. It deals only with the most important pathogens of honeybees and is in no way intended to be a comprehensive document covering all aspects of bee breeding. It is written with the end-users (honeybee queen breeders) in mind with the explicit ambition to extend current achievements in the fields of epidemiology, bee biology and bee genetics fields into applied apiculture. We discuss pathogen transmission and virulence because host-parasite co-adaptation ultimately is influenced by how we breed and maintain bees and because selection influences not only one part in a host-parasite system. The text is intentionally concise, lacks references and tries to summarise the current state of the art in breeding disease resistance in honeybees. For further in-depth studies suggested reading is provided. It is assumed that the reader is familiar with most aspect of honeybee biology and familiar with different systems for rearing queen bees. Grafting methods etc. are beyond the scope of this manual.

## THE HOST

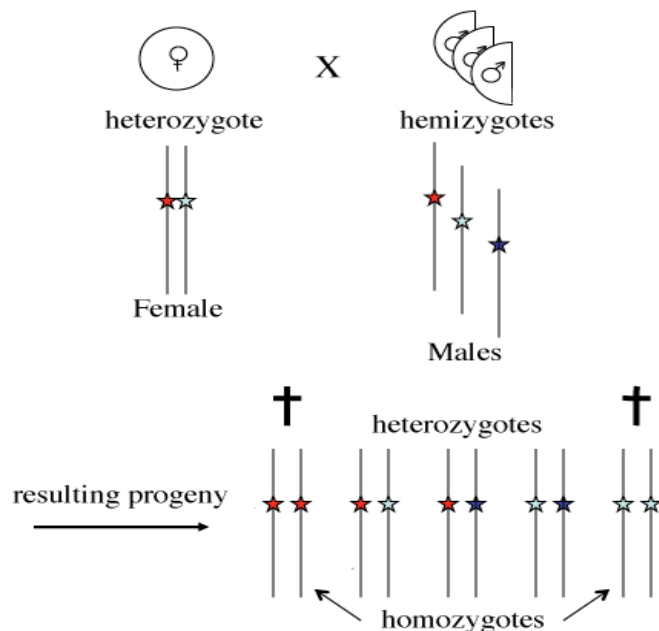
### Basic bee biology

For the purpose of this manual we only need to consider three main biology parameters, besides specific defence mechanisms: i) cast determination, ii) reproduction, and ii) sex determination.

### Sex determination

Sex determination in honeybees is not determined by eggs being fertilised or not. The sex is determined by heterozygosity (hetero=different) or homozygosity (homo=same) (zygote=fertilised egg) of sex alleles at a sex determining locus (location) on the chromosomes. There may be 15-20 sex alleles coding for sex floating around in a free mating population of honeybees. Heterozygosity at the sex determining locus results in females, whereas homozygosity at the sex determining locus results in diploid males (see figure below). Such diploid eggs with only one sex allele are eaten by the worker bees as they hatch to larvae. In unfertilised eggs (hemizygotes), there can only be one sex allele and, thus, the end result will be a haploid male (drone).

With a large number of sex alleles in the population, the probability of homozygosity at the sex determining locus in fertilised eggs is very low in a free mating population. However, breeding most often results in reduced genetic variation, and possibly loss

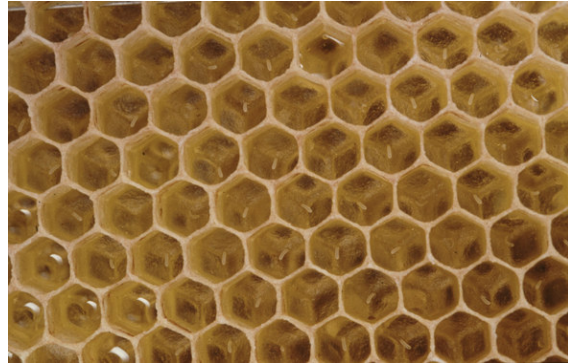


*Schematic representation of progeny in a haplo-diploidy system where sex alleles at the sex determining locus determine sex, with only three sex alleles (coloured stars) available.*

of sex alleles, making homozygosity at the sex determining locus more common. In effect, homozygosity acts as a lethal gene. This is why inbreeding beyond a certain level is even worse for honeybees compared to animals with sex determining chromosomes (such as mammals).

### Caste determination

Every fertilized egg laid by the honeybee queen has the potential of becoming a queen bee. What determines the cast (queen bee or worker bee) is the food composition. Sometimes during the third day as a larva, the food produced by nurse bees (royal jelly) is diluted with nectar and pollen for all progeny destined to be worker bees. Larvae destined to become honeybee queens receive undiluted royal jelly throughout their larval development. Thus, in queen rearing, larvae to be used should be 1-2 days old, that is 4-5 days after the eggs are laid by the queen.



*Every fertilized egg is a potential queen provided it is heterozygous at the sex determining locus. Photo: S. Camazine*

### Reproduction and mating

Honeybees reproduce at two different levels, i) the individual level and ii) the colony level. The queen's activity represents reproduction at the individual level, where fertilised eggs normally result in female progeny and unfertilised eggs result in drones (but see sex determination below).

Colony level reproduction occurs as colonies swarm by colony fission. It is the individual level reproduction that makes colonies strong enough to reproduce at the colony level. Without colony level reproduction (swarming) the honeybee as a species would perish.

