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DOCTORAL THESIS RESEARCH

INFESTATION AND DISTRIBUTION OF *VARROA DESTRUCTOR* IN HONEYBEE BROOD COMBS OF *APIS MELLIFERA LIGUSTICA*

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"If the bee disappeared off the face of the earth,
man would only have four years left to live."
— Maurice Maeterlinck, The Life of the Bee, 1901
Nobel Prize

INFESTATION AND DISTRIBUTION OF *VARROA DESTRUCTOR* IN HONEYBEE BROOD COMBS OF *APIS MELLIFERA LIGUSTICA*

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SUMMARY

INTRODUCTION

Recently, the decline of honey bees has focused much interest across the Planet. The bees are dying for several causes, not always completely identified. The so called Colony collapse disorder (CCD) is a combination of factors that include loss of habitat, climate change, immunodeficiencies, malnutrition, various pathogens, genetic factors, changing beekeeping practices and the indiscriminate use of pesticides, primarily neonicotinoids. Among these, the impact of the parasitic mite *Varroa destructor* and its ability to transmit viral pathogens has been long thought to be a major cause. At a World level, the mite *Varroa destructor* is considered to be the *Apis mellifera* number one enemy. *Varroa destructor*, of Eastern origin, was recently distinguished only by *Varroa jacobsoni*. Infact, studies conducted on mites from different parts of Asia (Anderson and Truemam 2000) at morphological and mitochondrial DNA have shown that under the binomial *V. jacobsoni* lurked a complex of about five species of which only two of these have been officially described.

The mite *Varroa destructor* has adapted the Eastern Europe *Apis cerana* bee, which has developed defense systems for a long time. It has been noticed, for example, that our Western honey bee, has more difficulty on mite parasite cleanse than the Eastern bee. Therefore, European bee results much less prepared in implementing physiological and behavioral mechanisms of self-defense (Rosenkranz et al., 2010). The mite transfers the virus from diseased bees to healthy ones. The saliva of the mite also creates immunodeficiency to the bee, which is therefore no longer able to implement defensive barriers. There are presently no cure for viral diseases.

In the environmental conditions that favour the constant presence of brood in hives, the only option is to maintain the infestation of Varroa to an acceptable level (threshold) by adopting a constant monitoring of the infestation, and a decision-making process for control of parasite according to Integrated Pest Management (IPM) or Best Management Practices (BMP's) strategies to prevent the collapse of the colony. This can be achieved also by adopting a model of mite population dynamics on bee brood that contemplates the current behaviors of Varroa mothers on bee brood infestation, rather than just the distinction between male and female brood. Though another aspect must be considered, namely the phenomenon of the mite multi infestation and its aggregate or contagious distribution (Floris, 1991, 1997) in the brood cells and the influence that plays in the reproduction of the mite.

The aim of this study was to investigate and better define the evolution of the Varroa infestation in the hives of *Apis mellifera ligustica*, and develop control strategies with the aim to prevent the negative effects due to this parasitic mite.

In the brood, the parasites may present different degrees or levels of infestation: less than 5% indicates a quantity of parasites low enough not to warrant treatment; 25% or more, indicates a severe infestation, which requires immediate treatment. However, if 10% or more of the inspected cells contains Varroa, the treatment is recommended (Parkman *et al.,* 2002).

CHAPTER I

THE MITE *Varroa destructor*

The *Varroa destructor* is an ectoparasitic mite and represents the most important parasitic disease of honeybees. It is responsible of serious damage to beekeeping, as it attacks either brood and adult bees. For unknown reasons, the mite first parasitizes the larva and then becomes phoretic. It is only involved with the Apinae species but not with the Meliponinae, better known as sting-less bees (Eichwort, 1994).

V. destructor mites (Anderson and Trueman, 2000) and its congeneric *V. jacobsoni* (Oudermans, 1904) are of Southeastern Asia origin and were natural parasites of Eastern bee, *Apis cerana*.

During the first half of the last century, due to the commercialization of honeybees (nucleus or packages of bees and queens) and because of the nomadism towards Southeastern Asia the *A. mellifera* came into contact with *V. destructor*, and this has caused the dispersion in the world.

The jump to *A. mellifera* probably took first place in the Philippines in the early 1960s where imported *Apis mellifera* came into close contact with infected *Apis cerana*. Until 2000 all the mites found on honeybees were erroneously classified as *Varroa jacobsoni*.

Indeed, after meticulous study, the scientific community came to the conclusion that the mite attacking the *Apis mellifera* was different from the one that bred on *Apis cerana*, and realized that they were two different species.

It followed that the *V. destructor*, on the *A. cerana* has maintained the old nomenclature, *V. jacobsoni*. The two species are morphologically similar but vary in size and shape of the body: *V. jacobsoni* is smaller and has a more circular shape compared to *V. destructor*.

Yet, to validate the study and demonstrate the presence of two different species of mites, tests on mitochondrial DNA, the gene sequence (CO-I) were carried out. About 18 haplotypes were identified (Anderson and Trueman, 2000; Warrit et al., 2006), but only two of them are considered dangerous to the honeybee: the Japanese/Thai mites imported into South America are considered less aggressive (de Guzman and Rinderer, 1999) and the Korean ones (in Europe, Africa and Asia) are considered more virulent (de Guzman et al., 1998; Anderson and Trueman, 2000; Garrigo et al., 2003).

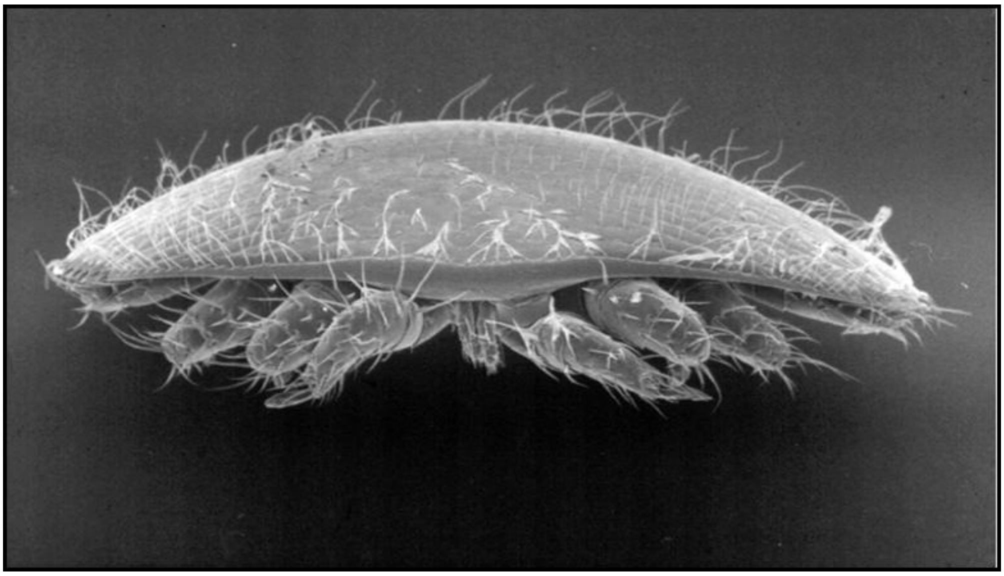

Fig. 1 - The female of *Varroa destructor* in frontal view

Classification of the mite *Varroa destructor*

Phylum: Arthropoda; **Subphylum** Chelicerata; **Class**: Arachnida; **Order**: Mesostigmata; **Family**: Varroidae, which included two **General** *Euvarroa* and *Varroa.*

Euvarroa Delfinado and Baker

- *Euvarroa sinhai* Delfinado and Baker
- *Euvarroa wongsirii* Lekprayoon and Tangkanasing.

Varroa Oudemans

The Genus *Varroa* is actually represented by four species, all ectoparasitic obligate mites:

1) *V. jacobsoni* Oudemans: ectoparassitic described on *A. cerana* in Djava (Oudemans, 1904) and on A. *nigrocincta* in Indonesia (Anderson and Trueman, 2000; Hadisoesilo and Otis, 1998).

2) *V. destructor,* described for *A. cerana* and for the new host*: A. mellifera,* wrongly considered *V. jacobsoni.* (Anderson e Trueman, 2000; Anderson, 2000) (Fig. 1)

3) *V. rindereri,* described on *A. koschevnikovi* in Borneo (De Guzman and Delfinado-Baker, 1996).

4) *V. underwoodi* was indicated on *A. cerana* in Nepal (Delfinado-Baker and Aggarwal, 1987).

Ontogenesis

The lifecycle of *V. destructor* (Fig. 2) starts when the female mite lays fertilized eggs into the bee brood cell (Donzé *et al.,* 1994, 1998). Eggs are white in colour and oval in shape, measure about 0,30 mm in lengthng and 0,23 in width. Eggs generally cannot be seen by the unaided eye. *V. destructor* develops from egg with different stages and moults before becoming adult. After 24 hours a hexapod larval develops inside the egg.

This larva becomes a protonymph hatching out from the egg after other 24 hours. During the protonymph phase the male and the female are undistinguishable without dissection. The stage lasts three days for the male mite and five days for the female mite. The protonymph has a circular and transparent white body, eight legs and pointed chelicerae which measure 0,70 mm in length. The protonymph passes through the deutonymph stage. This stage takes about two days for both sexes (Colin, 1982) and resemble the adult males (Smirnov, 1978; Hirschmann, 1990), but with a reduction in setae. The female mite has finished its development when it acquires a dark brown color. Approximately, since the egg deposition to formation of adult parasites it takes about 7-9 days for males and females.

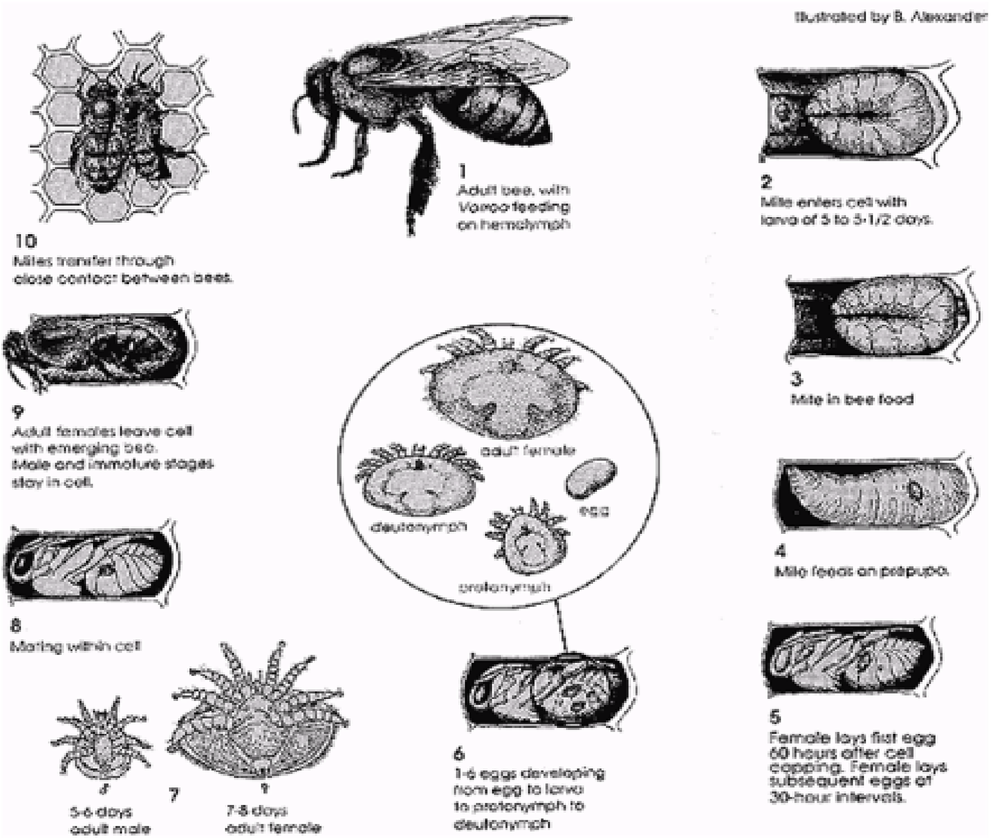

Fig. 2 - Life-cycle of *Varroa destructor*

Morphology:

The mite *V. destructor* is featured by sexual dimorphism (Infantidis, 1983) (Fig. 3) with a series of adaptations to their host. The Varroa female is reddish-brown to dark and oval in shape, measuring 1,00 to 1,77 mm in length and 1,50 to 1,99 mm in width. The body is sheltered by one dorsal hairy shield, highly sclerotised and different ventral shields, also haired. Ventral shields connected by the thin and flexible membranes permit to extend during feeding and egg formation.

Adult males are yellowish with lightly tanned legs, spherical body shape and weakly sclerotised, measuring 0,75 to 0,98 mm in length and 0,70 to 0,88 in width.

Fig. 3 - The female and the male of *Varroa destructor*
(http://www.regione.vda.it)

Anatomy:

The body of the mite is divided in two parts, the gnathosoma and the idiosoma in both sexes. The gnathosoma is the most changing part of an organism body in function of life type and food and which permits the adaptability to different environments. In female mite, the gnathosoma is situated antero-ventrally comprising two chelicerae and two sensory pedipalps. It is known as biting and sucking mouthparts. Chelicerae have different functions and shapes in male and female mite. The parts are formed by three segments: the basal, the middle and the distal digit. In the female mite, the chelicerae measure about 15 µm.

The idiosoma is the largest part of the mite body, which bears the legs, the respiratory pores, the peritreme, the genital and the anal openings and an variety of tactile and sensory structures.

In Varroa female, the locomotion structure consists of four short and strong pair of legs in the antero-laterally body position. The first pair of legs have a sensory pit organ, the other three pairs are used for movement. The last digits are transformed in the adhesive suckers, apotele, which permit to the female mite to move into the hive and to adhere to the host (Rujter and Kaas, 1983). In mesostigmatic mites, the respiratory structure is formed by the internal tracheal system which flows into a peritreme. The peritreme has several tooth-like projections. They are surrendered of special cells, located laterally over coxa III and protected (enclosed) within a peritrematal shield which has a opening at the end.

In addition, they increase in size to facilitate gas exchange into the brood cell or to adjust to different atmospheric conditions as in the case of increased dioxide carbonic (Liu, 1986).

