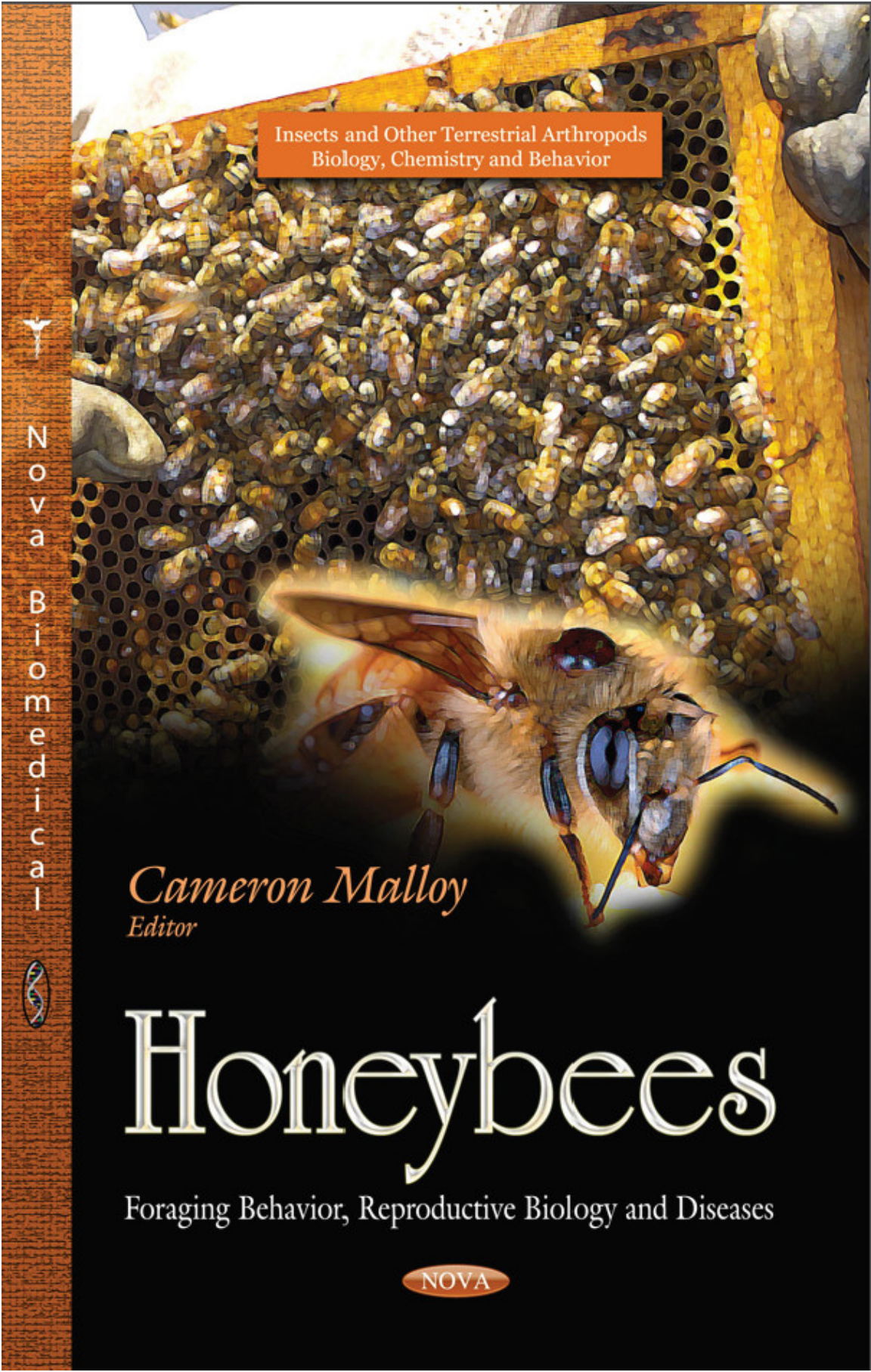
Insects and Other Terrestrial Arthropods
Biology, Chemistry and Behavior
Nova Biomedical
Cameron Malloy
Editor
Honeybees
Foraging Behavior, Reproductive Biology and Diseases
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INSECTS AND OTHER TERRESTRIAL ARTHROPODS:
BIOLOGY, CHEMISTRY AND BEHAVIOR

HONEYBEES

FORAGING BEHAVIOR, REPRODUCTIVE BIOLOGY AND DISEASES

INSECTS AND OTHER TERRESTRIAL ARTHROPODS: BIOLOGY, CHEMISTRY AND BEHAVIOR

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PREFACE

Honeybees are a subset of bees in the genus Apis, primarily distinguished by the production and storage of honey and the construction of perennial, colonial nests out of wax. In this book, the authors present current research in the study of the foraging behavior, reproductive biology and diseases relating to honeybees. Topics discussed include research into diseases affecting honeybees; the status of bee health and colony losses in Argentina; situational choices among alternative visual stimuli in honeybees and paper wasps when foraging; regulation of the crop content for foragers upon departing the hive; sex differences of dopamine control systems associated with reproduction in honeybees; and the roundtrip-structure of the foraging honeybee (Apis mellifera).

Chapter 1 - This chapter provides a general description of the major diseases affecting honeybees (*Apis mellifera L*), dividing them into two main categories: brood and adult bee diseases. Within the group of diseases that affect the honeybee brood are American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius)*, Chalkbrood (*Ascosphaera apis*), Stonebrood (*Aspergillus* ssp.), and, Sacbrood (*Morator aetatulas* or SBV).

Diseases that affect adult bees are produced by *Nosema apis* or *Nosema ceranae*, *Malpighamoeba mellificae*, protozoa, such as Gregarines, and flagellates (*Crithidia mellifera)*. Other adult bee diseases include septicemia (*Pseudomonas aeruginosa*) and, spiroplasmosis, produced by *Spiroplasma apis* and *Spiroplasma melliferum*, respectively, and adult bees can also be parasitised by such mites as *Varroa jacobsoni*, *Acarapis woodi* and *Tropilaelaps clareae*. Among the viruses that cause disease in adult bees are the acute paralysis virus of bees (ABPV), the chronic paralysis virus of bees

(CBPV) and Israeli paralysis virus (IAPV), filamentous virus (FV), black queen-cell virus (BQCV), bee virus Y (BVY) and bee virus X (BVX).

Beehives can also be adversely affected by pest organisms. These species include moths (*Galleria mellonella, Achroia grisella* and *Anagasta kuehniella),* the bee louse *Aethina Braula coeca*, *Senotainia tricuspis* and certain species of ants. Finally, there is a group of noninfectious factors that can cause dead larvae, pupae and adult bees, such as chilling, overheating, starvation, genetic lethality and plant and pesticide poisoning.

Chapter 2 - Honey bees are essential components to modern agriculture and economy. However, a continuos increment in colony losses and colony depopulation cases are being reported worldwide. This critical situation has put on the edge the fragil equilibrium between bees and plants, obligating to several scientists to redirect their research lines. Most researchers agree that there is no single explanation for the extensive colony losses but that interactions between different stresses are likely involved. Argentina is not the exception, several reports of colony losses and colony depopulation cases were informed by beeckepers around the country but still there is no accurate data registered. It is believed that the total number of colonies in Argentina suffered a 30% of reduction in the last years. In this way, this article intends to evaluate the current situation of honey bee health in Argentina. In this review, the authors evaluated the impact of the main parasites and pathogens affecting honey bee colonies and discussed the role of each over honey bee losses in Argentina. Also, the authors discuss the classical control forms applied in Argentina to reduce *Varroa* mite populations, going deeper in the problems related to acaricide resistance phenomena and bee product pollution. Second, the authors provide data about bee nutrition in Argentina and the main strategies used by beeckepers to manage their colonies. Third, the authors evaluate the impact of monocultures and pesticides associated to them over colonies survival. Finally the authors try to estimate the current status of colony losses through the data reported by official institutions devoted to the study of honey bees and also, by the beekeepers. This article aims to serve as a reference of the current status of honey bee health for Argentina and also, to serve as a comparison with future losses as well as providing guidance to future hypothesis-driven research on the causes of colony mortality.

Chapter 3 - Since 1960s the honeybee has been serving as a traditional model in studying intelligence/cognitive abilities in insects. In this chapter, new examples of cognitive tasks including planning are described. Paper wasps were shown to be capable of contextual learning as well. Common methods of free flying insect training in field experiments were used.

The decision depended on additional condition. The insects were presented with two visually different (by color or by shape) feeders placed on a horizontal table. The additional condition was background color. There were two randomly changed backgrounds. The rewarding depended on what background the stimuli were placed. At the background N1 the stimulus N1 was rewarded, and vice versa. In modified experiment, the background remained constant, while feeding places were changed randomly. There were two locations at the distance of several meters from each other. The experimental table was randomly positioned at one of these locations. At the location N1 the stimulus N1 was rewarded, and vice versa. Flying insects learned to check both locations very easily, and then the learning to make correct choices started. Majority of the individuals studied (but not all) solved the tasks.

The events described are very similar to so called "conditioned switching" well known in vertebrata. Traditionally, this phenomenon is investigated in the frame of "higher *nervous* activity" conception.

As a control, in special experiments, ability to recognize familiar colors at new background and at new place was studied. Supposition that the background color dependent task and the location dependent task are different by their innate predispositions is discussed.

Learning of regularity in alternations of feeding objects across foraging trials. The bees (wasps were not tested) were presented with two feeders, which differed by color. In consecutive bee visits, rewarded colors were altered regularly (N 1 – N 2 – N 1 – N 2 and so on), positions of the feeders being changed randomly. After long training period (up to some days), all experimental bees solved the task. The task may be considered to be a sort of planning: the bee remembered rewarded color in present visit and planed to choose the other color in the next one.

Chapter 4 - Honeybee foragers carry a small amount of honey when they leave the hive and consume it to produce energy for flight during foraging. In this review, the author examines how and why honeybees regulate the amount of honey taken from the nest for foraging. It is estimated that bees are able to fly 1 km using 1 μL of unripe honey. In nectar foragers, the amount of fuel loaded in the hive depends on the distance from the hive to the food source; thus, bees foraging on sites that are further afield carry more fuel. The amount of crop content on departure reduces as the foragers repeatedly visit a food source, suggesting that the informational state of the bees influences the amount of fuel carried. In addition, waggle dancers carry less honey on departing the hive compared with potential recruits leaving the hive after

following the dance. Foragers collecting other materials, such as pollen, water or resin, might have different ways of regulating their crop content upon leaving the hive. Examples described here indicate that the amount of honey loaded at departure is under complicated close regulation.

Chapter 5 - Dopamine is a key substance in the regulation of reproductive behaviors and sexual maturation in social hymenopterans. In honeybees (*Apis mellifera*), factors affecting brain dopamine levels appear to differ between male and female bees. The brain levels of dopamine in males are enhanced by juvenile hormone (JH) and the dynamics of dopamine in the brain are similar to those of JH. Both dopamine and JH have roles in promoting mating flight behavior in males. JH can also enhance the gene expression of a dopamine receptor, indicating a parallel regulation of dopamine supply and dopamine receptor expression. The brain levels of dopamine in honeybee workers are regulated by queen substances and increase in the absence of the queen to enable their transition to become reproductive workers. The queen substances can also regulate the expression of particular dopamine receptors in workers. Nutritional factors can influence the brain dopamine levels through the supply of dopamine precursors. However, JH might not regulate the levels of dopamine in the brain in both reproductive workers and queens, because these females have low titers of JH in their hemolymph. Thus, the regulatory systems of dopamine in the brain differ between male and female honeybees. Such differences might be unique to honeybees because they share few similarities with the regulatory systems of primitively eusocial species or highly eusocial ant species.

Chapter 6 - The foraging system of honeybees (Genus *Apis*), being socially and individually controlled, represents the most complex behavioral system known among invertebrates. This system had been most thoroughly studied in the European hive-bee (*Apis mellifera*), with major advances achieved in the previous century based on prize-winning discoveries of Karl von Frisch. Much of v. Frisch and collaborators' research on honeybees was closely linked to the analysis of the bees' communication dances inside the hive. Concerning the navigation system of individual foragers outside the hive, the author will review recent new findings and their theoretical integration.

Investigating the navigation system of a small insect, flying distances of up to several kilometers, is challenging. Innovative methods (e.g. use of harmonic radar) have yielded novel insights, useful to develop a new explanatory and synthesizing theoretical framework. In brief:

Simple straight journeys from the hive to a collecting site, or the reverse, are both constructed of three distinct sequential constituents: *Distal*

navigation, *peripheral navigation* and *focal navigation*. Dead reckoning, the use of compass and distance knowledge, dominates distal navigation, which may span kilometers. Peripheral navigation, being used in the less than 100m range around the respective target, is dominated by the use of remembered terrestrial cues in order to orient towards the chosen goal from different directions. Focal navigation prepares for touchdown, based on increasing fine-grained spatial visual knowledge close to the target location. The knowledge required to implement this three-part navigation system is acquired in reverse order during exploration: *Focal exploration*, *peripheral exploration* and *distal exploration*.

This relatively simple three-part navigation system increases in complexity by adding *navigation hubs*, locations at which the individual bee interrupts navigation and decides when and where to depart towards another location. There are two types of such hubs: the hive itself and some recently identified extra-hive hubs, located at some distal collecting sites where the forager decides to fly home or to fly to some other collecting site, if the current one is depleted. Honeybees are also capable to make iterative use of focal navigation to successfully traverse mazes.

Given comparative evidence, the honeybee's three-part individual navigation system is found exclusively in the monophyletic lineage of the Euaculeata, the mostly nest-provisioning stinging wasps and bees. The species rich family of the ants (Formicidae) is an offshoot inside this lineage, but their navigation system is somewhat differently structured in adaptation to navigation on the ground and even underground.

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Chapter 1

HONEYBEE DISEASES

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ABSTRACT

This chapter provides a general description of the major diseases affecting honeybees (*Apis mellifera L*), dividing them into two main categories: brood and adult bee diseases. Within the group of diseases that affect the honeybee brood are American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius)*, Chalkbrood (*Ascosphaera apis*), Stonebrood (*Aspergillus* ssp.), and, Sacbrood (*Morator aetatulas* or SBV).

Diseases that affect adult bees are produced by *Nosema apis* or *Nosema ceranae, Malpighamoeba mellificae*, protozoa, such as Gregarines, and flagellates (*Crithidia mellifera)*. Other adult bee diseases include septicemia (*Pseudomonas aeruginosa*) and, spiroplasmosis, produced by *Spiroplasma apis* and *Spiroplasma melliferum*, respectively, and adult bees can also be parasitised by such mites as *Varroa jacobsoni, Acarapis woodi* and *Tropilaelaps clareae*. Among the viruses that cause disease in adult bees are the acute paralysis virus of bees (ABPV), the

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chronic paralysis virus of bees (CBPV) and Israeli paralysis virus (IAPV), filamentous virus (FV), black queen-cell virus (BQCV), bee virus Y (BVY) and bee virus X (BVX).

Beehives can also be adversely affected by pest organisms. These species include moths (*Galleria mellonella, Achroia grisella* and *Anagasta kuehniella),* the bee louse *Aethina Braula coeca*, *Senotainia tricuspis* and certain species of ants. Finally, there is a group of noninfectious factors that can cause dead larvae, pupae and adult bees, such as chilling, overheating, starvation, genetic lethality and plant and pesticide poisoning.

1. INTRODUCTION

The importance of honeybees is not under debate, as it is well-known that the commercial farmer depends essentially on the work performed by a single species, *Apis mellifera* L., which also plays a vital role in maintaining the natural balance and biodiversity in ecosystems. Nevertheless, the situation for honeybees is complex because worldwide business practices have caused the spread of infectious agents to all continents. The pathogens to have first contact with honeybees in America and Europe can be a real danger to native honeybee populations.

Beekeepers and researchers have assembled extensive information about the diseases that can affect honeybees; in the 1900s, researchers and beekeepers first raised the alarm about a new disease that was reducing the hives' population in Europe and USA, and research into diseases affecting honeybees began to increase.

Currently, it is essential to understand the relationships between pathogens acting as vectors of other pathogens that provoke honeybee and hive diseases. It is also important to consider secondary infections aggravating sick honeybees and possible interactions of abiotic factors that can affect the bee immune system. The characteristics that make honeybees more susceptible to certain diseases are not yet fully understood; therefore, it is necessary to study the generalities that currently surround the infectious agents that can cause disease and the features that may soon truly endanger the population of the hive.

2. DISEASES THAT AFFECT THE HONEYBEE BROOD

2.1. American Foulbrood

American Foulbrood (AFB) is a disease distributed worldwide. It is considered one of the most severe and dangerous diseases affecting the brood of honeybees (*Apis mellifera L.*)[1], is highly contagious, difficult to control, may be latent in infected colonies and is capable of destroying a colony.[2] The conditions that cause the infection are unknown, and AFB is present throughout the year; however, a hive under stress or with a genetic predisposition is more vulnerable to suffer from this disease. [3]

It is believed that the oldest report of this disease was made by Aristotle (384-322 B.C.) in the ninth book of his History of Animals [4], but the first description of the organism causing the disease was performed by White in 1906, [5] classifying the etiologic agent as *Bacillus larvae*.

Later, Katznelson mentioned in 1950 [6] that other species of microorganisms may be related to the disease and indicated that the *Bacillus pulvifaciens* was another species that can cause AFB. Subsequent work reclassified both *B. larvae,* and *B. pulvifaciens* within a new genus called *Paenibacillus*. Heyndrickx, in 1992 [7], used various techniques, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), analysis of amplified ribosomal DNA restriction (ARDRA), analysis of random amplified polymorphic DNA (RAPD) and length polymorphism analysis of amplified fragments (AFLP), to reclassify the two microorganisms as subspecies of *Paenibacillus larvae (P. larvae* subs. *larvae* and *P. larvae* subs. *Pulvifaciens)*.

Subsequent molecular biology studies, such as those published by Genersh et al., [8] using rep-PCR and primers ERIC, concluded that *P. larvae* have 4 genotypes (two related to the subspecies *larvae* and two associated with subspecies *pulvifaciens*); and different phenotypes including the virulence level. Therefore, *P. larva* is now considered the only causative agent of AFB, without subspecies differentiation.

2.1.1. Pathology

The disease cycle begins when the larvae, eat food contaminated with spores of *P. larvae,* which germinate and grow in the midgut. In the first stage of infection, bacteria act as commensals. Once they fully penetrate the intestinal tissue, they move to the intracellular space, where they proliferate in the hemocoel. [9] It is believed that a combination of proteases secreted by *P.*

larvae destroy cell attachment structures, these proteases damage the tissues and give AFB its characteristic appearance. [10]

The spores also perform their infectious cycle in adult honeybees, but adults do not have symptoms of the disease, instead acting as disease vectors. [11]

It has been shown that the bees responsible for cleaning of the comb cells are able to reduce the incidence of infection through hygiene practices that they perform. In the case of an outbreak, they act quickly to remove the dead larvae and thereby prevent the proliferation of the disease. It is believed that honeybees have naturally developed this hygienic behaviour. We also know that certain lines have some genetic advantage and increased resistance to the disease so that larvae are less likely to develop the infection. [12] Therefore, it is important for honeybeekeepers to be able to choose queens that are able to pass these characteristics to their offspring.

2.1.2. Diagnosis

The AFB-affected hives have characteristics that can be identified visually by honeybeekeepers. Because AFB is considered extremely aggressive, it is crucial that the disease be detected in the early stages of infection. It is vital for the honeybeekeeper to examine the combs; this disease usually has symptoms such as perforated caps with dark and greasy appearance. Larva content becomes viscous and sticky. Dead or dying larvae adhere strongly to the base of the cells, have a brown or black colour, and emit a characteristic sour odour. [13]

As already mentioned, a careful and experienced beekeeper can detect an outbreak of AFB, but laboratory techniques can also be applied to diagnose the disease. The identification of *P. larvae* can be carried out by molecular techniques or basic microbiology techniques from infected tissue, honey, pollen, wax or inert material.

The techniques used to identify *P. larvae* include microscopic identification of the morphology of the spores and vegetative cells, evidence of proteolytic enzymes in milk, reduced nitrate in BHIT agar, presence of catalase, identification of antibodies, and growth in different growth media such as agar, BHIT supplemented with thiamine for vegetative forms, or enriched media with yeast, starch and glucose to stimulate sporulation. [14]

This disease is difficult to control because the microorganism that produces it forms spores that are known to be resistant to high temperatures and to disinfectants and that can remain viable for several years. The most drastic treatment is the incineration of the contaminated material and the death

of infected honeybees; however, this solution is not the most popular because it represents a loss for honeybeekeepers.

The best advice is to implement preventive solutions. First, keep the hive clean; use clean raw materials, maintain hygiene among the staff, and require suppliers to certify that bees are disease-free. The following are some of the techniques used for disinfecting contaminated materials: immersing the material in paraffin (10 minutes, 150 °C), using gamma radiation, ethylene oxide, lye bath (50 g NaOH dissolved in 38 L of water) and use of sodium hypochlorite. [13]

Some countries authorise the use of antibiotics such as oxytetracycline hydrochloride (OTC), sulfathiazole and tylosin for the treatment of AFB. Antibiotics do not act in the spores, thereby eliminating the clinical symptoms, nor cure the disease; their use creates resistant strains and decreases the longevity of adult honeybees and vitality of the brood; these substances are considered to cause contamination of honey and wax. [10],[13],[15],[16]

2.2. European Foulbrood (EFB)

In 1912, White [17] identified the causative agent of EFB and describes for the first time the European foulbrood disease and identified the etiological agent as *Bacillus pluton*. Later, in 1956, Bayle [18] reclassified the causative agent of European foulbrood as *Streptococcus pluton*, and, years later, this microorganism was reclassified in a new genus called Melissococcus. Today, *Melissococcus plutonius* is considered as the causative agent of European foulbrood. There was some initial confusion when trying to determine the causative agent of EFB because there is a group of disease-associated bacteria (*Bacillus alvei, Enterobacter faecalis, Achromobacter eurydice)* [19] that were each once identified as the primary pathogen. Some of these organisms have been reclassified, and others are currently considered secondary invaders once the honeybees have been affected by EFB. Among the secondary pathogens, *Enterobacter faecalis* and *Achromobacter eurydice* appear in high numbers in affected larvae. *Achromobacter eurydice* is naturally present in the bacterial flora of larvae; apparently, when it develops to a significant level, larvae become more susceptible to infection. [20], [21]

The saprophytes *Bravebaclus laterosporius* and *Paenibacillus alvei* were also once considered as the causative agent of EFB; however, there is no evidence in the literature that proves this hypothesis. These bacteria live and

multiply in tissue residues of larvae, and it is known that *P. alvei* is unable to grow in the gut of healthy larvae. [21], [22]

2.2.1. Pathology

The first stage of the infection begins when larvae ingest food contaminated with *M. plutonius*, and this stage is considered asymptomatic. Bacteria multiply and invade the midgut, first destroying the peritrophic membrane and then invading the intestinal epithelium. [23]

The infection is not always lethal; some infected larvae may survive and pupate, so that bacteria are discharged with the faeces and deposited on the walls of cells. In this mode, *M. plutonius* can remain infective for several years. [22]

The number of bacteria present appears not to be related to the severity of the infection. This may be because certain strains of *M. plutonius* are more virulent (atypical strains); or require the presence of certain factors that induce the expression of virulence genes; or due to the presence of microorganisms such as secondary *P. alvei* can cause more serious infection. Some authors consider that the larvae do not overcome the infection and practically starve because *M. plutonius* behaves as a parasite, competing against the larvae for food; if these organisms are found in large quantities, the larva is at a substantial disadvantage. However, there is no consensus on the cause of death in the larvae, nor is the role of secondary invaders precisely understood. McKee (2004) proposes that death caused by secondary invaders is due to tissue damage caused by the infection. [24]

It is noteworthy that the larvae are vulnerable at any stage of development, but older larvae are less prone to infection, and it is also considered that the death of the infected larvae may be related to other factors such as nutritional state, immune response, adult hygienic behaviour and interaction of the infectious agent with the normal bacterial flora of the larvae. [25], [26], [27]

2.2.2. Diagnosis

In this field, the honeybeekeepers diagnose the disease based on visual inspection. When the infection is severe, the worker honeybees cannot remove all the dead larvae and can be observed in cells with characteristic brown colour extremely similar to that presented by larvae affected by AFB. The infected larvae lose their bright white; yellow becomes opaque and finally appears brown. In the last stage of the infection, one can see the tracheal system of the larvae because there is an increase in the transparency of the tissues. The dead larvae are flaccid (non-viscous) in consistency and have a

sour smell. Once the tissue debris is totally dry, the larvae no longer adhere to cells and are easily removed from the combs, where they are observed as small traces falling from cells. In the combs, it can be observed an irregular pattern of empty cells alternating with cells containing diseased and developing larvae and larvae. [13], [28]

Other method for diagnosing this disease include the identification of *M. plutonius* or secondary invaders, such as *P. alvei*, by microscopic observation (nigrosin staining), [13] ELISA techniques for the detection of *M. plutonius*, [29] and isolation using modified basal medium, M110 agar (anaerobic or microaerophilic conditions, with 8-10% CO_2). [30] Detection techniques also include DNA amplification using PCR technique variations with different primers. [31], [32] [33]

With appropriate actions, honeybeekeepers can achieve a significant reduction in the effects of some of the stressors that affect honeybee colonies [13] and even prevent or limit outbreaks of European foulbrood. *M. plutonius* appears to be widespread in hives and resident in the colonies. In some countries, such as the UK and New Zealand, once there is an outbreak of European foulbrood seriously affecting a colony, the protocol is destruction by incineration. This procedure has dramatically reduced the incidence of EFB in the UK. Although *M. plutonius* is a non-spore forming bacterium, some bacteria can survive in the combs and cause disease in the following season. [13]

There is another treatment known as "shook swarm", in which adult honeybees infected with EFB are treated and placed in a new hive. [33] This greatly reduces the risk of recurrence of EFB. Some countries allow OTC application of antibiotics as therapy; the difficulties associated with this system include the cost to the honeybeekeeper, the problem of antibiotic residues left in the hive products and the problem of antibiotic-resistant strains. [32]

2.3. Sacbrood (SBV)

Sacbrood is another disease that can affect the brood of honeybees and is produced by the virus [SBV] of the family *Picornaviridae* and was the first virus affecting honeybees to be completely sequenced. In 1917, White attributed this disease to an infection caused by a virus; [34] not until 1964 was the virus characterised. [35] SBV is a single-stranded RNA virus; the virus particles are 28 nm in diameter, with no cover. [36]

It is known that this disease occurs most often in spring, when the colony is growing faster and larger numbers of vulnerable young adults and larvae are present in the hive. [37] The disease is less common and is considered less dangerous than other viruses that affect honeybees. Infected larvae have a high concentration of virus in the fatty tissue and muscle. [38] Adults are carriers of the virus, which is found in their pharyngeal glands, but show no signs of infection. Larval contamination takes place through feeding by adults. [39]

2.3.1. Pathology

The incubation period of the disease lasts a week. Larvae infected with SBV are similar in appearance to those infected with European foulbrood. An infected larva does not reach pupal development because its tissue is destroyed by the viral invasion, and the ecdysial fluid containing a large number of viruses accumulates under the skin or tissue and forms a fluid bag near the anus; that is why this disease is called Sacbrood. Another hallmark of the disease is the change in colour: infected larvae change colour and can be yellow, grey or brown, with the head being darker than the rest of the body. Their integument is opaque; larvae do not adhere to the walls of cells and can be easily removed from the hive. Once dry, larvae infected by the Sacbrood virus can be observed as a type of dark brown crust. [40]

This disease can kill the brood of a hive if not controlled. Nurse honeybees, being entirely responsible for maintaining the brood combs, clean so that the disease does not spread. Because this disease is of viral origin, it cannot be eliminated with antibiotics. This virus not only affects the young but also adult honeybees, but the disease in adults is less obvious and causes weakness and reduced life expectancy rather than death [41]. [37]

This infection is believed to spread vertically, via the queen, who transmits it to her descendants. [40] It is also believed that other viruses are present in healthy larvae and that there is some additional factor that triggers the disease. Recent data show that the virus-induced disease may be exacerbated and persistent infections present if the colonies are infested with the parasitic mite *Varroa destructor*, and it has been observed that the incidence of mite infestation is also increasing. [42]

2.3.2. Diagnosis

The ways to confirm the etiology of this virus are immunoassay techniques (ELISA), electron microscopy and serology. Most of these tests show a low sensitivity and specificity, [43] and differentiation between types of virus by such traditional methods is difficult.

Since the publication of the complete SBV nucleotide sequence, [44] the use of molecular biology techniques, including reverse transcription polymerase chain reaction (RT-PCR), real-time PCR, amplification of different regions of the SBV genome and Western blot, has been proposed as methods for identifying this virus. [42]

There is no treatment available for a colony infected with SBV. The proliferation of the virus can be stopped through providing abundant nectar, increasing artificial feeding, sanitation measures and the exchange of queens in the hives for younger queens. [40]

2.4. Stonebrood

Stonebrood is a disease that was first described in 1906 by Massen. [45] There are several species of fungus of the genus *Aspergillus* associated with this disease, most prominently *A. flavus*, *A. fumigates* and *A. niger*. [46] Stonebrood is distributed worldwide, and the spores of *Aspergillus* are found in such different substrates such as soil, air and water.

The larvae of honeybees are more susceptible to developing this disease if they are under some stress, and infection occurs through ingestion of spores or cuticular lesions. [47] Colonies can recover from Stonebrood depending on specific features such as hygienic behaviour of nurse honeybees and the genetic characteristics of the colony; in their natural environment, certain insect species show adaptations in their metabolism to remove small amounts of aflatoxin. Some species are sensitive to low concentrations of mycotoxins, but others have metabolic pathways that oxidise mycotoxins and reduce their toxicity, and it is proposed that this occurs via monooxygenase P450 enzymes, which can be induced by propolis.[48]

2.4.1. Pathology

The symptoms observed in colonies affected by Stonebrood are not very different from those presented by brood colonies affected by Chalkbrood, including the presence of an irregular pattern in the brood cells and affected larvae with the appearance and texture of small stones. Larvae residues are difficult to remove for honeybees that have the responsibility of cleaning the honeycomb, and infected larvae are covered with powder which can be of different colours depending on the species of *Aspergillus* that affects the colony. It is believed that cause of death in honeybees is aflatoxin poisoning; however *A. flavus* does not produce aflatoxin but produces all the symptoms of

stone breeding. The pupae can die of poisoning by aflatoxin from the fungi and structural damage from the mycelium. [47] Once infection has been observed, control is complicated because these fungi form resistant spores. However, Stonebrood is considered a minor disease because it has little effect and thus little economic relevance.