Honeybee queens mate in flight on drone congregation areas, often some distance from the colony of origin. During flight, she mates with 15-20 drones or even more. The nuptial flight may be repeated if the spermatheca is not sufficiently filled during the first flight, but most often, she only mates once.



*Swarming represents colony level reproduction. Photo: I. Fries.*

### Individual level defence

At the individual bee/larval level, the host-defence system is complex and involves both humoral and cellular aspects. Honeybees, like other insects, rely on an inducible systemic humoral immune response to counter infections. Synthesis and secretion of a number of different antimicrobial peptides in the fat body, active against both bacterial and fungal infections are released into the haemolymph and compose a general non-specific line of defence. Antimicrobial peptides isolated from challenged honeybees include two defensins, apidaecin, abaecin and hymenoptaecin, each with a rather broad activity spectrum. In the BEE SHOP project, a considerable genetic variance for drone larval resistance against AFB has been confirmed.

Different levels of antimicrobial peptides, may have led to the observed variance in larval tolerance to AFB.

In addition to the humoral response to infections, host-defence in insects relies on cellular reactions, which involve a number of specialized blood cell types, so called haemocytes. Phagocytosis (engulfing) of foreign objects and parasites, and participation in the control of infection through production of secreted molecules, occur through haemocyte activity. Furthermore, haemocytes are instrumental for wound healing.

Presently, selection of individuals based on genetic markers for specific disease resistance traits is not possible, because such markers are not available. As more genetic information becomes available this may be possible at least for some single loci traits. For traits with a more complex genetic background multiple markers and indexing of different genes will be required. Nevertheless, selection based directly on the genome, without phenotype complications, has enormous advantages and will undoubtedly increase in importance in the future.

Interestingly, the sequencing of the honeybee genome has revealed fewer genes for disease resistance compared to other insects. This should indicate that investment in an individual level of defence is less important in social insects, where there is also a colony level defence.

### **Colony level defence**

It is well documented, not only in honeybees, that there is an important social component in resisting various pathogens in social insects. Thus the term “social immunity” has been coined. The social component consists of behavioural activities of individuals, but where often no single individual need to include all needed traits for certain behaviours to be accomplished. The best example from honeybees is the hygienic behaviour (see p. 17), where different activities needed to remove diseased brood (detection, uncapping, removal) are controlled by different genes independently inherited. The gene frequency of these traits in the population will determine the efficacy of the behaviour, independent of the number of bees actually carrying all necessary traits. This is because the bees cooperate to achieve this social immunity. It is likely that the described behaviour is the most important line of defence for brood diseases, since colonies with a high level of hygienic behaviour can be impossible to infect with American foulbrood for production of clinical disease symptoms.

### **Host variations**

As demonstrated for individual larval AFB resistance in BEE SHOP, there is a considerable variation between bee strains in disease tolerance. This is also true for colony level defence, where there is a wide variation in how fast colonies can clean out diseased brood. For adult bee diseases, less is known about such variations, but most likely they exist. For breeding purposes, it is this variation in disease resistance that we want to take advantage of.

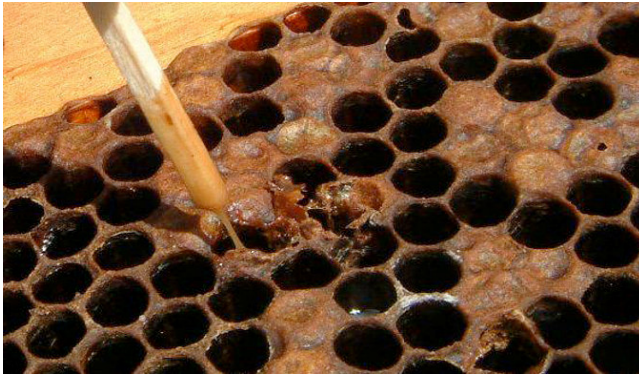
Variations in individual level pathogen resistance is difficult to measure, whether we discuss brood diseases or adult bee diseases. The best practical approach here is to simply not breed from colonies where diseases can be diagnosed. Variations in colony level disease mechanisms, on the other hand, are easy to monitor, at least for brood diseases. Measurements of the level of hygienic behaviour (see p. 18), should be part of any serious attempt to breed bees more tolerant to brood diseases (including the parasitic mite *Varroa destructor*).

## THE PATHOGENS

### Larval diseases

#### American foulbrood

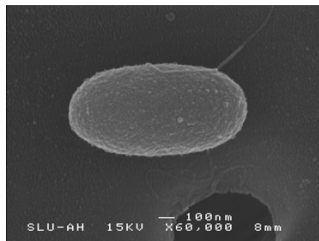
American foulbrood (AFB) is the most dreaded brood disease of honeybees world-wide. It is caused by the spore-forming gram-positive bacterium *Paenibacillus larvae*. Once a beekeeping operation has been thoroughly infected, the disease is very difficult to control, because of the extremely resilient spores that are easily spread with material, wax, honey or bees between



Field symptoms of honeybee brood killed by *Paenibacillus larvae*. Photo: I. Fries

colonies and between apiaries. On the other hand, healthy normal bee colonies do not easily contract clinical disease with limited amounts of spore in, for example, spore-contaminated honey. Actually, infection experiments in BEE SHOP demonstrate that even if feeding of viable spores of *P. larvae* results in loss of brood, suggesting that diseased larvae are removed, clinically visible symptoms often do not occur. The experiments in BEE SHOP have demonstrated that beekeepers must be effective in distributing the spores, for AFB to be a problematic disease.