In *Varroa destructor*, the mouthparts or gnathosoma is located between the first pair of legs. In male, the distal digit in chelicerae are modified into a spermatodactyl for transferring sperm to the female genital tract, during the mating. The males of Varroa are not capable to feed themselves therefore after various mating they will die.

Pedipalps of the female mite are used to anchor in the bee body. Chelicerae have two teeth useful to cut the bee adult chitin, larval and pupae cuticle.

The muscular pharynx allows the parasite to suck the haemolimph on the host. The female mite introduces the saliva, during this action it may transmit pathogens to bee hive. The amount of hemolimph swallowed depends on the period of the year.

The digestive apparatus consists of an oesophagus, of a median intestine with six blind intestines and Malpighi's tubuli which arrive to recto intestine until the anal orifice. The female mother mite constantly defecates in the brood cell. This action creates a particular site with protection role for son and daughters mites.

The nervous central system of Varroa is formed by a ganglion under the oesaphagus which is covered of cortical membrane (Giacomelli *et. al.,* 2013).

In relation to the biology of the mite, the sensory organs have great importance. This structures are represented by hairs, pits, setae and sensilla that are localized in the whole body including legs and mouthparts.

On the first pair of legs is located a complex sensory organ within a cavity, eighteen sensilla, divided in nine long hairs and nine internal sensilla. The structure is similar to the Haller's organ in ticks (Ramm and Böckeler, 1989) that are used like the insects antennae (Rickli *et al.* 1992). perception of volatiles. The sensilla have various functions and are divided in different groups according to the role: mechano and chemoreceptive functions (Milani and Nannelli, 1988; Ramm and Böckeler, 1989; Kirchner, 1993; Endris and Baker, 1993); hygro and thermo-receptors (Dillier *et al.,* 2006). Other studies are made to examine and described the female mite nymph's structure of the setae present on the leg I, associated with volatiles (Zakaria *et al.*, 2009).

Reproductive System of *V. destructor*

In male and female mite, the genital orifices are located between the second pairs of legs, in the genital-plate. The female reproducing apparatus is formed by two systems: the first one with the uterus, ovary and vagina which emerge towards the external opening to release the fertilized eggs. The second part contains: a pair of sperm induction pores, the solenostomes, that are located on each side between coxae III and IV. They are connected with two tubuli, two rami and a duct to a large sac-like organ, the spermotheca (De Ruijner and Kaas, 1983), which has the function to receive, store and mature the sperm received during the mating so it can fertilize the egg cells. This is particularly an advantage when there are a limited number of males in a population as in *V. destructor*. The camera spermatica causes the connection with the ovary, the uterus and the spermotheca. Attached to the ovary, close to the ventral spermotheca area, there are two lyrate organs which have a nutritional function for the oocytes, similar to telotrophic ovarioles of some insects (Alberti and Zeck-Kapp, 1986).

The male genital system consists by a single testis in the rear of the body. The testis is connected to two vasa deferentia which arrive to a ductus ejaculatorius up to the external edge of the opening. Furthermore, an accessory gland produces proteinaceous secretion (Alberti and Hänel, 1986; Di Palma *et al.*, 2013). The sperms are classified ribbon type (Alberti, 1980a, 1980b) matured after eigth stages, six in the body of the male and two on spermatheca of the female mite, after sperm transferral.

The fertility of *V. destructor*

The fertility of *V. destructor* is influenced by different factors. The real reproductive rate of *V. destructor* (= number of viable adult offspring per parental mite) depends on mite fertility (reproduction yes or no) and fecundity (= number of offspring per reproductive cycle), under natural conditions. Varroa mother throughout her life can lay inside the brood cell, up to 7 eggs at intervals of 30 hours, in laboratory conditions (De Ruijter, 1987). Mother mites can lay 2-3 eggs in natural conditions (Fries and Rosenkranz, 1996; Martin and Kemp, 1997).

The reproduction rate is of 1.3-1.45 in single infested worker brood and of 2.2-2.6 in drone brood. This fact is due to the capping in different periods (Martin, 1994, 1995a, 1995b).

In the original host, *Apis cerana,* the production of the two Varroa mites, *V. jacobsoni* and *V. destructor* is limited to drone brood, for the unknown causes (Anderson, 2000; Boot, et al., 1996; Garrido, 2004, Rath, 1999; Rosenkranz et al., 2010). Observations performed in Uruguayon colonies of *A. mellifera* belonging to a subspecies not well defined, showed that bees survived in absence of acaricides treatments due to the inability of varroa mite to produce adult offspring in worker bee cells (Ruttner *et al.,* 1984). Instead the two haplotypes of the *V. destructor* can reproduce on *A. mellifera* both on drone and worker brood (Muňoz *et al.,* 2008).

The mite *V. destructor* on *A. cerana*, reproduces mostly on male brood (Koeniger *et al.*, 1981; Anderson, 2000; Boot *et al.,* 1996; Garrido, 2004), rarely in female broods (De Jong, 1988).However, it doesn't represent any harm that may affect the survival of the hive, whiereas in *A. mellifera* the attack of mites appears to be catastrophic for the bees' survival.

This, according to some studies, is due to the fact that *A. mellifera* is a new parasitic host, whereas in the case of *A. cerana* there is a balanced host-parasite relation of co-evolution, established over time (Rath, 1999), a condition manifested by particular behaviors among Eastern bees against the mite that consists in «grooming behavior» (Büchler *et al.,* 1992) and «removal behavior» (Büchler, 1994). It was suggested that in workers of feral population of Africanized honey bees in tropical America, the fertility of varroa female is lower (Camazine, 1986; De Jong, 1996; Rosenkranz and Engels, 1994a). This phenomenon was not confirmed by other studies (Marcangeli *et al,* 1992; Medina and Martin, 1999; Medina et al., 2002). Recently, it has been seen that the fertility of the mite in the workers brood of Africanized bees has increased to similar levels of European bees (Carneiro *et al.,* 2007; Correa-Marques *et al.,* 2003; Garrido *et al.,* 2003). The causes of infertility of Varroa are unknown. However this is probably probably related to the host and to the fact that not all fertile Varroa females, i.e. female which lay at least one egg, are really reproducing succesfully. Female mites producing only one egg, with no males, or with delayed start of oviposition may not contribute to the growth of Varroa population; in 11-21% of the brood cells the male is missing (Donzè *et al.*, 1996; Martin *et al.,* 1997). Several studies have shown that the mortality of mites is influenced by climate, season and honey bee subspecies (Eguaras et al., 1995; Ifatidis *et al.,* 1999; Mondragòn *et al* 2005, 2006). The reproductive rate depends on the infestation of single brood cell; in fact in multiple invaded drone and worker brood cell the reproductive rate per female mite is significantly reduced (Fuchs and Langenbach, 1989; Martin, 1995b; Martin and Medina, 2004; Mondragòn et al., 2006; Rosenkranz *et al.,* 2010).

Fig. 4 - The female of *Varroa destructor* in ventral view

Mite orientation in the host

The female of this ectoparasitic mite lives attached to the body of its host, either adult or larva inside the capped bee cells, through its biting mouthparts with which it penetrates the cuticle inter-segmental exoskeleton membranes to suck the hemolymph. Frequently, it can also be localized between the head and the thorax. Most of her life is lived within the operculated cells, with a marked preference for the dark honey bee nest (Boot *et al.,* 1993). The sensory mechanisms by which the mites come in contact with its host are not well known. Instead,e their perception to light and vibrations are clearer (Kirchner, 1993). *V. destructor* preferably invades the colder cells, those located in the periphery with temperatures between 26 and 33 ° C (Le Conte and Arnold, 1987, 1988; Rosenkranz, 1988), compared to the most internal which have a temperature between 34.5 and 35 °C (Becher and Moritz, 2009; Rosenkranz and Engels, 1994), although more recent studies do not confirm this hypothesis (Dillier, 2006)

The effect of the ectoparasitic mite, *V. destructor* on emergence adult worker honeybee *A. mellifera* is on weight, reserve of water, protein, carbohydrate, and lipid levels (Bowen Walker, 2001). The mite leverages the resources taken away apparently inefficiently (Garedew *et al.,* 2004). In a recent study it has been observed that the subtraction of hemolymph alters the chemical composition of the cuticle of parasitized bees by depriving them of useful elements which prevent its protective function from outside dangers, such asdehydration, which could partly explain the reduced longevity of bees (Annoscia *et al.,* 2012).

Varroa is specialized in living in perfect synchronization with its host. For its spread it is dependant on adult bees, with a preference for the nurse bees shortly after living the brood cell on an emerging young bee for transport to the brood cells (Knaus, 1993; Kuenen and Calderone, 1997), which the nurses bees are the most haunted in the colony for. According to various studies on the choice of the mite towards hosts at different stages of development, the female mite prefers the brood of drones, which is from 8 to 10 times more infested than the workers (Fuchs, 1990; Boot *et al.,* 1995b; Calderone and Kuenen 2001). Usually, two to four adult females of Varroa come out from a drone cell, compared only to one or two from the worker cells. Varroa enters inside the drone cells about 40-50 hours prior to cell capping, whereas in worker cells just 15-20 hours before (Boot *et al.*, 1992). The attraction of the mite for different fractions of larval cuticle extracts is ascertained, in particular for anester secreted by the larva at 5th instar, when the cell is capped (Le Conte *et al.*, 1989). The greater attractiveness of the male brood and the greater time spent on this brood seem related to to the fact that the drone larvae produces slightly higher quantities of three methyl- and ethyl esters over a longer time period (Calderone and Lin, 2001; Le Conte *et al.,* 1989). Italian researches have detected the presence of a strong attractive content in the larval food,later identified as acid 2-hydroxyhexanoic (Nazzi *et al.*, 2004). The presence of octanoic acid in the royal jelly of queen larvae cells, has a repellent action (Nazzi *et al.*, 2009). This would explain the low infestation in queen cells (Harizanis, 1991). The production of specific hydrocarbon patterns in 4th and 5th instar larvae could differ considerably, and it coud be used by the mite for chemotactic recognition of the suitable host stage (Aumeier et al., 2002; Rosenkranz *et al.*, 2010). Therefore Varroa female would be able to choose the suitable 5th instar larva when it secretes the highest level of esters. Beside chemical cues, the choice of the mite could be influenced by non-chemical factors.

Other studies showed that the distribution of the mite inside the brood cells is affected by cell size and cell height, as well as by the age of the brood cell .

The behavior of the mite was assessed during tests on the manipulation of brood cells (Boot *et. al,* 1995a; De Ruijter and Calis, 1988; Kuenen and Calderone, 2000). It has also been shown that when a worker bee larva is placed in a drone cell, the mite infestation is lower than normal and probably this is related to the distance between the larva and and rim cell (Calderone and Kuenen, 2001). Considering the age of the honeycombs, those which have hosted more cycles of brood are more susceptible to infestation (Piccirillo and De Jong, 2004). However, in spite of all these tests, it is not yet clear the reason why Varroa is found under natural conditions most frequently in brood cells than on adults (Boot *et al.*, 1993; Martin *et al.*, 1998), whereas in laboratoryr tests adult bees were always more attractive than any larval stage (LeDoux *et al.*, 2000; Zetlmeisl and Rosenkranz, 1994). In other experiments performed under natural conditions, it was shown that during summer up to 90% of the mite population can be within the brood (Rosenkranz and Renz, 2003).

In infested brood combs, it may happen to find capped brood cells with 2, 3, or more mites (Fig. 5), next to brood cells uninfested. The distribution of mites on larvae is not random, but aggregated (Floris, 1991; Floris, 1997), also in laboratory (Milani and Chiesa, 1990). The reasons for the phenomenon of aggregation of the mites are not yet clear.

Fig. 5 - Mites feed on prepupa

Spread of the mite in different continents on colonies of *Apis mellifera*:

Diffusion in Italy.

In Italy the first discovery of *V. destructor*, then incorrectly called *V. jacbsoni* was in a apiary in Staranzano (Gorizia province) and it occurred in June 16, 1981.

Introduction around the world.

The mite was found in former USSR since 1953; in Pakistan, in 1955; in Japan, in 1958 (Smirnov, 1978); in China in 1959; in Bulgaria in 1967; in Brazil about in 1971; in Germany, in 1977 (Ruttner and Ritter, 1980); in Poland, in 1980; in France, in 1982; Switzerland and Spain, in 1984; in Portugal, in 1987; in USA, in1987 (Oldroyd, 1999); in Canada, in 1989; in United Kingdom, in 1992 ((Thompson, 2002); New Zealand (North Island), in 2000; New Zealand (South Island), in 2006 ("Varroa Mite, *Varroa destructor*". MAF Biosecurity New Zealand, 2009); in Hawaii, in 2007 (Oahu, Hawaii Island) (Wu, 2007; "Varroa Mite Information". State of Hawaii, 2013).No presence is yet recorded from Australia, except report on a ship docked in the port of Sidney, who was carrying thousands of Asian bees parasitized of the mite[1].

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[1] (http://www.abc.net.au/am/content/2012/s3639182.htm).

As of mid-2012, Australia was thought to be free of the mite (Holland, 2012; Jopson, 2012). In early 2010, an isolated subspecies of bee was discovered in Kufra (Southeastern Libya) that appears to be free of the mite (Paxton R. and Moritz R., 2010).

CHAPTER II

COLONY COLLAPSE DISORDER (CCD)

Colony Collapse Disorder (CCD) in USA.