2.4.2. Diagnosis

Identification of *Aspergillus* spp. requires laboratory cultivation and microscopic examination. Conidiophore structures (spore forming structures) are extremely important for the identification; it is not a difficult microorganism to grow, and it shows very good progress in standard fungal media such as potato dextrose agar (PDA), agar Sabouraud and agar Czapek dox supplemented with yeast extract. Conidia of the different species have different colours; proper identification of *Aspergillus* spp. requires experience [47] because there is no single method (morphological, physiological or molecular) that can be used to identify all species of Aspergillus (approximately 250). However, Jensen et al. have developed techniques for amplification of the internal transcribed spacer (ITS) and β-tubulin gene with primers specific for identification of individual species. [49]

2.5. Chalkbrood

Chalkbrood is caused by a fungus; the etiological agent was initially recognised as *Pericystis apis* in 1913, as described by Maasen. [49] Years later, in 1955, this fungus was reclassified by Spilor [50] as *Ascosphaera apis*.

This fungal infection affects only the brood of honeybees. Although it can be fatal, it does not result in the loss of entire colonies, but it may cause productivity losses of hives by the decrease in the number of adults, and it thus has an economic impact on honeybeekeepers. This disease is distributed worldwide, and its incidence has increased. [51]

It was previously believed that Chalkbrood could be developed when the larval cuticle was infected with ascospores, but it is now known that ascospores cannot germinate in larval cuticle; this disease can only be spread by adult bees feeding larvae [52]

2.5.1. Pathology

Once the contaminated food is ingested, the ascospores germinate in the intestinal lumen, where the concentration of CO_2 promotes their development.

This fungus produces enzymes that penetrate the intestinal wall, promoting the development of mycelium within the body cavity and thereby invading the tissues. [53] The deaths of the infected larvae are produced by enzymatic and mechanical damage in the tissues as well as by the presence of toxicosis in the haemolymph. [54]

Mycelial development begins at the back of the larva. Then, the body is totally covered with a layer of mycelium, and brown spots develop, indicating the formation of ascomata. Finally, when the larva dies and is dehydrated, it presents an aspect of a "mummy": dusty-looking larvae of this type can be found in the cells or at the entrance of the brood comb as they are removed by the worker honeybees responsible for cleaning the hive. Dehydrated larvae may be black when ascospores are present in infected tissue or may be white when viewing only cellular debris and fragments of mycelium. [55]

As with most fungi, the growth of *A. apis* is favoured by certain environmental factors that can occur in a hive, such as increased humidity, [56] inadequate ventilation and optimal growth temperature (33-36 °C). [57] This infection can occur at a higher incidence in spring, but there are other factors that can affect its development, such as the specific strain of *Ascosphaera apis*, its virulence and immune response and the hygienic behaviour of the honeybees responsible for cleaning in the colony.

2.5.2. Diagnosis

This disease is detected visually, by observing whether there are affected larvae in the honeycomb cells or dead larvae in the hive entrance, presenting the characteristic appearance of the infection.

The specific agent causing the disease can also be identified with optical microscopy to observe the characteristics of the *A. apis* structures. The culture media used for the growth of this fungus can be SDA, PDA, YGPSA or MY20. [58] For the description and identification of the typical morphology of the fungus and ascospores, the culture of A. *apis* can be observed by optical microscopy with lactophenol blue staining

The different *A. apis* strains can also be identified with PCR-based DNA amplification, using primers such as BOX, REP and ERIC, [58] for identifying isolates, and microsatellite markers [59] and selected intergenetic region sequences or introns for amplification have also been used for the same purpose. [60]

3. DISEASES THAT AFFECT ADULT BEES

3.1. Nosemosis

Nosema, or Nosemosis, is a disease caused by *Nosema* spp. is known to affect the queens, workers and drones, and it occurs worldwide. The etiological agent that causes the disease was first described in 1909 by Zander. [61] Until recently, it was believed that *Nosema* only affected the honeybee, *Apis mellifera* L., and that *Nosema ceranae* was a parasite that only affected the Asian bee, *Apis ceranae*. Now, it is known that *Nosema ceranae* can also infect *Apis mellifera* colonies, causing what is known as type c Nosema. [62]

3.1.1. Pathology

Both species of *Nosema* infect epithelial cells in the posterior part of the ventriculum, causing a large number of spores in a short period of time, and shed spores in the lumen of the intestine, where they mature and infect additional epithelial cells. Eventually, spores are released with faeces, which is the main source of infection. [63] Honeybees may show swollen abdomen and faecal brown marks in the hive. Additionally, heavily infected colonies show decreases in breeding rates and slow population growth, especially in spring. [64]

The physiological damage of nosemosis has been described, including impaired protein metabolism as indicated by a lower midgut proteolytic activity. [65] The smaller amount of amino acids in haemolymph causes reduced size [66] and lower levels of proteins in the fatty tissue. [67]

Disease transmission occurs through ingestion of spores in food, trophallaxis or perhaps after cleaning the body hairs; [68] the combs are also a source of infection because the spores are expelled in large numbers in the faeces of sick individuals and remain viable for more than a year. [69] The effects of nosemosis on honeybees include premature aging, which leads to reduced longevity. [63]

3.1.2. Diagnostic

The disease can be initially identified by examination of the hives, but other causes, such as pesticide poisoning and mite-induced diseases, must be excluded before a final diagnosis. During the winter, this disease can increase honeybee mortality, but a vital sign for the identification of the disease is that

honeybees exhibit a colour change in the ventricle, which is usually brown and turns white with the consistency change, becoming an extremely fragile tissue.

Another method of identifying the causative agent of Nosema is by microscopic examination (400X) of the abdominal contents of honeybees, which can reveal the presence of spores. The *Nosema apis* spores have an oval shape; *Nosema ceranae* spores are slightly smaller. The internal contents of *Nosema apis* spores can be observed after staining with Giemsa stain; spores of *Nosema apis* have a characteristic appearance, with a thick wall and blue inside, but the inner cores of the spores is not visible. The appearance of *Nosema apis* spores can be confused with yeast, fungal spores, and cysts of *Malpighamoeba mellificae*. [70]

An important set of techniques developed for molecular detection of *Nosema* spp. in honeybees (*N. apis*, and *N. ceranae)* can be found in the literature, usually based on PCR (uniplex or multiplex PCR, PCR-RFLP or qPCR) with a wide range of PCR primers specific for the species of Nosema. [71]

The antibiotic fumagillin has proved effective against both species of Nosema. [72] Fumagillin is one of the few drugs that are effective against microsporidia. [73] Thymol and resveratrol have been tested in honeybees; both significantly reduced the infection, and resveratrol increased the life expectancy of honeybees. [74] These products may be useful alternatives to control Nosema disease, although more studies under field conditions are required. Nosema disease can be prevented with proper apiary management; replacement of hives and queens and hygienic colony-handling actions appear to be useful in the control of Nosema. [75] For disinfection of tools and equipment, spores can be destroyed by heating at a temperature of at least 60 °C for 15 minutes. The combs can be sterilised by heating at 49 °C for 24 hours. [76] The vapours from a solution of acetic acid at least 60% inactivated spores in a few hours depending on its concentration; higher concentrations are even more effective and destroy spores within a few minutes. [77],[78]

3.2. Protozoa

3.2.1. Amoeba

Amoebiasis, a disease caused by the protozoan *Malpighamoeba mellificae* (Sarcodina order), is an infection that affects adult honeybees and is most prevalent in tropical regions. [79]

3.2.1.1. Pathology

This disease is contagious but not considered relevant. *Malpighamoeba mellificae* form cysts as resistant stages, are extracellular parasites and feed by pseudopods, although they seem to also possess flagella that allow them to reach Malpighian tubules. Cysts survive for more than 6 months in the faeces of honeybees but are susceptible to common disinfectants. Once ingested, the cysts reach the ventricles, where gastric juices bring on germination; germination then occurs in the pylorus, where solid matter accumulates. This material acts as a "plug", causing parasites to migrate into the Malpighian tubules (excretory organ similar in function to kidneys). In the Malpighian tubules, protozoa are attached to the epithelium and begin to feed the tissue damage in the process. The parasites multiply and, after 3-4 weeks, many tubular epithelial cells have been destroyed and released cysts, infecting other cells or passing cysts to the intestine to be excreted in the faeces. [80]

Damaged Malpighian tubes inhibit the excretion of metabolic waste and the exchange of solutes in the haemolymph, alter excretory capacity and lead to waste accumulation and nitrogen poisoning. [81] The disease process weakens honeybees, making them more susceptible to other infections. [82] The severity of the disease remains unclear, but it is severe in combination with Nosema. *M. mellificae* is also linked with dysentery symptoms in adult honeybees, and infected honeybees tend to "disappear inexplicably" from the hive. [83] The amoebiasis is almost exclusive to the worker honeybees, and modes of transmission and factors favouring disease development are essentially the same as those of Nosema. [80]

3.2.1.2. Diagnostic

To monitor the presence of amoebae in a colony, cysts can be observed by microscopic examination of the tubules in sick honeybees. Cysts measure 5 to 8 mm in diameter. *M. mellificae* can be observed with a dry objective or with an immersion objective for more detail. [84] There are currently no genetic markers for *M. mellificae*, and this limits their identification by molecular techniques. [85]

There is no effective treatment against amoebiasis. Disinfection with acetic acid as for Nosema has proven effective in decontamination of the combs. The disease can be controlled with Best Management Practices, such as changing combs, disinfecting materials (boxes, bottoms and tops) and transferring the honeybees to uncontaminated hives. [80], [86]

3.2.2. Gregarines

Gregarines are a diverse group of protists that parasitise many species of invertebrates including the honeybee, in which it causes premature death and colony loss [87]. Four species of gregarines (order Gregarinida) are associated with honeybees: *Monoic apis*, *Apigregarina stammeri*, *Acuta rousseaui* and *Leidyana apis*. These protozoa have two stages of development: cephalonts (immature), which measure an average of 16x44 µm, and sporonts (mature), which average 35x85 µm. [84]

3.2.2.1. Pathology

Once ingested, gregarines reproduce asexually, developing into trophozoites in the midgut. The trophozoites attach to the epithelium, where they encyst, destroying epithelial cells and absorbing nutrients from the midgut, reducing the honeybees' nutrition and creating tissue damage where opportunistic pathogens can attack. [85] The infection cycle begins again when the spores leave the host through faeces. Gregarines infect other species of honeybees and wasps, inhibiting feeding, reducing fertility and increasing queen mortality. [88] In tropical climates, colonies seem more susceptible. [87]

3.2.2.2. Diagnostic

Gregarines can be observed in a microscope low power objective from a sample of midgut epithelium of the adult honeybee suspected of being infected [84]. The same treatments can be used as for Nosema, as well as the management practices and disinfection previously mentioned for amoebiasis. [80]

3.2.3. Flagellates

The flagellates associated with honeybees are Crithidia species (Leptomonas). Honeybees worldwide have been identified as affected by this protozoan, [89] and there are two strains known to affect honeybees: *Crithidia mellificae* [90] and the strain called SF (San Francisco). [91] These flagellates are found living freely in the lumen or attached to the hindgut epithelium, as well as in the rectum of adult honeybees. [92] Trypanosomes have mobile flagellated and amastigote forms (non-flagellated, rounded stage); the latter produces surface incrustations in the intestinal epithelium. [90],[93]

3.2.3.1. Diagnostic

Crithidia can vary in size from 5 to 30 µm. [93]. These organisms can be observed by preparing a smear from a midgut macerate and rectum and viewed with a dry objective on a light microscope [84]. Although historically these flagellates have been associated with diseased honeybees, current involvement in the health of honeybees remains unclear.

The three castes in the colony of bees can be parasitized, but the mechanisms of transmission and distribution remain unknown because this infection has no symptoms and no treatment so far. [80]

3.3. Septicaemia

Burnside (1928) [94] described septicaemia as a disease of adult honeybees caused by the bacterium *Bacillus apisepticus*. Later, Landerkin and Katznelson (1959) [95] reclassified *B. apisepticus* as *Pseudomonas apiseptica*, which is now considered a synonym of *Pseudomonas aeruginosa.* [13]

Septicaemia is a fatal bacterial disease that is considered a secondary infection. [96] In honey a bee, this condition refers to any disease caused pathogenic bacteria or their toxins in the haemolymph. [13]

The bacterium that has been most associated with septicaemia infection in both adults and brood [95] is *Pseudomonas aeruginosa*. Although *Hafnia alvei* and *Serratia marcescens* have also been associated with the infection of the disease-bearing mite *Varroa destructor*, the vector [97] *P. aeruginosa* has not been associated specifically with honeybees and is commonly found in the environment (water and soil). Thus, it may infect honeybees as an opportunistic saprophyte, depending on primary pathogens to break cuticular barriers, allowing *P. aeruginosa* to access vulnerable tissues. [98] After this stage, the bacteria pass into the haemolymph where they replicate, generally causing infection and death of the honeybee. [99] The growth of *P. aeruginosa* is aided by the existence of other diseases but also by factors such as a high level of moisture in the honeycomb, improper feeding or artificial feeding in excess. [94]

3.3.1. Pathology

The main symptoms of septicaemia are a change in the colour of the adult honeybee haemolymph (brown to milky white) and a rapid degeneration of the muscles. [13] Therefore, this disease results in the destruction of the connective tissues of the thorax, legs, wings and antennae, and consequently

affect honeybees crumble when handled. Dead or diseased honeybees have a putrid smell, and diseased honeybees can be observed in the hive without moving, feeding or flying. [99]

3.3.2. Diagnostic

Septicaemia can be diagnosed by the reproduction of the disease symptoms in healthy honeybees through the preparation of an aqueous extract (macerating the equivalent of a suspicious honeybee per ml of water). First, bees are carefully anesthetised with CO_2, and then healthy honeybees are inoculated by direct microinjection into the thorax, between the third and fourth abdominal segments. Honeybees infected with septicaemia die within 24 hours, show the typical symptom odour and "rupture" after approximately 48 hours. [84]

For isolation of *P. aeruginosa*, either Agar F (Pseudomonas isolation agar) or King B agar is used. [101] The presence of *P. aeruginosa* can also be checked by optical microscopy with Gram stain; the smears are prepared from tissues of the wings, observing the morphology and characteristic grouping of bacteria (a Gram (+) rod-shaped bacterium measuring 0.5-0.8 x 1.5-3.0 µm). [84]

3.4. Spiroplasmosis

Spiroplasmas are a type of bacteria (Mollicute class) lacking a cell wall and having a helical configuration. [101] The spiroplasmas have been isolated from the haemolymph and gut of insects; [13] adult honeybees are affected by two species of spiroplasmas: *Spiroplasma apis* [102] and *Spiroplasma melliferum*. [103]

Spiroplasma melliferum measures 0.7-1.2 mm in diameter, and may have a length that ranges between 2 and 10 µm. [84] The disease caused by this bacterium most frequently affects colonies in late spring. [96]

Spiroplasma apis also causes a lethal infection called "May disease". The sick honeybees die, presenting symptoms such as bloating and restless movements. [103]

Infection of honeybees takes place through the mouth; [96] bacteria break the barrier of the gut, enter the haemocoel and invade the haemolymph, then multiply, causing a systemic infection that can eventually cause death. [85] Before dying, honeybees have a bacteria count of 10^{11} organisms per mL of haemolymph, and they tend to die after a week. [96]

Spiroplasmas are susceptible to tetracycline and can be grown in rich media containing bovine serum or in standard medium for mycoplasma. [96], [84] These bacteria can be observed by phase-contrast microscopy or dark-field microscopy from samples of diseased haemolymph of adult honeybees. [84]

3.5. Mites

3.5.1. Varroa Destructor

The first report of varroa mites was conducted in the year 1904 in Indonesia. A researcher named Oudemans [106] identified a parasite of the Asian honeybee *Apis cerana* as the Varroa mite. It was subsequently established that the genus Varroa comprises four species: *V. jacobsoni* [106] *V. underwoodi* [107] *V. rindereri* [108] *V. destructor*. [109]

In 2000, Anderson and Trueman [109] used molecular tools to demonstrate that the invasive population was not the species described by Oudemans in 1904[106], determining instead that the species of mite that affects both *Apis mellifera* and *Apis cerana* is *Varroa destructor*, so publications prior to 2000 refer to *V. jacobsoni* instead of *V. destructor* as the main invasive species of *Apis mellifera.*

The parasite *Varroa destructor* was initially limited to Asia, where colonies of *Apis cerana* are not significantly affected, most likely due to the co-evolution between the two species; [110] this adaptation probably caused the development of hygienic behaviour of *A. cerana* to keep the number of mites under control. Instead, *Varroa destructor* is a severe parasite of *Apis mellifera*, which is a less-resistant host, allowing an increased spread of the infection and ultimately the loss of colonies affected by the parasite. [111] The Varroa damage begins in offspring; and adult parasitised bees cannot carry out their tasks becoming unproductive members of the colony, [112] and consequently the colony is weakened.

Adult bees affected by the mite lose weight, become malformed, and die prematurely; *V. destructor* indirectly causes problems because it is considered a virus vector for SVB and BQVB. [113]

3.5.1.1. Diagnosis of Varroa

Mites are found below the abdomen of adult bees, attached by legs and oral structures in intersegmental membranes (phoretic phase, from Greek 'forest', load). It is during this period that they feed intermittently from the

haemolymph of the host through the intersegmental membrane perforation. The individual key to the Varroa development cycle is the adult female, as its life alternates between the reproductive stage and phoretic phase. [114]

The adult female is brown or reddish brown, oval and flat. Its dimensions are on average 1 mm long and 1.5 mm wide, while the female nymph stages and males are smaller and cream or white in colour. All stages are easily visible to the naked eye. [115]

Infective Cycle

A female Varroa (adult) enters the brood cell to begin its reproductive cycle. The entry into the brood cell must occur at a precise stage. Before the brood cell is capped, there is a significant risk of detection and removal by the bees. After brood cells are sealed, the female mite cannot enter.

The factors that cause and influence the entry of phoretic Varroa into cells are not yet well known. There appear to be some breeding chemical signals that are an essential element of the infestation. There is a hypothesis that the phoretic Varroa is guided by pheromones emitted by the larva, to penetrate the brood cells at the right time, but this idea remains controversial.

It is likely that other groups of molecules involved in infection encourage breeding. In addition, mechanical factors certainly have an influence in favour infestation. For example, the cell size and prominence or the distance between the larva and cell edge significantly influence the infestation, and these elements could partly explain the higher infestation of drone breeding cells.

The female Varroa infests a brood cell when all larvae have reached the fifth stage of larval development, stage L5. The female Varroa submerges in food for the larva until it starts pupal development, and only then does the Varroa female begin to lay eggs.

A few hours later, a Varroa larva appears inside the egg. This larva becomes a protonymph, a deutonymph and finally an adult. The whole development takes approximately 130 hours for a female, 150 hours for a male. The male mates with the female as soon as it reaches adulthood. When the second daughter becomes mature, the male leaves the first daughter to mate with her, repeating the same situation with other females. A female Varroa can be fertilised only in the cell where she was born.

Dissemination of Varroa

When it emerges from bee brood cell, much of the new Varroa mite remains in the cell. As soon as they leave the cell, these mites try to move onto the bees, to enter the phoretic stage and start the reproductive cycle. [115]

3.5.1.2. Diagnostics for the Treatment of the Colony Varroa

In colonies affected by Varroa, it is necessary to determine the degree of infestation in the colony, by sampling in the hives, to take the decision to implement an acaricide. One useful method is the sugar shake method. This method estimates the prevalence of mites within the colony (the percentage of adult bees with mites). The sticky board method allows the entire load of the colony (the overall number of mites in the hive), and the alcohol wash method is similar to the sugar shake; it requires that the beekeeper brush or shake the adult bees from a frame into a clear container to measure the prevalence of Varroa mites. [116] To evaluate the amount of Varroa in a hive, it is possible to extrapolate from weekly mortality, but the results are not very reliable. It is more reliable to use a sample to determine the rate of infestation and then extrapolate to the entire hive.

Currently, colonies infested with Varroa are treated with synthetic chemicals, mainly pyrethroids, fluvalinate, flumethrin, amitraz or coumaphos. While these have good efficacy and allow convenient control of parasites, their use has serious drawbacks. There are three possible alternative treatments of interest to use for beekeepers: formic acid, oxalic acid, and thymol. [117]

3.5.2. Tropilaelaps ssp.

These mites are often called Asian mites because their natural host is the bee species *Apis dorsata*. Of the four known species, *Tropilaelaps clareae* and *Tropilaelaps mercedesae* are the exclusive parasites of honeybee larvae. [118]

The life cycle of Tropilaelaps is similar to that of Varroa destructor but with slight differences. Tropilaelaps has a higher reproductive rate because it has a shorter life cycle. This is because they have a faster development time and shorter phoretic phase. Consequently, when both types of mites are present in the same colonies, Tropilaelaps populations increase faster than Varroa, by a factor of 25:1. [119]

For the adult reproductive phase, mites enter the cells containing larvae. Reproduction takes place within the cells with larvae that are already fully sealed. Usually, the mother mite lays three to four eggs in bee larvae, the eggs hatch after about twelve hours later, and the larvae pass through nymphal stages (protonymph and deutonymph) before reaching adulthood. Once hatched, male and female mites feed on the haemolymph of the developing bee, causing damage by depriving the developing bee essential nutrients required for growth. [120]

Tropilaelaps infestation kills approximately 50% of the larvae. Many adult bees that survive the larval stage infection have deformed abdomens,

deformed wings and short or missing legs. [121] The first sign of an infestation by Tropilaelaps species is the observation of the presence of reddish-brown mites in combs or on adult bees.

Tropilaelaps clareae (<1 mm in length) [122] can be easily recognised and differentiated from Varroa destructor using a 10 × magnifying glass. [123] The main methods used for Varroa detection can be applied to Tropilaelaps species: monitoring mite mortality and observing cells uncovered with larvae developing state to track the degree of infestation and the application of acaricides. Some Varroa miticides have been used to control Tropilaelaps, such as strips impregnated with Fluvalinate. Snuff smoke in the smoker causes the mites to fall off the bees. Also used are strips impregnated with an aqueous solution of potassium nitrate (15%) to which two drops of amitraz (typically 12.5%) are added [124]. These strips are inserted in the drying and burnt; the smoke causes mites to leave the hive. Another method is to use pads impregnated with 20 ml of 65% formic acid, placed in the hives, near the top. [125]

3.5.3. Acarapis Woodi

There are four described species in the genus Acarapis, *A. dorsalis* Morgenthaler, *A. externus* Morgenthaler, *A. vagans* Schneider and *A. woodi*. Of these four species, only *A. woodi* is found within the tracheal system [126] and is dangerous for honeybees.

The mite *Acarapis woodi* has been extensively studied since its discovery in 1919. [127] The life cycle of *A. woodi* is not well known; all of the mite stages live in the tracheal tubes of adult honeybees, and only adult females move outside the host to infest other bees. [128]

3.5.3.1. Pathology

Adult female mites enter the tracheae of adult bees and pierce the tracheal wall, where they feed on the haemolymph and reproduce. *Acarapis woodi* (Rennie) is an internal parasite that spends most of its life in the thoracic trachea.

After completing development and mating, a young adult female mite moves out of the tracheal system and onto the hairs of the bee until it comes into contact with a new bee, when it determines the suitability of the host; if appropriate, it moves to and enters the new host's prothoracic spiracle. Numerous studies have confirmed that mites strongly prefer young bees; [129] this preference appears to be influenced by differences in cuticular chemistry.

It is believed that the damage to the bees may be due to some or all of the following causes: a reduction in air flow due to the obstruction caused by the number of mites and their waste, especially in smaller tracheal branches; [130] loss of nutrients due to the haemolymph feeding by the mites; acquisition of secondary infections that appear more easily because of the wounds left by the punctures made by the mites; [131] injuries to muscles and nerve tissue near the trachea; [132] behavioural disorders; paralysis of the flight muscles due to toxins produced by the mite; [133] alteration of metabolism; interference with flight muscles due to rigid tracheae. This shortens the life cycle, [134] reduces the total number of the brood in spring, and increases mortality of hives during the winter. [135]

3.5.3.2. Diagnosis

The positive diagnosis of this disease can be made by microscopic examination of the tracheas with different dissection techniques (methylene blue staining), [136] discoloration (black dots) and the presence of eggs, nymphs, and mite adult stages [84] or more sophisticated techniques such as ELISA. [137], [138]

It is known that certain colonies are more resistant to these mite infestations, apparently because of the cleaning behaviour of the bees of the colony and certain components that may be present in the cuticle of young bees. [139], [140]

3.6. Diseases Caused by Viruses

3.6.1. Acute Paralysis (ABPV)

Acute bee paralysis is an infection caused by "acute bee paralysis virus" (ABPV), a virus from the family *Dicistroviridae*. This virus has widespread prevalence in honeybee colonies and a predominantly sub-clinical etiology that contrasts with the extremely virulent pathology encountered at elevated titres, whether artificially induced or encountered naturally. [141]

It has been postulated that ABPV causes death of honeybees once hives have been infected by the mite *Varroa destructor*. [142] Mites damage the tissues of bees, and, in doing so, can act as vectors for viral particle release into the haemolymph. [143] Despite the lack of evidence of viral replication in the mites, they are capable of transmitting ABPV. In nature, the virus spreads as an asymptomatic infection through salivary gland secretions of adult bees and food contaminated with these secretions. [144] Moreover, viruses were

recently detected in semen from infected drones sampled, indicating that ABPV can be transmitted vertically. [145]

ABPV was originally discovered in the UK. [146] The basic genome organisation of ABPV is typical of the *Dicistroviridae* family, containing a single strand of positive-strand RNA with two open reading frames (ORFs), separated by an intergenic region (IGR). The largest ORF is located at the 5' end of the genome and encodes the nonstructural proteins involved in viral replication. The shorter ORF is located towards the 3' end of the genome and encodes the capsid proteins found in the viral particle, and the genome is polyadenylated at the 3' end. [147]

3.6.2. Chronic Paralysis (CBPV)

A condition called chronic paralysis affects adult bees, and the virus causing the disease (chronic bee paralysis virus, CBPV) is distributed worldwide. [148] Signs that bees have contracted the disease include the following: the presence of tremors; bees cannot fly, crawl to move, and lose their hair; sometimes, bees acquire a black hue and creep into the of the hive entrance. [149] Once the disease has spread, a significant number of bees can be observed at the entrance of the hive, and this is reflected in the decrease in the honeybee population. [150]

CBPV was isolated first in 1963. [151] It is an anisometric particle, non-enveloped, and measures 30-60 nm in length and 20 nm in width. [152] It is classified as a positive single-stranded RNA virus and has a segmented genome comprising two RNAs, RNA 1 and RNA 2; [153] it has been suggested that the ORFs from RNA1 encode nonstructural proteins and ORFs in from RNA 2 encode structural proteins. [150]

Due to their genetic characteristics, CBPV seems to occupy an intermediate position between two families of viruses: *Nodaviridae* and *Tombusviridae*. These families already share common characteristics, [154] and CBPV has not yet been assigned to any family. Therefore, evaluation of the taxonomic position of CBPV and understanding how it infects bees requires a description of its proteins using molecular techniques. [150]

3.6.3. Israeli Paralysis (IAPV)

The Israeli Acute Paralysis Virus (IAPV) was detected for the first time in Israel [155] and distributed later to the rest of the world. Recently, the presence of IAPV has been correlated with the loss syndrome observed in the United States called Colony Collapse Disorder (CCD), [156] and it has been

suggested that IAPV could be a statistically significant marker for CCD, but this hypothesis is still under discussion. [157]

IAPV is a positive strand RNA virus containing two reading frames separated by an intergenic region. The 5' ORF encodes three non-structural proteins (helicase, protease, and RNA-dependent RNA polymerase), and the 3' ORF encodes two proteins of the capsid. [155] Based on homology and genomic structure, IAPV is classified within the family *Dicistroviridae* [158] and is believed to be closely related to Kashmir bee virus (KBV) and ABPV, although all three are genetically and serologically distinct. [155] In addition to this close genetic relationship; they share a number of biological characteristics, such as the main routes of transmission and life stage of the primary host. At low levels, infected colonies do not show clinical signs, but high levels of exposure to the virus cause high rates of mortality. The symptoms of IAPV are characterised by trembling, followed by progressive paralysis and death.

3.6.4. Filamentous Virus (FV)

Unlike other viruses known to affect the genus *Apis*, filamentous virus has a genome of DNA. [159] This virus was initially confused with rickettsia due to its size, and in some cases their symptomatology was similar to that presented by bees purportedly attacked by rickettsia. [160]

This virus replicates primarily in the fatty tissue and ovary in adult bees and remains asymptomatic in them; in pupae found in the cells, it induces changes in colour from white to black and brown hues. This virus is considered the least virulent of all known to affect bees. Because of its large size and the presence of viral particles in the haemolymph, identification can be performed with an optical microscope from haemolymph samples from sick or dead bees. Haemolymph-infected bees take on a milky white appearance, and virus particles can be observed in bar form by microscopy. [161]

Some cases have described the presence of these viral particles in healthy bees with no symptoms, and it is known that transmission of the virus can occur by inoculating food or through the action of a vector (*Nosema apis)*. [162]

3.6.5. Black Queen Cell Virus (BQCV)

BQCV was originally found in dead larvae and pupae of queen bees. [163] These virus particles have an isometric shape, 30 nm in diameter, and only single-stranded RNA forming the four capsid proteins. This virus was first isolated from prepupae and pupae of queens that were found dead in their

cells. [164] The virus is named for the appearance of darkened areas in the cell walls containing infected pupae. Evidence has been presented that suggests interactions between viruses and parasites of bees. BQCV is often associated with the microsporidian parasite *Nosema apis* and may be involved in the death of bees infested with that parasite. [165]

3.6.6. Bee Virus Y

Bee virus Y (BVY) was first isolated in Britain. This virus was found on dead or dying adult bees, and their condition was attributed to BVY. [166] As with BQCV, this virus is closely associated with the parasite *N. apis* and follows the same regular cycle of annual incidence, with maximal infections in spring and early summer. [167] However, in contrast to BQCV, BVY can infect young bees when they are fed with it, but the incidence of infection increases when young bees are fed with the virus and spores of *N. apis*. The nature of its association with *N. apis* is unknown, but similar to BVX, BVY multiplication can be largely restricted to the intestine of adult bees and can reduce the parasitic resistance of the intestinal cells to infection or facilitate viral entry. BVY has been detected in several European countries and may be the most commonly documented bee virus. [168]

3.6.7. Bee Virus X

Nothing is known of the natural history of this virus, except that it was isolated from adult bees in Arkansas. Like other viruses of bees, there are no symptoms associated with natural infections of BVX, but the lifespan of bees infected in the laboratory is significantly reduced. [167] BVX is often found in association with the parasite *Malpighamoeba mellificae* in dead bees in late winter; however, BVX is not dependent on the parasite, and it can infect bees and occurs naturally in the absence of *M. mellificae*. [169]

In nature, viruses and parasites may be transmitted via the same route. They could be eaten during cleanup activities after faecal contamination in the honeycomb. [170] BVX does not induce death quickly: infected bees can live for several weeks, but their longevity is reduced slightly, or significantly more if BVX is associated with other pathogens. This can be crucial when the adult longevity is essential for the survival of bees in the colonies, especially in late winter. [170] BVX is likely to accelerate the death of worker bees infected with it and *M. mellificae*, and because there are not enough young bees in winter to replace these losses, infected colonies may decline and die in early spring. [171]

3.7. Pests

3.7.1. Wax Moth

There are three species of moths considered pests in the colonies of honeybees, but the main cause of damage to a hive is the greater wax moth, *Galleria mellonella L*. Two species can also present problems for beekeepers causing minor damage; they are the lesser wax moth (*Achroia grisella* F.*)* and the Mediterranean flour moth *(Anagasta kuehniella* Zeller*)*. [84]

Although these moths are present in virtually all areas where beekeeping is practiced, they are not considered a threat to the colonies unless some other weakening factor is present, allowing the colony to be totally overrun and destroyed. [172]

Greater Wax Moth, *Galleria mellonella L*

The reproductive cycle of these moths begins when they lay their eggs in the combs. *G. mellonella* eggs are small yellowish-white and hard to see with the naked eye, but most are in groups of 100-150 eggs. They adhere strongly to the structure in which they are deposited. The eggs usually hatch in 8 to 10 days, but this period can be up to 30 days at low temperatures. After hatching, the larvae feed on the wax present in the combs, as well as honey, pollen and other impurities found in the combs. [172]

Lesser Wax Moth, *Achroia grisella*

Lesser wax moths are symbionts of the honeybee and are associated with colonies of honeybees during most of their lives. [173]

The egg incubation time depends on the temperature; it is only 5 days at 30 °C but 22 days at 16 °C. The number of eggs laid by an *A. grisella* female during her short life has been estimated at 250-300. [172]

Mediterranean Flour Moth, *Anagasta kuehniella*

Anagasta kuehniella can become a significant pest because it prefers to oviposit in stored pollen and can cause severe economic losses to beekeepers. Usually, *A. kuehniella* is detected infesting stored pollen. [174]

Paradichlorobenzene and ethylene dibromide have been used to control these insects. Other control measures include treatment with carbon dioxide, heat, or cold.