Recent comparative experiment of individual larval infectivity and virulence, between different genotypes of *P. larvae*, have demonstrate a surprisingly wide variation between different



*Paenibacillus larvae* spore. Photo: I. Fries

isolates, both regarding infectivity (infectious dose, ID<sub>50</sub>) and virulence (time required to kill infected larvae). It appears that the pathogen has the option of choosing strategy; i) kill larvae slow, build up a huge spore bank over time in infected colonies, and primarily rely on horizontal between-colony transmission. ii) kill larvae fast, allowing most diseased larvae to be removed before sealing, and primarily rely on vertical between colony transmission.

Field experiments in BEE SHOP, as well as modelling the evolution of virulence of AFB strongly support this description. Under apicultural conditions, the first genotype is probably favoured because beekeepers aid in promoting horizontal disease transmission and remove the negative influence for the pathogen of being virulent at the colony level by replacing colony losses (otherwise the pathogen would risk depleting itself of hosts). Under natural conditions it appears the second genotype could be favoured because of lower level of horizontal disease transmission and the possibility to transmit between hosts using mainly vertical transmission.

The most effective way to select for tolerance to AFB is to select for hygienic behaviour in the bees (see p. 17).

#### European foulbrood

European foulbrood is a bacterial disease caused by *Melissococcus plutonius* that colonise the gut of the bee and compete for nutritional resources. This disease appears to be factorial,

possibly related to bee colony density, since areas in Europe with low bee densities have less problems. Other environmental factors may also be important.

Nothing is known about individual level resistance to *M. plutonius*. Selecting for colony level hygienic behaviour will lower the risk of serious disease outbreaks.

### Chalk brood

Chalk brood is a fungal disease caused by the heterothallic fungus (sexual reproduction) *Ascosphaera apis*. Large variations in incidence occur between years without any plausible explanations available.

Nothing is known about individual level resistance to *A. apis*. Selecting for colony level hygienic behaviour will lower the risk of serious disease outbreaks.

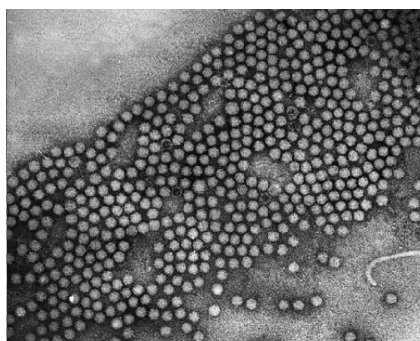


*European foulbrood. Photo: I. Fries.*

### Sacbrood

Sacbrood is a viral infection, infective for both larvae and adults. In adults the virus propagates in the hypopharyngeal glands, but without obvious clinical disease symptoms. When the brood is infected, through the infected brood food produced by infected adults, typical clinical disease symptoms appear as infected larvae die.

Nothing is known about individual level resistance to sacbrood. Selecting for colony level hygienic behaviour will lower the risk of serious disease outbreaks.



*Acute bee paralysis virus.  
Photo: B. Ball.*

### Virus infections associated with *Varroa* mites

An array of virus infections are described from honeybees. With the exception of sacbrood, described above, such infections have rarely produced serious visible symptoms or colony mortality. With the introduction of *V. destructor* into European honeybee populations, this situation has radically changed. Most virus infections that will multiply upon injection in honeybee haemolymph have become associated with the mite, where the mite triggers covert infections to become overt, and vectors the infections between adults and between adult bees and brood. At least one of these virus infections, deformed wing virus (DWV) also replicates in the mite as well as in the bee. Virus infections associated with the mite include; DWV (and related virus types), acute bee paralysis virus (ABPV), slow bee paralysis virus (SBPV), Kashmir bee virus (KBV).

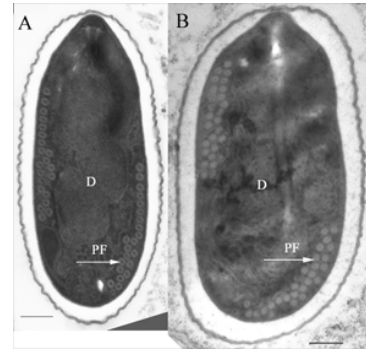
Nothing is known about individual level resistance to virus infections. Likewise, it is not known if selecting for colony level hygienic behaviour will lower the risk of serious outbreaks of virus infections.

### Diseases of adult bees

A range of different pathogens are described from adult honeybees. However, none except the nosema parasites appear to have the capacity to strongly impact colony vitality.

### ***Nosema* spp. disease**

Nosema disease of honeybees is caused by two microsporidian parasites, *Nosema apis* and *Nosema ceranae*. The former is known from European honeybee populations for over 100 years, whereas the latter probably is a more recent introduction into European honeybee populations from the Asiatic honeybee, *Apis cerana*. Reports from Spain suggest that *N. ceranae* is much more virulent than *N. apis*, killing infected colonies within a year if not controlled. However, it appears that this level of virulence may not appear in many other areas.