During ten or more years the beekeeping world is facing a serious problem due to the disappearance, for not well-known reasons, of large quantities of adult worker bees of *Apis mellifera* L. (foragers) (Ellis *et al.,* 2010). The honey bees go out to forage the nectar and the pollen, but they never return to the hive. The hive deprived of worker bees shows specific signs not associated with those produced by known pathogens and pests of bees (Annoscia, 2011). To identify the phenomenon, American scientists have coined the term Colony Collapse Disorder, CCD (VanEngelsdorp *et al.*, 2008). However, it must be emphasized that the acronym CCD is related to the decline in honey bee health which is a complex problem caused by a combination of stressors (VanEngelsdorp *et al.*, 2009) and not as the main cause of bees mortality. In fact, the syndrome is positioned in 8th place in the list of the possible adversities of honeybees (VanEngelsdorp *et al.*, 2010, Floris and Satta, 2013). The CCD was recognized for the first time in bee populations in North America at the end of 2006 (Oldroyd, 2007) they were events comparable to the current CCD, but to a lower level (Underwood and VanEngelsdorp, 2007). Some beekeepers began reporting losses of 30-90 percent of their hives. The winter season affects a certain amount of bees, as it is almost physiological, but the magnitude of reported losses was unusually high. However, it is not the first time that disappearance of honey bees occurs, in fact in scientific literature it is known that it occurred in the 1880s, the 1918s, the 1919s (Mraz 1977) and the 1965s (Oertel, 1965), but there are no evidence that can identify the event as CCD. Other losses due to unexplained events are those of about 2000 colonies of bees in 1903, in the Cache Valley in Utah, after a «hard winter and a cold spring» and 53 percent of the colonies in 1995-96, in Pennsylvania (Kaplan, 2012).

Moreover, from 1972 to 2006, in the USA a dramatic reduction in the number of feral honey bees (now almost extinct; *A. mellifera* is not a native species of the American continent, where it was introduced by man) was also recorded (Watanabe, 1994; Klein et al., 2007). There are at least 17.000 other bee species globally (Michener 2000) and many of these are known to pollinate crops, either in situ as an ecosystem service (Ricketts et al. 2008), or potentially as managed species (Kevan *et al.* 1990). In the past, pumpkins and other cucurbits were pollinated by wild bees who built the nest in the ground as the *Peponapis pruinosa* Dire (Tepedino, 1981), whose population has now been reduced, probably because of their sensitivity to pesticides and plowing action (Splawski, 2012).

Crops of modern agriculture, especially monocultures, depend on the work of bees, at least 39 plants grown on 59 (Klein *et al.*, 2007). Presently, the honeybee *A. mellifera* is the most economically important pollinator of crop monocultures worldwide (McGregor 1976; Watanabe 1994; Klein *et al.*, 2007), as for some fruit, seed and nut crops (Southwick and Southwick 1992; Klein *et al.*, 2007) and for coffee in Panama (Roubik, 2002; Klein *et al.*, 2007). Currently there are at least 90 crops of great commercial interest in North America: 87 of the main food crops, among the 115 taken into account (www.federapi.biz). During the last century, there was a increase of 50 % in honey production and an increase of 300 % of the service of pollination (Aizen and Harder, 2009; Floris and Satta, 2013). The use of the pollination service has an impact on the American economy, more than 14 million dollars for each year (Morse and Calderone, 2000; VanEngelsdorp *et al.*, 2008), with their value topping $ 215 billion worldwide (Gallai *et al* ., 2009; VanEngelsdorp *et al.*, 2008).

In the USA, the monitoring service on honey bees health is performed each year by the Agricultural Research Service (ARS), USDA's Internal Research Agency, the United States Departement of Agriculture, US Environmental Protection Agency (EPA), other Federal Agencies, the National Institute of Food and Agriculture (NIFA), in collaboration with 22 universities and several private research centres.

The processed values are published in the Annual Colony Collapse Disorder Research Progress Reports, related to the losses of bee colonies during winter season: 32% in 2006-2007; 36% in 2007-2008; 29% in 2008/2009; 34% in 2009-2010; 30% in 2010-2011; 21.9% in 2011-2012, 30.5% in 2012-2013 (www.ars.usda.gov.ccd). These values show the precariousness of bee health and the volume of suffered beekeeping damage, where at least a third of these losses are attributed to CCD by beekeepers. Recently, investigations carried out by COLOSS report that between 1th October 2013 and 1th April 2014, the levels of bees die-off were lower than average, and it suggests not to drop the guard down (VanEngelsdorp *et al.* 2014).

The decline of bees in Europe

In recent years, the phenomenon known as the CCD, whose causes are yet not known, has also been observed in Europe. That is the case of honeybee losses reported in the island of Wight (Oldroy, 2007; Underwood and VanEngelsdorp, 2007; Floris and Satta, 2013). Some other times the losses of bees have been improperly charged to the CCD (Ratnieks and Carreck, 2010). Major European beekeepers in Belgium, France, Nederlands, Greece, Italy, Portugal and Spain (Dupont, 2007), followed to a lesser extent by Swiss (Dainat, 2012) and Germany (Steinberger, 2007) complain of huge loss of honey bees colonies. In Europe, there are estimated about 15 millions of beehives, 700.000 beekeepers and a European average of about 2,8 hives per Kmq (Fao and Apimondial, 2008). In Italy, the data indicated 1.560.000 beehives at least, with a variable estimated number between 34.000 and 55.000 consisting of professional beekeepers and hobbyist beekeepers (National Observatory of Honey, 2013).

At a world level the presence of more than 65 millions of beehives belonging to 6,5 millions of beekeepers (Fao and Apimondial, 2008) has been estimated.

The international economic damage caused by the decline of bee populations has been really impressive. The first estimated losses reach one billion euro per year; of which 20 million of euro per year to US, 70 million euro per years to China, 500 million euro per year to Europe and 40 million euro per year to Italy (Date CE, 2008; www.meteowebcam.it). The first depopulation reports in Italian apiaries date back to 1999 (Porrini, 2008). It is very difficult to quantify the real amount of the losses; the estimate has been of 30-40%, with inhomogeneous delivery in the national territory (Mutinelli, 2008, Lodesani, 2013).

In Europe, the damage caused by the decline of bee populations have been on average 20% (by 1,8 to 53%) (Neumann and Carreck, 2010; Floris and Satta, 2013).

It is well known the importance of bees and pollinators for the food chain. The bees also pollinate the forage crops such as alfalfa and clover, which is crucial for the feeding of farmed animals destined to meat production (Celli, 2008).

If pollination is a noun referring to a transfer of pollen from anther (in angiosperms) or male cone (gymnosperms) to stigma or female cone, respectively (Lawrence, 2000; Samnegård, 2011), in Europe, it is estimated that 84% of the 264 crops need pollinating animals (Williams, 1994); about one-third of food crops is pollinated by insects (Kremen *et al.*, 2007), including bees.

In addition, the bees are not only pollinators, they also are producers of the products that the man has learned over the millennia and continues to derive from their breeding (Ratti *et al*., 2012).

The bees are also valuable as a bio-ecological indicators for the detection of heavy metals, radionuclides (Tonelli, 1990), pesticides (Celli, 1994; Celli *et al*., 1996), of *Erwinia amylovora* an bacterial of Rosaceae, pears, apples and other ornamental plants (Ghini *et al*., 2002; Sabatini *et al*., 2002).

The bee can be considered as an open biological micro modelling, suitable for the study of the interactions with the climate (Solimene, 2009).

On the other hand, in order to meet human and animal needs, it has been used like a type of intensive agriculture and monoculture, which leads to the risk that the service of pollination due to wild pollinators is also less exercised due to a reduction of the biodiversity.

The biodiversity is the whole and the variety of all living being, flora and fauna in the ecosystem of the planet and expresses it to level of genes, species and ecological unit. According to some experts, even if honeybees were to increase, they would hardly be able to carry out the activity of wild pollinators (Aizen and Harder, 2009). Recently, it has been estimated that the total value of related pollination-dependent crops amounted to about 265 billion euro (Lautenbach *et al*., 2012). Of course, if the pollinators disappear, the value would be higher. However, it is difficult to know exactly the current conditions and global quantity and diversity of pollinators (Lebuhn *et al*., 2013).

Signs and symptoms

According to the epidemiology, we can speak of a collapsed colony due to the CCD when it shows the following symptoms:

1) Presence of the queen bee. If the queen is not present, in fact, the death of the hive is not due to the CCD;

2) rapid loss of worker bees and the simultaneous presence of capped brood (larvae and pupae abandoned) within the colony; generally the bees never leave the hive until the cells have not been totally disclosed;

3) Lack of worker bees died inside or in proximity of the colonies in collapse phase;

4) Presence of food stocks, both pollen and honey, which have the following features: a) they are not immediately robbed by other bees, (kleptoparasitism), b) the late invasion of parasites and other intruders in the hive colonies collapsed (eg. the wax moth *Galleria mellonella* L. and the small hive beetle *Aethina tumida* Murray,);

5) Low levels (not harmful) of *Varroa* and / or *Nosema* spp.;

A weakening colony is noticed in case of CCD syndrome and it is manifested by:

a) A few bees are able to raise the brood present in the hive;

b) Presence of young bees;

c) Presence of the queen;

d) Difficulty of beekeepers to feed, with appropriate nutrients, the remaining bees (Ellis *et al.*, 2010).

In the event of this phenomenon, the Colony losses due from CCD are a very serious problem for beekeepers so that the number of managed US honey bee colonies dropped from 6 million in 1947 to just 2.5 million today, like in some other parts of the world (Williams *et al.* 1991; Matheson *et al.* 1996; Delaplane and Mayer 2000). There are several factors to consider when estimating the decline:

1) the beekeeping business ending due to the global crisis;

2) the retirement of beekeepers;

3) the human disturbance causing the loss of natural and semi-natural habitats (Kearns *et al.*, 1998; Aizen and Feinsinger, 2003; Goulson et al., 2008; Winfree et al., 2009). The negative effect of habitat loss on biodiversity in

general is well documented (Fahrig, 2003) and acts through a variety of mechanisms which result in decreased reproduction and survival;

4) the bees are negatively affected by human disturbance and pollinators are threatened by increasing human land use (Winfree *et al.*, 2009);

5) genetically modified (GM) crops;

6) stress management, due to the continuous transportation of the colonies;

7) scarcity of a variety of pollen and nectar sources.

In addition to this large list of adversity for the bees, there are further stresses produced by the arrival since 1980s of new pathogens and pests of different kinds (viruses, bacteria and fungi) (Kaplan, 2012), followed in 1990s by the arrival of other terrible mite *V. destructor* (Anderson, 2000) which produces the varroosis, by the tracheal mites the *Acapis woodi* (Downey & Winston 2001; Chen *et al.* 2004), the small hive beetle *Aethina tumida;* the nosemosis type C caused by the microsporidians of *Nosema ceranae* (Higes *et al.* 2006; Rinderer, 2012); accidental or intentional exposure to pesticides at lethal or sub-lethal levels, neonicotinoids: clothianidin, thiamethoxam, imidacloprid (Ingram *et al.* 1996) and various negative impacts of systemic insecticides on pollinators (Hopwood *et al.*, 2012).

Administration of contaminated food with tau-fluvalinate and imidacloprid pesticides has shown serious behavioural disturbances, compared to not contaminated control samples of bees (Bethany *et al.*, 2012). Moeover, it has been shown that exposure of imidacloprid in honey bees results in increased levels of the gut pathogen *Nosema* spp.(Pettis *et al.*, 2012). To face this alarming situation, the American government has allocated several million dollars per year. In 2014 8 million dollars were committed in Conservation Reserve Program (CRP) for farmers and ranchers to the 5 most important States managing honeybees(Michigan, Minnesota, North Dakota, South Dakota and Wisconsin), and 3 million dollars were added by USDA for the

States of the Midwest by the Incentive program of Natural Resources Conservation Service Environmental Quality (www.usda.gov/wps/portal/usda).

The aim of the allocation of this capital is to favour bees by planting specific flowers and plants to implement nectar and pollen sources, as well as public education programs regarding the behaviors to adopt in order to help honeybees. In addition, with funding of $ 50 million President Barack Obama asks for the Environmental Protection Agency and the Department of Agriculture of the United States to make an effort to determine the causes of the decline of bees and other pollinators and find a solution to protect them.

Other possible causes of stress

1) Global climate change

Recent studies on global climate change show that between the 40th and the 70th parallel. since the late nineteenth century to 2008, the average temperature has increased of about 1.3° C. This variation means a change enough to shift the balance between the biotic and abiotic components of the planet (Nanetti, 2008). It is estimated a further increase of 1.5 ° C over the next few decades. The temperature rise is

The 2006-2007 winter has been the warmest ever in the last few decades (Solimene *et al.*, 2009). The process of increasing temperatures is very evident at the North Pole, between December and February. It has been demonstrated that 78% of 542 species of flora and animals have well shown alterations in population dynamics (Menzel *et al.*, 2007).

This phenomenon is caused by an elongation of the Spring and Autumn season to the detriment of the winter season. This would have caused the discrepancies on the phases of flowering and the resumption of activities of the bees; in other words we assume that bees increase their working life from 20 to 35 working days over the year, which would lead to a deficiency of the immune system resulting in stress which makes them more easily attacked by pathogens. A stress process follows several steps: the adaptation phase, the compensation stage and the collapse of the system (Solimene *et al.*, 2009).

Usually after the winter, during the first flights, honeybees, adopt dressing mechanisms against the stress using the elder which is a strong anti inflammatory (Solimene *et al.*, 2009); but because of the mismatch of the biological cycles of plants this ability is lower. In addition, the increasing of temperatures has stimulated the presence of early and repeated brood in the bee hives, in a period during which the queen bee should be at rest. All this has encouraged the proliferation of diseases and parasites on bees, among them the mite *V. destructor* whose biological cycle is closely linked to that of bees. The more the bees breeding season is at long term, the more the mite has available food and their ability to proliferate increases also exponentially (Charrière, 2012, www. swissinfo.ch), making inefficient any formerly taken measures (Solimene *et al.*, 2009).

2) **The fragmentation and degradation of near- and semi-natural habitats**

Another factor of disturbance is the fragmentation and degradation of near- and semi-natural habitats can be detrimental to bee communities (Rathcke and Jules,1994; Kremen *et al.* 2002, 2004; Steffan-Dewenter *et al.* 2002, 2006; Larsen *et al.* 2005; Cane *et al.* 2006; Klein *et al.*, 2007). Wild bees that provide pollination services to vegetable crops depend on forage resources, nesting sites, and overwintering sites in the agricultural landscape (Smith *et al.*, 2013). Setting aside natural areas in the near vicinity of vegetable fields may be an effective way to support wild, crop-visiting bees and secure their pollination services (Smith *et al.*, 2013). The presence of wooded areas around farms, hedgerows and herbaceous field margin is essential for survival of wild bees (Allsopp *et al.*, 2014).

3) **Nutritional stress**

Generally, for their own welfare, bees require several varieties of pollen.

