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The impact of the natural honeycomb management on *Apis mellifera* colonies

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The mite ectoparasite *Varroa destructor*, poses a serious threat for the survival of the *Apis mellifera* colonies. The intensive use of acaricidal products is one of the most common methods for defending bees from *Varroa* that can cause the contamination of the wax foundation used in beekeeping. The natural honeycomb management could provide a solution for this problem, because it involves the use of frames without wax foundation which allows the bees to build a complete comb *ex novo*. On the other hand, colonies which are free to build cells of their choice, usually build a number of drone cells higher than colonies managed with the wax foundation. This could potentially lead to several negative consequences because the *V. destructor* reproductive success is greater on drone broods than on worker broods.

The aim of the present study was to examine the colony development, to evaluate the honey production and to monitor the growth of *V. destructor* infestations and associated virus infections in *Apis mellifera* colonies managed by using natural honeycombs compared with the conventional management.

Several colony parameters were measured in spring and summer. The strength of the colony was used to estimate the worker and drone populations. In order to measure the *V. destructor* infestations were used several methods, such as the natural mite fall, the powdered sugar roll, the soapy water and the brood cell uncapping. Molecular analysis was performed in order to measure the viral load of five *Apis mellifera* viruses. The honey produced was measured by collecting the honey stored in the supers, which are boxes placed on a beehive for bees to store. The results showed that the higher presence of drone brood in the colonies managed using the natural honeycomb did not negatively affect the colony development nor the mite *V. destructor* population compared to control colonies. The molecular analysis showed that the DWV was the most common virus found in bee samples, and its viral load was more influenced from the mite infestation rate than from the treatment.

The analysis carried out in this study showed that the natural honeycomb management can represent a valid alternative to the wax foundation. This kind of colony management thus appears to contradict our primary hypothesis which was that letting the bees build their own honeycomb would have led to a significant increase in the *V. destructor* infestation. Productivity data did not provide reliable results about the difference between the natural honeycomb and the conventional colony management due to climatic adversities. Further studies will be performed to better investigate this aspect.

Data about the natural mite fall and the estimation of the mite population in the phoretic/reproductive phases provided a useful starting point for further studies on the correct timing to carry out acaricide treatments both in conventional and natural honeycomb managed colonies.

CHAPTER 1. Introduction

After the global spread of the mite *Varroa destructor* Anderson & Trueman, 2000 (Delfinado, 1963; Matheson, 1996; Navajas, 2010), a parasite of *Apis mellifera* Linnaeus, 1758, beekeepers started to make an intensive use of different acaricidal products in order to control this mite, which has been one of the main problems of beekeeping in the last half century worldwide (Rosenkranz et al., 2010). Most of these products are fat-soluble synthetic pyrethroids which affect several aspects of both bees and human's health. A consequence of the use of acaricides in beehives is the contamination of the wax of the combs and consequently of the waxy foundations obtained from the processing of the wax of treated beehives. The contamination with acaricides of wax foundation is one of the causes of poor brood and colony development (Medici et al., 2012; Alkassab et al., 2020; Morales et al., 2020; Wilmart et al., 2021). These contaminants can transfer from the wax to honey and pollen causing a risk not only to bees but also to consumers of bee products. In addition, the persistent presence of accumulated small amounts of acaricides makes wax combs act as a persistent *Varroa*-killing treatment that may contribute to the development of their resistance to the chemicals (Bieńkowska & Konopacka, 2001). For example, cases of *Varroa* resistance to Apistan© (fluvalinate) were recorded only a few years after it was first registered for use in Italy (Milani, 1994; Lodesani et al., 1995; Watkins, 1997).

To prevent this problem, natural honeycomb beekeeping (even partial) could be adopted by beekeepers: this technique involves the use of frames without wax foundation (or provided with a small strip), in which bees can build their comb with a structure designed by themselves (Conrad, 2013). This natural approach allows the bees to build, beside the worker cells, a certain number of drone cells (Eckert & Shaw, 1960; Free, 1967). Many beekeepers consider drone breeding unnecessary and wasteful for the colonies and for this reason they prefer to use

frames with wax foundations of only worker cells. Indeed, drone breeding requires much more food than workers. Levenets (1956) found that it required 6.89 kg of honey to rear and feed 1000 drones during a season (Free, 1967; Johansson & Johansson, 1971). In addition, the *Varroa* mite reproductive success is greater on drones than on worker broods (Martin, 1998a; Nazzi & Le Conte, 2016). *V. destructor* is an obligatory ectoparasite mite of honey bees considered to be one of the main causes of colony mortality worldwide (Berthoud et al., 2010; Le Conte et al., 2010). The mite life cycle is closely connected to, and thus highly dependent on, the life cycle of the honey bee, since the mite reproduces in brood cells and feeds on the host hemolymph (Sammataro et al., 2000; Rosenkranz et al., 2010). In addition, mite infestations have also indirect pathological effects, including the spread and development of viral infections (Ball, 1988; Ribière et al., 2008), which significantly contribute to the collapse of honey bee colonies (Martin, 2001; Staveley et al., 2014). Several honey bee viruses linked to *Varroa* mite parasitism have been described (Ellis & Munn, 2005; Runckel et al., 2011). Some examples are the acute bee paralysis virus (ABPV), the black queen cell virus (BQCV), the chronic bee paralysis virus (CBPV), the deformed wing virus (DWV) and the sacbrood virus (SBV).

For a long time, beekeepers in many countries have tried to exploit drone broods in order to reduce the use of acaricides and delay the onset of pesticide resistance. The most popular method is the simple removal of *Varroa* mites from drone broods. Drone broods are, in fact, good candidates for trapping and removing *Varroa* mites because of the strong preference of *Varroa* mites for drone cells during the reproductive phase, when they invade cells for reproduction (Boot et al., 1995a). However, even if drone production may seem to be wasteful to beekeepers, it has an essential function for honey bee colonies: drones guarantee the spread of the genes of their mother queen and regulate the colony temperature, especially in the first part of the season, characterized by variable weather (Kovac et al., 2009). The drone population

reared in a colony might reach the 20% of a colony when beekeepers allow them to build the drone cells, which represents a considerable investment of resources (Rowland & McLellan, 1987). Indeed, colonies provided with only worker wax foundations produce drone cells in every available space (burr comb), thus disrupting their routine to find space for drone cells or to rebuild worker brood cells to satisfy this basic instinct (Johansson & Johansson, 1971).

In this regard, there are no studies concerning the development, productivity, and health status of colonies managed with natural honeycombs (i.e., allowing them to build proper drone cells), and scientific research on honey bees has been generally conducted on professionally-managed hives (i.e., in standard hives used with frames and wax foundations for worker broods). Therefore, there is still a lack of adequate data on the development and composition over time of honey bee colonies characterized by a more natural social structure than that actually observed in beehives raised in standard hives and managed with waxy sheets (Smith et al., 2016). Moreover, there is little information about the mite *V. destructor* development in these colonies with a more natural social structure. It is important to understand whether a larger drone rearing can affect parameters such as colony size and mite population growth, in order to understand how to manage this parasite in such beehives.

Several studies have attempted to model and quantify the *Varroa* mite population growth in order to understand and predict mite population dynamics when colonies rear more drone than colonies managed using wax foundation (Camazine et al., 1988; Fries et al., 1994; Boot et al., 1994; Boot et al., 1995b; Martin, 1998b; Calis et al., 1999b; Calis, 2001; DeGrandi-Hoffman & Curry, 2004; Wilkinson et al., 2002). On the contrary, there are very few field studies that monitor mite population growth or its infestation rate in *Apis mellifera* colonies and none of them was performed on colonies managed with the natural honeycomb and therefore in presence of a more natural social composition.

The aim of the present study was to address this knowledge gap by examining the development, assessing productivity and monitoring the growth of *V. destructor* infestation (and related viruses) in *Apis mellifera* colonies when using natural honeycomb management to allow them a higher drone brood production.

1.1 *Apis mellifera*

Apis mellifera Linnaeus, 1758 is an insect native to the old world (Europe, Africa and the Middle East) which has spread all over the world thanks to the trade in honey bee colonies which began in the XVI century. Common characteristic of all species belonging to the genus *Apis* is that they are eusocial insects and that the colonies they form are durable over time. There is a subdivision into castes in which we find the queen who is the only fertile female, workers who are sterile females that carry out the main jobs inside the hive, such as taking care of the offspring and foraging, and the drones, who are fertile males whose main role is to spread their genes mating with queens of other colonies. In addition, the honeycomb itself is generally considered to be the fourth caste, as it is a key part of a bee colony as a superorganism. The hive is made of the wax produced by the bees and it is shaped like hexagonal cells of various sizes in which bees rear their brood (the juvenile stages), and store pollen and nectar for the honey production (Contessi, 2016).

1.1.1 The queen

The queen is the mother of all the bees in the family. It is genetically identical to a worker bee however, unlike a worker bee, it is fed during the larval stage and throughout its entire life with the hypopharyngeal and mandibular glands secretion produced by worker bees, which is called royal jelly. The queen bee has a well-developed reproductive apparatus with a larger

abdomen than that of the workers, which makes it fertile. The specific diet is responsible for not only its morphology and fertility, but also its longevity. In fact, a queen can live even for five years, unlike a worker bee that lives about four weeks during the production season and no more than four-five months during the winter (Contessi, 2016).

The queen bee originates from a fertilized egg and is laid in a specific cell called the royal cell. The royal cell is created by worker bees and initially appears like an open dome downwards. After three days, a larva emerges from the egg and grows by going through five moults and eating only royal jelly. After the larva has spent five and a half days growing, the workers close the cell and strengthen the walls of the royal cell with wax. The larva creates a silky cocoon inside, undergoes one final moult, and turns into a pupa. After seven and a half days of the capping, the queen removes the wax cap and emerges. About 16 days after the egg is laid, the queen completes her development. From the 5th to the 15th day after its hatch, it starts orientation flight outside the hive until it is ready for the "mating flight" to the drone assembly place. The distance of the drone assembly place from its original beehive can range between three and 16 km. The queen mates with various drones multiple times during the wedding flight, and the mating takes place in the air. After mating, the spermatozoa move into the spermatic duct and reach the spermatheca, where they will be preserved for the entire duration of their life. The higher the number of drones that fecundate it, the longer will be the oviposition period, allowing for high genetic diversity among the bees in the colony to which they belong. Families of bees with slightly different characteristics may form over time due to the incomplete mixing of sperm from different males. As daughters of the same mother and different fathers, they should be considered stepsisters (Contessi, 2016).

The queen starts to lay eggs in the bottom of the cells two to five days after mating. Eggs that are provided with sperm are laid by the queen in both the workers' and royal cells, which

will produce workers bees and queens, respectively. Drones are a result of parthenogenesis in male cells, where the queen lays eggs without spermatozoa. In 1845, J. Dzierzon discovered the phenomenon of haplodiploidia in bees: females are born from fertilized eggs, while males are born through haploid arrhenotokous parthenogenesis from unfertilized eggs (Contessi, 2016).

The queen's role in the colony is more than just breeding cells to produce new offspring. The queen is responsible for the production of different pheromones, which contributes to the cohesiveness and functionality of the entire colony. The mandibular pheromone is one of them, and it is used to signal its presence to the rest of the colony. This pheromone enables families to better resist the honey robbing by bees from other colonies and promotes foraging bees to harvest. The absence of this pheromone indicates the absence of the queen in the colony. A series of actions are initiated in queenless conditions, such as the construction of royal cells which triggers the swarming phenomenon. The swarming is also caused by overpopulation, which prevents enough pheromone from circulating among all individuals of the colony. The pheromones are distributed among all the individuals in the colony for trophallaxis, which allows the bees to share food between different individuals of the colony by mouth. However, the amount of pheromone taken is drastically reduced during this process of exchanging substances from one bee to another; as a result, many bees do not receive an adequate amount of pheromone in colonies that are too large (Nauman et al., 1991).

1.1.2 Workers

The workers, as well as the queens, come from fecundated eggs. A larva emerges after three days from oviposition. For the first three days, it is fed with royal jelly, followed by honey and pollen. The larval stage lasts for 11 days, and during their growth, larvae undergo 5 moults. The cell in which it develops is capped by adult worker seven days from the hatching and then

the larva becomes a pupa. Finally, 12 days after the cell capping the adult bee emerges from the cell (Contessi, 2016).

Depending on her age, a worker can perform various tasks. During the first three days, the worker is responsible for cleaning the cells. From the fourth to the ninth day, it performs the function of a nurse bee, who has the task of feeding and supervising the brood. Initially, she takes care of the older larvae by providing them with water, honey and pollen, then it takes care both of larvae that are not more than three days old and larvae reared in royal cells. In fact, this period coincides with the development of the glands that produce royal jelly. Bees deal with the construction and repair of the honeycomb from the 10th to the 16th day, because this period coincides with the development of their glands which produce wax. Eight glands are located in the ventral part of the abdomen and allow bees to secrete thin flakes of wax. She spends the next three days to receive nectar and pollen collected from forager bees which will be stored into the honeycombs cells. At 20 days old, she is involved to the defense of the colony and from the third week she become a forager bee until death. During the production season, workers live for about 30-40 days, while those born in the fall can survive for 5-6 months. Unlike the queen, their ovaries are atrophied. However, they remain so only if the workers are in the presence of both the queen and the brood, which produce the mandibular pheromone and the brood's pheromone, respectively. In a queenless colony, the ovaries of the workers begin to develop again until some workers are able of laying eggs. Since these workers have never mated, they lay unfertilized eggs from which only males will be born, for this reason they are called workers drone layer (Contessi, 2016).

1.1.3 Drones

The drones are the males of the colony. They have larger eyes than workers, that almost touch the forehead, and a squared abdomen. Drones' development is slower than that of queens

and workers. The larva is born from an unfertilized egg after three days. It is fed with royal jelly for the first three days and then with pollen and honey. After six and a half days the larva is sealed by the workers with a convex cap. The larva creates a cocoon and develops into a pupa, and after 14 and a half days, the adult emerges. As a whole, the drone emerges after 24 days from egg laying. Their first orientation flights, lasting only a few minutes, occur nine to 12 days after the emergence. Drones are able to mate after 12-20 days, however they reach full sexual maturity at 30-40 days. Their lifespan is usually fifty days, but they are drive away or killed by the workers after the swarming period, depending on the season (Howell & Usinger, 1933; Fukuda & Othani, 1977).

It was once believed that the only purpose of drones was to fecund virgin queens. For this reason, beekeepers have always attempted to limit the presence of males by all means, as it does not lead to any practical advantage within the hive. Experiments have shown that if the number of drones is kept within the range of 2000 to 6000 individuals, they do not affect the production of honey. It is hypothesized that the honey they consume is compensated by other functions that allow the worker bees to perform other tasks (Contessi, 2016). The most recent research suggests that drones can rear larvae, heat the brood with the heat produced by their body, and participate in the maturation of honey through ventilation. Despite their inability to harvest, they are involved in the movement of nectar for trophallaxis, which is crucial for the maturation of the nectar in honey. Drone eggs normally first appear within a colony in spring, reach maximum numbers in June and cease to be laid in early autumn. Then, when foraging becomes more difficult, adult drones which have not died of old age are evicted from the colony by workers and soon perish (Allen, 1958; Free & Willams, 1975; Morse et al., 1967). The period and number of drones produced by a colony are determined by several criteria. Drones are not produced until there are sufficient workers to tend them, and the number of drones is regulated so they do not become excessive in a healthy colony. This first criterion not only

ensures that most drones reach maturity but also that their maximum number coincides with the occurrence of virgin queens with which they can mate, as the production of new queens is also connected with colony size (McLellan & Rowland, 1986). The second criterion is a compromise between the logistical burden of feeding and caring for large numbers of drones and the biological necessity for transmitting more genes to the general bee gene pool, which can only be achieved if there are sufficient drones from that colony able to mate with neighboring virgin queens (Free & Williams, 1975).

1.1.4 Economic importance

Bees play an essential role in the formation and conservation of the environment in which they live. Thanks to the mutual dependence relationship established with angiosperm plants about 90-100 million years ago, they contribute to the conservation of plant biodiversity and therefore of ecosystems in general, thanks to their pollination service. In addition, they have a crucial economic role as crop pollinators (Gallai et al., 2009; Potts et al., 2010; Calderone, 2012; Melathopoulos et al., 2015). Both managed and wild bees are responsible for about 70% of the global pollination and guarantee about 35% of global food production. An estimated 87.5% of the world's flowering wild plants (about 308.000 species) depend, at least in part, on animal pollination for sexual reproduction, and this value ranges from 94% in tropical plant communities to 78% in those of temperate zones (Schmeller, 2017). It has been shown that 70% of the 115 crops of global importance benefit from animal pollination (Klein et al., 2007); moreover, pollination contributes to the increase in the annual world monetary value of agricultural production amounts to about 260 billion euros (Lautenbach et al., 2012). In Europe, the production of about 80% of the 264 cultivated species depends on the activity of pollinating insects (Hendrikx et al., 2009).

1.2 *Varroa destructor*

1.2.1 Description and spread

Figure 1 – The mite *Varroa destructor*, Anderson & Trueman (2000). www.cabidigitallibrary.org

The mite *Varroa destructor* Anderson & Trueman, 2000 (Figure 1), is considered the most important ectoparasite of *A. mellifera* (Nazzi & Le Conte, 2016), causing devastation to honey bee populations throughout the world for the past thirty years. At the beginning it was confused with another mite species called *Varroa jacobsoni* Oudemans, 1904. In fact, the current name of this species was formalized only in 2000 (Anderson & Trueman, 2000). Both species infest their primary host, *Apis cerana* Fabricius, 1793 (or Asiatic bee), even though without causing negative effects on this species, which is able to remove the mites from the infested body thanks to a special grooming behavior adopted by nestmates (Rath, 1999). Probably the mite leaped from *A. cerana* to *A. mellifera* for the first time in the Philippines during the fifties and sixties of the 20th century, following the importation of *A. mellifera*

colonies. This event made the contact between these two species of bees possible and allowed *V. jacobsoni* to change host, to undergo a period of rapid evolutionary change and to speciate into the harmful parasite *V. destructor*. Molecular biology studies have shown that the *V. destructor* species is well differentiated from the *jacobsoni* one and that, among the various haplotypes observed within the species, only two are highly pathogenic for *Apis mellifera*: the Korean and the Japanese ones (Anderson & Trueman, 2000, Rosenkranz et al., 2010).

V. destructor was thus identified as *V. jacobsoni* for the first time on *A. mellifera* in 1958 (Mikawa, 1986) and the uncontrolled movement of *A. mellifera* by humans has led to the subsequent spread of the parasite to Europe (1967), North Africa (1975), South America (1971), and USA (1987) (De Jong et al., 1984; Needham, 1988; Matheson, 1995). The spread of the *Varroa* mite across the globe has considerably increased in recent years, with the invasion of countries such as Costa Rica, South Africa, Panama, and New Zealand (Van Veen et al., 1998; Calderon et al., 2000; Harman, 2000). *A. mellifera* colonies are moved all over the world for commercial reasons: transport of bees and queens, the migratory activity of beekeepers, and swarms that can be carried in aircraft and ships represent the *modus operandi* of its earlier spread (Shimanuki et al., 1980). The mite spreads between honey bee colonies through the robbing of hives by foraging bees, the drifting of foraging bees and drones, and by migrating honey bee swarms. The use of honey bee colonies in commercial pollination, which brings into close proximity colonies that were widely dispersed, accelerates the spread. In addition, the long-distance migration of honey bee swarms by beekeepers, for commercial pollination and to exploit honey-flows, results in the rapid wider dispersal of the mite.