*Spores of N. ceranae and N. apis. Photo: I. Fries.*

Nothing is known about individual level resistance to nosema infections of either species, nor is any colony level resistance described. However, biological variation seems likely and queen bee breeders in Sweden and Denmark report successively lower incidence of *N. apis* when breeder colonies have been tested for 15 years and queens bred only from colonies where the disease cannot be diagnosed using light microscopy.

### **Tracheal mites**

The introduction of tracheal mites in the US and Canada in the mid-1980's is a good example of both successful breeding for mite tolerance and for host-parasite co-adaptation. The effects from the parasite was devastating initially, but because of large scale breeding efforts with use of more mite tolerant stock, the negative effects diminished over the years. Over time, the problems have become negligible also in non-selected populations through natural selection. In Europe tracheal mites exist but are sometimes difficult to find and cannot be regarded as a problem for beekeeping.

### **Virus infections associated with *Varroa* mites**

See same heading above for larval diseases.

### ***Varroa* mites**

*Varroa* mites (*Varroa destructor*) are a relatively new introduction into European honeybee populations. The original host, *A. cerana*, is not damaged to any significant degree by the mites, whereas most colonies in European bee populations succumb if the mite numbers are not controlled. The mites feed on honeybee haemolymph, both from adult bees and from brood. Reproduction can only occur in the sealed brood cells, but the mites survive for extended periods on adult bees without access to brood. Where brood rearing occurs throughout the year, mite populations build up much faster compared to temperate climates where colonies have no brood for part of the year. Nevertheless, colonies are likely to collapse 3-4 years after mite introduction also in a Scandinavian climate.



*Female Varroa destructor. Photo: T. Sensenbaugh.*

## EPIDEMIOLOGY OF HONEYBEE DISEASES

### Disease transmission

How pathogens transmit from one host to the next is at the core of disease epidemiology in any system. Pathogens need not only to get access to a host and multiply within hosts, for increased fitness, they also need to transmit effectively between hosts. In honeybees, another level of complexity is added, because pathogens need not only to gain access to individual bees, multiply within infected bees and spread between individual bees (within colony multiplication). For increased fitness, pathogens also need to transmit between colonies. From the pathogen's perspective, it is of no use to multiply effectively and outcompete other strains within the colony, unless this also results in increased between-colony transmission.

Undoubtedly, between-colony transmission must be accomplished for pathogens to be successful in honeybees. Transmission can occur through two fundamentally different modes of transmission; horizontal and vertical transmission. The main mode of transmission will largely determine the level of virulence in pathogens that evolve in a specific host system. The complexity of honeybee reproduction (see above) means that we (or pathogens to be successful) need to consider transmission both between individual bees within colonies and transmission between colonies.

#### Horizontal transmission

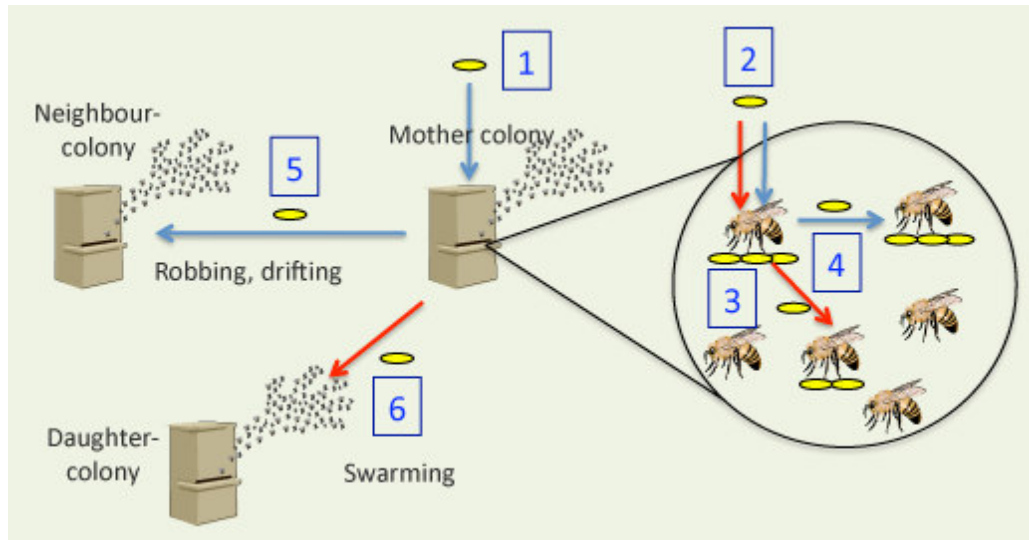
Horizontal pathogen transmission is defined as transmission between hosts within generation. In honeybees, within colony horizontal transmission occur whenever a pathogen transmits from one bee to the next.

Between-colony horizontal transmission occurs when robbing or drifting bees carry pathogens between colonies (or when beekeepers aid in that transport).

#### Vertical transmission

Vertical pathogen transmission is defined as transmission between hosts between generations. In honeybees, within colony vertical transmission occur whenever a pathogen transmits from the queen to her progeny. So far, only one virus infection (DWV) has been shown to be vertically transmitted from queen to progeny through infected eggs.

Between-colony vertical transmission occurs when colonies reproduce (swarm by colony fission) and pathogens are transmitted with the swarming bees to a new colony (from mother colony to daughter colony). Thus, all pathogens in honeybees that can be transmitted through adult bees can be vertically transmitted at the colony level.