The same methods of control are used for the three insects. The damage caused by these insects is quite similar, and it is necessary to identify the insects to ensure that they are causing the problem. [172]

3.7.2. Termites and Ants

In some regions, termites can damage parts of wooden hives, thus not directly affect honeybees. Although ants are occasionally found in hives, not all species cause problems. Species of ants that invade colonies to steal honey include *Formica rufa, Formica blood, Formica fusca, Lasius niger;* ants that steal pollen include *Crematogaster jherinil. Camponotus herculeanu* subsp. *pennsylvanicus* attack wood from the hives. [175] In general, the majority of ant species are not harmful, though they sometimes turn around inside the hive in search of food. [172]

3.7.3. Small Hive Beetle (Aethina tumida)

A. tumida (SHB) Murray (Coleoptera: *Nitidulidae*) is considered the most serious pest to recently affect the European honeybee in the United States. [176]

SHB was identified for the first time in the U.S. in the port city of Charleston, South Carolina, in the summer of 1996. [177] Subsequently, SHB was confirmed in the states of Florida, Georgia (1998), and North Carolina. Adult beetles have been identified in Minnesota, and Iowa; although SHB are not yet widely established in these states, the annual spring movement of colonies from south to north provides a method for the continued spread of SHB in the northern regions of North America. [178]

This species is a minor pest to beekeepers in Africa, in contrast to its destructive ability observed in Florida. The small hive beetle has had an economic impact in the U.S., through both colony losses and contamination of stored products of the hive. [179]

This beetle usually attacks weak or small colonies, causing minor damage to well-established colonies. Nonetheless, it has been observed to damage strong colonies through infestations that cause weakening. It can damage the colony because it digs within the combs in search of honey and pollen and defecates on the combs, causing fermentation of honey, which often drips off the combs and is observed as a viscous film below of the hive. Honey can be then unfit for human consumption, and there is also evidence that the beetle can feed on the young of honeybees.

For identifying beetles from different regions, a gene variation in the mitochondrial enzyme cytochrome oxidase I (COI) has been used to illustrate the close relationship between the hive beetles collected in the United States and hive beetles collected from South Africa. [178] In fact, two different haplotypes were found in the U.S. (NA1 and NA2), and they are different from most of the haplotypes found in South Africa.

3.7.4. Bee Louse

Coeca Braula Nitzsch (orders Anoplourae and Mallophagae), the "bee louse", is a fly without wings. This insect can be found on drones, workers and queens in the colony and is transported by honeybees (phoretic phase). Adult Braulidae (1.2 to 1.5 mm long) are Diptera and, similar to the other members of the family Braulidae, live as commensals.

The destructive phase of lice is the larva, which makes tunnels in the combs to steal honey and feed. No apparent damage is attributed to adult bee louse; the louse spends its life in the bodies of workers and queens. The bee louse is considered harmless but disrupts the postural queen and can cause death if present in large numbers. The apparent preference of bee lice for the queen is most likely because the queen is the permanent member of the colony and because of the frequency and quality of feeding compared to worker honeybees. [172]

3.7.5. Apimyiasis

Senotainia tricuspis Meigen is a well-known endoparasite of the honeybee and causes apimyiasis, which can sometimes be particularly serious. It is more common in sunnier regions; this parasite is distributed in Mediterranean countries (Spain, Romania, Italy, etc.). It behaves as a parasite only in the larval stage; *Senotainia tricuspis* females attack foraging honeybees and drones, and sometimes bumble bees and solitary honeybees. The female occupies a position on the roof of a hive. From there, the attacked bees are just taking off, and the fly deposits one or two small larvae that pass through the membrane between the head and thorax. The fly returns to position and eventually repeats this behaviour through the course of the daylight hours. *Senotainia tricuspis* can produce 700 to 800 larvae. [180]

After the larva penetrates the chest muscles, it develops toward the second larval stage, in which it feeds on haemolymph while the host survives. This larva is white with black markings, measuring 1.5 mm long and 0.5 mm wide. When the bee host dies (at 2-4 days after parasitisation), the larva feeds on the tissues, devouring the chest muscles and the soft tissues of the chest and head. When it reaches 8-9 mm in size, it leaves the dead body buried for metamorphosis and becomes an adult within 7-12 days. [181], [172]

3.8. Abiotic Factors

In addition to the various problems mentioned above, adults or breeding honeybees can die from temperature changes, hunger, effects of inbreeding and poisoning. The hive is a eusocial colony in which the honeycomb structure serves for the birth and development of new members. In addition to being home to all individuals and giving them protection, it is used as a food reserve and, when the combs are filled, they act as protective coatings and thermal insulation for the hive.

3.8.1. Chilling and Overheating

Honeybee adults and their young may die due to extreme fluctuations in temperature which can cause cooling or overheating of the hive. This situation is regulated by a number of mechanisms that tend to maintain temperature within a range that allows them to survive. [182]

Chilling

Hives maintain a balanced performance at 35 °C; this temperature must be maintained by the colony during the year. When the outside temperature is between 15 and 30 degrees, adult honeybees increase their body temperature by additional movements of the thoracic muscles and by forming clusters (winter ball). Honeybees generate heat and change position, rotating between the centre and edges, to avoid chilling of young that are in the combs and to prevent their death. Sometimes, honeybees stay in the hive without leaving to forage because the low temperature does not allow them to move with skill, and because the food collected does not exceed the extra energy consumption. When the effort to maintain the temperature is insufficient, their movements are clumsy, and they stand motionless, waiting to die. If the temperature is below 14 ºC, activity is not sufficient to keep the temperature, so bees brood and do not leave to find food, subsisting on stored reversals, minimising consumption.[182].

Overheating

In spring and summer, the temperature in the hive is maintained through ventilation by honeybees beating their wings to send a stream of air from the hive entrance inwards, while other honeybees ventilate in reverse, causing a current to flow through the inside of the hive and freshen the ambient air. On hot days and when the bee population is numerous, many of them move to the entrance of the hive and are grouped there to allow ventilation inside the hive

to be performed efficiently. Honeybees produce a circular motion with their wings (forced ventilation) to increase evaporation, lower heat and ventilate the hive.

Temperature increase in the hive increases the evaporation of water that normally occurs when transforming the nectar into honey. Evaporation of the droplets (or fine surface water) takes heat from the surface of the bodies, which are supported by the decreasing temperature. If necessary, honeybees carry water to the colony to increase evaporation, keeping the indoor humidity at appropriate values for the hydration of the larvae and the distribution of the odours generated by queen pheromones. [182]

3.8.2. Plant Poisoning

Of the extant varieties of plants, only a few species are dangerous to honeybees because they contain toxic elements in pollen or nectar that significantly reduce the number of honeybees in the colonies. The most important of these are California buckeye (*Aesculus californica)*, black nightshade (*Solanum nigrum)*, death camas (*Zygadenus venenosus)*, dodder (genus Cuscuta), leatherwood or titi (*Cyrilla racemiflora)*, locoweed (genus Astragalus), mountain laurel (*Kalmia latifolia)*, western false hellebore (*Veratrum californicum)* and yellow jessamine (*Gelsemium sempervirens)*. [183]

The species *Cyrilla racemiflora*, southern summer leatherwood or titi, blooms from May to July. It is considered undesirable because the nectar and pollen are responsible for a condition known as "purple brood" [184] that kills breeding, becoming a purple color. In areas where this plant abounds, beekeepers move their honeybees during its flowering season to areas away from these plants. [185]

3.8.3. Lethality Genetics

In some cases, gene mutations occur that make the individual nonviable, with its death occurring before the individual reaches maturity and reproduces. These genes are called lethal genes. Thus, a dominant lethal allele will never be heritable because the individual who possesses it will never reach maturity and cannot leave offspring. By contrast, recessive lethal genes are masked under conditions of heterozygosity.

Genetic Structure of Bees

Sex determination in bees is dependent on variation in a gene called csd (complementary sex determination locus) of which there are multiple alleles.

[186] MacKensen (1951) [187] was the first to propose that the sex of bees was determined by a locus with multiple alleles. Estimating multiple alleles in different populations may vary from 6 to 18. [188]

Individuals with a single copy or two identical copies of the csd gene (i.e., homozygous for this locus) are male. Drones are normally haploid, generated by parthenogenesis, i.e., from unfertilised eggs and with all the genetic load of the mother, but diploid males can be produced by homozygosity (inbreeding). Those with two different copies (heterozygous) are females, queens and workers. [189]

When bees are inbred, leading to genetic homozygosity, the effects can be observed in decreasing populations. However, for sex alleles in inbreeding conditions, almost half of the eggs are diploid drones. Most of these larvae are eaten within 72 hours after hatching, [190] and the presence of two identical sexual alleles is therefore fatal for larvae. [191]

3.8.4. Pesticides

Although bees are not the target of the agrochemicals used in crop protection, they are widely affected by pesticides. [192] These chemicals lower bee populations in two ways: first by causing their death directly, as many pesticides needed on crops are highly toxic, and because the use of herbicides can reduce the floral supply per unit area for foraging bees.

Pesticide toxicity can occur by ingestion of contaminated nectar or pollen or by contact poisoning (when flying through a cloud of pesticide or by contact with parts of a plant that has been treated with some agrochemical). [192]

The colonies in hives may be directly affected by pesticides, but most often only foraging bees are affected, whether by dying in the field or by subtler physiological impairment. If the foraging bees die, the colony as a whole is affected because the foragers are responsible for maintaining the entry of food into the hive. [193]

There are several levels of intoxication: acute and chronic. A pesticide may kill without directly harming the bee if it has sublethal effects. Sublethal effects can alter the bee's behaviour, nutrition, communication, thermoregulation, learning, or memory, causing the weakening of the immunity of the colony and lowering resistance to pathogens and parasites.

The toxicity of a pesticide is a result of its physicochemical properties, the method of preparation and the inherent ability of bees to address the material internally. Organophosphate and carbamate insecticides act on the nervous system of bees, causing regurgitation problems, distended abdomen,

aggressive behaviour, erratic movements, inability to fly, disorientation, lethargy, paralysis, disease and death. Pyrethroids, in turn, induce erratic movements, inability to fly, and stupefaction, often followed by paralysis and death. Organochlorine compounds act as neuroactive agents on the transmission of nerve impulses, inducing erratic movements, abnormal activity and tremors. [194] Insecticide growth regulators (IGRs) of the benzoylurea type (inhibitors of chitin synthesis) are considered relatively safe for bees. However, high doses of these compounds may also be harmful to adult bees. [195]

Until 1985, the use of pesticides on honeybees was focused on pesticides applied on crops that bees accidentally affected. The recent introduction of pests such as *A.woodi* (1984), *V. destructor* (1987) and *A. tumida* (1997) has resulted in the intentional introduction of pesticides into the hive, has caused the accumulation of residues in residents and products of the hive, and has put more pressure on the colonies because the immune system of honeybees must now respond to toxic compounds found in greater proportion and range in the environment.

CONCLUSION

There are many factors affecting a colony of honeybees, but whether the decline in population in a colony is due to some biotic or abiotic factor, the development of these diseases must be better understood and further investigated to suppress them most effectively.

It is still necessary to learn more about the organisational form and behaviour of a colony, and the physiology of honeybees in response to the presence of stressors and pathogens, as well as the effects that can produce genetically modified species in the environment and to design and enforce regulations that do not allow the spread of "exotic diseases". Although the major disappearance of a number of individuals in the population may be related to a known or unknown pathogen, the more worrying disappearances are those which are based on multiple factors for which the interrelationships are unknown, as it is even more complicated to show how to reverse such problems.

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Chapter 2

THE STATUS OF BEE HEALTH AND COLONY LOSSES IN ARGENTINA

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ABSTRACT

Honey bees are essential components to modern agriculture and economy. However, a continuos increment in colony losses and colony depopulation cases are being reported worldwide. This critical situation has put on the edge the fragil equilibrium between bees and plants, obligating to several scientists to redirect their research lines. Most

researchers agree that there is no single explanation for the extensive colony losses but that interactions between different stresses are likely involved. Argentina is not the exception, several reports of colony losses and colony depopulation cases were informed by beeckepers around the country but still there is no accurate data registered. It is believed that the total number of colonies in Argentina suffered a 30% of reduction in the last years. In this way, this article intends to evaluate the current situation of honey bee health in Argentina. In this review, we evaluated the impact of the main parasites and pathogens affecting honey bee colonies and discussed the role of each over honey bee losses in Argentina. Also, we discuss the classical control forms applied in Argentina to reduce *Varroa* mite populations, going deeper in the problems related to acaricide resistance phenomena and bee product pollution. Second, we provide data about bee nutrition in Argentina and the main strategies used by beeckepers to manage their colonies. Third, we evaluate the impact of monocultures and pesticides associated to them over colonies survival. Finally we try to estimate the current status of colony losses through the data reported by official institutions devoted to the study of honey bees and also, by the beekeepers. This article aims to serve as a reference of the current status of honey bee health for Argentina and also, to serve as a comparison with future losses as well as providing guidance to future hypothesis-driven research on the causes of colony mortality.

Keywords: *Apis mellifera* – Bee health – Colony losses – Argentina

INTRODUCTION

The sexual reproduction of many crops and most wild plants depends on animal pollination by insects, birds, and bats, among others. Insects play the most important role in this respect (Klein et al., 2007). Among them, solitary and social bees provide the greatest contribution to the development of angiosperms (Brown and Paxton, 2009). This is explained, in part, by the massiveness and homogeneity of modern agriculture, due to which most crops depend on honeybee-mediated pollination (Aizen et al., 2008). Even though global trends seem to indicate that bee population is growing (Ghazoul, 2005a), there is strong evidence that a marked decline in pollinator populations has taken place in different parts of the world (Potts et al., 2010). This critical situation has put on the edge the fragil equilibrium between bees and plants, obligating to several scientists to redirect their research lines.

The European honeybee *Apis mellifera* L. is the most important crop pollinator, with an exhaustively studied biology. Honey bees are essential

components to modern agriculture and economy. Its distribution is wide, spanning from Europe all the way to Africa and Asia (Smith, 1991). Currently it can also be found in America, owing to colonies transfer by beekeepers for production purposes (Delaplane and Mayer, 2000). The relevance of *A. mellifera* for humanity lies in its being responsible for pollinating 77% of the food resources that sustain human population worldwide (Buchmann and Nabhan, 1996). However, as social individuals, colonies exert a double attraction for the pests and pathogens affecting them: on the one hand, colonies represent a high density of potential hosts, and, on the other, a large assembly of individuals with similar genetic characteristics (Schmid-Hempel, 1995). Since 1998, individual beekeepers have been reporting unusual weakening and mortality in colonies, particularly in France, Belgium, Switzerland, Germany, the United Kingdom, the Netherlands, Italy and Spain. Mortality has been extremely high when activity is resumed at the end of winter and beginning of spring. Moreover, since 2006 disastrous colony losses have been reported in Europe and North America (Le Conte et al., 2010). The causes of the losses were not readily apparent and have been attributed to overwintering mortalities and to a new phenomenon called Colony Collapse Disorder (CCD). Most scientists agree that there is no single explanation for the extensive colony losses but that interactions between different stresses are likely involved. There are undoubtedly various causes for recent colony losses. However, CCD and wintering mortalities have been cited as the most frequent reasons. While it is well established that the ectoparasitic mite *Varroa destructor* is a major contributor following its arrival in Europe and the Americas in the 1970s, the drivers of more recent losses remain unclear (Stokstad, 2007). One interesting observation is that at the time of collapse, *Varroa* mite populations were not at levels known to cause economic injury or population decline (vanEngelsdorp et al., 2009a; 2009c). Probably, the best explanation for colony losses observed worldwide is the interaction of different stressors affecting honeybee colonies. In light of the importance of honey bees for pollination and human nutrition, recent major losses of honey bee colonies demand urgent scientific clarification.

Apis mellifera is affected by various living organisms. Among the most virulent ones, virus, bacteria, fungi, beetles and mites should be underlined (Genersch, 2010). Mites parasitizing *A. mellifera* have become a severe concern worldwide, as they threaten the survival of bee colonies and jeopardize commercial beekeeping development (Sammataro et al., 2000; Rosentkrantz et al., 2010). In view of the negative impact that *Acarapis woodi* (Rennie, 1921), *Tropilaelaps clareae* (Delfino & Baker, 1961), *Varroa*

jacobsoni (Oudemans, 1904) and *Varroa destructor* (Anderson & Trueman, 2000) exert on bee colonies, these mites species have attracted the attention of the scientific community. Among the above mentioned, *V. destructor* causes the most devastating effects on European bee colonies worldwide (Rosenkranz et al., 2010). The ectoparasitic mite *V. destructor* impairs both brood and adult bees causing a non-uniform disease pattern called varroosis or parasitic mite syndrome and including a specific form of brood damage termed "snotty brood" (Shimanuki et al., 1994). The indirect impact produced by the mite on bee larvae development and their survival (as a result of pathogens transmission to its host), is equal or more important than the direct effects produced by it through their alimentation episodes on adult or young bees. This virulence effects against bees are explained by the mite's ability to transmit different pathogens (Genersch, 2010). Virus transmission by *Varroa* mites was stated as one of the main threats for *Apis mellifera* colonies (Martin et al., 2012). Since *Varroa* mites have become in a new parasite for european honey bees, viruses are able to replicate themselves inside mite (without parasite's mortality) and consequently, infect their final host with higher loads of virus (Gisder, 2009).

Nosema ceranae is another emergent parasite for european honeybees and togheter with *N. apis* constitute the etiological agent of the nosemosis, a disease affecting the *A. mellifera* intestinal epithelium, causing serious damages to colonies (Higes et al., 2008). Originally described in *Apis cerana* (Fries et al., 1996), the spread of *N. cerana* from *A. cerana* to *A. mellifera* probably occurred within the last decade (Klee et al., 2007). Currently, the presence of *N. ceranae* is more common than that of *N. apis* in European honeybees (Chen & Huang, 2010) and this parasite was associated with massive colony losses (Higes et al., 2008). Fries et al. (2013) summarized the main aspects of the illnesses produced by this pathogen, providing data about the severe problems produced at the level of both colony and individual honey bee. In adittion, mite infestation could contribute to *Nosema* development (Mariani et al., 2012). Other studies suggested that recent losses of colonies observed in Europe and in USA may be caused by synergist effects between *N. ceranae* and *V. destructor* (Anderson and East, 2008; Cox-Foster et al., 2007). This interesting behavior could be the result of the action of stressors generated by *Varroa* that affect the peritrophic membrane of the bee, a physical barrier against microsporidia infections.

American Foulbrood (AFB) is one of the most severe bacterial diseases that affects larvae of honeybee *A. mellifera*, causing a decrease of bee population and honey production. The causative agent is *Paenibacillus larvae*,

a gram positive and sporeforming bacterium that is distributed worldwide (Genersch et al., 2006). The disease is usually not recognized until signs of infection are detected in hives during routine hive management procedures. In some cases, the disease may not be recognized until considerable damage has been done. It is characterized by the typical 'foul' putrescence emanating from hivescontaining infected brood and represents a serious, worldwide problem for apiculture (Ellis & Munn, 2005). However compared with other honey bee pest such as *Varroa destructor*, American Foulbrood generate lower risks hor honey bee survival. The cultural practices applied by beeckepers during last years have mantained under control this bacterial disease around the world and currently, only small "hot-spots" of this pathogen emerge among apiaries.

Bee nutrition is a key factor explaining colony losses. Adequate nutrition is a honey bee colony's basis for growth and development. Since long time ago, it was reported the nutricional requieriments for honey bee growth and development (De Groot, 1953; Haydak, 1970). The ussefull data provided by these researchers led to the development and formulation of special diets that support colony development (summarized in Brodschneider and Crailsheim, 2010). In adittion, in *A. mellifera* colonies nutritional deficiencies that affect the immune response can accelerate the spread of disease among nest mates, increasing pathogen levels and reducing adult longevity and survival (Mayack & Naug, 2009). What began as a nutritional deficiency could quickly develop into colony loss due to an infectious disease. By this pathway, nutrition is a key factor in resistance to pathogens (Rowley & Powell, 2007). A recent study on *A. mellifera* argues that poor nutrition depresses the immune system and consequently could drive colony loss (Van Engelsdorp et al., 2008b). In the same way, Allaux et al. (2010) found that bees fed with diets rich in protein modify both individual and social immune competencies. These authors suggest a link between nutrition and immunity in bees, underlining the fundamental role of the availability of resources for pollinators' health. Proteins, carbohydrates, minerals, lipids, and vitamins are supplied primarily by nectar, pollen and water. When these resources are depleted, bees must use proteins and lipids from their own tissues to produce larval food and survive for a short period of time. Therefore, stronger colonies could enhance survival, moreover when poor nutrition is explicitly identified as a probable contributing factor in recent colony losses (vanEngelsdorp et al., 2009b). Because landscapes have become increasingly characterized by agriculturally intensive monocultures, and since honey bee pollination services often occur within a human-defined ecosystem, bees nutritional needs may not be provided for properly (Naug, 2009). In agree with Brodschneider and

Crailsheim (2010), the question arises if and how bees should be provided with supplemental food when nutritional deficits occur.

Also, one potencial factor explaining colony losses is the spraying of pesticides on agricultural fields. To date, many studies have examined the adverse effects of neonicotinoids and other pesticides on honey bees (Oldroyd, 2007; Maini et al., 2010). The sub-lethal effects of neonicotinoids include impaired learning behavior, short and long term memory loss, reduced fecundity, altered foraging behavior, and motor activity of the bees. For example, a reduction in homing flights in the honey bee *A. mellifera* after a sublethal dose of neonicotinoid insecticides was reported by Matsumoto (2013). Taking into account that the proportions of agricultural crops that depend on honey bees are increasing because of their versatility, low cost, and the ease with which they are moved and managed, special attention should be focussed in how these crops are currently managed. For example, most honey bee losses from 1966–1979 in USA were attributable to organochlorine, carbamate, organophosphorus, and pyrethroid pesticide exposure (NAS, 2007). This data provide interesting aevidence about the negative effects that pesticedes exerts on bee colonies. Johnson et al. (2010) discussed the role that pesticides and their residues in hive products may play in colony collapse disorder and other colony problems. Although no single pesticide has been shown to cause colony collapse disorder, the additive and synergistic effects of multiple pesticide exposures may contribute to declining honey bee health.

Today, is widely accepted that the massive colony losses observed in the last years are produced by the synergistic effects that parasites/pathogens, pesticides and colony nutrition exerts on bees. However, still is hard to explain or to predict how bee populations will be affected at global scalle by these stressors. The Argentinean Republic is a wider territory. This region is large and highly diverse, with beekeeping being practiced over a wide range of climates (from tropical to temperates ones) and altitudes (from sea level to around 2000 m altitude), by very different beekeepers (who have from 15 colonies each in Mesoamerica up to 15 000 in northern Mexico or the Pampas region of Argentina) (Vandame & Palacio, 2010). Hence it is hard to draw a general picture that can take into account all of this diversity. Also, it is difficult to present consice information because there is scarce information published.

This article is intended to serve as a reference of the current status of honey bee health for Argentina and also, to serve as a baseline information for future scientists that decide develop their researchs in honeybee health and conservation.

THE MAIN CURRENT BIOLOGICAL HAZARDS FOR HONEY BEES IN ARGENTINA

American Foulbrood (AFB)

Probably, AFB was the first sanitary challenge to overcome by beekeepers (toghether with Varroosis) in Argentina. AFB was a real problem for argentinean apiaries during the 90s. During this decade, the bacterial disease has caused many colony losses in Argentina mainly as a consequence of the lack of chemical tools to control this pathogen and also to the scarce information related to the cultural practices or biotechnical methods that should be performed to maintain controlled this disease on apiaries.

AFB was early detected in 1989 in Argentina and was hypothesized that its entrance to the country was through alive material imported from USA (Alippi, 1992). It has been reported that AFB came to spread in most of the provinces of greater importance in beekeeping production, with incidences as high as 30% in some geographic areas of Argentina (Marcangeli et al., 2005). There is no official data published, but it is believed that at least, a 30-45 % of the colonies established in Argentina were lost as a result of the negative effects produced by the bacterial disease against bee colonies during those years (Eguaras, Personal Communication). Hence, beekeepers started to apply antibiotics, such as tetracycline hydrochloride as AFB preventive and curative treatments. However their extensive use have led to the aparition of antibiotic resistant isolates of *P. larvae* (Alippi, 1996). The concern for problems arising from microbial resistance is growing and the outlook for the future use of antimicrobial drugs is still uncertain in Argentina. So, our research group has been working during last years in the search of new alternative treatments against *P. larvae*, to be combined with biotechnical methods. These new molecules comprises essential oils, and/their components and vegetal extracts (Fuselli et al., 2006; 2007; 2009; Gende et al., 2008a; 2008b; 2009a; 2009b).

These days AFB is less of a problem than it used to be, due to biotechnical practices (Gende et al., 2009a). In adittion, genetic selection of local bee ecotypes with high hygienic behavior was started some years ago. Importance was given to disease tolerance, resulting in a bee strain that is now mainly resistant to AFB (Palacio et al., 2000). Also, interesting advances in AFB monitoring in honey bee colonies from Argentinean apiaries was reached (Fernandez et al., 2010, Gende et al., 2011). The aims of these studies were to establish a relationship between the number of spores per bee and the extent of

disease development in the colony. Also it was established a minimum number of spores (threshold) from which the clinical symptoms of AFB start to appear in the colonies. These methodological tools combined with "friendly" environmental molecules and selection of local ecotypes of bees resistant to AFB have put light on the control of the bacterial disease to finally reach a controlled status of the illness.

Nosemosis

Nosema apis and *N. ceranae* constitute the etiological agent of the nosemosis. These microparasites are Microsporidia, a group of fungi highly specialized, adapted to parasitism (Sina et al., 2005). Nosemosis is a disease affecting the *A. mellifera* intestinal epithelium, causing serious damages to colonies (Fries et al., 2013).

N. apis was early detected in Argentina in the middle 50s (Cornejo & Rossi, 1975). Although during the first years of this illness the nosemosis induced by *N. apis* has affected bee production, the effects of this parasite over *A. mellifera* colonies were never reported as "lethals"(Cornejo et al., 1970). Currently, surveys of *Nosema* spp. distribution in Argentina were performed. As in other countries, nosemosis is strongly affected by the environmental conditions in Argentina, so a constante survey of the disease has to be conducted to decide the control actions (Sarlo et al., 2005). The results obtained revealed a higher prevalences of *N. ceranae* infecting productive apiaries (Plischuk et al., 2008; Medici et al., 2012). Moreover, only in specific sites of Buenos Aires province co-infections of *N. apis* and *N. ceranae* were detected. In none of these apiares, single infections by *N. apis* were detected (Plischuk, 2011).

Nowadays, the situation of Nosemosis in Argentina is very different to that described by Cornejo and Rossi time ago. More recently, Sarlo (2011) conducted an extensive aproach of the critical impact of *Nosema* spp. in Argentina and through his study, a clear picture of the negative impact of *N. ceranae* against argentinean beeckeeping was reported. Sarlo (Personal Communication) informed that losses per year caused by the disease in the Argentinean Pampas reached values near to 50 %. Nevertheless, the virulence of *N. ceranae* in Argentine vary greatly through the argentinean territory and is hard to predict. Other studies have also monitored the sporulation dynamics of *N. ceranae* (Sarlo et al. 2005; Tiranti et al., 2011; Mariani et al., 2012; Plischuk, 2011). As it was reported in Table 1, the prevalence and abundance

of the microsporidium varies significantly and authors concluded that these parameter are strongly influenced by the evironmental conditions.

Nosemosis control in Argentina is mainly based in fumagiline aplications. Depending on the geographical site, it is recommended the administration of one or two fumagillin applications (fall or fall and spring) (Sarlo et al., 2011a). Porrini (2012) explained the meaning of the two strategies: (a) The autumn cure, is aimed to keep the colony alive during the cold periods and avoid accumulation of spores in the inner colony environment; (b) The spring cure is done to maintain healthy adults individuals, these individuals will take care the new brood population and consequently, expand the colony. As a final result, following this strategy a vigorous hive at the beginning of the next season will be obtained. However, *Nosema* control is not widespread among beekeepers due to:

1) each zone has an own flowering curve;
2) in many cases, colonies are transported by beekeepers when they practice transhumance and
3) the cost of fumagiline.

Research efforts to find effective and noncontaminant compounds against *N. ceranae* infections have been undertaken in Argentina using different substances, such as essential oils and/or their components, vegetal extracts, as well as bacterial metabolites. Porrini et al. (2011a) fed ad libitum newly emerged bees with enriched syrups after individual infection of the microsporidium. Diets consisted of ethanolic extracts obtained from *Artemisia absinthium*, *Allium sativum*, *Laurus nobilis*, and *Ilex*. However ten percent concentrations showed high toxicity on infected bees. In another study, Porrini et al. (2011b) have reported that spores exposed to direct contact with a particular surfactin revealed a significant infectivity reduction when inoculated on bees. This surfactin, administered ad libitum from the individuals' emergence, led to a significant reduction in parasitosis development when bees were infected with untreated spores 7 days postemergence. Recently, Maggi et al. (2013) have conducted different experiments to evaluate the effects of these bacterial metabolites on bees: In vitro administration revealed no toxic effects against bees. Colonies fed with the lactic acids incremented their beehive population and also the amount of fat bodies per bee. Finally, the organic acids reduced the intensity of the pathogen after the second application of treatment as well as enhanced the fumagillin efficiency. These studies constitute the first reports of antiparasitic activity of "new" molecules against the microsporidian *N. ceranae* and postulate natural substances as an alternative for antiparasitic treatment in Argentina.