1.2.2 Life cycle

Varroa destructor is an obligatory parasite, spending its entire life in the bees' nest either on immature stages or on adult bees (Rosenkranz et al., 2010; Sammataro et al., 2000). The life

cycle involves two distinct stages: the phoretic phase, which is spent on the adult bee, and the reproductive phase, which is spent inside a bee brood cell. The invasion of the brood cell, which represents the beginning of the reproductive phase, occurs some hours before a cell containing a bee larva is sealed. Fertilized female mites invade a cell containing a bee larva, attracted to the cell by volatiles released by the larvae prior to cell capping (Le Conte et al., 1989). Important note: they prefer drone brood cells to worker cells (Fuchs, 1990). Once the cell is capped, the mite feeds on the bee's hemolymph and lays eggs on the surface of the cell wall (Boot et al., 1992). The first egg laid develops into a male, and all subsequent eggs into female mites (Shimanuki et al., 1980). The offspring mate with each other until the emergence of the adult bee (Ifantidis, 1991). When the adult bee emerges, the male mite dies, whereas the mother mite with her mated daughters leaves the cell onto the young bee where they spend the phoretic phase before entering a brood cell to reproduce again. Adult mites live on the blood of adult bees, have a lifespan of 2-3 months, and a female may produce two or at most three broods (Fries et al., 1994).

1.2.3 Impact of Varroa mites and viruses on beekeeping and honey bees

There are several causes that lead to the loss of bees, such as pesticide residues, pathogen agents, climate change or cultivation systems, but the weight of each factor remains unclear (Chauzat & Faucon, 2007; Mullin et al., 2010; Serra-Bonvehí & Orantes-Bermejo, 2010; Smith et al., 2013; Goulson et al., 2015; Switanek et al., 2017; Flores et al., 2019). However, *Varroa destructor* can be considered the main pathogenic agent leading to the colony losses observed in most parts of the world. *Varroa destructor* and *Apis mellifera* have not co-evolved for a long period of time, for this reason they do not exhibit an adapted host parasite relationship, resulting in *Varroa* often killing its host. Its dangerousness can be attributed to its trophic activity which causes a weight loss of the infested adult bees, a reduction in the protein content in the

hemolymph and a drastic reduction both in longevity and vitality of the infested adult bees, causing weakness or stress on the bee health (De Miranda & Genersch, 2010; Di Prisco et al., 2011). At the colony level, untreated *Varroa* infested colonies usually die within six months to two years. High bee density combined with severe *Varroa* infestation accelerates bee death (Ritter et al., 1984). When adult bees are infected before overwintering, they survive a shorter time compared to mite-free bees, as a result transition from summer to winter bees does not occur. As a consequence, they do not live as long as winter bees and are less able to contribute to the increase of colony strength in the early spring. When the mite infestation is high, parasitized colonies collapse during the winter (Kovac & Crailsheim, 1988; Boecking & Genersch, 2008).

In addition, the mite plays a fundamental role in transmission of pathogens, mostly viruses (Neumann & Carreck, 2010; Rosenkranz et al., 2010; Francis et al., 2013), whose infection in turn may generate favorable conditions for more severe outbreaks of Nosema (Forsgren & Fries, 2010). Viruses are diagnosed with different frequencies, depending on the area in which the studies are conducted or on the honey bee subspecies. It was proved that different subspecies of *A. mellifera* may react differently to management practices or environmental conditions, including pathogens (Ruttner, 2013; Hatjina et al., 2014). The most widespread and dangerous bee viruses all over the world, able to cause severe disease in honey bees are the acute bee paralysis virus (ABPV), the black queen cell virus (BQCV), the chronic bee paralysis virus (CBPV), the deformed wing virus (DWV) and the sacbrood virus (SBV).

The ABPV infection in honey bee colonies might not be apparent, while the fatal infections are usually related to infestations of *V. destructor*, which is its vector, causing paralysis of adult bees and dead broods in a short period (Tentcheva et al., 2004).

BQCV is one of the most prevalent and widespread viruses in honey bee colonies, with a constant annual incidence in the adult population closely related to *Nosema apis* or *Nosema ceranae* infestations (Doublet et al., 2015). Although BQCV does not cause visible symptoms in infected adult bees, however, it clinically affects mainly pre pupae or pupae of the queen, especially in spring and early summer (Laitlaw, 1979). Infected queen pupae die and the cell walls get black. According to reports of beekeepers, BQCV can cause problems with queen rearing and may influence over-wintering honey bee losses (Faucon et al., 2002).

CBPV is transmitted through food or wounds, causing severe paralysis in adult bees and may contribute to colony losses. The clinical manifestation of the disease is severe; the adult bees are unable to fly, they tremble and crawl, and the wings are asymmetrically outspread. The bees look black due to cuticle melanisation and hair loss (black bees). Some clinical signs such as trembling may be confused with intoxication syndrome (Schurr et al., 2017). The viral outbreaks are often reported in the spring and summer months. There are indications that overwintering losses may be associated with CBPV infection (Faucon et al., 2002; Antúnez et al., 2005). Although in some cases up to 30% of worker bees are affected, CBPV infection sometimes remains undetected, and the colonies usually recover spontaneously from the disease (Bailey, 1967).

DWV is the most important virus vectored by *V. destructor*. It causes severe loss of bees leading to bee hives collapse (Highfield et al., 2009; Nazzi et al., 2012; Giacobino et al., 2016). Although DWV occurs in *Varroa*-free natural populations (Martin et al., 2012; Mouret et al., 2013), it appears to amplify in the presence of the mite, either because it can replicate in *Varroa* (Ryabov et al., 2014; Gisder et al., 2009) or because virus particles accumulate in the mites' guts (Erban et al., 2015). Indeed, the clinical morphological symptoms like deformed wings and shortened abdomens develop only when *Varroa* is associated with DWV, which is lethal

to the bees (Ball & Allen, 1988; Martin, 1998a; Bowen-Walker et al., 1999; Martin, 2001; Tentcheva et al., 2006). There is also evidence that *Varroa* not only acts as a vector but also increases the virulence of DWV infections, turning relatively asymptomatic infections into "overt" infections associated with clinical disease symptoms and increasing colony mortalities in winter.

SBV affects the brood of the honey bee and results in larval death. Infected larvae fail to pupate and ecdysial fluid aggregates around the integument, forming the "sac" from which the disease takes its name. Larvae change in color from a pearly white to pale yellow; after death, they dry out and change to a dark brown ship-shaped scab. Infection of adult bees is possible; the viruses are able to propagate in them, but the bees remain apparently healthy. Sacbrood virus appears mainly in spring, when the brood season begins and large numbers of infected young adults are present (Ritter, 1996).

1.3 Problems of the beeswax foundation used in the modern beekeeping

The honeycomb is a structure built by the bees using the beeswax. As above mentioned, it is also considered the biggest organ of the bee colony (Tautz, 2008) or the fourth cast of the bee colony (Fontana, 2021). Bees build the honeycombs on which they live with the wax produced by the glands present in their abdomen. The comb consists of two faces with hexagonal cells which, starting from the center, develop towards the outside. Cells are used to rear brood or alternatively to store honey and pollen.

The honeycomb used in modern beekeeping is often boarded in a wood structure called frame that contains a steel wire which crosses the inner structure to support it. In beekeeping, the conventional honeycomb is built starting with a beeswax foundation included into the frame

that the bees use to complete the cells. Conversely, a natural honeycomb is a comb that is fully built by bees without the use of beeswax foundations. It is also called “foundationless frame”. Beeswax foundation (or simply foundation) is considered to be one of the great beekeeping inventions. It was invented by Johannes Mehring in 1857, shortly after Langstroth introduced the moveable comb hive in 1852 (Hogg, 1980). The foundation is a flat sheet of beeswax that is embossed with the shape of the base of the cell, either made by casting or rolling. In this way, the beekeepers provide the bees with a "foundation" on which to build their comb. Foundation, basically, is used to guide the location and orientation of the comb. Patterned foundation also guides the size of the comb cells, which influences the size of the worker bees and discourages the production of drones.

From this descends that the foundation is not required, as bees will build comb without it. But considering that it takes 8-10 kg of honey to produce 1 kg of beeswax (Fontana, 2021), it is evident that most of the beekeepers prefer to add the foundations in order to improve the honey production. The typical foundation pattern has a 4.9 mm to 5.4 mm cell diameter, which encourages the production of worker bees instead of drones. Studies show that the worker cell’s size is closely related with the climatic area and the monthly temperatures. Size of the worker cells built by bees living in cool-climate are between 4,9 and 5,2 mm. In contrast, in warm-climate areas the cell diameter is between 4,7 and 5,0 mm and in tropical climates it is between 4,5 and 4,8 mm. The cell size influences the bee size and longevity, and the larvae life-cycle length, which might consequently condition the reproductive rate of *V. destructor* (Maggi et al., 2010). For these reasons, the use of beeswax foundation with an absolutely arbitrary size may not lead to a direct advantage for bee colonies except for a higher honey storage, because it does not consider the colony necessity (Fontana, 2021).

Recent research underlines that the commercial foundation is often contaminated. Acaricides used for the control of *Varroa* are a major source of pollution in a honey bee hive. Many surveys in Europe and in the USA have shown widespread use of acaricides, resulting in a high level of contamination in beeswax. The most frequently detected pesticide residues in beeswax samples were coumaphos and fluvalinate (Chauzat & Faucon, 2007; Lodesani et al., 2008; Ravoet et al., 2015; Calatayud-Vernich et al., 2017). The action of these pesticides presents different effects in the beehive. Studies conducted by Johnson et al. (2009) underlined the synergistic effects of fluvalinate and coumaphos showing that there is a marked increase in fluvalinate toxicity on younger bees previously treated with coumaphos. Medici et al. (2012) found that the presence of acaricides in the beeswax affected adversely brood survival. Bee broods developed in a contaminated environment are more sensitive to the effects of these acaricides than adult bees. Larvae developed in the beeswax with a concentration of coumaphos and fluvalinate of 6311.64 ± 757.39 µg / kg and 204 ± 30.75 µg / kg respectively, were associated with survival rates of 65 ± 8.6 %. Fluvalinate negatively affects the survival rate of drones. Experiments conducted by Rinderer et al. (1999) showed that in colonies treated with Apistan©, the survival rate of drones after one day, five to 11 days and 12 to 18 days from emerging were of 86.1, 43.0 and 33.0 % respectively compared with control colonies whose survival rate were of 97.5, 53.6 and 37.5 %, respectively. In addition, it reduces seminal vesicle weight and sperm counts production of drones. Experiments conducted by Burley (2007, 2008) confirm the negative effect of miticides on drone reproductive physiology. The author compared colonies treated by Apistan© (fluvalinate, Checkmite+® (coumaphos) and Apilife Var® (containing thymol, eucalyptus oil and L-methanol) with control colonies. He found that drones in treated colonies exhibited significantly lower sperm viability than control colonies. In addition, drone sperm counts were lower in colonies treated with fluvalinate, thymol and coumaphos, compared to drones in untreated colonies. Other field studies have reported that

apparently healthy hives showed very low survival rates of bee larvae, probably caused by a very high acaricide concentration in the wax (Orantes-Bermejo et al., 2010).

Adulteration of the beeswax foundation with cheaper vegetal or industrial waxes including paraffin or stearin have been increasing lately. Some researchers investigated the impacts of adulteration on colony development of honey bees. Studies carried by Castro et al., (2010) showed that the addition of paraffin wax to beeswax foundation decreased the area built in the combs and also reduced the queen acceptance for oviposition. Reybroeck (2018) showed clear brood losses and adverse effects on brood development at stearin concentration up to 7.5%.

1.4 Why it is important to evaluate the effect of the drone on the development and health status of the colonies

As is already known, the drone brood is strongly preferred by *V. destructor* for several reasons:

1) The period between a cell's invasion and the capp is longer in drone cells (45h) than in workers' cells (18h), making it therefore more suitable for invasion (Ifantidis, 1988; Boot et al., 1992).

2) The average number of female offspring produced by a single mother mite invading a worker cell is between 1.2 - 1.5, while this value rises to 2.2 - 2.6 in drone brood cells because the period that the drone pupa spends closed inside these cells is greater than in the worker pupae cells (Boot et al., 1995b; Martin, 1995a).

3) The weight of a drone and a worker larva during the capped of the cell is respectively 365 mg and 140 mg. Consequently, the nurse bees will spend more time visiting the drone cells than the worker cells. So, a mite has a much better chance to visit a drone cell than a worker cell (Martin, 1998a).

4) Chemical factors make the drone brood more attractive than the worker brood for the mite (Le Conte et al., 1989).

For these reasons, the number and duration of drone broods reared represents an important factor which could significantly increase the mite reproductive rate (Martin 1995a; Harris et al 2003). Nevertheless, there are relatively few studies on the variation in numbers of drones in a honey bee colony throughout the year (Allen, 1958, 1963, 1965; Fukuda & Othani, 1977) and none of them also consider their contribution in the mite infestation rate in open field tests. Several models have been created to predict the mite population growth related to drone rearing. All of them are based on the assumption that the mite population growth is strongly affected by the drone number and for this reason they predict a substantial mite increase in spring and summer, when the number of drone cells is the highest (Fuchs, 1990; Martin, 1995a). One of those (Wilkinson & Smith, 2002) is the model which accounts for the importance of drone brood by modeling the *Varroa* population according to the percentage of drones in the bee colony. The model explains that a relatively small percentage of drone's brood can contribute to a large proportion of the mites emerging from the brood after reproduction, suggesting that beekeepers have to prevent the development of a large quantity of drone brood.

1.5 Aims of the project

The aim of the present study is to obtain useful information about the development, the productivity and the health status of the *Apis mellifera* colonies by using natural honeycomb management to allow them a natural development of the combs and thus a higher drone brood production, compared with a conventional honeycomb management from the spring to summer.

The study was made both in Italy and in the Netherlands using several methods and techniques to collect data in the field and in the laboratory. The aims of the experiments carried out in both countries were the same, however they can be considered complementary to each other. To measure the development of bee colonies in Italy, the strength of the colonies was estimated (adult bees, worker brood and drone brood). The honey stored in the super was measured to collect data on honey production. The health status was evaluated by measuring the *V. destructor* infestation rate and the viral load of the viruses detected in the colonies. In particular, the *Varroa* infestation rate was estimated on adult bees and by using the mite fall method which gives an indication of the colony infestation. In the Netherlands experiments were set up to integrate information that was not obtained in Italy. The strength of the colony was estimated, and the mite infestation rate both on adult bees and in the brood, making a distinction between worker brood and drone brood. This information was used to estimate the *Varroa* population of the colonies and to obtain precise data of the *Varroa* distribution between the phoretic and reproductive phases.

Our starting hypothesis was that a drone production increase in colonies managed both by adding empty frames and drone foundation is expected compared to the colonies managed by adding frames with worker foundation. This higher drone production and the natural honeycomb managing could cause several consequences. Worker production could decrease in

those colonies drone broods are reared in higher quantity compared to the control colonies. Bees might use more space building drone cell honeycombs rather than worker cell honeycombs. In addition, drone larvae need much more food compared to the worker larvae, and therefore the colonies that invest more resources in drone rearing may limit the worker production, in particular during the season in which drones are reared in abundance. Finally, colonies managed by using the natural honeycomb have to invest more energy to build their combs *ex novo*, which could negatively affect the development of the colony. All these factors could negatively affect the development of these colonies compared to the colonies managed by using foundation.

In the experiment performed in Italy we expected that colonies which reared more drone broods increased the mite infestation rate due to the higher reproductive success of *V. destructor* into the drone cells. Consequently, in these colonies we expected to observe a higher infestation rate on adult bees and consequently also higher mite fallen and mortality during the bee emergence. For the same reasons, we also expected a higher viral load of those viruses related to *V. destructor* (i.e., ABPV and DWV).

CHAPTER 2. Experiment managed in Italy

2.1 Materials and methods

2.1.1 Setting up experimental colonies

The first part of the research was dedicated preparing the colonies for the experimentation in the field. The open field test was carried out near the city of Rovereto, in Northern Italy (45°52'43''N; 11°01'13''E), and lasted for three years. 60 experimental colonies were set up between June and July 2019, equally divided into three apiaries at about 700 m from each other and at the same altitude (204 m). To house all the honey bee colonies were chosen the Dadant beehives, commonly used by Italian beekeepers. They were prepared putting inside five starting frames with worker foundation and an equal number of bees using the artificial swarming technique. The old queen was removed and new queens reared from the same mother were used for each colony in order to minimize bee genetic variation. Colonies were feeded with sugar (1,25 Kg) and let them develop. Acaricide treatments against *V. destructor* were carried out in July and at the end of November with Api-Bioxal© (Chemicals Laif) by following the instruction on the label.

2.1.2 Colony management

In the second year, at the beginning of March 2020 the number of bees, combs with brood and food resources of each surviving colony were equalized, the latter using the method described by Delaplane et al. (2015). All colonies consisted of three brood combs, two frames with food resources and one division board, which is a special board in polystyrene included in a frame. It is used to divide the hive into two part which are the nest and the empty area. This tool allows to keep the nest wormed and prevents bees from starting to build the comb

from the roof of the hive.Each apiary (Figure 3) represented a different kind of colony management (Figure 2):

- C (n = 13): these colonies represented the standard method of colony management from beekeepers and thus our negative control: they were managed by adding frames with wax foundation with only worker cells during the seasons (i.e., no drone cells).
- T1 (n = 16): these colonies represented a natural honeycomb management and thus our treatment. They were managed by adding frames having at the top only a 3 cm strip of foundation in order to allow bees to build a regular comb according to their preference.
- T2 (n = 12): these colonies were managed by adding 2 frames with drone foundation and subsequently regular worker foundation and thus they could be considered as a positive control. In this way, we wanted to facilitate the bees to rear a higher number of drones than in the other conditions. In the meantime, the energetic investment made by the T1 colonies to build *ex novo* their comb was excluded.

Colonies were checked every week in order to evaluate whether adding or not a new frame with foundation (C and T2 colonies) or with the strip of foundation (T1 colonies) according to their needs. Frames were added from the beginning of April to mid-June, and the last frame was added in the 85% of the colonies at the middle of May. The honey super, the box that holds the frames where the bees stored part of their honey, was added on the top of the hive when the last frame added in the core hive was well built and the surface was covered with more than 50% with adult bees. Chemical treatments were carried out on August 5th with Apivar© (Amitraz), on September 22nd with Apistan© (Fluvalinate) and on November 26th with Apibioxal© (oxalic acid).

In the third year the surviving colonies plus 14 extra colonies were used in order to balance the number of colonies for each treatment. As well as in the second year, the number

of bees, brood cells and food resources were equalized in each colony before starting the data collection. The third year the only chemical treatment used was Apibioxal (oxalic acid). The acaricide treatment was carried out on July 28th after 30 days from the caging of the queens that was performed on June 28th in all the colonies.