For all three castes pollen is the main source of protein, fat, minerals and vitamins necessary for growth and development. After reaching the adult stage, the bees can, however, live only with honey or sugar, that is, a diet based exclusively on carbs. Studies have shown that without exception, plants that rely solely on insects for pollination produce the highest quality pollen, packing 65% more protein into their pollen than plant species that do not have to rely on insect pollinators (Hanley *et al.*, 2008). With the spread of the monocultures wrongdoing on many wild plants (Johnson *et al.*, 2010), this biodiversity is lower, making their diet deficient, getting nutritional stress and confirming the thesis of the death of the bees (Ognibene, 2007), that would compromise bee's immune system, making them very sensitive to the threat of pathogens (Oldroyd, 2007; Mutinelli and Granato, 2007). Many die-offs of bees are due to hunger, in the winter season (VanEngelsdorp *et al.*, 2010). Therefore the lack of nutrients can be balanced by the administration of nutritional supplements, pollens and proteins (Brodschneider and Crailscheim, 2010).

4) **Genetically modified crops**

Genetically modified crops (GMCs, GM crops, or biotech crops) are plants used in agriculture, the DNA of which has been modified using genetic engineering techniques. The first commercial cultivation of genetically modified on plants of tobacco in 1986, occurred in France and USA (Clive 1996). The technique consists to introduce new genetic material, or alter existing genetic material to introduce intended, new traits or characteristics or traits into organisms, in this case vegetable, as soybean, corn, canola, and cotton seed oil. These have been engineered for resistance to pathogens and herbicides and better nutrient profiles. Studies that independently assessed potential effects of Bt Cry proteins on honey bee survival (or mortality). Results show that Bt Cry proteins used in genetically modified crops commercialized for control of lepidopteran and coleopteran pests do not negatively affect the survival of either honey bee larvae or adults in laboratory settings (Duan *et al.,* 2008). Furthermore, GMOs are linked to new insecticides known as neonicotinoids, which are used for the tanning of seeds. Beekeepers of Ontario(Canada) pointed the finger at a crop of GM corn, for the loss of their 37 million bees. The bees had died a few days after sowing new fields of GM maize in the vicinity of the hives. As demonstrated by Di Prisco et al (2013) the use of neonicotinoid pesticides damages the immune system of bees and their capacity for direction, resulting in mass die-off.

5) **The pathogens and parasites**

Among the pathognes and parasites, a fundamental role is due to the spread of invasive pests such as the *V. destructor* mite, the microsporidians, *Nosema ceranae* and *Nosema apis* (Highes *et al.*, 2006); the tracheal mite *Acarapis woodi* Rennie, who fortunately was no longer reported in Italy for a long time (Mutinelli, 2008).

Among the various problems related to old and new pathogens and parasites spread the following factors are to be considered:

i) interchange between bee families in a territory which is not dependent on man or the individual beekeeper (Panella, 2008);
ii) irresponsible trade facilitated by the small size of the insect for research and / or commercial purposes, dependent occurrence on man or beekeeper. (Panella, 2008).

Even in Italy considerable efforts are been performed to clarify the causes of the bee colonies losses. The results of these activities are distributed by BeeNet program, formerly called ApeNet. Among the studies contemplated to fight against the adversities faced by the bees, the most significant ones to the interaction between the parasitic mite *V. destructor* and deformed wing virus, are DWV (Nazzi *et al.*, 2012; Floris and Satta, 2013), combined with the study of the chronic effects of pesticide residuals (Floris and Satta, 2013).

In the past, investigations have been metagenetically performed with the aid of PCR, in which they were compared to healthy beehives and apiaries presenting phenomenology of CCD type with the purpose of proving their correlation between Israel A PVirus and colonies collapsed (Cox-Foster *et al.* 2007). However, this investigation has not been reflected and there is no evidence on the correlation between IAPV and CCD, because the virus was present in CCD colonies without phenomenology (Anderson and East, 2008). Therefore, in that casethe phenomenon of CCD was not determined by

IAPV, but by the interaction between diseases and other causes of stress (VanEngelsdorp et al., 2009); which causes are hard to detect (Crailsheim *et al.*, 2009; Brodschneider and Crailsheim, 2010).

Recently some studies have shown that a plant-pathogenic RNA virus, the tobacco ringspot virus (TRSV), could replicate and produce virions in honeybees, *Apis mellifera*, resulting in infections that were found throughout the entire body. The same virus has been also found into the gastric cecum of *Varroa* mites, suggesting that Varroa mites may facilitate the spread of TRSV in bees but do not experience systemic invasion (Li *et al.*, 2013). In Italy, the periods of high vulnerability of bees, correspond to the resumption of beekeeping: the first period, from January to February, during the sowing of summer crops and in conjunction with the pesticide treatments and fruit plants for the tanning of the seed corn and the second period, in mid-June, when grapevine treatments against *Scaphoideus titanus* Ball, vector of Grapevine yellows (flavescence dorée) (Sgolastra et al., 2005) often causes high bee mortality in the 3rd period.

Another aspect to consider is the genetic erosion caused by inbreeding. A queen usually is fertilized by a maximum of a dozen drones, if this occurs frequently, it can cause weakness to the colony favoring the birth of workers or weaker queens and less productive. In Europe, there are several species and subspecies of honeybees. In France there is the *Apis mellifera mellifera* or black bee, in Italy the so called Italian bee *Apis mellifera ligustica.* Genetic improvement is practiced only in England where *Apis mellifera ligustica* and *Apis mellifera mellifera* are hybridized, but this does not happen in Italy (Guidorzi, 2014. http://www.salmone.org/).

There is a widespread importation of bees from non-EU countries, but not without consequences. It is estimated that the annual demand of queen bees of *A. mellifera ligustica* varies from 200,000 to 500,000 when there is high mortality of bees. By prudent estimates, not even a third of the Italian queen bees are required. The other queen bees are from the USA, South America, Isle Hawaii, Australia, New Zealand, Israel, Slovenia and even from Morocco and Algeria. In 2008, according to Ministry of Health data, about 5-6000 feature a passport, the other hundreds of thousands of bees are queens with clandestine spread of hybrids, which are less convenient due to the deposition of excessive brood, exaggerated consumption stocks and pollen and limited response to diseases. This import has started in the 80s at the arrival of the mite *Varroa destructor* Anderson in Italian apiaries (Cironi, 2008). This practice has also favored the entrance of the microsporidium *Nosema.*

In recent years the role of the use of pesticides to treat the parasitized bees and the use of new generation agricultural pesticides such as neonicotinoids (Sgolastra *et al*., 2012) that contaminate the pollen (Tudisca, 2014), have been put under the spotlight.

Residues of at least one of 53 pesticides (including 22 insecticides / acaricides, herbicides and two fungicides 29) were identified in trapped pollen and comb pollen samples (beebread) (Greenpeace Research Laboratory, 2014).

There are 22 chemicals specialty used in the tanning of the seed, for the treatment in the open field and for some treatments of ornamental plants (Cironi, 2008). According to the experts, two neonicotinoid insecticides (imidacloprid and acetamiprid) are suspected of causing the deaths of bees and have effects on the human nervous system during development (EFSA, the European Food Safety Authority, 2013).

A study on risk assessment for side-effects of neonicotinoids in bumblebees with and without impairing foraging behaviour resulted in a decrease of pollination, decrease in reproduction and death of the colony, due to lack of food after the sublethal exposure to neonicotinoids (Mommaerts *et al*., 2010; Bortolotti *et al*., 2002). The treatment of maize seeds with systemic molecules represent a significant threat to bees, especially when using thiamethoxam, one of the compounds with the more toxic effects(Tremolada *et al.*, 2010).

Analyzing various degrees of exposure of bees to thiamethoxam using the RFID (Radio-Frequency Identification), a decreased foraging success and survival in the honey bees was shown (Henry, 2012). Laboratory tests have shown increased toxicity of acetamiprid and thiacloprid in hungry bees (Laurino *et al.,* 2011). Interactions between *Nosema* microspores and a neonicotinoid puts at risk the life of the bee colonies honeybees (*Apis mellifera*) (Alaux *et al.*, 2010). Similarly, the confirmation on the high mortality of honey bees that were exposed to sublethal doses of fipronil and thiacloprid previously infected by *Nosema ceranae* was shown (Vidau *et al.*, 2011).

In presence of residual neonicotinoids, the diversity of pollinators, and also other invertebrate fauna, is severely compromised (Van Dijk *et al.* 2013).

Several stressors are able to reduce the efficiency of the immune system by acting on factor NF-kB, exposing the colonies to the risk of collapse induced by viral proliferation (Nazzi *et al.*, 2012).

As a cause of CCD, it has also been suggested the interference caused by technological products (cellular telephones and repeaters). The theory suggests that radiations from mobile phones interferes with bees' navigation systems, preventing the famously homeloving species from finding their way back to their hives (Lean and Shawcross, 2007).

A limited research indicated that radiation from mobile phones could be a possible cause of Colony Collapse Disorder (CCD) in North America and Europe, with bees’ ability to navigate interfered with by mobile phone radiation (Kuhn, 2005, 2006). This theory has been considered, scientifically, unfounded as the heterogenic results and the many uncontrolled variables in the study argue against a causal relationship (GSMA Environmental Insider).

Some initiatives for the study of the bee's burden

Throughout the world, organizations, universities and private structures are trying to investigate the mechanisms that lead to the depopulation of hives.

The European Union, spending 3 million euro, has given the responsibility for monitoring the honey bees to the European Union Reference Laboratory For Bee Health (ANSES).

The methodology of the monitoring carried out by French laboratory ANSES has been strongly criticized because it included only pathogens and not pesticides nor lack of food resources (Pesticide Action Network (PAN) Europe, 2014).

In Italy, this year a fundraising campaign in defense of bees and biodiversity has been set up to raise awareness against pesticides killer of bee and pollinator insects, and they were collected 55,023.83 EUR in the first 6 months of the year, with a total forecast by the end of the year of 80,000.00 euro (Bee Generation, Unaapi, Conapi, AAPI and other supporters, 2014).

Population dynamics

The infestation and subsequent parasitic disease caused by Varroa mites on honey bees is called Varroosis (Fig. 6).

The evolution of the infestation depends on complex multifactorial interactions both internal and external to the hive (Lodesani *et al.*, 2002). The dynamic development of the brood may considerably affect the growth of the infestation. The number of parasites in the bee colony increases with the availability of the brood, especially of drones, due to the presence of nectar flow and environmental conditions (Currie and Tahmasbi, 2008). The life expectancy of the adult female mite depends on factors such as temperature and humidity, and it can range from a few days up to a few months. In fact, studies confirm that under temperate conditions untreated colonies may collapse in two years after the initial 3-4 Varroa infestation (Buchler, 1994; Korpela *et al.*, 1993; Rosenkranz *et al.*, 2010), compared Italy and Northern Europe, where adverse weather conditions recorded brood blocks for longer or shorter periods.

Fig. 6 - Mites transfer through close contact between bees (Aralonline.org)

Pathogenesis

The mite propagation can occur for various reasons at different levels including the problem of transmission of secondary infections by the mite:

At apiary level

The dynamics of the population may be affected by the introduction of new mites in the colony by the forage or drones who move from one area to another; after robbing activities of looting (Greatti *et al.*, 1992), from the wax moth; infestation by a bee to another after the visit to the flowers successively or simultaneously; by contagion between neighboring hives; the transfer of the brood combs with or any material from a beehive to a healthy infested;

from the practice of nomadism with the transport of infested apiaries in free areas or healthy apiaries in infested areas.

At colony level

The evolution of the parasitosis is constant in those regions where is the presence of brood in the hives is recorded throughout the year, as in the case of Sardinia.

Apis mellifera is helpless against the mite; the biological cycle of the mite is 7-9 days with the possibility of realization of at least two complete cycles of evolution of the parasite on the drone brood (15 days) and worker bee (12 days); the mite is normally vulnerable at a nymphal stage, but it is protected within the brood cell; also, the adult mites have the ability to protect themselves during adverse times in the colony during winter in temperate zones and in the summer in the tropics with the possibility to switch from a bee mite to another within the same family. Another possibility is to spread from one country or one region to another, and it can occur by natural swarming or due to trade in swarms or families. The propagation can be facilitated between regions with no natural obstacles. It is estimated that from an apiary infested, in 2-3 years Varroa can infests all farms in a region. From seven hives in 1977, the infestation spread until it reached more than 10,000 in 1983 in Germany (Ruttner, 1983).

Macroscopic symptomatology

Direct damage

The evolution of the disease has a slow progress and long-term. We can distinguish three phases:

a) first stage is characterized by the appearance of a small number of mites, which does not affect the normal development of the colony. The disease course lasts one to three years and, since any appreciable events may be noticed, it often goes undetected .

b) second stage lasts approximately one year during which the amount of Varroa increases. The colony appears weakened and its productivity is lowered considerably. Typical identified symptoms are represented by the appearance of small bees, restless and unable to fly, phenomena of swarming, orphanhood and replacement of the queen. The infested forager bees also show a reduction in learning ability (Kralj *et al.*, 2006) their flights are longer lasting and they are unable to return to their hive (Kralj and Fuchs, 2006), drones and workers with deformed wings are also reported and finally high mortality in the sealed brood.

c) third stage: the colony collapses due to markedly infestation. Bees carrying one or more mites manifested high mortality of bees and brood, and, in the absence of proper treatment the family is about to discontinue .

Clinical feature

In summary, the invasion of the mite involves the death of a high number of nymphs and the hatching of adults with little viable and morphological alterations. In the most severe forms, the dead larvae are reduced to a whitish mass.

In front of the entrance door of the hive it is possible to find many dead bees or dying.

The decline of the longevity of queens and workers, of course, affects the ability to lay eggs and brood care. However, the most affected by the attack of the mite are the drones which are inhibited to their coupling capacitance (Duay *et al.,* 2002) and less swarms (Fries *et al.,* 2003).

Bees are feeling assaulted and agitated and try to break free from varroa. Symptoms related to brood hit by varroosis are similar to those of American and European Pesti: disorder, available mosaic with deformation and perforation of the cap. The capsules have holes of irregular shape with white edges. The larvae and nymphs death give off a characteristic odor. The weakening of the family is progressive and reaches its peak in late autumn (Gallo and Aiello, 1984).