Table 1. Mean prevalences and abundances of *N. ceranae* in Argentina

Site	Date of sampling	# of colonies sampled	*Nosema* Prevalence (%)	*Nosema* Abundance (spores/bee, mean values)	Study
Mendoza Province	2003-2004	160	Not provided	< 30.000	Funes (2004)
Southeast of Buenos Aires province	2004-2005	6000	Not provided	260.045 (May) 570.000 (June) 3.720.273 (July) 968.911 (August) 705.287 (September) 1.490.000 (October) 1.005.510(November) 106.231 (December)	Sarlo et al. (2005)
Córdoba Province	2006	172	72.5	68.389 (mean value for three months of sampling: August-September-October)	Tiranti et al. (2011)
Entre Ríos Province	2008-2009	250	Not provided	227.777 (June) 300.683 (July) 1.253.666 (August) 492.000 (September) 210.333 (October) 324.066 (November) 112.000 (December)	Mariani et al. (2012)

Site	Date of sampling	# of colonies sampled	*Nosema* Prevalence (%)	*Nosema* Abundance (spores/bee, mean values)	Study
				182.893 (January) 133.583 (February) 188.333 (March)	
Argentinean Pampas	2006-2009	1309	12.09 (season 2006-2007) 5.23 (season 2007-2008) 1.93 (season 2008-2009)	19.600.000 (season 2006-2007) 17.100.000 (season 2007-2008) 16.500.000 (season 2008-2009)	Plischuk (2011)

Varroosis

Like in most of the countries, *Varroa destructor* is the main biological hazard for honey bees in Argentina, specially in most temperate regions of the country. Despite that this parasite was identified in 1976 (Montiel & Piola, 1976), still exist serious problems related to colony losses in argentinean territory as a consequence of the devastating effects produced by the varroosis (Maggi, 2010). Time ago, when *Varroa* has bee recently introduced, the disease was controlled only with one cure per year using mainly, synthetic acaricides. Today, there are few areas in which parasite populations are kept under control with only once yearly treatment (Eguaras & Ruffinengo, 2006).

Varroa Reproduction and Virulence in Argentina

Argentinean honey bees are infested by the Korean haplotype of *V. destructor*, the most virulent haplotype of the parasite (Maggi et al., 2012a). Varroosis is a significant problem in temperate regions of the country due an unbalance interaction in the host-parasite interaction. In this way, colonies die if chemical treatments are not well performed. Only in tropical and subtropical regions of Argentina, *V. destructor* appears to be less virulent than in temperate regions (Eguaras & Ruffinengo, 2006). This situation is mainly explained by the ecotype of bee: africanized bees are commonly found in the northern regions of Argentine. Although some beekeepers from these regions have reported that their colonies are mantained alive without treatment applications against *Varroa* mites, most of them recognize that their honey production is affected over time by the disease. Besides that local stocks of bees were described as tolerant against *V. destructor* (Eguaras et al., 1995), the obtention of a genetic strain of tolerant bees against the mite is far away from the hands of beekeepers.

Varroa reproduction considering single mite infestations, was deeply studied in Argentina. The first reports were conducted by Marcangeli et al. (1992; 1995) and by Eguaras et al. (1988; 1993; 1994a; 1994b; 1995). Recently, the reproductive parameters were monitored by Maggi et al. (2010a). If data sets are compared, significant changes in reproductive parameters are visualized over time (Table 2). This suggest that the increased mite virulence observed in the last years, could be based on a reproductive change across time. In this sense, in last years it could be observed an increment in the reproductive rate and rate of increase. More studies should be conducted to test this hipothesis.

Table 2. Reproductive parameters of *Varroa destructor* considering single mite infestations in Argentina

Season	Number of parasitized cells	Prevalence (%)	Non-reproductive cells (%)	Rate of increase	Reproductive Rate	Study
Autumn	181	20.13	44.19	Not provided	Not provided	Marcangeli et al. (1995)
Spring	107	10.66	28.03	Not provided		
Autumn Winter Spring Summer	705 1353 416 216	13.73 25 7.15 3.67	Not provided	1.17 (pooled data for all seasons)	1.9 (pooled data for all seasons)	Eguaras et al. (1995)
Autumn Winter Spring Summer	Not provided	Not provided	28.1 18.2 35 18.6	1.04 1.17 0.97 1.18	2.48 3.41 2.59 3.42	Eguaras (1993)
Not provided	4510	16.01	Not provided	2.05	2.92	Marcángeli (2004)
Autumn	1159	40.87	26	0.96	Not provided	Maggi et al. (2010a)

Other topics also should be conducted related to *Varroa* biology. Maggi et al. (2009a), reported the presence of three main morphotypes of *Varroa destructor*. To date there is no enough biological knowledge to explain this morphological variability. Nevertheless different hipothesis were postuladed: (a) the morphotypes are the result of morphometric correlations between coexisting populations of *V. destructor* and *A. mellifera*. Relationships of this nature have been observed for others host–parasite systems (Poulin, 1998; Poulin et al., 2003). Recently, parasitological studies have interpreted these parasite–host interactions in terms of energy flux, showing that parasite biomass is controlled by food resources offered by the host, which metabolic rate and the size of the host (George-Nascimento et al. 2004). Within *A. mellifera* bee populations, morphological differences have been found (Buco et al., 1987; Radloff & Hepburn, 2000; Kandemir et al., 2000). The bee morphotypes could offer different energetic resources to maintain the parasite morphotypes. In this sense, future researches would analyze if a particular morphotype of *V. destructor* is related to a particular morphotype of *A. mellifera*. (b) The morphotypes observed for *V. destructor* are the expression of the interaction between genotypes of both, parasite and host ("Extended Phenotype Theory", Lambrechts et al. 2005). The potential importance of this concept was demonstrated by a hypothetical coevolutive model from parasite–host systems, which consider that epidemiologic features are controlled by the interaction between both components of the system (Restif & Koella, 2003). Several studies have documented the existence of different genotypes in *A. mellifera* in Argentina, reflecting different levels of Africanized bees in the country (De Santis & Cornejo, 1968; Dietz et al., 1985). In this way, the morphotypes observed in this study could represent the expression of the interaction between different genotypes of the parasite and its host. (c) The morphotypes found are the result of selective pressures produced by different intensities of acaricide use, which are characteristic of each geographic location. Clarke & McKenzie (1987) reported that insecticide-resistant phenotypes of the Australian sheep blowfly, Lucilia cuprina, showed greater fluctuating asymmetry (random differences between left and right sides of a normally bilaterally symmetric organism) than susceptible phenotypes. In *V. destructor* mites, relationships between mite survival rate and body size were observed under exposition to organic acaricides in laboratory: the parasites of bigger sizes showed higher survival rates than the ones of smaller sizes (Maggi pers. Obs.). Consequently, it is possible that geographic locations where the sampling was conducted, corresponded to different intensities of acaricide use, which selected the morphotypes encountered in *V. destructor*. Finally, the

morphotypes registered in this study would be the result of local and current conditions (bee body-size variability, selective pressures of acaricides, interactions between genotypes of host and parasite, or another hypothesis not discussed here) through small changes in body size, resulting in the life-history strategy best suited to those conditions. This phenotypic plasticity should be an immediate response to environmental changes and not result in changes in genotypic frequencies in the population. According to Stearns (1992) a true evolutionary response, involving a shift in gene frequencies in the population, can occur only when environmental changes persist in time. The parasite system *V. destructor/A. mellifera* is a "young system" because *V. destructor* colonized *A. mellifera* host in 1960 when beekeepers move colonies of A. mellifera from Europe to Asia and the parasite could switch its host (Anderson & Trueman, 2000). In this sense, morphotypes observed in this research could represent an immediate response to environmental changes.

Varroa and the Presence of Virus in Argentina

So far, 22 different viruses have been isolated from honey bees (Runckel et al., 2011) and for Kashmir bee virus, sacbrood virus, acute bee paralysis virus, Israel acute paralysis virus and deformed wing virus, it has been proven that they can be vectored by *V. destructor* (Genersch, 2010). Onlty bee paralysis virus (ABPV), deformed wing virus (DWV) and Israeli acute paralysis virus (IAPV) were implicated in winter losses in Germany and in colony collapse disorder (CCD) in the USA (Cox-Foster et al., 2007; vanEngelsdorp et al., 2007; 2008; 2009a; 2009c; Genersch et al., 2010). Although it has long been known that *V. destructor* is able to induce colony losses especially in combination with virus infections [see references in Genersch (2010)], the mite did not come into focus when inexplicable overwintering losses and seasonal losses were reported from different regions in the world in the recent past. One explanation used to exculpate *V. destructor* was that the mite has been around now for nearly 40 years and has spread around the world during this period, but increased and inexplicable colony losses—like CCD in the USA (vanEngelsdorp et al., 2007; 2008; 2009a)—have been reported only recently. Several studies were conducted to adress this question confirming that *V. destructor* is the main biological hazard for bee colonies around the world, and no other pathogen has a comparable impact on beekeeping [for more datail see (Genersch, 2010)]. Moreover, Martin et al. (2012) has proven how the global honey bee viral landscape is altered by the

mite. In this researchs the author reports that the mite increased the prevalence of a single viral species, DWV, from ~10 to 100% within honey bee populations, which was accompanied by a millionfold increase in viral titer and a massive reduction in DWV diversity, leading to the predominance of a single DWV strain. Currently, is widely accepted by researchers that colony losses is a consequence of multifactorial reasons, being the ectoparasitic mite the main explanation for this dramatic losses.

In Argentina the information about bee viruses is scarce and fragmented. Typical symptoms of deformed wing disease are vestigial and crumpled wings, bloated abdomens, paralysis, and a severely shortened adult life span for emerging worker and drone (Genersch, 2010). Marcangeli et al., (1992) reported deformed bees emerging from cells not parasitized by *Varroa* mites in the province of Buenos Aires. Besides not viruses identification were performed in this study, the symptoms described by Marcangeli and co-autohors could be perfectly correlated with the presence of large amounts of DWV as it was showed in other studies [(see references in Lanzi et al. (2006)]. Sacbrood bee virus (SBV) had previously been detected in larvae collected in Buenos Aires Province, in the mid 1990s from one of numerous colonies with symptoms (Message et al., 1996). Nevertheless, during 2010 more data about bee virus was published. Recently, Reynaldi et al. (2010) describe the first molecular report of the presence of chronic bee paralysis virus (CBPV), SBV and ABPV during a screening of 61 apiaries located in the main honey producer province (Buenos Aires) using a RT-PCR assay. In this study, they found a low rate of infection and few cases of co-infection with more than one virus in the studied apiaries when compared with other countries of South America (Antúnez et al. 2006; Teixeira et al. 2008). One year later, the same authors reported the precense of IAPV in samples taken from several Argentine provinces by using a reverse transcription Polymerase Chain Reaction assay. Our data indicate the existence of high frequency of IAPV in asymptomatic hives of Argentina (Reynaldi et al. 2011). Our research group also was determined the presence of DWV in from apiaries located at Buenos Aires province (Argentina) and Colonia Department (Uruguay) (Brasesco et al. 2013). Table 3 details the respective results for each apiary analyzed. The same ones were very interested due that most of the female mites studied were negative for all the virus analyzed.

These fragmented studies suggest the need of further epidemiological studies in order to determine the prevalence of honeybee viruses in Argentine apiaries and its role in the sudden collapse of colonies. Taking into account that most of these viruses are importnat pathogens of honey bee and are the

etiological agents of diseases, it is not surprising that some of them, have now been found in Argentina, considering its worldwide incidence and recent findings in neighboring countries (Antunez et al., 2006; Texeira et al., 2008; Freiberg et al., 2012). As it was stated by Freiber et al. (2012); with global trade and travel occurring at unprecedented rates, the global spread of viruses and other pathogens can occur faster than ever before. It is thus extremely important to monitor the global spread of pathogens, including those that infect honey bees. So, further and more extensive studies will be vital to understanding how the virus spreads, as well as the prevalence and impact of the virus in Argentina.

Table 3. Virus detected from samples colected in Uruguay and Argentina by means of RT-PCR real time

		Virus			
Apiary	**Samples**	**SBV**	**DWV**	**IAPV**	**ABPV**
A (Uruguay)	Adult bee	-	-	-	-
	Adult bee	-	-	-	-
	Pupae	-	-	-	-
	V. destructor (female)	-	-	-	-
	V. destructor (female)	-	-	-	-
B (Argentina)	Adult bee	-	+	-	-
	Adult bee	-	-	-	-
	Adult bee	-	-	-	-
	Adult bee	-	-	-	-
	Adult bee	-	+		
	Adult bee	-	-	-	-
	Adult bee	-	+	-	-
	Adult bee	-	-	-	-
	Adult bee	-	-	+	-
	Adult bee	-	-	-	-
	Pupae	+	-	-	-
	Pupae	+	-	-	-
	Pupae	-	-	-	-
	V. destructor (female)	-	+	-	-
	V. destructor (female)	-	-	-	-
	V. destructor (female)	-	-	-	-

Brasesco et al. 2013.

The Critical Situation of Varroa Control in Argentina: Control Failures, Resistance Episodes and Acaricide Residues

Probably, the main colony losses reported in the last years by beekeepers in Argentina, should be attributed to *Varroa* control failures on field more than biological phenomena such as CCD episodes. At present, high infestation levels of *V. destructor* are being found in bee colonies after treatment with coumaphos or amitraz.

Historically, synthetic acaricides have been the chemicals of choice for *V. destructor* control in Argentina. Since its detection in 1976, synthetic acaricides such as fluvalinate, coumaphos, flumethrin and amitraz were deliberately applied to restrict the population gorwth of *Varroa* mites (Eguaras & Ruffinengo, 2006). Although different commercial combinations of these acaricides are available in Argentina, most beekeepers prefer to use their own homemade formulations. This controversial situation is explained by a complete lack of reliance by beekeepers to the commercial formulations available for *Varroa* control in our country. Since 2008, our research group is studying the resistance phenomena in Argentina (Maggi et al., 2008). From a total of 43 cases surveyed, only a 40% of them were positive for resistance phenomena. Where no resistance phenomena were detected, other explanations such as commercial adulterated acaricides or wrong treatment applications performed by beekeepers, were detected (Maggi, 2010).

The widespread use of the synthetic acaricides throughout these years, combined with a lack of response by official institutions and also, with a lack of information available, placed a strong selective pressure on mite populations, so resistant mite populations to amitraz and coumaphos have emerged in our country (Maggi et al., 2009b; 2010c; 2012b). Fernández and García (1997) reported low efficacy of fluvalinate in field experiments. However, laboratory experiments were not performed at that time to corroborate the supposed resistance phenomena detected on field. Moreover, dose-response curve have been estimated in mite populations for fluvalinate and susceptible mite populations were detected in Argentina (Maggi et al. 2008). Until now, no cases of cross resistance between acaricide or multiple resistance were detected in Argentina. As some beekeepers use more than one acaricide in a unique control treatment, special attention should be paid on this topic in future studies.

In addition to resistance phenomena and control failures (even when *Varroa* mite populations still are susceptible), the beekeeping Argentinean industry is suffering dramatic negative effects due wax adulteration and contamination (Castro et al., 2010; Medici et al., 2011). In a survey performed during 2010 and 2011, gas chromatography studies revealed that 62.5 % of wax samples were contamined with coumaphos and 37.5 % with fluvalinate (Medici et al., 2011). More worrying was the fact that 87% of virgin commercial wax samples, were contaminated with coumaphos (Medici et al., 2011). Also, Medici et al. (2011) correlated the presence of acaricides in beeswax and how they affects the survival of breeding bees. In this study three types of recycled beeswax foundation containing paraffin wax in different proportions (0%, 20% and 40%) were used. The authors have reported that survival rate of bee bred was higher when using beeswax adulterated with paraffin: Recycled beeswax without added paraffin wax (0%) had high levels of coumaphos and fluvalinate contamination, and when paraffin wax was added in different percentages (20%, 40%) the concentration of these components was lower. The presence of acaricides in beeswax adversely affected brood survival: when the pesticide concentration decreased, an improvement in the survival rate was found. Larvae developed in beeswax foundation without paraffin wax, exposed at higher concentration of pollutant residues were more vulnerable to the toxic effects of the acaricides. Also, induction of immune related genes in response to synthetic miticides was reported by Garrido et al., (2012). This study suggests that acaricides could strongly impact on immune signaling cascades and cellular immunity.

Example of IPM for Varroa Destructor in Argentina

Integrated Pest Management (IPM) is a pest management system that, in the socioeconomic context of farming systems, the associated environment and the population dynamics of the pest species, utilizes all suitable techniques in as compatible a manner as possible and maintains the pest population levels below those causing economic injury (Dent, 1991). In terms of strategies for pest control, IPM is the most modern concept. Its main objective is to apply the least amount of toxic substances, combined with the implementation of cultural practices, with a view to minimizing risks and hazards for human beings and the environment. IPM is being successfully applied in more than fifty countries, and is focused on prevention and non-chemical treatments. To achieve this goal, researchers include continuous controls and reports about

environmental health, as well as pests recognition and their biology. Finally, with all this information, researchers are able to conduct a comprehensive analysis and implement the most appropriate and safe control strategy.

Even though IPM is not a basic biological pest control system using organic drugs, it is extremely important to include as many organic substances as possible in control tactics. Conceptually this entails a change of mind for beekeepers, and leads to the replacement of the improper use of synthetic pesticides with a complete program involving various substances and strategies to maintain parasite populations below the economic damage threshold. Currently, most beekeepers do not apply IPM to control *Varroa*, but stick to a scheduled treatment instead: the use of one or two pesticides, applied systematically in accordance with a rigid and predefined schedule, carrying out, in many cases, low parasite prevalence (percentage of parasite infestation) treatments and, hence, unnecessary.

According to Eguaras & Ruffinengo (2006), four main points remain the cornerstone for a successful IPM for *V. destructor* populations:

- Tactics to reduce the growth of *V. destructor* populations (biotechnical methods)
- Monitoring and control, if applicable
- Sanitary treatments with toxicologically and environmentally friendly substances
- Search for hosts (bees) tolerant to parasites.

Eguaras & Ruffinengo (2006) destailed an example of an IPM carried out for *V. destructor* in the southeast of Buenos Aires province. In periods subsequent to honey collection, a standard colony can reach a mite population with close to 4000-5000 individuals. This represents the economic damage threshold for the colony. Indeed, this phase is critical for the colony and, therefore, a treatment should be administered to reduce the mite population to tolerable values. Currently daily counts of dead mites per colony are close to 20 mites/day. To avoid colony collapse, a treatment with thymol (1 application of 25 g of thymol in alcohol solution embedded in a spongy matrix) was conducted with an efficacy between 85% and 90%. As a result, the parasite population fell abruptly to around 450/600 mites per colony. This treatment improves the colony condition and ensures the emergence of healthy bees to maintain a desirable colony population. However, getting through the winter and starting the spring in optimal conditions is not enough for the colony. The remaining number of mites in the hive, their continuous reproduction, coupled

with the reinfestation caused by the proximity of wild swarms or neighbor apiaries, can alter the colony development. A second treatment with oxalic acid (4.5 % in sugar solution at 60%, 5 ml per comb) should be initiated during the cold weather when bee queens end egg-laying (usually June, early July). If during these months brood does not develop inside the colony, this single treatment with oxalic acid will suffice to reduce mite population (60/120 per colony) until the following year (March), and no subsequent treatment will be required in spring. After oxalic treatment, mite population monitoring based on natural mite mortality is around 1 or 2 dead mites per hive. This value will increase to reach 20/25 mites per colony after harvest the following year. Conversely, if during winter breeding is significant, the oxalic treatment will have a reduced final efficacy (from 5o to 70 %), and the number of mites per colonies will only be reduced to 285/475 mites/colony. Indeed further monitoring should be implemented and possibly a new treatment with formic acid or a biotechnical method in early spring is required.

Perspectives for Varroosis in Argentina

V. destructor appear to be the main challenge for argentinean beekeepers. Not only based on the biological knowledge that they need to control this parasite, but also based on the difficulties that they have to overcome when decide to treat their colonies. Whatever the strategies performed by beekeepers, still exist too work to do by the official institutions to solve the colateral damages produced by the acaricide treatments. As resistance episodes were the main problem in Argentina from 2007 to date, a new problematic is arising in our country due the negative sublethal effects against honey bee colonies generated by the continuous use of the synthetic acaricides (Maggi et al., 2011b). Currently, our research group is assesing new molecules for *Varroa* control (Damiani et al., 2010; Eguaras et al., 1998; 2001; 2003; 2004; 2005; Maggi et al., 2010b; 2011a).

BEE NUTRITION, COLONY DEVELOPMENT AND BEE INMUNE SYSTEM

An adequate bee nutrition is crucial to ensure the survival of the colony. For example, deficiences in pollen nutrition may be responsible for CCD

(Brodschneider & Crailsheim, 2010). Also Van Engelsdorp et al., (2008) have reported that poor nutrition depresses the immune system of bees and consequently could drive colony loss. The pionner works conducted by Haydak (1970), provide crucial data about the development and formulation of special diets for the colony development.

Argentina present a high variability degree of climate and vegetation. Although there is an evident decrease in vegetation diversity from north to south in the country, still relicts with high diversity are present in some austral locations (Cabrera, 1976). So, it is essential to consider these variations when designing future feeding strategies to be carried out in the apiary: those ones located at geographic zones rich in vegetation will need a lesser pollen complementation than zones with poor flora diversity. Temperate climates from Argentina provide an “extra” challenge for beekeepers in terms of diets and food suplementation (protein and carbohidrates). After summer, colonies must be prepared to overcome winter due an strong reduction in terms of nectar fluxes. An usual strategy is to provide high amount fo syrup 2:1 (sugar and water) in a short period of time with the aim to "block" the colony with syrup reserves, and limit the queen oviposition. This action will ensure an adequate source of carbohidrates to pass the colder months until reach the new spring. In addition, after summer proteins have to be supplied to colonies in some areas were poor pollen diversity and quality is detected.

In last years, there is increasing scientific research directed to bee nutrition in Argentina. Sarlo et al., (2011b) determine if the supply of a protein supplement in corn syrup at different pH values affects Vitellogenin (Vg) expression and protein content in honeybees fat bodies. Vg production is strongly dependent on the availability of proteinaceous food. Vg expression in the first four days of life determins the age to begin foraging and whether it preferentially forages for nectar or pollen and is also part of a regulatoryfeedback loop that enables Vg and Juvenile Hormone to mutually suppress each other. Their results showed that the protein supplement a different pH had the same effects that pollen having a high Vg expression at the first days of lifeand then declining progressively. The same situation was observed when the total proteincontent was quantified. The acidification didn´t produce significant differences. These results suggest that the use of a protein supplement complete in free aminoacids has asimilar effect to the pollen, and it could be used as a substitute when there is a shortage of it.

However, some considerations should be made regarding to bee nutrition and parasites development: e.g.: Fries (1993) showed that good pollen supply reduced infection levels in colonies and Mayack & Naug (2009) demostrate

that nutritional deficiencies can accelerate the spread of disease among nest mates, increasing pathogen levels and reducing adult longevity and survival. However, the results published by Porrini et al., (2011b) showed that when bees are fed on pollen, the parasite *N. ceranae* develops quickly, exhibiting significantly higher intensities than under other treatments. Their data demonstrate a parasite development that depends on host-condition. Another molecules derivated of aminoacids could be used as stimulator of the inmune system of the hoey bees: Nitric oxide (NO) is a highly reactive multifunctional free radical generated during the oxidation of L-arginine to L-citrulline by the enzyme NO synthase (NOS). Numerous reviews have described central roles for NO signaling in host defense mechanisms against infections caused by viruses, bacteria, protozoan, and metazoan parasites. In addition, NO acts as a non-specific cytotoxicmolecule (Rivero, 2006). So far, there is scant information available about NO participation in *A. mellifera* immune defense, and the only report in this regard is the one by Negri et al. (2012a; 2012b; 2013). These studies demostrated that a free radical genetared from arginine could be used in feeding strategies in honey bee colonies to enhance their development and health status. Thus, these results should be considered when designing feeding strategies for bee colonies due nutrition, is a key factor in resistance to pathogens (Rowley & Powell, 2007). Currently, our research group is studying *A. mellifera* cellular-humoral immune responses. (Figure 1).
Microorganisms associated with *A. mellifera* have received special attention as a new option for integrated pest management (IPM). In this example, strains of bacteria and their products have become an interesting field for experimentation as they are commonly isolated from the hive environment and bees'digestive tracts (Audisio & Benitez-Aherendts, 2011). Their potential effects on bees have been evaluated in three different ways. First, bacteria strains have been selected and studied as probiotic supplements for bee consumption, testing for effects on colony development. In these studies the administration of *Lactobacillus* and *Bacillus* strains helped the development of bee colonies by enhancing the brood and also honey yield (Sabate et al., 2012). Second, bacteria (or their metabolites) were tested as alternative control methods for bee parasites. Sabate et al. (2012) have documented a negative impact against *N. ceranae* and *V. destructor* when bacterial administration was applied on beehives in field conditions. Also, Porrini et al. (2010) have reported that particular surfactins can alter spore structure. Third, bacterial strains were studied as activators of immune-competence in bees. Evans & Lopez (2007) have demonstrated activation of antibacterial peptide expression when bees were fed bacteria. These authors

also proposed that nonpathogenic bacteria could be used as a probiotic supply to enhance honey bee humoral immunity. Recently, Maggi et al. (2013) assess the effect of the oral administration of the metabolites produced by *Lactobacillus johnsonii* CRL1647 (mainly organic acids) supplemented in syrup, on: (I) *N. ceranae* sporulation dynamics before and after fumagillin application, and (II) performance of *A. mellifera* colonies. Colonies fed with the lactic acids incremented their beehive population and also theamount of fat bodies per bee. Finally, the organic acids reduced the intensity of the pathogen after the second application of treatment as well as enhanced the fumagillin efficiency. This study provided important information for the development of new control substances against nosemosis and also about new substances to be applied as stimulators of colonies development.

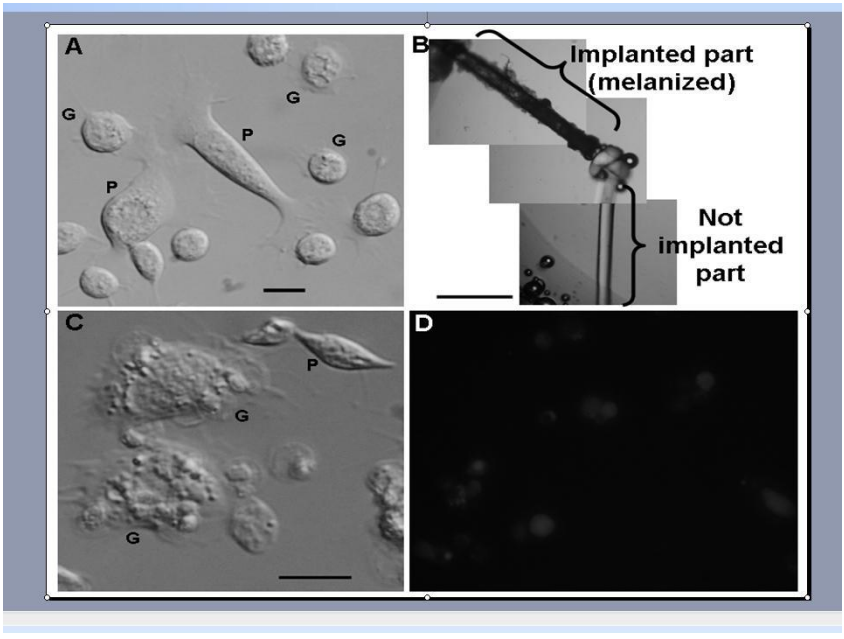

Figure 1. Studying *A. mellifera* cellular-humoral immune responses. A) Fifht instar larvae hemocytes in vitro. Scale bar = 10 micrometres. “P” means plasmatocyte-like hemocyte. “G” means Granulocyte-like hemocyte. B) Nylon thread implant used to trigger an encapsulation response. The brownish colour of the implanted part is the result of the melanization response (humoral). Scale bar = 0.5 millimetres. C) Newly emerged adult (worker) hemocytes in vitro. Scale bar = 10 micrometres. “P” means plasmatocyte-like hemocyte. “G” means Granulocyte-like hemocyte. D) Nitric oxide producing worker hemocytes. Nitric oxide production inside the cells is evidenced by the green fluorescence (here displayed in grey colour).

AGROCHEMICALS AND MONOCULTURES IN ARGENTINA

Another permanent threat for the bees coming from human manipulated environments is the exposure to pesticides used in crop production. Until the mid-20th century, pest insect control in agriculture relied on largely inorganic and botanical insecticides, which were inadequate. Then, the remarkable insecticidal properties of several organochlorines, organophosphates, methylcarbamates, and pyrethroids were discovered, leading to an arsenal of synthetic organics. The effectiveness of these insecticides, however, diminished over time due to the emergence of resistant insect strains with less sensitive molecular targets in their nervous systems. This created a critical need for a new type of neuroactive insecticide with a different yet highly sensitive target. Nicotine in tobacco extract was for centuries the best available agent to prevent sucking insects from damaging crops, although this alkaloid was hazardous to people and not very effective. The search for unusual structures and optimization revealed a new class of potent insecticides, known as neonicotinoids, which are similar to nicotine in their structure and action (Tomizawa & Casida, 2009).

Historically, Argentina has been among the world leaders in the production and/or export of agricultural products. The main reason for this is that it is a relatively sparsely populated country, but richly endowed with natural resources for production agriculture (Carballo et al., 2012). According to declarations from the sector Chambers, Argentina consumed 340 million litres of pesticides and herbicides in the last year; and this quantity is increasing 15% to 20% each year (Avila Vazquez, 2010). In aggrement with data provided by the Food and Agriculture Organization (FAO) (FAOSTAT database), in 2006 Argentina accounted for only 0.59% of the world's population, but for a much higher 2.10% of the world's total land area. Furthermore, Argentina's shares of the world's arable land and the planet's area with permanent meadows and pastures were even higher, at 2.23% and 2.96%, respectively (Lence, 2010). At present, Argentina is the top exporter of soybean oil and soybean meal and the third-largest exporter of soybeans. In addition, is the world's second-largest exporter of corn, sunflower meal, and sunflower oil and must be considered that the relative incidence of crops in exports is even larger (80% of the total) (Carballo et al., 2012). The most important development was the explosive growth of soybeans, which went from being essentially unknown in the early 1970s to becoming by far the most important crop. In 2005-07, more than half of the crop area and about 45% of the value of crops produced corresponded to soybeans. The evolving

patterns in crop output were induced by changes in the relative profitability of the various crops, largely arising from shifts in world supply and demand, the introduction of new technologies, and domestic agricultural policies (Carballo et al., 2012).