Figure 2 – Types of frames with wax foundation used. The image a) represents the frame used in C colonies with the standard wax foundation that colonies use to build worker cells b). The image b) represents the frame used in T1 colonies with the little strip of wax foundation that colonies use to build both worker and drone cells d). The image c) represents the frame used in T2 colonies with the drone wax foundation that colonies use to build mainly drone cells f).

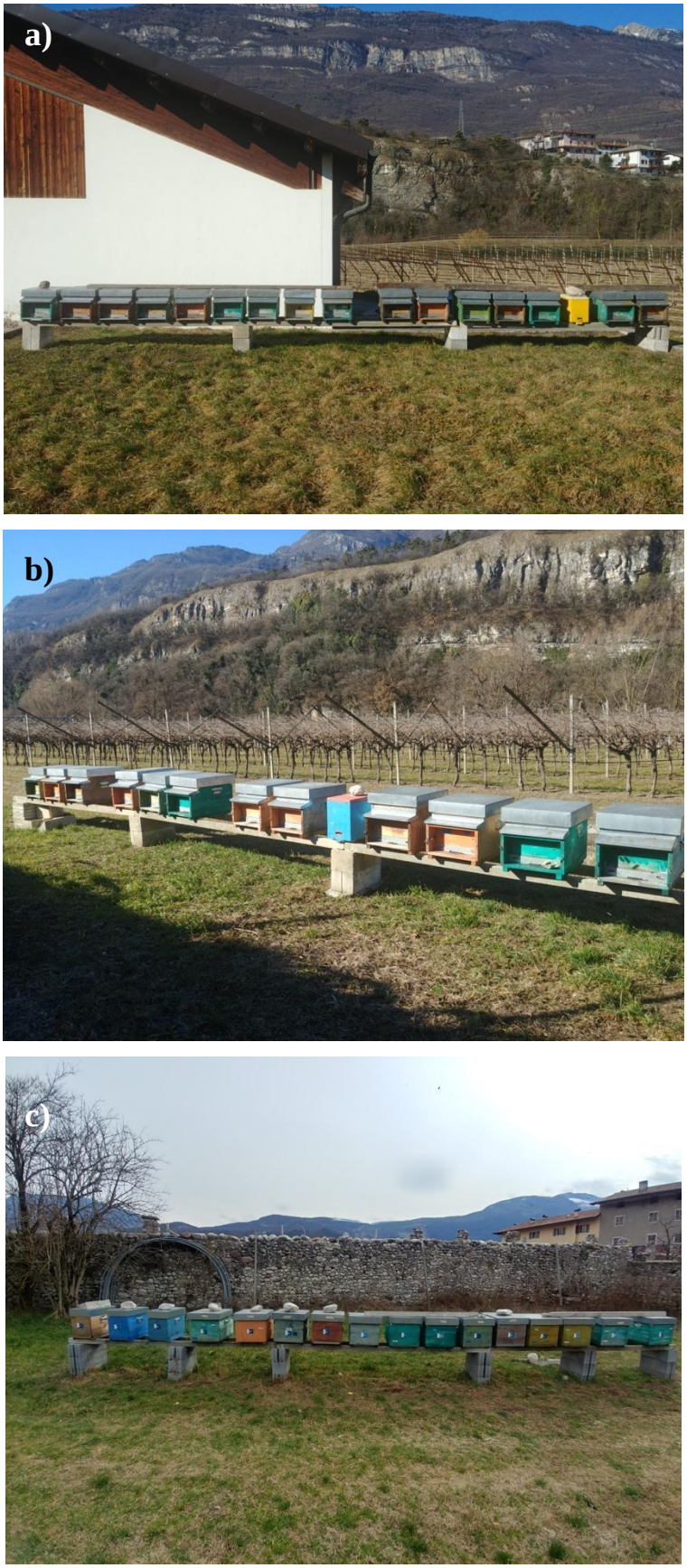


Fig. 3 – Experimental colonies divided in three apiaries; each of them represents a different treatment: a) C, b) T1 and c) T2.

2.1.3 Estimation of the colony strength

The estimation of the colony strength is a method which allows estimating the number of adult bees and brood cells in each colony. Every 3 weeks, images of each comb with and without bees were taken in order to estimate the number of bees and brood of each colony. Images were taken in a narrow time window of 2 - 4 days, randomly assigning the time of inspection so that the day effect is randomly distributed across treatments. The work was carried out by 2 operators; one of them took the frames from the hive while the other one acquired the images with the camera (Panasonic Lumix DMC-TZ20). In order to reduce the bias caused by the bees' flying activity, images with bees were taken in a time range from 7:00 am to 9:30 am according to the date (i.e., earlier in the summer, later in early spring and fall) and when the temperature was higher than 15°C. Images of the broods were taken after shaking the frames inside the hive to get rid of the bees.

The number of adult bees was evaluated through a subjective method estimating the percentage of area covered by bees on each comb side. This method involve that the human observer visually estimate the surface area of a comb covered by a target as well bees, brood, honey etc. (Delaplane et al., 2013). To do that, each side of the comb has been ideally divided into 10 parts, each of which represents 10% of the entire comb surface. In this way, the operator could take note in the field of the percentage of surface occupied by bees and this value was used to estimate the number of bees on each frame according to the table of Hernandez et al. (2020).

For the capped brood estimation an objective mode based on the computer-assisted digital image analysis has been used. This method consists in analyzing the images with the Java-based image processing software ImageJ (Schneider et al., 2012) in order to measure the brood surface. Every image was opened with the software and the scale calibrated (usually the

longer side of the frame was used as a reference scale). The brood area was selected using the freehand selection and the outside area was excluded. The threshold color was adjusted modulating saturation and brightness in order to select with a high accuracy the capped brood cell excluding the non-interest areas. The selected area was then measured in cm^2 using the appropriate command converted in number of worker and drone brood cells according to the table of Delaplane et al., 2015.

2.1.4 Infestation of Varroa measurement

Two sampling methods were performed to estimate the mite infestation rate: (1) by monitoring the natural fall of *Varroa* and (2) by using the powdered sugar roll method.

2.1.4.1 Natural mite fall

The natural mite fall method is based on the quantification of naturally dead mites. It was calculated by counting the number of mites fallen on the floorboard trap placed below the hive (Branco et al., 2006). A squared sheet of paper was placed on the top of the floorboard and covered with a thin layer of Vaseline (white soft paraffin EP, Merkur 500, A.C.E.F. s.p.a.) in order to trap the fallen *Varroa* and to prevent ants from preying the mites. Every week, the fallen mites were counted and removed, and the floorboards were cleaned and re-covered with Vaseline (Figure 4).

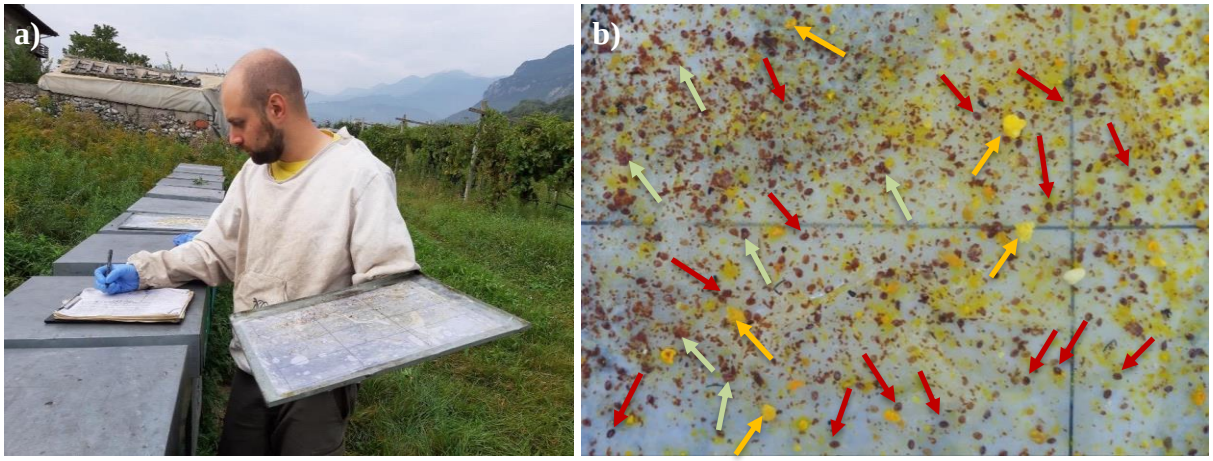


Fig. 4 – a) Tray used to trap and count the mite fall. b) The picture shows a portion of the tray, red arrows indicate the mites, yellow arrows indicate the pollen and the green arrows indicate pieces of cell cap of the comb which fall on the tray when the bees emerge from the cell.

2.1.4.2 Powdered sugar roll method

The powdered sugar roll method (Figure 5) was performed according to the protocol of Dietemann et al. (2013). To have a representative sample, about 300 adult bees were collected with a 100 mL container directly from the brood chamber of each colony covered with bees. Samples were taken during the day in a period from 10:00 am to 12:30 am. Bees were put in a jar with 35 g of powdered sugar and rotated for 180 s in order to cover the bee bodies with sugar. Then the jar was turned upside down and shaken vigorously for one minute over a sieve that does not permit the passage of the mites. To determine the infestation of adult bees with *Varroa* mites, the number of mites counted was divided for the number of bees in the sample and multiplied by 100 (Dietemann et al., 2013, Jack et al., 2020).

Figure 5 – Steps of the powdered sugar roll method. 1) Bee collection by moving the edge of the jar on the bee from the top to the bottom. 2) Icing sugar added into the jar with the bees. 3) A view of the bees covered with icing sugar. 4) Mixing process to cover the bee bodies with sugar. 5) The jar was turned upside down and shaken to collect mites. 6) Mites collected over a sieve.

2.1.5 Viral load analysis

2.1.5.1 Collection of honeybees

During the first year, three samplings were performed, one every two months (May, July, and September), while in the second year four samplings were performed monthly (April, May, June, and July). At least 40 bees per colony were collected from the hive with 50-ml falcon tubes. Tubes with bees were temporarily stored in wet ice and then at -80°C in the laboratory until total RNA extraction.

2.1.5.2 RNA isolation

During the entire procedure, the work was carried out in wet ice. Before the RNA extraction, 40 whole bees per colony were transferred to extraction bags (BIOREBA) with 4 mL of DEPC water and then crushed until the mixture was homogeneous. Two portions of 500 μL were transferred to 2 mL tubes: one was stored at -80°C and the other one was used for the RNA extraction.

RNA extraction was performed adding 900μL of Trizol to each tube, by vortexing for 1 min and incubating at room temperature for 5 minutes. Then, 180 μL of chloroform were added to remove proteins, lipids, and DNA and each sample was vortexed for 30 sec. Tubes were centrifuged at 12000 rpm for 15 min at 4°C, and 800 μL of the supernatant were transferred in a new 2 mL tube. Then, 450 μL of isopropanol were added and the tubes turned 4 times in order to mix the solution and incubated for 10 minutes at room temperature. After supernatant elimination, 1350 μL of ET-OH 75% (diluted with DEPC water) were added and each tube was centrifuged at 12000 rpm for 5 minutes. Supernatant was removed and the tube let dry under the hood. RNA samples were resuspended in 100 μL DEPC water and stored at -80°C before proceeding with the next step.

2.1.5.3 Reverse transcription

Before the quantification, the RNA extracted was diluted 1:20 (in nuclease-free water). Quantifications were performed with the Qubit™ fluorometer (Invitrogen, Thermo Fisher Scientific), using the RNA broad range kit. The concentration of the RNA samples was then equalized to 500 ng/µL (in a final volume of 50 µL), using DEPC water.

Reverse transcription was performed in 2 steps: in the first step a solution with 1 µL of Mix Random Primers + dNTPs (10 mM each) (1:1) (Invitrogen, Thermo Fisher Scientific), 4 µL of DEPC water and 7 µL of extracted RNA was prepared for each sample in a 200-µL tube. After 1 cycle in the thermocycler at 65°C for 5 min, the second step was performed adding a Retro Mix composed of 1 µL of M-MLV Reverse Transcriptase, 1 µL of RNase OUT, 2 µL of DTT and 4 µL of Buffer (Invitrogen, Thermo Fisher Scientific) to the sample. Then, 3 steps at 25°C, 37°C and 70°C were performed respectively for 10 min, 60 min and 15 min. The cDNAs obtained were stored at -20°C.

2.1.5.4 Real-time PCR

Real-time PCR was performed using primers designed to detect five honeybee viruses: acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), and sacbrood virus (SBV) (Locke et al., 2012; Mondet et al., 2014). For each virus, a standard curve was built starting from a pGEM®-T Easy plasmid (Promega), containing the target viral sequences, cloned in *E. coli* cells. One-step real-time RT-PCR was developed based on SYBR Green detection. The assays were run on an CFX96 Real-Time Detection System (Bio-Rad) thermocycler in 10-mL reaction volumes containing 7 µL of working solution and 3 µL of template (cDNA template or negative template or standard). The working solution for each virus was prepared with 1 µL of dd water, 5 µL of SYBR Green

PCR Master Mix, and 1 μL of each virus primer (forward and reverse). The primers used for the virus quantification are listed in Table 1.

Tab. 1 – The table show the information about the primers used for the virus quantification.

Target	Primer	Sequence	NT	Size
DWV complex	DWV-F8688	GGTAAGCGATGGTTGTTTG	19	143 pb
	DWV-B8794	CCGTGAATATAGTGTGAGG	19	
ABPV complex	AKIV-F6099	CCCATTGTGTATGGACAC	18	104 pb
	AKIV-B6164	GCTCCATAATTGCATTTTCACA	22	
BQCV	BQCV-qF7893	AGTGGCGGAGATGTATGC	18	294 pb
	BQCV-qB8150	GGAGGTGAAGTGGCTATATC	20	
CBPV	CBPV1-qF1818	CAACCTGCCTCAACACAG	18	296 pb
	CBPV1-qB2077	AATCTGGCAAGGTTGACTGG	20	
SBV	SBV-qF3164	TTGGAACTACGCATTCTCTG	20	335 pb
	SBV-qB3461	GCTCTAACCTCGCATCAAC	19	

The cycling parameters were an initial activation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 sec. The amplification reaction was followed by a melting curve analysis to determine the specificity of the amplification products by incubating them for 15 s at 95°C and 60 s at 60°C and then reading the fluorescence at 0.3°C increments from 60°C to 95°C.

Negative controls were included on each plate. Plasmids of known concentration containing inserts for each target were used to generate standards for absolute quantification,

obtained from 10-fold serial dilutions. Each plate contained eight different concentrations of each external standard covering 8 orders of magnitude. Two replicas for each sample, standard and negative control were performed on the plate. Standard deviation and average between two replicas were calculated. Only when the difference between the two replicates was lower than 25% of the mean viral load (standard deviation/average < 0.25), the qPCR results were considered valid. Otherwise, the qPCR run was repeated. To compare the viral load in the two treatments, the Mann-Whitney test was performed.

2.1.6 Data analysis

All statistical analysis and plotting were performed with R version 4.2.2 (R Core Team, 2022) and RStudio. Kruskal-Wallis test, followed by Dunn's post hoc test with Benjamini-Hochberg correction was used to compare the number of bees, drone brood, worker brood, fallen *Varroa* mites and recorded infestation rate among treatments and in the different sampling periods. Mann-Whitney test was used to compare viral load between treatments and in the different sampling periods.To model the infestation index, to allow for repeated measures of the same beehives for several weeks, we applied generalized linear mixed models (GLMMs) using ghmmTMB (Brooks et al 2017), where the beehive ID was used as a random factor. Since the collected data was skewed, we used Tweedie GLMMs with logit. The models included as covariates the number of bees, drone brood and worker brood, the treatment (C, T1 and T2), the year (2020 and 2021), and the week (only for GLMMs). To avoid collinearity among the covariates, once the models were fitted, the variance inflation factor (VIF) was used to drop correlated variables, considering a threshold value VIF<3 (Zuur et al 2010). Considering the high correlation between the number of bees and the number of worker brood, these two covariates were used in separate models. Model assumptions were verified following

the protocol presented by Zuur & Ieno (2016) and with DHARMa (Hartig, 2022). Akaike Information Criterion (AIC) was used for model selection and predictor effect plots (Fox & Weisberg, 2018) were used to visualize the fitted coefficients.

2.2 Results

2.2.1 Colony strength

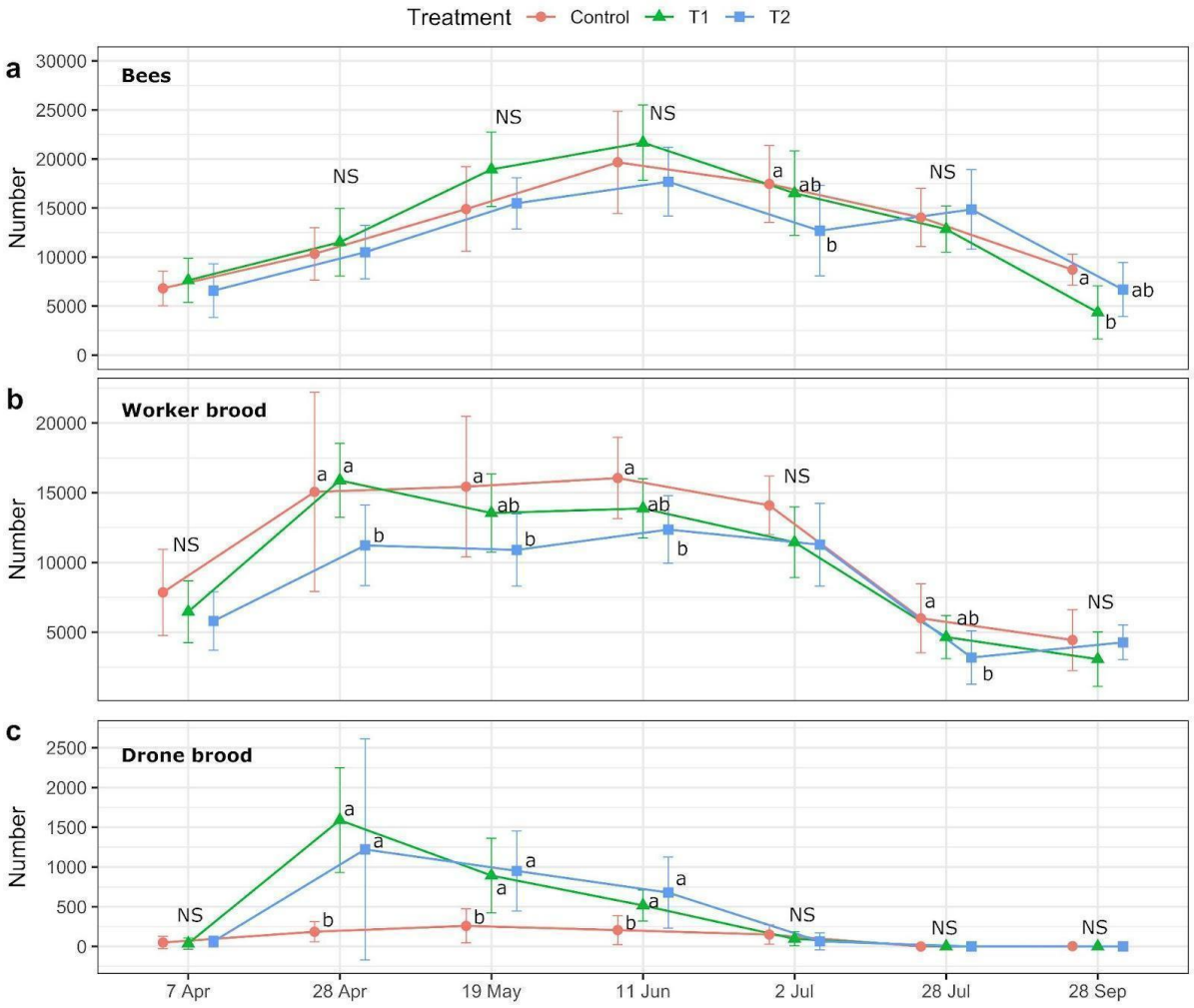


Fig. 6 – Mean number of bees (a), worker brood (b) and drone brood (c) recorded in 2020 per sampling period for each treatment. Vertical lines show the standard deviation while different letters indicate significant differences ($p < 0.05$) after Kruskal–Wallis test and post hoc Dunn's test with Benjamini-Hochberg correction.