*Transmission routes of pathogens in honeybees. 1. Entry into host colony. 2. Entry into individual bees. 3. Multiplication within individual bees. 4. Between bees transmission. 5. Between colony horizontal transmission. 6. Between colony vertical transmission. Blue arrows – horizontal transmission, red arrows – vertical transmission.*

### Evolution of virulence

The mode of transmission will largely determine the evolution of virulence in pathogens in a given host system.

Generally, pathogens that rely mainly on horizontal transmission are likely to develop more virulent host-parasite relationships. The rationale behind this is that when the pathogens have accomplished between-host transmission, the fate of the host is of no interest. Thus, virulence and host mortality can result as a side-effect. In other cases, where transmission is dependent on host mortality, it is an obvious advantage for the pathogen to develop high virulence.

Pathogens that rely mainly on vertical transmission are likely to develop more benign host-parasite relationships. The rationale behind this is that such pathogens rely on host fitness to accomplish between-host transmission. Thus, the negative fitness impact on the host must be kept to a minimum.

Interestingly, pathogens that rely mainly on vertical transmission, most often need an element of horizontal between-host transmission to maintain themselves. The rationale behind this is that over time, infected host lines would be outcompeted by non-infected host lines with only vertical pathogen transmission. This is provided pathogens have some negative fitness effect on the host.

### Implications for apiculture and breeding of honeybees

The reproduction system in honeybees, with colony level reproduction through swarming, generates the opportunity for all pathogens that can be transmitted on or in adult bees to have the option of vertical colony level transmission. In fact, all known honeybee diseases, whether infective for adults, larvae or both, can be transmitted with adult bees. The prediction from this is that honeybee diseases should be benign. At the individual level, high virulence is often present in honeybees, in particular for brood pathogens. However, colony level virulence is rare for pathogens in honeybees, where the host-parasite relationship has to some extent been moulded

by evolution (thus, recent introductions such as *Varroa* mites or *Nosema ceranae*) should perhaps not yet be considered in this context.

We hypothesise that the apparent colony level virulence of American foulbrood (caused by the spore-forming bacterium *Paenibacillus larvae*) is an apicultural artefact. Apiculture largely increases horizontal disease transmission through crowding of colonies (increased drifting and robbing). Horizontal disease transmission is further increased by apicultural manipulations such as shifting of comb material between colonies and shifting of brood and adult bees between colonies. Furthermore, vertical between colony disease transmission in apiculture is kept to a minimum because honeybee colonies are kept from swarming. The prediction from all this suggests that apiculture selects for more virulent pathogen strains. How to consider the normal formation of new nucleus colonies from brood and bees from several colonies in this context is difficult to evaluate. Possibly, the probable effect from apiculture in developing more virulent pathogens, can be counteracted by beekeepers, if queens in clinically diseased colonies are not used as breeding queens. Also, the destruction of colonies showing clinical disease symptoms of AFB may alleviate the negative selection impact from apicultural practices. If clinically diseased colonies are destroyed, the most susceptible hosts and the most virulent pathogen strains are eliminated. This last point is probably why stamping out policies have been so effective in the control of American foulbrood in countries where this is practiced (i.e. Sweden, Norway, New Zealand).



*Crowding of colonies in apiaries create increased between colony horizontal disease transmission.*

## BEE BREEDING FOR DISEASE TOLERANCE



*Breeding within race limits the genetic pool. Photo: S. Camazine*

Breeding of honeybees have a long tradition where racial considerations have, and still play, an important role in the breeding strategy. The ambition to preserve specific race characteristics were less pronounced in the successful breeding program developed by the Benedictine monk Brother Adam at the monastery Buckfast Abbey in the UK in the early 1900's. His breeding strategy specifically produced hybrid bees with the intention to find beneficial phenotypic characteristics, rather than race typical features. The ambition was to breed productive bees, but also to breed disease resistant bees. In particular, resistant to the tracheal mite *Acarapis woodi*.

The ambition in most bee breeding in Europe is to develop positive variants for further breeding within race. The honeybee races *Apis mellifera mellifera*, *Apis mellifera carnica* and *Apis mellifera ligustica* are examples where large scale breeding also for production colonies is focused not only on

characteristics linked to production, but to race typical characteristics. Generally speaking, the more parameters included in a breeding program, the slower is the progress in each individual trait included in the program. Furthermore, the focus on breeding of race typical bees limits the genetic base from which selection of breeding material can be made. A wide genetic base with genetic variation is fundamental for making genetic progress through selection, also from the disease resistance perspective. Thus, it can be expected that the focus on race, rather than on production or disease resistance characteristics, the breeders of race typical honeybees limit the possible progress. This does not mean that breeding of race typical bees should be avoided all together. From a conservation point of view, it is of high priority to conserve locally adapted strains, not only of bees, but also of other domesticated animals. However, for production purposes the use of a larger genetic base available for breeding will, for genetic reasons, yield the best genetic progress.

Nevertheless, breeding means in principle that the genetic variation is reduced. It should be clear that honeybees in particular are extremely dependent on genetic variation for their long term survival. A main reason for this is the sex determination system with sex alleles, which relies on genetic variation to produce female progeny. The extreme level of multiple mating we see within the genus *Apis* is also an indication of the importance of genetic variation in these social insects. In fact, empirical evidence now demonstrate that increasing the within colony genetic variation, also increases disease resistance.