There is little data published completely reliable or comparable about pestices and bees in Argentina. Taking into account that crop production is associated with pesticides and colony losses, there is a compelling need for studies in Argentina. Our research group have received several notification by beekeepers about colony losses induced by crop pesticides. But in Argentina, Stadler *et a.l* (2003) placed hives in the center of large fields of flowering sunflowers from seed treated again at a higher rate than the U.S. label, and confirmed that at least 20% of the pollen in the combs was sunflower, and that the colonies had stored sunflower honey. They could not detect residues of imidacloprid in the pollen, and found that the colonies in the treated field actually performed better than in the untreated. They then moved the hives to natural pasture, and checked them again after 7 months, and found no differences between the groups.

Finally, the effect of genetically modified (GM) crops on honey bees is a controversial but little studied topic in Argentina. Although they are permitted in Argentina for GM-soy, there is conflicting evidence about GMO and its impacts on bees (Rose et al., 2007; Duan et al., 2008; Han et al., 2010). Vandame & Palaciod (2010) conclude that small scale agriculture has protected honey bees, due to low exposure to chemical contaminants, which could be a third reason why CCD has not been reported in LA. There are however some changes in the practices that could convert to threats, like the continuous and evidently extension of GM crops in Argentina, or the increasing use of insecticides in all countries. Future studies should be performed to help to the official institutions with their action measures regarding to pesticide use and the advance of GMO crops.

ESTIMATING COLONY LOSSES IN ARGENTINA

Currently in Argentina, the strength of honey bee colonies is decreasing each year, thus requiring more intensive care and attention. Although there is an ongoing discussion whether or not we are really facing a “global pollinator crisis,” there is no question that many solitary and social bees are declining (Ghazoul 2005a, b; Steffan-Dewenter et al., 2005; Allsopp et al., 2008). A recent metastudy revealed that although the global number of managed honey

bee colonies increased by 45% over the last five decades, there is a marked decrease of such colonies in Europe and North America at the same time (Aizen & Harder, 2009). Since crop pollination in North America and Europe is highly and increasingly dependent on honey bees (Aizen et al., 2008), this development is alarming, although not all countries are equally affected. In Europe, for instance, Austria, Germany, Sweden and Switzerland are facing a critical decrease in the number of managed honey bee colonies, while other European countries like Greece, Italy, Portugal, and Spain even report a considerable increase (vanEngelsdorp & Meixner, 2010).

To date for Argentina, there have been no reports of massive colony losses or weakening of colonies due to adult bees losses, such as described by CCD, by official institutions, researchers, for beekeepers professional beekeeping organizations (Vandame & Palacio, 2010). Data related to parasite epidemiodology around the country is scarce. Only especific and local surveys were perfomed by official institutions in Argentina, and a true national sanitary program still is a debt for argentinian beekeepers. Currently, available global data and knowledge on the decline of pollinators are not sufficiently conclusive to demonstrate that there is an argentinean pollinator and related crop production crisis. However, every year serious problems related to colonies survival are being detected by our research group. It is believed that at least, a 30 % of colony losses are being detected every year in Argentina (Dr. Mariano Bacci, SENASA, Personal communication). From our point of view, this percentage value is alarming. The reasons for such colony losses are more than one and probably, are the result of synergistic effects generated by Varroa control failures (including resistance phenomena and pollution of bee products), monocultures (and their pesticides associated) and habitat fragmentation. According to a survey performed by SENASA (see http://www.senasa.gov.ar/Archivos/File/File3824-varroosis- aituacion-actual-argentina.pdf) the main threat for the argentinean bee colonies is *V. destructor*. Table 4 details the main results from a survey performed during 2007 by official institutions of Argentina. Most of the bee samples were taken after of control treatments against *V. destructor*. From the Table 4, it could be observed that a 46% of the samples presented values of mite infestation higher than 3 % even in colonies that had been received sanitary controls against varroosis. This is an alarming result, considering that not always the resistance phenomena are involved in the colonies where samples were collected. Control failures based on incorrect product application by beekeepers, adultered miticides and colateral effects induced by bee product

contamination are being the worst enemy these days for the argentinan beekeeping economy.

Table 4. *V. destructor* survey performed in several locations of Argentina during 2007

Provinces of Argentina	VARROOSIS				
	Number of samples analyzed	Number of positive samples	Infestation (%) on adult bees		
			0.1 - 1	1.1 - 3	> 3
RIO NEGRO	380	217 (57%)	39%	26%	35%
NEUQUEN	138	100 (73%)	37%	20%	43%
SANTA FE	725	489 (68%)	22%	23%	55%
MENDOZA	468	434 (93%)	45%	32%	23%
TUCUMAN	125	109 (87%)	16%	39%	45%
SALTA	60	51 (85%)	11%	10%	80%
TOTAL	1896	1400 (74%)	28%	25%	46%

Data extracted from http://www.senasa.gov.ar/Archivos/File/File3824-varroosis-aituacion-actual-argentina.pdf.

CONCLUSION

V. destructor was, is and will be, the main biological hazard for honey bee colonies in Argentina. Although the nosemosis and the american foulbrood caused colony losses years ago, these diseases are now well managed by means of biotechnical practices performed by beekeepers. It seems that a significant change in the reproductive biology of *Varroa* populations has occurred through time. Taking into account that only the Korean haplotype was detected in Argentina, this change would be partially responsible for the higher virulence against bee colonies detected across the country. The knowledge about viruses affecting honey bees is still scarce. Too much work should be conducted to answering which are the main factors determining the epidemiology of the viruses detected in Argentina.

The nutrition of bee colonies is currently, one of the main research field with more development these days. Our research group is working hard in the development of new molecules that enhance the inmune system of honey bees and consequently, improve the colony development even when foreign stressors are driving their negative impacts against it. In this sense, human

activities and their environmental impacts (such as pesticides) are being detrimental to *A. mellifera* colonies in Argentina. Pollination is not just a free service but one that requires investment and stewardship to protect and sustain it. There should be a renewed focus on the study, conservation and even management of native pollinating species to complement the managed colony tradition. Economic assessments of agricultural productivity should include the costs of sustaining wild and managed pollinator populations.

Taking into account that no CCD cases were detected in Argentina, it seems that the main enemy for the argentinean beekeeping is the human being. Too much effort should be done if our main goal is to reduce the high colony mortality that is being reported by the official institutions these days. A deeper study about the true impact induced by pesticides against honey bees still is a debt for us. From our point of view, and IPM program devoted to *V. destructor* together with the support of the government authorities is the logical way to ensure the bee health in Argentina.

Several field experiments have shown that IPM developed for mite control can be used to maintain *Varroa destructor* populations below colonies damage levels. Nonetheless, more time should be spent and periodic visits be made to the apiary in order to implement the program. The IPM developed for beekeeping is a suitable tool even in areas where bee brood is present throughout the year. Taking this into account, a single treatment cannot successfully control parasites and, therefore, should be periodically repeated. Biotechnical methods and low bee toxicity products that do not add foreign elements to hive products should be adopted. Forms of control such as those developed in this chapter can assist in the reduction of the longstanding use of synthetic acaricides, reducing wax and honey residues as well as the resistance phenomena detected in *V. destructor* populations. It has been demonstrated that the greater the effectiveness and success of arthropod pest management, the greater the likelihood of the pest developing resistance to that management tactics. This is particularly true when the goal of pest management is to reduce pest population and maintain it at a very low level. The probability of resistance evolution will be lower when goals emphasize damage and disease prevention, which sometimes can be accomplished without harming most of the pest population. In apiaries where Varroa mites are still susceptible, rotation between resistant and non-resistant acaricides (still effective in the control of the parasite) should prolong the effectiveness and prevent the occurrence of chemically resistant mites. In apiaries where *Varroa* mites are resistant, the introduction of Integrated Resistance Management (IRM) programs is essential. This includes selecting bees tolerant to the mite

concerned, monitoring mite population, implementing nonchemical control methods and rotating pesticides, whether natural or synthesized. Finally, achieving an integrated management of *Varroa destructor* entails a change of mind for beekeepers and the active participation of all those players involved in the industry. Producers should understand that the only way in which parasites can be managed is by implementing health strategies that address parasites and hosts biology, both of which are essential to attain an effective acaricide treatment. National and private scientific bodies should engage with the current issues faced by beekeeping and promote scientific activities aimed at discovering and developing new tools that could be implemented in an IPM. Finally, it is imperative that the political players responsible for national bee health ensure the linkage between the scientific and productive sectors so that the tools developed are implemented and honey bee preservation is ensured.

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Chapter 3

SITUATIONAL CHOICES AMONG ALTERNATIVE VISUAL STIMULI IN HONEYBEES AND PAPER WASPS WHEN FORAGING

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ABSTRACT

Since 1960s the honeybee has been serving as a traditional model in studying intelligence/cognitive abilities in insects. In this chapter, new examples of cognitive tasks including planning are described. Paper wasps were shown to be capable of contextual learning as well. Common methods of free flying insect training in field experiments were used.

The decision depended on additional condition. The insects were presented with two visually different (by color or by shape) feeders placed on a horizontal table. The additional condition was background color. There were two randomly changed backgrounds. The rewarding depended on what background the stimuli were placed. At the background N1 the stimulus N1 was rewarded, and vice versa. In modified experiment, the background remained constant, while feeding places were changed randomly. There were two locations at the distance of several meters from each other. The experimental table was randomly positioned at one of these locations. At the location N1 the stimulus N1 was rewarded, and vice versa. Flying insects learned to check both

locations very easily, and then the learning to make correct choices started. Majority of the individuals studied (but not all) solved the tasks.

The events described are very similar to so called "conditioned switching" well known in vertebrata. Traditionally, this phenomenon is investigated in the frame of "higher *nervous* activity" conception.

As a control, in special experiments, ability to recognize familiar colors at new background and at new place was studied. Supposition that the background color dependent task and the location dependent task are different by their innate predispositions is discussed.

Learning of regularity in alternations of feeding objects across foraging trials. The bees (wasps were not tested) were presented with two feeders, which differed by color. In consecutive bee visits, rewarded colors were altered regularly (N 1 – N 2 – N 1 – N 2 and so on), positions of the feeders being changed randomly. After long training period (up to some days), all experimental bees solved the task. The task may be considered to be a sort of planning: the bee remembered rewarded color in present visit and planed to choose the other color in the next one.

INTRODUCTION

In the beginning of the XX century, Karl von Frisch (Frisch, 1914) provided evidence that the honey bee is able to learn many characteristics of biologically important objects (such as color, odor, time of feeding and others). About 50 years later great convergent similarity between insect and vertebrata behavior was shown; this conclusion of primary importance was made independently in different scientific schools (Lobashov, 1955; Mazokhin-Porshnyakov, 1981; Bitterman, 1988). It was based mainly on the results of the investigation bee conditioning, orientation and memory. In parallel, new era of pattern recognition in honey bees commenced when people began to ask whether bees could learn abstract features, or properties, of patterns (Shrinivasan, 2010). Thus, not only simple conditioning, but also cognitive/intelligent capacities of insects occurred to be mainly as good as these of vertebrata too. The first who demonstrated bee abilities to categorize visual patterns (it was called "generalization of visual stimuli") was Mazokhin-Porshnyakov (1969; the first publication was in1968 in Russian, in the References see reviews in English: Kartsev, 1996; Mazokhin-Porshnyakov, Kartsev, 2000). Ants and paper wasps were shown to be able to generalize visual stimuli as well.

At present, research of insect cognition is very popular and fast developing branch of biology where honey bee remains a principal model

object (for review see Shrinivasan, 2010). In addition to behavioral events, nervous mechanisms underlying the learning process are successfully investigating (for review see Menzel, 2012). Following characters of cognition may be picked up: 1) categorization of the searched objects (visual patterns), 2) contextual learning (see below), 3) ability to use learned information in a novel situation, 4) making nonstandard decisions rejecting inborn rules of behavior (Kartsev, 1996), 5) planning future behavior, 6) also ant language may be considered to be a sort of cognitive operation, because it is very flexible and presumably is based on individual learning (Reznikova, 2007), in contrary to bee language, which is rather inborn. One of the most fascinating bee features is their ability to master abstract inter-relationships, such as "sameness" and "difference" (Giurfa et al., 2001). In "delayed symbolic match-to-sample" task, the experimental bee had to use the identity of a sample stimulus (which can be A or B) to choose between two other comparison stimuli (C and D) that were presented simultaneously at some distance from the first stimulus (Zhang et al., 1999; 2005). Details of the training procedure play very important role in cognitive experiments. Thus experiments, in which the distance (and therefore the delay) between the sample stimulus and the comparison stimuli systematically increased reveal that the sample stimulus can be held in working memory for a duration of up to 5 seconds (Zhang et al., 2005). Who could predict that the best learning will occur at the distance of 375 cm, while at the distance of 475 cm the bee choices will come to a random level?

It should be taken into consideration as well that each behavioral act depends on innate predispositions facilitating or inhibiting learning. So, two logically similar tasks may be quite different by their inborn base. We will touch on this problem briefly comparing our two experiments where bees chose a rewarded stimulus in the pair in dependence on the background color or in dependence on the feeding place. In real life, cooperation between inborn rules of behavior and learning (including cognition) must be an adaptation that increases evolutionary success of a given species. It sounds like an idle speculation, because evolutionary experiment is not possible. However comparative approach can cure this problem indirectly. One of the goals of this chapter was to provide evidence, that paper wasps are capable of contextual learning too. The next step could be to find species specificity, but this goal would require much more statistical data to analyze all the variety of individual ways of behavior in each experimental task. Individual behavioral differences are a constitutional feature of cognition, because it is individual

high-order learning – in contrary to complex forms of mainly inborn behavior, such as bee dances.

The experiments described in the first part of this chapter deal with contextual learning.

Two kinds of contexts can be mentioned.

The first one is concerned with different behavioral activities (motivations). For example, bees and wasps did not use directly the individual foraging experience when searching for nest entrance and vice versa (Kartsev, 1996). Then the results were confirmed for bumble bees. But: contextual isolation turned out not to be absolute; some indirect interrelationships between learning in contexts in question were found (Collborn et al., 1999; Fauria et al., 2002; Worden et al., 2005; Kartsev et al., 2005). The contextual isolation seems to be not concerned with cognition.

The second kind of context deals with one behavioral activity, in our case – with food searching in different situations/contexts. We call it situational choices (not to confuse with the first kind of context). A situation is an obligatory additional condition indicating the reward. In fact, our experimental tasks are nothing but "if..., then..." tasks well known in vertebrata research. Some examples of solving such tasks for bees are described. In one of the series of fruitful maze experiments, left and right turns were signaled by different colors placed on the back wall of each box of the maze where a turn had to be made: for example, if the blue mark was presented, then "turn left" and if the yellow mark was presented, then "turn right." The results revealed that bees learn this task well, just as well as the task of simply following a mark (Zhang et al., 1996). Another condition indicating the reward may be location of the discriminative stimuli. Collett and Kelber (1988) were perhaps the first who demonstrated bee ability to learn tasks in a context-specific way. They used square constellation of four cylinders (two blue cylinders on one side, and two yellow cylinders on the other) in two locations 33m apart. At the first location a feeder was placed between yellow cylinders and at the second location between blue ones.

Solving these tasks is rather an evidence of cognitive abilities (if only contextual isolation do not facilitate learning – see below). Thus the tasks of such kind may be called "situational choices"/"context-specific"/ "if ..., then..." tasks.

In the experiments described in the first part of the chapter, the tasks in question were studied in another way than those described earlier. Instead of landmarks in a maze or at a foraging area, two flat figures (rewarded and unrewarded) different by color or by shape were presented at a small

horizontal training table. Additional condition was color of the background or location of the training table (the distance between the locations was several meters). For example, at the yellow background stimulus A was rewarded and B was unrewarded, while at blue one – vice versa. To estimate the results obtained it is very important to know whether insects do constantly recognize discriminative stimuli in different experimental situations, or there exists a contextual isolation. Some preliminary attempts to investigate this problem are described below.

The experiment described in the second part of the chapter is concerned to some extent with a problem of insect ability to plan behavior. There are no obvious facts confirming this ability. As Menzel (2012) notes in the bee cognition review, it is unknown whether it is justified to assume that honeybees are capable of planning their actions according to "what", "where" and "when" categories of memory. In our experiment, a bee had to learn regularity of alteration of rewarded stimulus in the pair across consecutive bee visits (foraging trials). The simplest rhythm was presented: stimulus A – stimulus B – stimulus A – stimulus B and so on. So a bee had to behave in such a way as if she thought: "if now I am eating from the orange feeder, next time I will choose pinkish one". It may be considered to be a sort of planning. Bees are known to be able to remember tasks within a temporal context, as if they could "plan" their activities in time and space (Zhang et al., 2006), but it is rather an evidence of contextual isolation, but not "mental" planning.

There are publications (see papers reviewed in Mazokhin-Porshnyakov, Kartsev, 2000) where bee ability to learn regularity in alternations of feeding places was proved. However, when we tried to repeat this experiment, only one individual of about 40 solved the task. Certainly, it was due to some details of training procedure, which remained under our control; or the experimental bees were genetically different. It is a general problem of many complicated experiments, which usually is not discussed. Anyway, we used colors as alternative stimuli. As far as we can judge, color alteration is easier than place alteration, and results of the color experiment are better reproducible.

MATERIALS AND METHODS

In field experiments, individually marked free flying honey bees *Apis mellifera* L. and paper wasps *Paravesula* (=*Paravespa*) *vulgaris* L. and *P. germanica* F. were trained to discriminate between visual stimuli. The results

for both wasp species are considered together, because no behavioral species-specific features were revealed in the experiments. In case of the bees, Carpathian race characters prevailed; racial origin is well known to influence some characteristics of bee behavior. (In our other experiments (not described here) some differences depending on the race were found in the artificial flower visiting tasks.)

Common methods of insect training introduced by Karl von Frisch were used. They are as suitable for wasps as for bees. First of all an insect was attracted to a small table (25 cm x 25 cm, covered by glass, with a sweet lure placed on it) and then was marked by acryl dye. Later the marked experimental bee recruited new nest mates, one of which was chosen to participate in the next experiment. In each trial, only one insect took part in the training; all extra individuals were caged for the period of the experiment. Usually each individual was used in one experiment (with a few exceptions; for example, bees 2-08 and 3-08 took part in two experiments in different days – table 1 and table 9). The training table was positioned at a distance of 10 – 15 m from the hive; the locations of natural wasp nests were unknown.

When the insect began to perform regular foraging trips to the feeder on the table, a pair of visually different figures was presented. A small cup with 50% sugar water was placed at the center of the rewarded figure. The unrewarded one contained concentrated sodium chloride solution (which is a stimulus of aversive conditioning and facilitates learning – Prof. Z. Reznikova, unpublished data, personal communication). The chemicals used are known to be undistinguishable by bees and wasps from a distance. However, in the beginning of every new season odor control with unmarked cups was performed to exclude any odors absorbed by the sugar or by the salt.

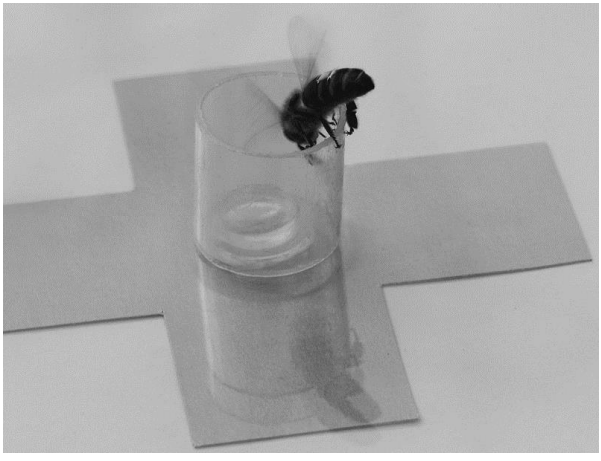
Each cup was placed inside a small cylindrical screen 1.5 cm high. Therefore, to taste the searched object, the insect had to penetrate into the cylinder. This was to ensure that the insect had really chosen the given figure. After every visit the figures were arranged randomly in 4 positions. The positions 1 and 2 were situated along the direction “further – nearer” in reference to the watcher (for the bees, it was approximately north – south direction), the positions 3 – 4 – along “left – right” (west – east) direction; if the position of the rewarded figure was 1, the other figure was placed in the opposite position 2 and so on. As a randomizer for the choice of a placement of the rewarded figure a rolling die (or a coin) was used. After several insect visits the feeders were replaced with new ones to prevent the insects from orienting by their own odor or by any possible uncontrolled markers (defects)

on the screen or on the cup. Thus, the discrimination between rewarded and unrewarded objects was possible only by visual stimuli.

A choice was considered to be made after landing at the feeder and dropping at least by head into the screen (Figure 1). Only the first choice in each foraging trip was recorded. The choice was "correct" or "incorrect" in dependence on what figure, rewarded or unrewarded, was chosen. The proportion of correct and incorrect choices was analyzed statistically, by chi-square test and by modified Fisher's test – φ-test.

The method described did not prevent access to the reward after a mistake, and random choices with 50% level of mistakes might be considered to be an adaptive, although not optimal, behavioral strategy. However, the insects usually did not adopt this strategy. Certainly, there were individuals that chose randomly, but they learned to inspect feeders very gently to minimize contact with the distasteful salt, and they learned to move to the alternative figure immediately after a mistake. (The phenomenon of unlearning, i.e. preferring the random choice strategy, might be as interesting as that of learning; however, nobody investigated this problem so far.)

Colors and shapes of the figures were used as discriminative stimuli. The colors looked from the human point of view like violet (450 nm), blue (470 nm), green (500 nm), yellow (580 nm), orange (605 nm), pinkish (620 nm) – we call it "pinkish", because it was not just diluted red indistinguishable by bees as a color – and white (Whitman paper).



Author of the photo: V. Kartsev.

Figure 1. Marked bee at the experimental feeder. A choice of the figure was considered to be made if the bee penetrated into the feeder at least by her head.

The colors were different by their intensity as well; however, in the frame of our experimental paradigm it is not important which specific visual character did the insect use when discriminating the figures. In the color experiments, the figures were circles 5 cm in diameter.

In the shape experiments, the two figures were a circle (3.5 cm or 6 cm in diameter) and a cross as if it was constructed of five squares 2.5 cm x 2.5 cm. These figures were black and they were placed at white background.

RESULTS

1. Learning in Reference to Additional Condition Presented at the Time of Decision Making

1.1. Choices between the Alternative Colors in Dependence on the Background Color

In consecutive insect visits, one of the two different training tables was presented to the individually marked bee or wasp, the table location remaining constant. The swap of the tables was random.

1.1.1. *Bees*

For the bees, the two tables were yellow and white respectively. Two circles – pink and orange were placed on each table. At the yellow table pink was rewarded while orange was unrewarded; at the white table – vice versa. Thus in decision making the additional/principal condition was the color of the background.

The experiment was carried out during three years with three different bee colonies. A variant with a blue table instead of the white one was carried out as well, different watchers being involved in the process. It did not change the paradigm of the experiment, but made sure that bee cognitive abilities in question did not depend on specific details of the training procedure.

Learning abilities. The results are summarized in tables 1 and 2. The column "P_3" indicates whether proportion of choices of the colors at the first training table – pink(+) : orange(-) – differs significantly from that at the second one – pink(-) : orange(+). In other words, it indicates whether bees are able to categorize two contrary tasks in reference to an additional condition/context.

Table 1. Proportions of correct and incorrect choices of the colored circles at the training tables differing by the color (yellow and white) for bees

Label of the bee	The yellow table: pink (+), orange (-)			The white table: orange (+), pink (-)			P_3
	+	–	P_1	+	–	P_2	
2-08*	23	7	<0,01	27	3	<0,001	<0,001
3-08	24	1	<0,001	20	5	<0,01	<0,001
4-08	20	5	<0,01	21	4	<0,001	<0,001
5-08	20	5	<0,01	23	2	<0,001	<0,001
6-08	22	8	<0,05	27	3	<0,001	<0,001

Signs (+) and (-) symbolize rewarded and unrewarded figures as well as correct and incorrect choices (choices of rewarded and unrewarded figure).

P is the statistical significance of differences:

P_1 is the probability that differences between empirical and theoretical proportion (+) : (-) are due to chance alone at the first (yellow) table; null hypothesis ratio is 1 : 1;

P_2 – the same at the second table;

P_3 is the probability that differences between empirical proportions of choices of the colors (pink : orange) at the first table and at the second one are due to chance alone.

* The labels of the bees are taken from the original experimental protocol. The extension after hyphen indicates the year (-08 is 2008).

For example, for the bee with label 2-08 (table 1) proportion pink(+): orange(-) at the yellow table is 23 : 7, while proportion pink(-) : orange(+) at the white table is 3 : 23. It is easy to calculate that differences are highly significant (P_3<0,001). Fourteen individuals out of 21 studied solved the task during about 80 visits in the first day and 4 individuals did it in the second day of the training period. In sum, 18 individuals among 21 demonstrated significant learning. Thus the main conclusion is that bees can solve tasks of such kind.

Memory and learning speed. The experiment was not aimed to study these questions; however there are some points worth mentioning. Seven individuals were studied during two days and one individual during three days. Four of them did not learn significantly in the first day: P_3>0,05 – ns (not significant) in the table, but improved their results in the second day (P_3 <0,001 or 0,01). It gives evidence that these individuals can learn, but do it slower than the other ones. It means as well that in the second day the bee remembered the task, in spite of pause between the training periods in the first and in the second days including the night and some hours of bee flight activity (during which the bee

had been waiting in the hive with periodical inspections of the site of the experiment, from which the tables were temporarily removed).

Table 2. Proportions of correct and incorrect choices of the colored circles at the training tables differing by the color (yellow and blue) for bees

Label of the bee	The yellow table: pink (+), orange (-)			The blue table: orange (+), pink (-)			P_3
	+	–	P_1	+	–	P_2	
22-09	31	12	<0,01	23	4	<0,001	<0,001
1-11, 22.06.11	18	21	ns	25	16	ns	ns
1-11, 23.06.11	35	14	<0,01	32	18	<0,05	<0,001
2-11, 24.06.11	20	15	ns	27	11	<0,01	<0,05
2-11, 25.06.11	22	18^{a}	ns	31	9^{b}	<0,001	<0,01
3-11, 26.06.11	25	10	<0,05	21	14	ns	ns
3-11, 27.06.11	26	4	<0,001	26	7	<0,001	<0,001
5-11	32	6^{A}	<0,001	23	19^{B}	ns	<0,001
7-11, 06.07.11	16	19	ns	18	16	ns	ns
8-11, 09.07.11	19	11	ns	14	17	ns	ns
8-11, 10.07.11	27	14	<0,05	26	13	<0,05	<0,01
8-11, 11.07.11	37	12	<0,001	27	14	<0,05	<0,001
9-11	26	14	<0,05	32	8	<0,001	<0,001
10-11, 15.07.11	21	8	<0,05	18	14	ns	<0,05
10-11, 16.07.11	24	8	<0,01	23	5	<0,001	<0,001
12-11, 18.07.11	23	7^{a}	<0,01	14	17^{b}	ns	ns
12-11, 19.07.11	20	4	<0,001	23	6	<0,001	<0,001
13-11	31	10	<0,001	27	12	<0,001	<0,001
14-11, 26.07.11	18	12	ns	17	13	ns	ns
14-11, 27.07.11	16	17	ns	23	15	ns	ns
17-11	25	7	<0,01	21	7	<0,01	<0,001
18-11	29	12	<0,01	32	7	<0,001	<0,001
19-11, 08.08.11	14	13	ns	17	16	ns	ns
19-11, 09.08.11	18	18	ns	19	15	ns	ns
20-11	23	6	<0,01	29	3	<0,001	<0,001

ns – not significant.

Different superscript letters in a line indicate cases when proportions (+) : (-) differ statistically significantly at the two training tables (small letters correspond to P<0,05, capital letters correspond to P<0,01).

The rest of the legend is the same as in table 1.

Obviously the information regarding the task must be kept in the long-term memory. Comparison of total proportions (+) : (-) in the consecutive days for the bees that demonstrated learning revealed the following facts: one case of the negligent small changing (+2% for the bee 2-11), three cases of about 15% increase which was not statistically significant because of insufficient amount of data (the bees 1-11, 3-11 and 10-11) and two cases of statistically significant increase (17 % for the bee 8-11 between the first an the third days, $P<0,05$; and 20% between two days for the bee 12-11, $P<0,05$). The last cases confirm the supposition regarding the bee memory.

Some individuals learned at one of the two training tables better than at the other one. In three cases, the differences are significant; these cases are marked by different superscript letters in corresponding lines in table 2. For example, the bee 2-11 during two days made correct choices more often at the blue training table than at the white one. It is significant in the second day of watching (see table 2) and by two days in total ($P<0,05$). Most likely, these events are concerned with interior bee tendency to color constancy and with their desire to follow the preferred color; if this tendency was not overcome, incorrect choices would prevail on one of the tables. Thus proportion (+) : (-) in ratio 1: 1 might be due not to random choices, but to uncompleted learning, if at the other table correct choices prevail. In the beginning of the experiment, some individuals tried to follow one color. However, this strategy was realized during short term only, and the statistics does not work here, individual variations being great.

No learning. The experimental task seems to be rather easy for bees. However, in case of three individuals the results are not significant. It may occur because of fundamentally different reasons: insufficiency of the training period and lack of the statistical data or random choice strategy adopted by an individual deliberately. The last reason seems to be the case for two individuals (bees 14-11 and 19-11, table 2). They did choose the colors randomly not only in the first, but also in the second day of the training (nevertheless they obtained the reward after mistakes – see above). And analysis of behavior dynamics did not reveal any increase of the portion of correct choices. So these individuals did not learn to discriminate the colors, but they learned to inspect the feeders briefly and to remove to the alternative feeder, if the first choice was incorrect.

1.1.2. *Wasps*

The honeybee is not unique in its ability to choose alternative colors in dependence on the background colors. The wasps were studied in the same

experiment as the bees; the only difference was that more contrast colors were used, because wasp color vision is not as perfect as the bee one. The results are presented in table 3. Four individuals out of 6 solved the task during the training period in 50 – 80 visits (as it is judged by P_3 value – see above). No obvious differences between bees and wasps were found.

Table 3. Proportions of correct and incorrect choices of the colored circles at the training tables differing by color (yellow and green) for wasps

Label of the wasp	The yellow table: violent (+), orange (-)			The green table: orange (+), violent (-)			
	+	–	P_1	+	–	P_2	P_3
6-08	18	12	ns	16	14	ns	ns
7-08	26	10	<0,01	29	8	<0,001	<0,001
8-08	20	5	<0,01	20	5	<0,01	<0,001
12-08	21	9	<0,05	25	5	<0,001	<0,001
16-08	16	20	ns	19	15	ns	ns
17-08	25	15	ns	29	12	<0,01	<0,01

The legend is the same as in the table 1.