In 2020 colonies started with a similar number of bees (C: 6808 ± 1769; T1: 7621 ± 2239; T2: 6573 ± 2726) when the apiaries were setted up. The graph (Figure 6) of the estimated number of bees showed an increase of the adult bee population from April in all 3 apiaries, reaching a plateau in mid-June (C: 19663 ± 5214; T1: 21675 ± 3852; T2: 17687 ± 3499). From June the number of bees started to decrease in all the colonies. All the treatments showed a similar trend during the year. Indeed, the number of bees estimated in each apiary was not significantly different from each other except for the fifth and the last check at the end of September. In the fifth estimation, the number of bees in T2 colonies was significantly lower than C colonies. But in the last estimation the number of bees estimated in T1 colonies was statistically lower than the C colonies (Table 2).

Colonies started with a different number of worker brood cells (C: 7858 ± 3083; T1: 6471 ± 2214; T2: 5804 ± 2098), in particular, the number of workers estimated in T2 colonies was statistically lower then both C and T1 colonies. The number of worker brood reared during the year in T1 colonies was not statistically different from the C colonies as shown in graph Figure 6. Despite T1 colonies built their extra combs ex novo, it did not influence the number of reared workers. T2 colonies reared a number of workers statistically lower than C colonies during the period from the end of April to the mid of May and at the end of July (Table 2).

The number of drones estimated in the second, third and fourth period were statistically higher both in T1 and T2 than in C colonies (Table 2). Drones were reared from April to the beginning of July in all the treatments (7 Apr: C=50 ± 78, T1=37 ± 71, T2=64 ± 67; 28 Apr: C=186 ± 128, T1=1589 ± 659, T2=1221 ± 1391; 19 May: C=259 ± 215, T1=892 ± 471, T2=951 ± 503; 11 Jun: C=206 ± 182, T1=517 ± 196, T2=678 ± 449; 2 Jul: C=151 ± 122, T1=96 ± 88, T2=64 ± 106).

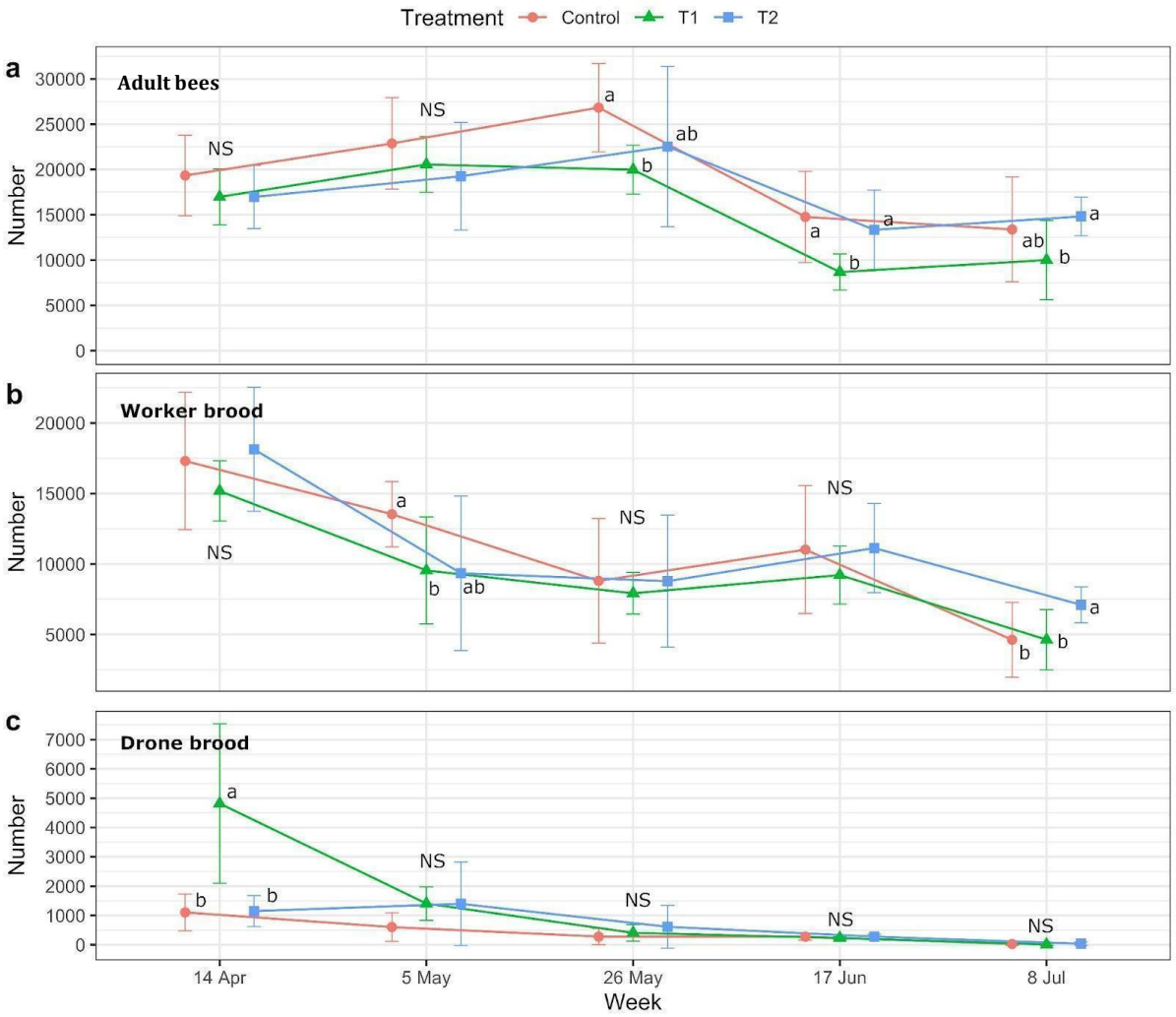


Fig. 7 – Mean number of bees (a), worker brood (b) and drone brood (c) recorded in 2021 per sampling period for each treatment. Vertical lines show the standard deviation while different letters indicate significant differences ($p < 0.05$) after Kruskal–Wallis test and post hoc Dunn's test with Benjamini-Hochberg correction.

In 2021, colonies started with the same number of bees (C: 19335 ± 4456, T1: 16976 ± 3080, T2: 16965 ± 3481), a quantity more than twice higher than the starting point of the previous year. The graph (Figure 7) of the estimated number of adult bees follows a similar trend of the previous year with a progressive increase of the number of bees from April to the end of May. T1 colonies reached the highest peak of population at the beginning of May (T1: 20556 ± 3072), while C and T2 reached the highest peak at the end of May (C: 26832 ± 4881, T2: 22532 ± 8841). Later on, the bees population decreased in each treatment by nearly half and remained constant from June, when the queens were caged, to July. The number of bees in T1 colonies

was statistically lower than C colonies in May and June, but in the last sampling there were no significant differences with the C colonies (Table 3).

Colonies of the three treatments started with a similar number of worker brood cells (C: 17303 ± 4876; T1: 15175 ± 2136; T2: 18129 ± 4396), even if it was more than twice higher than the value estimated the previous year during the first brood estimation. The higher number of worker brood cells of the year for each treatment was measured at the beginning of the experiment. In the fourth estimation, the average number of worker brood before the queen caging (carried out on June 28th) was 11020 ± 4529, 9218 ± 2056 and 11132 ± 3160 cells in C, T1 and T2 colonies, respectively. In the last estimation the average number of workers was 4632 ± 2651, 4636 ± 2144 and 7108 ± 1277 cells in C, T1 and T2 colonies respectively, which was lower than the estimation made the previous year in the same period probably due to the queen caging (C: t test, t=8.2212, df=1, p=3.886E-07; T1: t test, t=6.8469, df=1, p=1.1813E-06; T2: t test, t=4.025, df=1, p=0,00097) . During the year there were no significant differences among treatments and the trend was similar for each of them. Statistically, differences among the treatments were observed during the second and the fifth period (Table 3).

The number of drones brood cells in T1 colonies was statistically higher than in the two treatments in April as shown in Figure 7. Then, there were no statistically differences among the colonies in each period (Table 3).

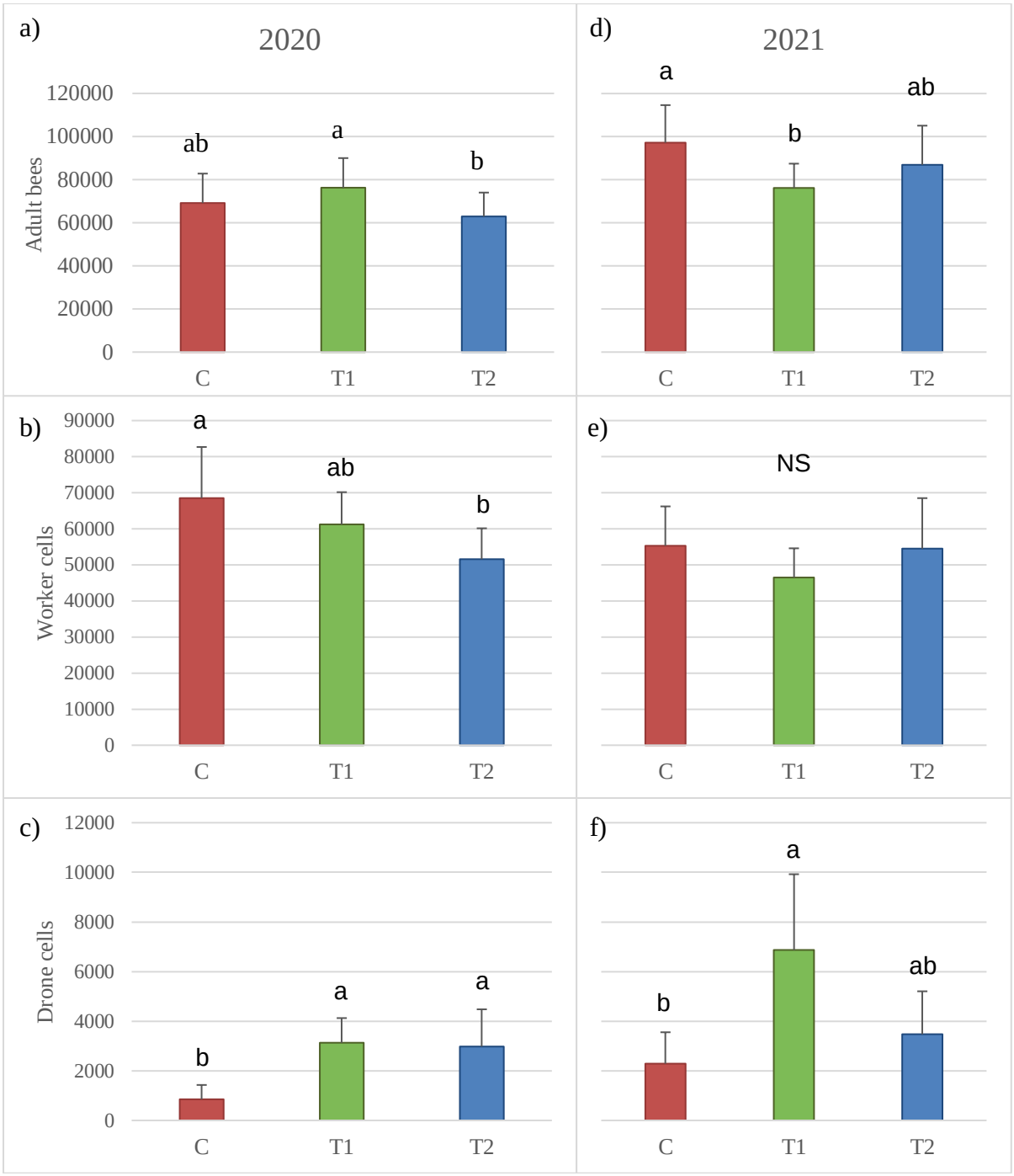


Fig. 8 – On the left column are represented the average number (± SD) of a) bees, b) worker and c) drone cells reared during the 2020 from April to July. On the right column are represented the average number (± SD) of d) bees, e) worker and f) drone cells reared during the 2021 from April to July. Different letters indicate significant differences ($p < 0.05$) after Kruskal–Wallis test followed by post hoc Dunn's test with Bonferroni correction.

The total population of adult bees of T1 and T2 colonies in 2020 was not statistically different from the C colonies (Figure 8), but in 2021 T1 statistically differs from the C colonies. In general, T1 colonies reared a total number of workers which was not significantly different

from the C colonies in both years. As for T2 colonies, only in 2020 they reared a total number of workers significantly lower than the C colonies. As for the drones, T1 colonies reared a total number of drones statistically higher than C colonies both in 2020 and 2021. Although all colonies reared more drones in 2021 than in 2020, the same rate (1 : 3) of drones between colonies C and T1 was maintained. T2 colonies reared on average the same number of drones during the two years, that was around 3000 drones. In addition, the number of drones reared by T2 in 2020 was statistically higher than C colonies but not in 2021 when the number of drones was not statistically different (Table 4).

2.2.2 Natural mite fall

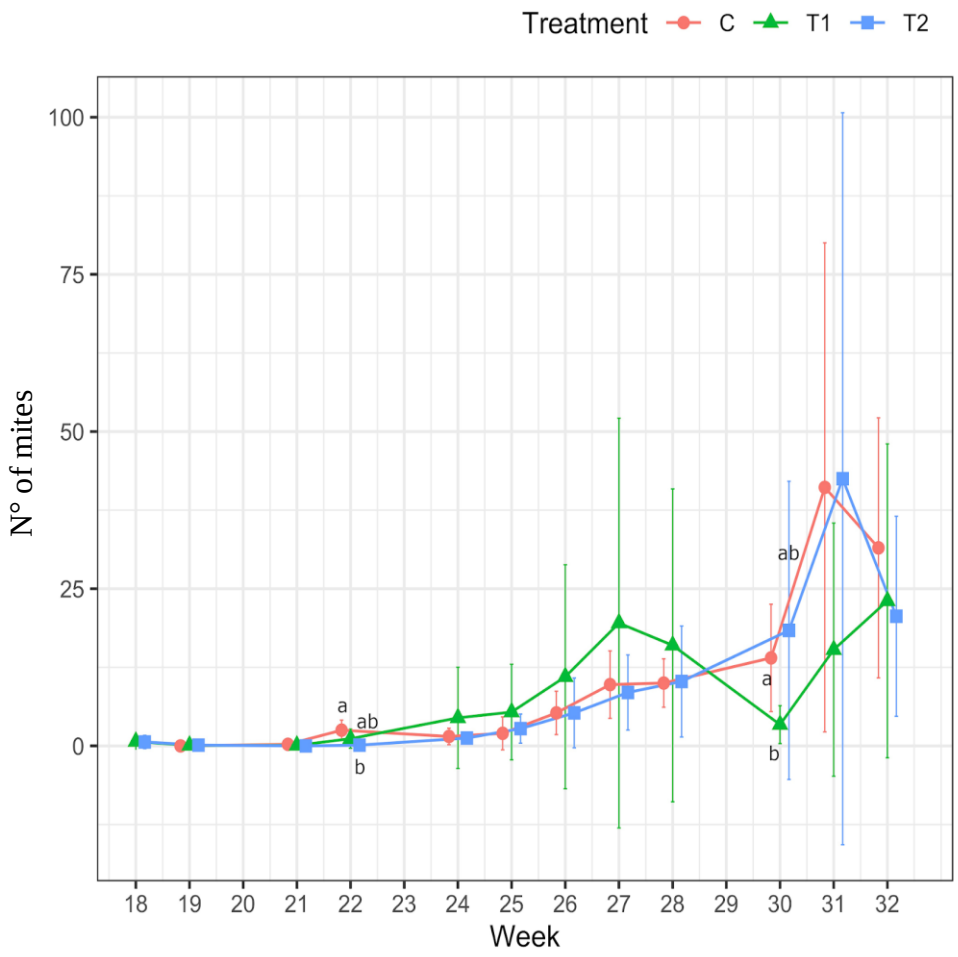


Figure 9 – Mean natural mite-falling per week before acaricide treatment recorded in 2020. Vertical lines show the standard deviation while different letters indicate significant differences ($p < 0.05$) after Kruskal–Wallis test and post hoc Dunn's test with Benjamini-Hochberg correction.

Natural mite fall (NMF) was counted during all the trials. A relevant NMF difference between the two years was observed. In 2020, the average weekly NMF ranged from 0.50 ± 1.05 at the beginning of the experiment and 24.72 ± 21.36 at the end of the experiment, with a min and max of 0 and 180 fallen mites. In 2021, this value ranged from 17.81 ± 23.15 at the beginning of the experiment and 654.48 ± 495.22 at the end of the experiment, with a min and max of 0 and 3029 fallen mites. This different fall of mites between the two survey seasons is partially attributable to the different methods of controlling the *Varroa* mite which in 2020 was based on the use of slow-release synthetic acaricides in the presence of brood while in 2021, the technique of caging the queen was adopted, followed by treatment with oxalic acid in the total absence of brood.

The NMF followed an exponential trend in all the 3 treatments during the 2020 as it is shown in the graph in Figure 9. C and T2 colonies showed a similar trend from July onward. The NMF in T1 colonies seemed to follow an up and down trend. It followed an exponential trend until the 27th week then started to decrease until the 30th week and then went up again to the last check before the acaricide treatment. There were no significant differences among the treatments except in correspondence of the 30th week when T1 colonies reached a NMF value statistically lower than C colonies (Table 5).