Diagnosis

The fight against *V. destructor* is complex, difficult and problematic due to its biological characteristics, to the incapacity to detect it in some stages of development and the trend of the evolution of the infestation.

An essential condition for the purpose of limiting the damage of propagation is early detection. The level of infestation can also be evaluated by checking the number of Varroa in the male brood (which is the most affected).

This can be completed with the use of an uncapping fork on kelp cells and performing a careful visual examination of larvae.

Finding more than 3 out of 10 small cells infested in the spring / early summer is indicative of a high degree of infestation.

Damages at the individual level

To assume the hemolymph, the ectoparasitic perforates the cuticle of the adult bee and the larva which causes lesions that allow pathogens to penetrate into their body.

The *V. destructor* female mite's body, compared to other ectoparasitic such as ticks, is not elastic and cannot inflate, therefore it is forced to assume continuously hemolymph (Gallo and Aiello, 1984). Also the actions performed by the mite on bees facilitate the emergence of numerous histological, physiological (Bowen and Gunn, 2001) and pathological alterations.

Histological and physiological alterations

The mite injects anticoagulant and immunosuppressive substances through saliva (Brossard and Wikel, 2004) it and can active potential latent virus infections. Furthermore, the saliva of the Varroa can damage the hemocytes and inhibit their ability to aggregate that is a fundamental process for both wound healing and for phagocytosis of pathogens (Richards *et al.*, 2011).

A very important protein in bees is the vitellogenin protein which acts as a reserve for the production of royal jelly, basilar during periods of poor reserves of pollen and the recovery spring brood and especially essential for the regulation of the immune system, aging and the conduct of the hive (Amdam *et al.*, 2004b).

In presence of Varroosis, the values of vitellogenin diminish and bees undergo changes in behavior; the foragers begin foraging early and are not fully immunocompetent (Wilson-Rich *et al.*, 2008). Some authors argue that a strong varroa infestation and low levels of vitellogenin in parasitized bees would be the cause of winter mortality of families (Amdam *et al.*, 2004a; Dainat *et al.*, 2012a). The attack of mites inhibits the action of the enzyme phenoloxidase (Alaux *et al.*, 2011; Gregorc *et al.*, 2012; Yang and Cox-Foster, 2005), which plays an important role in the process of pigmentation, scarring and wound healing, essential for insects (Kanost and Gorman, 2008).

In addition, on infested bees the synthesizing enzyme glucose oxidase is inhibited in the hypopharyngeal glands and secreted with the royal jelly, which would protect against contamination of the meal larvae.

Indirect or secondary damage

The Varroa interacts with the virus of honeybees, aggravating their pathogenicity. *V. destructor* could increase the pathogenicity of Deformed Wing Virus (DWV) acting as a vector (Martin *et al.* 2012) and enabling amplification of viral effects, without necessarily compromising the immune response of bees. Increased levels of DWV in the presence of a greater number of Varroa founders can be explained by the increase of action as a vector or with the proliferation of cuticular lesions on bees (Donzé and Guerin, 1994).

About 18 virus have been isolated from honey bees (Chen and Sieds, 2007) and the mite would be implicated in the transmission of many of them: Deformed wing virus (DWV) (Boecking and Genersch, 2008), Kashmir bee virus (KBV), Sacbrood virus (SBV), Acute bee paralysis virus (ABPV) (Di Prisco *et al.*, 2011).

Some of the viruses are transmitted horizontally and vertically (Boecking and Genersch, 2008).

In association with *V. destructor*, the pathogenicity of the virus may increase, for example, the symptoms of infection of DWV strongly depend on co-infection with V. destructor (Yang and Cox-Foster, 2005; Nazzi *et al.* 2012).

CHAPTER III

INTRODUCTION TO THE BIOLOGY OF THE BEE, PATHOGENS AND MAIN ENEMIES

Of the living species, only *A. mellifera* has been found in the fossil record from Pleistocene copal (Engel, 1998a, 1998b). According to a study, the bees are a derived, monophyletic group of the spheciform wasps and presumably arose sometime in the earliest mid-Cretaceous after the origin of angiosperms (Engel, 2001).

Honeybees are known for their organization in colonies, however, the fossil record have not demonstrated their propensity to social life. It is thought they have experienced a gradual process and that affected only certain groups of Hymenoptera.

A comparison between morphological Hymenoptera fossils and modern ones, lead us to observe that they retained the same morphology suitable to work in perfect harmony with the environment. The bees are the Hymenoptera that live in ordered and complex colonies. They may constitute a Super creature, which can be composed of 3,000 up to 80,000 bees.

Each creature of the hive has a precise assignment that can be more or less secure depending on whether you play inside or outside of the hive; young worker bees perform 'safe' tasks within the hive (nest), while later take on tasks that pose a risk outside the nest (Winston, 1987; Schmid-Hempel and Schmid-Hempel 1984; Visscher and Dukas, 1997). The bee, like all living things, during his short life, can undergo many hardships, due to attacks of numerous enemies who can be classified as parasites, predators, or bother diners, depends on the nature of the damage caused the hive and the relationship he has with the bee (Ben Hamida, 1999).

Their home is the hive. The internal organization is divided into three distinct castes. The fertile caste (the queen and the drones) and the sterile caste (the workers).

There are gathered in two types of bees, honeybees and wild bees.

The first are those that man has selected and used for thousands of years for the production of honey and other products of the hive (wax, propolis, royal jelly), to make source of income or for simple passion.

Those wild, however, only produce honey for their sustenance and do not store stocks.

The bees feed on nectar visiting several flowers A worker bee visits an average of 1,500 different flowers for a load of pollen (Benjamin, 2009).

These bees, thanks to the thick hair covering their bodies, carry pollen from one flower to another by promoting the reproductive process of plants and sustain biodiversity. This type of pollination is called "entomophilous” to distinguish by the “anemophilous”, that is favored by the wind.

The queen bee

The queen bee lives between 3 and 4 years in average, and represents the female reproductive organ of the hive. It can reach the dimension of 17 to 20 mm in length in 16 days. It is equipped with a sting.

To become a queen bee, the larva is fed with a special nutrient called royal jelly, a fluid secreted by the pharyngeal glands of the workers.

Its function is to procreate. A good queen, in the good season, can lay about 1,500 eggs a day, about 250,000 eggs per year and, during her life is capable to lay up to a million eggs.

After the nuptial flight, the queen is part of the hive and begins to lay her eggs inside the cells. After 3 days of laying, the egg hatches.

In the beginning, the larvae are fed by the nurse bees with royal jelly, then with a mixture of pollen and honey. After ten days, the larva has completed its growth, and the workers ensure them in an opercular cell (sealed with a set of pollen and wax). The larva meanwhile is closed in a cocoon.

Twelve days later, the cell exits a young worker bee who has the size and appearance of the final: since laying three weeks have passed.

In Italy and in northern Europe the queen deposition decreases with the arrival of the first cold, and it stops approximately at the beginning of October, to restart the activity at the beginning of the following spring. In regions with mild climate, as in the case of Sardinia, the queen laying eggs always throughout the year.

The eggs are deposited into hexagonal cells of different sizes depending on whether they are worker bees to be contained and/or drones. The queen, thanks to the secretion of specific pheromones produced by the gland Nasanoff, controls and can hold the family together; can lead to atrophy of the ovary of the other females, can choose which type of wax is to be produced (Winston, 2001) to adjust the size and the quantity of cells to be built (Ledoux, 2001), inhibit the breeding, by worker bees on another queen. When the queen bee gets older, the activity of the gland of his Nasanoff decreases the production of the pheromone. So she dies and in the absence of her pheromone, the workers begin to raise a new bee queen.

The drones

The drones are the male component of the hive, are haploid. In the hive they are present only during late spring and summer. Their main function is to mate with the queen bee.

Sexual maturity is reached after about a week of birth and die shortly after mating. They reach an average of 15 mm in length. They do not have to haul bodies and have no sting. The drones are recognized to perform those other tasks through trophallaxis, with the worker bees caring of rearing larvae, they warm the brood up with the heat from their body, and aerate and mature honey through the ventilation produced by the wings. (Harrison, 1987; Southwick and Moritz, 1987).

However, during the first autumn cold, when the nectar and pollen resources become scarce, the drones are pushed out of the hive and left to die. In exceptional circumstances, when the colonies are orphans of the queen bee, it can happen that the bees can allow drones in the hive to stay indefinitely.

The worker bees

The worker bees have their reproductive organs atrophied. They live an average of 40 days. They include a sting.

In the summer months a worker can live about 6 weeks and up to 6 months in the fall, this allows the colony to survive the winter and assist the younger generation in the spring, before they died.

Their tasks consist in feeding the queen bee and immature stages; building and defending the hive, collecting nectar, turning pollen into honey, keeping the pollen from which derive the protein, bringing water to drink and humidify the hive, they also collect resins that becomes propolis in the hive, taken from the trees and use as an antimicrobial to disinfect and paint the hive (Crowder and Harrell, 2012). Finally, the look after brood (with stages: egg, larva, prepupa and pupa) which represents the immature stage bee. Features of the bee are the dances that represent a symbolic language, interpreted to communicate with her sisters (Frisch, 1923).

Brief to the classification of bees

From the perspective of systematic classification, bees belong to: Phylum: Arthropoda; Subphyla: Tracheata; class: Exapoda; Order: Hymenoptera; Family: Apidae.

The family of Apidae, is divided into genres: *Apis, Bombus, Melipona* and *Trigona*. Belong to the genus *Apis* four species: *Apis* Linnaeus, 1758; *Apis mellifera* Linnaeus, 1758 (Check list of the fauna of Italy) *ligustic*. The honeybee (*Apis mellifera* L.) is native to Africa and crossing France it has spread across Europe, Africa, West Asia. It has been introduced in the Americas, Australia and New Zealand. Belong to the genus Apis other three species:*Apis florea* F. or "Midget bee" is smaller than the *A. mellifera*, is widespread in India, Indochina and Indomalesia

- *Apis dorsata* F. or "Giant bee" is widespread, also in India, Indochina and Indomalesia. It is a very aggressive species and for its remarkable size is similar to the bumblebee.

- *Apis cerana* or *A. indica* : indicates the species is very similar to *A. mellifera*, but is slightly smaller; is widespread in India, China, Japan and Afghanistan to the west comes in contact with Siberia honeybee. They build colonies of small entities and may be kept. Do not produce propolis.

The species *Apis mellifera* comprises various races that differ in the morphological characteristics due to their geographical distribution. However, the inventory is still to be established.

The main are:

European races

- *Ape mellifera mellifera* or black bee that populates the Western and Northern Europe. It develops in late spring but with good ability to spend the winter with very low temperatures.
- *Apis mellifera ligustica* or Italian bee has spread almost all over the Italian territory, from the foothills of the North up to Calabria.
- *Apis mellifera siciliana* n Sicily there is the dark

The *Apis mellifica ligustica,* is the host object of this doctoral thesis research. It differs from other races because the workers have the first segments of the abdomen pale yellow color, the queens are golden yellow, with a huge capacity of egg-laying. It is perfectly integrated in a Mediterranean climate, such as Sardinia, with short winters, mild, wet and dry summers with nectar flow of long duration. This breed is difficult to acclimate to climates of Nordic kind.

Despite all, it is the race most widespread in the world. The advantages are: industriousness of the colony, docility to the stables and not ready to swarming. The purer strains of the species are now only in an island across Australia, in Kangaroo Island, brought by Ligurian emigrants arrived here in the second half of the nineteenth century. The defect: the colonies tend to pillage and drifting[2].

- *Apis mellifera carnica*: the distribution area of this race covers the center Eastern Alps, Austria, Slovenia and the southern part of the former USSR. The color is dark and the size is larger than the

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[2] (http://www.winenews.it/print)

ligustica honeybee. Develops rapidly in the spring, but it is becoming strong swarming.

Other races are:

- *Apis mellifera cecropia* widespread in Greece
- *Apis mellifera adami,* in tribute to Brother Adam; is located in the island of Crete.

African races

- *Apis mellifera scutellata* originate in the areas of savanna in southeastern Africa. It is a subspecies of the Western honey bee
- *Apis mellifera intermissa* spread to Algeria Morocco, Tunisia, bee aggressive and prone to swarming
- *Apis mellifera adansonii* or African bee is widespread in Central Africa,
- *Apis mellifera monticola* live in the area of Kilimanjaro
- *Apis mellifera lamarckii* or Egyptian bee, aggressive and prone to swarming, building lots of real cells. *Apis mellifica sahariensis* lives in the oases
- *Apis mellifera capensis*, widespread in South-Africa.

The African bees, although subject to the same diseases and parasites that are collapsing our western bees, show no signs of decline. It's been suggested that the basis of their resistance to aggression is due to genetic reasons, and especially to the different practices in use in East Africa, where "industrial" beekeeping practically does not exist and the use of pesticides is very limited.

With the aim of obtaining docile and productive bees, in 1956 the Brazilian government authorized the hybridization between the Spanish bee, *Apis mellifera iberiensis*, and the African bee, *Apis mellifera scutellata.* Africanized honey bees have become legendary for their aggressivity, stinging, nest defense behavior which has let them won the media title of "Killer Bees" (Sears, 1999).

For about 40 years, they have in fact spread throughout the South and Central America, and recently they have started to expand into the southern United States. About Africanized bees there are conflicting versions, between those in favor and those who are opposed.

The public should stay informed about Africanized honey bees concerning issues, but not be unduly alarmed. Any future Africanized honey bee problems are not without solutions (Collins *et al.*, 1994; Collins et al. 1988). In a study on Africanized bees in Brazil many qualities are recognized after forty years of adaptation and success (De Jong, 1996). Also, Africanized honey bees have low infestation levels of the mild *Varroa destructor* in different ecological regions in Mexico (Medina-Flores *et al.*, 2014).

Races of Middle East and Eastern

- *Apis mellifera syriaca*, in Turkey, Syria, Israel, Jordan, Lebanon;
- *Apis mellifera remipes*, resembles to the *ligustica*, widespread in Iran and Turkey;
- *Apis mellifera jemenitica*, widespread in Yemen and South Arabia.