Table 4. Proportions of correct and incorrect choices between the cross and the circle at the training tables differing by color (yellow and white) for bees

Label of the bee	The yellow table: cross (+), circle (-)			The white table: circle (+), cross (-)			
	+	–	P_1	+	–	P_2	P_3
9-08*	15	11	ns	15	11	ns	ns
10-08**	31	17	<0,05	30	19	ns	<0,05
11-08*	29	11	<0,01	27	13	<0,05	<0,001
12-08**	27	13	<0,05	31	9	<0,001	<0,001
13-08**	28	12	<0,05	32	8	<0,001	<0,001

The legend is the same as in table 1.

* The circle 6 cm in diameter was used.

** The circle 3,5 cm in diameter was used.

1.2. Choices between the Alternative Figures Differing by the Shape in Dependence on the Background Color (Experiment with Bees)

The experiment was like the ones described above with exception of discriminative visual stimuli; instead of colored circles the figures differing by the shape and size were used. It was aimed at getting additional evidence that bee cognitive behavior studied does not depend on any sensory peculiarities of these insects, but on their brain. The results are presented in table 4. Four individuals out of 5 solved the task during 80 visits (as it is judged by P_3 value – see above). No fundamental differences between the color and the shape experiments were found.

Surprisingly, a few attempts to repeat this experiment with wasps were not successful. Two wasps studied did not discriminate between the cross and the circle even in the simple – without any additional condition – experiment. It is an "inconvenient" fact, because cross and circle used to be model figures in many experiments, and wasps are able to discriminate between them. (Usually such results are not published; however they must be as interesting as examples of successful learning, if we want to know the whole organization of cognition.) The bad learning was observed in the year 2008, in which the wasp density in Moscow suburb was very low and it remained so during the next few years.

1.3. Choices between the Alternative Colors in Dependence on the Feeding Location

The idea of this experiment was in general like that in the experiments described above. However an additional condition of decision making was not the color of the training table, but the location of it. There were two locations of the table at the distance of several meters from each other, which were swapped randomly. The rewarding and unrewarding of the visual stimuli discriminated depended on the location of the training table.

Before the experiment per se (colored circles discrimination) had started, the insect was trained to visit both possible feeding locations. When the watcher and the training table changed location, the insect was confused at first, but then founded the table at the new location and learned to check both feeding places. Flying insects do it easy. Usually an insect returned to the last visited place, however if the table was relocated, flied to the other location at once.

Table 5. Proportions of correct and incorrect choices of the colored circles at the training tables at different locations for bees

Label of the bee	The first location: pink (+), orange (-)			The second location: orange (+), pink (-)			
	+	–	P_1	+	–	P_2	P_3
1 m distance between the locations							
2-07, 22.06.07	15	13	ns	19	9	ns	ns
2-07, 23.06.07	35	30	ns	39	26	ns	ns
2-07, 24.06.07	36	17	<0,01	41	14	<0,001	<0,001
4-07, 25.06.07	22	23[a]	ns	32	13[b]	<0,01	ns
4-07, 26.06.07	31	29[A]	ns	50	10[B]	<0,001	<0,001
4-07, 27.06.07	4	11[A]	ns	13	2[B]	ns	ns
4-07, 28.06.07	14	17[a]	ns	23	7[b]	<0,01	ns
12-07	17	13	ns	20	10	ns	ns
7-08	10	15[a]	ns	18	7[b]	<0,05	ns
8 m distance between the locations							
5-07	15	15[A]	ns	26	4[B]	<0,001	<0,01
6-07*	18	12[a]	ns	26	4[b]	<0,001	<0,001

The legend is the same as in tables 1 and 2.

* For this bee, blue and yellow colors were used instead of pink and orange.

1.3.1. *Bees*

Learning abilities. The results are presented in table 5. Two individuals out of 6 solved the task during 60 visits in the first day and 2 others did it in the second or in the third day of the training period (as it is judged by P_3 value – see above). In sum, 4 individuals out of 6 demonstrated significant learning. Thus bees are able to solve the task making decision in dependence on the location of the discriminative objects by the distance of several (1 –8) meters.

The distance between the different locations seems to be important. None of the three individuals which took part in the 1 m distance variant solved the task during the first day of the training (about 60 visits), but both individuals in the 8 m distance variant did it. However the available data are not sufficient for well-grounded conclusion.

Memory and speed of learning. In general, the same behavioral peculiarities were found as the ones in the experiments with the altered backgrounds (see above). For example, for the bee 2-07 correct choice portion increased significantly ($P<0,05$) in the third training day in comparison with the second day. Therefore, she remembered her previous day experience. In

contrary, for the bee 4-07 no pair of days differed significantly, and no long-term learning dynamics was revealed. Nevertheless, this bee solved the task statistically significantly during the second day of the training (because of sufficient statistical data – tab. 5) and during the four training days in sum (total proportion pink(+) : orange(-) at the first location is 71 : 80, proportion pink(-) : orange(+) at the second location is 32 : 118; the differences are statistically significant: $P<0,001$). Thus there are individual peculiarities in bees, the speed of learning varies.

For 4 out of 6 individuals, proportions (+) : (-) differed significantly at different locations (marked by subscript letters in the corresponding lines in table 5). As it was supposed above, it is concerned with interior bee tendency to follow one preferred color. This tendency is more evident in the beginning of the training. For example, the bee 4-07 on 27.06 did not fly properly and performed only 30 visits preferring orange at both locations; at the first location it lead to prevailing of incorrect choices. If the training period was longer, this problem would be overcome thanks to learning.

1.3.2. *Wasps*

The results are presented in table 6. Three out of 4 individuals studied solved the task by the highest level of statistical significance during 70 – 80 visits. Thus in all tasks studied, the cognition in wasps seems to be at least as good as that in bees.

Contrary to the bees, in the case of wasps the portion of the correct choices was independent of the location of the training table (table 6) or of the color of the background (table 3). It might be supposed that the tendency to follow a single color is stronger for bees than for wasps.

Table 6. Proportions of correct and incorrect choices of the colored circles at the training tables at the distance of 8 m from each other for wasps

Label of the wasp	The first location: violet (+), orange (-)			The second location: orange (+), violet (-)			
	+	–	P_1	+	–	P_2	P_3
9-08	32	1	<0,001	28	9	<0,01	<0,001
10-08	20	15	ns	19	16	ns	ns
13-08	29	6	<0,001	26	9	<0,01	<0,001
14-08	22	8	<0,05	24	7	<0,01	<0,001

The legend is the same as in tables 1 and 2.

1.4. Recognition of Familiar Colors at a New Place and at a New Background

The results described above are considered to be an evidence of the individual comprehension of logical laws of the tasks by the bees and wasps. However, it may be supposed that insects do not recognize familiar stimuli at new places and at new backgrounds. In this case, they solve the logical task as two separate simple conditioning tasks by means of hypothetical mechanism of "automatic" contextual isolation, which might be called as "unconditioned switching" (in contrary to "conditioned switching"). In the absence of interaction between the two simple tasks, it would facilitate solving the whole task consisting of two contrary tasks. For example, an experience to choose orange and to reject pink at one place would not make difficulties for learning to choose pink and to reject orange at the other place. A priori this supposition seems to be rather unrealistic. Let us now describe the results of two other experiments aimed to answer the question: do bees recognize familiar colors at new locations and at new backgrounds?

Learning procedure. The experiments were like these described above, but without any additional condition. A pair of pink and orange circles was used as discriminative stimuli; they were presented at the blue or at the yellow background. The rewarded color, the background and the locations of the training tables remained constant during 30 visit training period.

Table 7. Proportions of correct and incorrect choices of colored circles before and after the relocation of the training table

Label of the bee	First 10 visits		Last10 visits before relocation		First10 visits after relocation	
	+	-	+	-	+	-
11-09	10	0	10	0	9	1
15-09	4	6	8	2	10	0
17-09, 30.06.09	5	5	10	0	4	4
17-09, 01.07.09	-	-	10	0	7	3
18-09	9	1	9	1	9	1
24-09	10	0	10	0	10	0
25-09	-	-	10	0	10	0
∑	38 (76% of +)	12^{A}	67 (96% of +)	3^{B}	61 (87% of +)	9^{AB}

The legend is the same as in tables 1 and 2.

Dashes in the column "First 10 visits" indicate that the individual had previous experience of discrimination of the experimental colors.

Then the table was relocated at the distance of several meters (5 m – 8 m) or the background was changed. The rewarded color in the pair and order of the background presentation varied for different individuals. Some individuals were studied repeatedly.

1.4.1. The Role of the Location of the Training Table

The results are presented in table 7. In the last 10 visits before the relocation of the training table and in the first 10 visits after the relocation the proportions (+) : (-) remained between 8 : 2 and 10 : 0 in five cases out of seven. Thus, no drastic influence of the location of visual stimuli on their recognition should be supposed. In sum (the last line of the table), the portions of correct choices before and after the change were 96% and 87%. Some decrease did occur, but it was not statistically significant.

The conclusion that bees recognized familiar colors at new place is rather predictable, because in nature they do it when foraging.

1.4.2. The Role of the Color of the Training Table

The results are presented in table 8. One bee (7-9) is not included in the table because she did not demonstrate statistically significant learning during the first 40 visits. This fact is rather unusual, because color discrimination is known to be one of the easiest tasks for bees.

Table 8. Proportions of correct and incorrect choices of colored circles before and after the change of the color of the training table

Label of the bee	First 10 visits		10 visits before table color change		10 visits after table color change	
	+	-	+	-	+	-
4-09	10	0	9	1	8	2
15-09	-	-	8	2	8	2
18-09	7	3	9	1	9	1
24-09, 23.07.09	7	3	9	1	9	1
24-09, 27.07.09	-	-	9	1	8	2
25-09, 29.07.09	6	4	9	1	4	4
25-09, 30.07.09	-	-	10	0	8	2
∑	30 (75% of +)	10^{A}	62 (89% of +)	8^{B}	51(75% of +)	17^{A}

The legend is the same as in tables 1, 2 and 7.

Like it was in the previous experiment, proportions (+) : (-) were remained between 8 : 2 and 10 : 0 irrespective of the change of the color of the background in five cases out of seven. Nevertheless, slight decrease of the correct choice portion was observed usually (for example 10 : 0 reduced to 8 : 2 and so on). In sum, the portion of correct choices decreased from 87% to 75%, the differences being statistically significant (the lower line in the table 8).

Thus at least sometimes, the color of the background influences the recognition of familiar colors of the food objects. However it would be early to conclude, that bees do not recognize learned colors at the new background at all.

Indirect evidence that bees do recognize the colors at new background is a tendency to follow one color at different backgrounds (see above; table 2, the lines with superscript letter markers). One more evidence was obtained in the experiment described below (in which bees learned to change colors regularly). The bee 27-09 successfully solved the task at the blue background (this passage is included in table 9). And then the background was substituted by the yellow one. In the first 10 visits after the change the bee chose the colors correctly all 10 times. None of the 17 individuals studied in the experiment in question did so in the first 10 visits. Total proportions of correct and incorrect choices were 23 : 7 at the blue background and 21 : 0 at the yellow one. Obviously, the bee used her initial experience in the new situation recognizing familiar colors at the new background.

Thus sometimes bees recognize colors irrespective of the background, but sometimes do not. The last fact is surprising. Most likely there are great individual differences in the reaction to background change. Additional experiments are necessary to investigate this problem.

2. Learning in Reference to the Information Obtained during the Previous Act of Feeding

In the pair of pink and orange circles (presented at the blue background), the rewarded color was alternated: pink – orange – pink – orange and so on after each bee visit. Thus the bee had to remember what color she had chosen in her last visit and had to choose the other one in the present visit. In this experiment, there was no cue like background color or training table location indicating the reward in each visit, as it was in the experiments described

above. But the additional condition was the regularity of the rewarded color alteration in consecutive visits.

Table 9. Proportions of correct and incorrect choices of colored circles when the rewarded color was regularly altered in consecutive bee visits

Label of the bee	Proportion of choices		Portion of (+), %	P
	+	-		
2-08	40	15	73	P<0,001
3-08	34	20	63	ns
7-08*	27	23	54	ns
8-08*	44	16	73	P<0,001
9-08*	39	13	75	P<0,001
13-08*	48	8	86	P<0,001
14-08*	39	11	78	P<0,001
1-09, 09.06.09	44	43	51	ns
1-09, 10.06.09	96	74	56	ns
1-09, 11.06.09	113	66	63	P<0,001
3-09, 12.06.09*	57	38	60	P≈0,05
3-09, 13.06.09*	130	86	60	P<0,01
3-09, 14.06.09*	47	22	68	P<0,01
3-09, 14.06.09	52	33	61	P<0,05
3-09, 15.06.09	98	58	63	P<0,001
12-09*	30	29	51	ns
13-09, 23.06.09*	68	32	68	P<0,01
13-09, 23.06.09	22	9	71	P<0,05
13-09, 24.06.09	51	17	75	P<0,01
13-09, 26.06.09	45	26	63	P<0,05
13-09, 27.06.09	103	44	70	P<0,001
13-09, 28.06.09	101	50	67	P<0,001
13-09, 29.06.09	82	44	65	P<0,001
16-09, 30.06.09*	76	43	64	P<0,01
16-09, 01.07.09*	100	60	63	P<0,01
16-09, 02.07.09*	70	41	63	P<0,01
16-09, 03.07.09*	52	37	58	ns
16-09, 07.07.09*	26	24	52	ns
16-09, 08.07.09	65	47	58	ns
16-09, 09.07.09	23	20	53	ns
16-09, 11.07.09	105	35	75	P<0,01
18-09	50	24	68	P<0,01
20-09, 14.07.09*	56	30	65	P<0,01
20-09, 15.07.09*	22	8	73	P<0,05
20-09, 15.07.09	75	29	72	P<0,001

Table 9. (Continued)

Label of the bee	Proportion of choices		Portion of (+), %	P
	+	-		
21-09, 16.07.09*	64	41	61	P<0,05
21-09, 17.07.09*	41	21	66	P<0,05
21-09, 18.07.09*	14	5	74	P<0,05
21-09, 18.07.09	97	46	68	P<0,001
21-09, 19.07.09	104	35	75	P<0,001
26-09, 05.08.09*	62	25	71	P<0,001
27-09*	23	7	77	P<0,05
28-09, 10.08.09*	63	37	63	P<0,01
28-09, 11.08.09*	77	23	77	P<0,001
29-09*	39	21	65	P<0,05
29-09	31	9	75	P<0,001

P is the probability that differences between empirical and theoretical proportion (+) : (-) are due to chance alone; null hypothesis ratio is 1 : 1.

The * indicates the "simple" variant (with two possible positions of the feeders at the training table) and the absence of * indicates the "complicated" variant (with four possible positions of the feeders).

The rest of the legend is the same as in table 1.

There were two variants of the experiment. In the first, simpler one, the positions of the circles at the training table remained constant. So the rewarded feeder could be predicted either by the color alteration or by the alteration of the reward positions. In the second, more complicated variant, the circles were rearranged randomly in one of the four possible positions at the training table (see description of methods above) after each visit. Thus the only way to solve the task was to learn the rhythm of rewarded color alteration. Usually a bee was presented first with the simple, then with the complicated variant. Actually the differences between the variants are not principal, because the main question of the experiment was the question if the honeybee is able to make present decision in dependence on the previous one, or in other words, to plan its next decision during making the present one. It does not matter a lot if bees take into consideration colors of the figures or their positions when solving the problem.

The results are presented in table 9. An individual was considered to solve the task, if correct choices prevailed statistically significantly (P<0,05 at least).

Learning abilities. In the first day of the experiment, the simple variant of the task was solved by 13 individuals out of 15; the training period varied widely – from 30 to more than 100 visits. Two individuals, who did not solve,

performed 50 and 59 visits respectively. The complicated variant of the task was solved by 2 individuals (during 55 and 74 visits respectively) out of 4 ones (the remaining two individuals, who did not solve the task, performed 54 and 87 visits respectively).

Some seven individuals were trained from two to eight days. All of them solved the complicated task. Thus the conclusion is: the honeybee is able to learn the regularity of alteration of two colors. That means that it is able to make present decision in dependence on the previous one.

Speed and dynamics of the learning The behavioral dynamics in consecutive days of the training period was rather sophisticated. For some individuals, the portion of correct choices gradually increased (not statistically significantly), but for the others it did not (for example, the bee 16-09 in table 9).

As usual, there were individual differences between the bees. For example, the bee 27-09 obtained 71% of the correct choices during 30 visits while the bee 12-09 had only 51% during 59 visits; the differences are statistically significant ($P<0,05$).

Comparison between the variants of the experiment. The variant with the two regularly changed positions of the rewarded feeder was supposed to be simpler than that with the four randomly chosen positions. Let us analyze what happened when just after solving the simpler task an individual was presented with the complicated one. The changes in the portion of correct choices were following: -7%, +3%, -1%, -6%, +10%. In all cases, prevalence of the correct choices remained statistically significant and there were no statistically significant differences between the proportions of correct and incorrect choices before and after the change of the variants. It means, it was the rhythm of the color alteration but not of the position alteration that the bee learned in the simpler variant. Thus both variants appeared equally easy for the learned bees. However some differences may be expected in the beginning of the learning.

CONCLUSION

The results obtained give new examples of insect cognitive abilities. The paper wasps were shown to solve the cognitive tasks in question as well as the honey bee. Thus the honey bee is not unique in its ability of contextual learning using symbolic cues indicating the reward. As far as we know, it is the first direct confirmation of this fact.

In general, the results described in the first part of the chapter are in good agreement with a range of modern works (for review see Menzel, 2012; Shrinivasan, 2010). Our experiments with the background as an indicator of the reward are related to the experiments, where bees successfully learned to negotiate mazes by using a symbolic cue; left and right turns were signaled by different colors (Zhang et al., 1996). Our experiments, where the insects had to choose one or the other stimulus from the pair in dependence on the location of the feeding place, are principally similar to these of Collett and Kelber (1988). However, all details of the training procedure were different (such as presentation of discriminative stimuli at vertical or at horizontal surface, kind of this stimuli, distance between the locations of feeding points and between hive, and many others). It is an important conclusion that in the frame of experimental paradigm, bee learning remains invariant to the details of training procedure.

An ability to solve a given "cognitive" task may be due not to a pure individual high-order learning (cognition), but to some inborn reactions facilitating learning. For example, if bees do not recognize familiar colors at different backgrounds, choosing of the colors in reference to the background divides into two simple tasks and deals rather with bee memory, but not with its cognition. It may be supposed that different mechanisms underline bee behavior in the task of color discrimination in reference to the background and in the task of color discrimination in reference to the location of the feeding. There are not sufficient data to make any well-grounded conclusion yet. However, it is the right time to draw attention to this problem. The complexity of the problem increases a lot because of great individual differences among bees. Most likely, in the case of the color recognition at a new background some individuals do it very well while the others perform poorly. Actually, each behavioral task may be characterized and categorized by a variety of individual ways of its solving and by frequencies of these ways.

On the one hand, we consider our results to be new examples of cognitive abilities in insects. On the other hand, "situational choices" are very similar to phenomenon, which might be called "conditioned switching". This phenomenon is well known in vertebrata. Traditionally, it is investigated in the frame of "higher *nervous* activity" conception established by Ivan Pavlov. Is conditioned switching an evidence of cognition? The answer is rather unclear. Maybe, there is phenomenon of "conditioned switching" and phenomenon of "unconditioned/contextual switching/isolation" The first one is concerned with individual learning – cognition, the second one – with some physiological mechanisms. Contextual isolation is sure to exist between

different behavioral activities such as foraging and nest searching (see introduction). However as our results allow supposing, it exists within one behavioral activity (foraging) as well.

In the experiment described in the second part of this chapter, bee learning was studied across the consecutive visits (foraging trials). Bees were shown to be able to alternate regularly feeding objects. Each time the bee had to make its decision in reference to the information obtained in the previous visit. We do not know of other work confirming this capacity. There are examples of insect abilities to learn rhythm of alteration, but within one foraging trial. There is evidence that bees are better at learning mazes that involve regular patterns of turns compared with random mazes (Zhang et al., 2000). Ants can tell each other about turn regularity using their fascinating language (Reznikova, 2007).

The question whether insects (as well as "higher" animals) can plan their behavior is of primary importance. The last experiment touches on this problem. So, after choosing rewarded pink circle during the present visit the bee had to plan to choose the orange one in the next visit. It is a sort of elementary planning. Zhang et al. (2006) showed that bees can learn in temporal context and thus "plan" their activities in time and space using context to determine which action to perform and when. However, the last phenomenon is rather an evidence of contextual isolation, but not of cognition, which is concerned with individual learning of logical laws of the task.

To sum up, the following items should be noted:

1. The honey bee and the paper wasps (*Parsvespla* spp.) are able to choose one of the two alternative feeders, which differ by color in dependence on the background color.
2. Wasp cognitive abilities in the contextual tasks are as high as the bee ones.
3. Honey bees are able to choose one between two alternative feeders different by shape and size (circle and cross) in dependence on the background color.
4. Honey bees and paper wasps are able to choose one of the two alternative feeders, which differ by color in dependence on the two possible locations of the feeders (at distances of several meters).
5. An ability to make decision in reference to additional condition seems to be similar to phenomenon "conditioned switching" well known for vertebrata.

6. Honeybees recognize learned colors at a new place (at the distance of several meters from the initial one), however, at least some individuals, do not do it at a new background (or do it poorly).
7. Honey bees are able to learn regularity of alteration of the rewarded color in the pair (color 1 – color 2 – color 1 – color 2 and so on) across consecutive visits. It may be considered to be a sort of planning.
8. Great individual behavioral differences between insects studied were observed not only in the cognitive tasks, but also in the simplest ones, such as color discrimination by bees.

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Chapter 4

FUEL FOR FORAGING: REGULATION OF THE CROP CONTENT OF FORAGERS UPON DEPARTING THE HIVE

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ABSTRACT

Honeybee foragers carry a small amount of honey when they leave the hive and consume it to produce energy for flight during foraging. In this review, I examine how and why honeybees regulate the amount of honey taken from the nest for foraging. It is estimated that bees are able to fly 1 km using 1 μL of unripe honey. In nectar foragers, the amount of fuel loaded in the hive depends on the distance from the hive to the food source; thus, bees foraging on sites that are further afield carry more fuel. The amount of crop content on departure reduces as the foragers repeatedly visit a food source, suggesting that the informational state of the bees influences the amount of fuel carried. In addition, waggle dancers carry less honey on departing the hive compared with potential recruits leaving the hive after following the dance. Foragers collecting other materials, such as pollen, water or resin, might have different ways of regulating their crop content upon leaving the hive. Examples described here indicate that the amount of honey loaded at departure is under complicated close regulation.

1. INTRODUCTION

Animals usually have energy reserves, such as fat, that they consume to survive a period when food is not available and that provides energy for various activities, including foraging. However, honeybee workers have little energy reserve in their bodies. If deprived of feeding and trophallaxis with nestmates, honeybee workers will die over a relatively short period of time.

The reduced energy reserve is probably an adaptation for flight. In flying birds, various anatomical, physiological and behavioral traits help maintain a low body weight. Honeybees might be able to reduce the energy reserves stored in their bodies because of the constant supply of honey from the hive; as long as they return to the hive, they will be able to access this highly concentrated energy source. Thus, honeybees might have evolved to exploit an energy source in the hive rather than in their own bodies.

It has long been known that foragers receive a small amount of honey from nestmates when they leave the hive to forage (Parker, 1926; Beutler, 1950). This honey is kept in the crop (honey stomach), which is a sac of stretchable membrane located before the mid-gut in the digestive tract (Snodgrass, 1956) (Figure 1). The honey is gradually consumed to generate energy for flight during the foraging trip (Gmeinbauer and Crailsheim, 1993) and, thus, is called ‘fuel’ honey. One might think that workers should carry the exact amount of fuel required for a flight from the hive to the food source, but this is not the case. During foraging, workers always encounter uncertainty. For example, bees might fail to forage because of the presence of competitors or fluctuations in nectar secretion. In such cases, they have to return to the nest or find other nectar sources using fuel carried from the nest. Although some bees search flowers based on information communicated through waggle dances, the rate of foraging success is not high as a result of such recruitment (Seeley, 1983; Biesmeijer and Seeley, 2005). The need for fuel might differ depending on the type of forage. Nectar foragers could fuel themselves for return flights at flowers, whereas bees collecting non-energy-source materials, such as pollen, water and resin, are unable to do so. Recently, it was revealed that there is finely tuned complicated regulation of the amount of honey loaded on departure of the nest (Harano et al., 2013). In this chapter, I review the usage and regulations of honey carried from the nest in honeybee foragers.

Figure 1. The crop of the honeybee, *Apis mellifera*, into which foragers load fuel in the form of honey.

2. Energetic Requirements for Flight

In the European honeybee *Apis mellifera* L., most workers forage in a 6-km radius from the nest (Visscher and Seeley, 1982), although some might fly more than 10 km to forage (e.g., Gary et al., 1978). How much honey is required for bees to fly over these distances? The energetic efficiency of their flight has been investigated by many researchers using free-flight bees, bees attached to a roundabout or bees kept in a flight chamber. Gmeinbauer and Crailsheim (1993) reviewed these studies and conducted own experiments using a roundabout, concluding that workers consume approximately 100 mg/h/g body mass of sugar irrespective of their flight speed. In other words, a single worker consumes 10 mg/h because their body weight is approximately 100 mg. Visscher et al. (1996) determined the flight speed of the bee to be 20 km/h and estimated that bees require 1 mg of sugar to fly 2 km. The concentration of honey that foragers receive from nestmates on leaving the hive is approximately 50% (A. Mitsuhata-Asai, M. Hayashi, K. Harano, and M. Sasaki, unpublished observation). These data provide an estimation that bees can fly approximately 1 km with 1 μL of unripe honey, which is loaded at the nest.

However, honeybees are heterothermic and their metabolic rate changes depending on their body temperature. Their energy requirement could be increased when they find a good food source and their body temperature increases as a result of such a discovery (Balderrama et al., 1992; Moffatt, 2000).

3. FUEL IN NECTAR FORAGING

3.1. Effect of Food-Source Distance

Ruth Beutler (1950, 1951) was the first researcher who experimentally demonstrated that the amount of fuel honey in honeybee foragers is tightly regulated. She hypothesized that bees carry a sufficient amount of honey to provide energy for foraging flight and tested the hypothesis by training bees to feed on syrup feeders placed at different distances (1–2000 m) from the hive. One hour after the bees started to fly between the hive and a feeder, they were caught at the entrance to the hive when they were leaving to go to the feeder and sacrificed to determine the weight and sugar content of the crop. The results showed that bees increased the weight and sugar content of their crop and the mid-gut if they foraged on feeders located further from the hive.

Based on these findings, Beutler (1950) further questioned whether bees inform nestmates of the energy requirements for traveling to a particular foraging site by means of dance communication (von Frisch, 1967). However, this question was not been addressed until recently.

3.2. Do Recruits Use Dance Information to Estimate the Energy Requirement for Foraging Trip?

Honeybee foragers perform waggle dances after returning from a good food source. The typical waggle dance comprises waggle runs, in which foragers move straight while shaking their abdomen, and return runs, in which they return to the starting point of the waggle run; the combination of waggle and return runs is usually repeated many times. The distance and direction to a food source from the nest are expressed by the duration and angle of the waggle run, respectively (von Frisch, 1967) and the information is interpreted by nestmates that follow the dance (followers) (Michelsen et al., 1992; Riley et al., 2005).

A recent study addressed Beutler's question of whether followers carry fuel honey that corresponds to the fuel requirement based on distance information communicated by waggle dances.

In the experiments, bees were allowed to forage freely in the field and their crop content was measured upon leaving the hive after the bees had performed or followed a waggle dance. The distance of each food source was estimated from the duration of the waggle runs in each waggle dance. As a result, a positive correlation was found between crop content and waggle-run duration of dance that the bees either performed or followed (Harano et al., 2013; Figure 2).

These results appear to support the hypothesis that followers use waggle-dance information to estimate their energetic need for a foraging trip. However, further research is needed to exclude the possibility that the positive correlation results from determining the fuel load need based on the memory of the follower.

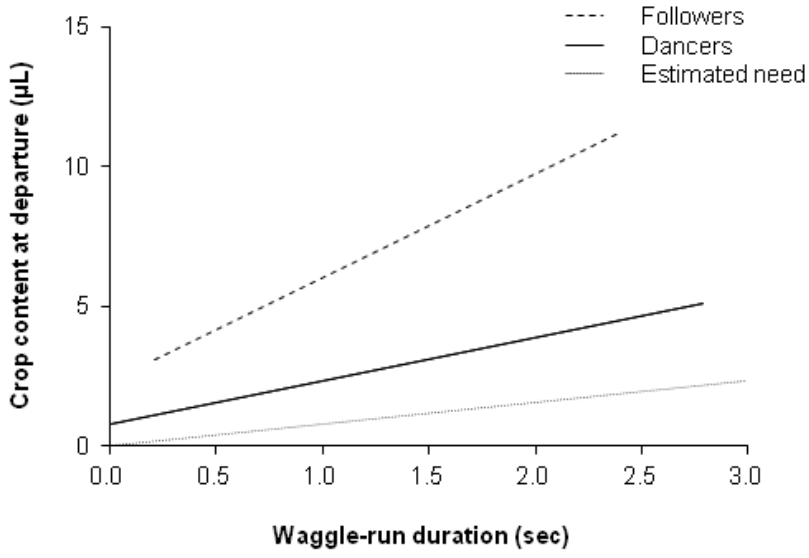


Figure 2. The relation between crop content at departure and waggle-run duration in dancers and followers of *Apis mellifera* [Regression lines based on Harano et al. (2013) are shown]. For a reference, the estimated need for honey (assumed to be 50% sugar) for a one-way trip to a food source is shown [the estimation is obtained based on Sasaki (2001) for the relation between food-source distance and waggle-run duration, and on Gmeinbauer and Crailsheim (1993) for the energetic efficiency].

Given that the previous experience of followers was not controlled for in the above experiment, it was possible that followers had already visited the food source indicated by a dance that they followed, and had learnt the energetic need for travelling to the food source. This possibility can be tested using artificial feeders located at different distances and with followers that have never visited them.

3.3. Effect of Experience

Once bees find a food source, they often commute between the source and their nest. During this repeated foraging, they learn the route from the nest to the source and gather information to collect food materials efficiently (e.g., visual and chemical cues of rewarding flowers). Consequently, they might change the amount of honey taken from the nest. Such an effect of experience was found in a comparison between dancers and followers. Dancers have usually made several successful trips to a food source, whereas followers might have never visited the indicated food source (but see above). In the experiment described above, followers had more crop content compared with dancers on leaving the nest even if their destinations were similar in distance from the hive (Figure 2). These results could reflect different informational states in the two types of bee. In contrast to dancers that will travel to the food source through a learnt route, followers might rely on information communicated by waggle dances to find the food source. Perhaps because waggle dances do not pinpoint the target but only the area around it (von Frisch, 1967; Gould, 1975ab; Towne and Gould, 1988), followers load extra fuel to enable them to search for the target food source within that area.