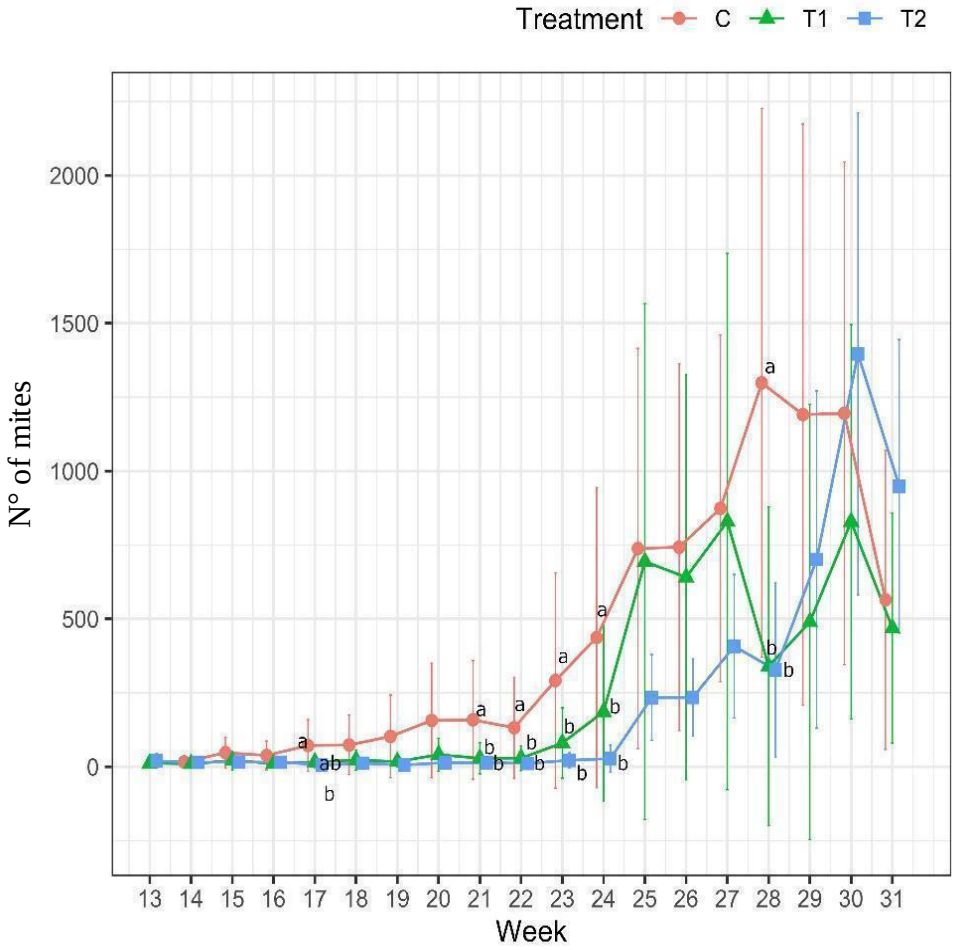


Figure 10 – Mean natural mite-falling per week before acaricide treatment recorded in 2021. Vertical lines show the standard deviation while different letters indicate significant differences ($p < 0.05$) after Kruskal–Wallis test and post hoc Dunn's test with Benjamini-Hochberg correction.

The NMF in 2021 followed an exponential trend in all the 3 treatments but with some differences compared with the previous year (Figure 10). The number of mite fallen during the pre-treatment period was considerably higher than in 2020, showing that the mite population growth in two year old colonies is much higher than in one year old colonies (C: t test, t=3.2428, df=2, p=0.005098; T1: t test, t=3.2647, df=2, P=0.003546; T2: t test, t=6.6775, df=2, p=5.3108E-06). C colonies show the same exponential trend of the previous year, in addition during the entire month of June and the 28th week mite fall was higher than the other colonies.

T1 colonies show an exponential trend until the 27th week, after that it quickly decreases and then increases as well as in the previous year (Table 6).

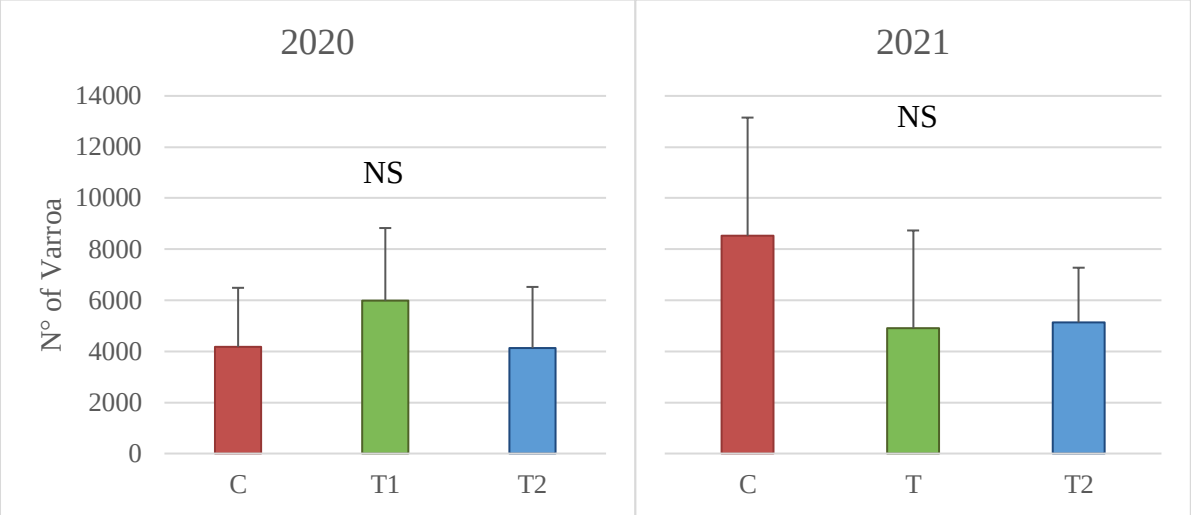


Fig. 11 – Total mite fall after in a) 2020 and b) 2021. Vertical lines show the standard deviation while different letters indicate significant differences ($p < 0.05$) after Kruskal–Wallis test and post hoc Dunn's test with Bonferroni correction.

Data of the total number of mite fall after acaricide treatment were considered as the total *Varroa* population present in the colony before the acaricidal treatment (Bieńkowska & Konopacka, 2001, Branco et al., 2006, Pietropaoli et al., 2021). As shown on the graph (Figure 11), there were no significant differences among the three treatments. Unfortunately, colonies started to collapse in the following two weeks as a consequence of the acaricide treatment; for this reason, we decided to consider the total number of *Varroa* fallen during the year instead of the total *Varroa* fallen after the acaricide treatment. Although the total number of mite fall in C colonies was almost twice as high than the other two treatments, there was not a statistically difference from C and the other two treatments (Kruskal-Wallis test. 2020: K=0.1604, df=2, C - T1 p=0.4961, C - T2 p=1, T1 - T2 p=0.2421. 2021: K=0.05656, df=2, C - T1 p=0.06058, C - T2 p=0.2691, T1 - T2 p=1).

2.2.3 Infestation rate on adult bees

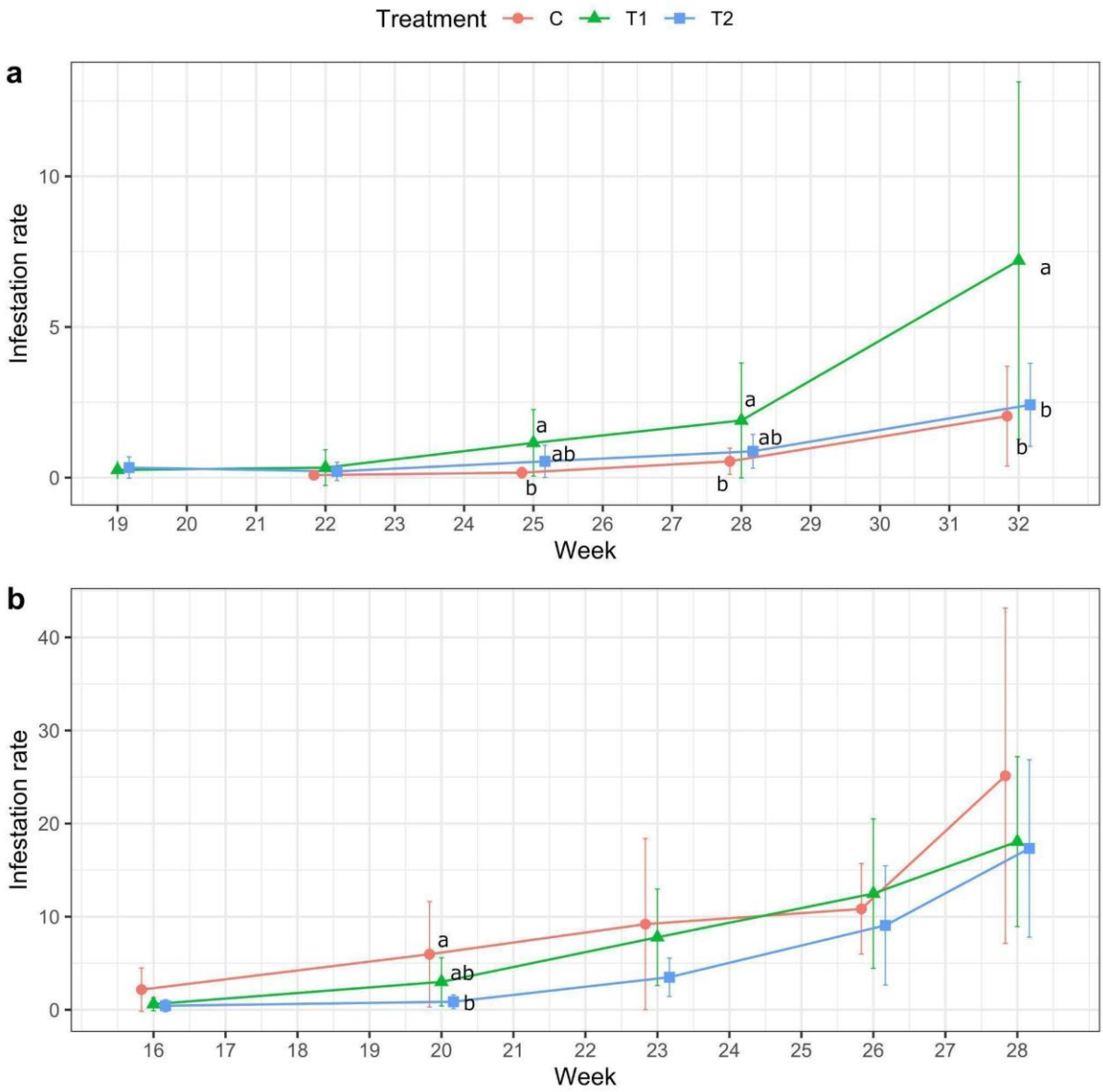


Fig. 12 – Infestation rate on adult bees recorded per week in 2020 a) and 2021 b). Vertical lines show the standard deviation while different letters indicate significant differences ($p < 0.05$) after Kruskal–Wallis test and post hoc Dunn's test with Benjamini-Hochberg correction.

The *Varroa* infestation rate on adult bees (Figure 12), calculated by using data of the powdered sugar technique, shows an increase of the mite infestation rate on adult bees during the year 2020. This increment was not similar among the 3 treatments. The infestation rate on T1 colonies increased exponentially from July to August and started to be statistically higher than C colonies from the 25th week. T2 colonies showed a mite infestation rate similar to the

C colonies. There were no significant differences among the treatments during the 2021, except on the second check. The infestation rate on adult bees in each period was statistically higher than the previous year (Table 7).

2.2.4 Models

Data of the infestation rate of adult bees were use in a GLMM analysis in order to model the infestation rate with the other covariates as the number of bees, drone brood and worker brood, the treatment (C, T1 and T2), the year (2020 and 2021), and the week. The two tables (Table 8 and 9) show the result of this analysis. It was necessary to create two models with the infestation index because there was a high correlation between the number of bees and the number of worker brood. For this reason, the first model was made using the worker and drone brood (Table 8) and on the other one was used the number of bees. The infestation index showed a similar pattern concerning the effect of year, treatment and drone brood (Table 8). The number of bees and the week of observation showed a significant positive effect (Table 9). The interaction between week and treatment showed a steeper relationship for T1 and T2 compared to C colonies. The treatment T2 had a significant negative effect on the infestation index, regardless of the interaction with the year (Table 9). These two tables indicate that the factor bees has an effect twice bigger than drone brood on the infestation index.

2.2.5 Viral load analysis

The presence of five honeybee viruses was assessed in this study. Only two viruses (ABPV and DWV) were detected repeatedly in honeybees from the colonies sampled. During the year 2020, three samplings were performed (May, July, and September). BQCV, CBPV, and SBV were not detected in any sample. ABPV was detected during the third sampling (carried out in September), and the percentage of infested colonies was 50% and 100% for C colonies and T colonies respectively (Table 10). DWV was detected at each sampling, with an

increase in the percentage of infested colonies during the season. The percentage of colonies infested by DWV in May, July and September was 0%, 37,5%, and 100% in C colonies, while 7,7%, 38,5%, and 100% in T colonies, respectively (Table 10). The statistical test showed a significantly higher ABPV viral load in T colonies (3.74E+07 ± 3.69E+07) compared to C colonies (1.96E+04 ± 2.41E+04) in September (Mann-Whitney, p=0,00019) as shown in figure 13. No significant differences between two treatments emerged in DWV viral load in July (Mann-Whitney, p=0,57426), but the test showed a significantly higher viral load in T colonies (9.19E+08 ± 1.55E+09) than in C colonies (2.93E+07 ± 7.01E+07) in September (Mann-Whitney, p=0,0066121) as shown in figure 14.

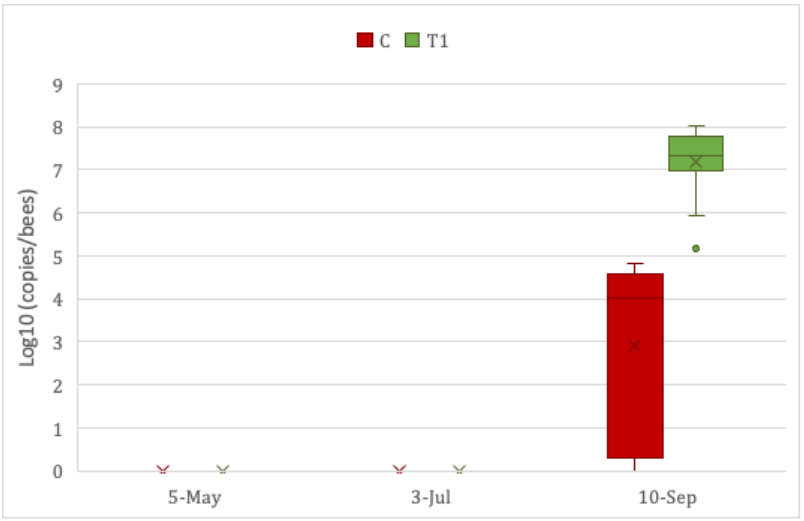
During the year 2021, four samplings were performed (April, May, June, and July). ABPV, BQCV, and SBV were not detected in any sample. CBPV was detected from April to June in a few samples of both treatments. The percentage of colonies infested by CBPV was 20%, 20%, 30% and 0% in C colonies, while 9,09%, 27,27%, 18,18% and 0% in T colonies in April, May, June, and July, respectively. DWV was detected in each sampling, and the percentage of infested colonies and the viral load increased over the season as well as the previous year. The percentage of colonies infested by DWV was 70%, 90%, 100%, and 100% in C colonies, while 27%, 100%, 100%, and 100% in T colonies in April, May, June, and July, respectively (Table 10).

It was not possible to compare the CBPV viral load between C and T treatments because there were not enough positive colonies in each period. The mean viral load of the only positive colonies for the virus was 1.77E+05 ± 4.71E+05 copies, with a minimum of 1,06E+02 and a maximum of 1,71E+06. The viral load trend of DWV shows an increase over time in both the treatments. The average viral load in C colonies was 2,16E+06 ± 5.14E+06, 6,09E+07 ± 1.58E+08, 8,89E+08 ± 1.64E+09 and 5,37E+08 ± 3.60E+08, while in T colonies was 1,95E+06 ± 6.45E+06, 8,87E+07 ± 2.36E+08, 3,74E+08 ± 4.72E+08 and 1,95E+08 ± 2.00E+08 in April,

May, June and July respectively. Statistical analysis showed a significant difference in April and July (Figure 14), in which the viral load in C colonies was higher than T colonies in both the sampling (Mann-Whitney: Apr, p=0.04042; May, p=0.75133; Jun, p=0.86026; Jul, p=0.01996).

Table 10 – Percentage of infested colonies by viruses in each period during the biennium 2020-2021.

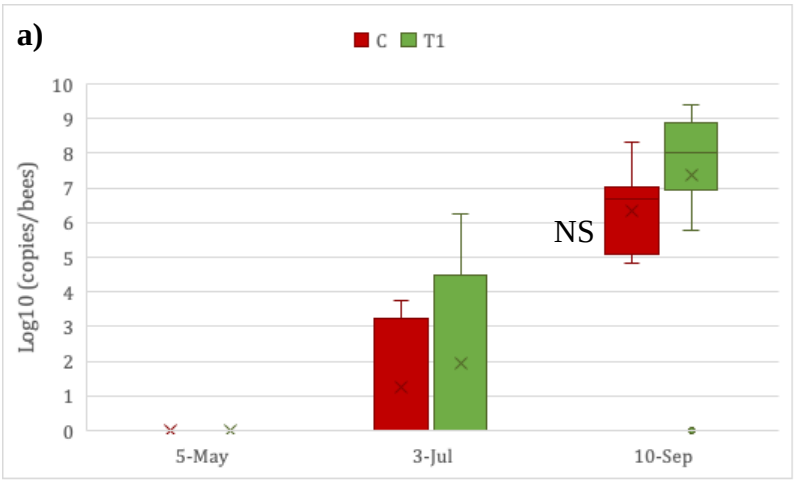
Virus		2020				2021			
		May	Jul	Sep		Apr	May	Jun	Jul
DWV	T	7,7%	38,5%	100%	T	27%	100%	100%	100%
	C	-	37,5%	100%	C	70%	90%	100%	100%
ABPV	T	-	-	100%	T	-	-	-	-
	C	-	-	50%	C	-	-	-	-
BQCV	T	-	-	-	T	-	-	-	-
	C	-	-	-	C	-	-	-	-
CBPV	T	-	-	-	T	9,09%	27,27%	18,18%	-
	C	-	-	-	C	20%	20%	30%	-
SBV	T	-	-	-	T	-	-	-	-
	C	-	-	-	C	-	-	-	-



a

b

Fig. 13 – Average (± SD) ABPV viral load during 2020. Different letters indicate significant differences ($p < 0.05$) after Mann-Whitney test.