### **Breeding systems**

A number of different approaches can be taken for breeding of honeybees. Some are extreme in the sense that the genetic variation is at risk. However, when specific traits are sought for, it can be advantageous to limit the genetic variation, not in the whole population of bees, but in a selected sub-set used to identify the trait of interest, for example hygienic behaviour. By including such selected variants in the population at large with use of free mating, the frequency of the desired traits can be increased in the whole population without significant loss of genetic variation. Another approach has been to maintain inbred lines that are crossed (through open mating or through AI) to produce production queens. Such hybrid bees show good vigour and production capacity, but the problems around maintaining highly inbred stock for such purposes have made this approach less attractive for breeders.

### **Artificial insemination**

The use of artificial insemination (AI) is today practical and highly successful, with several technical developments and increased understanding of bee biology/physiology and genetics, along the way. Since AI offers complete control of the mating design, as well as the number and genetic composition of the drones to use, the technique offers new opportunities to develop and maintain selected lines of bees of particular interest. Actually, breeders with focus on maintaining specific races of honeybees now have a tool where the racial purity can be completely controlled. The resulting lack of vigour when genetic variation is decreased can be somewhat counteracted by keeping separate lines of bees of interest and make the crosses that ensures that the largest number of sex alleles possible remain in the population used.

AI is no longer restricted to laboratories or advanced biologists/apiculturists. With the right equipment and proper training it is now a widespread and increasing practice in breeding bees. A good introduction to the technique and its possibilities can be found at [www.honeybee.breeding.com](http://www.honeybee.breeding.com).

### **Closed populations**

Closed population breeding was developed by American researchers in the 1980's. A closed population is, as the name implies, a population of bees where outside genetic influence is not

allowed or kept to a minimum. The size of the closed population need not be defined upwards, but there are limitations to the minimum size of a population that can be kept with maintained vigour and fitness. To some extent, the minimum sized population is determined by how the selection of queens (and drone producers) for the next generation is chosen. A closed population can be maintained by

1. *random selection of queens*

With random selection of queens (with respect to sex alleles) at least 50 breeder queens must be used to maintain 85% brood viability over 20 generations. If AI is used instead of natural mating, the use of pooled and homogenized semen from drones from all breeder queens in the population secures equal contribution from all breeder queens in the population.

2. *queen supersedure and natural mating*

If each breeder queen is replaced by one of her superior daughters, only 35 breeder queens are needed to maintain 85% brood viability over 20 generations.

3. *top crossing and AI*

Semen is collected from drones from a superior performing queen. This semen is used to inseminate a) all daughter queens from all breeding queens or b) to mix in pre-decided proportions with semen from drones from all breeder queens in the population. If new “blood” is needed in a closed population, this is an option for introduction of a new sex allele, to prolong the population’s life span.

4. *selection of brood solidness (AI and / or natural mating)*

Selection for high brood solidness in a population of 50 breeder queens can be effective in preventing loss of sex alleles from the population. This assumes random mixing of semen in the queen’s spermatheca.

### **Mating stations**

Mating stations are locations where the topography or other factors reduce the impact from drones not to be included in the breeding program. Truly isolated mating stations are difficult to achieve other than on islands surrounded with enough water. For reasons given above regarding honeybee genetics, the number of drone producing colonies should be as large as possible, and preferably unrelated. If the drone producing colonies are from superior stock, but unrelated, the use of mating stations does not greatly accentuate the risks for loss of sex alleles in the population of bees at large. A common mistake by beekeeper’s use of mating stations is the use of few and often related colonies for drone production. If a substantial proportion of queens are mated under such conditions the population at large is in danger of critical inbreeding problems and loss of sex alleles.

### **Free mating**

Free mating of honeybee queens means that that all the drone producing colonies within flight distance of the drone congregation area where the queens will mate, contribute to the gene pool. Because of the multiple mating, the risk of inbreeding is negligible provided

the honeybee population is not isolated. For queens to be used for honey producing colonies, freely mated queens are recommended for their low production cost and low risk of inbreeding problems. Such queens can very well be produced



*Drones provide genes from their grandmother’s mating.  
Photo: S. Camazine*

from inbred lines, as described above.

When free mating is practiced, freely mated queens still provide a source of selected material on the drone side. The genes transmitted from drones from a particular queen have nothing to do with the mating of this queen (since the drone is haploid and have no father). Thus, if the freely mated queens are from superior stock, the drones they produce also represent superior stock.

### **Evaluation of breeding value**

Any selection program must be based on performance testing. A traditional way of evaluating the breeding value of specific queens is to compare series of sister daughters to the average performance in the apiary. When several traits with different weights are of interest, a breeding index can be constructed. The queen that produces the progeny with the best combined and weighted average performance has the highest breeding value. However, this phenotype approach is suboptimal because it does not account for the maternal effects, nor the environmental effects. Since negative correlations exist between direct effects and maternal effects in honeybees, response to breeding efforts is reduced. Some success can be achieved also using the apiary average approach, but the larger the breeding program, the more sense it makes to adopt evaluation systems developed for breeding in general, and adapted to the peculiarities of bee genetics and biology. The best linear unbiased prediction (BLUP) approach includes the maternal effects and also considers the relatedness among colonies in the whole population. German data demonstrate that a shift from the apiary average approach to calculations of breeding values using BLUP significantly increases selection progress. The use of modern breeding evaluation systems is probably beyond most bee breeder's computing skills. However, a growing number of breeding organisations make use of the services offered by the Bee Institute in Hohen Neuendorf, Germany, a service available in several languages. See [www.beebreed.eu](http://www.beebreed.eu) for more information.