The extra fuel might increase the chance of accomplishing successful foraging in followers. This effect might be enhanced when a foraging site comprises profitable and unprofitable flowers of the same plant species that are distributed heterogeneously: that is, the proportion of profitable flowers is high in some patches but not in others (Figure 3). Such heterogeneity is likely to occur because the amount and concentration of nectar are influenced by floral stage, age and other physiological conditions of plant, as well as by the abiotic environment, such as soil and insolation conditions (Shuel, 1992). Conspecific and heterospecific competitors might also create heterogeneity among patches. Recruits guided by dances might consume a lot of energy to reach a patch with profitable flowers even if they had ever foraged in that area because the distribution of profitable flowers can change with time.

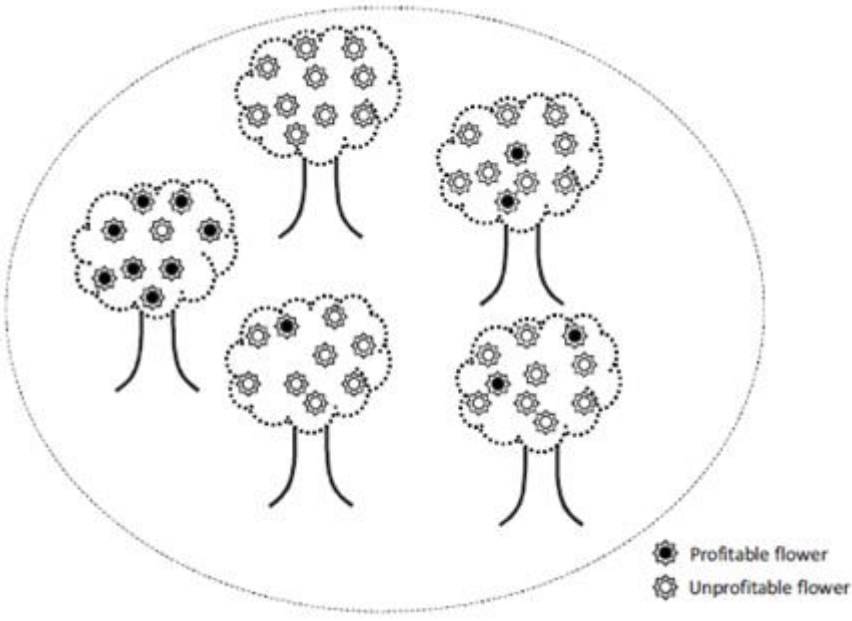


Figure 3. A hypothetical arrangement of flowers with heterogeneity in their profitability. Profitable flowers might be distributed heterogeneously owing to plant conditions and activity of competitors. A dashed circle shows a range to which waggle dances guide recruits. The recruits visit these flowers one by one and might consume much energy before finding a profitable patch.

If they have extra fuel, they are more likely to find profitable patches; otherwise they might have to return to the nest without a harvest.

However, there is another possible explanation for the large amounts of fuel in followers. Recent studies demonstrated that the waggle dance does not encode absolute distances to food sources but only the amount of optic flow (i.e., a flow of visual patterns created by the environment) that the dancers have perceived during a flight from the nest to a food source (Srinivasan et al., 2000; Esch et al., 2001).

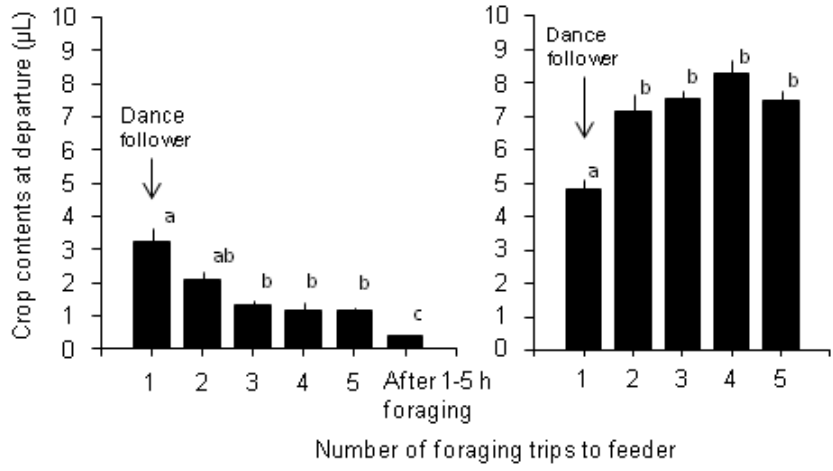
Followers can find the indicated food source by flying in a direction informed by a dance until perceiving the same amount of optic flow as the dancers did. However, followers are not informed of the energetic need for flight to the target because the rate of optic flow (i.e., amount of optic flow per unit distance) can vary depending on the environment over which the bees fly (Esch et al., 2001). Therefore, they might have to carry a larger amount of fuel than is in fact needed.

The amount of honey required to fly directly to a food source with a given distance can be estimated based on an energetic efficiency reported for honeybees (Gmeinbauer and Crailsheim, 1993) (see section 2). Taking into account the estimation, results of Harano et al. (2013) show that dancers loaded two to three times more honey than the requisite amount on average, although there was large variance in their crop contents (Figure 2). When the bees find nectar at flowers, they can fuel themselves for the return trip, but even if they fail to do so, they are probably able to return to the nest using fuel loaded in the crop at the nest and sugars present in the mid-gut and hemolymph. Followers leave the nest with more than five times the honey needed for a direct flight to an advertised food source.

As described above, the differential fuel loading between dancers and followers can be caused by the difference in their foraging experience. Given that followers can become dancers after they find a food source, the amount of fuel is expected to decrease with foraging experience. Such an effect of foraging experience was confirmed by an experiment with an artificial feeder (Harano et al., 2013). The bees left the hive with the largest amount of honey after following a dance (first trip to feeder) and then reduced the honey as they repeatedly visited the feeder. The honey load on departure decreased considerably during the first two trips and slightly thereafter (Figure 4a). Brandstetter et al. (1988) also observed a decrease in the sugar content of the whole body of foragers within 2–5 days of continuous foraging at a feeder and argued that it was caused by diminished motivation for a familiar food source.

4. Costs of Carrying Fuel

The regulation in fuel loading suggests that there is a mass-dependent cost of carrying honey. In birds, several costs are considered in relation to fat accumulation. Some birds migrate long distances, during which the energetic expenditure greatly overcomes the energetic income and they consume fat to compensate for this. However, the birds do not accumulate fat maximally, suggesting that fat accumulation has costs as well as benefits for the birds. According to a review by Witter and Cuthill (1993), the mass-dependent cost of fat accumulation is primarily associated with the metabolic expenditure and predation risk. Given that the power required for flight is a function of body mass, birds with more fat might have to consume more energy to fly over a unit distance than those with less fat.



a b

a - Sugar Syrup.
b - Pollen substitute.
Based on Harano et al. (2013).

Figure 4. Effects of foraging experience on crop content on departing the nest in *Apis mellifera*. Foragers were allowed to collect sugar syrup (a) or pollen substitute (b) from feeders.

Besides, birds with a lot of fat might have reduced maneuverability and agility in the air, leading to a higher risk of predation. In honeybee foragers, the fuel load might induce similar costs to those of fat accumulation in birds. The bees might control their fuel load to keep the metabolic cost of flight low and to maximize their net income. This possibility seems likely and, indeed, the increment of metabolic rate with increasing load has been shown experimentally (Wolf et al., 1989).

However, there are inconsistent observations with this hypothesis. In a flower patch, bees usually visit many flowerets and increase their body mass with collected nectar.

At this time, their metabolic rate does not necessarily increase with the load and only does so when the food source is highly profitable. Balderrama et al. (1992) and Moffat (2000) showed this and suggested based on their analyses that the changes in metabolic rate at food sources primarily depends on the motivation of the bee rather than on the load size.

Given that bees change their body temperature according to the quality of food source, which can substantially affect metabolic rate (Schmaranzer and

Stabentheiner, 1988; Stabentheiner and Hagmüller, 1991; Stabentheiner, 1996), motivation is likely to be an important factor affecting the energetic expenditure of a bee.

Does this mean that the amount of fuel loaded at the nest does not influence the energetic expenditure for foraging flight? Further study is needed to answer this, but, in my opinion, the load size is likely to affect it. The results of Balderrama et al. (1992) and Moffat (2000) clearly show that a factor other than load size primarily determines the metabolic rate but do not rule out the influence of load size on the metabolic rate.

It is still possible that bees with more fuel consume more energy under conditions with a given motivation or body temperature. The metabolic cost of carrying fuel might be small on an individual basis but when thousands of bees work on foraging, it would be large enough to be an object of natural selection.

In addition, nectar foragers might encounter bee-specific costs of carrying fuel. Given that bees carry fuel as well as collected nectar in their crop, if they bring excess fuel, it would remain in the crop when they arrive at the food source and reduce the room for loading nectar.

5. HONEY LOAD AT DEPARTURE IN POLLEN FORAGERS

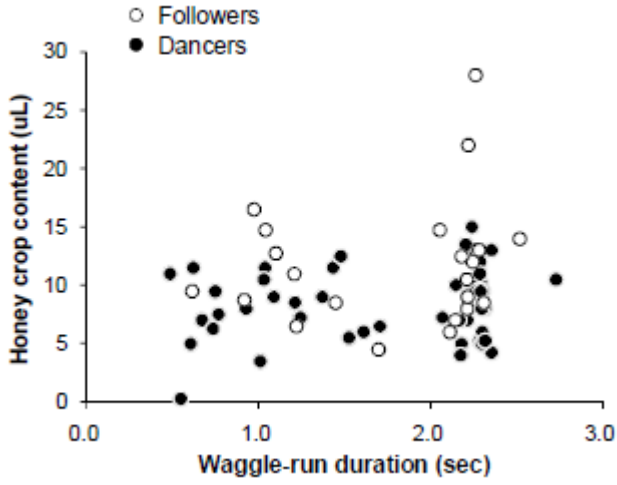
Honeybee foragers collect not only nectar, but also pollen as food. Workers tend to collect either of these even if flowers provide both (Parker, 1926; Free, 1960). Do pollen foragers have the same regulation of the amount of honey load carried from the nest as in nectar foragers? Beutler (1950) found that more honey is brought from the nest by pollen foragers than by nectar foragers and suggested that the honey was used to form pollen loads on the hind legs of the bees.

During pollen collection on flowers, bees regurgitate honey or nectar from the crop and mix it with the harvested pollen to give enough stickiness to form balls on their hind legs (Hodges, 1952). The honey or nectar used for this purpose is called ‘glue’ honey.

Although Beutler (1950) showed that pollen foragers have more honey on departing the nest than do nectar foragers, she did not investigate whether it increased with the distance to food source, as found in nectar foragers. It seems reasonable that both nectar and pollen foragers have the same regulation with respect to the distance because any bee must need more energy to fly further. However, when the relation between the honey load on departure and the distance to food source was preliminarily investigated in pollen foragers,

no positive correlation was found (Harano, unpublished data; Figure 5), although larger crop contents were found on departure in pollen foragers than in nectar foragers. Notably, no correlation was found even for dancers that were supposed to know the exact energetic expenditure for the flight to food source.

These results can be interpreted in several ways. They might suggest that honey load size does not affect flight metabolism (see section 4) and that its cost is only limiting the room for collected liquid (nectar or water) in the crop. If so, pollen foragers would not control the crop contents precisely at leaving the nest as long as they were able to load sufficient glue honey. However, the failure to detect a correlation might be caused by unknown factors influencing the amount of honey load greatly, independent of distance to food source. One of the candidates is the size of pollen load. As shown in Figure 6, the size of pollen load varies greatly among individuals. If pollen foragers take glue honey from the nest, they might adjust it to the expected size of pollen load. This hypothesis is now under investigation.



Harano, unpublished data.

Figure 5. Relation between crop content on leaving the hive and waggle-run duration for pollen foragers of *Apis mellifera*. The crop content was measured when each bee left the nest after performing a dance with pollen loads (dancers) or following the dance (followers). There was no significant correlation between them in dancers (N = 47, Spearman's correlation coefficient r_s = 0.07, P = 0.64) or followers (N =23, r_s = 0.18, P = 0.41).

Figure 6. Variation in the size of pollen load in *Apis mellifera*. Bees foraging at poor sites or those collecting nectar might have small pollen loads.

An experiment that investigated the effects of foraging experience clearly showed a difference in the regulation of honey load at departure between nectar and pollen foragers. As described above, foragers decreased honey load at leaving the hive as they repeatedly visited a sugar-syrup feeder (Figure 4a). When a pollen substitute was given at a feeder, bees increased the honey load at departure after they had visited the feeder once (Figure 4b). Although the reason why they increased the honey load is yet to be revealed, this might be caused by changes in the amount of glue honey, which is controlled differently from fuel honey.

One might wonder why pollen foragers bring glue honey from the nest given that nectar might be available at flowers. Although flowers of some plants produce abundant pollen, they might secrete little or no nectar (Parker, 1926; Shuel, 1992). When bees forage on such flowers, leaving the nest with glue honey is appropriate. By contrast, pollen foragers might not carry glue honey from the nest when foraging at flowers providing both pollen and nectar. Parker (1926) found that bees arriving at the prairie rose, which does not secrete nectar, had half-filled their crop with honey, whereas those arriving at the white clover, from which bees gather both pollen and nectar, had no honey in their crops. However, this result was not confirmed by Beutler (1950). It is possible that pollen foragers adjust the amount of glue honey taken from the nest depending on the species of the pollen source, rather than on the presence or absence of nectar in the flower.

It appears that bees are specialized either to nectar or pollen collection on some flowers, but that they collect both from other flowers. Close investigation is needed to examine the control of glue honey in relation to pollen-source plants. In summary, the regulation of honey load at leaving the hive appears to be more complicated in pollen foragers than in nectar foragers. Its detail is yet to be revealed and many questions remain.

6. FUEL FOR WATER COLLECTION

Honeybee foragers collect water in addition to nectar and pollen and use it to cool their nest and dilute honey for feeding larvae (Lindauer, 1954). Water collectors leave the nest with a little fuel as do nectar foragers and are suggested to load larger amounts of fuel when they are recruited to an unfamiliar water source (Visscher et al., 1996). However, unlike nectar foragers, they encounter the so-called 'water in the gas tank' problem. Given that they temporarily keep the collected water in their crop during a foraging trip, the fuel honey carried will be diluted to a great extent. If they consume the diluted honey as fuel, collected water also will be lost through their digestive tract. Therefore, how they gain energy for a return trip is an interesting question. According to Visscher et al. (1996), water foragers do not rely on fuel honey carried in the crop after loading water. By using isotopes, the authors showed that almost none of the crop content was sent to the mid-gut during the return trip. Their results suggest that bees obtain energy for the return trip from sugars in their mid-gut and hemolymph, and from glycogen in the muscle. However, such energy stores outside the crop are limited in water foragers as well as in other workers. Visscher et al. (1996) estimated the maximum distance over which bees can fly without using honey in the crop to be 4.2 km and argued that the problem of energy supply limits their foraging range of water. Honeybees usually collect water close to the nest, whereas nectar foragers sometimes visit flowers more than 10 km from the nest.

7. HONEY LOAD AT DEPARTURE FOR RESIN COLLECTION

A few workers of honeybees collect resin from buds or other parts of several plant species. The resin is deposited on the inner wall of the nest, mixed with comb wax or stuffed into cracks.

The resin found in honeybee nests is called propolis and has an important role in maintaining a hygienic environment in the colony because of its antibiotic properties (Winston, 1987).

Although no information is available for the amount of honey that the resin collectors take from the nest, it would be interesting because resin collection is carried out in a different way from any other type of foraging, such as nectar, pollen or water collection. Resin is brought home as balls on the hind legs, in a similar way to pollen, but bees do not need glue honey to make them because of the stickiness of the resin. Therefore, they might have a small fuel load, as do nectar foragers, on leaving the nest owing to there being no need for glue honey, or might carry as much honey as pollen foragers do because there is no concern about the room available for storing forage in their crop. Such a result would help to understand the benefits and costs of carrying honey from the nest.

8. When Do Recruits Decide on the Type of Material to Forage?

Followers tend to collect the same material as the dancers that they followed (Lindauer, 1953). Have they decided what material to collect before leaving the nest or do they do so at the foraging site? The data on honey load at departure support the former hypothesis. When the bees left to a pollen-substitute feeder, they took a large amount of honey from the hive, perhaps for glue, even when they had never previously visited the food source (Harano et al., 2013). Followers seem to be informed by dancers what material is available in the food source because pollen and resin foragers dance with their forage still on their hind legs.

Conclusion

Foragers carry fuel on departing the nest and gain energy for foraging activity by consuming it at least partially. The strategy to improve their foraging efficiency appears to include regulation of the size of honey load at departure, based on distance to food source, foraging experience and type of material that they collect.

Figure 7 summarizes the regulations of honey load on departure in honeybee foragers. When foragers have no foraging site to go, they follow waggle dances to obtain information about available resources. The waggle dances tell them the materials available in the foraging site as well as information about its location, and followers might change their control of honey load at departure depending on the information communicated. In nectar foraging, followers leave the nest with a certain amount of honey load according to the distance to food source. The amount of honey loaded at that time is greater than that required for a direct flight to the nectar source and probably includes extra fuel for searching for the food source. After the follower successfully finds the food source and acquires information about it (e.g., its exact location), they leave the nest with a reduced amount of honey. Pollen foragers appear to carry glue honey for making pollen balls in addition to fuel for a flight to the pollen source. They might increase their honey load on departure after visiting the food source. In water collection, the regulation might be similar to that of nectar foragers, but its details have not yet been revealed. There is no information relating to the fuel load in resin collectors.

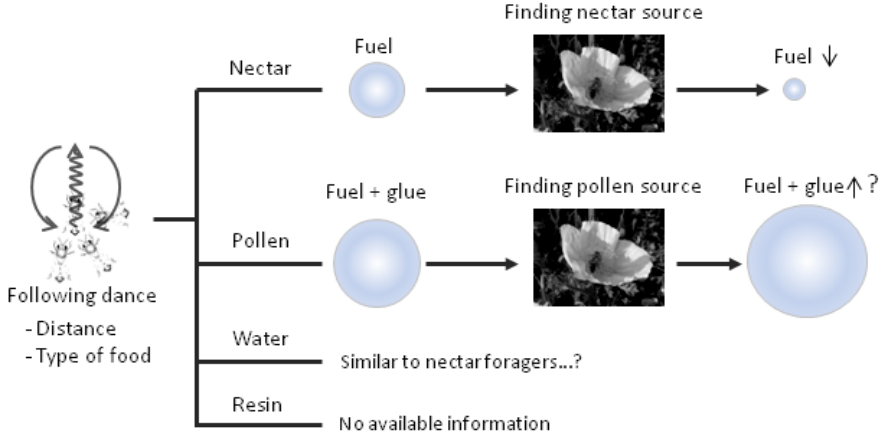


Figure 7. Summary of the regulations in the size of honey load on departing the nest in *Apis mellifera* (see main text for details).

It has been well documented that honeybees accomplish efficient foraging by taking advantages of abilities in information gathering, learning and memory, and communication among nestmates. However, the uncertainty or risk of failure is always present in their foraging and cannot be completely

eliminated by any such information. Foragers appear to manage this uncertainty by adjusting the amount of honey that they carry for foraging.

It is also suggested that they change its control depending on the materials for which they are foraging. Further studies of such regulations would shed light on new aspects of the foraging strategies of honeybees.

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Chapter 5

SEX DIFFERENCES OF DOPAMINE CONTROL SYSTEMS ASSOCIATED WITH REPRODUCTION IN HONEYBEES

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ABSTRACT

Dopamine is a key substance in the regulation of reproductive behaviors and sexual maturation in social hymenopterans. In honeybees (*Apis mellifera*), factors affecting brain dopamine levels appear to differ between male and female bees. The brain levels of dopamine in males are enhanced by juvenile hormone (JH) and the dynamics of dopamine in the brain are similar to those of JH. Both dopamine and JH have roles in promoting mating flight behavior in males. JH can also enhance the gene expression of a dopamine receptor, indicating a parallel regulation of dopamine supply and dopamine receptor expression. The brain levels of dopamine in honeybee workers are regulated by queen substances and increase in the absence of the queen to enable their transition to become reproductive workers. The queen substances can also regulate the expression of particular dopamine receptors in workers. Nutritional factors can influence the brain dopamine levels through the supply of dopamine precursors. However, JH might not regulate the levels of dopamine in the brain in both reproductive workers and queens, because these females have low titers of JH in their hemolymph. Thus, the

regulatory systems of dopamine in the brain differ between male and female honeybees. Such differences might be unique to honeybees because they share few similarities with the regulatory systems of primitively eusocial species or highly eusocial ant species.

1. INTRODUCTION

The honeybee (*Apis mellifera*) is a highly eusocial species of Hymenoptera, with reproductive divisions of labor among individual females. Queens specialize in reproduction, including mating and egg laying, whereas workers perform various tasks, including care of the larvae and queen, comb building, food storing, nest guarding and foraging. Workers are normally infertile, but can lay unfertilized eggs in the absence of a queen. Substances produced by the queen control the reproductive physiology and behavior of the workers. The physiological mechanisms underlying the reproductive inhibition of these workers by the queen are still unclear, but are important for understanding the evolution of the reproductive divisions of labor in highly eusocial bees.

Biogenic amines are neuroactive substances controlling social behaviors and reproduction in both males and females. These substances are synthesized in the neurosecretory cells in the brain or other ganglia and are secreted into the relevant neural circuit and other target tissues. In the target cells, the receptors bind with a particular amine and change the intracellular levels of the second messenger (cAMP), causing expression of relevant genes and changes of threshold in the neurons. These actions can result in physiological and behavioral modulations in response to variable environments in the honeybee nest. However, it is still not fully understood how the regulatory systems of these amines control social behavior in the honeybee. In this chapter, I introduce the regulatory systems of one important biogenic amine, dopamine, which has a role in reproduction by male and female honeybees.

2. MOLECULES INVOLVING DOPAMINE SIGNALING IN THE HONEYBEE BRAIN

The distribution of dopamine secretory cells in the brain has been investigated by histochemical studies using dopamine antibodies (Schäfer and Rehder, 1989; Schürmann et al., 1989). Given that dopamine is a precursor of

norepinephrine, which is a functional monoamine in vertebrates, the dopamine-like immunoreactive cells might contain both dopamine and norepinephrine secretory cells. However, the amount of dopamine in the brains is more than ten-fold that of norepinephrine (Brandes et al., 1990; Sasaki and Nagao, 2001). Therefore, many of the dopamine-immunoreactive cells in the brain of insects are thought to be dopamine secretory cells. Histochemical studies indicate that seven groups of dopamine-like immunoreactive cell exist within somata in the brain of worker bees (Figure 1 and Table 1). These cells project their neuropil to the central body, mushroom body, other areas of the frontal, lateral and caudal protocerebrum, dorsal deutocerebrum and antennal lobe (Table 1). However, there are no dopamine-like immunoreactive cells in the optic lobe.

Table 1. Localization of cell groups and their neuropilar projection areas in dopamine-like immunoreactive neurons in the brain of workers of the honeybee *Apis mellifera*[a]

Cell group[b]	Number of somata in one hemisphere	Localization of somata	Projection of neuropil
1	30–50	Laterofrontal protocerebrum, at rim of lateral calyx	Central body (partially)
2	5–10	Frontal protocerebrum, medial to lateral calyx	Mushroom bodies and surrounding neuropil
3	10	Frontal and caudal pars intercerebralis	Central body and caudal protocerebrum
4	40	Frontal border of proto- to deutocerebrum	Frontal protocerebrum around and within mushroom body α-lobes
5	2	Frontal border of proto- to deutocerebrum	Lateral and caudal protocerebrum
6	2–4	Lateral protocerebrum	Lateral protocerebrum
7	2–3	Lateral border of deuto- to tritocerebrum	Dorsal deutocerebrum, antennal lobe

[a] Based on Schäfer and Rehder (1989) and Schürmann et al. (1989).
[b] The cell group numbers correspond to the numbers in Figure 1.

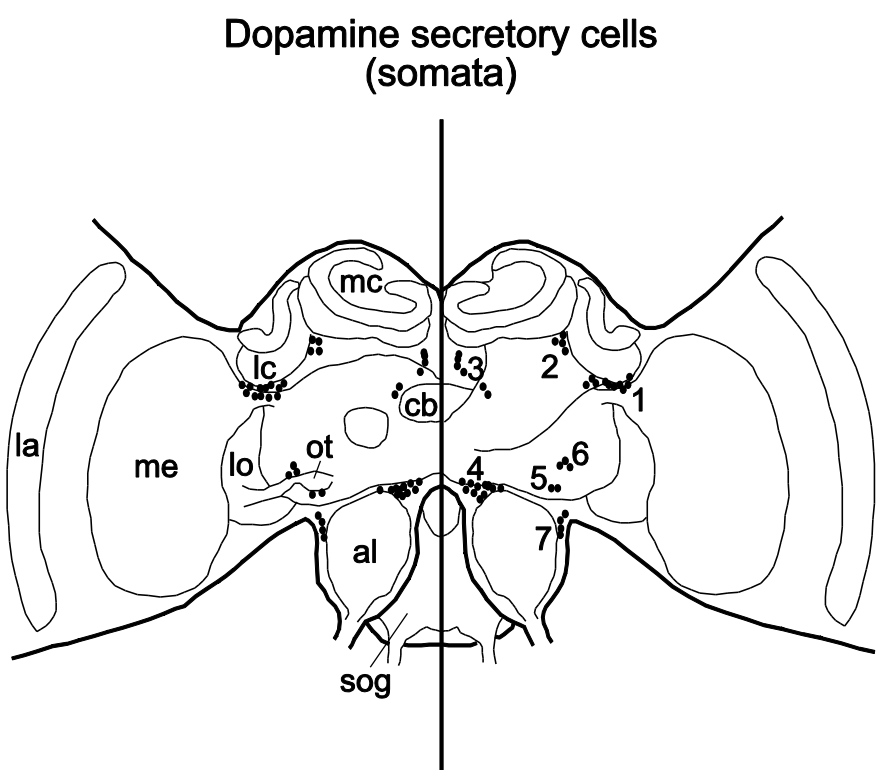


Figure 1. Distribution of dopamine-like immunoreactive cells in workers of the honeybee *Apis mellifera*. Numbers of cell groups correspond to the numbers in Table 1. Abbreviation: al, antennal lobes; cb, central body; la, lamina; lc, lateral calyx of mushroom body; lo, lobula; mc, medial calyx of mushroom body; me, medulla; ot, optic tubercle; sog, suboesophageal ganglion. Somata of dopamine secretory cells are illustrated based on Schäfer and Rehder (1989) and Schürmann et al. (1989).

In contrast to the distribution of dopamine-like immunoreactive cells, dopamine can be detected in areas out of reach of the projection of the immunoreactive cells, including the optic lobes (Sasaki and Nagao, 2001). This suggests that dopamine released into the projection area diffuses to non-projection area and acts on target cells in the brain. In fact, the dopamine receptors distribute the non-projection area of the dopamine-like immunoreactive cells (Beggs et al., 2005; Blenau et al., 1998; Kurshan et al., 2003, Table 2). Therefore, dopamine in the brain might act strongly on the receptors around the projection areas and weakly on the receptors not in the projection areas.

Table 2. Characteristics of dopamine receptors in the honey bee *Apis mellifera*

Receptors	Effects of activation	Regions of mRNA expressed in the brains in workers	Refs
AmDOP1	Upregulation of $[cAMP]_i$	• Optic lobes (somata within monopolar cell body layer and first optic chiasmata) • Mushroom body (somata of intrinsic neurons) • Antennal and dorsal lobes (somata around these lobes)	Blenau et al., 1998; Kurshan et al., 2003
AmDOP2 (AmBAR6)	Upregulation of $[cAMP]_i$	• Mushroom body (somata of intrinsic neurons)	Humphries et al., 2003; Kurshan et al., 2003
AmDOP3 (AmBAR3)	Downregulation of $[cAMP]_i$	• Optic lobes • Mushroom body (somata of intrinsic neurons) • Antennal lobes (somata around the lobes)	Beggs et al., 2005

Three types of dopamine receptor have been characterized and their distributions determined in honeybees (Table 2). *Am*DOP1 and *Am*DOP2 are D1-like receptors that cause cAMP elevations. The former is expressed on mushroom bodies, optic lobes, antennal and dorsal lobes in the brain (Blenau et al., 1998; Kurshan et al., 2003), whereas the latter is expressed on limited areas of the mushroom bodies (Humphries et al., 2003; Kurshan et al., 2003). *Am*DOP3 is a D2-like receptor causing cAMP depression and is expressed on mushroom bodies, optic lobes and antennal lobes in the brain (Beggs et al., 2005).

The dopamine transporter is expressed in the presynaptic membrane of dopamine secretory cells and re-uptakes dopamine released into the synaptic cleft. The ortholog of dopamine transporter gene (*Amdat*) in honeybees has been identified and its expression in brain has been investigated in two castes of females and males (Nomura et al., 2009). Its expression can affect the levels of dopamine and its metabolites in the brain, because the released dopamine can be re-uptaken by the transporters and re-used as a neurotransmitter or

neuromodulator, whereas the remaining dopamine can be inactivated by enzymes (mainly *N*-acetyltransferase in the honeybee brain).

Brain dopamine can diffuse via the hemolymph to act on peripheral targets. It has been reported that dopamine can be found in the hemolymph of queens and males (Harano et al., 2008a; Akasaka et al., 2010) and that dopamine receptors are expressed on the peripheral tissues, including the ovaries of reproductive workers (Vergoz et al., 2012).

3. FEMALE SEXUAL BEHAVIOR IN HONEYBEES

In honeybee females, the infertile workers have a pair of undeveloped ovaries that, if the relevant triggers are received, can mature so that the bees can lay unfertilized eggs in the absent of a queen in the colony. These reproductive workers do not mate with males, because the spermatheca does not develop during ovarian development. Virgin queens begin to fly from of the hive at 5–6-days old and mate with males in the air. Queens repeat the mating flight, mate with a total of 7–17 males (Winston, 1991). After the mating flight, spermatozoa from several males are transferred into the spermatheca and used for egg fertilization during egg laying.

Ovaries in queens develop quickly from the virgin to a mated state. A queen has a pair of ovaries with 150–180 ovarioles and can lay, on average, 1500 eggs per day during the summer (Winston, 1991). During oviposition, the queen inserts her head into the comb cell and then her abdomen, whereupon she lays an egg. By inspecting the cell before laying an egg, the queen can determine what type of cell it is and, thus, whether the egg should be fertilized before it is oviposited (Königer, 1970). Queens lay unfertilized eggs in the larger diameter cells (drone cells), whereas they lay fertilized eggs in the smaller diameter cells (worker cells and queen cells). When comb cells are not available, queens lay fertilized eggs, suggesting that the basic system of egg fertilization during egg laying is in place and that the queen is able to inhibit the fertilization of male-intended eggs (Sasaki and Obara, 1999). Queens can select either drone or worker comb cells for oviposition (Sasaki et al., 1996) and can select comb cells on the basis of the season and the nutritional state of the colony (Sasaki and Obara, 2001).