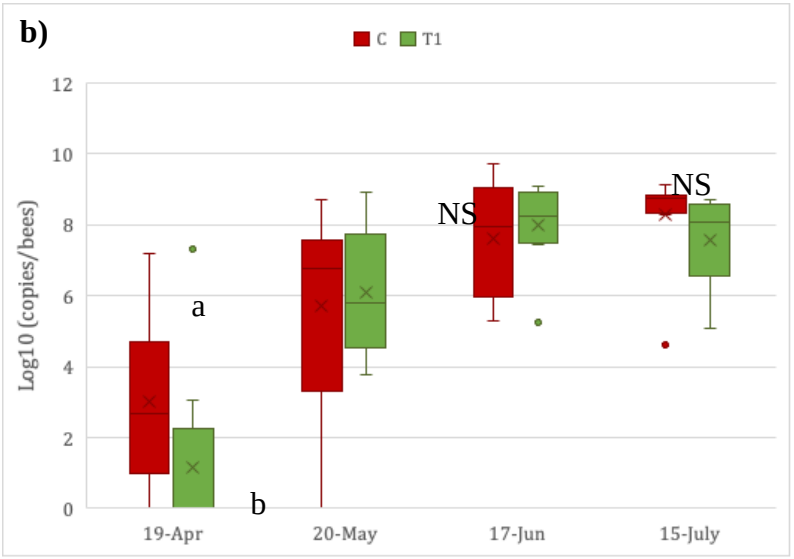


Fig. 14 – Average (± SD) DWV viral load during a) 2020 and b) 2021. Different letters indicate significant differences ($p < 0.05$) after Mann-Whitney test.

2.2.6 Honey production

In 2020, the first super was added during the month of May. Super was added when the core hive frames were covered with bees during the weekly check. supers were left on the top of the hives for all the productive season and removed for honey extraction on 8 July. C, T1 and T2 colonies collected an average of 6,83 ± 4,10 kg, 2,52 ± 2,58 kg and 3,82 ± 2,55 kg of honey respectively. The amount of honey produced varied from a minimum of 0 to a maximum of 12,94 kg.

In 2021, the first super was added during the month of April and the second one in the second week of June. supers were removed during the second week of July. Honey collected by the C colonies was on average the same as the previous year (6,59 ± 4,06 Kg). Honey was not collected by T1 colonies and T2 colonies collected an average of 0,83 ± 1,22 Kg.

The data on honey production only considers the honey extracted by centrifugation of super honeycombs only, but it does not consider the honey stored into the hive. In addition, this data does not consider the honey stored during the period before the removal of the super. The criticality of the summer season observed in both the years could have led to a large consumption and dislocation of honey from the super to the nest. This fact could explain why, at the time of the extraction, supers were almost empty. In the T colonies, when queens stop to lay eggs in drone cells, they could become available for honey storing. The relocation of honey from the supers to the nest could have been significant in these colonies, but unfortunately no measurement of honey stored in the nest was made, an aspect that should be taken into consideration in future studies for a correct evaluation of production.

2.3 Swarmed and died colonies

Of the starting 60 colonies set up in 2019, 19 died during the winter. The surviving 41 colonies were used for the experiment in 2020 riparted in the C (13), T1 (16) and T2 (12) apiaries. Before the first treatment on August 5, four colonies swarmed and one colony died in the C apiary, two colonies swarmed and one colony died on T1 apiary, and finally four colonies swarmed on T2 apiary. In the period between the first acaricide treatment and the end of the year 2020, one colony died on C apiary, eight colonies died on T1 apiary and three colonies on T2 apiary. Swarmed and dead colonies were excluded from the final analysis.

The surviving 17 colonies plus 14 new mature colonies set up the previous years were used for the third year (2021). In total 31 colonies were allocated in the 3 apiaries: 10, 11 and 10 colonies for C, T1 and T2 apiary respectively. At the beginning of March colonies were visited in order to equalize the number of bees and brood. Each colony started with 5 combs. There were no swarmed or orphan colonies during the year, but most of the colonies started to collapse between the end of July and the half of August. 7 colonies died on C apiary, 10 colonies died on T1 apiary and 7 colonies died on T2 apiary. A total of 7 colonies (3, 1 and 3 colonies on C, T1 and T2 respectively) survived after the winter.

2.4 Discussion

The aim of the present study is to examine the development of *Apis mellifera* colonies, productivity and the growth of *V. destructor* population associated to honey bee colonies managed using 3 colony managements to expand their populations: a conventional method which involve the use of the wax foundation, a natural honeycomb method which involve the use of empty frames to allow the bees to build the kind of cells they want and another one

which involve the use of two drone foundation and then worker foundation. In addition we want to investigate if a different number of drone brood reared could influence the presence of bee viruses (strictly related or not to *Varroa* mite) during the trials.

Colony strength: our starting hypothesis was that drone production increase in colonies managed by adding empty frames (T1 colonies) or drone foundation (T2 colonies) is expected compared to the colonies managed by adding frames with wax foundation (C colonies). In addition, workers' production could decrease in those colonies where a high quantity of drone brood is reared. In nature, colonies produce a high quantity of drone brood in their comb which could reach 20% of the total brood reared (Rowland & McLellan, 1987). It was observed also in managed colonies, where beekeepers add empty frames which allow the bees to build the kind of cells that they prefer. But it is possible only when the bee population is large enough and with a good number of workers that survived after the previous winter (Smith et al., 2016). Bee colonies start building drone comb when they possess at least 4000 workers according to Smith et al. (2014). A drone larva needs much more food than a worker to develop, and therefore the energy investment for the drone brood reared is higher than for the worker brood reared (Straus, 1911; Melampy & Willis, 1939).

In 2020, T1 and T2 colonies reared a number of drone brood statistically higher than C colonies, in particular during the period between April and June. These results are quite different from other studies in which it was found that colonies reach the maximum drone numbers in June (Mesquida, 1976) and cease to be laid in early autumn (Allen, 1958; Free & Williams, 1975). Our colonies reached the maximum drone numbers in April and started to decline the drone rearing after this data. Despite the T1 colonies rearing an amount of drone brood three times greater than C colonies in both the study years (Figure 8), it did not influence the colony development dynamic, which was the same in all the three kinds of treatments. This

data confirmed the Allen (1965) results, whose experiments showed that the increase of drone brood production does not lead to a decrease of worker brood production. Maybe because the additional drone brood was not high enough to cause an alteration on the performance of the colony. T2 colonies also reared a statistically higher number of drones than C colonies during the 2020, but the worker brood production was statistically lower than C colonies. The 2 drone foundations added in these colonies may have negatively influenced the colonies development. Just some of them used drone foundations to rear drones. It has been seen that several colonies modified this drone foundation producing a disrupted comb that was barely used by the queen for laying eggs and most of the time useless for the colonies. This fact is underlined by the great standard deviation of the number of drone brood cells measured during the second colony strength estimation of 28 April 2020 (Figure 6). For this reason, the addition of the drone foundation must be carefully evaluated in order to not worsen the colony condition, such as for instance letting them waste space and resources that could have been invested in a better way.

In 2021, T1 colonies were the only ones that reared more drones than C colonies. This data underlines how the quantity of drones reared by the colonies change each year. Colonies managed using both natural honeycomb (T1) and drone foundation (T2) do not always rear an average number of drones higher than the colonies managed using conventional foundation. T1 colonies followed the same trend of developing bee population and brood reared of C colonies, confirming what has already been seen the previous year.

Our results also show that the starting number of bees and brood influence the dynamic of the colony development. In 2020, the one-year honey bee colonies quickly expanded their brood area which contributed to increase the bee population. The bee population reached a plateau during the half of June and then started to decline. In 2021, colonies started in April with a higher number of bees which was more than twice higher than the starting point of the

previous year and as high as the maximum reached the previous year. Colonies for this reason started to decrease the number of worker brood reared instead of increasing that value. It is suspected that the number of bees estimated in the T1 and T2 colonies might have been overestimated due to the higher quantity of adult drones which cover a greater surface area than a worker bee. About the building behavior we observed that in each colony, bees began building comb immediately after frames being installed in their hive in April-May. Colonies in which were added frames with foundation, bees completed the comb in 3 - 5 days instead of the colonies T1 in which were added empty frames. In this case the comb was completed in 3 - 14 days, even if on average the 70% of each comb was built in 4 - 5 days. That ensures that, regardless of the kind of colony managing, queens of each colony have simultaneously almost the same surface comb in which eggs might be laid to produce brood.

Varroa infestation rate: understanding and predicting the mite population dynamics represent a crucial point to figure out how to manage this parasite and develop a successful countermeasure to avoid the colony's collapse. Most of the studies have attempted to model and quantify the mite population growth in order to understand and predict mite population dynamics (Camazine, 1988; Fries et al 1994; Boot et al 1994a; Boot et al 1995a; Martin 1998b; Calis et al 1999b; Calis 2001; DeGrandi- Hoffman & Curry 2004). However, there are few open field tests in which the growth of the mite population in colonies has been studied and monitored (e.g. Kokkinis & Liakos, 2004). One of these is the Calatayud & Verdu (1995) paper, in which they found exponential mite growth in *Apis mellifera iberiensis* Engel 1999, with the mite population doubling every month. *Varroa* mite population growth is generally exponential when the mite population is low (Fries et al 1994; Kraus & Page 1995a; Harbo 1996; Harbo & Harris 1999), even though there are several factors which influence the mite population growth. One of those is the drone brood production (Martin, 1998a; Nazzi & Le Conte, 2016). Most of the created models of mite population growth are based on reproduction in drone cells (Martin,

1995a). Martin 1995a and Harris et al., 2003 claim that the percentage and duration of drone brood in colonies and the percentage of reproducing mites is one of the major determinants of *Varroa* mite reproductive rates. Mite populations would be expected to increase in spring and summer, largely due the availability of drone cells (Fuchs, 1990; Martin, 1995a). The Wilkinson et al. (2002) model suggests that regular replacement of combs containing significant areas of drone brood, and the use of full sheets of foundation, should reduce the *Varroa* population growth rate.

Although several authors consider factors such as grooming, uncapping, removing and entombing behavior to be crucial for the parasite-host balance between *Varroa destructor* and *Apis cerana* (Van Alphen & Fernhout, 2020), it must be kept in mind that in *Apis cerana* the *Varroa* mite only rarely infests the female brood cells for the simple reason that the pre-imaginal development in this species of the genus *Apis* is only 19 days for worker bees and a good 23 days for drones (Koetz, 2013). This fact obviously determines reduced reproductive success of the mite in the female brood cells of *Apis cerana*. The recognition mechanisms of the male and female brood are therefore crucial for the reproductive strategy of *Varroa destructor*. With the spillover from *Apis cerana* to *Apis mellifera*, *Varroa destructor* has reasonably lost some fundamental reference points for its parasitic activity but was able to benefit from a much longer pre-imaginal development life cycle as regards the worker bees of the new *Apis* species with which she came into contact. In many of the subspecies of *Apis mellifera*, the life cycle of the worker bee requires in fact about 21 days from the deposition of the egg to the adult insect emerging and 24 days for the drones. In addition, we have to consider that most of the colonies in Europe and in the Americas are reared by beekeepers using the female wax foundation due to the deep-rooted negative consideration towards drones before the arrival of *Varroa* (Free, 1967; Johansson & Johansson, 1971). This attitude led to a serious damage to the colonies of *Apis mellifera* caused by the parasitic mite *Varroa*, which

nevertheless coexists quite peacefully with its original host species. In fact, for a colony of honey bees, without denying the great importance of drones in the colonies (Fontana, 2021), a parasitization mainly involving the male brood is not as crucial as it is if the worker brood suffers from the parasitic attack.

The preference for the drone brood has obviously been preserved even when the mite started to parasitize *Apis mellifera*. As is already explained in the introduction, in western honey bees *V. destructor* prefers drone brood, as well as for its atavistic reproductive strategy. For these reasons, in our experiment we might expect that colonies which rear more drone brood could increase the mite population due to the higher reproductive success of *V. destructor* into the drone cells. Consequently, in these colonies we should observe a major infestation rate on adult bees as a result of a higher offspring production, but also a higher mite fallen which reflects a higher cell invasion and then a high mortality during the uncapping of the bees.

In our paper we wanted to evaluate if the different drone brood production might have influenced the mite infestation rate during the years. We set up 2 apiaries with colonies managed using the natural honeycomb (T1 colonies) and drone foundation (T2 colonies) in which we gave the possibility to the bees to rear more drones compared to a conventional colony managing (C colonies). We measured the mite infestation rate during the year using two methods: mite fall of *Varroa* and the powder sugar methods. These two methods allow us to obtain information about the dynamic of the *Varroa* population growth during the year. Mite *Varroa* fall is used to estimate the total *Varroa* infestation rate within a colony. Even if several studies obtained contradictory conclusions about the accuracy of this method to determine total infestation rate, it could be considered a good indicator of the colony infestation (Branco et al., 2006) in order to understand the development of the dynamic of the mite population growth during the year. Different colony management partially influenced the dynamic of the *Varroa*

population growth during the year. Natural mite fall data showed an exponential growth in both the years of study and in addition it is characterized by a mite fall fluctuation which could coincides with a high and low bee emergence from the capped brood (Lobb & Martin, 1997). Natural mite fall quickly increased in T1 colonies in 2020 during the short period in which colonies reared drones, even if there were not significant differences compared with C colonies. During the 2021, the natural mite fall in T1 colonies was lower than in C colonies only at the end of May and at the beginning of June. The higher drone production in T1 colonies did not influence the mite fallen neither at the beginning nor at the end of the experiment when the acaricide treatment was performed. Also, the total mite fallen did not show a difference between T1 and C colonies. We just observed that T1 colonies had a drop of the mite fallen in both the two years of study during the 28th (in 2020) and 30th (in 2021) weeks. It is not clear the reason for this drop observed in these colonies; it is possible that mites change the behavior according to the kind of colony management. It might be interesting to examine in depth in future studies by analyzing the infestation rate both in worker brood and drones brood.

Analysis of natural mite fall in T2 colonies confirmed what we observed in T1 colonies. During the 2020, T2 colonies produced a higher number of drone brood than C colonies. Despite that, the mite fall in these colonies during the year was similar to the C colonies (Figure 11). In addition, there were no statistical differences about the total number of mites counted after the acaricide treatment between the T2 colonies and C colonies (Figure 11). This fact suggests that a higher drone brood production did not influence the *Varroa* infestation rate. During the 2021, T2 colonies did not produce a statistically higher number of drones compared to the C colonies, for this reason drone breeding cannot be considered as a variable that may have influenced the fall of *Varroa*. Total mite fallen graphs showed that there were no differences among the three treatments during the two years of study, even if in 2021 the average total mite fallen after the acaricide treatment in C colonies was particularly higher

compared to the other treatments. It could be explained by the fact that these colonies were more infested than the other colonies. Even if statistical analysis did not show any differences among the treatment when mite infestation rate was measured at the beginning of the experimentation both using mite fallen and powder sugar techniques. During the 2020, the *Varroa* infestation rate on adult bees measured using the powder sugar method shows that T1 colonies start to be significantly higher than C colonies from June (Figure 12) until the acaricide treatment was made. Nevertheless, during the 2021 the infestation rate on adult bees was similar in all the three treatments.

Viral load analysis**:** in order to collect information on the health status of the colonies, we investigated the presence, distribution and viral load of five viruses (ABPV, CBPV, BQCV, SBV, and DWV) in spring and summer. Out of the five viral pathogens tested, BQCV and SBV were not detected in any of the apiaries in the two years. These data do not confirm results shown in studies previously conducted in many European countries as Italy (Porrini et al., 2016; Bordin et al., 2022). Morawetz et al. (2019) reported a high prevalence of SBV in European honey bee colonies, particularly in spring and summer, when the colony is growing most rapidly and large numbers of larvae are available (Tentcheva et al., 2004). CBPV was detected only during 2021, but the infested colonies were not enough to allow a comparison of the viral load in the two treatments. ABPV was detected in both treatments only in September 2020. In T colonies, an average higher viral load was measured compared to C colonies. DWV was the most prevalent pathogen over the two years in all the apiaries investigated. During 2020, it was detected starting from May to September, and the viral load was higher in T colonies. During 2021, DWV was detected starting from April, and a higher viral load was measured in C colonies in April and July. Our results confirmed the findings of other authors, who described DWV as the most prevalent virus in Europe (Genersch et al., 2010) and suggested that its spread was most probably due to the *Varroa* mite infestation (Wilfert et al., 2016). However, little is

still known about the prevalence of DWV variants in Italy. Unfortunately, during 2021 most of the colonies died after the acaricidal treatment performed in August, and the surviving colonies were too weak, so we decided to stop the sampling. DWV viral load grew during the year in both treatments, but a correlation between viral load and mite fall was found only in 2020.

Production**:** several authors share the view that in a colony with hundreds of drones contributing just on temperature regulation (Free, 1980) and an occasional mate for a queen, there should be a loss of honey stores collected by worker bees (Johansson & Johansson, 1971). Beekeepers, considering drones wasteful, try to avoid this problem by preventing bees from producing their drone brood or destroying male brood. Indeed Levenets (1956) found that drone rearing requires much more resources than workers and they eat much honey when adults, in particular he calculated that it takes 6.89 kg of honey to rear and feed 1000 drones during the season. Despite these remarks there have also been some observations in favor of drones. Zander (1923) showed that stocks with the most drone brood produced more honey, Burdett (1933) concluded that drone comb does not reduce the crop of honey. Holick (1931) showed that colonies in which drones were removed developed slower, produced less surplus, lost interest in building comb, changed queens often, and were queenless more frequently. Since, unfortunately, this experimentation took place during the worst apiculture years that Italian beekeeping can remember, especially in northern Italy, the data concerning production can only be considered with great caution. Our data confirm data of Free, 1977. T1 and T2 colonies produced less honey than C colonies both in 2020 and 2021. The differences of honey stored into supers between T1 and T2 colonies gives an indication about how much honey T1 colonies have had to consume in order to produce ex novo wax for 5 comb construction. However, the honey produced in C colonies is completely irrelevant for a beekeeping company. The lower quantity of honey produced in T colonies compared to C colonies could be justified by the fact that bees in the T colonies needed more resources, and consequently more honey,

both to build comb ex novo and to rear many drones. Nevertheless, the data collected do not consider the quantity of honey stored into the hive, an aspect that will obviously be taken into consideration in future research.

2.5 Conclusions

Several model (Camazine, 1988; Fries et al., 1994; Boot et al., 1994a; Boot et al., 1995b; Martin, 1998b; Calis et al., 1999b; Calis, 2001; DeGrandi-Hoffman & Curry, 2004; Wilkinson et al., 2002) highlights the importance of some parameters relating to drone brood (percentage of drone brood, mite invasion of drone brood and mite reproduction in drone brood) in determining the *Varroa* population growth rate. Our data did not show a positive correlation between the number of drones produced and the increase of the mite infestation rate. Despite colonies managed using the natural honeycomb rearing drone brood numbers three times greater than the colonies managed using the foundation, there was not observed a higher mite infestation rate.

GLMM analysis results confirmed that the infestation rate on adult bees is not dependent on drone brood. The analysis showed that the year is one of the main parameters that have influenced the mite infestation rate. Indeed, the colony strength data showed that the starting number of adult bees and brood in the two years was different, in particular the adult bee population and the number of worker brood was higher during the 2021 than 2020 in both the treatments. This data suggests that the starting population of a colony has a higher influence on the infestation rate than the number of drones reared. This might confirm the hypotheses of Wilkinson et al., 2002 who found that this is probably because the drone brood capacity for mites reaches a limit before the worker brood, since there is less drone brood and the mites invade drone brood preferentially. As mites complete 2-3 reproductive cycles on average

during the period in which drones are reared (Martin & Kemp, 1997), there is not so great a dependence on reproduction in drone brood as was suggested to be the case in some *Varroa* population models (e.g., Fries et al., 1994). But the numbers of worker bee brood become an important factor determining the mite population growth rate when the mite population is high (Wilkinson et al., 2002). However, we have to say that our analysis did not take into consideration the infestation in the brood but only that in the phoretic phase. Further studies that also take into account the mite infestation in drone and worker broods will have to be carried out. This will help the understanding of those factors that most influence the growth of the mite.