Spread of pathogens in hives

It is known from the literature that some honeybees (*Apis mellifera*) collect nectar and pollen sources, but take on the responsibility, extremely risky, to raid the honey from other hives, giving rise to the phenomenon of looting, especially in famine times; *Apis mellifera* can plunder the hives of *Apis cerana*. It was also shown that the workers looting and those that collect water run more risks than those that collect nectar (Woyciechowski, 2007), live shorter than the foraging of pollen and nectar, while those bees that collect in unfavorable weather conditions are more at risk in regards to those operating in good weather conditions (Woyciechowski and Zozlowski 1998).

Unfortunately interspecific and intraspecific looting (kleptoparasitism) affects the lifespan of the looting bees as well as it may facilitate entry into the hive of origin or looted of parasites and pathogens. Frequent phenomenon also for all other pollinators who become infected is through the floral nectar contaminated.

In addition, the trypanosome protozoa *Crithidia bombi* can spread through the floral nectar contaminated with other pollinators infected (Manson *et al.*, 2010).

Main enemies of bees

Among the predators are mentioned many Arthropods: Arachnids such as poisonous spiders of the *Latrodectus* genus (black widow spiders)(Rayment, 1917; Botha, 1970; Smith, 1960; Ben Hamida, 1999), *Theridion* and *Achearanea* genera of the Theridiidae family (Kaston, 1978), *Aranaeus* spp (orb weavers) of the Araneidae and the "crab spiders" of the Thomisidae family, which are real or potential enemies of the honeybees. Mites of Parasitidae, Thrombididae and Sarcoptidae family and the damaging tracheal mite of Tarsonamidae family such as *Acarapsis woodi.* Among the insects some predatory insects Diptera such as the carnivorous Asilidae (robber flies), the *Senotainia tricuspis* of Sarcophagidae family, which is a well known thoracic endoparasite of honeybees. (Simintzis, 1949; Giordani, 1956, Boiko, 1959)

Braula coeca o bee lice of Braulidae family which lives as a commensal in the colony and is trasported by bees (phoresy) (Nixon, 1982 Bradbear, 1988),. Hymenoptera: ants as *Formica* spp and *Crematogaster jheringji* that disturb the colony.

Predators wasps of five genera so *Provespa, Vespa* (hornets), *Dolicovespula, Paravespula* and *Vespula* and *Philanthus* spp. of Sphegidae family. Lepidoptera such as *Galleria mellonella* (greater wax moth), *Achroea grisella* (lesser wax moth) and *Acherontia atropos* (deathhead moth) of Sphingidae family that are harmful to honey combs and stocks (Beljasky, 1927; Ben Hamida, 1999; Fois *et al.*, 2007).

Among other predatory birds such as *Merops apiater* (bee-eater), which feed on large amounts of bees. Carnivorous mammals as mice *Mus musculus*, *Apodemus sylvaticus* that damage apiaries and stored feeding and contaminating with the urina stored (Langstroth, 1860), bears. Occasionally racoons are enemies of bees. Amphibians as Bufonidae (toads) and Ranidae (frogs) families (Ben Hamida, 1999).

Notes on the immune systems of bees, individual immunity and social immunity.

The individual immunity

Bees can be attacked by various diseases. They have inner and outer protections, individual and, being social insects, also social immunity. In this regard, the first barrier to protect individual is the exoskeleton of chitin that covers the body while protecting the digestive tract is the peritrophic membrane (Antunez *et al.*, 2007).

Regarding the internal individual defense, the task is entrusted to the immune system which, in insects is divided into two categories: cellular immunity with defensive responses such as phagocytosis, nodulation, encapsulation and humoral immunity (Gillespie et al. 1997; Lavine and Strand, 2002; Boman, 2003) with the synthesis of proteins with antibacterial, antifungal, etc. (Hetru *et al.*, 1998; Lamberty *et a*l., 1999; Yamauchi, 2001; Klaudiny *et al.*, 2005).

The social immunity

This type of immunity is very important and, in fact, recent studies have shown that the social behavior can, in some cases, lead to a reduction of the parasite load due to immune responses density-dependent or collective defensive strategies (Hughes *et al.*, 2002; Traniello *et al.*, 2002; Baracchi *et al.*, 2013).

The immunity is a social defense system compound that affects the entire family and involves the production of a range of substances able to have a microbicidal effect which is produced by the nurses as the defensin (Klaudiny *et al.*, 2005) and many other antiseptics. These essential substances present in honey (Kwakman *et al.*, 2010) useful to individual well-being and the whole colony are synthesized from pollen; so it is clear that in case of lack of pollen, bees, have difficulties in defending themselves from attacks by pathogens with lethal consequences for both adult bees and for the brood.

In addition, a low diversity of available pollen can be a major limitation for the development of the hive (Alaux, 2010).

The protein food produced by the nurses, commonly referred to as jelly (real or workers) contains several substances with antibiotic activity, cannot be considered as part of the immune system and individual defensin, making the immune system instead of individual (Klaudiny *et al.*, 2005).

A) Brief history of beekeeping

Man has been using honey from at least 12,000 years.

The oldest evidence of honey harvest dates back to about 7,000 years ago and it is represented by a cave painting found in Europe, in the Cueva de Araná, Valencia, in Spain, portraying a character, with around bees, climbed on the vines in a rocky wall, who is intent on collecting the honeycombs of a ravine.

Another witness, also prehistoric, is that of the graffiti found in the Matopos Hills in Zimbabwe, in Africa and may be the oldest documentation of the use of smoking in treating bees (Crane, 2001).

It is, therefore, clear that the man's initial prehistoric activity was oriented toward the looting; practice still in use in many primitive peoples today.

In the Neolithic, approximately 7-8,000 years ago, with the stabilization of the current state-climate flora and fauna, the man from hunter-gatherer errant become sedentary and began to practice agriculture, the domestication and breeding of animals (dog, ox, pig, sheep, goats) and also using bees for their hospitalization rustic containers made with different materials in order to control them and exploit their products.

Beekeeping, was practiced regularly in Ancient Egypt in 2400 to C .; witnessed by hieroglyphics Abusser to present at the temple of Niuserra, belonging to the V Dynasty of Egypt.

The honey in Ancient Egypt was initially a luxury food, it was then used as a sweetener, flavoring and packaging for fish and vegetables, fruit jams and syrups, buns and preservative used along with fruits such as apples, quinces and pears. Along with milk, it was a food given to children of higher social strata. Was also identified as a food ration in commercial shipments, as spoils of war, payment of taxes, and temples and votive offerings.

By its fermentation mead was produced, which continued to be popular in the Middle Ages. Another popular drink was the honey wine, for which the finest wines and cheeses were used, as Falerno and Massico.

Then, the use of honey was extended to cosmetics, among the Sumerians (aromatic oils, perfumes), medicine and food by the Babylonians and, as an antiseptic, healing, purgative, up to crafts (dives to give brilliance to the color purple tissues or precious stones).

The famous Code of Hammurabi (1792-1750 BC) reported even among the crimes for which they were provided for severe penalties, emptying hive honey content, which makes us assume that this population was no longer limited to the search of wild honey, but they already practiced beekeeping.

The term "Melit" appeared for the first time contemporary to this people, among the Hittites, thanks to the sources handed down through reprocessing of their writing..

In Greece, thanks to Homer and Pythagoras it spread substantial news on the beneficial properties of honey and divination have.

Large consumers of honey were also the ancient Romans, who were able to conceive hives similar to those of recent use.

B) **Brief beekeeping in Sardinia**

In Sardinia, probably, the first apiary collecting a swarm was born in the wild, in tree trunks and natural cavities.

In the history of Sardinian beekeeping, are particularly important the cork skeps hive or ”bugni”, obtained from the bark of the cork oak *Quercus suber*, very abundant in the island.

The history of beekeeping in Sardinia orbits around the cult of Aristaeus, a minor god in Greek mythology.

According to the current interpretation of his cult in the island, it would have ancient origins proven by a bronze figurine of nuragic age kept in the "Museum Sanna" in Sassari, showing a male character, who would represent the local incarnation of "divine benefactor", which carries on his shoulders a bag with three jars containing liquids or pumpkins donated to men (milk, wine and honey) (Nicosia, 1985), although there are conflicting opinions on the subject (Sanna, 1998).

The devotion to Aristaeus dates back to the VIII and VII century b.C. (Breglia Pulci Doria, 1981).

The myth tells that Aristaeus arrived in Sardinia, he became the lord and introduced the practice of fruit growing, agriculture and perhaps beekeeping as it is shown by the discovery of another bronze figure at Oliena in 1855, dating back to the II-III century A.D.. (Roman period) and, in the Museo Archeologico Nazionale in Cagliari, which appears as a young man standing in the body which are visible five insects related to bees (Didu, 2003; Angiolillo, 1990; Zedda Macciò, 2010).

Being Sardinia an island in the Mediterranean Sea it also underwent the influence of the Phoenicians with the business of the wax in the tenth century. A.D. and Carthaginians III to the sixth century. A.A., which were considered large producers of honey and wax by the Romans. Subsequently, with the Roman domination also Sardinian apiculture products became renowned.

Quoted from famous people, to the Sardinian honeys were ascribed medicinal properties: Dioscorides lib. II, Cap. CII (40 approximately 90 A.D..), and also of good judgment by Virgilio in the Bucoliche (Ecloga VII). However, not wanting the unfavorable assessments of Marcus Tullius Cicero (106-43 b.C.) and Horace (65-80 b.C.). Later, in the Byzantine period (337-1267 A.D.), breeding, as well as also spread in the farmhouses and in particular in the monasteries, as well as in the countryside.

Beekeeping became intensive, during the Middle Ages, due to the request of wax for lighting and for the constant use of honey as a sweetener.

In the Judicial period 9th – 14th century, the beekeeping is regulated under the collection of rules known as Carta de Logu (code of laws promulgated by the State of giudicati, female Judge Eleanor, in 1392 in force until 1827) in which hives theft were subject to punishment with severe penalties and, depending on the severity of the offense, by fines of up to docking of one ear (Casula, 1995).

However, the penalties for those who stained the crime of hive thefts reached the utmost severity during the Spanish period, under Philip II in 1583.

In the centuries to come, beekeeping in Sardinia will be increasingly enhanced and modernized, even if it found resistance from beekeepers islanders.

In 1851 the American beekeeping was revolutionized by the invention of the rational beehive, by Lorenzo Lorraine Langstroth. The innovations continued, and from England, they spread throughout Europe, especially in France and Italy, by Charls Dadant, the beehive Dadant-Blatt attic with top mobile (Fig. 6). While in Italy we witnessed a modernization of beekeeping, according to a census of 1928, beekeepers still preferred to use the rustic hive or beehive.

In Sardinia time for the beekeeping modernization arrived in 1951 when the Beekeepers of Sardinia Consortium was established and the Regional Council voted the first law to regulate beekeeping in 1954, so assigning contributions and encouraging the growth and spread of the apiaries, with particular interest treatment of diseases.

Only around the 80s a real answer to the attempt to change the method was given and a considerable effort to keep bees no longer in the cork was done, but in traditional beehives.

The rejuvenation real happened in the years between 1980 and 1990 with the establishment of several cooperatives that acquired local innovative materials in the region and the Peninsula.

CHAPTER IV

CONTROL OF VARROOSIS

In Sardinia, the main acaricides experimentally tested are: Folbex Va (bromopropylate); Perizin (coumaphos); Apitol (cimatolo hydrochloride); Apistan (fluvalinate), Bayvarol (flumethrin); Apivar (amitraz); Rotenone, among those of natural origin: Apilife (thymol, menthol and eucalyptol); Apiguard (thymol gel) and organic acids (formic acid and oxalic acid).

Levels of effectiveness, biological effects and the most appropriate method of use in the island in relation to the possible accumulation of residues in products of the hive were ascertained on these miticides.

On some of these acaricides, those containing fluvalinate and flumethrin, there have been highlighted more or less high levels of drug resistance by the mite, which have led to a situation of strong crisis because they are long-acting formulations, in the form of strips (for es.di fluvalinate), suitable for the treatment of hives with the constant presence of brood.

With the ineffectiveness of such products, beekeepers have used on one hand an improper and indiscriminate use of other miticides, on the other, the use of natural products consisting of essential oils obtained from medicinal plants (eucalyptol, menthol, thymol) and from organic acids, which, however, show not always satisfactory levels of effectiveness , and especially variable even within the same apiary.

Furthermore thymol due to its lipophilic properties accumulates in the wax, but also in honey and pollen which are the main food resources of the larvae, and may affect genes involved in detoxification and in the development of adult in a more relevant fluvalinate (Charpentier *et al.*, 2013).

Though , in the context of natural acaricides used to control the mite, some organic acids play an important role in integrated pest management strategies. Among these, the most used are oxalic acid, formic acid and lactic acid.

These substances are components present in honey, then their use does not result in residues of toxicological significance, although the use of oxalic acid and formic require precautions in handling as considered caustic substances in case of contact and inhalation.

Between the two organic acids, formic acid is by far the most effective (Bolli *et al.*, 1993; Fries, 1989; Hoppe and Ritter, 1989; Hoppe *et al.*, 1989; Imdorf *et al.*, 1996; Lindberg *et al.*, 2000; Satta *et al.*, 2005), both against phoretic mites and against those in reproductive phase (inside the brood cells), with no effect of accumulation in the wax also thanks to its high volatility.

A field trial was conducted to test the efficacy of two different acaricides, the Apiguard (based thymol) and a new formulation based on formic acid, Biotab (Mereu Piras *et al.*, 2011).

Many studies are oriented towards the genetic selection of resistant strains of mite bees, but it is still far from reaching this focus .

Two unique sub-rural populations of European honey bees (in Gotland, Sweden and in Avignon, France) have adapted to survive for extended periods (over ten years) without the use of mild control treatments. This has been achieved through a natural selection process with unmanaged mild infestation levels enforcing a strong selection pressure (Locke, 2012). One research has been carried out on two distinct behaviors that have bees, ie, in the first case the ability to liberate dead individuals or dying in the brood from the hive in a considered time period (12-24-48 hours) through the test of the frozen brood (freeze killed brood or FKB).

The removal of the diseased brood is one of the main means of defense of the superorganism hive.

In fact this method proves to be very effective against fungus, American foulbrood and has a moderate action towards Varroa destructor. The second behavior is the Varroa Sensitive Hygiene, which allows a very high resistance to the mite and is expressed by some strains of *Apis mellifera* (Parker et al., 2012). Genetic tests were also carried out using the two forms of selection set yet it has not led to any feedback (Danka et al., 2013).