Reproductive workers show similar egg-laying behaviors to those of the queen. Reproductive workers inspect the comb cells for egg laying and select drone cells preferentially for unfertilized eggs (Sasaki, 2011). However, they compete aggressively among themselves for egg laying (Sakagami, 1954). Reproductive workers produce queen-like substances (Crewe and Velthuis,

1980) that can attract non-reproductive workers in a queenless colony, resulting in a 'royal court' surrounded by non-reproductive workers (Slessor et al., 1988). The reproductive potential of reproductive workers is lower than that of the queen, because of their fewer ovarioles (2–12) compared with the queen (Winston, 1991).

4. Reproductive Roles of Dopamine in Workers and Queens in Honeybees

Dopamine has multiple functions as a neuromodulator and neurohormone in insects. In honeybee workers, levels of dopamine in the brain increase with age in queenright colonies (Taylor et al., 1992). However, it is unclear whether the increased levels of dopamine are age or task dependent. For example, the expression levels of the dopamine transporter gene *Amdat* in the brain tend to increase with age, but are significantly higher in foraging bees than in nurse bees of the same age (Nomura et al., 2009). This suggests that the change in dopamine transporter systems in queenright colonies is task rather than age dependent. However, the question remains as to the reason for the changes in levels of dopamine in the brain.

Dopamine has a reproductive function in reproductive workers in queenless colonies. Brain dopamine levels are correlated with the ovarian diameter or degree of ovarian development in queenless workers (Harris and Woodring, 1995; Sasaki and Nagao, 2001). Oral applications of dopamine enhance the levels of dopamine in brain (Sasaki and Nagao, 2007) and promote ovarian development (Dombroski et al., 2003; Sasaki and Nagao, 2007). Such dopamine effects have also been reported in females of other hymenopteran species (Bouley et al., 2001; Sasaki et al., 2009). Whether dopamine acts directory on the ovarian tissues or on the brain with mediation of other substances for ovarian development remains to be determined. The expression of the gene encoding the dopamine receptor (*Amdop1*) is enhanced in the ovarian tissues of reproductive workers, whereas that of *Amdop3* is depressed (Vergoz et al., 2012). Given that the expression of *Amdop3* is activated in queenright workers, its receptor might mediate the inhibition of ovarian development. In fact, one of the substances produced by the queen, homovanillyl alcohol (HVA), can bind with *Am*DOP3 and act as a dopamine receptor agonist (Beggs and Mercer, 2009). If HVA is taken orally into the hemolymph, it can act directly on *Am*DOP3 in the ovaries. In the brain, expression of these genes changes depending on the reproductive state of the workers (Beggs et al., 2007; Vergoz et al., 2012), although the trends in the

changes reported differ. Beggs et al. (2007) reported the activation of *Amdop1* in 2-day-old queenless workers, whereas Vergoz et al. (2012) reported the inhibition of *Amdop1* in 10- and 15-day-old queenless workers and *Amdop2* in 6-day-old queenless workers. Therefore, expression of the genes encoding the dopamine receptors in the brain of reproductive workers remains controversial.

Dopamine is involved in the mating behavior of the queen. The brain levels of dopamine in virgin queens are higher than those of normal workers (Brandes et al., 1990; Sasaki et al., 2012a), but reduce as the queens begin to mate with the males (although the levels remain higher than those in worker bees) (Harano et al., 2005, 2008a). Expression of *Amdat* increases within 5 days of emergence and decreases over a few days following mating (Nomura et al., 2009). The changes in expression of *Amdat* parallel the changes in levels of dopamine in the brain of the queens. This suggests that the expression level of *Amdat* reflects the activity of dopamine secretory cells in the brain. The higher dopamine levels in the brain of virgin queens might result in the high mating activities that have been recorded before mating occurs. Injections of the dopamine-receptor agonist (6,7-ADTN) into virgin queens enhanced their locomotor activities, whereas injection of an antagonist (flupentixol) reduced such activity (Harano et al., 2008a). The increased locomotory activity might be necessary to maintain the mating flight, as well as the ability of the queens to attack aggressively rival virgin queens within the colony.

5. Regulation of Dopamine Systems in Females

Levels of dopamine in the brains of worker bees are influenced by the presence of a queen in the colony (Harris and Woodring, 1995; Sasaki and Nagao, 2001). Substances produced by the queen inhibit the reproduction by workers, including their ovarian development and egg-laying behaviors, as well as the elevation of dopamine levels in their brains. HVA has a similar chemical structure to that of dopamine and can bind with a dopamine receptor, *Am*DOP3, causing cAMP depletion in a similar manner to dopamine (Beggs and Mercer, 2009). It also inhibits the increase in levels of dopamine in the brain (Beggs et al., 2007). There are two possible mechanisms underlying the regulation of dopamine in the brain by HVA. The first is that HVA is taken orally into the hemolymph and acts on *Am*DOP3 in the brain. The second is that HVA binds with *Am*DOP3 expressed on the surface of the antennae, probably as a pheromone receptor and the sensory signals then influence the levels of dopamine in the brain. However, further studies are required to test these hypotheses.

Another factor affecting the level of dopamine in the brain is the nutrient supply, especially of the dopamine precursor, tyrosine. In the synthetic pathway of dopamine in the brain, tyrosine is converted into 3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase and is then metabolized into dopamine by DOPA decarboxylase (Sasaki, 2008). Given that tyrosine can be taken from royal jelly (Townsend and Lucas, 1940; Haydak, 1970), queens fed royal jelly by nurse bees are more likely to be able to maintain high levels of tyrosine in the hemolymph (Hrassnigg et al., 2003). In queens, the levels of DOPA, dopamine and dopamine metabolites (*N*-acetyldopamine and norepinephrine) in the brain are higher than in workers (Brandes et al., 1990; Sasaki et al., 2012a), indicating the upregulation of the dopamine synthetic and metabolic pathways. Given that the high level of dopamine in the brain of queens is not caused by the enzymatic activities of DOPA decarboxylase (Sasaki et al., 2012a), tyrosine intakes can influence the dopamine level in queens. Reproductive workers in queenless colonies might receive royal jelly from nurse bees, which would enhance their level of dopamine. In preliminary experiments, queenless workers fed royal jelly had higher levels of tyrosine, dopamine and tyramine in their brains compared with those fed honey or a sucrose solution (Matsuyama et al., unpublished), suggesting that one of the important factors regulating brain dopamine is the intake of tyrosine via consumption of royal jelly.

Tyramine is a precursor of octopamine and is synthesized from tyrosine by tyrosine decarboxylase (Sasaki, 2008). Tyramine injections or oral applications caused an elevation of dopamine in the brain of queenless workers (Sasaki and Harano, 2007). In reproductive workers, the levels of tyramine in the brain increase with ovarian development as well as with the increase in levels of dopamine (Sasaki and Nagao, 2002). These observations support the possibility that tyrosine intake via royal jelly in queenless workers causes the elevation of both tyramine and dopamine levels in the brain.

6. MALE SEXUAL BEHAVIOR IN HONEYBEES

Honeybee males mate with a queen once and die immediately because their genitalia are removed from their abdomen. Given that the males mate while flying, mating flight activity is an important factor for their mating success. Flight activity gradually increases with age (Akasaka et al., 2010) and males begin their orientation flights when 6–8-days old. The reproductive organs of males also mature once they are 8-days old.

7. Reproductive Roles of Dopamine in Males

Dopamine has a role in the elevation of mating flight activity in males. Dopamine levels in the brain, thoracic ganglia and hemolymph increase with age up to 7–8-days of age, and the brain and hemolymph levels decrease thereafter (Harano et al., 2008b; Akasaka et al., 2010). The expression of *Amdat* increases progressively for at least 15 days after emergence (Nomura et al., 2009). Locomotor activities (i.e. walking) increase with age and are enhanced by a dopamine-receptor agonist (6,7-ADTN) and inhibited by the relevant antagonist (flupentixol) (Akasaka et al., 2010). Flight-initiation and flight-maintaining activities are also enhanced by dopamine injections (Mezawa et al., 2013). Effects of dopamine on the development of the reproductive organs remain unknown. Given that hemolymph dopamine levels change in parallel with those in the brain, the dopamine circulated in the hemolymph might act on other tissues involved in the mating flight and copulation.

8. Regulation of the Dopamine System in Males

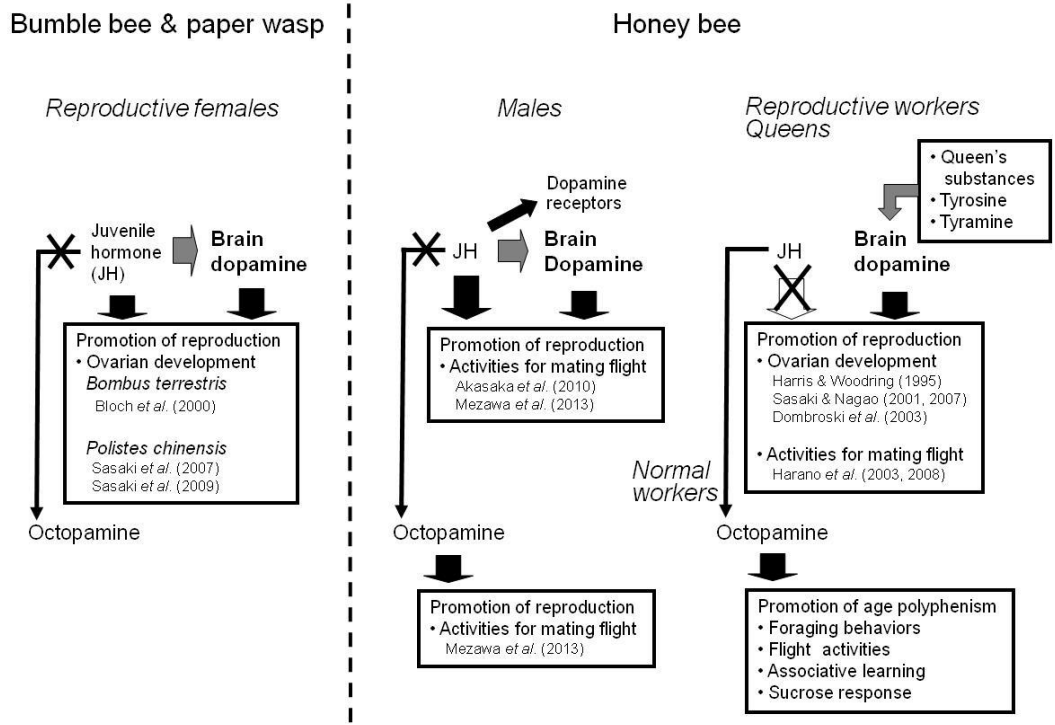
Dopamine levels in the brain and hemolymph of males change in parallel with those of juvenile hormone (JH) in the hemolymph (Tozzeto et al., 1995; Giray and Robinson, 1996; Harano et al., 2008b; Akasaka et al., 2010). JH can promote the mating flight in males (Tozzeto et al., 1997; Giray and Robinson, 1996) and has a role similar to that of dopamine. Applications of a JH analog (methoprene) to immature males enhanced the levels of dopamine (Harano et al., 2008b; Mezawa et al., 2013), and also the expression of *Amdop1*, in the brain (Sasaki et al., 2012b), suggesting that JH regulates both dopamine supply and dopamine reactions. Given that the increase in the levels of dopamine in the brain accompanies a decrease in the levels of DOPA in the brain (Mezawa et al., 2013), it might be that the upregulation of DOPA decarboxylase activities is age dependent. In preliminary experiments, the expression of genes encoding enzymes involved in dopamine synthesis increased with age up to 8-days old, although it was not determined which genes were upregulated by JH. It is also unclear whether the dopamine in the brain of males can be regulated by substances produced by the queen or by tyrosine intake, as seen in workers.

9. REGULATION OF DOPAMINE SYSTEM IN PRIMITIVELY EUSOCIAL SPECIES

The reproductive roles of dopamine in ovarian development in females have been reported in other eusocial species, including the paper wasp, *Polistes chinensis* (Sasaki et al., 2007, 2009) and the fire ant, *Solenopsis invicta* (Bouley et al., 2001). In a bumble bee *Bombus terrestris*, ovarian development in females is correlated with the levels of dopamine in the brain (Bloch et al., 2000), although the effects of dopamine have not yet been tested. In females of these species, JH can promote ovarian development (Brent and Vargo, 2003; Röseler, 1977), suggesting a relationship between JH and brain dopamine. In our unpublished experiments, JH III enhanced the level of dopamine in the brain in workers of *P. chinensis* and accelerated ovarian developments (Tsuchida et al., unpublished). Thus, JH is one of the regulators of dopamine in the brain of females of primitively and highly eusocial Hymenoptera, except honeybee females. These findings suggest that the regulation of dopamine in the brain by JH is different in honeybee females because there is a low titer of JH in the hemolymph (Robinson et al., 1991) and this hormone might have lost its reproductive function in this species. In honeybee males, the regulation of dopamine in the brain by JH is the same as that recorded in females of primitively eusocial hymenopterans.

CONCLUSION

In this chapter, I have introduced and compared the regulatory systems of dopamine in the brains of primitively and highly eusocial species and of the sexes in the honeybee. The comparisons between them are illustrated in Figure 2. Dopamine regulation by JH in the brain appears to be a prototype in social Hymenoptera. In honeybee females, dopamine in the brain is regulated by several physiological factors, including substances produced by the queen, tyrosine intake and tyramine. This change might be the result of the loss of reproductive function of JH and could lead to the regulation of octopamine for the promotion of age polyphenism (Figure 2, Schulz et al., 2002). More detailed investigations of such regulation in other hymenopteran species are required.



Abbreviation: JH, juvenile hormone.

Figure 2. Factors affecting brain dopamine levels and reproductive roles of dopamine in primitively [bumble bee (*Bombus terrestris*) and paper wasp (*Polistes chinensis*)] and highly eusocial species [honeybee (*Apis mellifera*)].

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Chapter 6

ROUNDTRIP-STRUCTURE OF THE FORAGING HONEYBEE (*APIS MELLIFERA*)

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ABSTRACT

The foraging system of honeybees (Genus *Apis*), being socially and individually controlled, represents the most complex behavioral system known among invertebrates. This system had been most thoroughly studied in the European hive-bee (*Apis mellifera*), with major advances achieved in the previous century based on prize-winning discoveries of Karl von Frisch. Much of v. Frisch and collaborators' research on honeybees was closely linked to the analysis of the bees' communication dances inside the hive. Concerning the navigation system of individual foragers outside the hive, I will review recent new findings and their theoretical integration.

Investigating the navigation system of a small insect, flying distances of up to several kilometers, is challenging. Innovative methods (e.g. use of harmonic radar) have yielded novel insights, useful to develop a new explanatory and synthesizing theoretical framework. In brief:

Simple straight journeys from the hive to a collecting site, or the reverse, are both constructed of three distinct sequential constituents:

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Distal navigation, peripheral navigation and *focal navigation*. Dead reckoning, the use of compass and distance knowledge, dominates distal navigation, which may span kilometers. Peripheral navigation, being used in the less than 100m range around the respective target, is dominated by the use of remembered terrestrial cues in order to orient towards the chosen goal from different directions. Focal navigation prepares for touchdown, based on increasing fine-grained spatial visual knowledge close to the target location. The knowledge required to implement this three-part navigation system is acquired in reverse order during exploration: *Focal exploration, peripheral exploration* and *distal exploration*.

This relatively simple three-part navigation system increases in complexity by adding *navigation hubs,* locations at which the individual bee interrupts navigation and decides when and where to depart towards another location. There are two types of such hubs: the hive itself and some recently identified extra-hive hubs, located at some distal collecting sites where the forager decides to fly home or to fly to some other collecting site, if the current one is depleted. Honeybees are also capable to make iterative use of focal navigation to successfully traverse mazes.

Given comparative evidence, the honeybee's three-part individual navigation system is found exclusively in the monophyletic lineage of the Euaculeata, the mostly nest-provisioning stinging wasps and bees. The species rich family of the ants (Formicidae) is an offshoot inside this lineage, but their navigation system is somewhat differently structured in adaptation to navigation on the ground and even underground.

INTRODUCTION

Workers of honeybees (Genus *Apis*) spend the last part of their life on foraging, their cognitively most demanding task. Like all complex behaviors, foraging is punctuated by sequences of adaptive decision making among possible behavioral alternatives. Thus, the roundtrips in this review are bracketed by a number of decisions: to leave the hive to navigate to some collecting site where the foragers decide to search and collect. Thereafter the roundtrip continues with the decision to leave the collecting cite to navigate to some other target, another feeding site or the hive, places where target-specific decisions interrupt or finally terminate the roundtrip.

In the European honeybees (several subspecies of the *Apis mellifera*) roundtrips regularly cover distances of several kilometers and the total foraging range of a colony covers some 100 square kilometers (Seeley 1985). Learning and implementing the navigation flight over such distances across

such a huge area is an enormous cognitive achievement, even more so, considering the tiny size of the bee-brain and short space of life-time, some two weeks, dedicated to this activity. For comparison, it takes us humans at least ten years to become proficient independent navigators (Learmonth & Newcombe 2010)

A behavior so demanding and complex as the honeybee's roundtrip navigation must have a long evolutionary history. The deep roots are largely unexplored. The particular roundtrip structure as found in the honeybees is a shared derived homology (synapomorphy) of most, if not all, flying and nesting Hymeoptera, the monophyletic taxon of the Euaculeata (Jander 1997). The adaptive radiation of this lineage started some 160 million years ago (Grimaldi and Engel 2005, Michener 2007). Well known Euaculeata are all the social and solitary bees (Anthophilia or Apiformes), the spider wasps (Pompilidae), the diggerwasps (Sphecidae and Crabronidae), the paper wasps (Vespidae) and the ants (Formicidae). However, in the diverse and ecologically successful ants the worker cast has lost flight; consequently, their roundtrip structure is a modified version of that of their flying ancestors (Hölldobler & Wilson 1990).

Among the thousands of euaculeate species the European honeybee (*Apis mellifera*) has the best-studied roundtrip structure. This will be the focus of this review; findings in other species will only be mentioned if they add additional insight not yet available for the honeybee. First I will cover the simple navigation between hive and one feeding site of the experienced forager; then the exploratory learning process on which this navigation skill is based; and finally, I will show how various constituents of honeybee navigation can be recombined in various adaptive ways.

EXPERIENCED NAVIGATION BETWEEN HIVE AND FEEDING SITE

The simple roundtrip comprises two parts, the outward journey and the return journey. Each one is composed of the same three distinct, sequentially deployed constituents, *distal navigation*, *peripheral navigation*, and *focal navigation* (Palikij et al. 2011). This recent insight was preceded by Dyer's (1996) distinction between distal and proximal navigation, the latter now being subdivided into peripheral and focal navigation. In the following the

discriminating characteristics of these three navigation mechanisms will be explained.

Distal Navigation. The decision to journey home, or out from there, is followed by distal navigation: the forager departs straight in a remembered direction. The underlying mechanism has been convincingly revealed by displacing an individual about to depart. This was first done with harvester ants (*Messor*) starting to home (Piéron 1904) and with similar outcome in a number of other species of ants (Brun 1914): the displaced ants travelled in the changed environment in the same direction and distance as if they had not been displaced. Such place-independent navigation performance had been aptly called *virtual orientation* (Brun 1914). Similarly, in numerous subsequent matching experiments with homing honeybees these displaced bees also displayed virtual orientation (e.g. Wolf 1927, Meder 1958, Geiger et al. 1994, Menzel et al. 2012). Corroborating for the outward journey, foragers about to leave the hive had been similarly displaced, where after they too displayed virtual orientation towards an imaginary feeding site (Renner 1959, Menzel et al. 1900, Menzel et al. 2006, Menzel et al. 2005).

What is the mechanism of virtual orientation (navigation) in experienced ants and bees? Darwin's (1873) hypothesis still best explains virtual orientation to this day: *dead-reckoning*. The navigating bee knows from previous exploration its navigation vector (direction and distance) towards its respective goal. The direction is kept by a celestial light compass and the diminishing distance from the goal is measured by the passing optic flow while flying straight. During the approach the expected distance to the goal still to be travelled is measured by the decremented vector length represented in the working memory. Arriving at the goal point, the initially recalled goal-vector is decremented to zero; after this event Cruse and Wehner (2011), among many others, refer tellingly of "zero-vector bees or ants."

The numerous and consistent results, showing virtual orientation after displacing honeybees about to initiate distal navigation, gives the strong impression that they completely ignore or even don't know any distal terrestrial navigation cues. Both is wrong on two counts.

First, if there is a conflict between compass cues and extremely salient visual terrestrial cues, like a continuous forest edge parallel to the flight direction, such terrestrial cues can overrule the bee's use of the celestial compass (v. Frisch & Lindauer 1954).

Second, given the finding that virtual navigation is used for homing, the knowledge base of which depends on the experience of the preceding outward journey, without such a journey, a "zero-vector" bee, when displaced from the

nest (hive), should not be able return home. The prediction proved to be wrong, many times. The initial discovery was made long time ago by Fabre (1879) who displaced mason bees (*Chalicodoma muraria*) up to several kilometers form their nest location, and these bees managed to return. Honeybees accomplish the same feat, given they had a chance to first explore their home range (e.g. Romanes 1885, Wolf 1926, Becker 1958, Capaldi & Dyer 1999, Menzel 2011).

What is the solution to this puzzle? Back-up: If an overcast sky eliminates the celestial compass cues, honeybees successfully navigate with terrestrial navigation cues (Dyer & Gould 1981, Schöne & Kuehme 2001). Otherwise, if there is no reliable fully self-controlled journey away from the hive, the information for dead-reckoning back home cannot be inferred and then previously explored terrestrial cues are there to help out. Details of what constitutes such terrestrial cues are still uninvestigated.

Peripheral navigation. The recently identified and labeled peripheral navigation takes over from distal navigation at a distance of less than 100m from the hives (Palikij et al. 2011). On sunny days peripheral navigation strikingly differs from distal navigation by switching from dead-reckoning to the use of known terrestrial navigation cues that surround the hive. This is experimentally demonstrated by displacing bees within the periphery: instead of virtual navigation, as in distal navigation, the displaced bees recognize their new location and then aim correctly in the direction of their hive (Palikij et al. 2011). From this performance one can infer the existence in the bee's memory the representations of places and associated compass directions. But few details are known.

By far the most thorough research on euaculeate peripheral navigation is that of Baerends (1941) on the sandwasp (*Amophila pubescens*). This species cares simultaneously for several larvae in several nests. The wasp inspects each nest and renders them invisible by plugging and spreading sand over them in order to prevent robbing. Depending on need, the larvae are fed with caterpillars hunted in the surrounding heath vegetation. The hunting area is about the same size as the peripheral navigation area of the honeybee. Displaced within the hunting area the prey carrying wasps can return straight to their nests nest from any direction. Displacing artificial terrestrial cues near the nest during absence of the wasp causes the wasp to search for its nest on a false place away from its true nest, matching the displacement distance of the artificial cues. Similarly in honeybees: displacing a single conspicuous landmark near a hive in an otherwise barren surrounding misguides the homing foragers (Gasbichler 1968)

The area of peripheral navigation in honeybee has been called *peripheral correction area*, assuming that its function is to correct inevitable long-distance dead-reckoning errors (Palikij et al. 2011). This idea is supported by the observation that satiated displaced homing bees switch near the hive from dead reckoning to a correcting, landmark-based approach to the hive (Geiger et al 1995).

Peripheral navigation near a feeding location, similar to the peripheral navigation near the hive, is suggested by some displacement experiments of Dyer et al 1993) Bees departing from the hive were displaced to three locations near a previously visited feeder. Instead of the expected virtual orientation (dead reckoning) after such a displacement they turned from all three places directly toward the feeder, suggesting peripheral, landmark-based navigation. By contrast, when displaced far from the known feeder location they deployed dead reckoning as expected.

Focal Navigation. Focal navigation controls the final approach and touchdown on the target, nest or feeder. The close distance to a known goal location greatly facilitates experimental research and more details are known than can be reviewed here. The distinction between peripheral and focal navigation is reminiscent of Collett's (1992) suggestion of two types of landmark memory: One of distal cues to find the approximate goal location and one for close, focal visual landmarks for pinpointing the goal.

THREE DISTINCT PATTERNS OF EXPLORATION

A well-honed foraging roundtrip requires considerable spatial knowledge, which is systematically acquired in distinct flight patterns of exploration, which are typical and exclusive for flying Euaculeata. The three sequential phases of navigation are matched in reverse order by three distinct phases of exploration*: focal exploration*, *peripheral exploration* and *distal exploration*. These three phases of exploration have modular properties. They can be chained together sequentially or can be deployed in isolation, depending on need. Observing but one of the three phases in a particular context never rules out the optional use of the others in a different context.

The first to observe and correctly explain aculeate exploration flights was the traveling naturalist Bates (1863) who observed this "circling" behavior in the digger wasp *Microbembex monodonta* after finishing its nest and before leaving for a hunt. Since then, similar and fairly stereotyped focal and peripheral exploration flights have been described exclusively in such a large

number of different flying euaculeate species that these action patterns can be safely called shared, derived characters (synapomorphies) of this taxon (Jander 1997). Due to difficulties of tracking high flying insects, reports about distal exploration are rare and incomplete.

Focal exploration. Focal exploration in the honeybee takes place close to the hive entrance or close to a rich feeding resource. It is the easiest exploration phase to observe and hence most thoroughly studied (e.g. Vollbehr 1975, Capaldi & Dyer 19990, Lehrer 1991, Lehrer 93). Still, research on honeybee focal navigation is no match to the meticulous, in depth research by Zeil (2003a,b) on the near-nest focal exploration and navigation of digger-wasps of the genus *Cerceris*, and by Philippides et al. 2013) of the near-nest focal and peripheral exploration of the bumblebee *Bombus terrestris.* In the latter's terminology "zigzag motive" stands of focal exploration and "looping motive" for peripheral exploration.

Focal exploration takes time and energy. Environmental novelty is required to motivate it. Honeybee focal exploration may tacitly take place while approaching a target, but is really conspicuous when leaving the hive or a just discovered food source. The departing bee immediately turns around and faces the point of departure. It then laterally flies left-right alternating arcs while continuously and approximately facing the point of departure. Gradually the alternating arcs increase in amplitude and height up to a point when the focal exploration terminates. Somehow during this process the bees gains knowledge about the near-goal cues used in focal navigation. To be reliable, several bouts of focal navigation might be required. At the end of a focal navigation bout the bee may decide to return to the starting location, or it may depart straight, or it may start peripheral exploration.

Peripheral Exploration. Peripheral exploration in honeybees may follow focal exploration or starts immediately in a novel environment prior to departing. The flight pattern is sharply different from that of focal exploration. The bee flies forward instead of laterally and circles around the departure location instead of oscillating between left and right. The incomplete circles (arcs) alternate between clockwise and counterclockwise rotation with the switch made by a tight-turns. The circles increase in diameter and height which makes it easy to lose sight of the bee. Video-analysis of the initial near-nest peripheral exploration circles in a bumblebee disclosed that at the switch-points between the peripheral exploration arcs the bee faces in the direction of its nest and may at this points sample and encode discrete sets of terrestrial cues to be used later in guiding peripheral navigation home (Phillipides et al. 2013).

Convincing evidence for the link between peripheral exploration and peripheral navigation came from a study by Opfinger (1931). She discovered that foraging bees at her feeding stations learned about focal navigation cues (odor, visual pattern, color) during the approach flight and conducted peripheral exploration when departing. Taking advantage of this fortuitous separation of focal and peripheral exploration she displaced 123 marked feeding bees from the arrival location to a novel location some 15m away where they all engaged in peripheral exploration before heading towards their hive. When retuning thereafter to the feeding area, 101 bees indeed searched at the displaced new location for the missing focal navigation cues, undoubtedly guided there by the peripheral navigation cues explored prior to the preceding flight home; the few remaining displaced bees ignored their recent peripheral experience and returned directly to their old feeding location.

Distal Exploration. Until recently there was no way to investigate distal exploration because exploring bees quickly moved out of sight. This limitation changed dramatically with the introduction of harmonic radar tracking of freely flying bees. First recordings of distal exploration are now available. Bees in a novel environment explore greater distances from their hive by flying out in hairpin loops. Each excursion is restricted to a narrow sector. Gradually loops cover greater and greater distances (Capaldi et al. 2000). Much has still to be learned about distal exploration such as the transition from exploration to foraging. Also, is distal exploration restricted to the hive? Is distal exploration similarly shaped in other species?

HIGHER ORDER NAVIGATION SKILLS

The navigation performance of individual honeybees can be more complex than the simple shuttling between two places. Various components of the navigation system can be combined into higher-order navigational structures. Three cases are worth mentioning:

The Multi-Hub System. A hub in the navigation system of the honeybee is a place where the bee is apt to choose one among two more outward journeys towards different goals, contingent on various types of information. The hive is the primary hub. Here bees may choose between different simple roundtrips depending on the time of the day or some reminding odor (Wahl 1932, Lindauer 1960, Reinhard et al. 2006).

Foraging bees may also establish secondary hubs away from the hive. A secondary hub can be a feeding site where the bees can learn to depart towards

one or more alternative feeding site if the current one is depleted or they decide to fly back home if they could fill their crop locally (Najera et al. 2012).

Honeybees are fast and flexible in establishing new primary hubs when establishing a swam site or when a swarm had moved into a new nesting site (Dyer 1993, Robinson & Dyer 1993).

Trap-Lining. Nectaries keep secreting. For this, it makes sense for a nectar foraging bee to journey sequentially to the same blossoms and single flowering plants at different locations, a foraging strategy referred to as trap-lining. In honeybees trap-ling has not yet been thoroughly studied, instead, such an exemplary research on the bumblebee *Bombus terrestris* provides valuable insight that most likely applies to honeybees as well (Lihoreau et al. 2012). Over repeated roundtrips individual bumblebees combine exploration with navigation. They discover thereby continuously productive artificial blossoms, gradually reduces errors of repeat visits and compute shortcut routes from blossom to blossom. It is reasonable to assume that the trap-lining bee iteratively repeats the distal-peripheral-focal navigation sequence.

Traversing Mazes. Traversing mazes, like trap-lining, requires repetitive deployment of navigation skills. In mazes the bees keep repeating focal navigation, which involves the use fine-grained focal navigation vision in order to link distinct visual stimuli with left-right choices at branches (e.g. Weiss 1953,1954a, Zhang et al. 1992, Zhang et al. 2000). Entrance and return routes in mazes have to be learned independently (Weiss 1954b).

Due to the iterative use of high-resolution pattern vision in focal navigation, mazes are excellent tools in studying pattern vision and even concept learning in honeybees (Srinivasan & Zhang 1998, Zhang 2006, Horridge 2009).

OVERALL CONCLUSION

The three-component theory of navigation, as advanced here, applies to honeybees and the other nesting and flying bees and wasps (Euaculeata). This theory is a powerful descriptive, predictive terminological and conceptual tool that coherently links together a large body of ethological (behavioral) experimental findings. There is no other competing theory similarly covering the same body of knowledge.

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