The productivity data was measured only weighing the honey stored and subsequently extracted from the super. Although 2020 and 2021 honey production in Trentino was not abundant, the data show that in the colonies managed using the natural honeycombs the honey produced is lower than in the colonies managed with the foundation use. However, this data only gives us a partial indication of the productivity of the two colony management systems. In fact, the supers were extracted at the same time from all the colonies and subsequently weighed to measure the quantity of honey. It cannot be excluded that in the two management systems the colonies distribute the resources between the nest and the super in a differential manner over time. Further investigations will have to be carried out to understand how in colonies managed with natural honeycombs bees organize the storage of honey between the nest and the super in order to understand the best moment in which to insert and subsequently extract the super to increase the quantity of honey collected by the beekeepers.

During the experiment carried out in 2020 we deliberately delayed the acaricidal treatment to obtain information of both the colony development and mite infestation rate over a longer period. In addition, the acaricidal treatment with Apivar© (Amitraz) was preferred to treatment with oxalic acid precisely to avoid the queen caging at least 3 weeks before the

acaricidal treatment, losing information on the development of *Varroa*. However, this choice has led to a considerable *Varroa* growth in the colony. In addition, we suspected that the acaricidal treatment with Apivar© did not work properly, which allowed the *Varroa* leaving the brood to survive and continue to infest new brood cells. This forced us to carry out two further acaricidal treatments respectively with Apistan© (Fluvalinate) and Apibioxal© (oxalic acid) to eliminate most of the *Varroa* that was still alive. In 2021 we adopted the standard method for acaricide treatment, which is to cage the queen for at least 3 weeks and then proceed with the acaricide treatment with oxalic acid. However, this led to an exceptionally high growth of *Varroa* in the period before the acaricide treatment which determined the collapse of most of the colonies concurrently with the acaricidal treatment. This abnormal mite growth could have been avoided by carrying out an acaricidal treatment with oxalic acid between January and February to eliminate most of the *Varroa* in phoretic phase. However, we did not carry out the acaricidal treatment at the beginning of the year in order not to compromise the development of the *Varroa*, a choice which probably caused an abnormal growth of the *Varroa* which led to the collapse of most of the colonies. Further experiments will have to be carried out to understand the best time to treat colonies against *Varroa* without compromising the production of honey by beekeepers.

CHAPTER 3. Experiment managed in the Netherlands

The aim of this experiment was to estimate the strength of the colony and measure the *Varroa* mite infestation rate in both worker brood and drone brood in order to evaluate what is the contribution of these broods to the *Varroa* development in colonies managed using the natural honeycomb and the conventional foundation. In addition, we aimed to estimate the total *Varroa* population growth associated with these two types of colony management using the information of the colony strength estimation, the infestation rate measured from adult bees and from the brood. In addition, we made this experiment using a different kind of beehive in a climatic context different from Italy.

3.1 Materials and methods

3.1.1 Setting up experimental colonies

The experiment was carried out at the apiary of Droovendal, 500 m north of the WUR Wageningen campus. 20 experimental colonies were setted up on 25th March 2022 through the artificial swarming of 10 colonies from the site of Almere and 10 colonies from the site of Lelystad. The colonies were set up using beehives (mod. Simplex) with 3 brood combs and 2 combs with honey stock. Colonies were fed with sugar and left there until they were moved to the apiary of Droovendal (51°59’28’’N, 5°39’39’’E) on 22nd April 2022 to start the data collection.

3.1.2 Colonies management

Similarly to the Italian experiment, colonies were divided in two groups as a standard and natural type:

- C: managed by adding frames with wax foundation during the season.
- T: managed by adding frames with only a 2 cm strip of foundation at the top to allow bees to build a regular comb with the kind of cells that they prefer.

In this case, we did not add the drone wax foundation treatment (T2 of the Italian experiment). Acaricide treatments against *V. destructor* were carried out in August and at the end of November of the previous year with Api-Bioxal© (Chemicals Laif) by following the instructions included on the label.

Colonies were checked every week in order to evaluate whether to add or not a new frame with foundation (C colonies) or with the strip of foundation (T colonies) according to the needs. A new beehive box with frames was added on the top of the previous one when the colonies completed to build the combs of their nest. Super were added to give the bees more space for the colony development according to their necessity.

3.1.3 Measuring colony parameters

During the years we collected the following data: as the strength of the colonies measure and the mite infestation rate in phoretic and reproductive phase. The colony strength estimation was measured every 21 days using the subjective mode described by Delaplane et al., 2013 during the year 2022 from May to August. Data were collected in a relatively short time window of 2 - 4 successive days, randomly assigning the order of inspection such that the day effect was a random effect across treatments. The work was carried out by 2 operators; one of them took out the frames from the hive and visually estimated the percentage of the comb surface covered by bees of brood, the other one recorded the measurement. According to the seasonal period, bees' estimation was taken in a time window from 7:00 am to 9:30 am in order to reduce the bias caused by the bees' flying activity and when the temperature was no lower

than 15°C. Subsequently estimation of the surface covered with worker and drone brood was taken shaking before frames with bees inside the hive. The percentage coverage estimated of bees and comb then was transformed into the number of bees or number of worker and drone cells according to the table of Delaplane et al., 2013.

3.1.4 Infestation of Varroa measurement

Two sampling methods were performed in order to estimate the mite infestation rate both in phoretic and reproductive phase: the soapy water method and the brood cell uncapping. Both methods were carried out according to the protocol of Dietemann et al., 2013. Samples of bees and brood were collected every 21 days after that the colony strength estimation was carried out.

3.1.4.1 Soapy water method

In the apiary, a sample of 200 – 300 bees from each colony was collected and immediately frozen at -20°C. In the laboratory, the bee samples were defrosted and counted. Bees and warm soapy water were inserted into a jar and shaken for 60 s to dislodge the mites from the bee body. The content of the jar was poured through a first plastic sieve (aperture: 3-4 mm) to collect all the bees and a second sieve (aperture < 0.5 mm) was put under the first one to collect the mites. Bees and mites were rinsed with water. Mites were then counted (Figure 15). The infestation rate on adult bees was calculated dividing the number of mites by the number of bees in the sample to determine the proportion of infested bees.

Fig. 15 – Stages of the soapy water method: 1) bees and warm soapy water inserted into a jar, 2) the jar at the end of the mixing process, 3) bees on the sieve ready to be rinsed with water, 4) mites collected at the end of the procedure.

3.1.4.2 Brood cell uncapping

Samples of comb with capped worker (150 – 200 cells) and drone (about 50 cells) brood were cut from a frame of each colony. Pieces of comb were frozen at -20°C until the *Varroa* inspection (Figure 15). In the laboratory, combs were thawed and all cells were uncapped to check for the mite presence. When an infested cell was found, the number of female mites and the presence of male mites were recorded. In addition, the mite stages and the pupae stage were recorded according with the tables of Dietemann et al., 2013. The infestation rate in the reproductive phase was calculated dividing the number of infested cells by the total number of uncapped cells in the sample to determine the proportion of infested cells both on worker brood and drone brood.

The values of the strength of the colony and of the infestation rate both on adult bees and in the reproductive phase were used to calculate the population size in the colony. The mite population size in the colony was calculated using the following formulas of Martin, 1998a:

- $Varroa\ population\ size\ in\ phoretic\ phase = a \times \frac{b}{c}$
- $Varroa\ population\ size\ in\ reproductive\ phase = d \times \frac{e}{f}$

a = total number of bees in the colony (Measure of the colony strength)
b = number of varroa counted phoretic phase (Soapy water method)
c = number of bees in the sample (Soapy water method)
d = total number of sealed brood cells in the colony (Measure of the colony strength)
e = number of infested cells (Brood cell uncapping)
f = number of opened cells (Brood cell uncapping)

To obtain the size of the *Varroa* population in the phoretic phase, the total number of bees in the colonies was multiplied by the proportion of infested adult bees in the colony. To obtain the size of the *Varroa* population in the reproductive phase in the colony, the total

number of sealed worker and drone brood cells were multiplied by the proportion of infested worker and drone cells respectively in the colony. The total mite population size of the colony was calculated adding the mite population on adult bees plus the mite population in the brood.

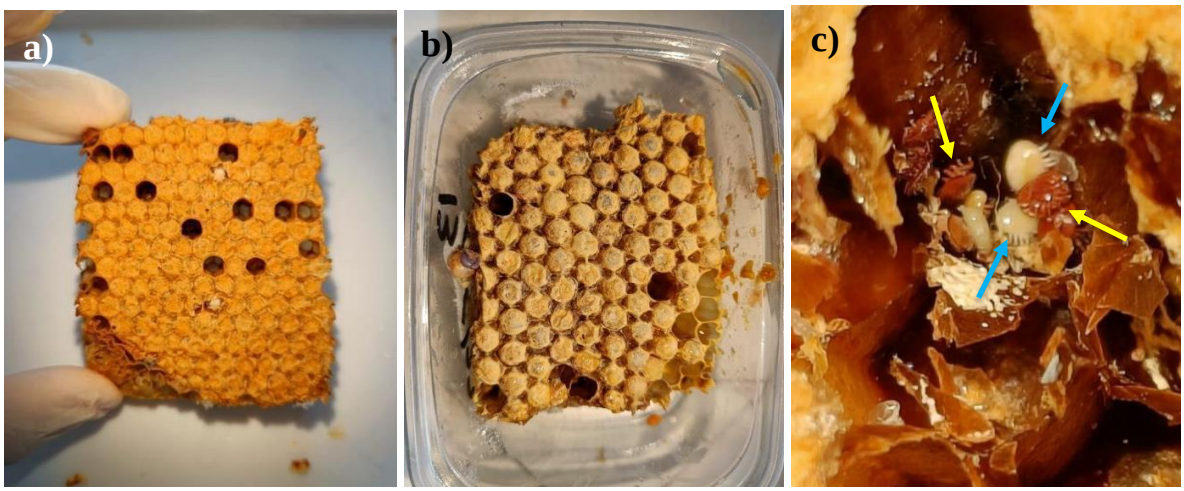


Fig. 16 – The image shows a) a piece of comb with uncapped worker cells, b) a piece of comb with uncapped drone cells and c) an infested drone cell with inside different mite stages. Blue arrows indicate deutonymph stage of female mites, the yellow arrows indicate adult stage of female mites.

3.1.5 Data analysis

Statistical analyses were performed with PAST version 4.12 (Hammer et al., 2001). Mann-Whitney test was used to compare the number of bees, drone brood, worker brood, and *Varroa* population between treatments and in the different sampling periods.

3.2 Results

3.2.1 Colony strength

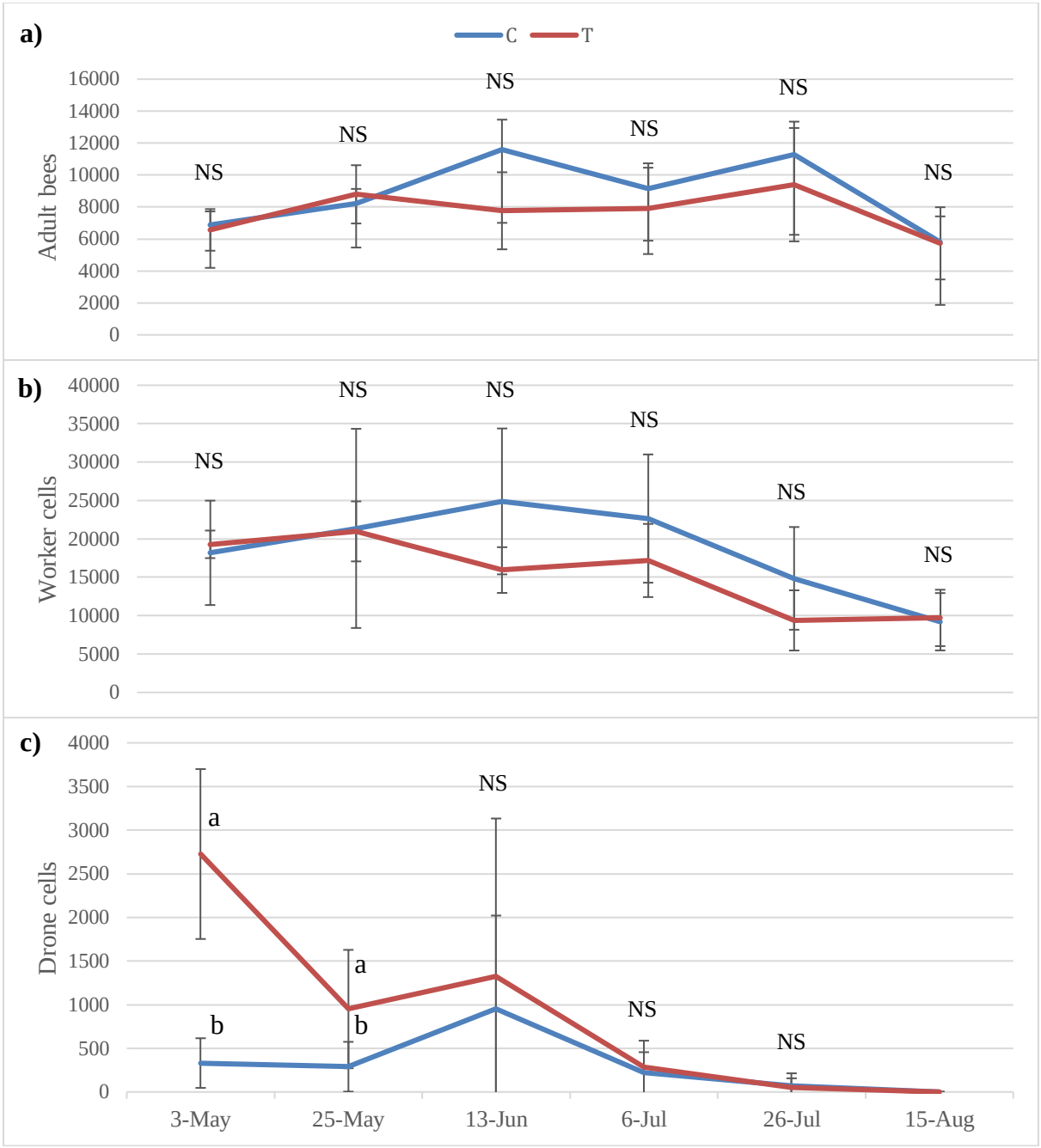


Fig. 17 – Mean number of bees (a), worker brood (b) and drone brood (c) recorded in 2021 per sampling period for each treatment. Vertical lines show the standard error while letters indicate significant differences ($p < 0.05$) after Mann-Whitney test. The blue line represents the C colonies and the red line represents the T colonies.

The colonies started with a similar number of bees (Foundation: 6875 ± 2683; No Foundation: 6569 ± 1302) and worker brood (Foundation: 10766 ± 4091; No Foundation: 11762 ± 1048) when the data collection started. The estimated number of bees (Figure 17) in the T colonies reached a plateau by the end of May and the value remained rather stable until the end of July. The bee populations in C colonies reached two peaks, the first one in June and the second one at the end of July, then they decreased from August similarly to what we observed for the T colonies. The statistical analysis showed no significant differences between the two kinds of colony management during the entire period. The average number of worker broods reared (Figure 17) in C colonies increased during the spring reaching a peak of 24874 ± 9501 worker cells in the middle of June and then it decreased. As for the T colonies, this value was lower than C colonies during the first part of the season, even if this value was not statistically different from C colonies during the entire period considered. Finally, the drone production (Figure 17) in the T colonies was statistically higher than in the C colonies in May. In the first estimation of May, T colonies reached a peak of 2726 ± 973 drones, while C colonies reached this peak in June with 953 ± 1068 drones. For the rest of the season there were observed statistically differences between the two treatments (Table 11).

3.2.2 *Varroa population size*

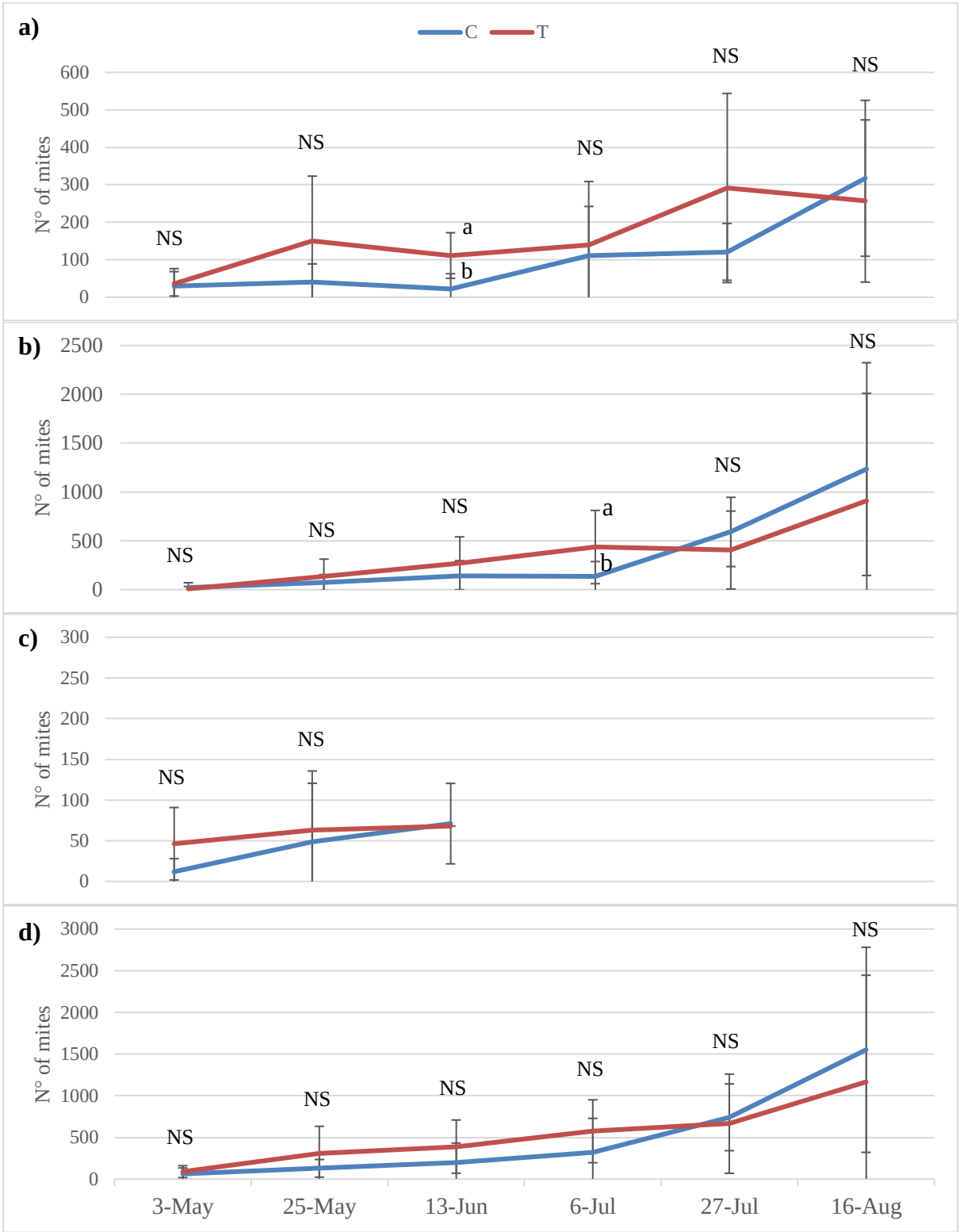


Fig. 18 – Average number of mites *V. destructor* a) on adult bees, b) into the worker brood c) into the drone brood and d) the total mite population recorded in 2021 per sampling period for each treatment. Vertical lines show the standard error while letters indicate significant differences ($p < 0.05$) after Mann-Whitney test. The blue line represents the C colonies and the red line represents the T colonies.