Other studies have addressed the evaluation of the index of the relationship varroa adult/youth and varroa mite adult/varroa total in relation to the observation of varroa falls to the bottom of the beehive (Rinderer et al., 2013); the more the total population of Varroa increases, the more you can rely varroa young; the decrease of the index, , therefore, will be seen as a good signal (Ordonneau, 2013).

The mite *Varroa destructor* in Sardinia

Although Sardinia is an island, it has not been immune to the invasion of the mite *Varroa destructor*, which was early reported, as *Varroa jacobsoni*.

Although there existed a measure of the Region of Sardinia which banned the import of domestic and foreign bees and bee products, the mite still managed to "land" on the island. In this regard, it has been hypothesized that the introduction has been perpetrated through trade with the mainland or with neighboring Tunisia.

The mite has made an appearance for the first time in the province of Cagliari, in 1983 (Prota, 1983). Initially, the invasion spread throughout the south-west area of the island then, through the channels of the frequent moves to nomadism, has spread into other areas, from the center-north to the south-east, facilitated in those years also by the spread of breeding rustic type, difficult to control. According to processing data presented by AA.SS.LL. in 2012, in despite of the different and significant hardships encountered, again demonstrates a reasonable level of dynamism.

To the adversities regarding various diseases and parasites that can afflict bees, are also added the environmental conditions related to drought in spring/summer and low temperatures manifested in February 2012, and the attack from the Hemiptera *Glycaspis brimblecombei* blooms of *Eucalyptus*, fundamental source of pollen to bees, in the summer. In Sardinia, it is estimated the presence of about 589 companies (+ 5% compared to 2011), although the number is considered underestimated with a total asset of about 50,000 colonies. The average corporate hives (about 80 units) has remained constant over the past 5 years (Laore, Regional Agency for Development in Agriculture, 2014).

Fig. 6 – Hives Dadant-Blatt model

CHAPTER V

INFESTATION AND DISTRIBUTION OF *VARROA DESTRUCTOR* IN HONEYBEE BROOD COMBS OF *APIS MELLIFERA LIGUSTICA*

Scientific premises and general aims

A reliable distribution model of the number of mother mites per honey bee brood cell is useful for many aspects:

1. First of all, it may be used to correctly estimate the infestation level of the brood, representing a valid support to control varroa, allowing to establish the most appropriate timing for control treatments, according to the principles of integrated pest management;

2. Secondly, damages suffered by larvae and pupae parasitized by Varroa mites depend on the number of mother mites penetrated into the cell, so that the distribution pattern of mites may be useful to define the damage on bee brood as a function of the infestation level.

3. An accurate estimate of the percentage of cells with a specific number of mites allows to accurately assess the reproductive rate of Varroa. In fact, the increased competition between daughter mites for food and space in a multi-infesteted cell induces a decrease in fertility on the mother mite.

The accuracy of a simulation model of the mite population could be improved including also this aspect.

The available forecasting models (Fries et al, 1994, Martin, 1998, 2001; Calis et al., 1999; Wilkinson and Smith, 2002; Degrandi Hoffman and Currey, 2004) completely neglect this aspect. In these models , the mite growth rate is related to the simple availability of brood (eventually taking in account the distinction between male and female brood), or refer to a Poisson distribution that has proven not to be sufficiently accurate to represent the actual behavior of mothers varroa in the bee brood infestation (Fuchs, 1988).

Some studies have shown an "aggregate" or "contagious" distribution of Varroa in brood (Floris, 1991 and 1997), explaining accentuation on multi-infestation with increasing population density, with obvious effects on the growth rate , on mite reproduction and on damage level to bee colonies.

In the environmental conditions that favor constant presence of brood in hives, with the seasonal variations, a correct estimate of the percentage of infested cells with one or more mites is considered to be an essential presupposition to define a more realistic simulation model of mite development dynamic and suited to its purpose of statistical distribution.

In this PhD thesis, on the basis of the data acquired by inspecting numerous female and male brood combs, with percentage of infested cells oscillating between 1,7% and 74,3%, regression equations of second degree were drawn, that ultimately allow to estimate, for each level of infestation, the percentage of cells invaded by one, two, three and four mother mites. In this work the implications of the aggregate distribution on the rate of reduction of fertility of Varroa and longevity of adult bees are also discussed.

Materials and Methods

Data collected in a previous study on the characteristics of infestation by varroa mites in operculated female brood combs (Floris, 1991) have been reanalysed.

20 brood combs with levels of infestation by *V. destructor* varying between 1,7% and 74.3% were examined.

The honeycombs (Fig.7) were removed from the central nest position of 50 hives in summer and autumn, in absence of male brood. After removal, the combs had been stored and frozen at - 20 ° C until analysis time.

The analysis concerned:

- The spatial position of each cell, which was reported on a sheet of clear plastic of the honeycomb size (Mapping) (Fig. 8);

- Examination of the cells was carried out through the removal of opercula of bees, that were classified according to their developmental stage as: pre-pupa, pupa with clear eyes and white body, pupa with dark eyes and pigmented body, and adult (Fig. 9-10);

- Number of Varroa mites present in every brood cell was counted distinguishing the development stage and its sex (son mites, daughter mites and mother mites).

The same observations were repeated in 2011 and 2013 on male brood combs removed from different hives during Spring and Summer, using the same work protocol. In addition, also 30% of female cells have been inspected.

For each comb containing female and/or male brood the following descriptive statistics were considered:

- Total number of brood cells.
- Total number of mother mites*
- Mean number of mites per cell brood mother.
- Number and percentage of cells containing 1, 2, 3, 4 mother mites per cell.

*(it represents the number of mites that can shift from a phoretic phase to the reproductive phase) .

Related to the total number of mother mites it is necessary to specify that it represents the number of mites that pass from the phoretic stage to the reproductive stage. This parameter, in predictive models on Varroa dynamics population is calculated as a fraction of the number of Varroa being in phoretic stage using an equation developed by Boot (1994, 1995), which identified a relationship between the exponential rate of invasion and the ratio brood/adult bees. In particular, amount of varroa that pass every day from phoretic stage to reproductive stage invading brood cells are defined by the following equation:

Total number of varroa passing from phoretic stage to reproductive stage = x 1-exp [- (rd + rw)] where:

rd = - 6.49 (D / B);

rw = - 0.56 (W / B);

B = is the weight of adult bees, assuming that individual bee weights 0,125 g.

D and W = represent the amount of respectively male and female available cells, to be invaded on a given day.

Therefore, for the calculation of this parameter in forecasting models, the number of adult bees and brood cells available for invasion by Varroa must be known (cells after a day from brood capped, corresponding about to the eighth day of the development of bee's life cycle) or, in other words, the dynamic development of honeybees colony must be known.

Fig. 7 - A honeycomb

Fig. 8 - Mapping of brood cells

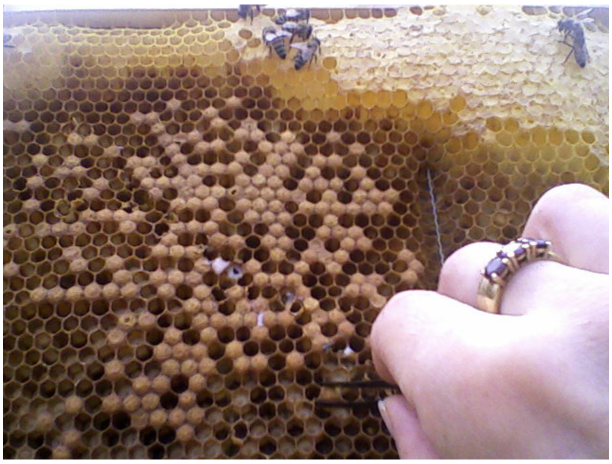

Fig. 9 - Removing of opercola

Fig. 10 - The developmental stage of bees

Data processing

By relating the following parameters, some 2nd degree regression equations were obtained:

1)
X = average number of mites per infested cell;
Y =% of cells infested;
2)
X = average number of mites per infested cell;
Y =% of cells with one mother mite;
3)
X = average number of mites per infested cell;
Y =% of cells with two mother mites;
4)
X = average number of mites per infested cell;
Y =% of cells with three mother mites;
5)
X = average number of mites per infested cell;
Y =% of cells with four mother mites;

The percentage of cells containing 5 mother mites as the difference between total number of available cells and amount of the percentages of cells inhabited by one, two, three and four mother mites was also calculated.

CHAPTER VI

RESULTS, DISCUSSION AND CONCLUSIONS

Results and discussion

In figure 11 the regression curve that relates the number of mites in the reproductive stage compared to the number of cells capable of being invaded with the percentage of cells infested is reported.

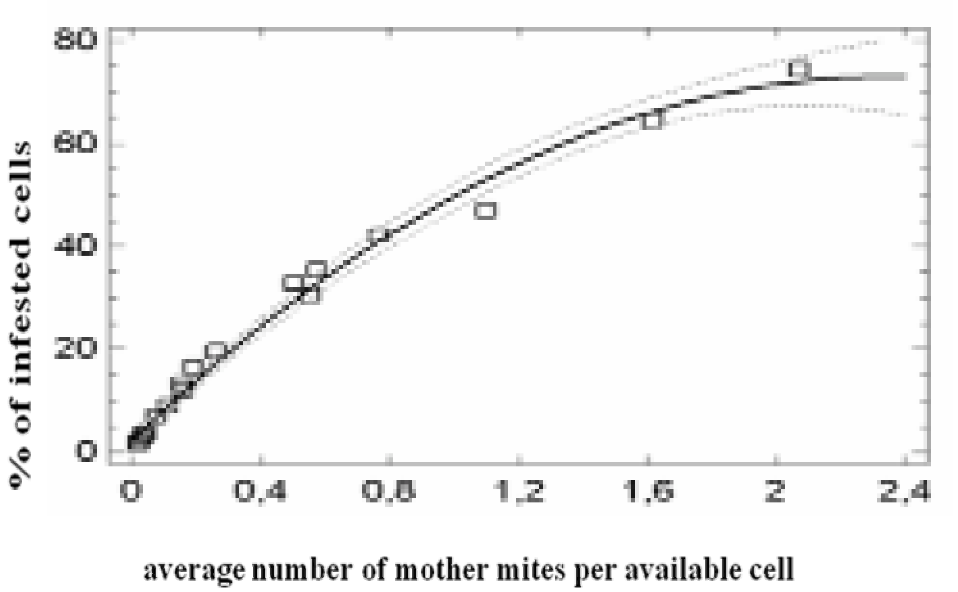

Figure 11 - Relation between average number of mother mites per available cell and the percentage of infested cells.

Between the two variables, there was a highly significant relationship (P <0.0000) with more than 98% of the variability of percentage of cells infested explained by the average number of mites per cell available.

The relationship between the two variables is also showing a progressive decrease in the rate of infested cells while increasing the average number of mites in the reproductive stage per available cell, with the result that even in the presence of a double number of mites per the suitable cells, the percentage of infested cells remains well below 80%.

It is clear that this type of trend finds its justification in the biological phenomenon of multi-infestation, namely in the invasion of the same cell by more mother mites, propensity that increases the average number of mites per cell provided according to a linear relationship highlighted in figure 12, also this extremely significant (P <0.0000).

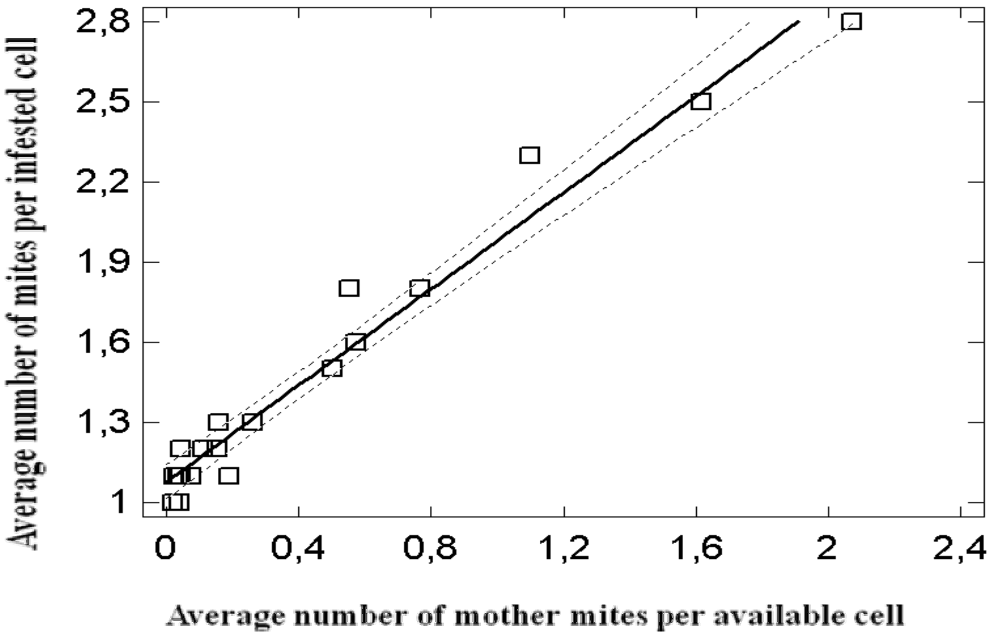

Fig. 12 - Relationship between average number of mother mites per available cell and the average number of mites per infested cell.

Figures 13-16 represent the relationships between the average number of mother mites and the mother mite infesting cell and the percentage of cells occupied respectively by one, two, three and four mother mites.

The same relationship has not been calculated for the percentage of occupied cells by five or more mother mites because this fraction represents only 3.7% of the cells infested out of the inspected combs , and the available data were limited. However it is also clear that the fraction of occupied cells by five or more mother mites can be calculated as the difference between the total of the cells invaded and the total number of cells occupied by one, two, three and four mother mites.