### **Breeding for disease resistant bees**

#### **Larval diseases**

Many diseases of honeybee brood are lethal to infected larvae. However, with one exception, brood diseases rarely kill honeybee colonies. The exception is AFB, which may be a result linked to apiculture, as discussed under "Implications for apiculture".

#### **AFB**

Breeding for resistance to AFB can be accomplished through identification of traits or genes linked to resistance to infection in individual larvae. BEE SHOP has identified one such gene, which is a first step in this direction. Nevertheless, selection for colony level resistance based on behavioural traits (hygienic behaviour, see below) is still the most well proven and useful method to select for suitable AFB resistant breeder queens.

Besides being effective for AFB, hygienic bees also become more tolerant to other brood diseases such as chalkbrood and sacbrood.

#### **Adult bee diseases**

In contrast to larval diseases, adult bee diseases are normally not lethal. They may shorten the life span of the bee and make infected colonies less productive, but diseases of adult bees rarely kill colonies. An exception is when new pathogens are introduced into previous disease free areas, such as tracheal mites (*Acarapis woodi*) or Varroa mites.

***Nosema spp.***

There are no records on documented tolerance to infection with *N. apis* nor to the new microsporidian *N. ceranae*. Field experiences suggest that by checking potential breeding stock for infection with *N. apis*, and avoiding using queens from infected colonies as breeders, the general infection level in the population can be reduced. If the same applies for *N. ceranae* remains to be seen.

***Varroa mites***

A number of characteristics have been identified with potential to influence the population build up of mites. These include:

- 1) Grooming behavior. The adult bees can damage adult mites and the proportion of damaged mites in the debris has been used for selecting breeder queens. However, bees also damage dead mites and selecting for this trait has not been successful.
- 2) Post capping period. With a shorter post capping period the average number of mated daughters per cell entry may be reduced. However, selecting for shorter post capping period will also most likely select for faster mite development.
- 3) Brood attractivity. With less attractive brood, the mites will stay longer on the bees and a slower population build-up. Presently, there is no practical way of screening for this characteristic.
- 4) Cell size. It has been suggested that the cell size influences mite reproduction. However, if there is such an effect, it is small and of little practical value.
- 5) Worker-drone brood ratio and brood dynamics. Colonies that produce less brood, in particular drone brood will produce fewer mites.
- 6) Mite reproduction/ infertility. Where mother mites do not reproduce or have delayed egg-laying the mite population grows slower. The character can be registered but requires substantial labor efforts.
- 7) Mite juvenile mortality. Where mite juvenile mortality is increased the mite population grows slower. The character can be registered but requires substantial labor efforts.
- 8) Hygienic behavior. Honeybees selected for hygienic behavior have lower mite levels in field colonies compared to non-hygienic bees.

From the list above it is obvious that breeding for single characters in the host population is very difficult and in some cases unlikely to be of any use. Selecting for hygienic behavior will have some positive effects on *Varroa* population build up, while effectively controlling a range of brood diseases.

To avoid looking at single characters, the mite population build up over time can be monitored in standardized units. Then all relevant parameters will be included without being specified. This approach has had some but limited success in Germany. This way of evaluating breeding material is costly and labour intensive.

Decades of selection based on one or several characteristics as mentioned have not produced mite tolerant bees that survive without mite control. However, such mite tolerant colonies exist

nevertheless. The common characteristic for such populations of bees is, also in Europe, that they have been exposed to natural selection, rather than directed selection efforts in apiculture. Thus, we know that co-existence between mites and bees can be achieved.

Unfortunately, the removal of mites through mite control, also removes any selective disadvantage for the mite of killing the host. And likewise, by removing mites through mite control any selective advantage for the bees of being mite tolerant, is disguised. However, it is not realistic to suggest large scale natural selection to be the method of choice for breeding more mite tolerant bees. The reason for this is the devastating effects it would have on honeybee populations, and even worse, on the eco service they provide, pollination. A practical solution to this dilemma is firstly to breed for hygienic bees, which has some positive effects, and then allow mite numbers to grow enough for comparative differences between different breeding lines to be exposed. In short, this means mite control but not enough to cover existing differences between potential breeder queens in mite population growth. Ultimately, such an approach is likely to cause some damage and possibly even some loss of colonies. But the alternative is probably a never-ending dependence on mite control for colony survival.

## **HYGIENIC BEHAVIOUR**

### **Background**

Hygienic behaviour is the uncapping of brood cells containing dead or diseased brood and the subsequent removal of the remains of this brood. This task is performed by the adult bees of a couple of weeks of age. Although hygienic behaviour is governed by a rather complex set of genes, the behaviour has a high heritability and is therefore well suited as a target for breeding.