The mite population on adult bees (Figure 18) in C colonies grew continuously from May reaching a mean number of 317 ± 208 individuals in August. On the contrary, the trend of the mite population on adult bees in the T colonies showed a less clear pattern during the entire period. The number of mites in these colonies increased faster compared to the C colonies, reaching value that was significantly higher in June (figure 18a). Colonies reached a peak of mites at the end of July, when the number of mites estimated was 292 ± 252. But at the end of the considered period, the number of mites in the T colonies was not significantly higher than in C colonies. The graph b in figure 18 shows that the trend of the mite population growth in worker brood was exponential in both the treatments, even if there were no statistically differences between two treatments during the period, excluding the fourth check during the first week of June in which the mite population of T colonies was higher than C colonies. The graph c in figure 18 shows that there are no statistically different differences between the two treatments about the estimated mite population in drone brood. The graph d in figure 18 shows the total mite population growth trend between the two treatments. The mite population in both the treatments grew during the entire period reaching a peak in August of 1552 ± 1230 in C colonies and 1015 ± 1377 in T colonies. During the entire period there were no statistically different differences between the two treatments. These graphs also show that most of the mites are distributed inside the worker brood instead of on the adult bees. It is particularly evident in July and August in which most of the 70% of the mite population was located inside the worker brood (Table 12).

3.3 Swarmed and died colonies

From May to August there were no swarmed colonies. 8 colonies lost the queen, and two of these colonies died during the last week of July and the second week of August. These colonies were excluded from the data analysis.

3.4 Discussion

This experiment performed in the Netherlands substantially confirmed the result obtained in Italy about the development of the colony. Indeed, significant differences were not observed between the colonies managed by using the wax foundation [C] and colonies managed by using the natural honeycomb management [T] about the number of bees and worker cells during the period considered. The amount of drone brood reared was higher in T colonies than C colonies in accordance with the expected results. This value was higher in particular in May and June.

Differently to what was done in Italy, in which only the infestation rate on adult bees and the natural mite fall were measured, here both the infestation rate on adult bees and the brood were assessed. This allowed an estimate of the *Varroa* population in each life stage every three weeks, obtaining important information about the contribution of the worker and drone brood on the *Varroa* development.

The population of the *Varroa* in the phoretic phase follows a different trend in each treatment. While in C colonies the mite population growth seems to follow an exponential trend, in T colonies the population of the *Varroa* seems to fluctuate up and down but with a constantly increasing trend. The mite population in T colonies tends to be higher than in C colonies during the entire period considered, resulting also statistically higher in mid-June sampling as shown in the graph in figure... . However, in mid-August the *Varroa* population in both treatments was almost the same.

The population of *Varroa* in the reproductive phase is differently distributed between the worker and drone's brood depending on the period. During the first week of May, in which the drone rearing reaches the highest level, the *Varroa* population is mainly concentrated in the drone brood, while in subsequent samplings it progressively increased in the worker brood. However, this increase appears to be faster in T colonies than in C colonies, resulting also statistically higher in the first week of July. But by the end of July the *Varroa* developed in the worker's brood increased exponentially until it reached the same level developed in the T colonies.

The mite population increase both in the phoretic phase and in the worker's brood during the first months of sampling could be partly explained by initial drones rearing increase. In fact, during the first sampling in May, most of the *Varroa* was sampled in the drone brood. As demonstrated by precious studies (Boot et al., 1995b; Martin, 1995a) the development of *Varroa* in the drone brood is much higher than that in the worker's brood due to its greater reproductive success in the drone brood. This therefore leads to a higher number of *Varroa* individuals leaving the drone brood, which would justify a greater, even if not significantly different, number of *Varroa* observed in the phoretic phase at the end of May in T colonies compared to C colonies.

However, the overall contribution of the drone's brood to the *Varroa* development is negligible compared to the *Varroa* developed in the worker's brood. In fact, the drone brood is reared in a very short period, restricted mainly in the spring season. Therefore, most of the *Varroa* grows up mainly in the worker's brood. The rise of population density starts its exponential phase from the first week of July in the C colonies and towards the end of July in the T colonies.

CHAPTER 4. Conclusions

In this PhD work, the effects on colony development, health status and honey production in colonies managed using a natural honeycomb were investigated. To be more specific, a partial use of the natural honeycomb was tested, because only half of the boxes supplied to the colonies did not have the complete wax foundation, but they only had a small strip of foundation to allow the colonies to build a regular honeycomb. Natural honeycomb beekeeping is a more sustainable alternative to modern conventional beekeeping, which uses the wax foundation. The improvement of this kind of colony management is represented by the fact that the honeycomb on which the colony develops will consist of clean wax, free from chemicals, which can increase the fitness of the colony since the colony can rear the brood inside uncontaminated wax. Moreover, this could lead to a partial economic benefit for the beekeeper who will no longer have to purchase foundations. For the beekeeper who wants to produce honey, the savings may be even greater, because the "purified" foundation without chemicals, in addition to being not easily available in the market, is also quite expensive compared to standard foundation. But this is not the end of the potential benefits that this kind of management could offer to the *A. mellifera* colonies managed by beekeepers. They often forget the very important role played by drones, which is reproduction. The gene flow could be improved by allowing colonies to rear an adequate number of drones, which contribute to improving the genetic pool of the species.

In fact, despite the evidence that genetic factors can play a significant role in species extinction (Saccheri et al., 1998; Frankham et al., 2002; Spielman et al., 2004; Hanski and Saccheri, 2006), genetic aspects of bee decline have not been adequately investigated. Nevertheless, several studies suggest that genetic diversity increases resistance against a wide range of parasites and disease affecting colonies. Conversely, a low genetic diversity in honey bee

populations may contribute to disease susceptibility (Delaney et al., 2009; Meixner et al., 2010). The genetic diversity reduces the risk of producing diploid males, which are produced when closely related drones and queens mate due to the sex determination system where fertilized eggs (normally female) that share the same allele for sex determination produce diploid males (Page, 1980; Tarpy & Page, 2002). A colony that is genetically diverse has the ability to respond positively to highly variable environmental conditions with more flexibility. Conversely, colonies with low genetic diversity have a narrow range of behavioral thresholds among their workers. For example, genetically similar colonies may allocate inappropriate numbers of workers to tasks required by the colony because the occurring genotypes may not have an optimal task threshold (Myerscough & Oldroyd, 2004). Moreover, the foundation forces the bees to build cells with a predetermined size, which does not always reflect the real need of the colony. Indeed, the size not only determines the kind of bees developed (workers and drones), but it also determines the size of the bees, which is depending on the climatic region and the season in which the colonies exist (Fontana, 2021).

This experiment showed that the partial use of the natural honeycomb did not negatively influence the colonies. In fact, the colony strength data collected in tested colonies are very aligned with those obtained in the standard colonies management, except for the results on the drones reared. The mite infestation of the colonies seems to be more related to the size of the colony than to the type of colony management. The higher number of drones reared in natural honeycomb colonies leads only to an early *Varroa* population increase, but this does not appear to be significantly different from the infestation observed in colonies managed with wax foundation. Consequently, the viral load of the viruses considered in this study does not seem to be affected by the different colony managements as well as the presence of *Varroa*. In particular, the deformed wing virus follows a trend that is strongly linked to the infestation of *Varroa* in the colonies as already demonstrated in other studies. The knowledge of the temporal

trend of *Varroa* infestation measured through natural mite fall or in phoretic and reproductive phase using appropriate sampling techniques provides a useful starting point for further studies on the correct timing for acaricide treatments both in colonies managed in a conventional way and using the natural honeycomb.

The downside is productivity. Although the data on honey production were obtained during two disastrous years for the local honey production, the result of this work gives us a clear indication. Natural honeycomb management negatively influences the honey production, maybe due to the high energetic investment both to maintain drones and to build their combs *ex novo*. However, our data were obtained only from honey stored into the super, while more complete data on the amount of honey stored in the nest are still missing. Hence further studies have to take in account the total honey production of the entire colony to understand how the colony distributes the resources between nest and super and better understand the best moment to remove the super in order to maximize the honey collected by beekeepers.

It will be interesting to improve the study of the effect of natural honeycomb management by testing the influence of a complete transition from the wax foundation use to a total natural honeycomb management.

This thesis has therefore certainly contributed to fill a huge scientific gap, which concerns the study of colonies managed through the partial use of the natural honeycomb and of the effects associated to an "abnormal" presence of drones in modern beekeeping (but absolutely normal in a natural and ecological perspective) on the development, health status and productivity of *Apis mellifera* colonies managed.

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APPENDIX I: Experiment managed in Italy (tables)

Tab. 2 – Kruskal-Wallis test result comparing the number of adult bees, worker cells and drone cells among C, T1 and T2 colonies during the year 2020 in each sampling date.

Sample	Date	K	df	p value		
				C – T1	C – T2	T1 – T2
Adult bees	7 Apr	0.4406	2	0.9884	1	0.7646
	28 Apr	0.6001	2	1	1	1
	19 May	0.0921	2	0.1649	1	0.1789
	11 Jun	0.0895	2	1	0.6203	0.0841
	2 Jul	0.2660	2	1	0.0381	0.0793
	28 Jul	0.2337	2	0.8452	1	0.3015
	28 Sep	0.0011	2	0.0009	0.5434	0.0966
Worker cells	7 Apr	0.5348	2	0.9218	1	1
	28 Apr	0.005936	2	1	0.007663	0.0365
	19 May	0.01873	2	0.6666	0.01552	0.1964
	11 Jun	0.02877	2	0.7184	0.02458	0.2562
	2 Jul	0.05078	2	0.07936	0.1163	1
	28 Jul	0.04214	2	1	0.04513	0.1822
	28 Sep	0.002954	2	0.0046	1	0.05024
	7 Apr	0.4014	2	1	1	0.5478
	28 Apr	0.003272	2	0.002408	0.07948	1

	19 May	0.003565	2	0.007081	0.01284	1
Drone cells	11 Jun	0.004606	2	0.019	0.01284	1
	2 Jul	0.149	2	1	0.1631	0.5536
	28 Jul	NA	2			
	28 Sep	NA	2			

Tab. 3 – Kruskal-Wallis test result comparing the number of adult bees, worker cells and drone cells among C, T1 and T2 treatments during the year 2021 in each sampling date.

Sample	**Date**	**K**	**df**	**p value**		
				C – T1	**C – T2**	**T1 – T2**
	14 Apr	0.2663	2	0.4568	0.4789	1
Adult bees	5 May	0.1398	2	0.1721	0.4183	1
	26 May	0.006035	2	0.00574	0.9503	0.1164
	17 Jun	0.002324	2	0.005049	1	0.01694
	8 Jul	0.02083	2	0.2113	1	0.02047
	14 Apr	0.05734	2	0.2116	1	0.07593
Worker cells	5 May	0.01916	2	0.01816	0.1603	1
	26 May	0.5969	2	1	1	1
	17 Jun	0.05935	2	0.1113	1	0.1376
	8 Jul	0.02674	2	1	0.08314	0.04205

	14 Apr	0.0007345	2	0.003606	1	0.003019
Drone cells	5 May	0.0655	2	0.06019	0.9397	0.588
	26 May	0.409	2	0.6688	0.8184	1
	17 Jun	0.05935	2	0.1113	1	0.1376
	8 Jul	0.1754	2	0.8561	1	0.1912

Tab. 4 – Kruskal-Wallis test result comparing the total number of bees, worker cells and drone cells among C, T1 and T2 treatments from April to July during the 2020 and 2021.

Year	**Data**	**K**	**df**	**P value**		
				C – T1	**C – T2**	**T1 – T2**
2020	Adult bees	0.05	2	0.72	0.7747	0.04493
	Workers	0.01395	2	0.898	0.0132	0.09925
	Drones	0.00044	2	0.00047	0.008238	1
2021	Adult bees	0.01288	2	0.0105	0.7738	0.2342
	Workers	0.08082	2	0.2206	1	0.1237
	Drones	0.00114	2	0.00092	0.6565	0.05629

Tab. 5 – Kruskal-Wallis test result comparing the number of natural mite fall among C, T1 and T2 treatments during the 2020 in each sampling date.

	p value

Data	K	df	C – T1	C – T2	T1 – T2
18th	0.1846	2	0.1654	0,16540	0,81432
19th	0.6181	2	0,67822	0,67822	0,67822
20th	NA				
21th	0.2497	2	0,32095	0,32007	0,58072
22th	0.005206	2	0,08612	0,00357	0,08804
23th	NA				
24th	0.9478	2	0,90077	0,90077	0,90077
25th	0.2049	2	0,22635	0,42611	0,55802
26th	0.7182	2	0,77402	0,77402	0,77402
27th	0.8787	2	0,74002	0,74002	0,77402
28th	0.6179	2	0,58983	0,58983	0,94174
29th	NA				
30th	0.01566	2	0,02457	0,60631	0,05759
31th	0.04921	2	0,07307	0,65932	0,11738
32th	0.3287	2	0,35115	0,35115	0,93186

Tab. 6 – Kruskal-Wallis test result comparing the number of natural mite fall among C, T1 and T2 treatments during the 2021 in each sampling date.

Data week	K	df	p value		
			C – T1	C – T2	T1 – T2

13th	0.5129	2	0,56419	0,85299	0,56419
14th	0.4148	2	0,40696	0,94103	0,40696
15th	0.3304	2	0,43072	0,48713	0,40696
16th	0.3077	2	0,37426	0,77700	0,37426
17th	0.02151	2	0,16141	0,01676	0,16141
18th	0.2405	2	0,33209	0,31198	0,65904
19th	0.05577	2	0,19338	0,05238	0,36048
20th	0.06937	2	0,16574	0,07303	0,47852
21th	0.002707	2	0,00601	0,00600	0,77547
22th	0.004203	2	0,02127	0,00469	0,43284
23th	0.002179	2	0,03687	0,00164	0,19708
24th	0.00568	2	0,03343	0,00555	0,36752
25th	0.2537	2	0,48301	0,29810	0,48301
26th	0.09923	2	0,35880	0,09715	0,30487
27th	0.2514	2	0,34041	0,32974	0,66870
28th	0.006897	2	0,00739	0,03118	0,50839
29th	0.1018	2	0,09770	0,29145	0,29145
30th	0.2653	2	0,45952	0,57163	0,32755
31th	0.9367	2	0,60336	0,18181	0,10568

Tab. 7 – Kruskal-Wallis test result comparing the number mite in phoretic phase (on adult bees) among C, T1 and T2 treatments during the 2021 in each sampling date.

Year	Data week	K	df	p value		
				C – T1	C – T2	T1 – T2
2020	19th	0.2508	2	0,32264	0,32148	0,58027
	22th	0.7709	2	0,79652	0,79652	0,92659
	25th	0.00577	2	0,00414	0,13956	0,13956
	28th	0.04986	2	0,04868	0,34288	0,26630
	32th	0.004172	2	0,00868	0,64786	0,02024
2021	16th	0.07416	2	0,16558	0,07992	0,50130
	20th	0.01883	2	0,50559	0,02119	0,05473
	23th	0.123	2	0,79659	0,16017	0,16017
	26th	0.5595	2	0,83231	0,63597	0,63597
	28th	0.5835	2	0,64653	0,64653	0,82606

Tab. 8 – Estimated regression parameters, standard errors, z-value and P-values for the Tweedie GLMM: Infestation index= Drone brood + Worker brood + Treatment*Week + Treatment*Year, AIC= 1210.

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-5.140	0.703	-7.309	<0.001
Drone brood	0.202	0.096	2.099	0.036
Worker brood	0.226	0.128	1.762	0.078
Treatment T1	-0.231	0.739	-0.312	0.755

Treatment T2	-1.525	0.881	-1.731	0.084
Week	0.157	0.027	5.753	0.000
Year 2021	3.570	0.251	14.245	<0.001
Treatment T1: Week	0.055	0.024	2.288	0.022
Treatment T2: Week	0.079	0.028	2.794	0.005
Treatment T1: Year 2021	-1.459	0.305	-4.782	<0.001
Treatment T2: Year 2021	-1.098	0.343	-3.198	0.001

Tab. 9 – Estimated regression parameters, standard errors, z-value and P-values for the Tweedie GLMM: Infestation index= Number of Bees + Treatment*Week + Treatment*Year, AIC= 1200.

	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
Intercept	-3.214	0.917	-3.504	<0.001
Number of bees	0.536	0.132	4.064	<0.001
Treatment T1	-0.857	0.746	-1.148	0.251
Treatment T2	-1.831	0.852	-2.150	0.032
Week	0.083	0.033	2.488	0.013
Year 2021	2.996	0.268	11.183	<0.001
Treatment T1: Week	0.078	0.024	3.244	0.001
Treatment T2: Week	0.091	0.028	3.300	0.001
Treatment T1: Year 2021	-0.821	0.318	-2.579	0.010
Treatment T2: Year 2021	-0.823	0.312	-2.635	0.008

APPENDIX II: Experiment managed in the Netherland (tables)

Tab. 11 – Mann-Whitney test result comparing the number of adult bees, worker cells and drone cells among C and T treatments during the year 2022 in each sampling date.

Data week	p value		
	Adult bees	**Workers**	**Drones**
3 May	0.96983	0.90972	0.00024
25 May	0.72393	0.85982	0.019159

13 Jun	0.13361	0.053784	0.94306
6 Jul	0.68892	0.092696	0.93607
26 Jul	0.37848	0.12821	0.65531
15 Aug	0.93723	1	1

Tab. 12 – Mann-Whitney result comparing the number of *Varroa* in photetic phase (adult bees), in reproductive phase (worker cells and drone cells) and the sum of this values among C and T treatments during the year 2022 in each sampling date.

Data week	**p value**			
	***Varroa* on adult bees**	***Varroa* into worker cells**	***Varroa* into drones cells**	**Total *Varroa***
3 May	0.48212	0.58425	0.1841	0.38107
25 May	0.90013	0.62723	0.66252	0.17066
13 Jun	0.02474	0.42949	NA	0.25245
6 Jul	0.63036	0.044951	NA	0.092696
26 Jul	0.29455	0.59403	NA	0.93619
15 Aug	0.47117	0.91511	NA	0.37848