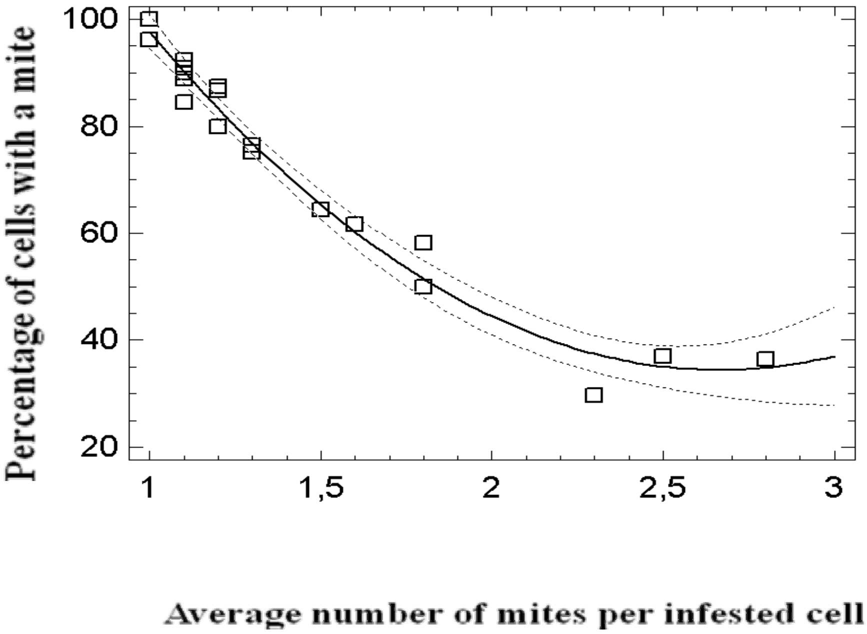

Fig. 13 - Relationship between average number of mites per infested cell and the percentage of cells with a mite.

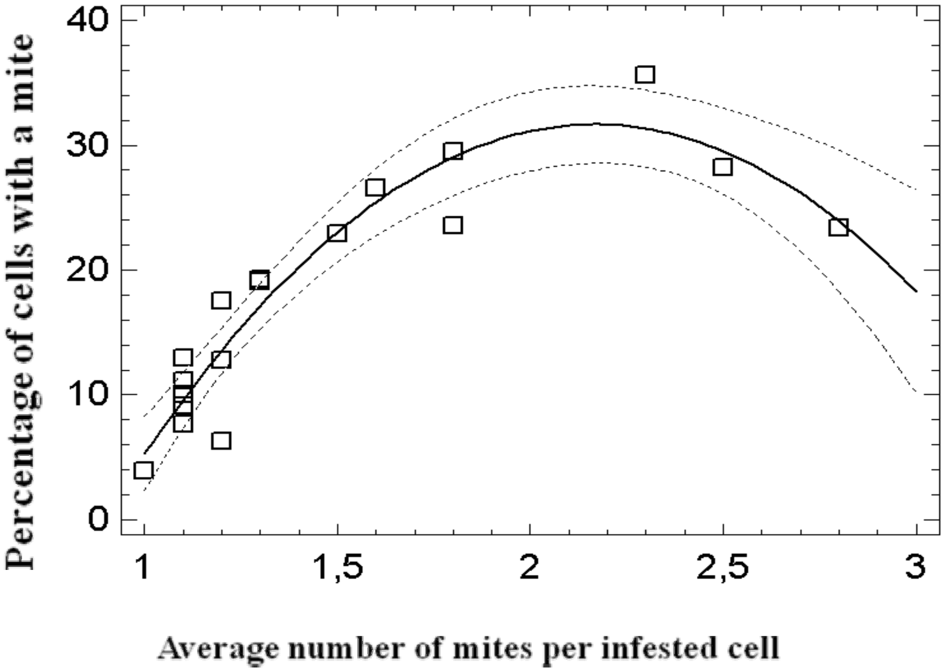

Fig. 14 - Relationship between average number of mites per infested cell and the percentage of cells with two mites.

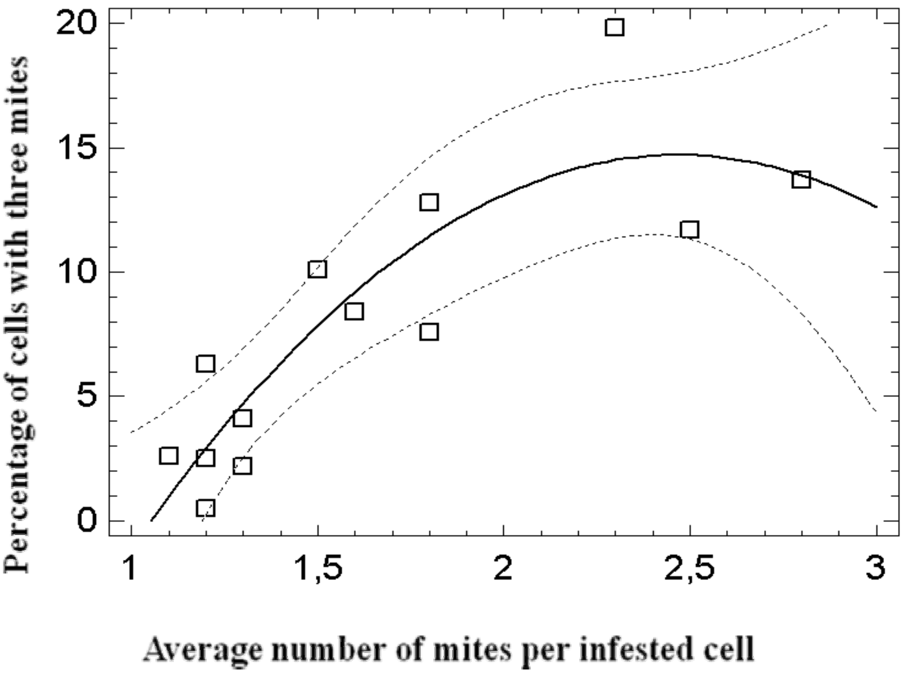

Fig. 15 - Relationship between average number of mites per infested cell and the percentage of cells with three mites.

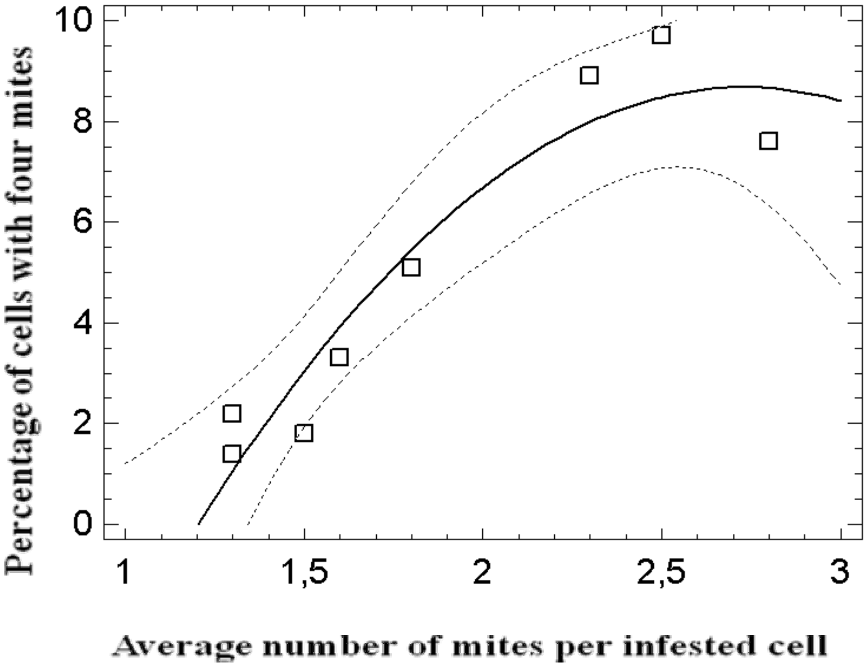

Fig. 16 - Relationship between average number of mites per infested cell and the percentage of cells with four mites.

The identified curves, all highly significant, assess the distribution of mother mites inside the brood cells according to different levels of infestation.

The progressive rate reduction of single mother mite cells showed in these curves tells us that a high level of infestation is compensated by the increased number of cells containing two or more mother mites.

The limit of applicability of these curves is represented by the range of the observed values (<2.8).

The reproductive rate of Varroa decreases with increasing multi infestation.

If for any given level of infestation the number of cells containing a specific number of mites is known then it will be possible to calculate the average rate of varroa reproduction.

For this purpose, we have simulated a series of mite distribution to increasing levels of infestation (n = 16) and converted it into a regression curve that expresses the prolificacy weighted average for mother mite in function of the average number of mites per infested cell (Fig . 17).

This information may obviously help in the implementation of a model on the varroa dynamics or to improve existing models. The same method was applied to derive a regression curve that describes the decrease in the longevity of adult bees always in function of the average number of mites per infested cell (Fig. 18). In this case, the information that is obtained can be used to predict what may the effects be on bee population dynamics for increasing levels of infestation.

The regression equations obtained were also used to derive the other two 2nd regression equations, useful to describe the percentage average reduction in fertility of mothers varroa and longevity of adult bees parasitized by mite during pre-imaginal stage. For this purpose, we used the data on fertility reduction of Varroa and longevity of adult bees reported in the literature (Martin, 1995; Degrandi- Hoffman and Currey, 2004).

In particular, for the fertility reduction of female mother mites the adopted values are as follows:

a) 9%, 14%, 40% and 100% in female brood cells with 2, 3, 4 and 5 mother mites, respectively;

b) 16%, 34%, 35% and 100% in male brood cells 2, 3, 4 and 5 mother mites, respectively.

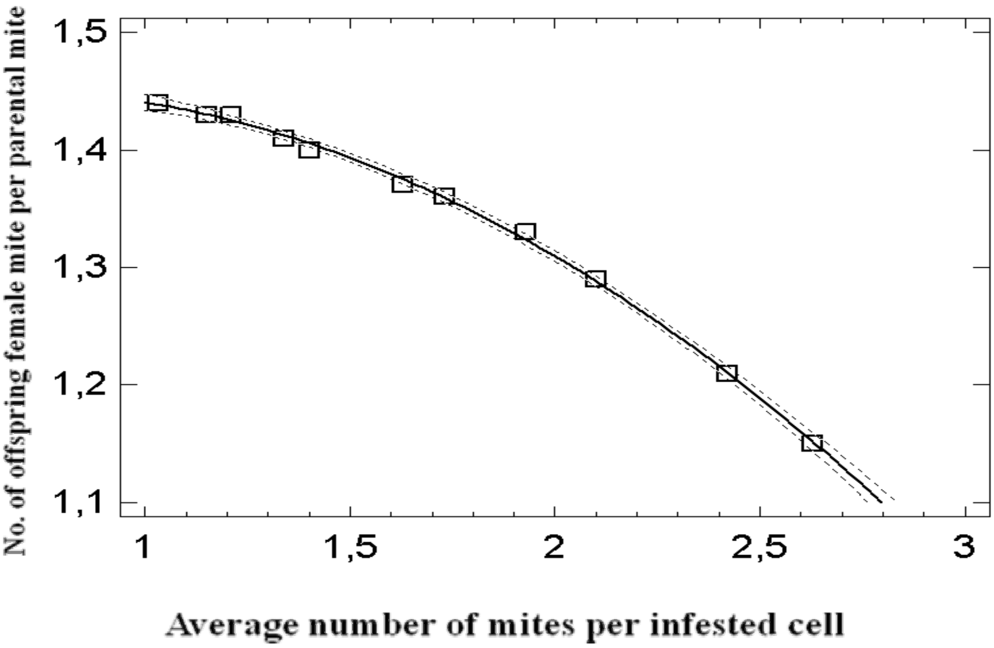

Fig. 17 - Relationship between average number of mites per infested cell and the number offspring female mites per parental mite.

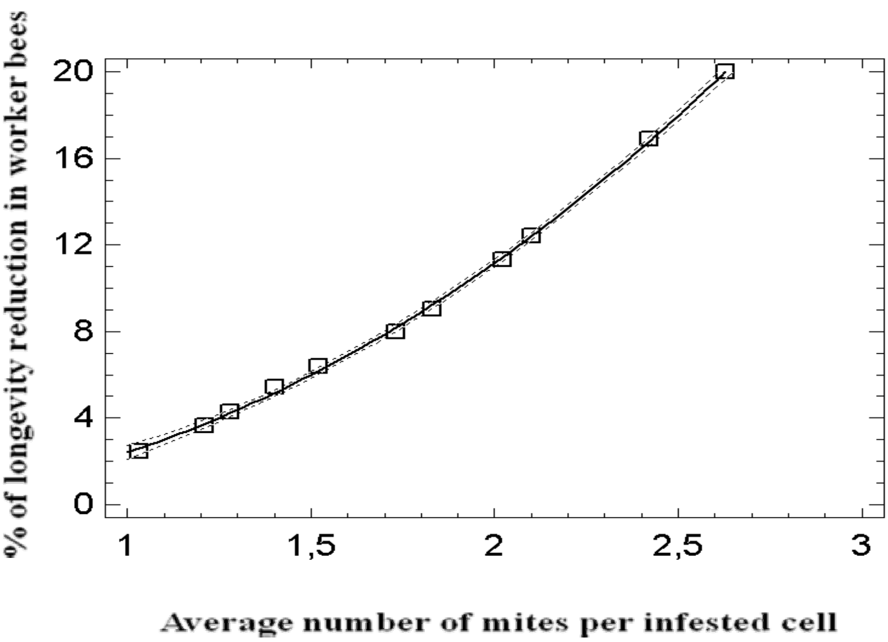

Fig 18 - Relationship between average number of mites per infested cell and the percentage of longevity reduction in worker bees.

For the longevity reduction of adult bees, were instead adopted the following values:

a) 10%, 20%, 40% and 80% in cells with 2, 3, 4 and 5 mother mites, respectively.

Conclusion

In conclusion with these regression equations, established the number of mites that pass from phoretic stage to reproductive stage and known the number of available cells to be invaded by the mite (using a forecasting model), it is possible to define the distribution of mother mites in brood cells for each infestation level (from 1 to 5 mites per cell). If these two parameters are not known, the equations could also be used to define the infestation brood level through sampling and obtaining from equation nr. 1, the average number of mites per infested cell.

Beside elucidating some relevant aspects of Varroa mites biology when it parasitizes *Apis mellifera,* these results are useful in the view to establish the most appropriate timing to perform treatments to control this serious pest.

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