### **Testing for hygienic behaviour**

The principle for testing the hygienic behaviour involves the killing of a number of pupae, usually 50-100 per colony. The rate of removal of the killed pupae per time unit is the measurement of hygienic behaviour, where a higher removal rate per time unit corresponds to a more efficient hygienic behaviour. Sealed brood should be chosen, irrespective of method used to kill the brood. Furthermore, brood should be of uniform age, preferably dark-eyed pupae. The measurements should be repeated at least twice but it should be clear, that comparisons between different regions or at different times of the year may not be reliable. The speed at which dead brood is removed is heavily influenced by the foraging conditions. With a good honey flow, brood is cleaned out faster compared to bad weather conditions or no honey flow.

### Pin-killing

The pin-killing method involves piercing of the pupae with a thin needle. The thin insect needles used for pinning insects are well suited for this. The needle is inserted through the cell capping, through the pupae to the bottom of the cell. In order to give a fair result, care must be taken to disrupt the capping as little as possible. Drops of haemolymph should be avoided on the capping since that may act as a cue to the bees that something is wrong.

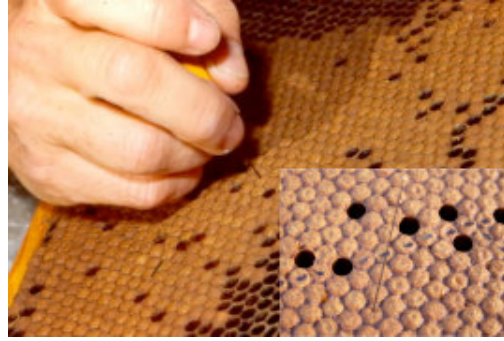
The pinning of 100 cells can be done quickly after a little practice. A small proportion of the pupae may survive the treatment and emerge with a melanized spot in the forehead where the needle entered. Therefore the pinning can be repeated with retained care to avoid damaging the cell capping. Field data from BEE SHOP experiments suggest that pin-killing is the method most closely correlated to removal of AFB deceased brood.

### Freezing – cut out comb

Freezing of cut out pieces of brood comb is relatively labour intensive. Approximately 100 sealed brood cells with dark eyed pupae are cut out from the comb with a sharp knife and put in a deep freezer over night. After freezing, the piece of comb is refitted to the comb from which it was taken. Thus, the colony has to be opened at least three times in order to perform the test, while the other methods described here only require the colony to be opened twice. A disadvantage with cutting out comb is that the knife kills a relatively large number of cells that may trigger the bees to clean out also the test cells. Nevertheless, investigations performed in BEE SHOP show that freezing of cells result in slower brood removal compared to pin-killing. Of the three methods described here cutting out and replacement of frozen brood is the method that yields the slowest brood removal.

### Freezing - liquid N

Sealed brood will be instantly frozen in contact with liquid nitrogen. Special containers are needed to handle liquid nitrogen and care should be taken to protect skin and eyes from contact. Nevertheless, when large number of colonies should be tested, the use of liquid nitrogen can be advantageous because of the handling speed. To apply the liquid nitrogen to the brood, a plastic cylinder of approximately 10 cm diameter is pushed into the brood comb and the nitrogen is poured into the cylinder to cover the brood. The nitrogen is allowed to evaporate and then the cylinder can be removed. Bees remove brood killed with liquid nitrogen faster than they remove cut out and replaced frozen brood, but not as fast as



*Using a thin insect needle inserted straight into pupae is a simple way of killing brood.*



*Hygienic bees will remove pin-killed brood within 12-24 hours. Photo: I Fries.*



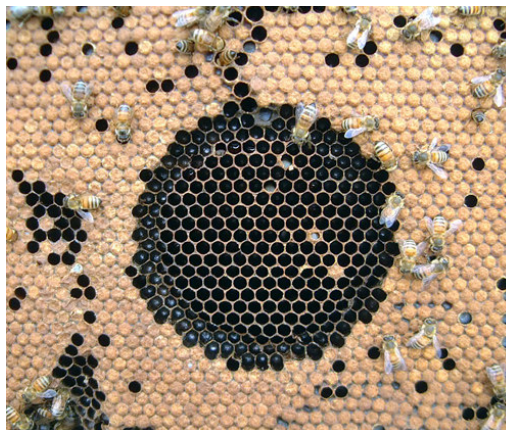
*Pouring liquid nitrogen into tubes can be done quickly on many combs. Note! Use gloves! Photo: M. Spivak.*

brood killed with a needle.

### Recording hygienic behaviour

Independent of method used for killing brood, records are taken at predetermined intervals of the proportion of cells that have been uncapped and the content removed by the bees. To increase precision, notes can also be taken on number of cells that have not been completely removed, but have been manipulated by the bees. Depending on the method for killing bees that have been used, the timing of the first reading may have to be different. For pin-killed brood it is often necessary to make a first reading already 12 hours after pinning the brood. For freeze killed brood it is often enough to start

recording 24 hours post freezing. However, it should be noted that with high levels of hygienic behaviour, a shorter interval may be needed for sufficient resolution of the results.



*Hygienic bees will remove freeze killed brood within 24 hours. Photo: M. Spivak.*

### FINAL REMARKS

The best tool for breeding of disease tolerant honeybees is presently to select for breeder queens exhibiting a pronounced level of hygienic behaviour. This leads to increased resistance to all known brood diseases and also to decreased Varroa population build up. Irrespective of breeding system chosen it is possible to increase the gene frequency through selection of traits linked to hygienic behaviour. Breeding for more disease resistant bees provides an effective way to reduce the chemical treatment of diseases and of reduced needs for pest control.



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